Analgesic effects of AAV vector expressing GAD65 in the rat dorsal root ganglia on neuropathic pain

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Analgesic effects of AAV vector expressing GAD65 in the rat dorsal root ganglia on neuropathic pain

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The Master's Thesis submitted to
the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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June 2006

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June 2006

ACKNOWLEDGEMENTS

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앞으로의 인생이 어떻게 펼쳐질지 지금은 알 수 없지만, 앞으로의 시간을 준비해 나갈 수 있는 소중한 밑거름이 되었음을 감사하게 생각하며, 이러한 시간이 있게 한 모든 이들에게 감사드립니다.

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2006년 6월

김재형

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Abstract

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Neuropathic pain is a chronic pain syndrome of unknown ethology that has been associated with drug, disease, injury-induced damage or destruction to the sensory afferent fibers of the peripheral nervous system (PNS). Neuropathic pain caused by peripheral nerve injury is clinically very common, yet with a few clinical treatments. γ-aminobutyric acid (GABA) is a principal inhibitory neurotransmitter used by many neurons in the spinal dorsal horn, and these transmitters have an

important role in spinal pain mechanisms. Several studies have shown that a substantial loss of GABA-immunoreactive neurons from the dorsal horn in nerve injury models, and it has been suggested that this may be caused with a loss of inhibition, which contributes to the behavioral signs of neuropathic pain.

To determine if release of GABA in spinal dorsal horn could reduce neuropathic pain that construct a rAAV-based vector encoding one isoform of human glutamic acid decarboxylase (GAD65) expression cassette consisting of the JDK promotor. Directly injected rAAV-mediated vectors into the DRGs of sciatic nerve injury group was observed that the GAD65 was transduced into the DRG and indicated significant analgesic behavior as compared to the vehicle group. Moreover, the magnitude of the pain relief maintained for entire experimental period, where GAD65 expression was also noticed with no substantial reduction in its immuno-reactive intensity. Finally, the significant amount of enhancement in GABA release following rAAV-GAD65 delivery was identified *in vivo* by HPLC.

These results suggest that rAAV-JDK-GAD65 gene transfer to DRGs could be used to useful treatment for peripheral neuropathic pain after sciatic nerve injury.

Key words : neuropathic pain, recombinant adeno-associated virus (rAAV), γ-amino butyric acid (GABA), glutamic acid decarboxylase (GAD), dorsal root ganglion (DRG)

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Ⅰ. Introduction

Neuropathic pain is defined as pain initiated or caused by primary lesions in or from the dysfunction of various neural systems and is potentially driven by multiple ethological factors.[1] Peripheral neuropathic pain, characterized by a wide spectrum of pathological processes, is composed of a number of

phenomena occurring at different sites and times, depending on disease states.[2] Multiple mechanisms appear to be responsible for this injury-induced neuropathic pain, including ectopic activity in primary afferent, induction of central sensitization in the dorsal horn in response to such inputs, and sprouting of the central terminals of large myelinated primary afferents. Another mechanisms that has been proposed to contribute to neuropathic pain is peripheral nerve injury-induced loss of inhibition in the spinal cord.[2] Among the complex mechanisms underlying neuropathic pain, partial nerve injury seems to result in a selective loss of GABAergic inhibitory synaptic currents in the spinal cord. This feature then contributes to the phenotypes of the neuropathic pain syndrome.[3,4,5,6] γ-aminobutric acid (GABA), the product driven by glutamate decarboxylase (GAD), is amain inhibitory neurotransmitter in the dorsal horn of the spinal cord and also plays an important role in the central nerve system (CNS).[7]

Genetical modification of neurons by introduction of a transgene can be an effective treatment for various neurological disorders with few current therapeutic strategies, such as Alzheimer's, Parkinson's disease, or chronic pain syndromes.[8,9,10] Previous reports have suggested that vector-mediated exogenous GAD and resultant GABA production transiently attenuates neuropathic pain after spinal cord injury[11] and peripheral nerve injury. So far, the human adeno-associated virus (hAAV) -based vector system has been most widely applied to neural systems,[12] largely due to several advantages, such as efficient long-term gene transfer and minimal side-effects.[13,14,15,16] Additionally, recombinant AAV2 (rAAV2) preferentially transduces primary neurons in the central nervous system.[15,17,18,19,20]

Two isoforms of mammalian GAD have been identified and are encoded by two distinct genes.[21] GAD65 is present as a membrane-associated form in the synapses, and is primarily involved in producing synaptic GABA for vesicular release. By contrast, GAD67 is distributed throughout the cell body and is mainly responsible for the production of cytosolic GABA by releasing GABA through a non-vesicular mechanism.[22,23,24,25] Moore *et al.*[3] reported that GAD65 concentrations are significantly reduced in the dorsal horn ipsilateral at the site of nerve injury in a rat pain model, whereas GAD67 expression is not. Recently, we constructed a rAAV2-JDK-GAD65 (rAAV-GAD65) vector encoding rat GAD65 whose expression is governed by the modified CMV promoter, called JDK.[9] Using the rAAV-GAD65 vector, which expresses higher levels of both GAD65 and GABA than does rAAV2-CMV-GAD65, we demonstrated that rat parkinsonian symptoms can be significantly improved concomitantly with the production of GAD65 near rAAV injection sites in the brain.

Based on these facts, this study explored the beneficial effects of exogenous GAD65 expression in the dorsal root ganglion (DRG)s of rats suffering from neuropathic pain by sciatic nerve injury. rAAV-GAD65 was directly administered into the DRGs, and the alleviation in mechanical allodynia and hyperalgesia was monitored for 8 weeks following virus injection. GAD65 expression and GABA release were determined by immunohistochemistry and HPLC analysis, respectively. These data suggests that administration of rAAV-GAD65 can induce consistent GAD65 expression in DRGs and resultant GABA release into the dorsal horn of the spinal cord, which in turn improves neuropathic pain symptoms after peripheral nerve injury.

Ⅱ. MATERIALS AND METHODS

1. Preparation of rAAV vector

Recombinant adeno-associated viruses 2 (rAAV2s) were constructed and produced based on the AAV helper-free system obtained from Stratagene (Kirkland, WA, USA). rAAV-GAD65 encodes rat GAD65 under the control of the JDK promoter[9] and rAAV-GFP encodes humanized recombinant renilla GFP (hrGFP) under the control of the universal CMV promoter in the backbone of the pAAV vector (Stratagene, Kirkland, WA, USA)(Figure 1). rAAVs were produced in 293 T cells using the calcium phosphate method by triple-transfection of pRepCap or pHelper, together with pAAV2-GAD65 or pAAV2-GFP.[26] To acquire a large and pure preparation of rAAVs, 293 T cells in 10x10 cm dishes were transfected and the rAAVs in the cells were liberated and purified by the SSCP method.[27] Following dialysis against PBS buffer (pH 7.4), highly concentrated rAAVs were stored in PBS buffer containing 5% glycerol at -80°C.[28] Total virus particles and infectious virus particles were estimated by analysis with an ELISA kit (Progen Inc., Heidelberg, Germany) and immunocytochemistry for GAD65 (Chemicon, CA, USA), respectively.[26]

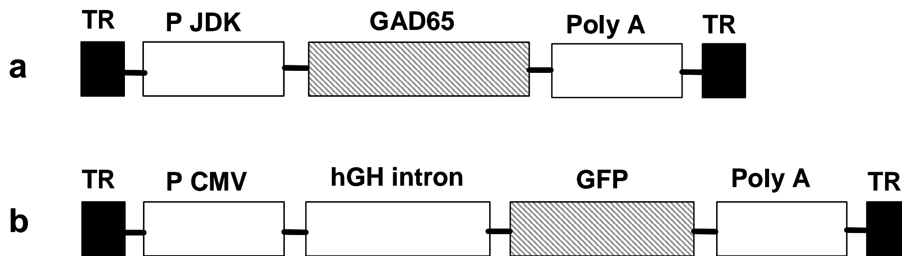

Figure 1. Schematic representation of rAAV2 vectors in the present study. (a) rAAV-JDK-GAD65, and (b) rAAV-CMV-GFP. Vectors shown contain the wild type AAV2 TR sequence.

2. Experimental animals

Every effort was attempted to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. Male Sprague-Dawley rats, each weighing 180-200 g, were housed in groups of five per cage, with food and water available ad libitum under a light-dark cycle of 12hr / 12hr, were used in this study. All experiment were performed during the light phase. Animals were allowed to acclimate at least for a week before surgery and behavioral testing. Experimental procedures were followed in accordance with the NIH regulations for animal care and with the approval of the Institution Animal Care and Use Committee of Yonsei University, Seoul, Korea. Subjects were randomly divided into four groups : Naive (n=8), Neuropathic pain only (n=6), Neuropathic pain + saline injection (n=6),

Neuropathic pain + rAAV2-JDK-GAD65 injection (n=11), Neuropathic pain + rAAV2-JDK-GFP injection (n=4).

3. Operative procedures

The present study was conducted according to the guidelines of the Ethical Committee of the International Association for the Study of Pain.[29] The rats were anesthetized with phentobarbital sodium solution (50mg / kg) and then a segment of left sciatic nerve was exposed between the mid-thigh levels. Surrounding tissues were carefully removed and the sciatic nerve gently held in place with forceps for the sciatic nerve was not damaged. Under surgical microscope (Olympus, Japan) observation, the three major division of the sciatic nerve (tibial, sural and common peroneal nerves) were cleary separated (Figure 2). To generate an efficient neuropathic pain model, the tibial and sural nerves were completely ligated and tightly transect, while the peroneal nerve was left intact.[33] Hemostasis was completed and the cut was closed with muscle and skin sutures.

4. AAV injection

Two weeks following the operation, the ipsilateral lumbar L4 and L5 dorsal root ganglion (DRG) of the anesthetized rats were surgically exposed by

removing part of the transverse process. Three $\mu\ell$ of rAAV were delivered to each DRGs through a glass micropipette connected to a hamilton syringe for ten minute wait.

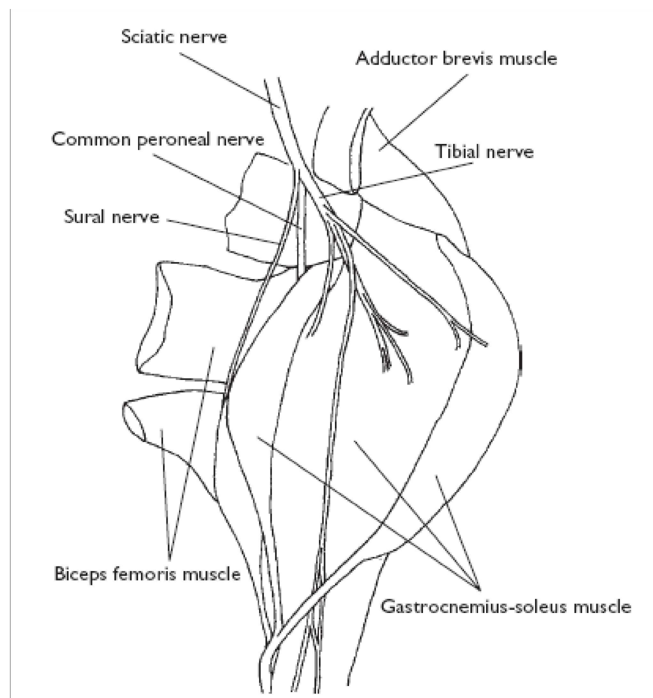

Figure 2. Anatomical schema of the sciatic nerve branches in the rat hind limb. (Lee B et al. Neuroreport 2000)

5. Behavior test

A. *von Frey* test

To measure mechanical allodynia, rats were placed inside acrylic cages (8×10×20cm) on top of a wire mesh grid, which allowed access to the paws. After 30min of adaptation, innocuous mechanical stimulation were applied with a *von Frey* filament (8mN bending force) 10 times to the lateral edge of the left and right hind paws.[30,31] The total frequency each animal lifted both the left and right hind paw was counted. The frequency of foot withdraw in percentage was used the final readout.

B. The pinprick test

To measure mechanical hyperalgesia, the pinprick test was performed. A pinprick test was using a bent needle (22-gauge) attached to a syringe. Rats were placed inside acrylic cages on top of a wire mesh grid, which allowed access to the paws. After 30min of adaptation, the lateral edge of plantar surface of the left and right hind paws was stimulated with the needle with sufficient intensity to produce a reflex withdrawal response in naive rats, but at an intensity which was insufficient to penetrate the skin. The duration of the paw withdrawal was recorded with a stopwatch.[32]

C. Hot plate test

To measure thermal allodynia, the animals were placed on a metal hot plate (45 ±0.5℃, dimensions 30×30) of 5 min. The total duration each animal lifted both the left and right hind paw was measured.

All animals were tested pre-operatively for a baseline responses and subsequently at days 1, 4, 7, 14, 21, 28, 35, 42, 49 and 56.

D. Acetone test

To measure mechanical allodynia, rats were placed inside acrylic cages (8 × 10 × 20cm) on top of a wire mesh grid, which allowed access to the paws. After 30min of adaptation, 100㎕ of acetone was sprayed onto the plantar surface of the rat's hind paw from below the grid with a syringe with a blunt needle. The spray was repeated 5 times on each paw with an interval of 5 min between each test. The frequency of foot withdrawal expressed in percentage was used as the cold allodynia index.

6. Microdialysis

The amount of *in vivo* GABA release in the dorsal horn was determined by HPLC following microdialysis. Male Sprague-Dawley rats were anaethetised under urathane (1.25mg/kg) and core body temperature maintained at around 37°C by a homeothermic blanket. Rats were mounted on a streotaxic frame and the dorsal surface of

vertebra T13 was surgically exposed and held immobilised on the horizontal plane by use of a spinal clamp. The dura was opened carefully and a microdialysis probe (CMA/11, Sweden) was inserted into the spinal cord.[32] The probe was perfused with an artificial cerebrospinal fluid (aCSF), which consisted of 145 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl, 1.0 mM MgCl, and 2.0 mM NaHPO at pH 7.4. The flow rate was carefully controlled to 1.0㎕ per min using a CMA/102 pump (CMA/Microdialysis, Sweden).

7. HPLC assay

The amount of GABA was determined using HPLC. 30 ㎕ of sample was mixed with 60㎕ of working solution, which was composed of 3 ml OPA stock solution (2.7mg O-phthaldialdehyde in 1ml MeOH, 5㎕ of 2-Mercaptoethanol, and 9 ml of 0.1M sodium tertraborate) and 1 ml sodium tetraborate. A reverse phase column (AccQ-Tag, 3.9×150mm, Waters for amino acid analysis) was used for separation. The composition of mobile phase was 0.02M sodium acetate buffer containing 30% acetonitrile at pH 4.6. The peaks were detected at 30℃ with a flow rate of 0.7ml/min using RF-10Axl (Shimazu Corp., Japan) at excitation and emission wavelengths of 340nm and 460 nm, respectively.

8. Immunohistochemistry

In order to acquire immunohistochemical data, the rats were anesthetized with urethane (1.25 mg/kg) and transcardially perfused with 125 ml normal saline, followed by 250 ml ice-cold 4% paraformaldehyde. The L5 segment of spinal cord and L4, L5 DRGs were removed, postfixed for 10 hours, and transferred to 30% sucrose, until equilibrated. Sections were cut to a thickness of 10 μm, frozen, and immunoreacted with a primary polyclonal antibody against GAD65 (1: 300; Chemicon, CA, USA) for 24 hours at 4℃. FITC-conjugated secondary antibody (1:1000, Santa Cruz Biotechnology, Inc., CA, USA) and cy3-conjugated secondary antibody (1:1000, Jackson Immuno Research, Inc., PA, USA) was then added to each section, and the sections were incubated for 1 hour at RT in darkness. The sections were washed with PBS, mounted on coverslips, and visualized by fluorescence microscopy.

9. Statistical analysis

Data was presented as means ± SEMs. Statistical analysis was performed using analysis of variance or Kruskal-wallis one-way analysis of variance protocols, followed by Mann-Whitney U-tested for the comparison of behavioral data from each group. The p-value of <0.01 and <0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS (version 11.5:SPSS Inc., Chicago, IL, USA).

Ⅲ. RESULTS

1. Direct injection of a recombinant adeno-associated virus can efficiently transduce DRGs in a rat neuropathic pain model.

To investigate the transduction efficiency of rAAV in DRGs, GFP expression was monitored after directly injecting rAAV encoding GFP gene (rAAV-GFP). Rats suffering from neuropathic pain were prepared by inducing peripheral nerve injury as described in the Materials and Methods. Two weeks after the operation, the three $\mu\ell$ of rAAV-GFP (1.3×10^7pfu/ml) directly injected into both L4 and L5 DRG neurons of Sprague - Dawley rats (n = 4). Figure 3 shows that both DRGs became brightly fluorescent due to the accumulation of GFP within 3 weeks after the injections. Moreover, the GFP signal remained positive without any noticeable decrease at 8 weeks, indicating sustained synthesis of GFP since the introduction of rAAV-GFP into the DRGs.

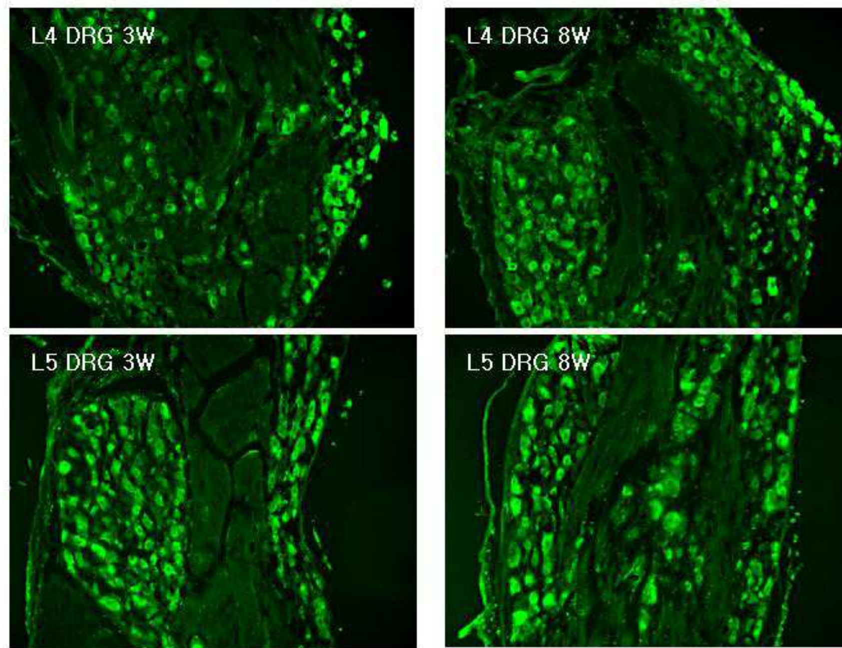

Figure 3. GFP expression 3 or 8 weeks after direct injection of rAAV-GFP into DRGs. Three ㎕ of rAAV-GFP (1.3 × 10^7 infectious particle/ml) were directly deilvered to L4 and L5 DRGs. The degree of GFP expression was examined at 3 or 8 weeks post-injection. The GFP signal was consistently observed at 3 and 8 weeks in both DRGs (Original magnification; ×100)

2. GAD65 can be readily detected following direct rAAV-GAD65 injection into the DRGs of a rat neuropathic pain model.

To examine GAD65 production in the DRGs in a neuropathic pain model, rats suffering from neuropathic pain were prepared by tight ligation and transection of th tibial and sural nerves, leaving common peroneal nerve intact which are major division of the sciatic nerve of Sprague - Dawley rats. Two weeks after the nerve injury operation, rAAV-GAD65 (2.4×10^6 pfu/ml, n=11) or saline (n=6) was directly introduced into either L4 or L5 DRGs by through a glass micropipette connected to a hamilton syringe.

Immunohistochemical staining specific to GAD65 implied that GAD65 could be readily detected with rAAV-GAD65 administration by immunochemical staining using both FITC or Cy-3 in the DRG neurons at 8 weeks post-injection (Figure 4). The intensity of GAD65 expression in DRGs injected with rAAV-GAD65 was significantly stronger than that of the saline injected neuropathic pain models. The number of GAD65 positive cells / section was higher in rAAV-GAD65 injected animals than saline injected animals (Figure 5). Therefore, the data shows that rAAV-GAD65 treatment induces the expression of GAD65 in the DRGs, which closely associated with the reduction of pain even after surgery.

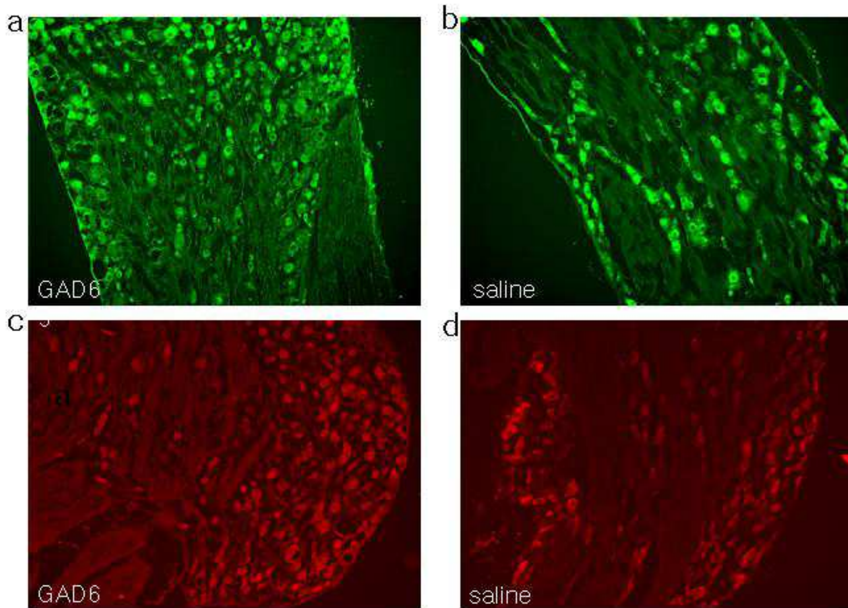

Figure 4. Immunohistochemical detection of GAD65 in the DRG 8 weeks after rAAV-GAD65 administered to DRGs. GAD65 expression was readily identified by immunohistochemical staining using primary GAD65 and FITC - conjugated secondary antibodies (a, b) and cy3 - conjugated secondary antibodies (c, d) compared with those injection with saline. (Original magnification; ×100)

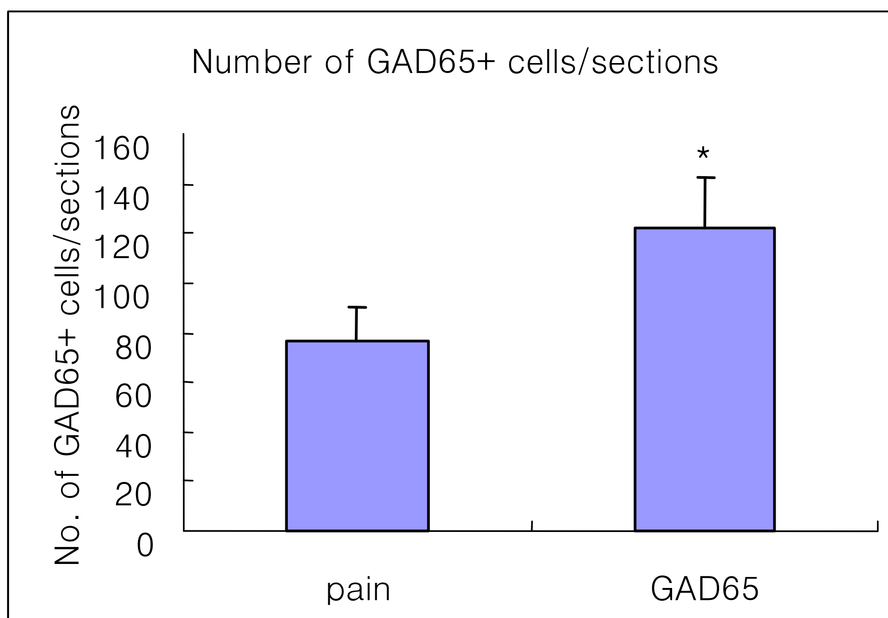

Figure 5. Number of GAD65 positive cells / section in DRGs at 10 weeks after injection rAAV-GAD65 and saline. The GAD65 positive cells / section of the GAD65 injected animals were higher than saline injected animals. Asterisk (*) indicates that the rAAV-GAD65 group differs significantly from the pain group at each time point. *P < 0.05.

3. GAD65 expression in DRGs leads to a sustained reduction in both mechanical allodynia and hyperalgesia induced by neuropathic pain.

The beneficial effect resulting from GAD65 synthesis in DRGs injected with rAAV-GAD65 was blindly estimated by performing *von Frey* testing. For this purpose, GAD65 was introduced into DRGs using rAAV2-JDK-GAD65 (rAAV-GAD65) vector encoding rat GAD65, which is previously generated our group.[9] The frequency of foot withdrawals to 10 times mechanical stimuli was plotted against as a final readout time hiring in 4 independent groups, which were either groups injected with rAAV-GAD65 (n=11), rAAV-GFP (n=4), saline (n=6) or a normal unoperated groups (n=8) (Figure 6). Within a week after rAAV-GAD65 administration, mechanical allodynia dramatically decreased down to 4.19 $\pm$ 0.2 incidences times per 10 stimuli ($P < 0.01$), while there were 8.75 $\pm$ 0.18 and 8.5 $\pm$ 0.3 incidences times per 10 stimuli in the rAAV-GFP and saline-injected groups, respectively. More importantly, the improvement in mechanical allodynia was significantly sustained throughout the entire experimental period in the group injected with rAAV-GAD65. By contrast, no reduction in symptoms was seen in both the rAAV-GFP and saline-treated groups. In addition, the control group with no operation did not suffer from any mechanical allodynia.

The positive effect of rAAV-GAD65 injection was examined in mechanical hyperalgesia using a pinprick test. The cumulative foot-lift duration was

blindly examined following similar surgical treatments to each experimental group as described in Figure 6. Two weeks after surgery for induction of neuropathic pain models, all three pain model groups experienced significantly increased foot-lift duration times before the treatment with rAAV-GAD 65, rAAV-GFP, or saline of 9.8 ± 0.4 sec, 11 ± 0.7 sec, or 10.5 ± 1 sec, respectively (Figure 7).

Among them, only the group treated exclusively with rAAV-GAD 65 showed improvements in symptoms resulting from hyperalgesia including a sharp reduction in the duration time one week after virus injection (5.5 ± 0.435 sec, $P < 0.05$). The improvement by rAAV-GAD 65 was sustained during the entire experimental period, with no observed diminishing effect both in the rAAV-GFP and saline-treated groups. And there was no significant difference of foot-lift duration with control animals (normal group) during the entire experimental period. And additionally hot plate test and acetone test (thermal allodynia test) were carried out, but there was no significant changes with all groups (data not shown).

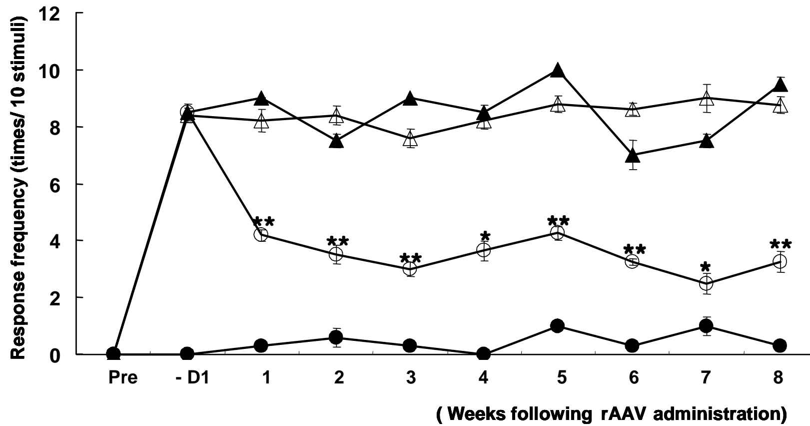

Figure 6. Effect on mechanical allodynia following rAAV-GAD65 administration in neuropathic pain models. Two weeks following neuropathic surgery, rats were either unoperated (●), or operated upon in order to administer rAAV-GAD65 (○), rAAV-GFP (△), or saline (▲). Once a week from 1 to 8 weeks, response rates to the *von Frey* filament were monitored as mechanical allodynia.

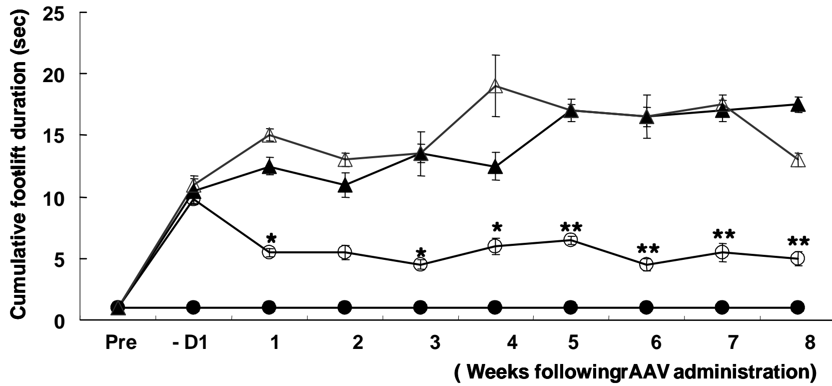

Figure 7. Effect on mechanical hyperalgesia following rAAV-GAD65 administration in neuropathic pain models. Rats were treated as described in figure 5 and tested for hindpaw withdrawal. (●), unoperated or operated upon, followed by the administration of (○) rAAV-GAD65, (△) rAAV-EGFP, or (▲) saline. Asterisk (*) indicates that the rAAV-GAD65 group differs significantly from the saine group at each time point. *$P < 0.05$, **$P < 0.01$.

4. The introduction of rAAV-GAD65 into DRGs results in an increased level of GABA in the dorsal horn.

In this study, HPLC analysis was carried out to determine whether any significant increase in the level of GABA in the dorsal horn following GAD65 transgene expression in DRGs. In order to do so, the microdialysis of the dorsal horn was performed at 8 weeks after rAAV-GAD65 or saline injection. The data indicated a significant enhancement in the level of GABA in the dorsal horns of the group treated with rAAV-GAD65 (0.619 $\pm$ 0.064 pmol/$\mu\ell$, $P < 0.01$), but not in the group treated with saline (0.284 $\pm$ 0.065 pmol/$\mu\ell$) (Figure 8).

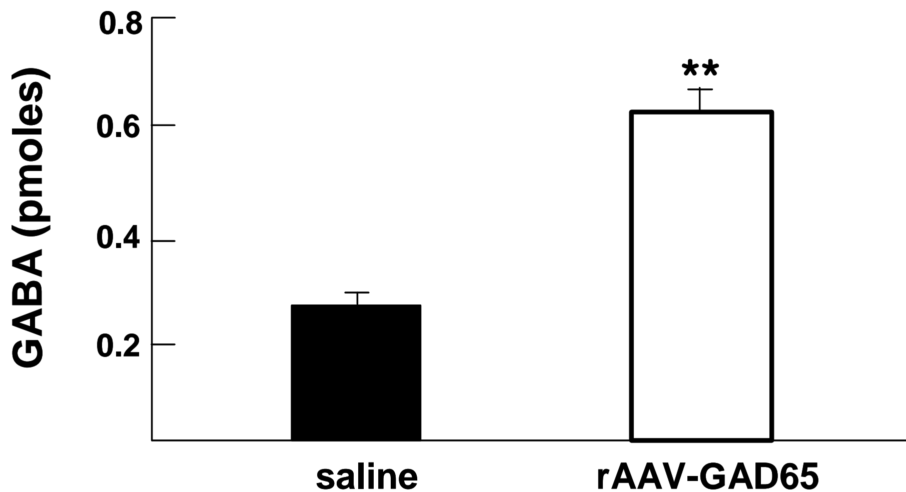

Figure 8. GABA concentration in the dorsal horn. Using HPLC and microdialysis, the amount of GABA was estimated from the CSF recovered from nerve terminals *in vivo*. Eight weeks after the direct injection of rAAV-GAD65 or saline into DRGs, the GABA concentration was found to be statistically increased in the rats with rAAV-GAD65, compared with that of the rats in the saline-injected group. **P < 0.01.

Ⅳ. Discussion

This study showed here that using rAAV-GAD65, the prolonged enhancement of GABA concentration was acquired in the spinal dorsal horn, the direct introduction of rAAV-GAD65 into the DRGs leading to significant long-term reduction of neuropathic pain. By transecting and subsequently ligating the tibial and sural sciatic nerves, we mimicked a peripheral nerve injury. Allodynic *von Frey* and hyperalgesic pinprick tests in this pain model suggested that the animals indeed suffered from evident neuropathic pains during the entire experimental period (Figure 6, 7). Previous reports indicated that this kind of neuropathic pain model provides a clear-cut response to various behavioral tests to validate different types of pain symptoms.[33,34] In agreement with previous studies, our data implied that the tibial and sural transection method fulfills two necessary criteria as a neuropathic pain model: the ease of preparation and the reproducibility of an end-point.

Recently, Liu *et al.*[11] constructed a recombinant herpes simplex virus (HSV) -based vector encoding human GAD67 (QHGAD67). Taking advantage of HSV characterized high-affinity retrograde transport in sensory neurons, they showed that subcutaneous footpad administration of QHGAD67 attenuates both below-level central by incomplete spinal cord injury[7] and peripheral neuropathic pain by the spinal nerve ligation model.[35] Concomitantly, the increase in GAD/GABA expression and down regulation of indirect biological markers (c-Fos, phosphorylated p-ERK1/2) was identified.[36,37] Yet, the pain alleviation maximized within few weeks following QHGAD67

administration only lasted for 2 to 5 weeks, and then drastically disappeared. On the contrary, the substantial decrease in neuropathic pain was readily observed within one week following rAAV-GAD65 administration in the current study. Furthermore, the magnitude of attenuating the pain symptom was retained throughout the entire experimental period. The experimental group with rAAV-GAD65 experienced 62.9 $\pm$ 0.32 % in *von Frey* test and 71.4 $\pm$ 1.24 % in pinprick test, compared to those of the saline control group. Furthermore, the data suggest that improved mechanical behavior initially detected within 1 week after virus inoculus persists throughout the experimental period up until 8 weeks post-injection. GAD65 protein was readily observed in the DRGs even at 8 weeks post-injection with no noticeable decrease in its intensity by immunohistochemistry, where the significant release in GABA was observed. Thus, these results highlight the beneficial effects of rAAV as a gene delivery vehicle for constitutive gene expression.[14,38]

Several studies have illustrated that rAAV2 preferentially transduces neurons in the DRGs, as well as in the peripheral axon.[38,39] Additionally, GAD65 in this study was transcriptionally controlled by the JDK promoter, which is 10 times stronger *in vivo* than the CMV promoter,[40,41] which may have resulted in the substantial GAD expression. One of the drawbacks of this study is, however, the requirement of complicated surgery to directly introduce the vehicle to DRGs. Recently, there has been a report demonstrating that rAAV can be administrated into the sciatic nerve of rats with ease in operation and persistent gene expression in DRG neurons.[42] Based on this study, we have injected rAAV-GFP into the sciatic nerve and found the decent amount of GFP positive neurons in DRGs (data not shown).

Currently, we are attempting to administer rAAV-GAD65 via the sciatic nerve and investigate its beneficial effects over direct DRG administration. Moreover, Xu *et al.*[35] developed the genetically-modified rAAV system preferentially homing rAAV to neuron cell bodies after being taken up by axons. The rAAV characterized by enhanced neuro-tropism, along with effective retrograde transport from axon terminal, would then allow for subcutaneous administer of rAAV for the substantial transgene expression, such as GAD65.

The inhibitory neurotransmitter GABA is synthesized by both GAD65 and GAD67, each of which is encoded by a distinct and differentially regulated gene.[22] According to Eaton *et al.*,[44] GAD65 protein levels declined significantly in all laminas of the ipsilateral dorsal horn in partial nerve injury models. This may reflect the reduced transcription/translation of GAD65 and a loss of GABAergic interneurons caused by cell death. Liu *et al.* have constructed a non-replicating HSV vector designed to express human GAD67 (QHGAD67), and they have demonstrated that subcutaneous inoculated QHGAD67 reduced below-level mechanical allodynia and thermal hyperalgesia after 5 weeks in a partial spinal cord injury model.[11] Peripheral nerve injury results in a decrease in GABA levels in the dorsal horn[45] and in a primary afferent-evoked inhibitory postsynaptic current in the dorsal horn after partial nerve injury.[3] HPLC analysis of microdialysis samples from the dorsal horn in the current study clearly showed the dramatic increase in GABA release, exclusively in the rAAV-GAD65 injected group. Thus, the data support the idea that the decrease in GABA level in the dorsal horn may be associated with the pain symptom following peripheral nerve injury.

In conclusion, our results indicate that direct injection of the rAAV2vector

expressing GAD65 into DRG neurons sharply alleviates deteriorated symptoms in peripheral neuropathic pain model. The data also implies that the therapeutic effects are promptly achieved and last for months, with no substantial decrease in its effects. Taken all together, the present study demonstrates that rAAV2-GAD65 holds a promising potential for the treatment of neuropathic pain. Furthermore, improvement in its administration technique would allow this strategy to be more practical, which is currently under the investigation.

Ⅴ. Conclusion

These experiments demonstrate that expression of GAD65 in DRG neurons, achieved by rAAV-mediated gene transfer in vivo, reduces mechanical allodynia and mechanical hyperalgesia in the sciatic nerve transection rat model of neuropathic pain. The direct administration of rAAV-JDK-GAD65 to dosal root ganglia (DRG) induced the constitutive GAD65 expression and release the GABA in spinal dorsal horn. Both allodynic and hyperalesic behavior tests suggested that neuropathic pain markedly decreased, along with the transgenic GAD65 expression. Moreover, the magnitude of the pain relief maintained for entire experimental period, where GAD65 expression was also noticed with no substantial reduction in its immuno-reactive intensity.

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ABSTRACT (IN KOREAN)

흰쥐의 후근 신경절에서 AAV 벡터를 통한 GAD65 의 발현이 신경병증성통증에 미치는 진통효과

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신경병증성 통증은 그 기전이 명확히 알려지지 않은 행동학적 만성통증 증후군으로써 약물, 질병, 부상에 의해 유발된 말초신경계의 구심성 감각신경 섬유의 손상이나 파괴와 연관되어 있다. 말초 신경 손상에 의해 유발되는 신경병증성 통증은 임상에서 매우 흔한 질병이지만 아직까지 그 치료법에 대해선 잘 알려져 있지 않다.

감마아미노 낙산 (γ-amino butyric acid, GABA)은 척수 후각 (spinal dorsal horn)에의 많은 신경세포에서 작용하는 주요 억제 신경전달물질로서, 이 신경전달물질은 척수 통증 작용에서 중요한 역할을 수행한다. 최근 몇몇 연구에서 밝혀진 바에 의하면 신경손상 동물모델에서 척수 후각 신경세포에 대한 감마아미노 낙산 면역반응에 상당한 감소가 나타나는 것이 관찰되었다. 그리고 이러한 현상

은 통증억제효과의 감소로 이어지며 이것은 나아가 신경병증성 통증에서 나타나는 행동학적 징후로 연결된다고 시사하고 있다.

만약 척수의 후각에서 감마아미노 낙산이 분비된다면 신경병증성 통증을 감소시킬 수 있을 것이라는 가정을 검증하기 위해 본 실험에서는 재조합 아데노 부속 바이러스 (rAAV)를 기반으로 사람의 글루탐산 탈탄산효소 (glutamic acid decarboxylase, GAD)의 이성질체중 하나인 GAD65 발현 유전자를 JDK 유발인자와 함께 암호화 하여 벡터를 제작하였다. 재조합 아데노 부속 바이러스 벡터를 좌골신경 (sciatic nerve)을 손상시킨 실험군 집단의 후근 신경절 (dorsal root ganglion, DRG)에 직접 주입하여 GAD65를 후근신경절로 형질도입 시킨 결과 재조합 아데노 부속바이러스 벡터를 주입하지 않은 비교 집단과 비교했을 때 확실히 구별되는 통증경감 효과가 있음을 관찰할 수 있었다. 더 나아가 전체 실험 기간 동안 통증 경감효과가 지속됨도 관찰할 수 있었다. 그리고 GAD65의 발현 또한 실험 기간 동안 눈에 띄는 감소 없이 지속되는 것을 관찰할 수 있었다. 이러한 결과들은 rAAV-GAD65에 의해 in vivo 상으로 전달된 GAD65에서 분비된 감마아미노 낙산에 의해 눈에 띄게 증가한 감마아미노 낙산의 양을 HPLC 를 통해 측정함으로써 확신할 수 있었다.

본 실험에서 관찰된 이러한 결과들은 후근신경절로의 rAAV-JDK-GAD65에 의한 유전자 도입이 말초 좌골신경 손상에 의한 신경병증성 통증 증후군에 효과적인 치료법이 될 수 있다는 것을 시사한다.

핵심되는 말 : 신경병증성 통증, 재조합 아데노 부속바이러스, 감마아미노 낙산, 글루탐산 탈탄산효소, 후근 신경절