HHS Public Access

Author manuscript

Gene Ther. Author manuscript; available in PMC 2016 July 11.

Published in final edited form as:

Gene Ther. 2016 April ; 23(4): 340–348. doi:10.1038/gt.2016.3.

HSV vector-mediated GAD67 suppresses neuropathic pain induced by perineural HIV gp120 in rats through inhibition of ROS and Wnt5a

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Abstract

Human immunodeficiency virus (HIV)-related neuropathic pain is a debilitating chronic condition that is severe and unrelenting. Despite the extensive research, the exact neuropathological mechanisms remain unknown, which hinders our ability to develop effective treatments. Loss of GABAergic tone may play an important role in the neuropathic pain state. Glutamic acid decarboxylase 67 (GAD67) is one of isoforms that catalyze GABA synthesis. Here, we used recombinant herpes simplex virus (HSV-1) vectors that encode *gad1* gene to evaluate the therapeutic potential of GAD67 in peripheral HIV gp120-induced neuropathic pain in rats. We found that 1) subcutaneous inoculation of the HSV vectors expressing GAD67 attenuated mechanical allodynia in the model of HIV gp120-induced neuropathic pain, 2) the anti-allodynic effect of GAD67 was reduced by GABA-A and-B receptors antagonists, 3) HSV vectors expressing GAD67 reversed the lowered GABA-IR expression, and 4) the HSV vectors expressing GAD67 suppressed the upregulated mitochondrial superoxide and Wnt5a in the spinal dorsal horn. Taken together, our studies support the concept that recovering GABAergic tone by the HSV vectors may reverse HIV-associated neuropathic pain through suppressing mitochondrial superoxide and Wnt5a. Our studies provide validation of HSV-mediated GAD67 gene therapy in the treatment of HIV-related neuropathic pain.

Keywords

HIV pain; GAD67; gene therapy; mitochondrial superoxide; Wnt5a

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THE CONFLICTS OF INTEREST: All authors declare no conflicts of interest for this study.

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INTRODUCTION

Chronic pain is still a significant problem for HIV patients[1]. Little is known about molecular mechanisms of HIV-associated neuropathic pain. Many conventional agents utilized as pharmacologic therapy for neuropathic pain are not very effective for providing satisfactory analgesia in patients with HIV-related neuropathic pain[2]. Gama-amino butyric acid (GABA)-mediated inhibition, acting both presynaptically and postsynaptically, exerts a tonic modulation of nociceptive neurotransmission between primary afferents and second-order spinothalamic tract neurons[3, 4]. Pharmacologic antagonism of spinal GABAergic neurotransmission results in mechanical hypersensitivity similar to that found in neuropathic pain[5–7]. Hypofunction of GABAergic inhibitory mechanisms after nerve injury has been reported[7, 8]. GABA concentrations in the cerebrospinal fluid were decreased in the SIV-infected rhesus monkeys[9], suggesting the decrease in GABAergic tone.

Although spinal GABAergic systems suppress neuropathic pain, it is not very known about the downstream mechanisms of GABA. Oxidative stress causes activation of a number of complex and interrelated signaling events[10]. Mitochondria are the main source of reactive oxygen species (ROS) in the spinal dorsal horn; scavengers of ROS have been shown to produce a strong antinociceptive effect on persistent pain[11]. Superoxide generated from mitochondrial oxidative phosphorylation is a major source of neuronal ROS[12]. Analgesic effect of ROS scavengers is observed in capsaicin-induced secondary hyperalgesia[13]. Oxidative stress has been suggested to play a role in the pathogenesis of neuroAIDS[14]. Our recent studies show that intrathecal injection of ROS scavenger reduces neuropathic pain induced by perineural HIV gp120[15]. Wingless-type mousemammary tumor virus integration site (Wnt) signaling is essential for neuronal development and the maintenance of the nervous system[16]. In the central nervous system, specific Wnt ligands such as Wnt5a (the prototypic Wnt ligand that activates the noncanonical pathways) are predominantly expressed in the neurons[17]. Recent studies suggest a critical role of Wnt signaling in the spinal cord[18–20] in the development of pathological pain. Wnt5a is involved in the intrathecal gp120-induced neuropathic pain[17, 21].

Many conventional agents utilized as pharmacologic therapy for neuropathic pain are not very effective for providing satisfactory analgesia in patients with HIV-related neuropathic pain[2]. We and others have previously demonstrated that replication-defective herpes simplex virus (HSV)-based vectors delivered by subcutaneous inoculation can be used to express neurotransmitters/neuropeptides in the dorsal root ganglion (DRG) neurons and to produce a pain-relieving effect in rodent models of different pain models[22–26]. Glutamic acid decarboxylase 67 (GAD67, or *gad1*) gene encodes for the rate limiting enzyme of GABA synthesis in presynaptic neurons. GAD67 mRNAs pertain to inhibitory neocortical neurons and were significantly decreased in frontal neocortex in subjects with HIV encephalitis[27]. In this study, we examined the antinociceptive effect of recombinant HSV-vectors encoding *gad1* in the peripheral gp120-induced neuropathic pain in rats, and tested whether mitochondrial superoxide and Wnt5a were involved in the antinociceptive effect.

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RESULTS

The anti-allodynic effect of GAD67 mediated by HSV vector on neuropathic pain induced by perineural gp120

Previous studies have demonstrated that the peripheral gp120 application into the sciatic nerve, results in neuropathic pain characterized by mechanical allodynia[28–30]. In this study, we examined whether overexpression of GAD67 mediated by the HSV vectors reduced neuropathic pain induced by perineural HIV gp120. Subcutaneous inoculation with QHGAD (30 μl containing 1×10^9 plaque-forming units/ml) was carried out in the plantar surface of the hind foot. Treatment with QHGAD caused a statistically significant elevation of mechanical threshold that was apparent on day 3 post vector inoculation compared with the control vector; the anti-allodynic effect of the HSV vector lasted for more than 28 days ($F_{(1,10)}$=19.29, P=0.001, two way ANOVA, Figure 1A). The comparison of the differences at individual time points between two groups was shown in Figure 1A). The area under curves (AUC) in the QHGAD group was significantly higher than that in the Q0ZHG group (P =0.002, *t* test, Figure 1B). The loss of GABAergic tone may play important role in the neuropathic pain[31]. Previous studies reported that the non-replicating HSV vector QHGAD produces GAD67 in primary DRG neurons in *vitro*[32], and in the lumbar DRG *in vivo* following subcutaneous inoculation with the vectors into the hindpaws of rats[32, 33]. Similarly, in the current study, GAD67 in the DRG or SDH in gp120 neuropathic rats with Q0ZHG was significantly lowered than that in the sham surgery group; there was a significant increase in GAD67 in the gp120+QHGAD compared with that in the gp120+Q0ZHG group in the DRG or SDH (data not shown).

The effect of intrathecal GABA antagonists on anti-allodynia produced by QHGAD in neuropathic pain

We tested whether intrathecal administration ofbicuculline (competitive antagonist of GABA-A receptor) and CGP35348 (selective antagonist of GABA-B receptor) antagonized QHGAD analgesia. For intrathecal administration of bicuculline and CGP35348, intrathecal catheters were implanted under isoflurane anesthesia[34, 35] (see the detailed description in Method). Seven days post intrathecal catheter implantation rats received gp120 application into the sciatic nerve. Then, seven days post gp120 application, rats received QHGAD. Two weeks after QHGAD, intrathecal bicuculline, CGP35348, or saline 10μl was injected. Mechanical threshold was measured using Von Frey fibers at 30, 60, 90, 120, 180, and 300 min post intrathecal injection. Intrathecal bicuculline (0.3μg) significantly lowered mechanical threshold for 3 hours compared with vehicle group ($F_{(1,11)}$=19.84, P = 0.001, two-way ANOVA) (Supplementary Figure S1.A). The AUC in the bicuculline group was significantly lower than that in vehicle group (P = 0.002, Supplementary Figure S1.B). Intrathecal CGP35348 (30μg) significantly decreased mechanical threshold for 2 hours compared with vehicle group ($F_{(1,10)}$=8.34, P=0.016, two-way ANOVA) (Supplementary Figure S1.A). The AUC in the CGP35348 group was significantly lower than that in the vehicle group (P =0.016, Supplementary Figure S1.B).

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The effect of GAD67 mediated by the HSV vector on GABA positive neuron expression in neuropathic pain

Evidence has shown that a reduced spinal GABAergic inhibitory function is involved in the neuropathic pain state[31, 36, 37]. Intrathecal GABA agonists reduce mechanical allodynia in the nerve injury pain model[7, 8]. In this study, we investigated whether the expression of GAD67 mediated by the HSV vector increased GABA neurons in the SDH in the neuropathic pain state. Neuropathic animals receiving the HSV vectors were perfused at 2 weeks after vector injection. GABA immunoreactivity was carried out using immunohistochemistry. The number of GABA immunoreactivity positive neurons was accounted in the SDH. GABA immunoreactivity positive imaging in sham (Supplementary Figure S2.A), and neuropathic rats with Q0ZHG (Supplementary Figure S2.B), and neuropathic rats with QHGAD (Supplementary Figure S2.C) was shown. There was a significant decrease in the number of GABA positive neurons in the gp120+Q0ZHG group compared with that in the sham+Q0ZHG ($P < 0.001$ *vs.* sham+Q0ZHG, Supplementary Figure S2.D). GABA positive neurons in the gp120+QHGAD group were markedly increased compared with that in the gp120+Q0ZHG ($P < 0.001$ *vs.* Q0ZHG, Supplementary Figure S2.D).

The effect of GAD67 mediated by the HSV vector on mitochondrial superoxide expression in neuropathic pain

Although the increased ROS in the spinal cord may induce pain by reducing GABAergic tone on neurons that are involved in pain transmission[36, 38], it is not clear whether increasing GABAergic tone reduces ROS in the spinal cord during the neuropathic pain state. Mitochondrial ROS is involved in the pain state[39]. In this study, we investigated whether the expression of GAD67 mediated by the HSV vector decreased mitochondrial superoxide in the SDH in the HIV gp120-related neuropathic pain state. Neuropathic animals receiving the HSV vectors were perfused at 2 weeks after vector injection. MitoSox Red (a mitochondrial superoxide indicator) was intrathecally administered 70 min prior to perfusion. MitoSox positive imaging was detected under a fluorescent microscope with a rhodamine filter as described previously[39]. The MitoSox positive imaging in sham, neuropathic rats with Q0ZHG, and neuropathic rats with QHGAD was shown in Figure 2A, 2B, and 2C, respectively. The number of MitoSox positive neurons was accounted in the SDH. We found that there was a significant increase in the number of MitoSox positive cells in the gp120+Q0ZHG group compared with that in the sham+Q0ZHG (Figure 2D). MitoSox positive cells in the gp120+QHGAD group were markedly decreased compared with that in the gp120+Q0ZHG (Figure 2D).

To determine the cellular localization of mitochondrial superoxide, immunostaining was carried out in spinal cord sections from neuropathic animals injected with Q0ZHG. We found that mitochondrial superoxide marker MitoSox Red signals were not colocalized with immunostaining of glial markers GFAP or OX42 (Figure 3A), however, almost all MitoSox signals colocalized with immunostaining of neuron marker NeuN (Figure 3B), indicating that mitochondrial superoxide was expressed in neurons, but not glia.

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The effect of GAD67 mediated by the HSV vector on the expression of Wnt5a in neuropathic pain

Recent studies suggest a critical role of Wnt signaling in the spinal cord[18–20] in the development of pathological pain. Wnt5a is involved in the intrathecal gp120-induced neuropathic pain[17, 21]. In the current study, we investigated whether GAD67 mediated by the HSV vector change Wnt5a in the SDH in the HIV-related neuropathic pain state. The immunoblots of Wnt5a was carried out using western blots. We found that there was a significant increase in Wnt5a in the gp120+Q0ZHG group compared with that in the sham +Q0ZHG ($P < 0.01$, Figure 4). Wnt5a in the gp120+QHGAD group were markedly decreased compared with that in the gp120+Q0ZHG ($P < 0.01$, Figure 4).

Double immunostaining showed that almost all Wnt5a colocalized with NeuN, but not glial markers GFAP or OX42 (Figure 5). The results above indicated that Wnt5a were located on the neurons in the spinal dorsal horn, which is in line with previous reports[40].

DISCUSSION

Previous studies have shown that HSV-mediated human proenkephalin expression in primary afferent axons at the dorsal root entry zone of the dorsal horn was reported[23]. We and others have demonstrated that replication-defective HSV-based vectors delivered by subcutaneous inoculation express enkaphalin or GAD67 in the DRG neurons and spinal dorsal horn, and produce a pain-relieving effect in rodent models of different pain models[25, 26, 33, 41]. The present studies demonstrate, 1) that subcutaneous inoculation of the HSV vectors expressing GAD67 attenuated mechanical allodynia in the model of HIV gp120-induced neuropathic pain, 2) that the anti-allodynic effect of HSV vectors expressing GAD67 was mediated through GABA-A and-B receptors, 3) that HSV vectors expressing GAD67 reversed the lowered GABA-IR expression, and 4) that the HSV vectors expressing GAD67 suppressed the upregulated spinal mitochondrial superoxide and Wnt5a.

Elucidating the molecular mechanisms of neuropathic pain is an important prerequisite for the rational development of novel analgesic drugs for the therapy of neuropathic pain. Nerve trauma models rely on direct injury to nerve. The predominance of traumatic nerve injury models does not fully match the clinical situation, as shown by an analysis of the randomized clinical trials[42, 43]. It is logical to select preclinical models that reflect more closely the precise pathophysiological condition studied in humans[43]. A multitude of disease-specific models have been developed in recent years, including for example, rodent models induced by anti-cancer chemotherapy, multiple sclerosis, or HIV-related painful neuropathy (see review[43]).

HIV coat protein gp120 binds with CD4 glycoprotein and chemokine co-receptors on the immune cells[44]. HIV-infected brain macrophages/microglia release potentially neurotoxic substances. These substances induce neuronal injury, dendritic and synaptic damage, and apoptosis[44]. The most common histological feature of painful HIV-neuropathy is characterized by loss of DRG sensory neurons, Wallerian degeneration of the long axons in distal regions, DRG infiltration by HIV-infected macrophages, and a 'dying back' sensory neuropathy, and loss of unmyelinated sensory fibers (see review[45–47]). The model of

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peripheral gp120 can induce neuropathic pain behavior, inflammatory cell infiltration into DRG, spinal gliosis, and reduction in intraepidermal nerve fiber density, which correlates well with the clinical scenario, therefore, the gp120 model is considered as HIV-related painful neuropathy[48].

In the mammalian spinal cord, tonic GABAergic inhibition plays an important role in normal sensory processing by increased behavior responsiveness[5, 7, 49]. Spinal GABA-A and GABA-B receptors modulate spinal systems activated by low threshold mechanoreceptors which mediate the allodynia observed following peripheral nerve injury[7, 8]. Electrophysiological studies show that GABAergic systems contribute to the tonic modulation of nociceptive neurotransmission at the spinal level[3]. A significant loss of GABAergic tone in lamina I-III of the rat spinal cord occurs in the somatotopic area of projection of the sciatic nerve after nerve transection[50, 51]. Previous evidence shows that peripheral injury decreases the GAD67 in the SDH[52].

GABA agonist drugs are approved for treatment of selected neuropathic pain syndromes, but the ubiquitous distribution of GABA receptors in the central nervous system results in side effects that impose severe restrictions on the dose of baclofen, even when administered intrathecally in attempting to control pain[33]. Previous reports that the GAD-expressing HSV vector reduces pain-related behaviors over the course of several weeks in different models of neuropathic pain[25, 33, 41, 53]. In our first report using the vectors expressing GAD67, we used spinal cord injury (SCI) model induced by T13 spinal cord hemisection[33], which is a direct spinal cord mechanic injury-related motor and sensory dysfunction, including neuropathic pain. Human painful neuropathies are very diverse. Peripheral HIV gp120-induced painful sensory neuropathy is a neuroimmunologic/neuro-inflammatory response. Our recent studies showed that in the gp120/ddC model, GAD67 expression mediated by the HSV vectors decreased mechanical allodynia[54]. In the present studies using the gp120-induced neuropathic model, we found that there was a down regulation of spinal GAD67 protein, and that the HSV-mediated gene transfer of GAD67 reduced mechanical allodynia and increased the lowered the expression of GAD67 in the SDH.

In mammals, GABA synthesis depends on two forms of the enzyme glutamic acid decarboxylase GAD65 and GAD67-that may serve distinctive functions within GABA-producing cells. GAD65 and GAD67 are encoded by two differentially regulated genes[55, 56]. In the mature neuron, GAD67 is widely present in both terminals and the cell body[55, 57]. In contrast, GAD65 is primarily localized to nerve terminals. In brain extracts, almost all GAD67 is in an active holoenzyme form, saturated with its cofactor, pyridoxal phosphate. In contrast, only about half of GAD65 (which is found in synaptic terminals) exists as active holoenzyme[57]. It has been shown that thermal and mechanical pains are mediated by different molecular mechanisms[58]. GAD65 knockout mice show significant reduction in response latency measured by the hot plate test but there is no genotype-specific difference when measured by the von Frey test, and GAD65-mediated GABA synthesis plays relatively small roles in nociceptive processing via supraspinal mechanisms[59]. In mouse diabetic model of neuropathic pain, GAD65 vector inoculation most significantly relieves thermal pain compared to mechanical allodynia by Von Frey filaments[25]. Introduction of either GAD65 or GAD67 by HSV or AAV vectors to the DRG/spinal cord has been shown to

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effectively relieve different neuropathic pain states[25, 32, 33, 41, 60, 61]. Clinical data show that patients with HIV-related painful neuropathy do not usually present with thermal hypersensitivity[62]. Moreover, mechanical pain thresholds are significantly decreased (mechanical allodynia) in the HIV pain patients[63]. In our studies, we focus on mechanical allodynia in the HIV pain state, therefore we used the HSV vector expressing GAD67.

Primary nociceptors are pseudounipolar neurons with cell bodies in the DRG and axons that terminate peripherally in the skin and project centrally to terminate in the spinal dorsal horn. HSV particles are taken up by sensory nerve terminals and then carried by retrograde axonal transport to the neuronal perikaryon in the DRG, where the wild-type virus may either re-enter the lytic cycle, or establish a latent state from which the (wild-type) virus can subsequently reactivate and spread to other individuals. However, recombinant HSV vectors establish a persistent state similar to latency characterized by the inability to reactivate or re-establish active virus growth, but retain the DRG-targeting properties of the wild-type virus and remain active in their ability to express transgene products[22, 64]. HSV vectors delivered by subcutaneous inoculation can be used to express neurotransmitters in the DRG and the spinal dorsal horn through central axons to produce a pain-relieving effect in different pain models in rodents[65]. The HSV vectors expressing GAD67 provide an analgesic effect in the neuropathic pain in the spinal injury or diabetic animals[25, 66].

In the current studies, we found that HSV vectors expressing GAD67 increased GABA-IR expression. The mechanisms by which GABA-IR neurons were increased by HSV-mediated GAD67 is not clear. Evidence shows that GABA can be synthesized via activity of GABA receptors[67]. Activation of GABA receptors in neural precursors may be induced by non-synaptic paracrine and/or autocrine released GABA[68]. GABA is synthesized by GAD in neurons where it functions as a autocrine signaling molecule[69, 70]. Recent studies show that there is a GABA autocrine feedback mechanism at nociceptive nerve terminals[71]. Spinal interneurons utilizing GABA as their neurotransmitter, also express GABA receptors, which may be activated in an autocrine manner[72]. GABA(A)-receptors can be activated by ambient GABA by an autocrine action[73]. Thus, it is possible that GABA mediated by GAD67 released from the central terminal of the DRG, may bind on the GABA receptor of dorsal horn neurons to enhance GABA product (an autocrine mechanisms in the dorsal horn GABAergic neurons).

Although spinal GABAergic systems suppress neuropathic pain, it is not known about the downstream mechanisms. As a part of the signaling pathways for the induction of persistent pain, ROS are critically involved in the spinal sensitization[74, 75]. Superoxide generated from mitochondrial oxidative phosphorylation is a major source of neuronal ROS[12]. Mitochondrial oxidative stress causes activation of a number of complex and interrelated signaling events in the pathogenesis of chronic pain[76]. A significant analgesic effect of ROS scavengers is observed in capsaicin-induced secondary hyperalgesia[13]. Furthermore, ROS accumulation is observed primarily in the mitochondria of the SDH neurons in different pain models[39, 77–79]. HIV gp120 has been implicated in initiation and/or intensification of ROS[80]. While the increased ROS in the spinal cord may induce pain by reducing GABA inhibitory influence on the spinal neurons that are involved in pain transmission[36, 38], little is known about whether GABAergic systems suppress ROS. In the gp120/ddC model, GAD67

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expression mediated by the HSV vectors reversed the increased signals of mitochondrial superoxide in the spinal dorsal horn[54]. In the present studies, we found that gp120 application increased spinal mitochondrial superoxide, and that overexpression of GAD67 mediated by the HSV vectors suppressed spinal mitochondrial superoxide, suggesting GABAergic systems negatively regulated mitochondrial superoxide.

Wnt signaling is highly evolutionarily conserved and is indispensable in animal growth, development, metabolism, and maintenance of stem cells[81]. Wntsignaling is essential for neuronal development and the maintenance of the nervous system[16]. In the central nervous system, specific Wnt ligands such as Wnt5a (the prototypic Wnt ligand that activates the noncanonical pathways) are predominantly expressed in neurons[17]. Wnt proteins are upregulated in the spinal cord in the development of pathological pain[18–20, 82]. Blockage of Wnt5a signaling impairs intrathecal gp120-induced allodynia, whereas activation of Wnt5a signaling facilitates allodynia, suggesting that Wnt5a signaling plays a critical role in the HIV pain pathogenesis. Consistent with this notion, Wnt5a is upregulated in the SDH of HIV patients with chronic pain[40]. NMDARs control metalloproteinase expression via a Wnt/MAPK signaling pathway in the primary cortical neurons[83]. Removal of GABAergic inhibition allowed for PKA-mediated NMDARs phosphorylation and synaptic accumulation, thus exaggerating NMDARs-dependent nociceptive transmission and behavioral sensitization[84]. Activation of GABA-B receptor by baclofen attenuates diabetic neuropathic pain, which may partly be accomplished via down-regulating the expression of NMDA receptors[85]. Thus, it is possible that GABA systems suppressed Wnt5a through inhibiting NMDA receptors.

In summary, we found the reduced spinal GABAergic tone in the painful HIV pathogenesis. Restoring the GABAergic tone by the HSV vectors reduced HIV neuropathic pain and reduced ROS and Wnt5a. Our studies prove the validation of HSV gene therapy in the treatment of HIV neuropathic pain.

MATERIALS AND METHODS

Construction of the HSV vector expressing the GAD67 and delivery

The vector QHGAD contains the encoding sequence of human *gad1* gene under the control of the human cytomegalovirus immediate early promoter (HCMV IEp) in the UL41 locus of an HSV recombinant defective for HSV genes ICP4, ICP22, ICP27, and ICP47. QHGAD was generated as described previously[33]. Control vector Q0ZHG is defective in the same genes but contains the Escherichia coli *lacZ* reporter gene in the same position. Animals were inoculated subcutaneously in the footpad of the hind paws with 30 μl containing 1.0×10^9 pfu with either QHGAD or the control vector Q0ZHG 1 week after the peripheral gp120 application.

Animals

Male Sprague-Dawley rats weighing 210 to 230 g were housed 1 to 3 per cage approximately 7 days before beginning the study. Rats were maintained with free access to food and water and were on a 12:12 light:dark schedule at 21°C and 60% humidity. A

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randomization was used to determine animals to experimental groups. All housing conditions and experimental procedures were approved by the University Animal Care and Use Committee and were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain.

Perineural gp120 neuropathic pain model

Under anesthesia with 2% isoflurane inhalation, the rat left sciatic nerve was exposed in the popliteal fossa without damaging the nerve construction as described previously[28, 48, 86]. Briefly, a 2 × 6 mm strip of oxidized regenerated cellulose was previously soaked in 250 μl of a 0.1% rat serum albumin (RSA) in saline, containing 400 ng of gp120 (Immunodiagnostics, Bedford, MA) or 0.1% RSA in saline for the sham surgery. A length of 3–4 mm of the sciatic nerve was wrapped loosely with the previously soaked cellulose, proximal to the trifurcation not to cause any nerve constriction and left *in situ*. The incision was closed with 4/0 sutures.

Intrathecal catheter implantation

For studying the effect of intrathecal administration of chemicals, chronic intrathecal catheters were implanted using isoflurane anesthesia as described in our previous studies[86]. Briefly, through an incision in the atlanto-occipital membrane, a polyethylene (PE-10) catheter, filled with 0.9% saline, was advanced 8.5 cm caudally to position its tip at the level of the lumbar enlargement. The rostral tip of the catheter was passed subcutaneously, externalized on top of the skull, and sealed with a stainless steel plug. Animals showing neurological deficits after implantation were excluded. Animals were used within 5 days after implantation of the catheter.

Mechanical threshold

The mechanical threshold was determined using calibrated von Frey filaments (Stoelting, Wood Dale, IL) introduced serially to the hindpaws in ascending order of strength, and animals were placed in non-transparent plastic cubicles on a mesh floor for an acclimatization period of at least 30 minutes on the morning of the test day. A positive response was defined as a rapid withdrawal and/or licking of the paw immediately on application of the stimulus. Whenever a positive response to a stimulus occurred, the next smaller von Frey hair was applied, and whenever a negative response occurred, the next higher force was applied. In the absence of a response at a pressure of 15.1 g, animals were assigned to this cutoff value. The mechanical threshold was determined according to the method described previously with a tactile stimulus producing a 50% likelihood of withdrawal determined by using the up-and-down method[87]. The experimenters were blinded to the groups during behavioral test.

Western blots

Under deep anesthesia, the L4–5 DRG and SDH ipsilateral to the gp120 application were removed rapidly, frozen on dry ice and stored at −80 °C. These tissues were homogenized in protein lysis buffer (150mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0) containing protease inhibitors (Sigma, St Louis, MO) and

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phosphatase inhibitors (Phosphatase Inhibitor Cocktail 1 and 2) (Sigma, St Louis, MO). The homogenate was centrifuged at 18,000 g for 20 min at 4 °C. The supernatant was collected and assayed for protein concentration using the DC protein assay (Bio-Rad, Hercules, CA). Aliquots containing 30 μg of protein were dissolved in Laemmli buffer and denatured at 95 °C for 5 min; proteins were separated by 10 to 12% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with rapidblock solution (Amresco, USA) and then incubated with primary antibodies for overnight at 4 °C, including mouse anti GAD67 (1:2000, sc7512, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Wnt5a (1:1000, ab72583, Abcam, Cambridge, MA) and mouse anti-β-actin (1 : 10000, A5441, Sigma, St Louis, MO). The blots were incubated with secondary antibodies (Santa Cruz Biotechnology) and developed in a chemiluminescence solution (Pierce Biotechnology, Rochford, IL, USA). Quantification of western blots was done from the obtained chemiluminescence values (Bio-Rad ChemiDoc, Bio-Rad). Target protein bands were normalized using the amount of β-actin.

Mitochondrial superoxide imaging in the SDH

MitoSox Red (Cata #, M36008, a mitochondrial superoxide indicator, Invitrogen) was dissolved in a 1:1 mixture of dimethylsulfoxide (DMSO) and saline to a final concentration of 33μM as described previously[54, 78]. MitoSox (30μl) was injected intrathecally. Approximately 70 min after injection, rats were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer, and the L4–L5 segments of the spinal cord were removed, postfixed in the same fixative solution for overnight, and cryoprotected with 30% sucrose in PBS for 2 days. The 35μm sections were examined under a fluorescent microscope with a rhodamine filter. Two different regions of the dorsal horn were photographed from 4–6 randomly selected sections from each animal: laminas I–II and laminas III–V. The number of MitoSox-positive cellular profiles with distinctive nuclei (dark oval shaped space surrounded by red granules) was counted from the pictures.

Immunohistochemistry

The distribution of GABA in the SDH was determined by immunohistochemistry. Rats were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer, and the L4–L5 segments of the spinal cord were removed, postfixed in the same solution for overnight, and cryoprotected with 30% sucrose in PBS for 2 days. Cryostat sections (35μm thickness) were incubated overnight at 4 °C with rabbit anti-GABA (1:1000; a gift from Dr. Yuan Zhu, Department of Medicine, University of Michigan, MI), mouse anti-GFAP antibody (1 : 2000, cata# G3893, Sigma, St. Louis, MO), mouse anti-OX42 antibody (1:1000, cata# CBL1512, Millipore, Billerica, MA), mouse anti-NeuN monoclonal antibody (A60) (1 : 5000, cata# MAB377, Millipore, Billerica, MA), or rabbit anti-Wnt5a (1:1000, cata# ab72583, Abcam, Cambridge, MA) followed by fluorescent IgG (Alexa Fluor 488, 1:1000 or Alexa Fluor 594, Molecular Probes, Eugene, OR, USA) for 2 hours at room temperature. Fluorescence images were captured by a fluorescent microscopy (Fluorescent M Leica/ Micro CDMI 6000B)[29, 88].

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Drug and data analysis

Bicuculline methiodide (0.3μg intrathecally) was purchased from Sigma, dissolved in saline and 10 μl were injected[89,90]. CGP 35348 hydrate (30μg) was purchased from Sigma, dissolved in saline and 10 μl was injected intrathecally. To compare the difference between the time-course curves of the behavioral testing, we used two-way ANOVA with one within-subjects factor (time) and one between-subjects factor (group) of a general linear model with repeated-measures analysis with IBM SPSS 21 (IBM, Armonk, NY, USA). The statistical significance of the differences of neurochemical changes was determined by the two-tailed *t*-test or one-way ANOVA *post hoc* test following Fisher's protected least significant difference (StatView, Cary, NC). All data were presented as mean±SEM. and *P*-values of < 0.05 were considered to be statistically significant. The sample size estimate was based on our previous studies[15,54]. For the detailed numbers of animals in each group, see Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The study was supported by the NIH DA026734 (S.H.), DA025527 (S.H.), NS066792 (S.H.) and DA34749 (S.H.). H.K. and M.K. were supported by Professor Hiroshi Iwasaki (Anesthesiology, Asahikawa Medical University, Japan). R.C.L was supported by NIH DE022903. We greatly acknowledge Dr. David Fink and Dr. Marina Mata providing the high-quality HSV vectors and the excellent technical assistance of Vikram Thakur (University of Michigan, Ann Arbor, MI). Authors thank Professor Yuan Zhu (Department of Medicine, University of Michigan, MI) for his anti-GABA antibody. We thank the helpful comments of Drs. Goins William and Joseph C. Glorioso (Microbiology and Molecular Genetics, University of Pittsburgh, PA).

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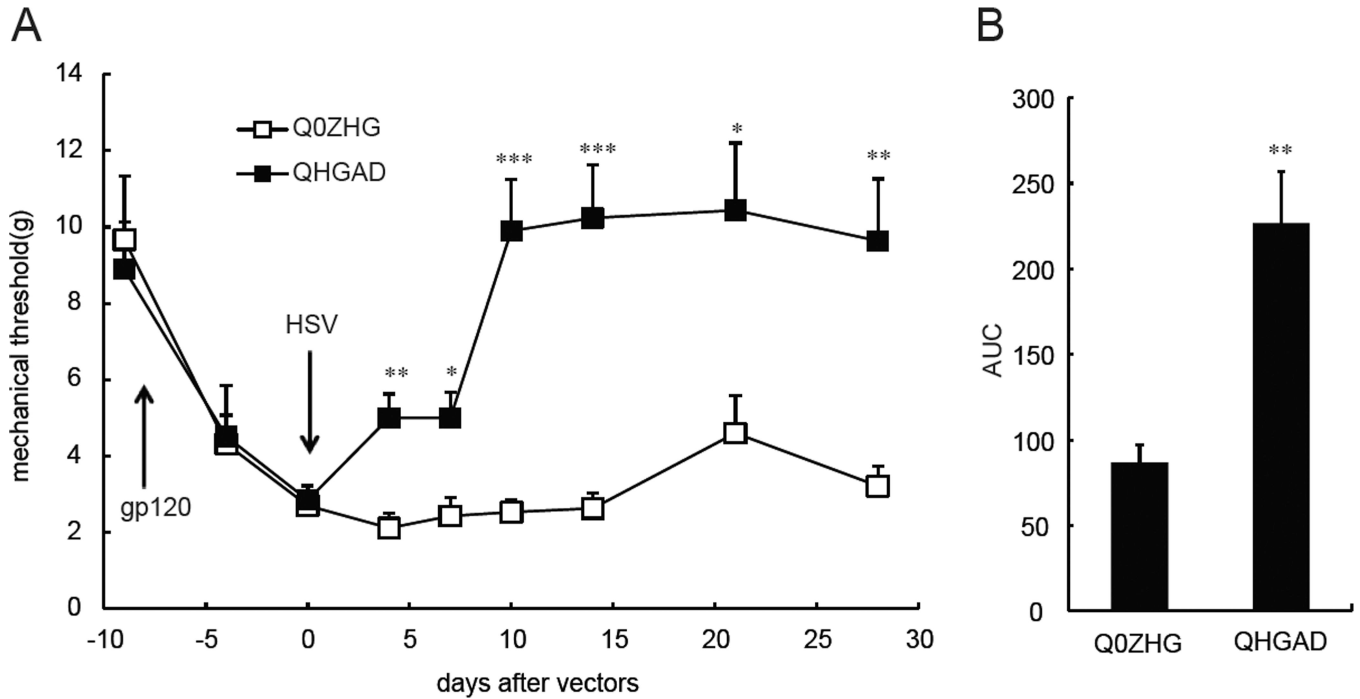

Figure 1.

The anti-allodynic effect of GAD67 mediated by the HSV vectors on neuropathic pain induced by HIV gp120. **(A)** Mechanical allodynia in rats was shown 1 week post the gp120 application (gp120). The times of gp120 and HSV vector inoculation were indicated by arrows. QHGAD resulted in a statistically significant elevation of the mechanical threshold (g) compared with the control vectors($F_{(1,10)}$= 19.29, P = 0.001, two way ANOVA repeated measures, n=6). The comparison of differences at individual time points between two groups was shown, *P < 0.05, **P<0.01, ***P < 0.001 *vs.* Q0ZHG, *t* test, n=6.**(B)** The area under the time-effect curves (AUC) in QHGAD group was significantly higher than that in the Q0ZHG group, **P < 0.01 vs. Q0ZHG, *t* test, n=6 rats.

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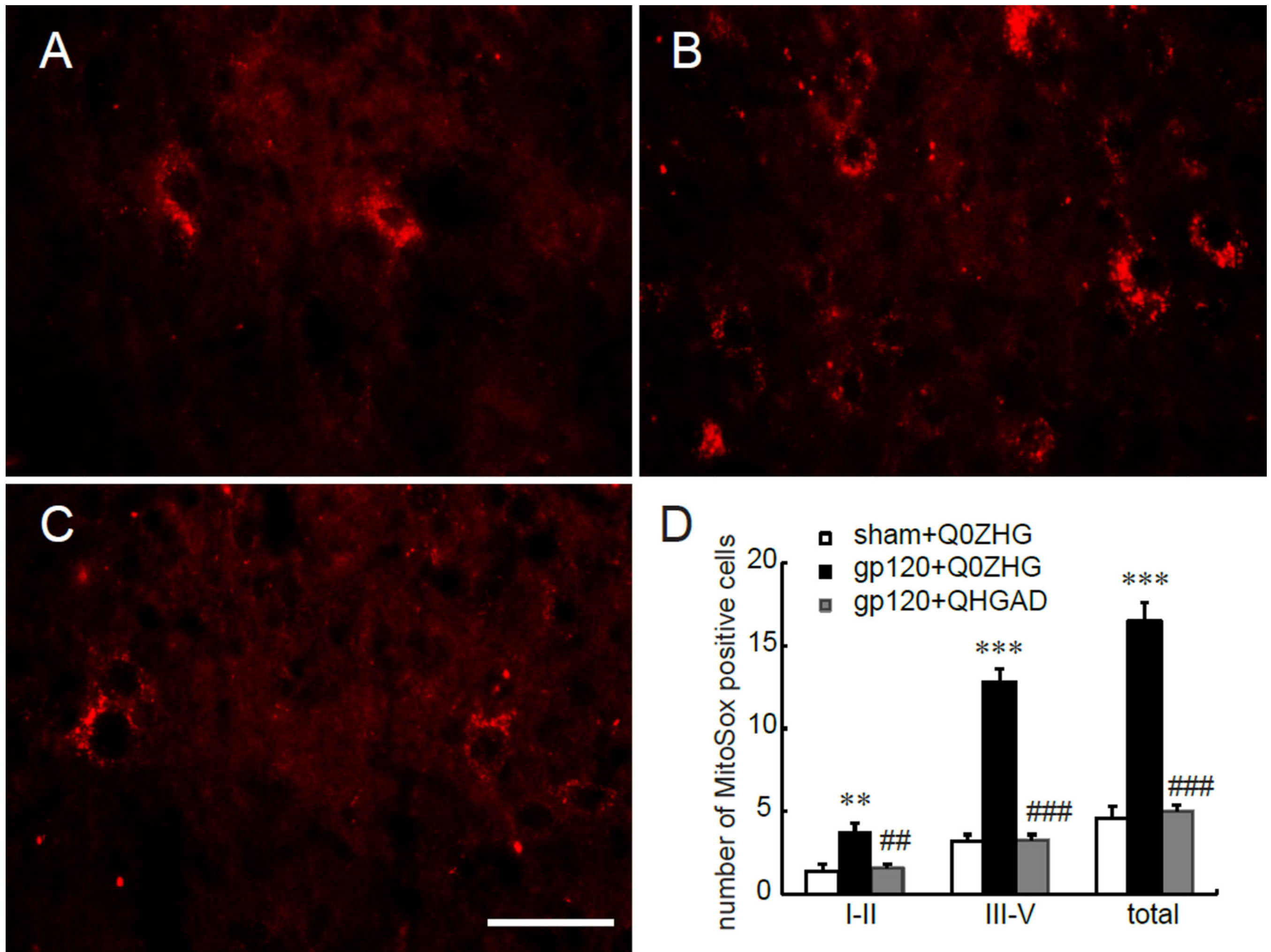

Figure 2.

The effect of GAD67 mediated by the HSV vectors on mitochondrial superoxide in the SDH. Rats with neuropathic pain were inoculated with QHGAD or Q0ZHG 1 week post gp120. In the control group, rats received the sham surgery and Q0ZHG (sham+Q0ZHG). Two weeks post vector injection, rats received intrathecal MitoSox Red, then perfused, and the spinal cord was harvested for mitochondrial superoxide image. The image of mitochondrial superoxide in sham+Q0ZHG, gp120+Q0ZHG, and gp120+QHGAD, was shown in Figure A, B, and C, respectively. (D) The number of mitochondrial superoxide positive cells in the SDH lamina I-II and III-V was shown, ** $P < 0.01$, *** $P < 0.001$ vs. sham group, ## $P<0.01$, ### $P < 0.001$ vs. gp120+Q0ZHG, one way ANOVA, n=6 rats. Scale bar, 50μm.

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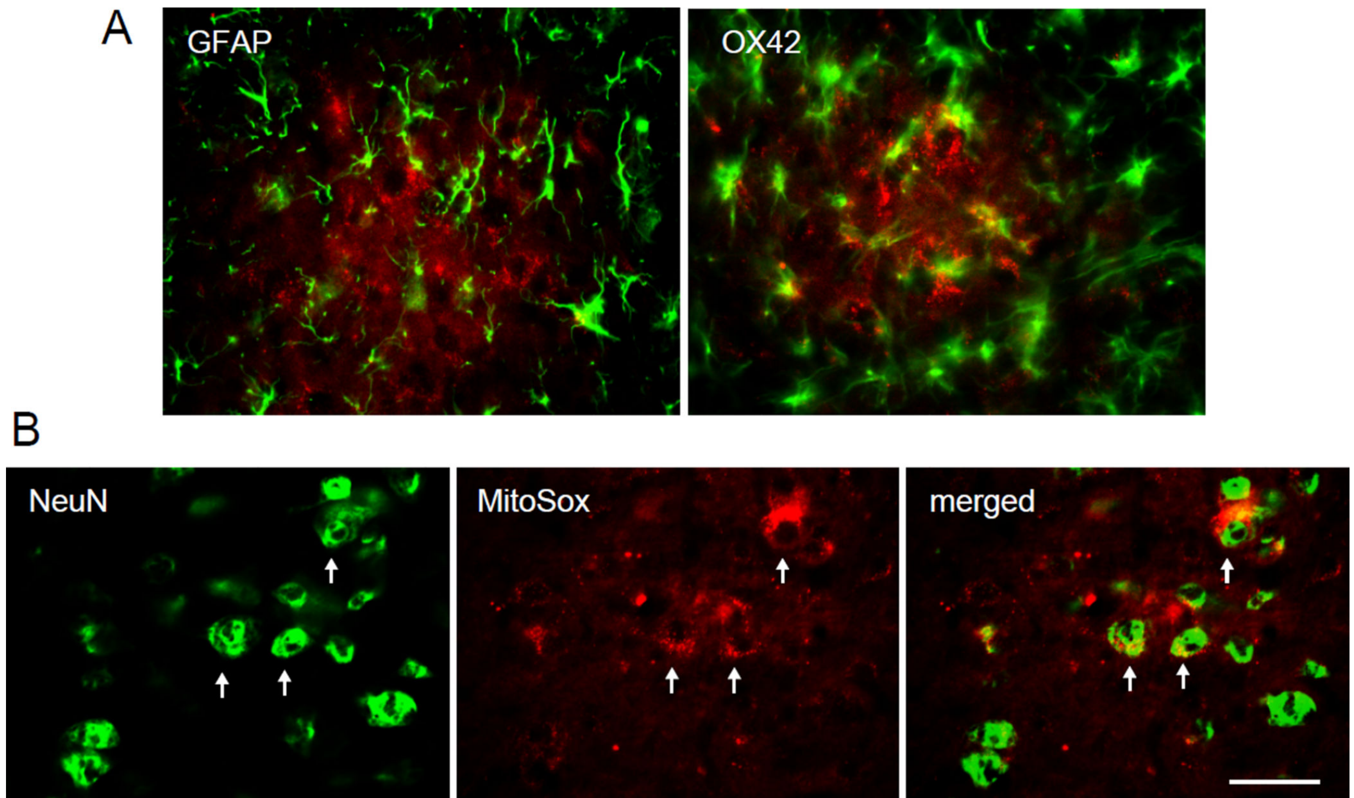

Figure 3.

Colocalization of MitoSox Red signals with immunostaining of GFAP, OX42 or NeuN in the spinal dorsal horn in the neuropathic rats. *(upper panel)* MitoSox Red signals were colocalized with immunostaining of GFAP (a marker of astrocytes) or OX42 (a marker of microglia) in the deep spinal dorsal horn. *(lower panel)* MitoSox Red signals was colocalized with immunostaining of NeuN (a marker of neurons) in the spinal dorsal horn, scale bar, 50 μm.

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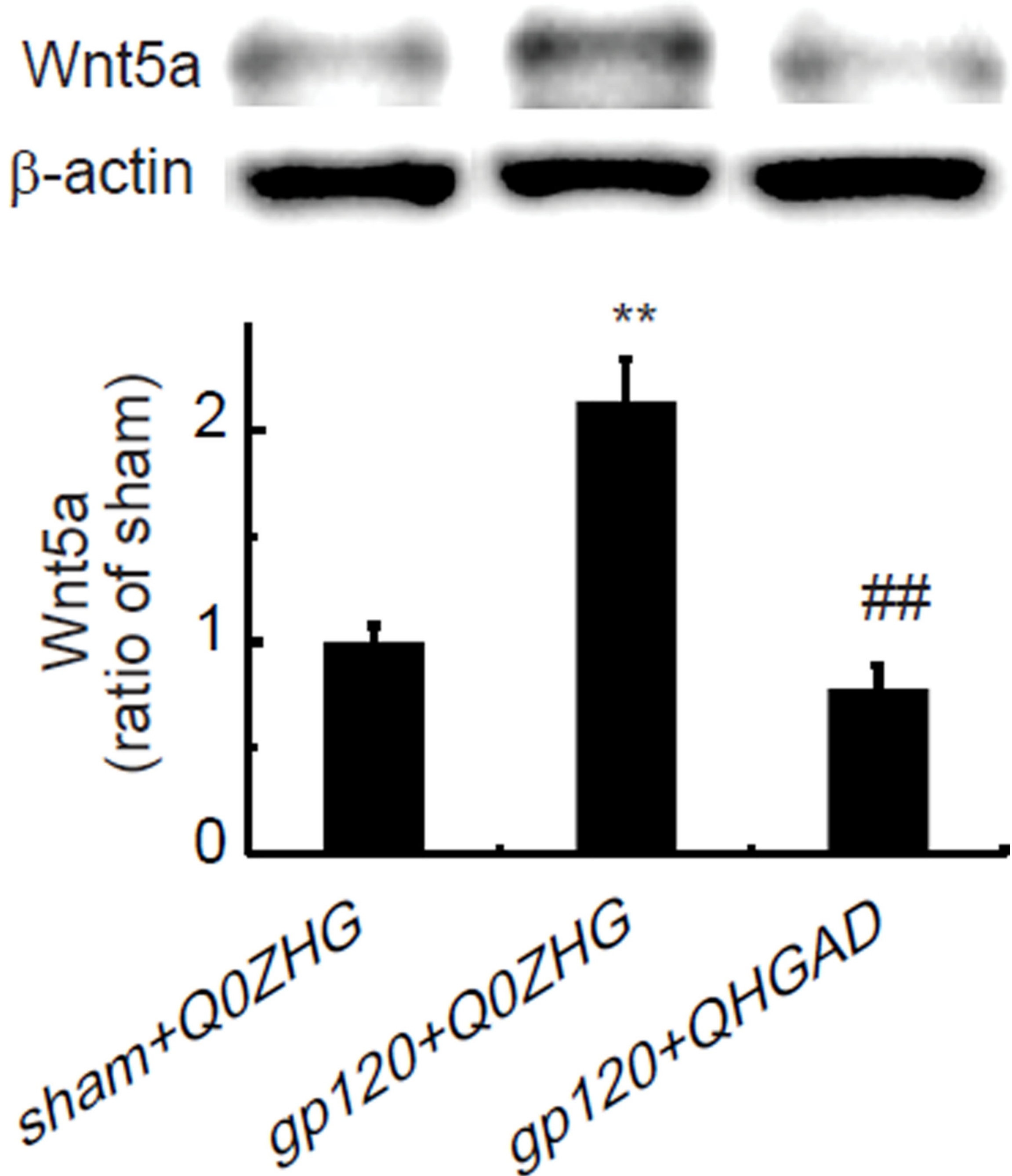

Figure 4.

The effect of GAD67 mediated by the HSV vectors on Wnt5a in the SDH. Rats with neuropathic pain were inoculated with QHGAD or Q0ZHG 1 week post gp120 application. In the control group, rats received the sham surgery and Q0ZHG (sham+Q0ZHG). At day 14 after vectors, the spinal dorsal horn were harvested and western blot assays were carried out for Wnt5a. The values from each test group were graphed as mean ± SEM, $^{**}P < 0.01$ *vs.* sham+Q0ZHG, $^{\#\#}P<0.01$ *vs.* gp120+Q0ZHG, one way ANOVA, n=4–5.

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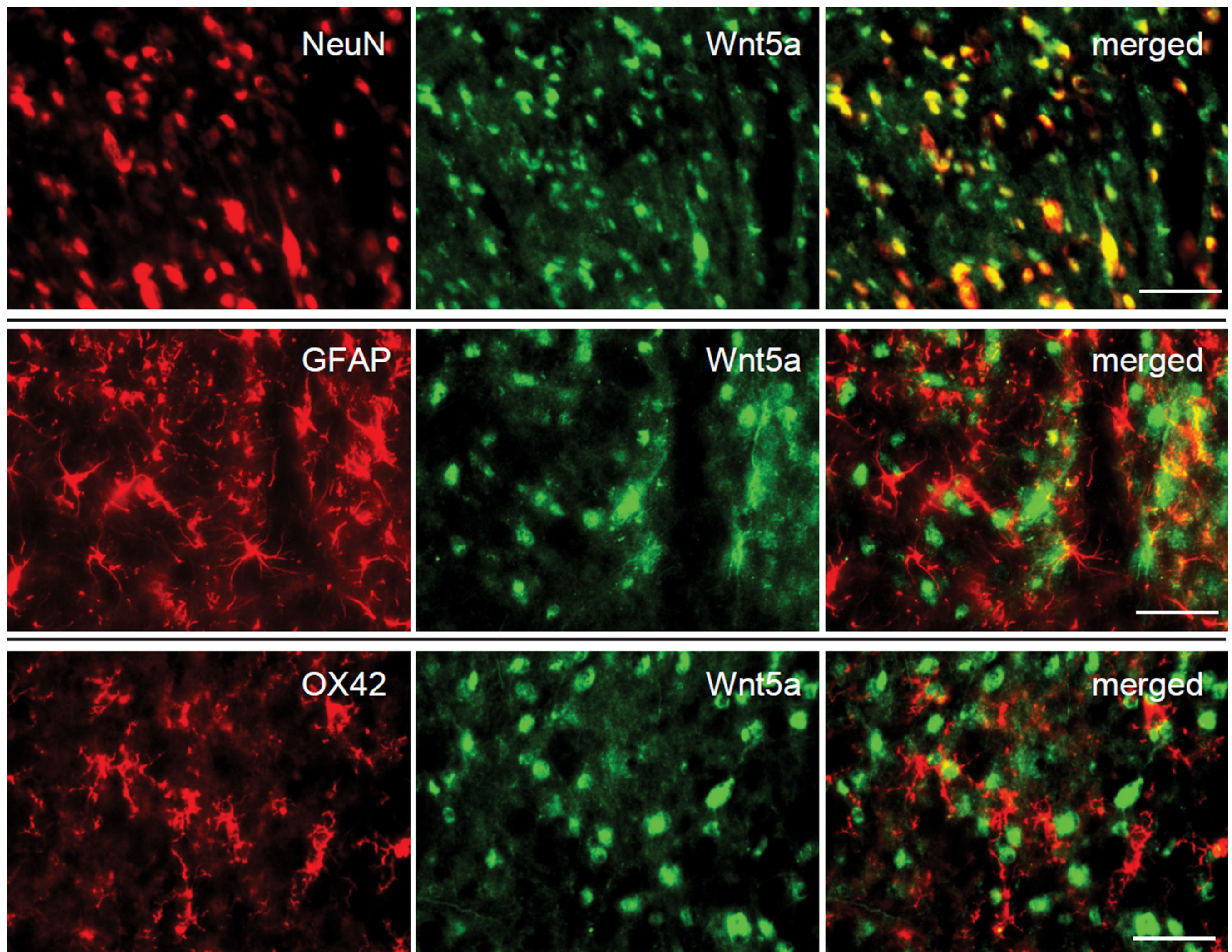

Figure 5.

Colocalization of Wnt5a with NeuN, GFAP or OX42 in the spinal dorsal horn. Animals received gp120 application with HSV vector Q0ZHG. *(upper panel)* Wnt5a was colocalized with NeuN immunostaining in the spinal dorsal horn, scale bar, 50 μm. *(middle panel)* The double immunostaings showed that Wnt5a was not colocalized with GFAP (a marker of astrocytes) in the spinal dorsal horn, scale bar, 50 μm. *(lower panel)* The double immunostaings showed that Wnt5a was not colocalized with OX42 (a marker of microglia) in the spinal dorsal horn, scale bar, 50 μm.

Table 1

The number of samples examined

behavior		**GAD67 Westen blots**		
Q0ZHG	6		DRG	SDH
QHGAD	6			
		sham+Q0ZHG	5	4
vehicle	5	gp120+Q0ZHG	4	4
bicuculline	8	gp120+QHGAD	5	4
CGP35348	7			
		MitoSox		
		sham+Q0ZHG	6	
GABA immunostaining		gp120+Q0ZHG	6	
		gp120+QHGAD	6	
sham+Q0ZHG	6	**Wnt5a Western blots**		
gp120+Q0ZHG	6			
gp120+QHGAD	6			
		sham+Q0ZHG	5	
		gp120+Q0ZHG	4	
		gp120+QHGAD	4	

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