RESEARCH ARTICLE

Beta-nerve growth factor gene therapy alleviates pyridoxine-induced neuropathic damage by increasing doublecortin and tyrosine kinase A in the dorsal root ganglion

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Abstract

Beta-nerve growth factor (β-NGF) is known to be a major leading cause of neuronal plasticity. To identify the possible action mechanisms of β-NGF gene therapy for sciatic nerve recovery, experimental dogs were randomly divided into control, pyridoxine, and pyridoxine + β-NGF groups. We observed chronological changes of morphology in the dorsal root ganglia in response to pyridoxine toxicity based on cresyl violet staining. The number of large neurons positive for cresyl violet was dramatically decreased after pyridoxine intoxication for 7 days in the dorsal root ganglia and the neuron number was gradually increased after pyridoxine withdrawal. In addition, we also investigated the effects of β-NGF gene therapy on neuronal plasticity in pyridoxine-induced neuropathic dogs. To accomplish this, tyrosine kinase receptor A (TrkA), βIII-tubulin and doublecortin (DCX) immunohistochemical staining was performed at 3 days after the last pyridoxine treatment. TrkA-immunoreactive neurons were dramatically decreased in the pyridoxine group compared to the control group, but strong TrkA immunoreactivity was observed in the small-sized dorsal root ganglia in this group. TrkA immunoreactivity in the dorsal root ganglia was similar between β-NGF and control groups. The numbers of βIII-tubulin- and DCX-immunoreactive cells decreased significantly in the pyridoxine group compared to the control group. However, the reduction of βIII-tubulin- and DCX-immunoreactive cells in the dorsal root ganglia in the β-NGF group was significantly ameliorated than that in the pyridoxine group. These results indicate that β-NGF gene therapy is a powerful treatment of pyridoxine-induced neuropathic damage by increasing the TrkA and DCX levels in the dorsal root ganglia. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University, South Korea (approval No. SNU-060623-1, SNU-091009-1) on June 23, 2006 and October 9, 2009, respectively.

Key Words: *β-nerve growth factor; βIII-tubulin; doublecortin; gene therapy; neuron-glial antigen 2; neuropathy; pyridoxine*

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Introduction

Nerve growth factor (NGF), which was named for its effects on the growth and differentiation of cells in the peripheral and central nervous systems, also affects the neural plasticity that allows the adult nervous system to adjust its function and phenotypic features in response to stimuli; therefore, it has been widely investigated because of its potential clinical benefits (Mearow and Kril, 1995; Aloe et al., 2012). NGF consists of three subunits, α, β, and γ, with β chains solely responsible for the trophic activity of NGF (Ullrich et al., 1983). Although some studies have been conducted to clarify the effects of β-NGF in animal models of peripheral neuropathy (Apfel, 1999; Chung et al., 2008b), a specific role of β-NGF in the adult peripheral nervous system has not been established.

NGF binds to two different receptors, p75 neurotrophin receptor ($p75^{NTR}$) and tyrosine kinase receptor A (TrkA) (Barbacid, 1994). Neuroblasts expressing elevated levels of $p75^{NTR}$ cause increases in cell apoptosis, even when sufficient serum is added to culture medium (Bunone et al., 1997). In contrast, treatment with NGF facilitates the homodimerization of TrkA and promotes neuroblast differentiation and cell survival via signaling pathways including mitogen-activated protein kinase (MAPK), Akt, and protein kinase C (PKC) (Wehrman et al., 2007).

During central and peripheral nervous system development, doublecortin (DCX) is associated with the migration of neuroblasts and widely expressed by immature neurons (Francis et al., 1999; Gleeson et al., 1999). In addition to its role in the developmental stage, DCX expression in adults

is predominantly confined to areas in which neurogenesis occurs, such as the subgranular zone of the dentate gyrus and the subventricular zone of the lateral ventricle (Alvarez-Buylla and Garcia-Verdugo, 2002; van Praag et al., 2002; Kempermann et al., 2003). As a result, DCX has been used as a classical marker for differentiating neuroblasts. One study also showed that distinct subsets of neurons are immunoreactive for DCX in the dorsal root ganglia (DRG), as well as in the central nervous system in mice and rats (Dellarole and Grilli, 2008). Accordingly, DCX is a possible candidate marker for plasticity of DRG.

Although changes in plasticity with DCX expression in the dog's brain with age have been reported (Hwang et al., 2008; De Nevi et al., 2013), no studies have investigated changes in DCX under neuropathic conditions or the response to neuroprotective agents in the DRG of dogs. Therefore, in this study, we investigated the changes in TrkA and DCX in the DRG of dogs following β-NGF gene therapy to identify the possible action mechanisms of β-NGF gene therapy.

Materials and Methods

Animal models

Twenty-four dogs (12 beagles and 12 mongrels) were used in this experiment (12 males and 12 females). The mean age of these animals was about 2 years old and the body weight was 4–6 kg. To investigate pyridoxine-induced neuronal damage in DRGs, a control group and three pyridoxine groups (animals were sacrificed at 0, 1, and 4 weeks after the last pyridoxine treatment, namely PDX-0 week, PDX-1 week, and PDX-4 weeks groups, respectively) were designated (n = 4/group). To investigate the action mechanism of β-NGF gene therapy, control, pyridoxine, and pyridoxine + β-NGF groups were designated (n = 4/group). In both experiments, the same animals were used for the control group. All dogs were clinically judged to be in good health and neurologically normal. The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University, South Korea (approval No. SNU-060623-1, SNU-091009-1) on June 23, 2006 and October 9, 2009, respectively. Pyridoxine chloride (Sigma, St. Louis, MO, USA) was prepared in an iso-osmotic sterile aqueous solution of sodium chloride (100 mg/mL) immediately before injection and administered at 150 mg/kg subcutaneously once a day in the morning for 7 days as described in a previous study (Chung et al., 2008a).

Recombinant dog β-NGF gene therapy

Twenty-four hours before pyridoxine intoxication, all dogs in the pyridoxine + β-NGF group were anesthetized by intramuscular injection of 10 mg/kg Zoletil 50® (Virbac, Carros, France) (Chung et al., 2008b). The dogs were then put in a head down position under sedation and had cisterna magna puncture performed using a 27-gauge needle. After the cerebrospinal fluid was drained via gravity flow with the 27-gauge needle, the prepared solution was injected into the cisterna magna. A 400 μL vehicle (cationic polymer transfection reagent, Polyplus Transfection, France) was administered to the control group and pyridoxine group, while single administration of constructed recombinant dog β-NGF plasmid solution (40 μg/400 μL) was administered to the pyridoxine + β-NGF group through the intrathecal region. Preparation and treatment of recombinant dog NGF plasmids were carried out in accordance with a previous study (Chung et al., 2008b).

Tissue processing

To observe the pyridoxine-induced neuronal damage in DRGs, dogs were anesthetized by intravenous injection of 10 mg/kg Zoletil 50® (Virbac) and 6 mg/kg propofol (Myungmoon Pharm., Seoul, Korea) at 0, 1, and 4 weeks after the last pyridoxine treatment. For β-NGF gene therapy, dogs were sacrificed with the same anesthetics at 3 days after the last pyridoxine treatment for histological observation, then perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (pH 7.4). The DRG of the lumbar level (L5–7) and brain were separated and post-fixation was performed with the same fixative for 12 hours, after which samples were dehydrated with graded concentrations of alcohol for embedding in paraffin. Thereafter, paraffin-embedded tissues were sectioned into 5-μm coronal sections using a microtome (Leica, Wetzlar, Germany) and then mounted into silane-coated slides. For positive and negative staining for DCX, the brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter, the frozen tissues were serially and transversely sectioned on a cryostat (Leica) into 30-μm coronal sections that were subsequently collected into 6-well plates containing PBS.

Cresyl violet staining to confirm the pyridoxine-induced cell damage

Cresyl violet staining was conducted as described in a previous study (Yoo et al., 2015). Briefly, DRG sections were rinsed in 1% cresyl violet acetate solution (Sigma-Aldrich, St. Louis, MO, USA) containing glacial acetic acid (Sigma-Aldrich). Before and after overnight staining at 25°C, the sections were washed twice in distilled water. Samples were then dehydrated and mounted using Canada Balsam (Kanto, Tokyo, Japan). Digital images of the DRG were captured with a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP72, Olympus) connected to a computer monitor.

Immunohistochemistry for TrkA, DCX, and βIII-tubulin

The DRG sections were placed 150-μm apart from each and then hydrated and treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 minutes. For antigen retrieval, the sections were placed in 400 mL jars filled with citrate buffer (pH 6.0) and heated in a 2100-retriever (Prestige Medical, Lancashire, UK). After antigen retrieval, slides were allowed to cool to room temperature, and then washed in PBS. For positive and negative staining for DCX, hippocampal sections were treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 minutes. After washing, sections were incubated

in 10% normal horse serum in PBS for 30 minutes. Sections were then incubated with diluted rabbit anti-TrkA (1:100, Abcam, Cambridge, UK), goat anti-DCX (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-βIII-tubulin (1:2000, Covance, Emeryville, CA, USA) for 48 hours at 4°C. For the negative control, pre-immune horse serum was used instead of DCX antibody. Sections were subsequently exposed to biotinylated horse anti-rabbit IgG or horse anti-goat IgG (diluted 1:200, Vector Laboratories, Inc., Burlingame, CA, USA) and streptavidin peroxidase complex (diluted 1:200, Vector Laboratories) for 2 hours at 25°C, respectively. Finally, sections were stained with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 0.1 M Tris-HCl buffer (pH 7.4).

To assess the antibody specificity for DCX, a positive and negative control test was conducted using the hippocampus because DCX immunoreactivity is mainly found in the neuroblasts in the hippocampal dentate gyrus (Hwang et al., 2007, 2008).

Quantification of data

All experimental measurements were performed under blinded conditions by two observers to maintain objectivity, and all experimental samples were analyzed under identical conditions. The number of cresy violet-positive neurons and DCX- or βIII-tubulin-immunoreactive cells in each group of sections was counted in the DRGs using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ, USA). DRG neurons were classified into three types according to their sizes: small (area 1000 μm^2), medium (area 1000–2000 μm^2), and large (> 2000 μm^2) (Kang et al., 2016). The number of these neurons in each group was measured in the DRG using the aforementioned image analyzing system. Cell counts were obtained by averaging the numbers of DRGs per section (10 sections) counted under a light microscope with a 10× lens.

TrkA immunoreactivity was evaluated using an image analysis system and the ImageJ software v. 1.5 (National Institutes of Health, Bethesda, MD, USA). Digital images of the DRG were captured with a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP72, Olympus) connected to a computer monitor. Images were calibrated into an array of 512 × 512 pixels corresponding to a tissue area of 600 μm × 450 μm (100× primary magnification). Each pixel resolution consisted of 256 gray levels, and the intensity of TrkA immunoreactivity was evaluated by the relative optical density (ROD), which was obtained after transformation of the mean gray level using the following formula: ROD = log(256/mean gray level). The ROD for background staining was determined in an unlabeled part of the sections using the Photoshop CC software (Adobe Systems Inc., San Jose, CA, USA), then subtracted to compensate for nonspecific staining using the ImageJ v. 1.50 software (National Institutes of Health). Data were expressed as a percentage of the control group values (set to 100%).

Statistical analysis

Statistical analyses of all data were performed using the GraphPad Prism (Ver 5.01) statistical analysis software (GraphPad Software, San Diego, CA, USA). The values shown represent the means of experiments performed for each experimental area. Differences among means were identified by one-way analysis of variance followed by Bonferroni's *post-hoc* test. A P value of < 0.05 was considered to indicate significance.

Results

Confirmation of neuronal damage in DRGs after pyridoxine intoxication

In the control group, abundant cresyl violet stained neurons were observed in the DRG (**Figure 1A**). In the PDX-0 week group, the number of large-sized neurons positive for cresyl violet was significantly decreased in the DRGs compared to that in the control group (P = 0.008), while the numbers of small- and medium-sized neurons in the DRGs were similar to those in the control group (**Figure 1B** and **E**). Abundant large-sized neurons positive for cresyl violet in the DRG were observed in the PDX-1 week group compared to PDX-0 week group (**Figure 1C**). In the PDX-4 weeks group, many cresyl violet-positive neurons were found in the DRG and there was a significant increase in the number of cresyl violet-positive neurons compared to the PDX-0 week group (P = 0.0024) (**Figure 1D** and **E**).

Effects of β-NGF gene therapy on TrkA-immunoreactive DRG neurons in pyridoxine-induced neuropathy

To elucidate the NGF-TrkA-mediated cell survival pathway, immunohistochemical staining of the DRGs for TrkA was conducted. In the control group, TrkA immunoreactivity was observed in the DRGs, especially in the medium- and large-sized DRG neurons (**Figure 2A**). In the pyridoxine group, TrkA expression was significantly decreased in the DRG compared to the control group (P < 0.05) because of loss of large-sized neurons (**Figure 2B** and **D**). In this group, strong TrkA immunoreactivity was found in the small-sized neurons. In the pyridoxine + β-NGF group, TrkA immunoreactivity was significantly increased in the DRGs compared to the pyridoxine group (P < 0.05), and TrkA immunoreactivity was similar between pyridoxine + β-NGF and control groups (**Figure 2C** and **D**).

Effects of β-NGF gene therapy on DCX-immunoreactive DRG neurons in pyridoxine-induced neuropathy

In the control group, the number of DCX-immunoreactive cells was detected in small-, medium-, and large-sized neurons (**Figure 3A**). In the pyridoxine group, the number of DCX-immunoreactive large-sized cells in the DRG was significantly decreased than that in the control group (P < 0.05) (**Figure 3B** and **D**). In the pyridoxine + β-NGF group, the number of DCX-immunoreactive cells were significantly increased compared to the pyridoxine group (P < 0.05), but the number of DCX-immunoreactive cells were slightly decreased compared to the control group (**Figure 3C** and **D**).

Antibody specificity of DCX in hippocampal dentate gyrus and DRGs

Immunohistochemical staining for DCX was conducted in the tissues of dentate gyrus and the DRG at the same time. Strong DCX immunoreactivity was found in the subgranular zone of dentate gyrus, while DCX immunoreactivity disappeared from the hippocampal dentate gyrus following treatment with the pre-immune serum instead of DCX antibody (**Figure 4A** and **B**). Similarly, DCX immunoreactivity was found in the DRG neurons, while DCX immunoreactivity was not detected in the DRG treated with pre-immune serum instead of DCX antibody (**Figure 4C** and **D**).

Effects of β-NGF gene therapy on βIII-tubulin-immunoreactive DRG neurons in pyridoxine-induced neuropathy

In the control group, abundant βIII-tubulin-immunoreactive neurons were detected throughout the DRG (**Figure 5A**). In the pyridoxine group, βIII-tubulin immunoreactivity was significantly decreased in the large-sized DRG neurons compared to the control group ($P < 0.05$) (**Figure 5B** and **D**). The numbers of small- and medium-sized DRG neurons were not significantly different between pyridoxine and control groups (**Figure 5D**).

In the pyridoxine + β-NGF group, the numbers of βIII-tubulin-immunoreactive small- and medium-sized DRG neurons were not significantly different from those in the control or pyridoxine group. However, the number of βIII-tubulin-immunoreactive large-sized DRG neurons in the pyridoxine + β-NGF group was significantly higher than that in the pyridoxine group ($P < 0.05$), although the number of βIII-tubulin-immunoreactive neurons in the pyridoxine + β-NGF group was lower than that in the control group (**Figure 5C** and **D**).

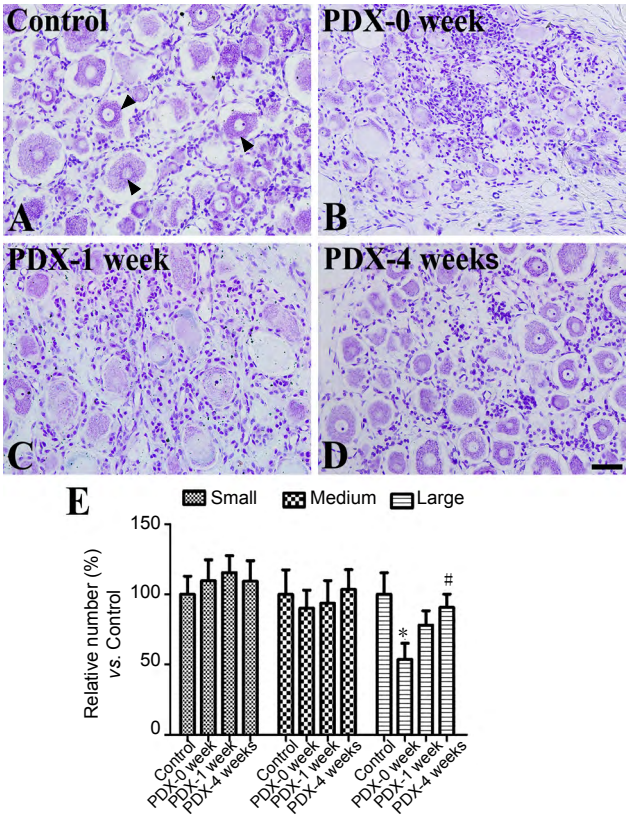

Figure 1 Cresyl violet staining in the dorsal root ganglia of the control (A) and PDX groups at 0 (PDX-0 week, B), 1 (PDX-1 week, C), and 4 weeks (PDX-4 weeks, D) after the last pyridoxine treatment.

(A–D) Many cresyl violet stained cells (arrowheads) are found in the control group. Pyridoxine intoxication causes decreases in large-sized neurons positive for cresyl violet and discontinuation of pyridoxine treatment gradually increases the number of large-sized neurons positive for cresyl violet in the dorsal root ganglia. Scale bar: 50 μm. (E) The percentage of the number of cresyl violet-positive cells in the dorsal root ganglia of the control and PDX groups ($n = 4$ per group; $*P < 0.05$, *vs.* control group; $\#P < 0.05$, *vs.* PDX-0 week group). The bars indicate the mean ± SD. PDX: Pyridoxine.

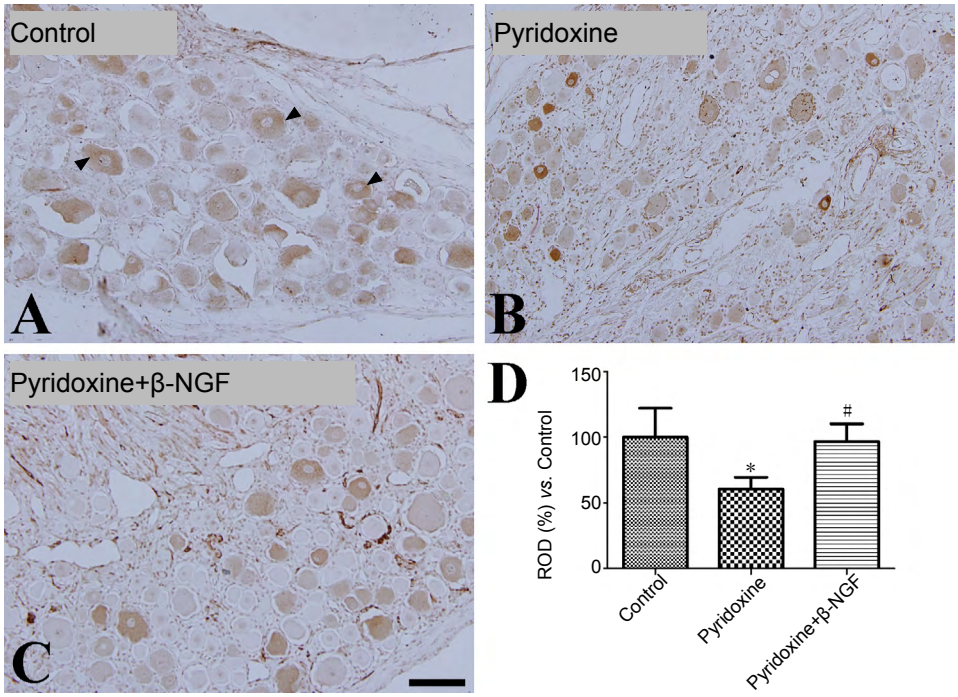

Figure 2 TrkA immunohistochemical staining in the DRG of the control (A), pyridoxine (B) and pyridoxine + β-NGF groups (C).

(A–C) TrkA immunoreactivity (arrowheads) in the control group was mainly found in the large-sized neurons in the DRG. In the pyridoxine group, strong TrkA immunoreactivity was detected in the small-sized neurons, but not in the large-sized neurons. β-NGF gene therapy significantly reduced the number of TrkA-immunoreactive cells in the DRG. (D) ROD was expressed as a percentage of the value representing the DCX immunoreactivity in the DRGs relative to the control group ($n = 4$ dogs per group; $*P < 0.05$, *vs.* control group; $\#P < 0.05$, *vs.* pyridoxine group). The bars indicate the mean ± SD. β-NGF: Beta-nerve growth factor; DRG: dorsal root ganglia; ROD: relative optical density; TrkA: tyrosine kinase receptor A (TrkA).

Discussion

In this study, we used dogs as an experimental model to identify the effects of dog β-NGF gene therapy. In a previous study, we confirmed the neuroprotective effects of β-NGF gene therapy against pyridoxine-induced sensory neuropathy in dogs (Chung et al., 2008b); however, factors responsible for neuroplasticity were not revealed. Therefore, in the present study, the effects of β-NGF gene therapy on the DRG were analyzed. First, we observed chronological changes in morphology of DRGs after pyridoxine intoxication. Pyridoxine intoxication significantly reduced the number of large-sized, but not small- or medium-sized, neurons in the DRG. This result is consistent with that of our previous studies showing that pyridoxine significantly decreased the number of large-sized neurons in dogs (Chung et al., 2008a) and chickens (Sharp and Fedorovich, 2015). In the present study, the discontinuation of pyridoxine treatment led to gradual reduction of neuronal damage in the DRG and resulted in a slightly lower number of DRG neurons at 4 weeks after the last pyridoxine treatment.

In our previous study, we observed the reduction of large-sized neurons positive for cresyl violet (51.7% *vs.* control group) in the DRG, while β-NGF gene therapy reduced the damage to large-sized DRG neurons by 78.1% relative to the control group (Kang et al., 2016). In the present study, we investigated the effects of β-NGF gene therapy at 3 days after the last pyridoxine treatment because discontinuation of pyridoxine treatment gradually ameliorated neuronal damage. First, we detected TrkA expression in the DRG to elucidate the effects of β-NGF gene therapy on the NGF-TrkA-mediated cell survival pathway because NGF binds to its high affinity cell surface receptor TrkA, which facilitates activation of intracellular signaling pathway related to neuronal differentiation and survival such as the MAPK, Akt and PKC pathways (Ibanez et al., 1990; Yoon et al., 1998; Wehrman et al., 2007). TrkA immunoreactivity was found in all DRG neurons, but TrkA immunoreactivity was strong in the medium- and large-sized DRG neurons. Pyridoxine caused neuronal damage in large-sized neurons and we did not detect TrkA immunoreactivity in damaged neurons, while strong TrkA immunoreactivity was observed in the small-sized neurons. β-NGF gene therapy significantly increased TrkA expression in the DRG. These results suggest that relatively low expression of TrkA in large-sized neurons may be associated with pyridoxine-induced cell damage, while strong TrkA immunoreactivity in small-sized neurons may be related to resistance to pyridoxine-induced neurotoxicity. In addition, β-NGF gene therapy facilitates NGF-TrkA signaling and reduces the neuronal damage in the DRG.

In this study, pyridoxine treatment significantly decreased the number of βIII-tubulin-immunoreactive large-sized DRG neurons compared to the control group. It has been reported that peripheral nerve degeneration, especially of large neurons, was associated with pyridoxine intoxication in rodent models (Kuntzer et al., 2004; Perry et al., 2004). In the present study, β-NGF gene therapy significantly reduced the loss of βIII-tubulin-immunoreactive large-sized neurons in dog DRGs that had been damaged by pyridoxine. These findings are consistent with the results of previous studies that showed NGF rescued DRG neurons from oxidative stress (Goins et al., 1999; Podratz and Windebank, 2005).

Pyridoxine, which is vitamin B_6, is closely related to neuronal signaling through the synthesis of neurotransmitters (Percudani and Peracchi, 2009), and vitamin B6 deficiency increases the risk of polyneuropathy (Spinneker et al., 2007). Paradoxically, vitamin B6 has also been identified as a neurotoxicant, and severe sensory neuropathological conditions caused by chronic abuse of oral vitamin B6 supplements have been described (Schaumburg et al., 1983; Dalton and Dalton, 1987). In a previous study, we made an animal model for neurotoxicity induced by vitamin B and observed significant reduction of large-sized neurons in the DRGs after pyridoxine intoxication (Chung et al., 2008a). However, the mechanism of this toxicity is not clear.

Few studies have investigated DCX immune responses under the condition of nerve damages. In the present study, DCX immunoreactivity was detected in the DRG to elucidate the role of DCX in the DRG after damage in dogs. Pyridoxine-induced DRG damage significantly reduced the number of DCX-immunoreactive large-sized cells, and DCX immunoreactivity was concentrated at the center of the DRG cells. However, one group showed the opposite phenomenon, in that DCX immunoreactivity was significantly increased in the central nervous system after various insults, including ischemia (Jin et al., 2001; Tonchev et al., 2003). The other groups also showed that DCX has abilities such as path-finding function of the growth cone and stopping the synapse signal in the peripheral nerve system (Jean et al., 2012; Bourgeois et al., 2015). Based on these data, we can assume that the paradoxical results depend on the time point of expression of DCX in the DRG during nerve damage. A previous study showed that intraventricular infusion of NGF to infants with hypoxic-ischemic damage significantly increased DCX expression (Chiaretti et al., 2008). In this study, we also confirmed that β-NGF gene therapy significantly ameliorated the reduction of DCX-immunoreactive neurons in the DRG. Based on these result, we can assume that β-NGF gene therapy may preserve DCX to maintain dendritic remodeling and synaptic maturation in the pyridoxine-damaged DRG. In-depth spinal cord studies are needed to assess the efficacy of β-NGF gene therapy in pyridoxine-induced neuropathic damage in this experimental model.

Overall, the results of this study indicated that β-NGF gene therapy could be a powerful method for treatment of pyridoxine-induced neuropathy by facilitating NGF-TrkA signaling-mediated neuronal plasticity and resuming DCX expression in the DRG.

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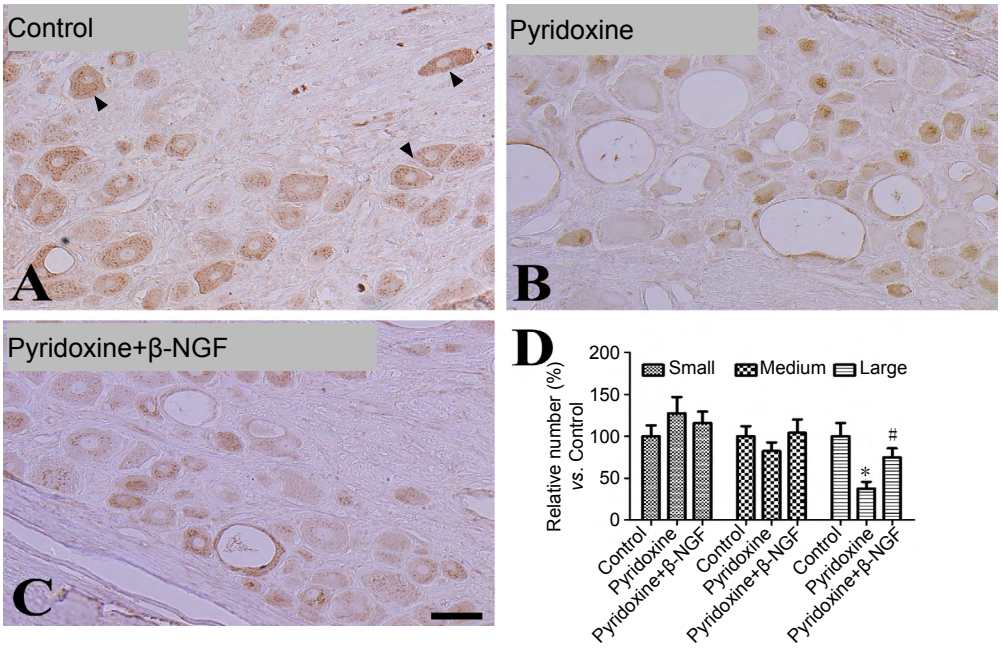

Figure 3 Immunohistochemistry for DCX in the DRG of the control (A), pyridoxine (B) and pyridoxine + β-NGF groups (C).
(A–C) The number of DCX-immunoreactive cells (arrowheads) was markedly decreased in the pyridoxine group, while the number of DCX-immunoreactive cells was significantly higher of the pyridoxine + β-NGF group than in the pyridoxine group. Scale bar: 100 μm. (D) The percentage of the number of DCX-immunoreactive cells in the DRG of the pyridoxine and pyridoxine + β-NGF groups, *vs.* the control group (n = 4 per group; $*P < 0.05$, *vs.* control group; $\#P < 0.05$, *vs.* pyridoxine group). The bars indicate the mean ± SD. β-NGF: Beta-nerve growth factor; DCX: doublecortin; DRG: dorsal root ganglia; ROD: relative optical density.

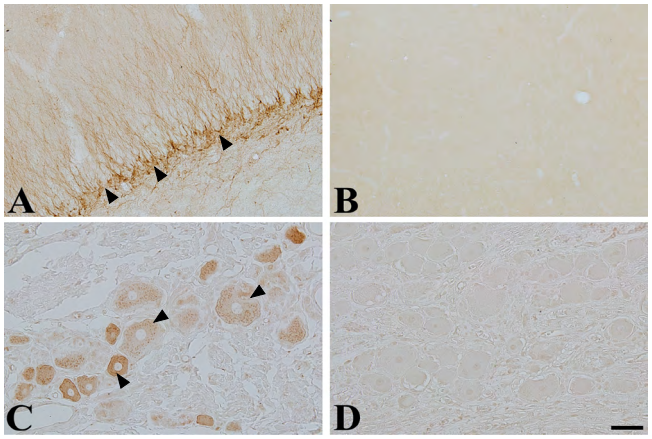

Figure 4 Confirmation of antibody specificity in the dentate gyrus and DRG tissue of dogs.
DCX immunoreactivity (arrowheads) was found in the subgranular zone of the dentate gyrus (A) and the DRG (C), while the negative control reveals no DCX-immunoreactive structures in the dentate gyrus (B) and the DRG (D). Scale bar: 100 μm. DCX: Doublecortin; DRG: dorsal root ganglia.

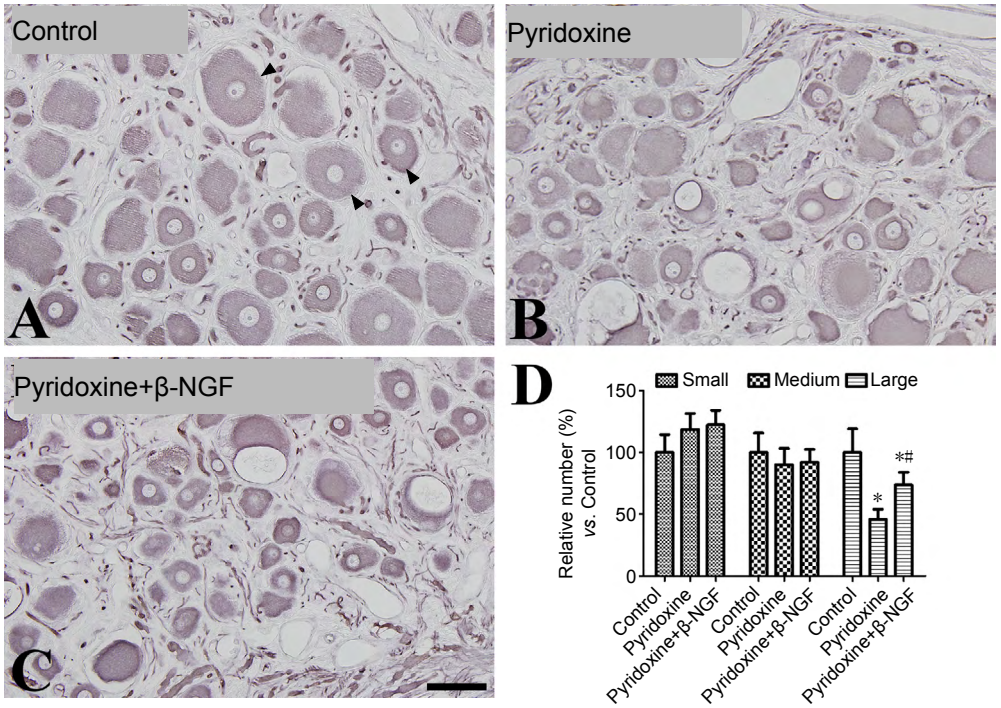

Figure 5 βIII-Tubulin immunohistochemical staining in the DRG of the control (A), pyridoxine (B), and pyridoxine + β-NGF groups (C).
(A–C) There were few βIII-tubulin-immunoreactive cells (arrowheads) in the pyridoxine group, even though many vacuoles (arrows) were detected. However, β-NGF gene therapy markedly ameliorated the reduction of βIII-tubulin-immunoreactive large-sized neurons in the DRG. Scale bar: 50 μm. (D) The percentage of the number of βIII-tubulin-immunoreactive cells in the DRG of the pyridoxine and pyridoxine + β-NGF groups, *vs.* the control group (n = 4 per group; $*P < 0.05$, *vs.* control group; $\#P < 0.05$, *vs.* pyridoxine group). The bars indicate the mean ± SD. β-NGF: Beta-nerve growth factor; DRG: dorsal root ganglia.

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