学位論文

Gene transfer of glutamic acid decarboxylase 67 by HSV vectors suppresses neuropathic pain induced by HIV gp120 combined with ddC in rats

(Herpes simplex virus ベクターを用いたグルタミン酸デカルボキシラーゼ（GAD67）の遺伝子導入は、HIVエンベロープ蛋白 gp120 と抗レトロウイルス薬 ddc により誘発されたラットの神経障害性疼痛を抑制する)

旭川医科大学大学院医学系研究科博士課程医学専攻

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Gene Transfer of Glutamic Acid Decarboxylase 67 by Herpes Simplex Virus Vectors Suppresses Neuropathic Pain Induced by Human Immunodeficiency Virus gp120 Combined with ddC in Rats

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BACKGROUND: Human immunodeficiency virus (HIV)-related painful sensory neuropathies primarily consist of the HIV infection–related distal sensory polyneuropathy and antiretroviral toxic neuropathies. Pharmacotherapy provides only partial relief of pain in patients with HIV/acquired immune deficiency syndrome because little is known about the exact neuropathological mechanisms for HIV-associated neuropathic pain (NP). Hypofunction of γ-aminobutyric acid (GABA) GABAergic inhibitory mechanisms has been reported after peripheral nerve injury. In this study, we tested the hypothesis that HIV gp120 combined with antiretroviral therapy reduces spinal GABAergic inhibitory tone and that restoration of GABAergic inhibitory tone will reduce HIV-related NP in a rat model.
METHODS: The application of recombinant HIV-1 envelope protein gp120 into the sciatic nerve plus systemic ddC (one antiretroviral drug) induced mechanical allodynia. The hind paws of rats were inoculated with replication-defective herpes simplex virus (HSV) vectors genetically encoding *gad1* gene to express glutamic acid decarboxylase 67 (GAD67), an enzyme that catalyzes the decarboxylation of glutamate to GABA. Mechanical threshold was tested using von Frey filaments before and after treatments with the vectors. The expression of GAD67 in both the lumbar spinal cord and the L4-5 dorsal root ganglia was examined using western blots. The expression of mitochondrial superoxide in the spinal dorsal horn was examined using MitoSox imaging. The immunoreactivity of spinal GABA, pCREB, and pC/EBPβ was tested using immunohistochemistry.
RESULTS: In the gp120 with ddC-induced neuropathic pain model, GAD67 expression mediated by the HSV vector caused an elevation of mechanical threshold that was apparent on day 3 after vector inoculation. The antiallodynic effect of the single HSV vector inoculation expressing GAD67 lasted >28 days. The area under the time–effect curves in the HSV vector expressing GAD67 was increased compared with that in the control vectors ($P = 0.0005$). Intrathecal GABA-A/B agonists elevated mechanical threshold in the pain model. The HSV vectors expressing GAD67 reversed the lowered GABA immunoreactivity in the spinal dorsal horn in the neuropathic rats. HSV vectors expressing GAD67 in the neuropathic rats reversed the increased signals of mitochondrial superoxide in the spinal dorsal horn. The vectors expressing GAD67 reversed the upregulated immunoreactivity expression of pCREB and pC/EBPβ in the spinal dorsal horn in rats exhibiting NP.
CONCLUSIONS: Based on our results, we suggest that GAD67 mediated by HSV vectors acting through the suppression of mitochondrial reactive oxygen species and transcriptional factors in the spinal cord decreases pain in the HIV-related neuropathic pain model, providing preclinical evidence for gene therapy applications in patients with HIV-related pain states. (Anesth Analg 2015;XXX:00–00)

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Patients with human immunodeficiency virus (HIV) infection have numerous complications, including neurological disorders. Painful HIV-associated sensory neuropathies (HIV-SNs) are the most common form of neuropathies, affecting about 30% of adults and children with acquired immune deficiency syndrome.[1,2] Antiretroviral therapies (ARTs) have lengthened survival among HIV-infected individuals, but ART is neurotoxic and causes a painful peripheral neuropathy as well.[3,4] Loss of γ-aminobutyric acid (GABA) inhibitory systems is thought to have an important effect in the spinal cord in the complicated mechanisms[5] that contribute to abnormal pain sensitivity and the phenotypic features of the neuropathic pain syndrome after nerve injury.[6,7] Although GABA concentrations in the cerebrospinal fluid were decreased in the simian immunodeficiency virus–infected rhesus monkeys,[8] the exact mechanism of spinal GABAergic tone associated with painful HIV peripheral neuropathies is unknown.

Mitochondria are suggested to be 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.[9] HIV infection and

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ART exert rapid toxicity by directly inhibiting the function of mitochondrial bioenergetics.[10] Oxidative stress has been suggested to play a role in the pathogenesis of neuroacquired immune deficiency syndrome.[11] Transcriptional factor cAMP response element–binding protein (CREB) in the dorsal horn neurons is related to central sensitization.[12] Phospho-CREB (pCREB) is upregulated in the spinal dorsal horn of pain-positive HIV patients.[13] The CCAAT/enhancer-binding proteins (C/EBPs) are transcriptional factors involved in cell development and the induction of several inflammatory mediators in various tissues, including central nervous system.[14] The C/EBPβ system may be involved in a variety of HIV disease stages.[15] The expression of C/EBPβ mRNA are elevated and its protein isoforms differentially expressed in total brain tissue of HIV-infected or HIV encephalitis patients.[16]

Pharmacotherapy provides only partial relief of painful symptoms in HIV patients.[17–19] Viral vector–mediated gene therapy may be a promising approach for treatment of persistent pain.[20] Recombinant herpes simplex virus (HSV)-based vectors delivered by subcutaneous inoculation can be used to express neurotransmitters in neurons of the dorsal root ganglia (DRG) and to produce a pain-relieving effect in different pain models in rodents.[21] Glutamic acid decarboxylase 67 (GAD67 or *gad1*) gene encodes for GABA synthetic enzyme in presynaptic neurons. HSV vectors encoding the GAD67 gene product can suppress detrusor overactivity in rats with spinal cord injury[22] and neuropathic pain in diabetic animals.[23] In this study, we examined the antinociceptive effect of GAD67 expressed by the replication-defective HSV-based vectors on the peripheral HIV gp120 combined with ddC (one of ART drugs) (gp120/ddC)-induced neuropathic pain in the rats, and we tested whether spinal mitochondrial superoxide, pCREB, and pC/EBPβ were involved in the antinociceptive effect of GAD67 in the gp120/ddC neuropathic pain state.

METHODS

Construction of the HSV Vector Expressing the GAD67 and Delivery

The vector QHGAD67 contains the encoding sequence of human GAD67 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. QHGAD67 was generated as described previously.[24] 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 QHGAD67 or the Q0ZHG control vector, 1 week after the peripheral application of gp120 with systemic ddC.

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. 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.

Neuropathic Pain Model Induced by Perineural gp120 Combined with ddC (gp120/ddC)

Under anesthesia, the rat left sciatic nerve was exposed in the popliteal fossa without damaging the nerve construction as described previously.[25–27] 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 40 ng of gp120 (Immunodiagnostics, Bedford, MA) or 0.1% RSA in saline for the sham surgery. A length of 3 to 4 mm of the sciatic nerve was wrapped loosely with the previously soaked cellulose, proximal to the trifurcation so as not to cause any nerve constriction and left in situ. The incision was closed with 4/0 sutures. Then animals received intraperitoneal injection of ddC (20 mg/kg, ChemGenes Corp, Wilmington, MA). The ddC was freshly prepared in saline on the same day of the gp120 surgery. Animals in the sham group received peripheral RSA and saline injection after surgery.

Mechanical Threshold

The mechanical threshold was determined using calibrated von Frey filaments (Stoelting, Wood Dale, IL) introduced serially to the hindpaw in ascending order of strength, and animals were placed in transparent plastic cubicles on a mesh floor for an acclimatization period of at least 30 minutes in 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 there was a positive response to a stimulus, the next smaller von Frey hair was applied, and whenever there was a negative response, 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.[28,29]

Intrathecal Catheter Implantation

For studying the effect of intrathecal administration of drugs on neuropathic pain, chronic intrathecal catheters were implanted using isoflurane anesthesia.[30] 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. The GABA receptor agonists muscimol and baclofen, dissolved in saline, were purchased from Sigma (Sigma, St Louis, MO). Spinal agents were delivered over a 60 seconds in a volume of 10-μL solution. Drug injection was immediately followed by a 10-μL physiologic saline to flush the catheter.

Western Blots

Under deep anesthesia, the L4-5 DRG or spinal dorsal horn ipsilateral to the gp120 application was removed rapidly, frozen on dry ice, and stored at −80°C. These tissues were

homogenized in protein lysis buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease inhibitors (Sigma) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 1 and 2, Sigma). The homogenate was centrifuged at 18,000*g* for 20 minutes 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 minutes; proteins were separated by 10% to 12% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with rapid block solution (Amresco) and then incubated with primary antibodies for overnight at 4°C, including mouse anti-GAD67 (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-β-actin (1:10000, Sigma). The blots were incubated with secondary antibodies (Santa Cruz Biotechnology) and developed in a chemiluminescence solution (Pierce Biotechnology, Rockford, IL). Quantification of western blots was performed using 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 Spinal Dorsal Horn

MitoSox Red (a mitochondrial superoxide indicator, Invitrogen) was dissolved in a 1:1 mixture of dimethylsulfoxide and saline to a final concentration of 33 μM as described previously.[31] MitoSox (30 μL) was injected intrathecally. Approximately 70 minutes after injection, rats were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer, and the L4-5 segments of the spinal cord were removed, postfixed in the same solution for overnight, and cryoprotected with 30% sucrose in phosphate-buffered solution 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 5 randomly selected sections from each animal: the lateral part of 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 as described previously.[31,32]

Immunohistochemistry

The distribution of GABA, pCREB, and pC/EBPβ in the spinal dorsal horn was determined by immunohistochemistry. Rats were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer, and the L4-5 segments of the spinal cord were removed, postfixed in the same solution for overnight, and cryoprotected with 30% sucrose in phosphate-buffered solution for 2 days. Cryostat sections (35 μm thickness) were incubated overnight at 4°C with rabbit anti-GABA (1:1000; gift from Dr. Yuan Zhu, Department of Medicine, University of Michigan, Ann Arbor, MI) or rabbit anti-pCREB (1:100, Cell Signaling Technology) or anti-pC/EBPβ (1:200, Santa Cruz Biotechnology) followed by fluorescent anti-rabbit IgG (Alexa Fluor 488, 1:1000, Molecular Probes, Eugene, OR) for 2 hours at room temperature. Fluorescence images were captured by a fluorescent microscopy (Fluorescent M Leica/Micro CDMI 6000B).[33,34] For immunostaining analysis, 5 sections were selected randomly and scanned using the fluorescence microscope. The number of GABA-positive cellular profiles with distinctive neuron cells was counted in the spinal dorsal horn laminas I–V. Images of pCREB immunoreactivity (pCREB-IR) and pC/EBPβ immunoreactivity (pC/EBPβ-IR) were then captured with the microscope, and we analyzed the image densities of pCREB-IR and pC/EBPβ-IR using the Image J software (version 1.46, National Institutes of Health).[33,35] The percentage change of staining density in sham groups was calculated as 100%.

Data Analysis and Statistics

The results describing behavioral responsiveness to von Frey stimulation that indicates mechanical sensitivity were given as a mechanical threshold. Differences in mean changes at the same time points were evaluated individually for the assessment of treatment effects on hypersensitivity with 2-tailed *t* tests. The area under the time–effect curves (AUC) data, depicting the mechanical threshold (g) over time, were calculated by the trapezoidal rule to express the overall magnitude and duration of effect, and analyzed between 2 different treatments using a *t* test. The values from each test group were graphed as mean ± SEM. The actual *P* values were shown in the figures.

Data for the effects of the HSV vectors on the neurochemical changes among 3 groups were compared with 1-way ANOVA with post hoc Fisher PLSD (Protected Least Significant Difference) test (software StatView5, SAS Institute Inc., Cary, NC) while the numbers of groups and sample sizes were considered following the information of Fisher least significant difference.[36]

We used strict statistic method to treat the $0.01 < P < 0.05$ as very great trends and the $P < 0.01$ as statistically significant because some group sample sizes are <6.[37]

RESULTS

The Antiallodynic Effect of GAD67 Mediated by HSV Vector on Neuropathic Pain Induced by gp120 with ddC

Previous studies have demonstrated that the peripheral gp120 application into the sciatic nerve results in neuropathic pain characterized by mechanical allodynia.[25,34,38] In this study, we examined whether overexpression of GAD67 mediated by the HSV vector reduced neuropathic pain induced by HIV gp120 with ddC. Subcutaneous inoculation with QHGAD caused an elevation of mechanical threshold that was apparent on day 3 after vector inoculation compared with the control vector; the antiallodynic effect of the HSV vector lasted for >28 days. For the comparison of the differences at individual time points between 2 groups, we used a 2-tailed *t* test (actual *P* values were shown in Fig. 1A). The AUC in the QHGAD group was higher than that in the Q0ZHG group ($P = 0.00049$, *t* test, $n = 6–7$, Fig. 1B).

Expression of GAD67 Mediated by the QHGAD Vector In Vivo

GAD67 is a key enzyme for GABA synthesis. The loss of GABAergic neurons may be an important element of neuropathic pain.[39] Previous studies report that the nonreplicating HSV vector QHGAD produces GAD67 in primary

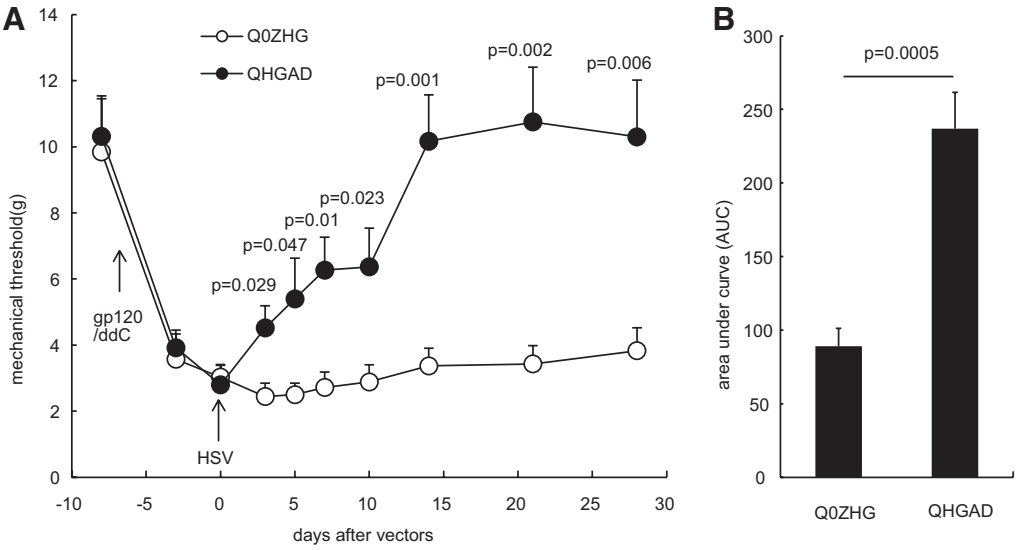

Figure 1. The effect of glutamic acid decarboxylase 67 (GAD67) mediated by the herpes simplex virus (HSV) vectors on neuropathic pain induced by HIV gp120 combined with ddC (gp120 + ddC). (A) The times of gp120/ddC and HSV vector inoculation are indicated by arrows. The comparison of differences at individual time points between 2 groups is shown, 2-tailed *t* test, mean ± SEM. (B) The area under curves (AUC) in QHGAD group was higher than that in the Q0ZHG group, *t* test, mean ± SEM, *n* = 6–7 rats.

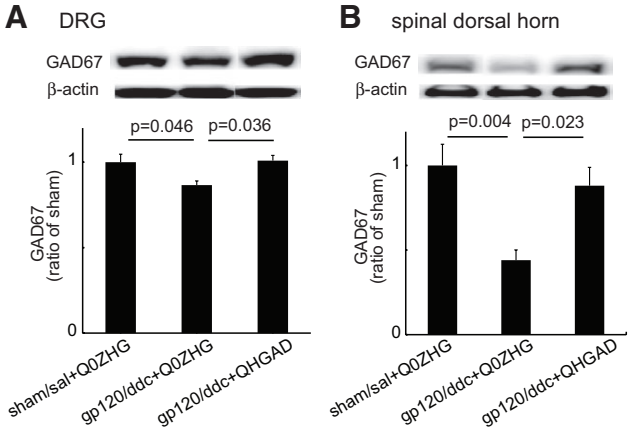

Figure 2. The expression of glutamic acid decarboxylase 67 (GAD67) mediated by the herpes simplex virus (HSV) vectors in the gp12/ddC model in the L4-5 dorsal root ganglia (DRG) (A) and spinal dorsal horn (B). The application of gp120/ddC lowered the expression of GAD67 compared to sham group in the DRG (A) and spinal dorsal horn (B); QHGAD reversed the GAD67 decrease compared to gp120/ddC + Q0ZHG in the DRG (A) and spinal dorsal horn (B). The data were analyzed using 1-way ANOVA with post hoc protected least significant difference (PLSD) test, mean ± SEM, *n* = 4–5.

DRG neurons in vitro,[40] and in the lumbar DRG in vivo after subcutaneous inoculation with the vectors in the hindpaws of rats.[24,40] Here, we tested whether GAD67 was decreased in the spinal dorsal horn and DRG in the gp120/ddC pain model and whether vectors QHGAD reversed the loss of GAD67. Rats received the inoculation with the vectors QHGAD or control vectors Q0ZHG in the hindpaws of rats with the gp120/ddC-induced neuropathic pain state. The ipsilateral L4-5 DRGs and spinal cords were harvested 2 weeks after inoculation with the vectors, and western blots were performed for the expression of GAD67 in the pooled L4-5 DRG or spinal dorsal horn. The expression of GAD67 in the DRG (Fig. 2A) or spinal dorsal horn (Fig. 2B) in neuropathic rats injected with Q0ZHG was lower than that in the sham group; there was an increase in the expression of GAD67 in the gp120/ddC + QHGAD compared with that in the gp120/ddC + Q0ZHG group in the DRG (Fig. 2A) or spinal dorsal horn (Fig. 2B). We designed sample size of 6 in each group. During the experiment, 1 or 2 rats died or were sick and we excluded them from the experiments. Actual *P* values were shown in the figure, 1-way ANOVA, *n* = 4–5.

Antiallodynic Effect of Intrathecal GABA Agonists

The time course of mechanical threshold produced by intrathecal injection of GABA-A agonist muscimol (Mus) and GABA-B agonist baclofen (Bac) is illustrated in Figure 3. Threshold was maximally increased within 90 minutes after injection 1 μg of Mus; the peak effect of baclofen was in 30 minutes and then gradually decreased over a 5-hour period, consistent with a previous report.[6] For the comparison of the differences at individual time points between Mus or Bac and control group, we used a 2-tailed *t* test (actual *P* values were shown in Figure 3A). The AUC in the groups of Mus and Bac was higher than that in the control group, *t* test, *n* = 5–6 (actual *P* values were shown in Fig. 3B).

The Effect of GAD67 Mediated by the HSV Vector on GABA-Positive Neuron Expression in Neuropathic Pain

Loss of spinal GABAergic inhibitory function is involved in the neuropathic pain state.[39,41,42] In this study, we investigated whether the expression of GAD67 mediated by the HSV vector increased GABA neuron in the spinal dorsal horn in the neuropathic pain state. Neuropathic animals receiving the HSV vectors were perfused at 2 weeks after vector injection. GABA immunoreactivity (GABA-IR) was performed using immunohistochemistry. The number of GABA-IR-positive neurons was accounted in the spinal dorsal horn. GABA-IR-positive imaging in sham (Fig. 4A),

neuropathic rats with Q0ZHG (Fig. 4B), and neuropathic rats with QHGAD (Fig. 4C) is shown. The number of GABA-IR-positive neurons in the gp120/ddC + Q0ZHG group was more than that in the sham/sal + Q0ZHG (Fig. 4D). Actual *P* values were shown in Fig. 4D, 1-way ANOVA, $n = 6$.

The Effect of GAD67 Mediated by the HSV Vector on Mitochondrial Superoxide Expression in Neuropathic Pain

Mitochondrial ROS is reportedly involved in the pain state.[43] Although we know that ROS is involved in reducing GABA inhibitory function on neurons in pain transmission,[41,44] less is known about whether GABA reduces ROS in the spinal cord in the HIV-related neuropathic pain state. In this study, we investigated whether the expression of GAD67 mediated by the HSV vector decreased mitochondrial superoxide in the spinal dorsal horn in the gp120/ddC pain state. Neuropathic animals receiving the HSV vectors were perfused at 2 weeks after vector injection. MitoSox Red was intrathecally administered 70 minutes before perfusion. MitoSox-positive imaging was detected using a fluorescent microscope with a rhodamine filter as described previously.[32,43] The number of MitoSox-positive neurons was accounted in the spinal dorsal horn. MitoSox-positive imaging in sham, neuropathic rats with Q0ZHG, and neuropathic rats with QHGAD is shown in Figure 5A, 5B, and 5C, respectively. The number of MitoSox-positive cells in the gp120/ddC + Q0ZHG group was more than that in the sham/sal + Q0ZHG (actual *P* values were shown in Fig. 5D, 1-way ANOVA, $n = 5–6$). There was a decrease in the MitoSox-positive cells in the gp120/ddC + QHGAD group compared with that in the gp120/ddC + Q0ZHG (actual *P* values were shown in Fig. 5D, 1-way ANOVA, $n = 5–6$). F5

The Effect of GAD67 Mediated by the HSV Vector on the Expression of pCREB in Neuropathic Pain

The phosphorylated CREB protein level is involved in the spinal dorsal horn in diabetic neuropathy rats.[45] In the

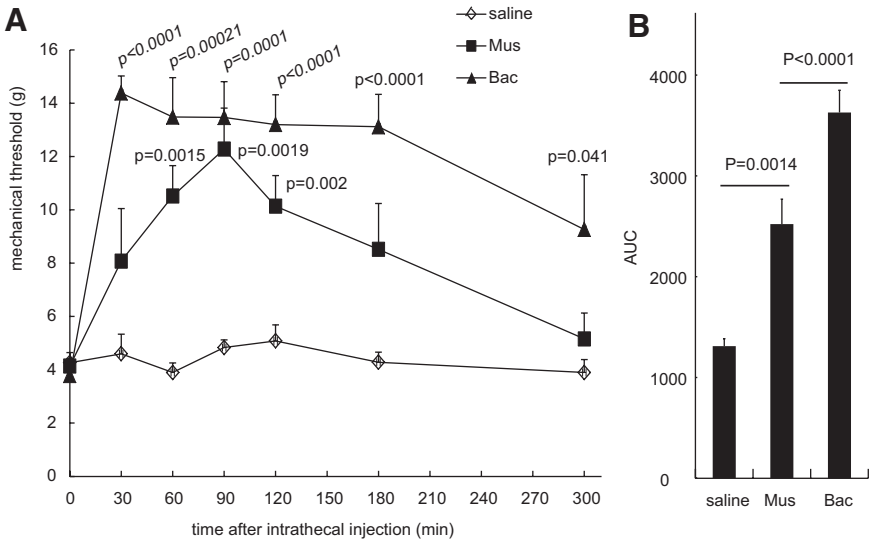

Figure 3. The antiallodynic effect of intrathecal γ-aminobutyric acid (GABA)-A and GABA-B agonists on the model of gp120/ddC. Time course of the antiallodynic effects of effects of muscimol (Mus, filled square) and baclofen (Bac, filled triangle) administered intrathecally in rats made allodynic by gp120/ddC. The data were analyzed using *t*-test, $n = 5–6$ rats.

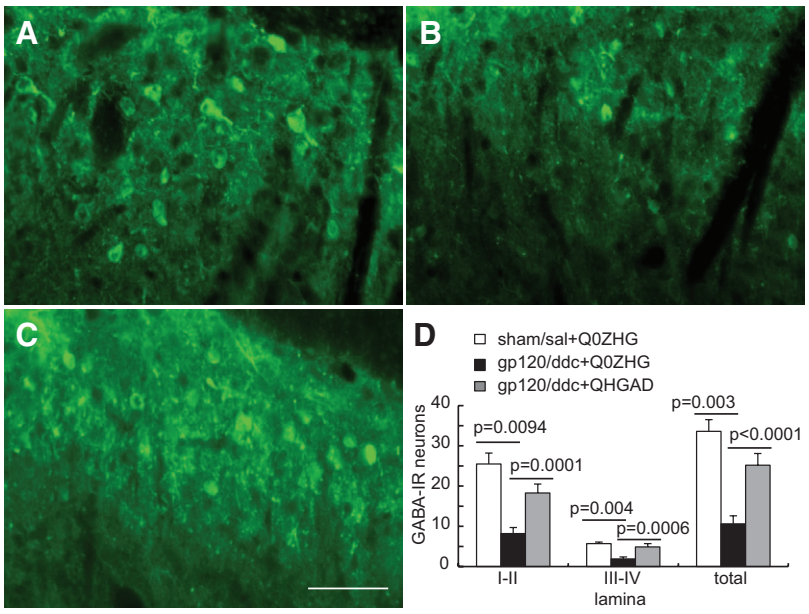

Figure 4. The effect of glutamic acid decarboxylase 67 (GAD67) mediated by the herpes simplex virus (HSV) vectors on the expression of GABAergic-immunoreactivity (IR) in the spinal dorsal horn. The representative image of (A) GABAergic-IR in sham/ddC + Q0ZHG, (B) gp120/ddC + Q0ZHG, and (C) gp120/ddC + QHGAD is shown. (D) The number of GABAergic-IR positive cells in the spinal dorsal horn laminas I–II and III–IV is shown. The data were analyzed using 1-way ANOVA with post hoc protected least significant difference (PLSD) test, $n = 6$ rats. AQ12

current study, we investigated whether the expression of GAD67 mediated by the HSV vector changes pCREB in the spinal dorsal horn in the HIV-related neuropathic pain state. Neuropathic and sham animals receiving the HSV vectors were perfused at 2 weeks after vector injection. The pCREB-IR was performed using an immunofluorescence staining. The images were then captured with the microscope, and image densities of pCREB in the spinal dorsal horn were analyzed. The pCREB-IR in sham, neuropathic rats with Q0ZHG, and neuropathic rats with QHGAD is shown in Figure 6A, 6B, and 6C, respectively. There was an increase in the densities of pCREB-IR in the gp120/ddC + Q0ZHG group compared with that in the sham/sal + Q0ZHG (P = 0.0018, 1-way ANOVA, n = 6, Fig. 6D). The densities of pCREB-IR in the gp120/ddC + QHGAD group were decreased compared with that in the gp120/ddC + Q0ZHG (P = 0.0021, 1-way ANOVA, n = 6, Fig. 6D).

F6

The Effect of GAD67 Mediated by the HSV Vector on the Expression of pC/EBPβ in Neuropathic Pain

The expression of C/EBPβ mRNA are elevated, and its protein isoforms differentially expressed in total brain tissue lysates of HIV-1–infected and HIV-1 encephalitis patients.[16] The expression of constitutively active CREB strongly activated C/EBPβ promoter–reporter genes, and induced expression of endogenous C/EBPβ in preadipocyte cells.[46] However, little is known about the downstream pathway involved in the GABA system. To better understand the mechanism underlying the reduction of HIV-related mechanical allodynia associated with GAD67 overexpression mediated by the HSV vector, we investigated whether the HSV vector decreased pC/EBPβ in the spinal dorsal horn in the HIV-related neuropathic pain state. Neuropathic animals receiving the HSV vectors were perfused at 2 weeks

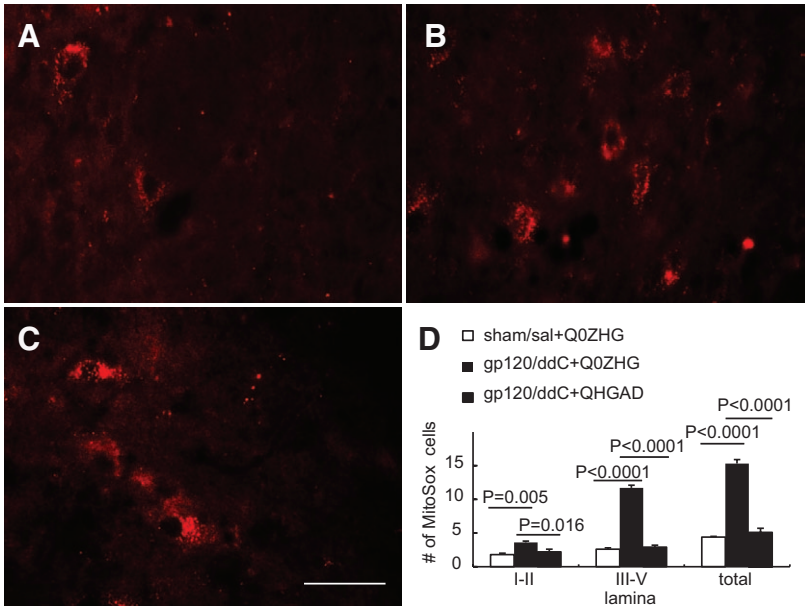

Figure 5. The effect of glutamic acid decarboxylase 67 (GAD67) mediated by the herpes simplex virus (HSV) vectors on mitochondrial superoxide in the spinal dorsal horn. The representative image of MitoSox Red for mitochondrial superoxide in (A) sham/ddC + Q0ZHG, (B) gp120/ddC + Q0ZHG, and (C) gp120/ddC + QHGAD is shown. (D) The number of mitochondrial superoxide-positive cells in the spinal dorsal horn laminas I–II and III–V is shown. The data were analyzed using 1-way ANOVA with post hoc protected least significant difference (PLSD) test, mean ± SEM, n = 5–6 rats.

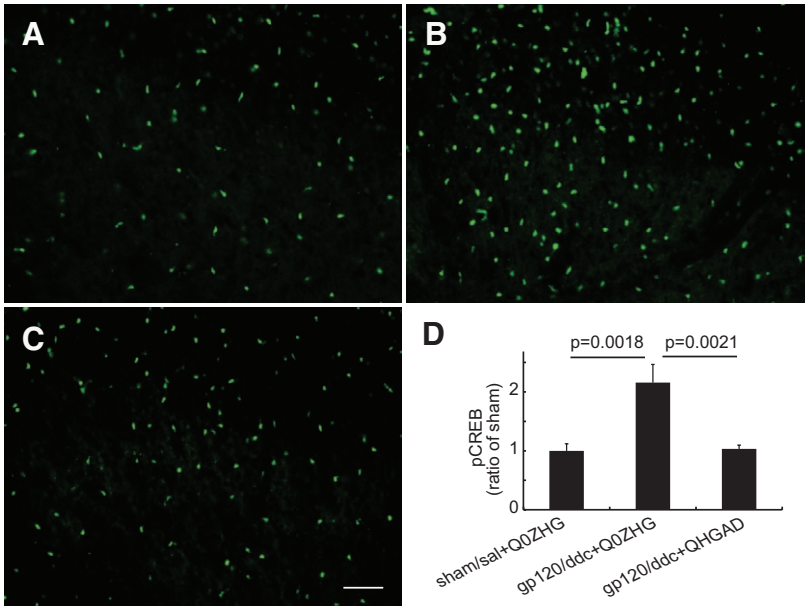

Figure 6. The effect of glutamic acid decarboxylase 67 (GAD67) mediated by the herpes simplex virus (HSV) vectors on phosphorylation of cAMP response element–binding protein (pCREB) in the spinal dorsal horn. The representative image of pCREB-immunoreactivity (IR) in (A) sham/ddC + Q0ZHG, (B) gp120/ddC + Q0ZHG, and (C) gp120/ddC + QHGAD is shown. (D) The quantitative signals of pCREB-IR in the spinal dorsal horn are shown. There was an increase in pCREB-IR in the group of gp120/ddC + Q0ZHG compared to sham/saline + Q0ZHG (P = 0.0018, 1-way ANOVA with post hoc protected least significant difference (PLSD) test); the expression of pCREB-IR in gp120/ddC + QHGAD was lower than that in gp120/ddC + Q0ZHG (P = 0.0021), 1-way ANOVA with post hoc PLSD test, mean ± SEM, n = 6 rats.

after vector injection. The pC/EBPβ-IR was performed using immunostaining. The images were then captured with the microscope, and image densities of pC/EBPβ-IR in the spinal dorsal horn were analyzed. The pC/EBPβ-IR in sham, neuropathic rats with Q0ZHG, and neuropathic rats with QHGAD is shown in Figure 7A, 7B, and 7C, respectively. There was an increase in the densities of pC/EBPβ-IR in the gp120/ddC + Q0ZHG group compared with that in the sham + Q0ZHG (P = 0.0002, 1-way ANOVA, n = 6, Fig. 7D). The densities of pC/EBPβ-IR in the gp120/ddC + QHGAD group were lower than that in the gp120/ddC + Q0ZHG group (P = 0.0048, 1-way ANOVA, n = 6, Fig. 7D).

F7

DISCUSSION

Recombinant HSV-based vectors delivered by subcutaneous inoculation can be used to express neurotransmitters in neurons of the DRG and to produce a pain-relieving effect in different pain models in rodents.[47] The HSV vectors expressing GAD67 provide an analgesic effect in the neuropathic pain in the spinal injury or diabetic animals.[22,23] In the current study, we demonstrated that (1) subcutaneous inoculation of the HSV vectors expressing GAD67 attenuated the behavioral manifestations of mechanical allodynia over the course of several weeks in a model of HIV gp120/ddC-induced neuropathic pain in rats, (2) intrathecal GABA-A and GABA-B receptor agonists reversed mechanical allodynia, (3) HSV vectors expressing GAD67 reversed the GABA-IR reduction in the relevant spinal dorsal horn in neuropathic rats, and (4) HSV vectors expressing GAD67 suppressed the upregulated spinal mitochondrial superoxide, pCREB, and pC/EBPβ associated with the gp120/ddC neuropathic pain state.

HIV-SN mainly contains the HIV infection–related distal sensory polyneuropathy (DSP) and antiretroviral toxic neuropathies. The most common complaint of HIV-SN is pain on the soles in HIV patients. The most common histological feature of HIV-SN 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[19,48,49]). Unfortunately, many conventional drugs used in pharmacologic therapy for neuropathic pain are not effective for providing satisfactory analgesia in painful HIV-related DSP because the exact molecular mechanisms of the painful HIV-DSP are not clear. In animal studies, peripheral nerve exposure to HIV protein gp120 induces persistent painful sensory neuropathy that may be sustained and magnified by long-term spinal neuropathology.[25,26] Joseph et al.[50] developed a model of nucleoside analog reverse transcriptase inhibitor–induced painful peripheral neuropathy in the rat using systemic administration of ddC, etc. Wallace et al.[38] showed that the model of combination of gp120 with ddC 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/ddC model is considered as HIV-related painful neuropathy. Thus, in the current study, we investigate the spinal effect of HSV vectors expressing GAD67 in the HIV-related neuropathic pain model.

In the spinal cord, GABA neurons are found primarily in interneurons of the superficial dorsal horn predominantly in lamina I–III that form axoaxonic synapses on primary afferent terminals and axodendritic synapses on projection neurons. Tonic GABAergic inhibition has an important function in normal sensory processing by increased behavior responsiveness.[22,23] Among the complex mechanisms underlying neuropathic pain, one is that nerve injury results in a selective loss of GABAergic inhibitory function in the spinal cord[5] that contributes to abnormal pain sensitivity and the phenotypic features of the neuropathic pain syndrome.[42] The rate-limiting step of GABA synthesis is catalyzed by GAD67. In the present studies, we found that there was a downregulation of GAD67 and GABA-IR in the gp120/ddC-induced neuropathic pain model, suggesting that the dysfunctional GABAergic tone is also important in the neuropathologic mechanisms underlying the development of HIV-related painful peripheral neuropathies.

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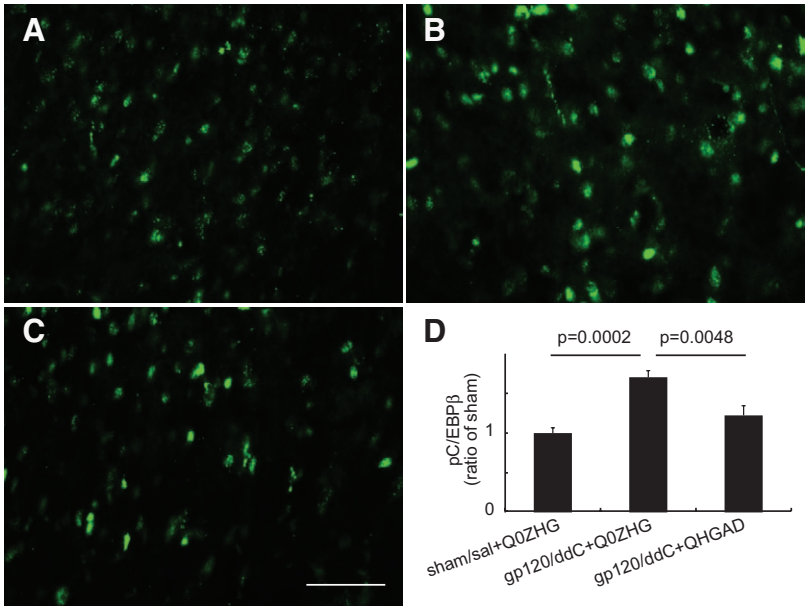

Figure 7. The effect of glutamic acid decarboxylase 67 (GAD67) mediated by the herpes simplex virus (HSV) vectors on phosphorylation of CCAAT/enhancer-binding proteins-beta (pC/EBPβ) in the spinal dorsal horn. The representative image of pC/EBPβ- immunoreactivity (IR) in (A) sham/ddC + Q0ZHG, (B) gp120/ddC + Q0ZHG, (C) and gp120/ddC + QHGAD is shown. (D) The quantitative signals of pC/EBPβ-IR in the spinal dorsal horn are shown. There was an increase in pC/EBPβ-IR in the group of gp120/ddC + Q0ZHG compared to sham/saline + Q0ZHG (P = 0.0002); the expression of pCREB-IR in gp120/ddC + QHGAD was lower than that in gp120/ddC + Q0ZHG (P = 0.0048), 1-way ANOVA with post hoc protected least significant difference (PLSD) test, mean ± SEM, n = 6 rats.

Although spinal GABAergic systems suppress neuropathic pain, little is known about the downstream mechanisms. Recent work has shown that ROS are involved in the development and maintenance of neuropathic pain. Oxidative stress causes activation of a number of complex and interrelated signaling events.[51] Accumulating evidence has demonstrated that free radicals are implicated as mediators of chronic pain.[31,52–56] Superoxide generated from mitochondrial oxidative phosphorylation is a major source of neuronal ROS.[57] Capsaicin-induced pain can be used to identify the site of action for ROS.[31] Analgesic effect of ROS scavengers is observed in capsaicin-induced secondary hyperalgesia,[58] suggesting ROS involvement in the spinal cord. Furthermore, ROS accumulation was observed primarily in the mitochondria of dorsal horn neurons in different pain models.[31,32,43,59] HIV gp120 or ddC has been implicated in initiation and/or intensification of ROS.[60,61] Although the increased ROS in the spinal cord may induce pain by reducing GABA inhibitory influence on substantia gelatinosa neurons involved in pain transmission,[41,44] little is known about whether GABAergic systems suppress ROS. In the present studies, we found that gp120/ddC increased spinal mitochondrial superoxide, which was reversed by GAD67 overexpression mediated by the HSV vectors, suggesting that GABAergic systems suppressed spinal mitochondrial superoxide.

In response to neural activity, phosphorylated CREB (pCREB) binds to promoters to trigger the expression of specific genes, that result in long-lasting synaptic plasticity.[62,63] Nociceptor afferent activation contributes to central sensitization through posttranslational and CREB-mediated transcriptional regulation in the spinal dorsal horn neurons.[12] Descalzi et al.[64] reported that genetic enhancement of CREB-mediated transcription increased behavioral responses to nonnoxious stimuli (or allodynia) in the CFA model or nerve injury, providing direct evidence that CREB-mediated transcription contributes to behavioral allodynia in animal models of chronic inflammatory or neuropathic pain. Nociceptive behavior induced by sciatic nerve injury is attenuated by intrathecal CREB antisense oligonucleotide during the period of injection, suggesting that spinal pCREB may contribute to the development of neuropathic pain.[65] Importantly, pCREB is upregulated in the spinal dorsal horn of pain-positive HIV patients.[13] Furthermore, GABA agonist significantly decreases pCREB in painful diabetic neuropathy rats.[45] Nonetheless, the relationship between GABAergic systems and pCREB in the HIV gp120/ddC neuropathic pain model remains unclear. Our results show that gp120/ddC increased spinal pCREB, which was reversed by GAD67 mediated by the HSV vectors.

C/EBPs are transcriptional factors involved in cell development and induction of several inflammatory mediators in various tissues, including central nervous system.[14] A variety of disease stages are described in which a dysregulation of the C/EBPβ system may be involved.[15] The expression of constitutively active CREB strongly activates C/EBPβ promoter–reporter genes and induces the expression of endogenous C/EBPβ in preadipocyte cells.[46] The expression of C/EBPβ mRNA is elevated, and its protein isoforms are differentially expressed in total brain tissue lysates of HIV-1–infected and HIV-1 encephalitis patients.[16] In the present study, we found that HIV gp120/ddC induced the upregulation of spinal pC/EBPβ, which was suppressed by GAD67 mediated by the HSV vectors. To our knowledge, we are the first to demonstrate the role of C/EBPβ in the GABAergic systems in pain models.

To produce a long-term analgesic effect, viral vectors expressing GAD67 may represent a promising approach in a variety of pain states. Previous studies have demonstrated that replication-defective HSV-based vectors delivered by subcutaneous inoculation can be used to express neurotransmitters/neuropeptides in the DRG neurons and produce a local pain-relieving effect in the spinal cord level in rodent models of different pain models.[23,66–69] The rate-limiting step of GABA synthesis is catalyzed by glutamic acid decarboxylase GAD67. We and others have found that transduction of the DRG neurons in vivo, achieved by subcutaneous inoculation of the HSV vectors in the foot, results in production of transgene-encoded GAD67 in the DRG neurons, increases the release of GABA in the spinal cord,[24] and suppresses mechanical allodynia induced by spinal cord injury, spinal nerve injury, and diabetic neuropathy.[23,24,40,70] GAD67 mRNAs pertain to inhibitory neocortical neurons and were decreased in frontal neocortex in subjects with HIV encephalitis.[71] In this study, we found that HSV vectors encoding GAD67 gene suppress mechanical allodynia induced by gp120/ddC in rats, increased the lowered GABA-IR neuronal cells, and inhibited spinal mitochondrial superoxide, pCREB, and pC/EBPβ in the spinal dorsal horn. The exact mechanisms of GAD67 mediated by HSV are not clear. Previous studies have identified oxidative stress as an inducer of posttranslational protein modification, resulting in transcriptional activation. For example, oxidative stress causes pCREB expression.[72–74] The expression of constitutively active CREB strongly activates C/EBPβ promoter–reporter genes and induces the expression of endogenous C/EBPβ, suggesting that C/EBPβ be the downstream factor of CREB.[46] Thus, it is possible that GABAergic tone suppresses ROS and then downregulated the expression of phosphorylation of CREB and C/EBPβ (see Fig. 8). F8

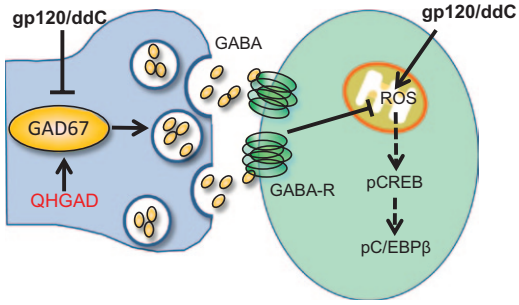

Figure 8. The hypothesized GABAergic signaling contributions to gp120/ddC-related neuropathic pain in the spinal cord. HIV gp120 has been implicated in disruption of mitochondrial transmembrane potential, initiation and/or intensification of reactive oxygen species (ROS), and decrease in glutamic acid decarboxylase 67 (GAD67).[60,75] Antiretroviral therapy drugs (e.g., ddC) induce mtDNA depletion and result in mitochondrial respiratory chain, and reduction in ATP and increased ROS.[76–78] ROS causes phosphorylation of cAMP response element–binding protein (pCREB) expression.[74,79] CCAAT/enhancer-binding proteins-beta (C/EBPβ) may be the downstream factor of CREB.[46] Thus, it is possible that an increase in GABAergic tone by the herpes simplex virus (HSV) vectors suppresses ROS and then downregulates the expression of phosphorylation of CREB and C/EBPβ.

In summary, except for the reduced spinal GABAergic tone, the upregulation of ROS, pCREB, and pC/EBPβ is involved in the HIV gp120/ddC-induced neuropathic pain. Increasing GABAergic tone by the HSV vectors reduced HIV-related neuropathic pain, likely through suppressing spinal ROS, pCREB, and pC/EBPβ. Our findings suggest that this gene therapy approach may prove useful in the treatment of HIV-associated neuropathic pain. ■

AQ14

DISCLOSURES

Name: Megumi Kanao, MD.
Contribution: This author conducted the study, analyzed the data, and participated in writing the manuscript.
Attestation: Megumi Kanao reviewed the original data and the analysis of the data, and approved the final manuscript.
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Contribution: This author conducted the study, analyzed the data, and participated in writing the manuscript.
Attestation: Hirotsugu Kanda reviewed the original data and the analysis of the data, and approved the final manuscript.
Name: Wan Huang, MD, PhD.
Contribution: This author conducted the study, analyzed the data, and participated in writing the manuscript.
Attestation: Wan Huang reviewed the original data and the analysis of the data, and approved the final manuscript.
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Attestation: Shue Liu approved the final manuscript.
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Contribution: This author designed the whole study, analyzed the data, and wrote the manuscript.
Attestation: Shuanglin Hao is the archival author/corresponding author and approved the final manuscript.
This manuscript was handled by: Jianren Mao, MD, PhD.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

AQ1—Please confirm whether the correspondence address has been set correctly.

AQ2—Please confirm whether "γ-aminobutyric acid (GABA) GABAergic inhibitory mechanism" is OK as given. Or should only "GABAergic inhibitory mechanism" be retained, without GABA, as given in the text?

AQ3—Please confirm the expansion of CNS.

AQ4—Please confirm whether "30 (L" is OK as set.

AQ5—Please provide the city and state name for Amresco.

AQ6—Please provide the city and state name for Invitrogen.

AQ7—Please confirm the expansion of "PBS."

AQ8—Please provide the city and state name for Cell Signaling Technology.

AQ9—Please provide the manufacturer's name, city, and state or country (if not US) for "Fluorescent M Leica/Micro CDMI 6000B."

AQ10—Please note that "lamina I–V" has been changed to "laminas I–V." OK?

AQ11—In the sentence "Actual P values were shown in the figure," can "the figure" be changed to "Figure 2"?

AQ12—Please confirm whether placement of part figure labels (A, B, and C) in the Figure 4 to 5 legends is OK.

AQ13—Please confirm whether the edit made in the sentence "The rate-limiting step… GAD67" is OK.

AQ14—Please check the disclosure information for accuracy.