胎児脊髄移植による神経再構築の実験的研究

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はしがき

平成４年度から、文部省科学研究費補助金（基盤研究C）の助成のもとに行われた「胎児脊髄移植による神経再構築の実験的研究」は２年間の研究期間を終了し、ここに研究成果をまとめることになった。研究計画のすべてが達成されたわけではないが、いくつかの新しい有用な知見が得られたと考えられる。報告書の作成にあたり、各分野の専門家の方々からの率直な御批判を願うものである。

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研究成果

1. Introduction

In avulsion injuries of spinal ventral roots and spinal cord injuries in which motor neurons are involved, regeneration of motor axons and muscle reinnervation cannot be expected [6]. However, there have been several reports of functional reconstruction using peripheral nervous system grafts or central nervous system grafts at the spinal cord level for reinnervation of the denervated muscles caused by these injuries [11,13,21,24]. Of special note is reconstruction using fetal central nervous system grafts, with which axonal regrowth by fetal neurons can be expected [22,23]. However. when transplanting nerve tissue to the spinal cord, it is difficult to assess whether the transplanted neurons are actually functioning.

We devised a simple model for transplantation of fetal spinal cord tissue to a peripheral nerve stump which has no contact with the central nervous system. Using this model, we investigated whether, at the peripheral nerve level, axonal regrowth by fetal spinal neurons could form new neuromuscular junctions.

2. Materials and methods

2.1. Preparation of the experimental model. (Fig.1).

Adult (6-8 week old) Sprague-Dawley rats weighing 150-250g were used. The sciatic nerve was exposed under intraperitoneal anesthesia with sodium pentobarbital (30mg/kg). About 20mm of the sciatic nerve was removed from the proximal level of the greater trochanter to the mid thigh level. The proximal stump was ligated to prevent regeneration. This condition was the model of denervation, and used for the control group. About 10mm of an autologous femoral vein was removed from the contralateral side. The vein was then sutured to the epineurium of the distal stump to prepare a cavity for transplantation of the fetal spinal cord. Fetuses were excised from rats on the 14th day of pregnancy under intraperitoneal anesthesia with sodium pentobarbital (30mg/kg) for transplantation of the fetal

spinal cord. About 20 fetuses can be obtained from one pregnant rat. The dura mater of the fetus was excised under a surgical microscope, and the fetal spinal cord from the cervical to lumber region was excised in a single piece. The dorsal root ganglion and the sympathetic ganglion were not included in the fetal spinal cord. The excised spinal cord was tentatively suspended in Hank's balanced salts solution. The suspension containing several spinal cords was aspirated into a microsyringe, and injected into the cavity prepared previously with the vein. As the fetal spinal cord passed through the needle of the syringe, it was gently minced. About 30 μl of fetal spinal cord tissue stayed in the vein cavity, facing to the distal stump of the sciatic nerve. This condition was the experimental group. The procedure from excision of the spinal cord to transplantation took approximately 5 minutes.

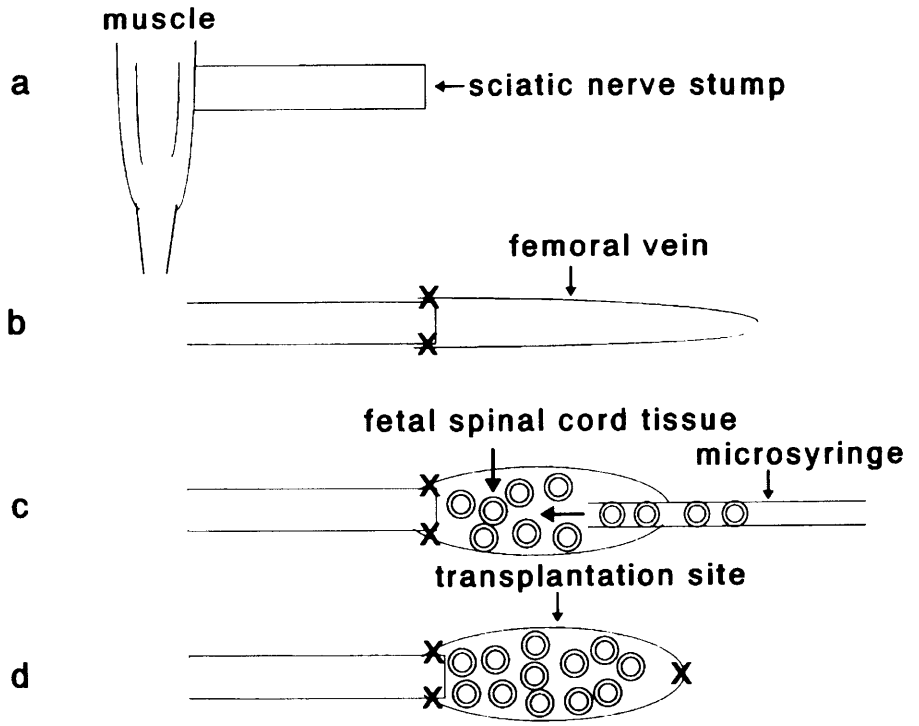


Figure 1. Preparation of the experimental model.

In the adult rats, a 20mm length of sciatic nerve was removed and the proximal stump ligated (a). The autologous femoral vein (10mm in length) was transplanted to the distal stump of the sciatic nerve (b). Then the spinal cord taken from the rat fetus (day 14 of gestation) was transplanted into the cavity of the vein (c, d). In the control group, only sciatic nerve transection was performed.

This transplantation was performed in 23 rats, with 11 rats serving as controls. After 3-6 months, electrophysiological and histochemical evaluations were carried out. Electrophysiological analysis and evaluation of the wet weight of the muscles were performed in 13 experimental and 11 control rats. Muscle histochemistry was performed in 3 of the 13 experimental rats. Regarding the additional 10 experimental rats, immunohistochemistry for choline acetyltransferase (Chat) was performed in 3 rats and fluorescein labeling using cholera toxin B subunit was performed in 7 rats.

2.2. Electrophysiological analysis.

The tibial nerve and common peroneal nerve were exposed under intraperitoneal anesthesia with sodium pentobarbital (30mg/kg), and they were directly stimulated electrically (0.1-0.6 mA, 0.2 msec). The bipolar electrode used for stimulation was hook-shaped and the nerves were isolated to avoid the current spreading to surrounding tissues. Macroscopic muscle contraction of the gastrocnemius and tibialis anterior muscle was observed with recording of evoked muscle action potential by means of a needle electromyograph (Neuromatic 2000 M/C, DANTEC).

2.3. The wet weight of the muscles.

After electrophysiological analysis, the muscles were excised postmortem. To evaluate muscle atrophy, the wet weight of the gastrocnemius and tibialis anterior muscles was measured, and the weight ratio of treated muscle to contralateral non-treated muscle was calculated.

2.4. Muscle Histochemistry

The soleus muscles on the treated and non-treated sides of the experimental rats were excised, and frozen. Ten μm thick serial sections were made. Transverse serial sections were stained by a battery of histochemical methods including adenosine triphosphatase (ATPase) with preincubation at pH 10.7, 9.4, 4.6, 4.5, 4.3, 4.2. Type 1, 2A, 2B, and 2C fibers were identified according to the criteria of Brooke and Kaiser [4].

2.5. Immunohistochemistry for choline acetyltransferase(Chat)

The experimental group were anesthetized with sodium pentobarbital (30mg/kg) and perfused transcardially with 0.01M phosphate buffered saline (pH 7.4) followed by 4% paraformaldehyde, 0.2% picrate and 0.35% glutaraldehyde in 0.1M phosphate buffer (PB, pH 7.4) at 4℃. The site of the transplantation was removed and post-fixed overnight with 4% paraformaldehyde and 0.2% picrate in 0.1M PB at 4℃, followed by immersion for at least 4 hours in 0.1M PB containing 15% sucrose at 4℃. Twenty μm thick sections were cut with a cryostat. The sections were put through the following steps in a free-floating state: 1) incubated for three days with rabbit Chat antiserum (diluted 1:5000, Chemicon) at 4℃; 2) incubated for two hours with biotinylated goat anti-rabbit IgG (diluted 1:1000, Vector) at room temperature; 3) incubated for one hour with avidin-biotin-peroxidase complex (diluted 1:4000, Vector) at room temperature; 4) reacted for 10 minutes with a mixture containing 0.02% 3,3'-diaminobenzidine, 0.005%H_2O_2 and 0.3% nickel ammonium sulfate in a 0.05M Tris-HCL buffer (pH 7.6); 5) mounted on gelatin coated glass slides, dehydrated and cover-slipped with Entellan. All sections were examined under a light microscope equipped with a bright-field condenser.

2.6. Fluorescein labeling by cholera toxin B subunit (CTB, List Biological Lab), [15].

A solution of 0.5mg of CTB in 100μl of distilled water was prepared. Fifty μl quantities of the solution were injected into the muscle belly of gastrocnemius and tibialis anterior muscles of the experimental rats using a microsyringe. Forty-eight hours later, transcardial perfusion was performed under anesthesia with 0.01M phosphate buffer, followed by fixation with 0.1M phosphate buffer containing 1.0% paraformaldehyde and 1.25% glutaraldehyde. After the site of the transplantation was excised, it was frozen and 13μm thin sections were prepared using a cryostat. The sections were observed under a light-down fluorescence microscope (OPTIPHOT-2, Y2B-EFDA2, Nikon) at Excitation B. The rats which underwent fetal spinal cord transplantation but did not receive a CTB injection were observed

as controls. Observations were performed in rats which received CTB injections and 2 control rats.

3. Results

There were no problems in the experimental animals caused by removal of the femoral vein.

3.1. Electrophysiological analysis

Analysis was performed in 13 rats from the experimental group and all 11 control rats. Macroscopic muscle contraction and an evoked muscle action potential were observed in the gastrocnemius and tibialis anterior muscles of all rats in the experimental group in response to electrical stimulation of the tibial nerve and common peroneal nerve (Fig.2).

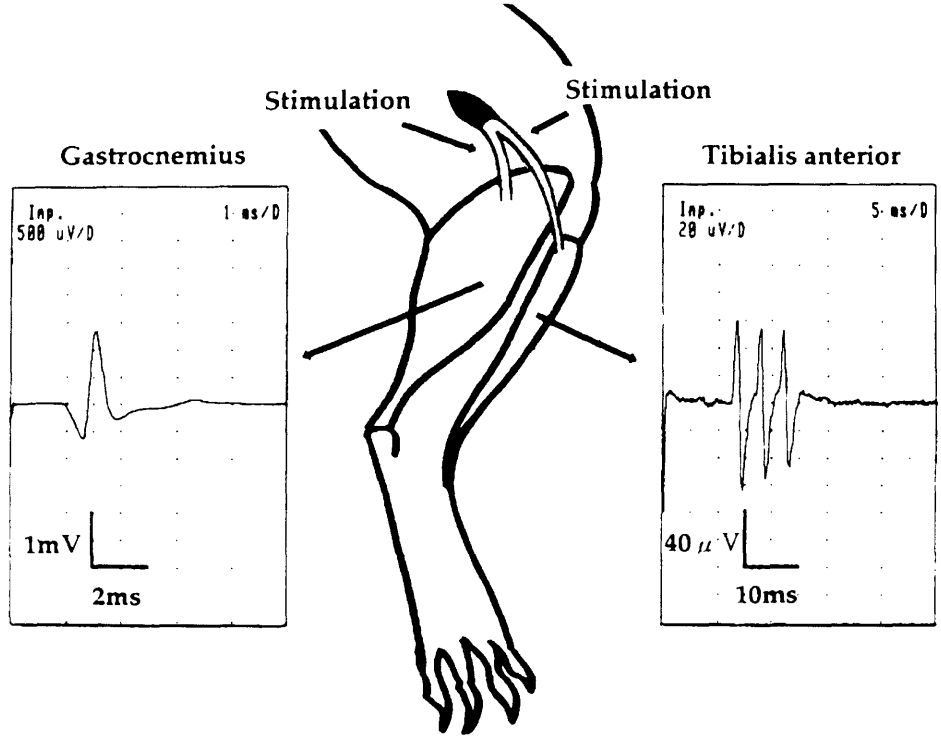


Figure 2. Electrophysiological analysis.

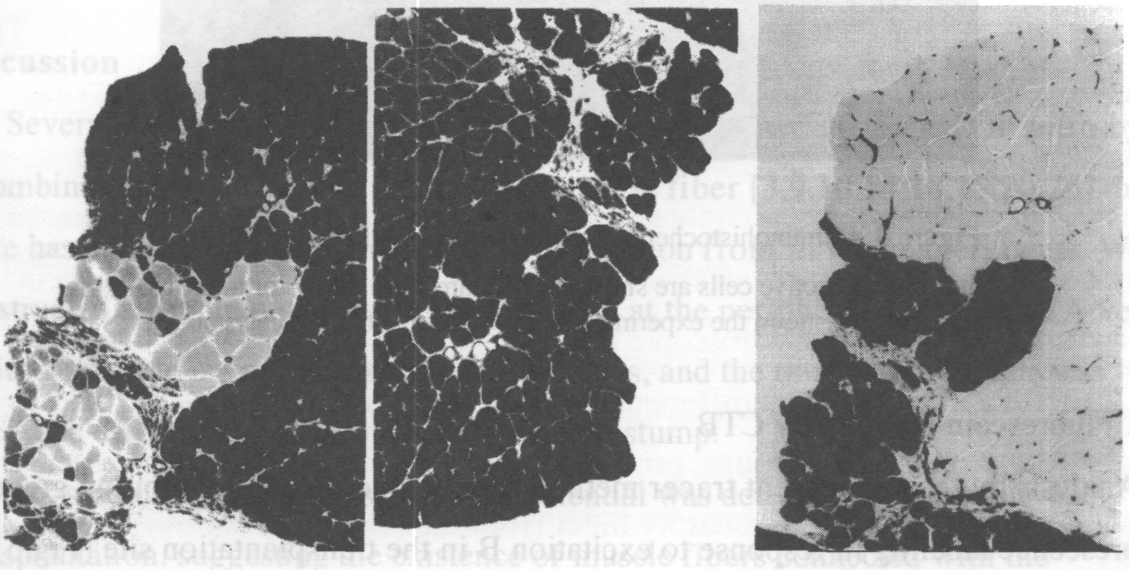
Evoked muscle action potentials recorded from the experimental group are shown. In this case, the tibial nerve was stimulated at 0.4 mA, and an action potential of 2 mV was obtained from the gastrocnemius muscle using a needle electrode. When the common peroneal nerve was stimulated at 0.5 mA, an action potential of 120 μV was detected from the tibialis anterior muscle.

The amplitude of the gastrocnemius muscles was 0.80±1.07 mV and that of the tibialis anterior muscles was 1.82±1.86 mV (means±SD). In contrast, neither muscle contraction nor evoked potential were observed in any of the control rats.

3.2. The wet weight of the muscles.

The wet weight of the gastrocnemius and tibialis anterior muscles was determined in 13 rats from the experimental group and in all 11 control rats. The wet weight was expressed as percentages of values for contralateral unoperated muscle. The mean weight ratio of the gastrocnemius muscle of the experimental group was 26.0±4.2% and that of the tibialis anterior muscle was 25.6±4.5%. The corresponding ratios of gastrocnemius and tibialis anterior muscles in the control group were 24.4±3.0% and 19.0±7.8% (means±SD), respectively. Student's t test showed there was no significant difference between the experimental group and the control group.

3.3. Muscle Histochemistry



a b c

Figure 3 a-c. Muscle Histochemistry

Muscle fiber grouping with Type 2 fibers was observed by routine (pH 10.7) ATPase staining in the soleus muscle on the treated side of the experimental group (a). Type 2C fibers that were dark at all pHs (arrow) were also observed (a-c). Transverse serial sections. The scale bar indicates 200 μm. a-routine ATPase, b-ATPase with preincubation at pH 4.6, c-The same at pH 4.3.

A grouping of Type 2 muscle fibers was observed on routine ATPase staining of the soleus muscle on the treated side of the experimental rats (Fig.3a). Type 2C fibers that were heavily stained at all pHs was also noted (Fig.3a-c). The majority of the soleus muscle fibers on the non-treated side were Type 1 fibers.

3.4. Immunohistochemistry for choline acetyltransferase(Chat)

At the transplantation site of the experimental rat, Chat immunoreactive cells were shown to be present (Fig.4). These cells were distributed widely in the transplantation site, but no significant pattern of localization was noticed. About 50 cells were present in each section.

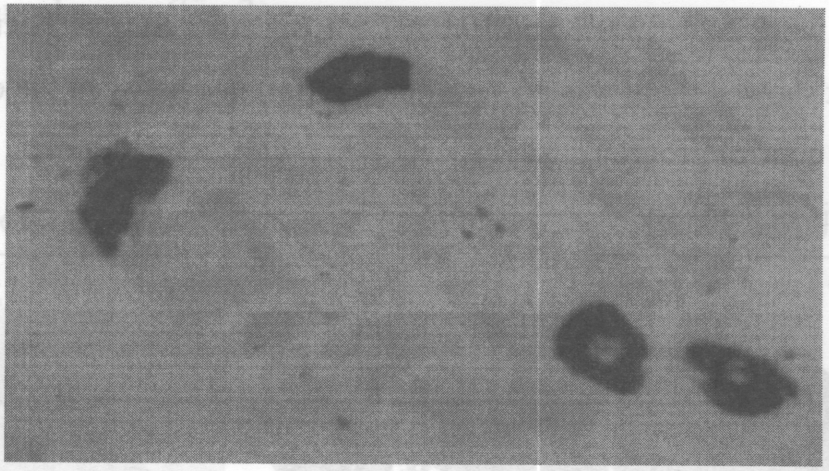


Figure.4. Immunohistochemistry for choline acetyltransferase(Chat)
Chat immunoreactive cells are shown. There are survival motor neurons at the transplantation site in the experimental rat. The scale bar indicates 50 μm.

3.5. Fluorescein labeling by CTB

Analysis by the fluorescent tracer method revealed nerve cells exhibiting green fluorescence labeling in response to excitation B in the transplantation site (Fig.5). The number of retrogradely labeled cells ranged from 5 to 8 in each section. The number of non-labeled cells ranged from 15 to 34 in each section. The ratio of retrogradely labeled cells to all cells ranged from 15% to 25%. No fluorescein-labeled cells were detected in any of the rats which did not undergo CTB injections.

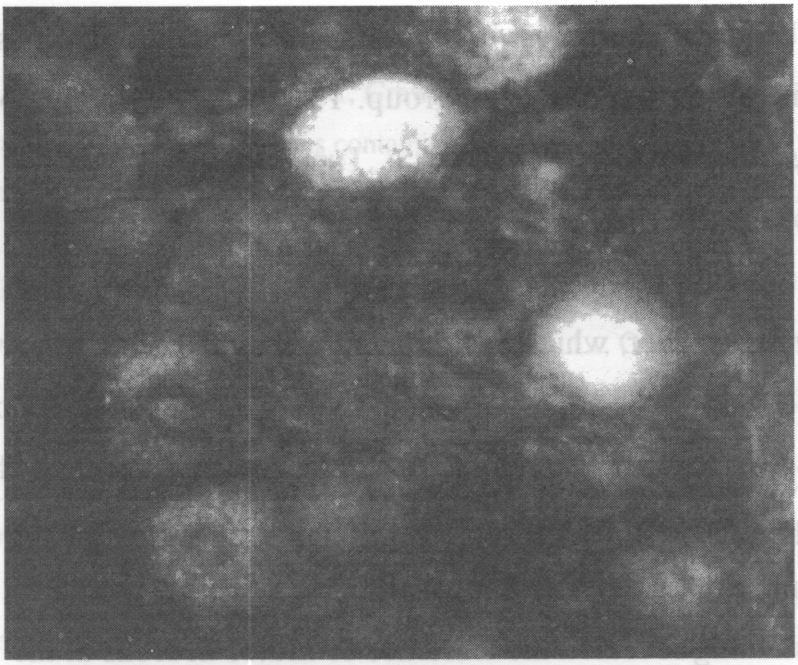


Figure 5. Fluorescein labeling by CTB.

Nerve cells with cell bodies that exhibit green fluorescence were observed at the transplant site of rats injected with CTB in the experimental group. The cells are ovoid. The scale bar indicates 50μm. Cells exhibiting similar fluorescence were not observed in the experimental rats which did not undergo a CTB injection.

Discussion

Several studies have shown new neuromuscular junction formation in vitro by recombination of fetal spinal neurons and muscle fiber [3,9,10,14,18,19,20,25], but there has been no definite evidence of such junction from in vivo experiments. We constructed a simple model for transplantation at the peripheral nerve level. A vein cavity was made using microsurgical techniques, and the transplanted tissue was able to be directly apposed to the sciatic nerve stump.

In our study, an evoked muscle action potential was detected in all 13 rats with transplantation, suggesting the existence of muscle fibers connected with the regenerated motor axons from the transplanted fetal spinal cord. Although the total number of neurons reinnervating each muscle was not counted, it is thought that the amplitude of evoked action potentials depends on the number of reconstructed motor units.

Histochemical study of muscle revealed reinnervation of denervated muscle,

illustrated by fiber type grouping with Type 2C fibers existing in the soleus muscle on the treated side of the experimental group. The soleus muscle in normal rats is composed almost entirely of red muscle, i.e.,Type 1 muscle fibers [4]. However, determination of muscle fiber type depends on the motor neuron with the fiber type changing when the muscle is reinnervated by different types of motor neurons after denervation. Type 2C fiber, which has characteristics intermediate between those of Type 1 and Type 2 fibers, is almost absent in mature animals, but is observed when denervated muscle fibers are reinnervated and the muscle fiber type changes [5]. Thus the findings of the experimental group indicate reinnervation by different types of motor neurons.

Chat immunostaining revealed the existence of motor neurons at the transplantation site. It is thought that these Chat immunoreactive cells are alpha-motoneurons [16] or preganglionic sympathetic neurons [1,7,8] as they were able to form a neuromuscular junction [2,12,17].

In the experimental rat, there were cells in the transplantation site which were retrogradely labeled with a fluorescein labeling substance injected into muscle. This procedure demonstrated a direct axonal communication between the muscle and transplanted neurons. Although the relationship between labeled cells and electrophysiological findings could not be precisely analyzed. We thought that the number of labeled cells was sufficient to evoke not only significant amplitude of action potentials but also macroscopic muscle contraction.

There was no significant difference in wet weight of the muscles between the control and experimental groups, probably because no voluntary muscle action occurred in the absence of excitatory input from the central nervous system against the transplanted nerve cells.

In conclusion, the axon regenerated from spinal neurons located at the peripheral nerve stump was able to reinnervate the denervated muscle. This procedure may therefore indicate a new possibility for motor unit reconstruction.

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おわりに

生後約6～8週，体重150～250gのS-D系ラットの坐骨神経を切断し，その末梢部神経約20mmを切除した。神経末梢側中枢端に約10mmの自家大腿静脈を盲管として縫着し，その内部に胎生14日目の胎児脊髄を注入した。術後約3か月で移植部末梢を電気刺激すると、全例において前脛骨筋と腓腹筋の誘発筋電図が確認できた。また、脊髄移植ラットに対して術側の前脛骨筋と腓腹筋にCTB 0.5mgを蒸留水100 μlに溶解したものをmicrosyringeでそれぞれ50 μlずつ注入した。約48時間後，経心臓的に0.01M phosphate buffer にて潅流後1.0%paraformaldehyde,
1.25%glutaraldehydeを含む0.1M phosphate buffer にて固定した。移植部を摘出後，凍結し cryostatで13 μmの薄切切片を作成し，B励起にて落射式蛍光顕微鏡で観察した。蛍光標識にてCTB注入ラットの移植部にB励起により緑色の蛍光を発する神経細胞を認めた。この神経細胞は楕円体型および多角体型を呈していた。一方CTB非注入ラットには同様の蛍光を一切認めなかった。CTB注入群，CTB非注入群，各群内で個々の所見は同等であった。
in vitroにてfetal spinal neuron と muscle fiberの再結合による新たなneuromuscular junction 形成の報告はあるが，これまでin vivoにて移植された脊髄神経細胞が機能的再建に貢献したという報告はない。筋に投与された蛍光標識物質が軸索流によって逆行性に移植部細胞に取り込まれたことより，筋と移植部神経細胞間に直接の線維連絡が証明された。この神経細胞は，移植した胎児脊髄にはdorsal root gangliaおよびsympathetic ganglia を含まないことと，muscleとneuromuscular junctionを形成したことよりanterior horn motoneuronまたはpreganglionic sympathetic neuron[1]であることが考えられる。今後の展望としてはいかにこの運動単位をcontrolするかであるが，電気的刺激や化学的刺激により興奮させることが考えられる。以上より末梢神経レベルで静脈を用いた盲管を作成することにより神経断端に位置する脊髄神経細胞から再生した軸索が脱神経筋を再支配したと考えられる。すなわちこの手法により運動単位再建が成功したといえる。