The Effects of Electroacupuncture on Peripheral Nerve Regeneration in Rats
Motohiro Inoue, Tatsuya Hojo, Tadashi Yano, Yasukazu Katsumi

Summary
This study was designed to examine the effects of electroacupuncture with direct current (DC) on peripheral nerve regeneration. The left sciatic nerve of 55 7-month-old rats was crushed at the thigh. They were randomly allocated to four groups: distal cathode DC group (n=15), distal anode DC group (n=14), sham operated group (n=13), and control group (n=13). In the distal cathode DC group, a cathode electrode was connected to an insulated acupuncture needle inserted at 1 cm distal to the injured site, while an anode electrode was connected to a needle inserted at 1 cm proximal to the lesion. In the distal anode DC group, the anode and the cathode electrode were connected to the needle at 1 cm distal and proximal to the lesion respectively. In the sham operated group, no electrical stimulation was given to the insulated needle inserted at the same site, and in the control group, no treatment was given. Regeneration of the sciatic nerve was evaluated by the number of evoked EMGs recorded at 12 sites in the plantar region, by their latency, and by the weight ratio of the tibialis anterior at four weeks after the crush injury. Regeneration of the peripheral nerve was faster and more accelerated in the distal cathode DC group than in the other groups, while in the distal anode DC group the regeneration was delayed. This result suggested electroacupuncture with cathode distal orientation might be a useful treatment having the advantage of enabling deeper insertion with minimal tissue damage.

Keywords
Peripheral nerve, nerve regeneration, direct current, electroacupuncture, sciatic nerve, rat.

Introduction
If a peripheral nerve is injured, Wallerian degeneration takes place distal to the injury site, and the regenerating axons sprout and elongate from this site. The neural growth cones of the sprouting axons require a scaffold to grow along. For example, they can adhere to laminin of the basal laminae on the surface of Schwann cells. A great number of in vivo and in vitro experiments have been performed to identify intrinsic factors that enhance the regeneration of peripheral nerves and have demonstrated that Schwann cells, neurotrophic factor and nerve growth factor are important. Extrinsic factors have also been investigated extensively. Electric stimulation and electro-magnetic field stimulation have been reported to physically stimulate axonal regeneration, but detailed mechanisms for this effect have not yet been clarified. Pomeranz et al reported that motor neuron regeneration was affected by direct application of weak direct current (DC) to the sciatic nerve of rats, and that this effect was altered by the polarity of the DC. Acupuncture has been used as an adjunct for the treatment of various types of neuropathy, but there is no report on its effects on neuronal regeneration.

Electroacupuncture can deliver an electric current of low frequency via needle electrodes. In contrast to transcutaneous electric nerve stimulation (TENS), electroacupuncture can be used to apply electric currents directly to deep tissue, and like TENS it is mainly used for pain control. We hypothesized that a modification of the method of Pomeranz et al with DC electroacupuncture would allow DC stimulation of the injured part of a deep nerve, and we tested the hypothesis in this animal study.
Methods

Animals
Fifty-five 7-month-old male Wistar rats, weighing 470-500g, were used. We used the rats at seven months of age because Campbell et al. and Black et al. recommended the use of adult rats for obtaining precise nerve regeneration data because of much faster nerve regeneration in infant rats (aged 2-3 months) than in adult rats (aged 6-10 months). Furthermore, rats with a sciatic nerve of 11cm +/- 5mm length between the greater trochanter and the third digit were selected for use for more precise assessment of the regeneration of the sciatic nerve. Under anaesthesia with pentobarbital sodium (50mg/kg, i.p.), the sciatic nerve was exposed at a site on the left femur and a 2mm crush site was produced at the center of the femur (about halfway between the greater trochanter and the lateral femoral condyle). A 30 second crush of the sciatic nerve was performed twice using forceps of 2mm width (Pean’s type haemostatic forceps, Natsume). After crushing the sciatic nerve, rats were randomly divided into four groups. Ethics approval was granted by Meiji University.

Experimental Groups
Distal cathode DC group (n=15)
Distal cathode DC application was performed daily for four weeks commencing the day of crushing the sciatic nerve until final assessment. In each rat, distal cathode DC was delivered via two acupuncture electrodes insulated except for the tip (0.2mm in diameter, 40mm in length), an anode electrode was placed at 1cm proximal and a cathode electrode was placed at 1cm distal to the crush injury. The distance from the skin to the sciatic nerve had previously been measured to determine the depth of the tips of the electrodes. Using an electric stimulator (SEN-3301, Nihonkohden), 500usec square pulses of 10Hz were delivered at 10 volts for 15 minutes. Rats were anaesthetised with halothane (Japan, Hoechst) and immobilised with a cloth with four holes through which extremities were pulled out during each electroacupuncture procedure.

Distal anode DC group (n=14)
Distal anode DC application was performed

Figure 1 Twelve recording electrode insertion sites in the plantar region used in the evoked electromyogram (EMG) procedure. (Based on the figure from Campbell, J.J. and Pomeranz, B. A new method to study motor neuron regeneration using electromyograms shows that regeneration slows with age in rat sciatic nerve. Brain Res 1993;60:265.) The extent of reinnervation was determined by the proportion of sites showing an evoked EMG upon electrical stimulation of the sciatic nerve. Scores ranged from 0/12 to 12/12. The twelve closed circles are sites overlying the flexor digitorum brevis, the lumbrical and interosseus muscles.

Figure 2 The figure shows a typical recording of evoked EMG detected at the plantar region by electrical stimulation at 5mm proximal to the crush injury. The spike at the left is the stimulus artifact, which we used to determine the latency.
daily for four weeks commencing the day of crushing the sciatic nerve until the final assessment. In each rat, distal anode DC was delivered via two insulated acupuncture electrodes, a cathode electrode was placed at 1cm proximal and an anode electrode was placed at 1cm distal to the crush injury. Using an electric stimulator (SEN-3301, Nihonkohden), electric stimuli were delivered. Electric stimulation parameters and other experimental conditions were the same as those for the distal cathode DC group.

Sham group (n=13)

After crushing the sciatic nerve, rats were treated daily for four weeks until final assessment in the same manner as the distal cathode/anode DC groups except that they did not receive electric stimulation.

Control group (n=13)

No treatment was given.

Assessment

Electrical and functional assessments were conducted at one, two, three and four weeks after crushing the sciatic nerve.

Number of EMGs recorded from 12 sites in the plantar region

According to the method of Campbell et al., the sciatic nerve was exposed at the left femur under anaesthesia with pentobarbital sodium (50 mg/kg i.p.). An electric stimulation was directly delivered at the site 5mm proximal to the crush injury and the EMGs from 12 sites of the left plantar region (evoked EMGs) were recorded in a bipolar fashion on an oscilloscope (VC-11, Nihonkohden) using a pair of closely placed needle electrodes (0.1mm in diameter), which had previously been inserted transcutaneously to the depth of 2mm. The sciatic nerve stimulation (500μsec square pulses, 1Hz) was performed with an electric stimulator (SEN-3301, Nihonkohden). Prior to the measurement of evoked EMGs, a threshold voltage, which produced the M wave in the triceps surae muscle, had been determined. At one, two, three and four weeks after crushing the sciatic nerve, the evoked EMGs induced by sciatic nerve stimulation at the threshold and twice the threshold voltage were measured five times. When regenerative evoked EMGs with multiphase and the same latency were induced five times by the stimulation at the threshold and/or at twice the threshold voltage (Figure 2), the regenerative innervation was considered to have been completed. The number of regenerative evoked EMGs was counted. When regenerative evoked EMGs appeared, the time to the EMGs after the electric stimulus was measured as the latency.

Behavioral test score

Behavioral test score (BTS) was determined without anaesthesia on the same day, prior to the assessment of evoked EMGs. This scoring system was developed by Campbell et al. for the assessment of motor function of rats. This is a combination of two methods previously described by Berenberg et al. and DeMedinacelli et al. that assess the physiological toe extension and spreading upon rapid downward movement (toe stretch reflex) and during walking (sciatic nerve index), respectively. As described by Campbell et al., the BTS ranged from 1 to 5 points, where ‘5’ represented complete functional recovery and ‘1’ indicated no functional recovery (Table 1).

Weight of the anterior tibial muscle

At four weeks after crushing the sciatic nerve, the left and right anterior tibial muscles were excised under anaesthesia with pentobarbital sodium (50mg/kg i.p.). The muscles obtained were weighed using an electronic balance (GR-120,

| Table 1 The scoring criteria for behavioral test score (BTS). |
|-----------------|-----------------|-----------------|
|                | W EXT | W SPR | TSR EXT | TSR SPR |
| 5               | +     | -     | +       | +       |
| 4               | +     | -     | +       | -       |
| 3               | +     | -     | +       | -       |
| 2               | +     | -     | -       | -       |
| 1               | -     | -     | -       | -       |

The BTS visually assessed the ability of the awake animal to both extend (EXT) and spread (SPR) toes upon elicitation of the toe stretch reflex (TSR) and during walking (W). The score ranged from 1 to 5, where ‘1’ was no functional recovery (W: EXT, SPR; TSR: EXT, SPR) and ‘5’, complete functional recovery (W: EXT, SPR; TSR: EXT, SPR). Details of each scoring criteria are shown in the table with signs that indicate the observation of each motion (+ for observed, – for not observed).
Table 2: Summary of the number of the evoked EMGs, latency, behavioral test score (BTS), and the weight ratio of the tibialis anterior muscle at one, two, three and four weeks after crush injury.

<table>
<thead>
<tr>
<th></th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
</tr>
</thead>
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<tr>
<td><strong>Number of evoked EMG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0</td>
<td>1.0&lt;0.8</td>
<td>2.0&lt;0.8</td>
<td>2.0&lt;0.8</td>
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<tr>
<td>Sham group</td>
<td>0</td>
<td>1.1&lt;0.8</td>
<td>3.1&lt;0.0</td>
<td>5.9&lt;1.6</td>
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<td>0</td>
<td>5.2&lt;0.3*</td>
<td>7.8&lt;1.1*</td>
<td>11.4&lt;0.9*</td>
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<tr>
<td>Distal anode DC group</td>
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<td>3.5&lt;0.8</td>
<td>5.1&lt;1.2*</td>
<td>6.5&lt;1.5*</td>
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<tr>
<td><strong>Latency (ms)</strong></td>
<td></td>
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<tr>
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<td>35.6&lt;2.4</td>
<td>30.7&lt;5.5</td>
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<tr>
<td>Sham group</td>
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<td>35.0&lt;2.7</td>
<td>30.3&lt;5.6</td>
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<td>25.9&lt;5.8*</td>
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<td>36.1&lt;2.5</td>
<td>30.5&lt;4.8</td>
<td>28.0&lt;7.9</td>
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<tr>
<td><strong>Behavioral test score (BTS)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>1</td>
<td>1.3&lt;0.5</td>
<td>1.8&lt;0.6</td>
<td>3.3&lt;0.6</td>
</tr>
<tr>
<td>Sham group</td>
<td>1</td>
<td>1.2&lt;0.4</td>
<td>1.8&lt;0.6</td>
<td>3.3&lt;0.6</td>
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<tr>
<td>Distal cathode DC group</td>
<td>1</td>
<td>2.3&lt;0.7*</td>
<td>3.1&lt;0.5*</td>
<td>4.6&lt;0.6*</td>
</tr>
<tr>
<td>Distal anode DC group</td>
<td>1</td>
<td>1.2&lt;0.4</td>
<td>1.6&lt;0.7</td>
<td>2.6&lt;0.9**</td>
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<tr>
<td><strong>Weight ratio of tibialis anterior (%)</strong></td>
<td></td>
<td></td>
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<td></td>
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<td>NA</td>
<td>NA</td>
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<td>NA</td>
<td>50.4&lt;4.4*</td>
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<tr>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>32.6&lt;3.7*</td>
</tr>
</tbody>
</table>

Mean ± SD
*p<0.01 vs. control group
NA indicates that the data in the all are not available.

A and D), and the percentage of the muscle weight on the injured side (left) relative to the intact side (right) was calculated.

Statistical analysis
Statistical analyses were performed using the Statview 4.5 program (SAS Institute Japan). For all parameters assessed (number of and latency to evoked EMGs, anterior tibial muscle weight, and BTS), data were compared among the four experimental groups using one-way ANOVA followed by Bonferroni/Dunn adjustment for multiple comparisons. Data were considered significant when the ‘p’ value was less than 0.05.

Results
Number and latency of evoked EMGs after stimuli
At one week after crush injury, no regenerative evoked EMGs were recorded in any rats in any of the four experimental groups. The numbers of regenerative evoked EMGs (mean ±/ SD) recorded in individual groups at two, three and four weeks after crush injury are shown in Table 2. At any of these times, the numbers of evoked EMGs recorded in the control and sham groups did not differ significantly. At all these times, the values observed in the distal cathode DC group were significantly different (p<0.01) from those observed in every other group. At three and four weeks after the crush injury, there was a significant difference (p<0.01) between the distal anode DC group and the control or sham group (Figure 3).

Data on the latency to the evoked EMGs after stimuli (mean ±/ SD) obtained at specified times in individual groups are shown in Table 2. At two weeks after crush injury, there was no significant between-group difference in the latency to the evoked EMGs. At three and four weeks, a significant difference (p<0.01) was observed between the distal cathode DC group and every other group, while the value observed in the distal anode DC group did not differ significantly from that observed in the control or sham group (Figure 4).

Behavioral test score (BTS)
In all experimental groups, the BTS showed similar changes over time to those observed in the numbers of evoked EMGs. BTS data (mean ±/ SD) obtained at specified times in individual groups are shown in Table 2. At any specified time, there was no significant difference between the control and sham groups. At two, three and
four weeks after crush injury there was a significant difference (p<0.01) between the distal cathode DC group and every other experimental group. At four weeks, the value observed in the distal anode DC group was significantly different (p<0.05) from that observed in the control or sham group (Figure 5).

Weight of the anterior tibial muscle
At four weeks after crush injury, there was no significant between-group difference in the weight of the right anterior tibial muscle (intact side); the mean ± SD was 0.805 ± 0.119g in the control group, 0.803 ± 0.097g in the sham group, 0.804 ± 0.078g in the distal cathode DC group, and 0.815 ± 0.112g in the distal anode DC group. The weight of the left anterior tibial muscle (injured side) in the respective groups was 0.275 ± 0.020g, 0.280 ± 0.038g, 0.405 ± 0.057g, and 0.264 ± 0.04g. The percentages of the muscle weight on the injured side relative to the intact side (mean +/- SD) are shown in Table 2. The relative muscle weight on the injured side did not differ significantly between the control and sham groups. There was a significant difference (p<0.01) between the distal cathode DC group and every other experimental group, while the value observed in the distal anode DC group did not differ significantly from that observed in the control or sham group (Figure 6).

Discussion
If an axon is injured, Wallerian degeneration takes place distal to the injury, while the proximal site (neuronal cell side) often escapes the degeneration and allows regenerating sprouting from intact axons. Most of regenerating sprouts occur from Ranvier’s nodes proximal to the injury and travel towards their target organ along the same route as before the injury. If there is no lesion on the regenerating route and if the target organ is not irreversibly damaged by denervation, behavioral function will recover nearly to the previous level, provided that the distance between the injury site and the target organ is not too long. If this distance is long, the previous function will be difficult to restore because axonal regeneration is very slow (1-4mm/day). If peripheral nerve regeneration could somehow be accelerated, and if the regenerating axons could be guided to more rapidly regain their former connection at the periphery, much better functional recovery
Figure 4 Changes in the latency of evoked EMGs in each experimental group. The latency shortened as time elapsed after crush injury in all experimental groups. In the distal cathode DC group, mean latency was significantly shorter than those of the other groups at week three and four. The number of recordings are not always the same because the number of evoked EMGs increased as time elapsed (distal cathode DC group: n=78, 117 and 171 at week two, three and four respectively, distal anode DC group: n=7, 24, and 44 at week two, three and four respectively, Sham operated group: n=14, 41, and 82 at week two, three and four respectively, control group: n=14, 40, and 79 at week two, three and four respectively).

may be achieved. In vitro studies have shown that, after application of electric stimulation, regenerating axons elongate in the direction of the cathode. Recently, Pomeranz et al reported that the regeneration of an injured nerve was enhanced by a continuous cathode DC stimulation at the site of the injury via chronically implanted electrodes. Frequent or continuous application of an electric stimulation is required for enhancement of nerve regeneration, but it is difficult in clinical practice to directly apply a continuous electrical stimulation to the exposed nerve. Electroacupuncture may be a simple method of indirectly applying an electrical stimulation to deep tissue. Considering the very small (about 0.2mm) diameter of an acupuncture needle used for this purpose together with the shape of its tip, electroacupuncture will cause only minimal tissue damage. However, electroacupuncture can only apply electric stimulation around the injury of a nerve and it remained unclear whether this treatment could produce an enhancement of axonal regeneration similar to that reported by Pomeranz et al. Thus, using a similar method to that used by Pomeranz et al, we examined whether application of DC stimulation via electrodes placed distal and proximal to a crush injury could enhance the regeneration of the injured nerve.

The control and sham groups did not differ significantly with respect to the number of or latency to evoked EMGs, BTS, or weight of the anterior tibial muscle. This confirmed that the needle insertion and the restraint of rats had no effect on the regeneration of the crushed sciatic nerve. At two, three and four weeks after crush injury, the mean number of evoked EMGs and the mean value of BTS in the distal cathode DC group were significantly greater than those recorded in the three other groups. These results indicate that cathode DC application via acupuncture needles placed distal to the injury accelerated the elongation of regenerating axons in the direction of the target tissue (interosseous muscle, lumbral muscle, flexor digitorum brevis muscle), and enhanced the functional contact of the nerve terminal with the motor endplate. The number of evoked EMGs at three and four weeks and the mean value of BTS at the 4th week after crush injury in the distal anode DC group were
significantly smaller than those of the control and sham groups. These results indicate that anode DC application via acupuncture needles placed distal to the injury delayed the elongation of regenerating axons in the direction of the target tissue and impaired the functional contact of the nerve terminal with the motor endplate. As shown in Figure 4, the latency to the M wave in the evoked EMGs at the third and fourth week after crush injury in the distal cathode DC group was significantly shorter than that of the other groups. As the outgrowth rate and diameter of axons are known to affect the latency to the neuronal response, distal cathode DC application to the injury was suggested to produce an enhancement of the outgrowth of regenerating axons.

The relative weight of the anterior tibial muscle at the fourth week after crush injury in the distal cathode DC group was also significantly greater than that of the other groups, suggesting that distal cathode DC application to the crush injury might facilitate the recovery of the nervous control of innervated muscles compared with the other treatments studied. This finding was in accordance with the increased innervation of the muscles in the plantar region.

The results of these experiments indicate that peripheral nerve regeneration was accelerated by distal cathode DC application but was rather delayed by distal anode DC application and therefore, support the findings reported by Pomeranz et al. and Roederer et al. The results obtained also indicate that electroacupuncture, which applies DC stimulation, may provide a simple and safe method of enhancing nerve regeneration in a non-invasive manner. How the cathode DC application enhances the regeneration of peripheral nerves remains to be determined, but the following two hypotheses have been presented. First, an electrophoretic movement of substances constituting membrane in the direction of a cathode may play a role in the enhancement of nerve regeneration. For the outgrowth of regenerating axons in the fascicle and for the arrival of the growth cones at the tip of the regenerating axons to the target tissues, axons are required to elongate along the scaffold on the surface or basement membrane of Schwann cells, and adhesion of membrane molecules to growth cones is thought to be an important event. This hypothesis is based on the findings obtained by electrophoretic studies that growth-associated substances move toward the cathode, including adhesion associated proteoglycan of regenerating axons; receptors for neurotrophic factor, which is necessary for neuronal cell growth; and ion channels. Siskin et al reported that neurite

![Figure 5 Changes in BTS after crush injury in each experimental group. In the distal cathode DC group, BTS scores were significantly higher than those in the other groups at week two, three and four. In the distal anode DC group, BTS scores were significantly lower than those in the other experimental groups at week four.](image-url)

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growth of chick embryos occurred in the direction of the cathode and suggested that this phenomenon might result from an increase in the level of receptors for neurotrophic factor.18

The other hypothesis is that cathode DC application to the injury indirectly enhances nerve regeneration by suppressing Ca2+-induced nerve injury rather than by inhibiting Ca2+ entry into the cells proximal to the injured site. If an axon is injured, an injury current occurs between the normal and injured sites, and Ca2+ entry increases at a site proximal to the injury. This may damage the cytoskeleton of normal axonal cells.19 While Wallerian degeneration usually develops at or distal to the injury site, increased Ca2+ entry into the site proximal to injury causes retrograde degeneration, leading to the so-called ‘die-back’ phenomenon.18 In an experiment using lamprey axons, it was confirmed that the ‘die-back’ phenomenon was suppressed by cathode DC application to the site distal to the injury but was enhanced by the same treatment applied to the site proximal to the injury.19 The enhancement of axonal regeneration by distal cathode DC application in contrast to its delay by distal anode DC application via an acupuncture needle observed in the present study might have both or either of the two mechanisms mentioned above.

In this study, distal cathode DC application to a crush injury was shown to produce an enhancement of the regeneration of the injured nerve. The cathode DC application was performed daily after the crush injury until the final assessment. Further studies are required to determine the optimal conditions for the enhancement of nerve regeneration, including the duration of DC application and the sites of stimulation. Nerve stimulation during the period of the ‘die-back’ phenomenon, or during the passage of growth cones through the crushed site may be enough if the ‘die-back’ phenomenon is caused by increased Ca2+ entry into the cells proximal to the injury site20,21 or if electrophoretic movements of neurotrophic and growth factors enhance nerve regeneration.18,21-23 Compared with exclusive stimulation at a crush injury, stimulation at serially changing sites from the injury to the periphery following an indication of Tinel’s sign, which appears at the tip of each developing nerve cell process, may produce a greater enhancement of the regeneration of the injured nerve. Further investigation is also necessary to determine the optimal stimulation frequency, intensity, and duration. The results of this study indicate that distal cathode DC application to a crush injury using acupuncture needles enhances
nerve regeneration and suggests that such electroacupuncture treatment might be a new and applicable method for current clinical practice.

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