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

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INTRODUCTION

Chronic pain is still a significant problem for human immunodeficiency virus (HIV) patients.1,2 Little is known about the 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 have 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 mouse mammary 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 cord18–20 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 different pain models in rodent.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 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.
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. 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 × 10⁸ plaque-forming units ml⁻¹) 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 4 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 analysis of variance (ANOVA), Figure 1a). The comparison of the differences at individual time points between the two groups was shown in Figure 1a. The area under curves in the QHGAD group was significantly higher than that in the Q0ZHG group (P = 0.002, two-way ANOVA). The area under curve in the bicuculline group was significantly lower than that in vehicle group (P = 0.016, two-way ANOVA; Supplementary Figure S1A). The area under curves in the CGP35348 group was significantly lower than that in the vehicle group (P = 0.016, Supplementary Figure S1B).

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. Intrathecal GABA agonists reduce mechanical allodynia in the nerve injury pain model. 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. Neurrophathic 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 S2A), and neuropathic rats with Q0ZHG (Supplementary Figure S2B), and neuropathic rats with QHGAD (Supplementary Figure S2C) 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 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 of bicuculline (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 (see the detailed description in Materials and methods). Seven days post intrathecal catheter implantation, rats received gp120 application into the sciatic nerve. Then, 7 days post gp120 application, rats received QHGAD. Two weeks after QHGAD, intrathecal bicuculline, CGP35348 or saline 10 μl was injected. Mechanical threshold was measured using Von Frey fibers at 30, 60, 90, 120, 180 and 300 min post intrathecal injection. Intrathecal bicuculline (0.3 μg) significantly lowered mechanical threshold for 3 h compared with vehicle group (F(1,11) = 19.84, P = 0.001, two-way ANOVA; Supplementary Figure S1A). The area under curves in the bicuculline group was significantly lower than that in vehicle group (P = 0.002, Supplementary Figure S1B). Intrathecal CGP35348 (30 μg) significantly decreased mechanical threshold for 2 h compared with vehicle group (F(1,11) = 8.34, P = 0.016, two-way ANOVA; Supplementary Figure S1A). The area under curves in the CGP35348 group was significantly lower than that in the vehicle group (P = 0.016, Supplementary Figure S1B).

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 analysis of variance repeated measures, n = 6). The comparison of differences at individual time points between the 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. HIV, human immunodeficiency virus; HSV, herpes simplex virus.

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GABAergic tone reduces ROS in the spinal cord during the neuropathic pain state. Mitochondrial ROS is involved in the pain state. 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 before perfusion. MitoSox-positive imaging was detected under a fluorescent microscope with a rhodamine filter as described previously. The MitoSox-positive image in sham, neuropathic rats with QOZH and neuropathic rats with QHGAD was shown in Figures 2a–c, 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+QOZH group compared with that in the sham+QOZH (Figure 2d). The MitoSox-positive cells in the gp120+QHGAD group were markedly decreased compared with that in the gp120+QOZH (Figure 2d).

To determine the cellular localization of mitochondrial superoxide, immunostaining was carried out in spinal cord sections from neuropathic animals injected with QOZH. 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.

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 in the development of pathological pain. Wnt5a is involved in the intrathecal gp120-induced neuropathic pain. In the current study, we investigated whether GAD67 mediated by the HSV vector changed 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+QOZH group compared with that in the sham+QOZH (P < 0.01, Figure 4). Wnt5a in the gp120+QHGAD group were markedly decreased compared with that in the gp120+QOZH (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 the previous reports.

**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. We and others have demonstrated that replication-defective HSV-based vectors delivered by subcutaneous inoculation express enkephalin or GAD67 in the DRG neurons and spinal dorsal horn, and produce a pain-relieving effect in rodent models of different pain models. 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 few of disease-specific pain models have been developed in recent years, including, for example, rodent models induced by anticancer chemotherapy, multiple sclerosis or HIV-related painful neuropathy (for review, see ref. 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 the 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 (for review see refs 45–47). The model of 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 GABAAergic inhibition has an important role in normal sensory processing by increased...
behavior responsiveness. Spin GABA-A and GABA-B receptors modulate spinal systems activated by low-threshold mechanoreceptors that mediate the allodynia observed following peripheral nerve injury. Electrophysiological studies show that GABAergic systems contribute to the tonic modulation of nociceptive neurotransmission at the spinal level. 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. Previous evidence shows that peripheral injury decreases the GAD67 in the SDH. GABA agonist drugs are approved for the 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. Previous reports showed that the GAD-expressing HSV vector reduces pain-related behaviors over the course of several weeks in different models of neuropathic pain. In our first report using the vectors expressing GAD67, we used spinal cord injury (SCI) model induced by T13 spinal cord hemisection, which is a direct spinal cord mechaninjury-related motor and sensory dysfuction, including neuropathic pain. Human painful neuropathies are very diverse. Peripheral HIV gp120-induced painful sensory neuropathy is a neuroimmunologinuro-inflammatory response. Our recent studies showed that in the gp120/ddC model, GAD67 expression mediated by the HSV vectors decreased mechanical allodynia. In the present studies using the gp120-induced neuropathic model, we found that there was a downregulation 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. The mature neuron of GAD67 is widely present in both terminals and the cell body. 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. It has been shown that thermal and mechanical pains are mediated by different molecular mechanisms. 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 has relatively small roles in nociceptive processing via

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 immunostainings 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 immunostainings showed that Wnt5a was not colocalized with OX42 (a marker of microglia) in the spinal dorsal horn, scale bar, 50 μm. HSV, herpes simplex virus.
supraspinal mechanisms.59 In mouse diabetic model of neuropathic pain, GAD65 vector inoculation most significantly relieves thermal pain compared with mechanical allodynia by von Frey filaments.52 Introduction of either GAD65 or GAD67 by HSV or AAV vectors to the DRG/spinal cord has been shown to effectively relieve different neuropathic pain states.5,32,33,41,60,67 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 patients with pain.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 neurotransmitter-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)-receptor-mediated current 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 onto the GABA receptor of dorsal horn neurons to enhance the GABA product (an autocrine mechanism 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.74-76 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 Although 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 expression mediated by the HSV vectors reversed the increased signals of mitochondrial superoxide in the spinal dorsal horn.81 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 that GABAergic systems negatively regulated mitochondrial superoxide.

Wnt signaling is highly evolutionarily conserved and is indispensable in the animal growth, development, metabolism and the maintenance of stem cells.81 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 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 has 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 NMDA receptors (NMDARs) control metalloproteinase expression via a Wnt/β-catenin 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 The activation of GABA-B receptor by baclofen attenuates diabetic neuropathic pain, which may partly be accomplished via downregulating the expression of NMDARs.85 Thus, it is possible that GABA systems suppressed Wnt5a through inhibiting NMDARs.

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 QOZHG 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 × 1012 p.f.u. with either QHGAD or the control vector QOZHG 1 week after the peripheral gp120 application.

Animals. Male Sprague-Dawley rats weighing 210 to 230 g were housed one to three per cage approximately 7 days before beginning the study. The 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 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 left sciatic nerve of the rat 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 in saline, containing 400 ng of gp120 (Immunodiagnostics, Bedford, MA, USA) or 0.1% rat serum albumin 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. 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. The animals showing neurological deficits after implantation were excluded. The 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, USA) introduced serially to the hindpaws in the ascending order of strength, and the animals were placed in non-transparent plastic cubes on a mesh floor for an acclimatization period of at least 30 min 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, the 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. The experimenters were blinded during the 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 (150 mM sodium chloride, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease inhibitors (Sigma, St Louis, MO, USA) and phosphatase inhibitors (phosphatase inhibitor cocktail 1 and 2; Sigma). The homogenate was centrifuged at 18 000 g at 4 °C. The supernatant was collected and assayed for protein concentration using the DC protein assay (Bio-Rad, Hercules, CA, USA). 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, Solon, OH, USA) and then incubated with primary antibodies overnight at 4 °C, including mouse anti GAD67 (1:2000, sc7512, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-ACT (1:1000, ab72583, Abcam, Cambridge, MA, USA) and mouse anti β-actin (1:10 000, A5441, Sigma). The blots were incubated with secondary antibodies (Santa Cruz Biotechnology) and developed in a chemiluminescence solution (Pierce Biotechnology, Rockford, 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 (catalog #M36008, a mitochondrial superoxide indicator, Invitrogen, Eugene, OR, USA) was dissolved in a 1:1 mixture of dimethylsulfoxide and saline to a final concentration of 33 μM as described previously. Mitosox (30 μl) 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 overnight and cryoprotected with 30% sucrose in PBS for 2 days. The 35 μm sections were examined under a fluorescent microscope with a rhodamine filter. Two different regions of the dorsal horn were photographed from four to six randomly selected sections from each animal: laminae I–II and laminae 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 overnight and cryoprotected with 30% sucrose in PBS for 2 days. Cryostat sections (35-μm 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, USA), mouse anti-GFAP antibody (1:2000, catalog #G3893, Sigma), mouse anti-OK antibody (1:1000, catalog #G811512, Millipore, Billerica, MA, USA), mouse anti-NeuN monoclonal antibody (A60) (1:5000, catalog #MAB377, Millipore) or rabbit anti-Wnt5a (1:1000, catalog #ab72583, Abcam) followed by fluorescent IgG (Alexa Fluor 488, 1:1000 or Alexa Fluor 594, Molecular Probes, OR, USA) for 2 h at room temperature. Fluorescence images were captured by a fluorescent microscope (Fluorescent M Leica/Micro CDMI 6000B).

**Drug and data analysis.** Bicuculline methiodide (0.3 μg intrathecally) was purchased from Sigma, dissolved in saline and 10 μl were injected. CGP35348 hydrate (30 μg) 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.

| Table 1. The number of samples examined |
|----------------------------------------|
| **Behavior test** | Q0ZHG | QHGAD |
| Vehicle | 5 | 6 |
| Bicuculline | 8 | 7 |
| CGP35348 | 6 | 7 |
| **GABA, immunostaining** | | |
| sham+Q0ZHG | 6 | |
| gp120+Q0ZHG | 6 | |
| gp120+QHGAD | 6 | |
| **GAD67, western blots** | | |
| sham+Q0ZHG | 5 | 4 |
| gp120+Q0ZHG | 4 | 4 |
| gp120+QHGAD | 5 | 4 |
| **Mitosox** | | |
| sham+Q0ZHG | 6 | |
| gp120+Q0ZHG | 6 | |
| gp120+QHGAD | 6 | |
| **Wnt5a, western blots** | | |
| sham+Q0ZHG | 5 | |
| gp120+Q0ZHG | 4 | |
| gp120+QHGAD | 4 | |
following Fisher's protected least-significant difference (StatView, Cary, NC, USA). All the data were presented as mean ± s.e.m. and P-values < 0.05 were considered to be statistically significant. The sample size estimate was based on our previous studies.\textsuperscript{13,14} For the detailed numbers of animals in each group, see Table 1.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)