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Electrical stimulation modulates injury potentials in rats after spinal cord injury

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Research Highlights

(1) The injury potential is used as the index for modulating electrical stimulation intensity, through various electrode locations and stimulation intensities.
(2) Electrical stimulation using a reversed electrical field polarity can effectively modulate the injury potential and benefits the spontaneous repair of the cell membrane in rats with spinal cord injury.
(3) Taking the injury potential value as the parameter of the electrical stimulator, a method for fining the stimulation intensity is proposed, thus promoting the application of electrical stimulation in spinal cord injury.

Abstract

An injury potential is the direct current potential difference between the site of spinal cord injury and the healthy nerves. Its initial amplitude is a significant indicator of the severity of spinal cord injury, and many cations, such as sodium and calcium, account for the major portion of injury potentials. This injury potential, as well as injury current, can be modulated by direct current field stimulation; however, the appropriate parameters of the electrical field are hard to define. In this paper, injury potential is used as a parameter to adjust the intensity of electrical stimulation. Injury potential could be modulated to slightly above 0 mV (as the anode-centered group) by placing the anodes at the site of the injured spinal cord and the cathodes at the rostral and caudal sections, or around –70 mV, which is resting membrane potential (as the cathode-centered group) by reversing the polarity of electrodes in the anode-centered group. In addition, rats receiving no electrical stimulation were used as the control group. Results showed that the absolute value of the injury potentials acquired after 30 minutes of electrical stimulation was higher than the control group rats and much lower than the initial absolute value, whether the anodes or the cathodes were placed at the site of injury. This phenomenon illustrates that by changing the polarity of the electrical field, electrical stimulation can effectively modulate the injury potentials in rats after spinal cord injury. This is also beneficial for the spontaneous repair of the cell membrane and the reduction of cation influx.

Key Words

neural regeneration; spinal cord injury; injury potential; electrical stimulation; electric parameters; cations; resting membrane potential; neural regeneration; electrode; stimulator; charge balance; grants-supported paper; neuroregeneration

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INTRODUCTION

An injury potential, first discovered by Galvani in late eighteenth century, is a direct current potential gradient between the intact spinal cord and injured site[1]. It is induced by an electrical current flowing into and around an injured nerve. Its appearance is an important indicator of changes in the microenvironment of damaged spinal cord. However, its importance has not been recognized until Borgens *et al* [2-3] investigated injury currents after spinal cord injury and suggested that compensation of the injury potential by applying an electrical field might enhance axonal regeneration in lamprey larvae. Since then, injury potentials after spinal cord injury have been investigated in rats, cats and guinea pigs[4-7]. All these studies show that injury potentials form immediately after injury and the initial absolute value of the injury potential is positively correlated to the severity of injury. The measured injury potentials, as a function of time, were fit with a logarithmic function[5]. As a result, it is deduced that if the absolute values of injury potential are reduced by applying an electrical field, the prognosis of spinal cord injury might be improved.

There are two methods to reduce the injury potential. Firstly, it is easy to assume that if the injury gap is plugged by biomaterials, such as polyethylene glycol- and chitosan-based nanoparticles, then the current flowing into the cell would be eliminated[8-14]; however, the biocompatibility of such particles should still be investigated. Conversely, it is known that the injury potential can be controlled by applying an electrical field. Because the formation of injury potentials is caused by the movement of extracellular and intracellular ions, which is also the major initiator of the secondary injury process, ions, such as Na^+ and Ca^{2+}, were carefully examined as well as injury potentials. Furthermore, preventing the influx of the free calcium immediately after injury to the spinal cord could inhibit secondary degeneration. One approach for inhibiting this influx of ions is by using an applied electrical field to modulate the movement of extracellular and intracellular Ca^{2+}, as previously proposed by Borgens[15]. In the *in vitro* study of Strautman and colleagues[16], the movement of Ca^{2+} was greatly reduced by an externally-applied electrical field, where the cathode was placed distal to the lesion, and the inhibition was increased by an applied field of the opposite polarity. However, the intensity of stimulation was not investigated and an optimal value was not recommended, nor the combination of injury potential and applied electrical field. In contrast, *in vivo* experiments concentrating on direct current stimulation and oscillating field stimulation could induce regeneration of injured axons after spinal cord injury[17-21]. Furthermore, the oscillating field stimulator has been used in a phase I clinical trial[22], and results showed that all the patients attending the phase I trial had some improvement in sensory and motor function. Unfortunately, the success of oscillating field stimulator in treating spinal injury was not based on *in vitro* experiments and considerable research will be required if the conditions are to be optimized for mammalian spinal injury[23-25].

Although Borgens[15,19-20] previously reported the mechanism of electrical stimulation for promoting axonal regeneration under the presumption that the use of electrical field stimulation could neutralize the injury current, his following studies using an oscillating field stimulator (changing the polarity of electrical field every 15 minutes) are not consistent with the previous presumption. In these studies, the oscillating field stimulation was performed several hours after spinal cord injury, where the injury potential may have already disappeared. So, the compensation of injury potential is not the mechanism of action of the oscillating field stimulator, and as the stimulation intensity cannot be defined without knowing the mechanism, this greatly limits the development of a therapeutic oscillating field stimulator.

To seek the mechanism underlying the regeneration of injured axons by electrical stimulation and to define appropriate stimul-

ating parameters, this study regards injury potential as a bridge between the stimulation intensity and the severity of spinal cord injury. It is believed that the injury potential could be adjusted to slightly above 0 mV to prevent the influx of extracellular cations, or –70 mV to reconstruct the normal resting membrane potential. The injury potential after stimulation was measured and compared with those measured immediately after injury in rats without stimulation. The optimal parameters of electrical stimulation were also investigated.

RESULTS

Quantitative analysis of experimental animals

A total of 30 adult female Sprague-Dawley rats with spinal cord injury, weighing 250–350 g were used in these experiments and randomly divided into three groups as follows: anode-centered group, cathode-centered group and control group. Ten rats in the anode-centered group received electrical stimulation through anodes placed at the injury site, while cathodes were placed at the rostral and caudal section. Another 10 rats in the cathode-centered group received electrical stimulation by inverting the electrode polarity to the anode-centered group. The other 10 rats in the control group received no electrical stimulation. Some rats were excluded because of excessive hemorrhage, error in surgery and unpredicted errors in the process of injury. No additional rats were recruited into the experiment. Finally, 25 rats were included in the statistical analysis, 8 in the control group, 8 in the anode-centered group and 9 in the cathode- centered group, respectively.

Injury potential in the control group (initial voltage data)

The rostral and caudal injury potential measured in the control group is shown in Figure 1. The initial value of rostral and caudal injury potential was –17.4 mV and –20.8 mV, respectively. The absolute value of injury potential reduced with time in a logarithmic manner.

Comparison of injury potential in the anode-centered group, cathode-centered group and control group

As shown in Figure 2, the injury potential was measured from immediately after spinal cord injury to 4 hours post-injury in the control group, and measured from immediately after 30 minutes of electrical stimulation to 4 hours post-injury in the anode- and cathode-centered groups. In the cathode-centered group, the injury potential is modulated to around –70 mV, which is about 3-fold the initial value. To clearly display the injury potential after electrical stimulation post-injury, the value between 0–30 minutes in the cathode-centered group is not shown. Two-way analysis of variance results showed that there were no significant differences in injury potentials between any two groups from all three groups from 30 minutes to 4 hours post-injury ($P > 0.05$). At 30 minutes, when the electrical stimulation was halted, the rostral and caudal injury potential in the anode- and cathode-centered groups were significantly higher than the control group ($P < 0.05$), whereas no significant difference was found between the two electrically-stimulated groups ($P > 0.05$). The absolute values of injury potential in both the anode- and cathode-centered groups were much less than initial value of 100%.

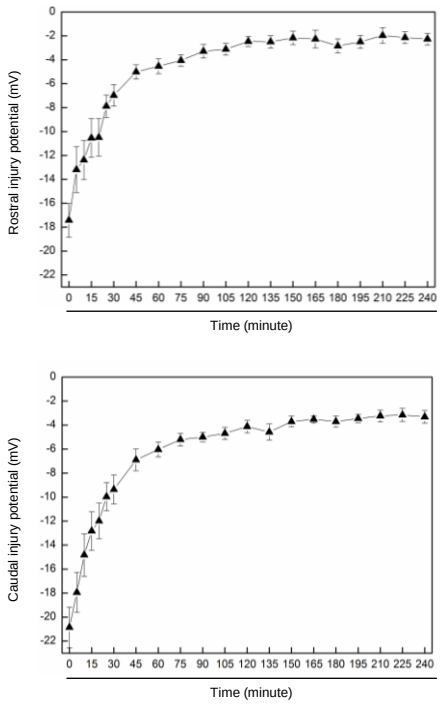

Figure 1 Rostral and caudal injury potentials measured in rats of the control group.

Data are expressed as mean ± SD, and the number of rats in the control group is 8.

Figure 3 shows the absolute values of rostral and caudal stimulating voltages at different periods of stimulation in both the anode- and cathode-centered groups. The stimulating voltages are adjusted to make the injury potential slightly above 0 mV in the anode-centered group or around –70 mV in the cathode-centered group every 10 minutes. Two-way analysis of variance results showed that the absolute values of rostral and caudal stimulating voltages in the cathode-centered group are larger than those in the anode-centered group during the three periods: 0–10 minutes, 10–20 minutes, and 20– 30 mi-

nutes ($P < 0.05$). However, there are no significant differences between the rostral and caudal stimulating voltages in the same period in either the anode-or cathode-centered groups ($P > 0.05$).

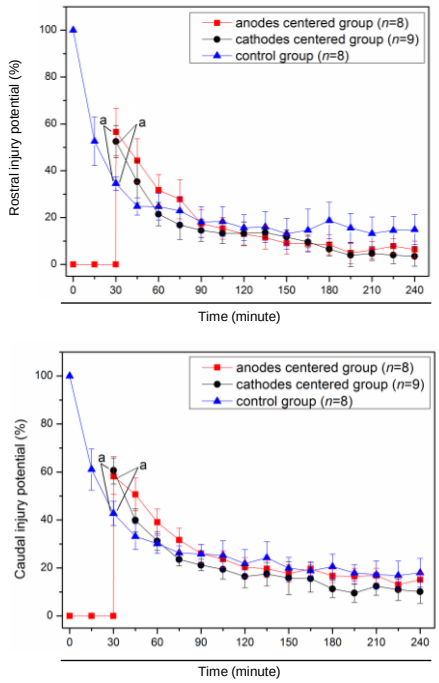

Figure 2 Influence of electrical stimulation with different polarities on rostral and caudal injury potentials.

Data are expressed as mean ± SD, the number of rats in the control group, anode-centered group and cathode-centered group are 8, 8 and 9, respectively. [a]$P < 0.05$, *vs*. control group using two-way analysis of variance and two-sample *t*-test. The unit of longitudinal coordinates is %, a normalized value equals the ratio of the injury potential at the measuring time point to the initial amplitude of the same rat. The horizontal coordinates describe the time after spinal cord injury in minutes.

Calculation of charges flowing into cells

The permeability of the extracellular space is 0.3 S/m (S/m is the unit of permeability, that is the reciprocal of resistivity)[26]. The distance between electrodes and the area of the spinal cord cross-sections are 15 mm and 10 mm^2, respectively. The results of calculation are listed in Table 1.

The charges of rostral and caudal influx in the anode- and cathode-centered groups were all smaller than the control group. The quantity of positive charges flowing from the extracellular space into the cells is about 0.48 × 10^{-3} C in the control group, which was much larger than the influx charges in the anode- and cathode-centered groups (0.37 × 10^{-3} C and 0.31 × 10^{-3} C, or 30% and 55%; $P < 0.05$). However, there were no significant differences in the rostral influx and caudal influx of positive charge, as well as the total influx between the anode- and cathode-centered groups.

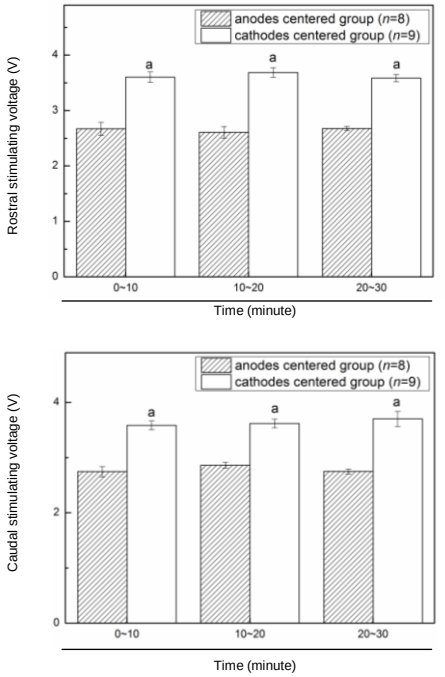

Figure 3 Influence of electrical stimulation with different polarities on rostral and caudal stimulating voltages in different periods of stimulation.

Data are expressed as mean ± SD, and the number of rats in the anode-centered group and cathode-centered group is 8 and 9, respectively. [a]$P < 0.05$, *vs*. anode-centered group using two-way analysis of variance and two-sample *t*-test. The horizontal coordinates describe the time period after spinal cord injury.

Table 1 Charges flowing into the cells after injury (×10^{-3} C)

Influx	Control group	Anode-centered group	Cathode-centered group
Rostral	0.20±0.01	0.14±0.01[a]	0.13±0.01[a]
Caudal	0.28±0.02	0.23±0.02[a]	0.18±0.02[a]
Total	0.48±0.03	0.37±0.03[a]	0.31±0.03[a]

[a]$P < 0.05$, *vs*. control group using two-way analysis of variance and two-sample *t*-test. Data are expressed as mean ± SD, and the number of rats in the control group, anode-centered group and cathode-centered group is 8, 8 and 9, respectively.

DISCUSSION

This study was undertaken to measure the injury potential after spinal cord injury and determine how this potential or injury currents could be modulated. When the spinal cord is injured, the axonal membrane is also destroyed and leads to the disappearance of the resting membrane potential. Thus, high concentrations of

extracellular Na^+ and Ca^{2+} flow from the intact tissue into the intracellular space of the injured axons through the site of injury, resulting in the formation of an injury current as well as an injury potential. The most obvious explanation for the difference in potentials between the injured and healthy tissue is that the potential at the injury site is smaller than the normal tissue. This was in fact confirmed by the results of this study. The initial value of the injury potential is about –20 mV. As time increases, the absolute value of the injury potential decreases logarithmically. At 120 minutes after injury, the absolute value of the injury potential reduces to only several mV, which is similar to previous findings[4-5].

After injury, the damaged axons have a certain ability to self-repair and seal the destroyed membrane. This leads to a decrease in the injury severity and quantity of injured axons. However, injury leads to the influx of extracellular Ca^{2+} into the intracellular space of the damaged axons, which is sufficient to prevent sealing of the membrane and may contribute to axonal dieback, retrograde cell death, and secondary axonal inhibition[11]. In contrast, voltage-dependent calcium channels play a critical role in membrane resealing, and there is likely an appropriate range of calcium influx through these channels to ensure effective axonal membrane resealing[27]. Thus, modulation of Ca^{2+} flow by electrical stimulation might be a solution for promoting membrane repair. In this study, the injury potential could be modulated to slightly higher than 0 mV in the anode-centered group by placing anodes at the site of injury and cathodes at the rostral and caudal section, whereby a weak electrical field, directed away from the site of injury, existed in the extracellular space. Thus, the Ca^{2+} will flow along the electrical field preventing the influx of ions. In the cathode-centered group, the injury potential was modulated to –70 mV, which is the normal resting membrane potential. It was noticed that membranes at the site under the anodes were intact and the membrane potentials were also at resting membrane potential. In addition, the membrane at the injury site was destroyed to make the intracellular and extracellular electrical potential the same. So the electrical potential of the intracellular space under the anodes equaled that of the extracellular space of the injury site, which may also lead to few influx of Ca^{2+}. Moreover, the extracellular concentration of Na^+ is also much higher than the intracellular space, meaning that electrical stimulation has the same effect on Na^+ as it has on Ca^{2+}. Therefore, the main components of injury currents can all be modulated by electrical stimulation, whether in the anode- or cathode-centered groups, whereas other methods of electrical stimulation provided different effects on extracellular cations. However, this study did not provide direct evidence of the direction or concentration of Ca^{2+} flow, which needs to be investigated in a future study.

Based on our results, there was no Ca^{2+} or Na^+ influx when electrical stimulation was applied after injury. However, when the electrical stimulation is ceased, the formation of the injury potential is believed to restart and its value should be equal to the initial value before electrical stimulation. However, in this experiment, the absolute value of the injury potential measured immediately after electrical stimulation in both the anode- and cathode-centered groups were much lower than the initial absolute value and higher than the control group. Taking into account an earlier finding that the grade of injury positively correlates to the initial amplitude of the injury potential, this contradiction could be explained if the membrane of injured axons has a certain capacity for self-repair. Therefore, when the injury potential begins to reform, the grade of injury would already be reduced, resulting in a decrease in the influx of extracellular Ca^{2+} and Na^+. This is important for clinical applications, whereby if the injury potential caused by spinal cord injury is modulated by electrical stimulation immediately after injury, the process of injury could be delayed, allowing for more self-repair and a better prognosis. However, the immediate application of electrical stimulation after injury is not possible in the clinical setting unless the patient is already present. Modulation of the injury potential at different periods after spinal cord injury should be investigated to examine its therapeutic potential in full.

Measurement of charges flowing into the cell illustrates the imbalance between intracellular and extracellular charges caused by the collapse of the normal membrane. In the anode- and cathode-centered groups, the total charges flowing into the cell from the extracellular space were 0.37×10^{-3} C and 0.31×10^{-3} C, 77% and 65% of the influx charges in the control group. This means that electrical stimulation reduces the influx charges after injury, which is good for maintaining the balance of intracellular and extracellular ions. Because the positive ions flowing into the cell after the collapse of membrane mainly consist of sodium and calcium, the modulation in injury potential can reduce the influx of these positive ions. As excess sodium can lead to cell edema and excess calcium can increase secondary damage, our finding that electrical stimulation reduces the intracellular concentration of these ions in

injured cells could improve the prognosis of spinal cord injury.

Furthermore, choosing the correct intensity of electrical stimulation is an important issue in the study of neural regeneration. Although the published clinical trials of Shapiro *et al* [22] showed that electrical stimulation was capable of enhancing spinal cord regeneration, the stimulating intensity they used was not based on *in vitro* experiments and had no theoretical basis. Thus, the development of their therapy with electrical stimulation was confined and there have been no subsequent reports using this technique. However, we propose in this paper that injury potential could be an indirect parameter in electrical stimulation therapy. In this study, the rostral and caudal injury potentials were all modulated to –70 mV in the cathode-centered group, whereas the rostral and caudal injury potentials were all modulated to slightly higher than 0 mV in the anode-centered group. Although both methods of electrical stimulation could prevent Ca^{2+} influx, there was a significant difference between the stimulating voltages of the two groups. The relatively high intensities in the cathode-centered group should be avoided, because they might cause the tissue near the electrodes to be burned. Therefore, the anode-centered method is preferred for future studies.

Moreover, there are no significant differences between rostral and caudal stimulating voltages in the same period. This suggests that it is advisable to use only one stimulator with the same rostral and caudal stimulating voltages and the rostral and caudal injury potentials can be modulated to slightly above 0 mV at the same time.

As long periods of direct current stimulation may cause tissue damage, a stimulator that provides charge balance is needed. In this study, extracellular Ca^{2+} could be modulated by both the anode- and cathode-centered groups, which may provide a solution for determining the charge balance by setting the product of the stimulating voltage and stimulating time to be a constant. However, the absolute value of the stimulating voltage will change when the polarity of the electrodes are reversed, which differs from the well-known oscillating field stimulator designed by Borgens *et al* [5, 19-20]. Because this stimulating strategy is based on the hypothesis that extracellular Ca^{2+} modulation is important for spinal cord injury repair, much work must be performed in the future to validate this hypothesis, in hope that a new kind of stimulator may be designed to help patients with spinal cord injury.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experimental regarding neuroelectrophysiology.

Time and setting

All experiments were performed at the Beijing Key Laboratory of Bioelectromagnetism, Institute of Electrical Engineering, Chinese Academy of Sciences in China from April to May, 2012.

Materials

A total of 30 female, specific pathogen-free, 3-month-old Sprague-Dawley rats, weighing 250–350 g, were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, China (license No. SCXK (Jing) 2005-0013). All experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[28].

Methods

Electrical stimulator and injury potential measuring system

The proposed electrical schematic of the stimulator for injury potential modulation is shown in Figure 4.

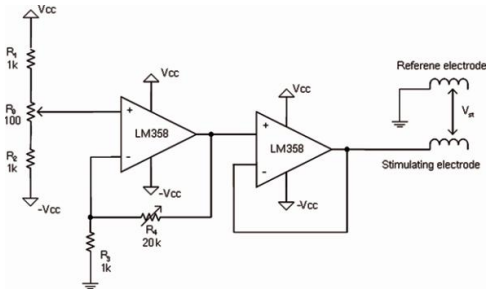

Figure 4 Circuit diagram of the stimulator for injury potential modulation.

A 100 Ω variable resistor, R_0, and two 1 kΩ resistors, R_1 and R_2, provide the following amplification circuit a reference voltage, which can be both positive and negative. A differential amplifier, which consists of an operational amplifier, LM358, a 1 kΩ resistor, R_3, and a 20 kΩ variable resistor, R_4, is used to amplify the reference voltage, and the amplification factor could be adjusted by altering the resistances of both R_0 and R_4. Then a voltage follower is used to avoid the possible variance of the amplification factor of the former amplifier. The stimulating electrode is connected to the output of the voltage follower while the reference electrode is connected to the ground electrode.

A 100 Ω potentiometer, R0, and two 1 kΩ resistors, R1

and R2, are series-connected between the two terminals of the ± 9 V power source, which contains two series-connected 9 V batteries. The electrical potential of the central terminal of the potentiometer is amplified by a non-inverting amplifier, which contains a 1 kΩ resistor, R_3, a 20 kΩ variable resistor, R_4, and a two-channel operational amplifier, LM 358 (Texas Instrument, Texas, USA). The amplification factor of the non-inverting amplifier, which is described as $1 + R_4/R_3$, can be adjusted by varying the resistance of R_4. The voltage follower, which has large input impedance, is used to eliminate the influence of the impedance caused by the tissue between electrodes on the former non-inverting amplifier. The voltage between the outputs of U2 is connected to the stimulating electrode, while the reference electrode is connected to the ground of the circuit.

Two stimulators were used for both rostral and caudal injury potential modulation and the ground electrodes of both stimulators are connected to each other. Both reference electrodes of the stimulators are placed beside the site of injury to make the potential of the site of injury the same. For rats in the anode-centered group, the output electrical potential of the central terminal of the potentiometer, R_0, is adjusted to below 0 to make the electric potential of the stimulating electrode smaller than 0, thus making the reference electrodes become anodes. In contrast, for rats in the cathode-centered group, when the output electric potential of the central terminal of the potentiometer, R_0, is adjusted to above 0, the central reference electrodes will become cathodes. The amplitude of stimulating voltage can be adjusted by changing the resistance of both R_0 and R_4. Electrodes with helix profiles are fashioned from 0.2 mm diameter Pt-Ir (90/10) wire (Nanjing Damai Co., Ltd, Nanjing, Jiangsu Province, China) and connected to the circuit through copper wires.

The potentials produced by the injury current are measured using glass electrodes (Nanjing Jiaoyuan Analytical Instrument, Nanjing, Jiangsu Province, China) as a function of time in the region of injury. The upper half of the glass tube, which contains a mercurous chloride electrode, is filled with 3 mol/L KCl and the lower half with 0.9% saline, of which the tip is plugged by a porous ceramic. The two solutions are separated by agar. The electrode is connected to differential amplifiers, from which the output is recorded by a data-collecting card and displayed on a computer.

Experimental procedure of injury potential modulation and measurement

All rats were anesthetized with an intraperitoneal injection of 2% pelltobarbitalum natricum (0.2 mL/100 g). The spinal cord was exposed by two small laminectomies placed at two vertebral segments (8^{th} and 12^{th} thoracic vertebrae) rostral and caudal to a centrally located laminectomy (10^{th} thoracic vertebrae). Two stimulators of the same kind were used for each rat in the anode- and cathode-centered groups. The electrodes were secured to the paravertebral musculature with silk sutures in such a way that the insulated tips of the electrode did not touch the spinal cord. In the anode-centered group, two anodes were placed beside the central laminectomy, whereas the two cathodes were placed beside the rostral and caudal laminectomies. In the cathode-centered group, the polarities of the electrodes were opposite to those in the anode-centered group. Figure 5 shows the positions of the stimulating electrodes in the experiment. No electrodes were used in the control group.

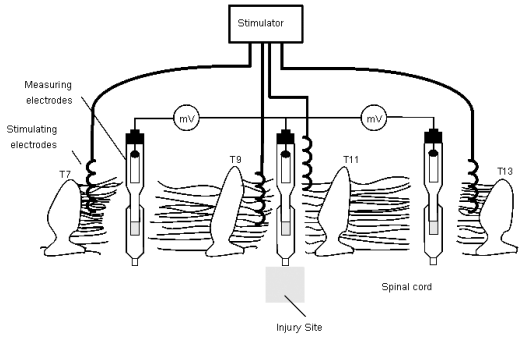

Figure 5 Positions of stimulating electrodes and measuring electrodes in the experiment of injury potential modulation.

The measuring electrode at the injury site was connected to the negative input of the amplifiers, while the other two measuring electrodes were connected to the positive inputs of two amplifiers. The voltage between the left two measuring electrodes and the right two were recorded as rostral and caudal injury potentials. In the anode-centered group, the rostral and caudal stimulating electrodes were cathodes, while the two adjacent to the site of injury were anodes. In the cathode-centered group, the polarities of the electrodes were the opposite of the anode-centered group in the corresponding position.

Rat models of spinal cord injury were created by a modified method for spinal cord contusion, as previously described by scholars[29-30]. A 10 g weight was dropped from 50 mm height to impact onto an organic glass impounder centered on the cord at T_{10}. Initial injury potentials were measured immediately after injury, following which electrical stimulation was applied (Figure 5). In the cathode-centered group, rostral and caudal stimulating voltages were adjusted to make both the rostral and caudal injury potential to be –70 mV, and the stimulating voltages were recorded. In the anode-centered group,

rostral and caudal stimulating voltages were adjusted to make both the rostral and caudal injury potentials slightly higher than 0 mV, and the stimulating voltages were also recorded. The stimulating voltages were adjusted and recorded every 10 minutes for 30 minutes in both the anode- and cathode-centered groups. Rats in the control group received no stimulation. For all rats, injury potentials were recorded every 15 minutes for 4 hours after injury.

Calculation of charges flowing into the cells

The charges that flow into the intracellular space could be calculated from the measured data. U_R (t) and d are the rostral injury potential and the distance between central electrode and rostral or caudal electrode, respectively. The rostral extracellular electric field, E_R (t), and rostral current density, J_R (t), can be approximately described as

$$E_R(t) = -U_R(t)/d \quad (1)$$

$$J_R(t) = \sigma E_R(t) \quad (2)$$

where σ is the extracellular permeability. Let Q_R and S be the total charges flowing from rostral extracellular space into the cell after injury and the cross-section of the spinal cord. Then

$$Q_R = \int_0^T J_R(t) S dt \quad (3)$$

where T is the total time of experiment. From the three equations above, the charges flowing into the cells from rostral extracellular space can be estimated as:

$$Q_R = -\int_0^T \frac{\sigma S U_R(t)}{d} dt \quad (4)$$

If the injury potentials between two measuring time points are considered to be varied linearly, the integration of rostral injury potential can be regarded as

$$Q_R = -\frac{\sigma S}{d} \sum_{i=1}^{N-1} \frac{\tau}{2} (U_{R,i} + U_{R,i+1}) \quad (5)$$

where τ is the time interval between two measuring points, $U_{R,i}$ is the injury potential acquired at the i^{th} measuring point, and N is the total measuring points. In addition, the charges flowing from the caudal extracellular space into the cell, Q_C, can also be calculated according to (1) – (5) if the rostral injury potential is substituted by the caudal injury potential. So the total charges flowing into cell is $Q = Q_R + Q_C$.

Statistical analysis

All the injury potentials recorded were normalized, where the injury potentials were described as the ratio of injury potential of a single rat at each moment to the initial amplitude of injury potential of the same rat. All statistical data were analyzed using SPSS 15.0 software (SPSS, Chicago, IL, USA). Injury potentials and stimulating voltages were compared between two groups, using all combinations from the total three groups, using two-way analysis of variance, where one factor is group (control, anode- or cathode-centered groups) and the other factor is time. Caudal and rostral stimulating voltages in both the anode- and cathode-centered groups are also compared by two-way analysis of variance, where one factor is measuring site (rostral or caudal) and the other factor is time. At each measuring time point, injury potentials measured at the same site and stimulating voltages of the same site are compared between two groups using two-way analysis of variance and two-sample *t*-tests. Rostral and caudal stimulating voltages at the same time point were compared between the anode- and cathode-centered groups using two-way analysis of variance and two-sample *t*-tests. In all the statistical analysis, $P < 0.05$ is regarded as significantly different. In all figures, the error bars express the standard error.

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