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Brief Communication

Motor nerve graft is better than sensory nerve graft for survival and regeneration of motoneurons after spinal root avulsion in adult rats

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ABSTRACT

In the present study, we compared the effects of implanting peripheral sensory nerve and motor nerve on motoneuron survival and regeneration after spinal root avulsion in adult rats. Our results showed that 116% more motoneurons regenerated axons into the motor than the sensory nerve graft and 59% of motoneurons survived in the motor nerve-implanted group compared to 48% in the sensory nerve-implanted group. We demonstrated by real time PCR that levels of BDNF and GDNF mRNA were significantly higher in the motor than the sensory nerve five days after implantation into the spinal cord. This may account for the superiority of motor over sensory nerve in promoting motor axon regeneration and motoneuron survival. Lastly, we also showed that implanting two sensory nerves enhances motoneuron regeneration over implanting a single nerve.

We previously showed that implantation of peripheral nerve or reimplantation of avulsed ventral root rescues the injured motoneurons from degeneration and provides a conduit for regrowing axons after avulsion (Gu et al., 2004, 2005; Wu et al., 1994, 2004). In this study, we compared the effects of implanting sensory nerve with those of ventral root after avulsion. The identification of sensory and motor Schwann cells and their differential trophic factor regulation after transection (Hoke et al., 2006) suggested that motoneurons might respond differently to sensory and motor nerves containing these two phenotypes of Schwann cells. Also, our goal was to determine whether sensory nerve is a suitable choice for grafting after avulsion injuries. Current treatments for avulsion patients include nerve transfer (Midha, 2004), reimplantation of avulsed roots, or implantation of sensory nerve graft(s) to form a bridge from the spinal cord to distal nerves (Carlstedt et al., 1995, 2000). The latter two methods rescue the injured motoneurons and allow reconnection with the peripheral circuitry. However, no experimental study has yet compared the effects of the modality of the graft in this type of injury. Our study aimed to shed light on this issue.

All procedures were approved by the Committee for the Use of Live Animals in Teaching and Research at the University of Hong Kong. Twenty-four adult male Sprague–Dawley rats weighing 280–320 g were used for morphological study.

Animals underwent surgical procedures similar to our previous study (Chu and Wu, 2006) with minor modifications. Briefly, under deep anesthesia the seventh cervical (C7) spinal roots (both dorsal and ventral) were avulsed with a fine hook. The avulsed roots together with the dorsal root ganglion were removed. To determine the effects of sensory and motor nerve implantation, two types of nerve graft were used: size-matched allogeneic saphenous nerve (sensory nerve, $n=6$) and allogeneic ventral root from the fourth or fifth lumbar segment (motor nerve, $n=6$). The nerve grafts were about 20 mm long and were marked by 10-0 suture (Ethicon; Johnson & Johnson) on the epineurium at 10 mm from the proximal end. We also compared the effects of implanting a single autologous saphenous nerve ($n=6$) with co-implantation of two autologous saphenous nerves ($n=6$). After avulsion, the nerves were implanted into the lateral funiculus and secured by 11-0 suture (Ethicon; Johnson & Johnson). The distal end of the graft was implanted into a juxtaposed skeletal muscle. Four animals receiving no further treatment after avulsion served as lesion control. Muscles and skin were closed in layers. Animals were kept for four weeks before perfusion.

The immunosuppressant cyclosporine A (CsA) was injected intraperitoneally (20 mg/kg) immediately after surgery in allogeneic nerve-implanted animals. The animals were then injected subcutaneously with 10 mg/kg CsA daily. Two days before the animals were killed, the nerve graft was exposed at the 10 mm position, guided by the 10-0 suture, and 0.5 μl of 6% Fluoro-Gold (FG; Fluorochrome, LCC) was

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injected into the nerve graft with a 10 μl Hamilton syringe to retrogradely label regenerated motoneurons.

At the end of the survival period, the animals were given a lethal dose of sodium pentobarbital. They were then perfused intracardially with normal saline followed by fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The spinal cords were harvested and post-fixed with fresh fixative overnight and subsequently placed in 30% phosphate-buffered sucrose. After the samples had sunk, they were cut into 40 μm sections by microtome and collected in 0.01 M phosphate-buffered saline (PBS; pH 7.4). They were subsequently mounted on microscopic slides for fluorescence microscopy and FG-positive motoneurons with visible nuclei were counted. To assess motoneuron survival, every alternate section from all samples was counterstained with 1% neutral red and motoneurons on the ipsilateral and contralateral sides of the C7 segment were counted under a light microscope following the method described previously (Wu et al., 1994, 2003).

To study the differences in trophic factor regulation in the two nerve types, thirty-six rats that underwent the same surgical procedures as above were used for measuring the changes in brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) mRNA levels. Additional rats were used to harvest normal un-operated saphenous nerves and ventral roots. Three groups including implantation with allogeneic saphenous nerve (with CsA), allogeneic ventral root (with CsA) or autologous saphenous nerve (without CsA) were studied. Five days after implantation, the nerves (twelve in each group, four nerves pooled into one sample, $n=3$) were harvested and 2 mm from both ends was removed to exclude regenerating axons in the proximal and infiltrated muscle cells in the distal end. Total RNA was extracted using TRIzol reagent (Invitrogen). The cDNA synthesis was performed using an Advantage® RT-for-PCR kit (Clontech) and oligo (dT) primers with 2 μg of total RNA. Primer sequences used to study the gene expression of BDNF and GDNF are listed in Table 1. Quantification of mRNA levels was performed by real time RT-PCR using SYBR® Green (Invitrogen). An iCycler thermal cycler (Bio-Rad) was used, with cycling parameters of 95 °C (2 min) and 32 cycles of 94 °C (30 s), 57 °C (30 s) and 72 °C (40 s) followed by a final elongation step at 72 °C (5 min). The samples were run in triplicate and the experiments were repeated at least twice. Relative targeted gene expression was normalized to the internal control gene, β-actin, in the same PCR reaction. Changes in mRNA levels five days after implantation were normalized to mRNA levels in corresponding nerve types from normal un-operated animals. PCR products were analyzed in 2% agarose gel to confirm a single band with the correct size.

All results are expressed as mean ± SD. Student's *t*-test or one-way ANOVA, where appropriate, was used to determine the statistical significance of differences among the means. A p-value of <0.05 was considered significant.

We used saphenous nerve instead of dorsal root because, clinically, cutaneous peripheral nerve is used instead of dorsal root. This also allowed us to cross-check our data with our previous results. We chose an allogeneic ventral root from the fourth or fifth lumbar segment instead of re-implanting the avulsed root because we needed a motor nerve as long as 2 cm and a cross-sectional area comparable to that of the saphenous nerve (58,512 μm^2 for saphenous nerve and 52,199 μm^2 for ventral root from a lumbar segment). Results of the present study found that double the number of motoneurons regenerated into ventral root than saphenous nerve (190 ± 18 *vs*. 88 ± 14 motoneurons, *t*-test, $p<0.001$, Figs. 1A, B, C) and significantly more motoneurons survived in the ventral root-implanted group (59 ± 4% *vs*. 48 ± 3%, *t*-test, $p<0.01$, Fig. 1C).

Trophic factor up-regulation is well-documented upon denervation in the distal nerve stump after peripheral nerve injury. We therefore compared the expression of two well-known neurotrophic factors for motoneurons, BDNF and GDNF in these nerves. We harvested the nerves five days after implantation because motor axons grow into the nerve graft between three and seven days post-implantation (unpublished data), thus the expression of these trophic factors should still have remained high at the time of nerve harvest. By normalizing with their corresponding nerve types from normal animals, both allogeneic sensory nerve and ventral root showed up-regulation of BDNF and GDNF mRNA. CsA administration in allograft-implanted animals did not seem to affect the expression of these genes because mRNA up-regulation in the autologous nerve graft did not differ from that in the allograft with CsA treatment. We found a markedly higher expression of these genes in motor than in sensory nerve (Figs. 1E and F, one-way ANOVA, $p<0.01$ in BDNF and $p<0.05$ in GDNF mRNA level).

We postulated that different trophic levels in the sensory and motor nerves accounted for the difference in degree of regeneration. The low level of regeneration into a sensory nerve might be overcome by implanting an additional sensory nerve at the avulsed site. We tested this idea by implanting two autologous sensory nerves and compared the results with only a single saphenous nerve. An additional nerve did not further promote survival, but more motoneurons regenerated into the two-nerve-implanted group (285 ± 73 *vs*. 179 ± 32 motoneurons, *t*-test, $p<0.05$, Fig. 1D). Autologous nerves were used here because we could harvest two saphenous nerves from one animal and avoid the use of CsA. Our results indeed showed that an extra graft did promote more motoneurons to regenerate. Besides having more trophic factors available, increased contact area with the spinal cord also increased the chance for axons to grow into the graft. Further experiments are needed to distinguish between these two factors.

Our results showed that motor nerve is superior to sensory nerve in supporting motoneuron survival and axonal regeneration. The results are consistent with other studies in peripheral nerve injury models (Lago et al., 2007; Nichols et al., 2004; Hoke et al., 2006). Hoke's study showed that Schwann cells can be subdivided into motor and sensory phenotypes with differential regulation of trophic factors after denervation and reinnervation. We tested two of the most commonly studied neurotrophic factors. BDNF and GDNF are potent motoneuron survival factors which work in the nanomolar range (Henderson et al., 1994; Yan et al., 1992). We previously showed that a single application of BDNF or GDNF rescues most of the injured motoneurons from death (Wu et al., 2003). Numerous *in vitro* and *in vivo* studies show that they promote axonal growth (Keller-Peck et al., 2001; Blits et al., 2004; Fine et al., 2002; Boyd and Gordon, 2003). Results of the present study showed that both BDNF and GDNF mRNA levels were up-regulated in sensory and motor nerves five days after implantation when degeneration occurs and only a few, if any, axons have entered the grafts. The greater regenerative properties of motor nerve are likely due to higher expression of these two potent trophic factors in motor nerve than in sensory nerve. Our results differ from Hoke's findings which indicated that GDNF is predominant in motor nerve whereas BDNF predominates in sensory nerve (Hoke et al., 2006). They showed that the BDNF mRNA level in ventral root did not change after denervation or reinnervation by sensory or motor axons over a study period of one month. The different outcomes in the two studies are probably due to the use of different injury models. The nerves in our study were grafted between spinal cord and skeletal

Table 1
Primer sequences used to study the gene expression of BDNF and GDNF in different types of nerves

Gene	Primer pair	Genbank accession number
BDNF	Sense: 5'-GGTCACAGCGGCAGATAAAAAGAC-3' Antisense: 5'-TTGGGTAGTTCGGCATTGCGAG-3'	NM_012513
GDNF	Sense: 5'-GACTCCAATATGCCCGAAGA-3' Antisense: 5'-TCAGTTCCTCCTTGGTTTCG-3'	NM_019139
β-actin	Sense: 5'-AGCCATGTACGTAGCCATCC-3' Antisense: 5'-CTCTCAGCTGTGGTGGTGAA-3'	NM_031144

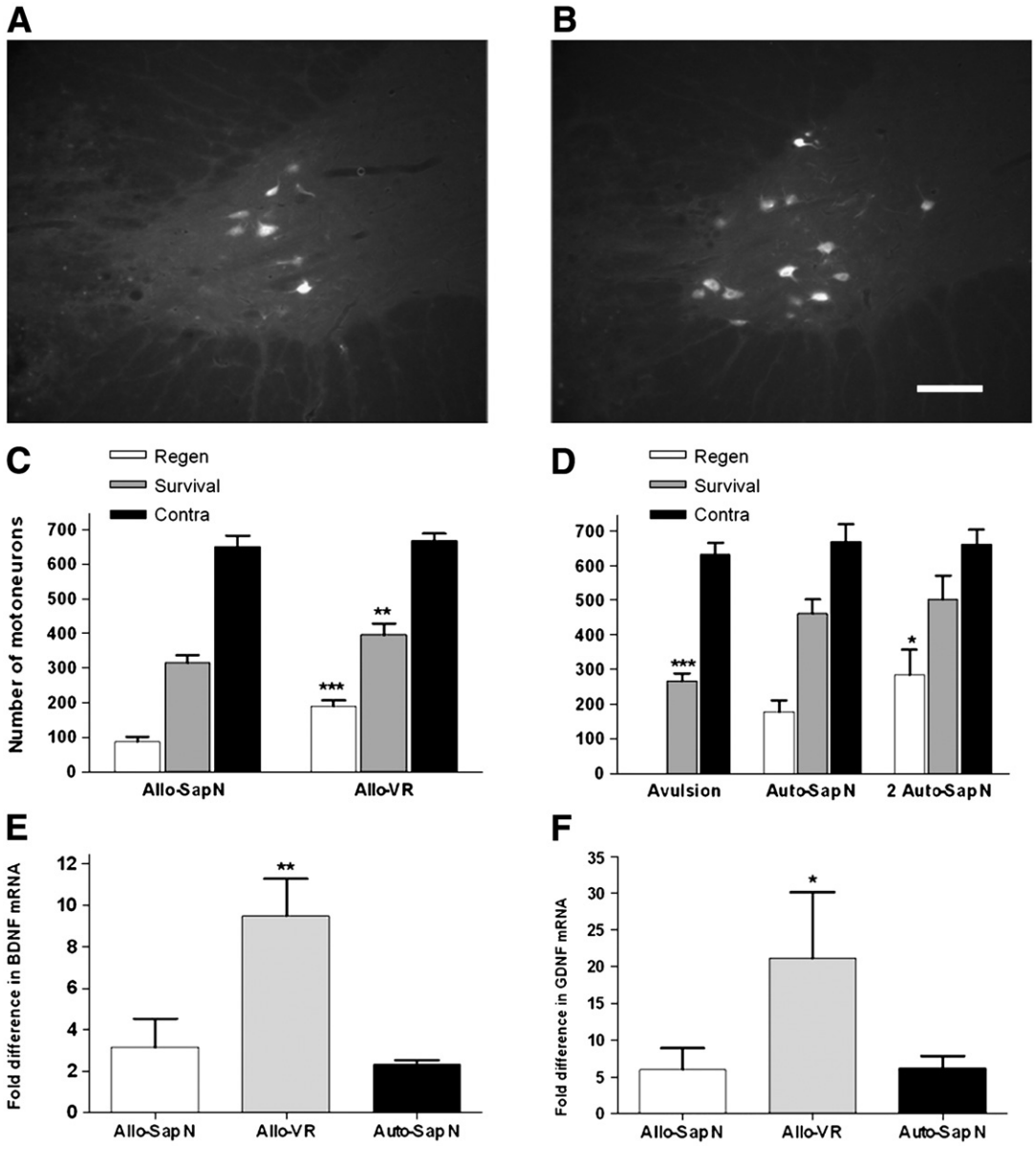

Fig. 1. (A and B): Representative pictures showing Fluoro-Gold labeled motoneurons from saphenous nerve-implanted (A) and ventral root-implanted (B) animals. Scale bar = 200 μm. (C): Number of regenerated (regen) and survived (survival) motoneurons at ipsilateral side in allogeneic saphenous nerve (Allo-Sap N) and ventral root (Allo-VR)-implanted animals. Number of motoneurons at contralateral side (contra) is shown in solid bar. (D): Number of regenerated and survived motoneurons in a single autologous nerve (Auto-Sap N) and two autologous saphenous nerve (2 Auto-Sap N)-implanted animals, number of survived motoneurons after avulsion is also given. Significantly more motoneurons survived after nerve implantation ($p<0.001$). (E and F): Fold increase of BDNF (E) and GDNF (F) mRNA levels in different nerve grafts relative to corresponding sensory or motor nerve from normal animals. * denotes $p<0.05$; ** $p<0.01$; *** $p<0.001$.

muscle after root avulsion, without the influences from the host nerves in the peripheral nerve injury model. Another possibility is that a different rate of revascularization of the grafts resulted from the different surgical procedures. However it is not known whether ischemic conditions affect trophic factor gene expression in Schwann cells. It would be of interest to investigate whether such differences also exist for other trophic factors in these two models.

Differences in the levels of trophic factors are not the only reason for preferential reinnervation by motor axons. Axonal regeneration adopts a form of redundancy to ensure correct innervation. It was shown recently that PSA-NCAM is expressed predominantly in motor axons and is required for the specificity of the motor axon–motor Schwann cell partnership (Franz et al., 2005). Besides reducing axon–axon adhesion by the polysialic acid moiety, an interaction between the motor axons and the sensory Schwann cells is also possible. Correct reinnervation is eventually based on the mutual interaction between axons and Schwann cells.

It is noteworthy that the degree of regeneration was much reduced in allogeneic nerve implantation compared to autologous implantation. CsA administration is unlikely to affect the expression of trophic factors, at least BDNF and GDNF, as shown in our study. In addition, CsA is not likely to decrease axonal regeneration, since the same CsA treatment protocol in autologous nerve-implanted animals did not show any reduction in the number of regenerated motoneurons ($n=4$, data not shown). The negative effects were most likely caused by the immunogenicity of allogeneic nerve (Evans et al., 1994), particularly in the outbred animals used in the study. Similar results have been obtained in experimental and clinical studies (Evans et al., 1994; Rodriguez et al., 2000; Siemionow and Sonmez, 2007). These data highlight the importance of avoiding the use of allografts in clinical settings. Therefore, despite our finding that motor

nerve is superior to sensory nerve in promoting regeneration, it is not suitable to use allogeneic motor nerve for grafting purposes. Reimplantation of avulsed roots is the best option when possible (Gu et al., 2004, 2005), otherwise, autologous saphenous nerve should be used.

In summary, our results showed that motor nerve is better than sensory nerve in promoting survival and regeneration. The beneficial effects are due to the higher levels of neurotrophic factors released by the motor Schwann cells in the motor nerve. However it is clinically impractical to use motor nerve for grafting purposes: implanting two or multiple sensory nerves is another alternative. A further possible alternative is to deliver trophic factors into the sensory nerve to promote regeneration across the central nervous system border.

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