The Effects of Generally Administered Anti–Nerve Growth Factor Receptor (p75NTR) Antibody on Pain-Related Behavior, Dorsal Root Ganglia, and Spinal Glia Activation in a Rat Model of Brachial Plexus Avulsion

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Purpose To investigate the effect of intraperitoneal administration of an anti-p75 neurotrophin receptor (p75NTR) antibody on reducing neuropathic pain in a rat model of brachial plexus avulsion (BPA).

Methods We randomly assigned 40 male Wistar rats to 4 groups. In the BPA group, the C8-T1 roots were avulsed from the spinal cord at the lower trunk level, and saline was administered intraperitoneally. In the anti-p75NTR groups, 1 μL or 50 μL anti-p75NTR antibody was administered intraperitoneally after avulsion. In the sham-operated group, the lower trunk level was exposed, and saline was administered intraperitoneally. Mechanical hyperalgesia and pain-induced walking patterns were measured using von Frey filaments and CatWalk gait analysis at various time points until 15 days after administration.

At 3 and 15 days after administration, sensory neurons involved in pain perception and satellite glial cells in the ipsilateral C7 dorsal root ganglia were immunolabeled with antibodies against calcitonin gene-related peptide and glial fibrillary acidic protein (GFAP), respectively. At both time points, microglial and astrocyte activation, indicative of spinal pain transmission, were immunohistochemically examined in the ipsilateral dorsal horn of the spinal cord (C7) using anti-ionized calcium-binding adaptor molecule 1 and anti-GFAP antibodies, respectively.

Results The gait pattern was significantly improved in both anti-p75NTR groups compared with the BPA group. There were significantly fewer calcitonin gene-related peptide-immunoreactive (IR) neurons, neurons encircled by GFAP-IR satellite glial cells, and GFAP-IR astrocytes in both anti-p75NTR groups compared with the BPA group at both time points. Fewer ionized calcium-binding adaptor molecule 1-IR microglia were quantified in both anti-p75NTR groups compared with the BPA group, but this was only significant at 15 days after administration.

Conclusions Systemic application of the p75NTR inhibitory antibody suppressed neuropathic pain after BPA.

Clinical relevance p75NTR may be a potential therapeutic target for the clinical treatment of neuropathic pain in BPA injury. (J Hand Surg Am. 2015;40(10):2017–2025. Copyright © 2015 by the American Society for Surgery of the Hand. All rights reserved.)

Key words Brachial plexus avulsion, DRG, glial cell, neuropathic pain, p75NTR.

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Received for publication December 3, 2014; accepted in revised form June 12, 2015.

No benefits in any form have been received or will be received directly or indirectly to the subject of this article.

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Previous studies have reported a high incidence of severe neuropathic pain in patients with brachial plexus avulsion (BPA). This type of pain is rapid in onset, long-lasting, and often described as a constant crushing sensation with a superimposed lightning-like shooting pain that can be difficult to treat. In clinical practice, patients with neuropathic pain receive nonsteroidal anti-inflammatory drugs, opioids, anticonvulsants, antidepressants, surgery, or a combination of these options. However, these opioids, anticonvulsants, antidepressants, surgery, or... receptors would be viable treatment options for pain. Thus, antibodies targeting NGF or its cognate NTFS, nerve growth factor (NGF) plays a crucial role recognized as potential therapeutic targets. Among NTFS, nerve growth factor (NGF) plays a crucial role in pain. Thus, antibodies targeting NGF or its cognate receptors would be viable treatment options for pain. In clinical studies using an antibody against NGF, patients with osteoarthritis reported significant and clinically meaningful pain reduction; however, certain adverse effects were also described.

NGF has 2 structurally distinct receptors: tropomyosin-related kinase A receptor and p75 neurotrophin receptor (p75NTR); p75NTR binds to all NTFS with equal affinity, whereas tropomyosin-related kinase A receptor is specific in its binding. We previously showed that local application of the anti-p75NTR antibody directly onto the avulsed brachial plexus at the time of the brachial plexus avulsion surgery could reduce neuropathic pain and suppress glial cell activation in a BPA rat model. However, in clinical practice, it is difficult to directly apply drugs onto the avulsed brachial plexus at the time of injury. We hypothesized that systemic application of the anti-p75NTR antibody 1 week after BPA injury could reduce neuropathic pain. We selected this time point based on the assumption that it would take at least 1 week for patients to be referred to hand specialists after BPA injury.

In the current study, we investigated the effect of different systemic doses of the anti-p75NTR antibody on nociceptive behavior, neuronal calcitonin gene-related peptide (CGRP) expression, satellite glial cell (SGC) activation in the dorsal root ganglia (DRG), and spinal glial cell activation in a rat model of BPA.

Materials and Methods

Experimental animals

The ethics committee of our institutions approved all experiments for animal procedures, which were in accordance with the 1996 revision of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Forty 6-week-old male Wistar rats (170–200 g) were used.

Experimental groups and surgical procedure

Wistar rats were assigned to 4 groups: (a) sham, (b) BPA, (c) 1.0 μL anti-p75NTR, or (d) 50 μL anti-p75NTR. We used 20 rats (5 per group) for immunohistochemical analyses 3 days after anti-p75NTR antibody or saline administration. We used the other 20 rats (5 per group) for behavioral tests and immunohistochemical analyses 15 days after administration. After anesthesia with sodium pentobarbital (40 mg/kg intraperitoneally), the brachial plexus was approached through a horizontal incision parallel to the clavicle that ran from the sternum to the axillary region. The pectoralis major muscle was displaced, and the lower trunk of the brachial plexus was isolated from the surrounding tissues.

In the sham group, the right brachial plexus was exposed without any injury to the nerves, and 1 mL saline was administered intraperitoneally 1 week after surgery. In the BPA group, the right lower trunk was extracted from the spinal cord by traction using forceps, and the skin was closed with 4-0 nylon sutures; 1 mL saline was administered intraperitoneally 1 week after surgery. BPA model rats were generated as previously described by Rodrigues-Filho et al. In the anti-p75NTR groups (1.0 and 50 μL), the right lower trunk was avulsed and either 1.0 μL anti-p75NTR antibody (Millipore, Temecula, CA) + 999 μL saline or 50 μL anti-p75NTR antibody + 950 μL saline was administered intraperitoneally 1 week after surgery.

Behavioral tests

Von Frey withdrawal thresholds: Tactile allodynia was assessed using von Frey filaments (Stoelting, Wood Dale, IL) ranging from 0.008 to 180 g. Allodynia was assessed before and 3 days after surgery, and at 1, 6, 12, and 48 hours and 3, 6, 9, 12, and 15 days after the administration of the anti-p75NTR antibody or saline. Rats were placed in plastic cages with a raised wire mesh floor. The area tested was the plantar aspect of the right front paw from the first digit to the third digit in the C6 and C7 dermatome distribution. Von Frey monofilaments were applied vertically to the paw through the mesh floor until it just bent, and was kept in this position for 6 to 8 seconds. The filaments were applied in ascending order, and the smallest filament that elicited a paw withdrawal response was considered the threshold stimulus. Each rat was tested 3 times at 5-minute intervals in a blinded fashion, and the average threshold stimulus was calculated.
CatWalk: We used the CatWalk XT gait analysis system (Noldus Information Technology, Wageningen, the Netherlands) to evaluate pain-induced walking patterns on the same day as von Frey testing. This system has been described previously and has been used for the assessment of pain in models of neuropathic pain. The CatWalk device consisted of a glass plate on which a rat is allowed to walk freely from one side to the other in a darkened room. Light was injected into the glass plate and totally reflected internally. When a rat’s paws made contact with the plate, light was reflected on the opposite side, producing a series of illuminated footprints. These footprints were captured by a video camera (Sony 3CCD Color Video Camera; DXC-990/990P, Sony, Tokyo, Japan) and sent to a computer that ran CatWalk software 9.0 to record gait patterns and perform automated analyses. Because gait speed and consistency can affect data, the following requirements had to be met: rats needed to walk across the walkway at a constant pace, and a minimum of 3 steps per crossing was required. Three proper crossings were recorded at each time point, and the data were averaged. In this study, the print area (size of the paw in contact with the floor) was analyzed in a blinded fashion.

Tissue preparation: Rats were anesthetized intraperitoneally with sodium pentobarbital (40 mg/kg) and transcardially perfused with 500 mL 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) at 3 or 15 days after the administration of the anti-p75NTR antibody or saline. The ipsilateral C7 DRG and C7 spinal cord were removed, postfixed in the same fixative solution overnight at 4°C, soaked in 20% sucrose solution in 0.01 M phosphate-buffered saline for 20 hours at 4°C, and embedded in Optimal Cutting Temperature compound (Sakura Finetek, Tokyo, Japan). The cryoprotected samples were frozen and kept at −80°C until use.

Immunofluorescence staining for the DRG: Immunofluorescence, using an antibody against CGRP to label sensory neurons involved in pain perception and glial fibrillary acidic protein to label SGCs, was performed in the ipsilateral C7 DRG. DRG samples were cut transversely at 12 μm thickness and mounted onto poly-L-lysine-coated slides (Matsunami, Tokyo, Japan). The specimens were incubated for 90 minutes at room temperature in a blocking solution of 0.01 M phosphate-buffered saline containing 0.3% Triton X-100 (Nacalai Tesque, Kyoto, Japan) and 3% skim milk. The sections were then incubated overnight at 4°C with rabbit antibodies against CGRP (1:1000; Immunostar, Hudson, WI) and GFAP (1:400; DAKO, Glostrup, Denmark) as primary antibodies. Next, the sections were incubated in a fluorescent-conjugated secondary goat anti-rabbit antibody overnight at 4°C (Alexa Fluor 488, 1:1000; Invitrogen, Eugene, OR). Ten sections (2 per animal) in

FIGURE 1: Von Frey filament testing. Rats in the BPA group had significant mechanical hyperalgesia compared with the sham group throughout the postsurgical period ($P < .01$). Compared with the BPA group, mechanical hyperalgesia was significantly improved at 3 ($P < .05$), 9 ($P < .01$), and 12 days ($P < .05$) in the 1.0 μL anti-p75NTR group and at 6 ($P < .05$) and 48 hours ($P < .05$) and 3 ($P < .01$), 9 ($P < .01$) and 12 days ($P < .05$) in the 50 μL anti-p75NTR group. Ab, Antibody; NS, normal saline.
each group were observed at 400× magnification using a fluorescence microscope (BX51, OLYMPUS, Tokyo, Japan).

We evaluated the proportion of CGRP-immunoreactive (IR) neurons by counting the number of small CGRP-positive neurons in a blinded fashion and dividing it by the number of all small neurons in the ipsilateral C7 DRG. We evaluated the proportion of neurons encircled by activated SGCs by counting the number of the neurons surrounded by GFAP-IR SGCs in a blinded fashion and dividing it by the total number of neurons in the ipsilateral C7 DRG.

**Immunofluorescence staining for the spinal cord:** Immunofluorescence, using antibodies against ionized calcium-binding adaptor molecule 1 (Iba1) to label microglia and GFAP to label astrocytes, was performed to study glial reactivity in the ipsilateral dorsal horn of the spinal cord (C7).

Samples of C7 spinal cord were cut transversely at 30 μm thickness and mounted onto poly-L-lysine-coated slides. The specimens were incubated for 90 minutes at room temperature in a blocking solution of 0.01 M phosphate-buffered saline containing 0.3% Triton X-100 and 3% skim milk. The sections were then incubated in a primary rabbit antibody against Iba-1 (1:1000; Wako, Osaka, Japan) and a rabbit antibody against GFAP (1:400, DAKO) overnight at 4°C. The sections were then incubated in a primary rabbit antibody against Iba-1 (1:1000; Wako, Osaka, Japan) and a rabbit antibody against GFAP (1:400, DAKO) overnight at 4°C. Next, the sections were treated with a fluorescent-conjugated secondary goat anti-rabbit antibody for 24 hours at 4°C, and IR signals were observed using a fluorescence microscope.

Using the ImageJ software (National Institutes of Health, Bethesda, MD), Iba1-IR microglia and GFAP-IR astrocytes were counted in 0.5 mm² visual fields of the ipsilateral

**FIGURE 2:** CatWalk (print area). The BPA group had a significantly smaller print area than the sham group throughout the postsurgical period (P < .01). The print area in the 1.0 μL anti-p75NTR group was significantly improved at 1 (P < .05), 6 (P < .05), and 48 hours (P < .05) and after 9 (P < .05) and 12 days (P < .01). In the 50 μL anti-p75NTR group, the print area was significantly improved at 1 (P < .01), 6 (P < .05), and 48 hours (P < .01) and 3 (P < .05), 6 (P < .05), 9 (P < .05), and 12 days (P < .01) after anti-p75NTR antibody administration compared with the BPA group. Ab, Antibody; NS, normal saline.

**FIGURE 3:** Percentage of CGRP-IR neurons (C7 DRG ipsilateral side). The proportions of CGRP-IR neurons among all small neurons in the ipsilateral C7 DRG at 3 and 15 days after administration were 41.5 ± 3.8% and 43.8 ± 2.9% in the sham group, 59.5 ± 4.3% and 62.8 ± 5.7% in the BPA group, 50.8 ± 4.1% and 41.1 ± 2.3% in the 1.0 μL anti-p75NTR group, and 47.0 ± 2.9% and 44.7 ± 2.0% in the 50 μL anti-p75NTR group, respectively.
dorsal horn of the spinal cord (C7) were counted in 5 randomly selected sections from each group in a blinded fashion. These counts were then averaged.

Statistical analysis

Results of the behavioral tests and immunofluorescence staining were compared among the 4 groups with Kruskal–Wallis tests followed by Mann–Whitney U tests. Bonferroni corrections were performed, and \( P < .05 \) was considered statistically significant.

RESULTS

Behavioral tests

Von Frey withdrawal thresholds: Rats in the BPA group displayed significantly greater mechanical hyperalgesia compared with rats in the sham group throughout the postsurgical period (\( P < .01 \)). Mechanical hyperalgesia in the 1.0 \( \mu \)L anti-p75NTR group was significantly improved at 3, 9, and 12 days, whereas hyperalgesia in the 50 \( \mu \)L anti-p75NTR group was significantly improved at 6 and 48 hours and 3, 9, and 12 days after the anti-p75NTR antibody administration compared with the BPA group (Fig. 1).

CatWalk: Rats in the BPA group had a significantly smaller print area compared with the sham group throughout the postsurgical period (\( P < .01 \)). The print area in the 1.0 \( \mu \)L anti-p75NTR group was significantly improved at 1, 6, and 48 hours, and 9 and 12 days. In the 50 \( \mu \)L anti-p75NTR group, the print area was significantly improved at 1, 6, and 48 hours and 3, 6, 9, and 12 days after the administration of the anti-p75NTR antibody compared with the BPA group (Fig. 2).

Immunofluorescence staining: The number of CGRP-IR neurons was significantly increased in the BPA group compared with the sham group at both 3 and 15 days after administration (\( P < .01 \)). In both anti-p75NTR groups, the numbers of CGRP-IR neurons were significantly reduced compared with the BPA group at both time points (\( P < .01 \)). There was also a significant difference between the 1.0 and 50 \( \mu \)L anti-p75NTR groups for CGRP-IR neurons at 3 days (\( P < .05 \)) (Figs. 3, 4).

The number of neurons encircled by GFAP-IR SGCs was significantly increased in the BPA group compared with the sham group at both 3 and 15 days.
after administration ($P < .01$). However, the numbers of neurons in both anti-p75NTR groups were significantly reduced compared with the BPA group at both time points ($P < .01$) (Figs. 5, 6).

The number of Iba1-IR microglia was significantly increased in the BPA group compared with the sham group at both 3 and 15 days after administration. Iba1-IR microglia in both anti-p75NTR groups were only significantly reduced compared with the BPA group 15 days after administration ($P < .01$). There was no significant difference between the BPA group and either anti-p75NTR group 3 days after administration (Figs. 7, 8).

The number of GFAP-IR astrocytes in the ipsilateral dorsal horn of the spinal cord (C7) was significantly increased in the BPA group compared with the sham group at both 3 and 15 days after administration. GFAP-IR astrocytes in both anti-p75NTR groups were significantly reduced compared with the BPA group at both time points ($P < .01$) (Figs. 9, 10).

**DISCUSSION**

In the developing nervous system, the primary role of NGF is in neuronal survival; however, this role shifts to the generation of pain and hyperalgesia in several acute and chronic pain states in adults. Therefore, pharmacological agents were developed to block NGF signaling at the receptor level, and antibodies against NGF. A number of clinical trials using antibodies against NGF, such as tanezumab, fulranumab, REGN 475, PG 110, and MEDI 578, have been performed. Among these compounds, clinical trials with tanezumab are the most advanced and have demonstrated significant and clinically meaningful improvements in pain and function. However, adverse side effects that are generally mild to moderate in intensity have also been reported. Thus, we investigated whether the anti-p75NTR antibody could reduce neuropathic pain in a BPA rat model. Although we did not investigate adverse events associated with this antibody, Iwakura et al reported no adverse events in a rat CFA-induced pain model.

For gait analysis using the CatWalk system, we found that the print areas in both anti-p75NTR groups were significantly improved compared with the BPA group. The CatWalk system has been used for assaying impaired gait function in the spinal cord, sciatic nerve, and knee. As previously reported...
by Matsuura et al., walking patterns in animal models of neuropathic pain such as chronic constriction injury may be influenced not only by paw pain but also by paw paralysis. However, improved walking patterns observed in BPA models likely correspond to a reduction in pain because the paralysis caused by complete BPA is never recovered without operative treatment. Therefore, the improvement of the print area in this study was likely due to pain reduction from the anti-p75NTR antibody.

We found that CGRP-IR small neurons and neurons encircled by GFAP-IR SGCs in both anti-p75NTR groups were significantly reduced compared with the BPA group. Calcitonin gene-related peptide is a neuropeptide that is not only related to inflammatory pain but also to neuropathic pain. SGCs, which surround neurons in the DRG, play a critical role in pain after inflammation or nerve injury. After nerve injury, SGCs are activated and provide sources of cytokines such as tumor necrosis factor-α and NTFS. In addition, SGCs in the rat DRG possess p75NTR and tropomyosin-related kinase A receptor receptors that enable SGCs to be influenced by NGF released from neurons. Therefore, we suspected that one of the possible sites of anti-p75NTR antibody interactions in our model was between neurons and SGCs in the DRG.

Paszcuk et al reported that glial cells are important in modulating BPA-induced neuropathic behavior. Many studies have demonstrated the important roles of microglia and astrocytes in neuropathic pain in releasing proinflammatory cytokines, chemokines, and other mediators that facilitate pain signaling, such as CGRP, substance P, and glutamate. In our current study, we found that numbers of Iba1-IR microglia in both anti-p75NTR groups were significantly reduced compared with the BPA group at 15 days, but not at 3 days, after the administration of an antibody. In contrast, the numbers of GFAP-IR astrocytes were significantly reduced after the administration of the anti-p75NTR antibody at both time points. There are several possible reasons for these findings. After nerve injury, microglia in the spinal cord undergo more rapid proliferation than astrocytes, and this proliferation is already prominent 2 days after spared nerve injury. Therefore, the microglial proliferation that occurred 3 days after the administration of an antibody in this study was too intense to be effectively inhibited. Although astrocytes are similar to microglia in some aspects of local immune signaling, the functions of the 2 cell types probably differ with respect to the local immune response when nerve injuries progress from acute to more chronic stages. For example, microglia might

**FIGURE 8:** Iba1-IR microglia (C7 ipsilateral side).

**FIGURE 9:** Number of GFAP-IR astrocytes (C7 ipsilateral side). The numbers of GFAP-IR astrocytes in the ipsilateral dorsal horn of the spinal cord (C7) at 3 and 15 days after administration were 431.1 ± 83.2 and 448.0 ± 104.6 in the sham group, 1299 ± 151.9 and 901.1 ± 158.8 in the BPA group, 893.6 ± 69.6 and 688.6 ± 149.0 in the 1.0 μL anti-p75NTR group, and 828.7 ± 51.2 and 619.8 ± 111.0 in the 50 μL anti-p75NTR group, respectively.
contribute to the genesis of neuropathic pain, whereas astrocytes might contribute to its maintenance.  

The systemic administration of the anti-p75NTR antibody to a rat model of BPA improved pain. p75NTR may be a potential therapeutic target for the clinical treatment of neuropathic pain in BPA injury. However, anti-p75NTR antibody treatment could impair nerve regeneration in patients with BPA injury who rely on spontaneous recovery or undergo nerve transfers and/or grafts. Further research will be necessary to compare the risks and benefits of this treatment to determine its clinical applicability. The use of this treatment in a clinical setting will require basic scientific studies to investigate adverse effects for nerve regeneration in an incomplete brachial plexus injury or after nerve repair surgery. Specifically, the optimal dose, number of doses, and application methods should also be evaluated.

There are some limitations to this study. We did not investigate the mechanisms of pain or sites of anti-p75NTR antibody action in the BPA rats. However, we speculate that 2 possible mechanisms could underlie pain development. First, BPA directly activates glial cells and aids in the development of pain. Second, increased CGRP and activated SGCs in the DRG serve as transmitters for glial cell activation in the spinal cord and subsequent pain development in the BPA model. With regard to possible sites of anti-p75NTR antibody action, we speculate that intraperitoneal administration and subsequent hematogenous spreading first affect the DRG, followed by glial activation in the spinal cord. However, it could be that intraperitoneal administration of this antibody could directly act on the spinal cord because the blood-spinal cord barrier is ruptured after BPA. Moreover, we did not confirm anti-75NTR antibody binding to cellular targets, so it is unclear whether the anti-p75NTR antibody exerted its effects by binding p75NTR. Additional studies are needed to clarify this point. Finally, because this experiment was completed 3 weeks after BPA induction, future investigations will be needed to identify an optimal treatment paradigm for the chronic phase.

ACKNOWLEDGMENTS

The authors would like to thank Takane Suzuki, MD, PhD, Kenichi Murakami, MD, PhD, Ken Hashimoto, MD, PhD, Masataka Shibayama, MD, PhD, Seiji Okamoto, MD, PhD, Yasufumi Ogawa, MD, Koji Sukegawa, MD, Nahoko Iwakura, MD, PhD, Sumihisa Orita, MD, PhD, Miyako Suzuki, MD, PhD, Go Kubota, MD, PhD, Yoshihiro Sakuma, MD, PhD, Kazuhide Inage, MD, and Takeshi Sainoh, MD, for technical assistance.

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