

Decreased dynorphin A (1–17) in the spinal cord of spastic rats after the compressive injury

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Abstract

Spasticity in rat hindlimbs was induced by compressing cervical spinal cord with a wax ball. Ashworth score and H-reflex were measured 1 week after the surgery. The results showed that: (1) muscle spasm was detected in the hindlimbs a week after the operation and maintained at least 8 weeks, (2) in the spastic animals, dynorphin A (1–17)-ir decreased significantly in thoracic and lumbar segments of the spinal cord and (3) peripheral administration of κ receptor agonist U50488H and electrical stimulation at 100 Hz effectively relieved the muscle spasm. Our data supported the note that the reduction of endogenous dynorphin A (1–17) might play an important role in the pathogenesis of spinally induced muscle spasticity and the replenishment of its shortage might relieve the spasticity.

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Keywords: Dynorphin A (1–17); Spasticity; Radioimmunoassay (RIA); Ashworth score; H-reflex; Transcutaneous electric nerve stimulation (TENS)

1. Introduction

Spinal spasticity is often caused by spinal cord injury or multiple spinal scleroses and is associated with serious neuropathic pain [5,19]. Our understanding of this debilitating syndrome is very limited because of lacking an easily manipulated animal model, which restricted the effective drug discovery. Although several classes of compounds, including baclofen and tizanidine, showed some efficacy against spasticity, they were not appropriate for regular application because of the requirement of intrathecal administration and the side effects manifested after the long-term usage [2,6,9,18]. Recently, electrical stimulation has been widely accepted due to its efficacy, safety and technical simplic-

ity [10,21,22,23,29]. Studies showed that high frequency electrical stimulation is more effective than low frequency [10,16,24], though the underlying mechanism is not determined. We postulated that it might be related to the acceleration of the release of the spinal dynorphin A (1–17), a major factor in mediating electroacupuncture analgesia at high frequency [3,8,11,13]. Additionally, intrathecal (i.t.) administration of NDAP, an analogue of dynorphin A (1–8), has been found to successfully relieve spasticity in rabbit model [28]. In the present study, we established a spinal spasticity model in the rat, and then measured immunoreactivity (IR) of spinal dynorphin A (1–17) during the induction of the spasticity in order to further explore the substantial roles that dynorphin A might play in the spinal spasticity.

2. Materials and methods

2.1. General

Female Wistar rats weighing 250–300 g were provided by the animal supply center of Beijing Medical University. The

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rats were housed six per plastic cage with standard chow and tap water available ad libitum. Upon arrival in the lab, the animals were allowed to acclimate to the facilities and the examiner for at least one week. All procedures were conducted according to the Guide for the Care and Use of Experimental Animals of Beijing Medical University adopted from NIH USA.

2.2. Animal surgery

The whole procedure was performed in the sterilized condition and the details were described in an earlier study [27]. Briefly, rat was anesthetized with chloral hydrate (400 mg/kg). After removing the hair with razor, the animal was bathed in body cleaner, followed by iodine and 70% alcohol successfully. The skin in the back of the neck was cut, and subcutaneous tissue and muscle were bluntly separated until the exposure of atlanto occipital membrane. Opening the membrane carefully with a needle, and a 2 mm diameter wax ball was inserted into the spinal subarachnoid space with a guide tube about 1.5–2 cm below the incision of the atlanto occipital membrane, the membrane was then closed and the skin was sutured. To ensure the correct placement of the wax ball in the region of C3–C4, each animal was subjected to autopsy after the experiment. Animals with the wax ball located outside area C3–C4 were discarded in our study. Similar surgical operation was performed on the sham animals except for the insertion of the wax ball.

2.3. Evaluation of Ashworth score

All rats were trained 1 week before executing the formal experiments in order to adapt the animals to the environment and the procedures. Rats were put in the prone position during the examination. The left hand of the examiner held the rat head and masked its eyes lightly. After the rats relaxed completely, the passive movements of the limbs were made [1]. The resistance against the passive movement was measured by Ashworth score, which is defined as follows:

- 0, no increase in tone;
- 1, slight increase in tone giving a ‘catch’ when the limb is moved in flexion or extension;
- 2, more marked increase in tone but the limb is easily flexed;
- 3, considerable increase in tone-passive movement difficult;
- 4, limb rigid in flexion or extension.

All the examinations were performed in a blind manner by a single experimenter unaware of the treatment. The Ashworth score in each rat was measured on 3 different days a week, for a total of 8 weeks. The average of the three measurements was recorded as the final Ashworth score for that week.

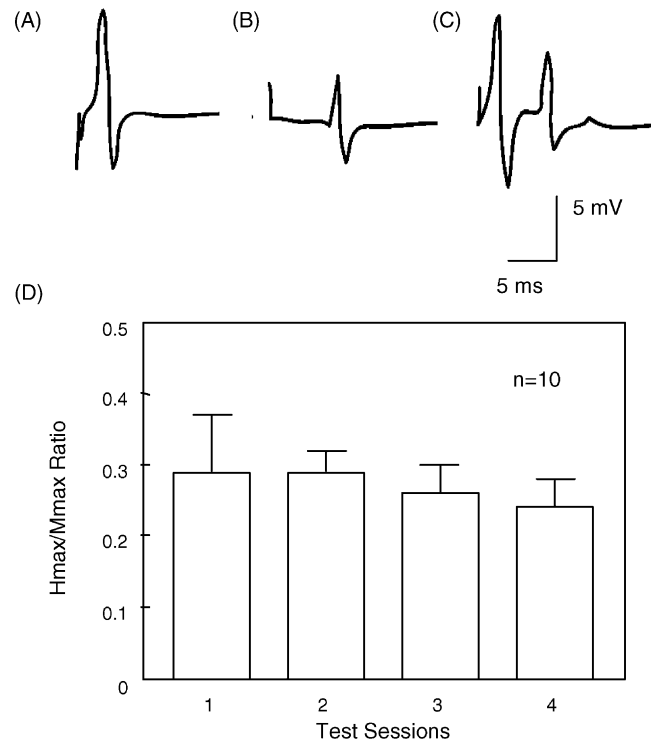


Fig. 1. Measurement of H-reflex and M response. (A) Maximum response of M wave with latency of 7.21 ms. (B) Maximum response of H wave with latency of 0.69 ms. (C) Average of 10 traces of maximum M response and H response. (D) Each bar represents the average of the ratio of Hmax/Mmax measured on 4 different consecutive days. The maximum H amplitude and maximum M amplitude was measured by the difference between the highest peak and the lowest peak. The data was expressed as mean \pm S.E.M.

2.4. Measurement of H-reflex and M response

The rat was loosely immobilized in a unique plastic cylinder with the hind legs protruding. Before applying the recording electrode, the hair in the right leg was removed to expose the skin completely. A pair of fine silver pad (2 mm in diameter) was vertically stuck on the skin on the top of gastrocnemius muscle with 0.5 cm apart. A pen-like stimulation electrode was placed along the sciatic nerve located in the middle lateral of the leg. The animal was grounded by inserting a silver wire in its tail.

When the EMG baseline was stable, a single-pulse (1 mA, 0.2 ms) was released from the computer that was connected to the MacLab/4e physiological recorder, with stepwise increment of 1 mA until achieving maximum response. The stimulation stopped automatically when the stimulus intensity reached 5 mA. H and M wave were sampled at 10 Hz and filtered at 10 Hz–10 kHz. In the low stimulus intensity, only H wave was evoked. In the high intensity stimulation, both H and M waves were evoked simultaneously (Fig. 1C). Fig. 1A is a sample of M wave with short latency and Fig. 1B is a sample of H wave with long latency. Ten traces with the maximum H and M response were averaged. Their amplitudes were obtained by subtracting the lowest peak from the

highest one. The ratio of Hmax/Mmax was calculated for further statistical analysis.

In order to test whether Hmax/Mmax ratio kept stable in the same animal ($n=10$) in different consecutive sessions, we performed the same experiment on 4 consecutive days. As shown in Fig. 1D, no significant differences can be seen among the four testing sessions, which showed the consistency of the measurement of H-reflex and M response.

2.5. Radioimmunoassay of dynorphin A (1–17)

Rats were rapidly decapitated and cut at sacral vertebra. The entire spinal cord was removed by flushing 10 ml normal saline rapidly into the sacral canal. The cervical, thoracic, and lumbar segments were collected, respectively, and then the tissue was heated in boiling water for 10 min. After homogenization at 4 °C for 10 min, the homogenate was centrifuged at $10,000 \times g$ (4 °C) for 20 min. The supernatant was lyophilized and reconstituted in 0.5–1.5 ml of 0.05 M phosphate buffer (pH 7.6). The protein content in the tissue was assayed by using Coomassie blue method. Dynorphin A (1–17)-ir was detected using the radioimmunoassay kits provided by Phoenix Company. The antibody showed no cross-reactivity with dynorphin A (1–13), Leu-Enkephalin and Met-Enkephalin. Standard curve was plotted and IC_{50} was shown to be 51 pg/tube in the standard curve, from which the immunoreactivity of dynorphin A (1–17) was calculated. The content of dynorphin A (1–17) was calibrated by dividing the protein content in the corresponding tissue and expressed as ng/mg protein.

2.6. Assessment of transcutaneous electric nerve stimulation (TENS)

Before each experimental session, Ashworth score in the rat was measured and its hair was removed. The rat was then loosely immobilized in a plastic cylinder with the hind legs protruding. Two pairs of gold foil electrode pads were attached to the point ST36 (located near the knee joint, 5-mm lateral and lower from the anterior tubercle of the tibia) and SP-6 (located near the ankle joint in front of the Achilles tendon at the level of the superior border of medial malleolus). The two pads in each leg were connected with the output terminal of Han's acupoint nerve stimulator (HANS, manufactured by the Beijing Medical University), which delivered square waves of 0.2 ms pulse at a frequency of 100 Hz. The intensity started at 1 mA, increased stepwise by an increment of 1 mA, and ended at 3 mA, with each intensity lasting for 10 min. After the stimulation, the rat was set free and the Ashworth scores in both hindlimbs were evaluated.

2.7. Drugs and statistics

U50,488H and Naloxone were purchased from Sigma (St. Louis, MO). Data was expressed as mean \pm S.E.M. Mann–Whitney test was employed to compare the Ashworth

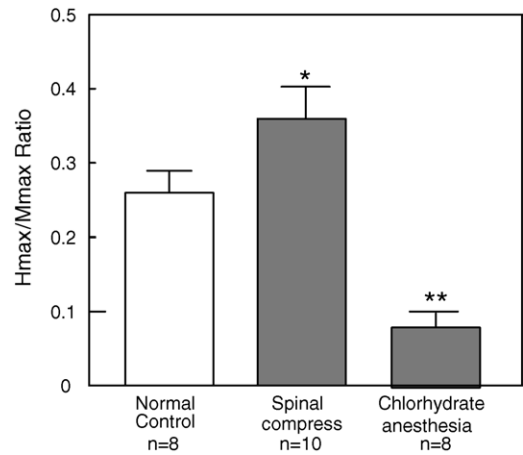


Fig. 2. The influence of spinal compression and anesthesia on the H-reflex and M response. The number under each bar represents the animal number in each group. The measurement of Hmax/Mmax ratio was described in Fig. 1. * $p < 0.05$ and ** $p < 0.01$ when comparing with animals without any pretreatment, t -test. The data was expressed as mean \pm S.E.M.

score between different groups. Continuous data were analyzed using ANOVA followed by Student's t -test. $p < 0.05$ or less is considered statistically significant based on two-sided hypothesis test.

3. Results

3.1. Increased Hmax/Mmax ratio

Ashworth score and Hmax/Mmax ratio were considered as two critical parameters in evaluating spasticity [1,4,10]. The ratio of Hmax/Mmax increased in the spastic animal and it decreased when the animal acquired anesthetic drugs [14,15,19]. Twenty-six rats were randomized into three groups in our study. In the first group ($n=10$), H-reflex was measured 4 days after the compressive surgery. The second group ($n=8$) was subjected to sham surgery, and H-reflex measured 4 days after the surgery. The third group was subcutaneously injected with 10 mg/kg chlorhydrate before the assessment of H-reflex. As shown in Fig. 2, the average Hmax/Mmax ratio in the sham group was 0.26 ± 0.03 . The ratio showed a significant increase (0.37 ± 0.04 , $p < 0.05$) in the surgery group, and a significant reduction in the group with administration of anesthetic drug (0.08 ± 0.02 , $p < 0.01$).

3.2. Increased Ashworth score

Ashworth score was measured in both hindlimbs three times a week for 8 weeks in the compressive and sham group, respectively. The average of Ashworth score measured in compressive animal ($n=6$) and sham animal ($n=8$) in the 1st, 2nd, 4th and 8th week were plotted and indicated in Fig. 3A. Ashworth score started to rise on the 4th day after the surgery and remained in the high level for 8 weeks. When compared in each week, we found the Ashworth score in

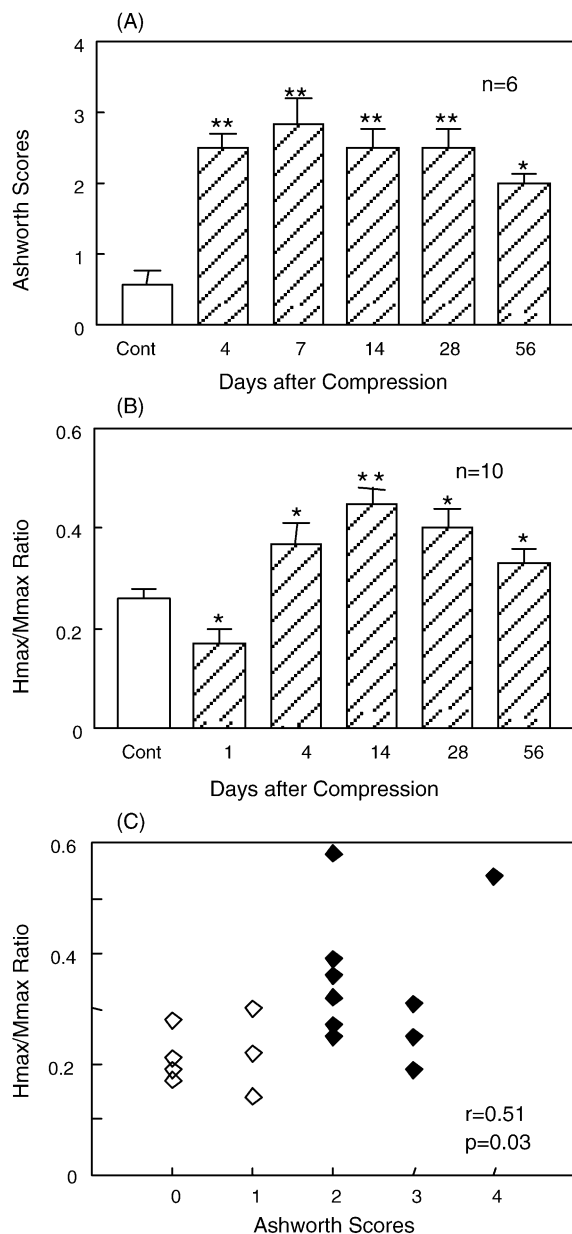


Fig. 3. Evaluation of spasticity induced by spinal cord compression. (A) Ashworth score measured on the different days after surgery. Blank bar represents the average of Ashworth score in sham animals. Slant bar represents the average of Ashworth score in the animal with compressive surgery. * $p < 0.05$ and ** $p < 0.01$, Mann–Whitney test, when compared with control group. (B) Change of Hmax/Mmax ratio on the different days after the surgery. * $p < 0.05$ and ** $p < 0.01$ when compared with control group, ANOVA. (C) Correlation between Ashworth score and Hmax/Mmax ratio.

compressive animals is significantly higher than that in sham animals ($p < 0.05$, Mann–Whitney test). Simultaneously, H-reflex and M response were measured on the 1st, 4th, 14th, 28th and 56th day after the surgery. The ratio of Hmax/Mmax showed significant decrease on the first day after surgery (Fig. 3B, $p < 0.05$, ANOVA). However, it started to go up from day 4, achieved its peak on day 14, and remained at the high level until day 56. The similarity between the patterns of the two parameters for muscle spasm suggest that one

may use only one of them to monitor the muscle spasticity (Fig. 3C, $p < 0.05$, regression analysis). Therefore, Ashworth score was employed to evaluate the spasticity in the following experiments.

3.3. Decreased dynorphin A (1–17)-ir in the spinal cord after the compressive surgery

Out of 50 rats subjected to compressive surgery, 26 showed an increase of Ashworth score 4 days after the surgical operation at or higher than 2. They were used for the determination of dynorphin A (1–17)-ir by radioimmunoassay. A group of 5–6 rats was sacrificed at the end of 1, 2, 4 and 8 weeks after surgery to obtain the time course of the changes of dynorphin A content in cervical, thoracic and lumbar segments.

Rats receiving sham operation were served as the control. A group of two to three rats were sacrificed in the same time period (1, 2, 4 and 8 week after the surgery) and the spinal cord was obtained for dynorphin radioimmunoassay. Since there were no significant changes over time, they were averaged to serve as the control.

One week after the compression surgery, dynorphin A (1–17)-ir displayed a significant decrease in the thoracic and lumbar segment (Fig. 4B and C, $p < 0.01$, ANOVA). In thoracic segment, the reduction lasted 8 weeks (Fig. 4B). In lumbar segment, dynorphin A (1–17)-ir reached the lowest level at week 4 (1.85 ± 0.71 ng/mg protein). By week 8 there is an increase in dynorphin content (7.9 ± 0.96 ng/mg protein). However, it was still significantly lower than the control level (11.42 ± 0.99 ng/mg protein, Fig. 4C, $p < 0.05$, ANOVA). In the cervical segment, there was a tendency of gradual decrease over time, although this change was statistically not significant (Fig. 4A).

3.4. Therapeutic effect of U50,488

Eighteen rats subjected to compressive surgery showing an Ashworth score at least 2 or over were used and randomized into three groups on the 14th day after surgery. They were intraperitoneally injected with (1) κ receptor agonist U50,488 H (10 mg/kg), (2) the κ receptor antagonist naloxone (20 mg/kg) and (3) an equal volume of normal saline. Then, Ashworth scores were measured 30 min after the drug administration, for a total of 90 min. The relative Ashworth score was calculated by the difference of Ashworth score measured before and after drug injection. Thus, the positive value means Ashworth score increased after the drug administration and vice versa. The result showed that administration of U50, 488H significantly decreased the Ashworth score and the reduction maintained for 50 min (Fig. 5, $p < 0.05$). However, pretreatment of naloxone has no significant effects.

3.5. Therapeutic effect of TENS

Rats subjected to compressive surgery showing an Ashworth score at least 2 or over were used and randomized into

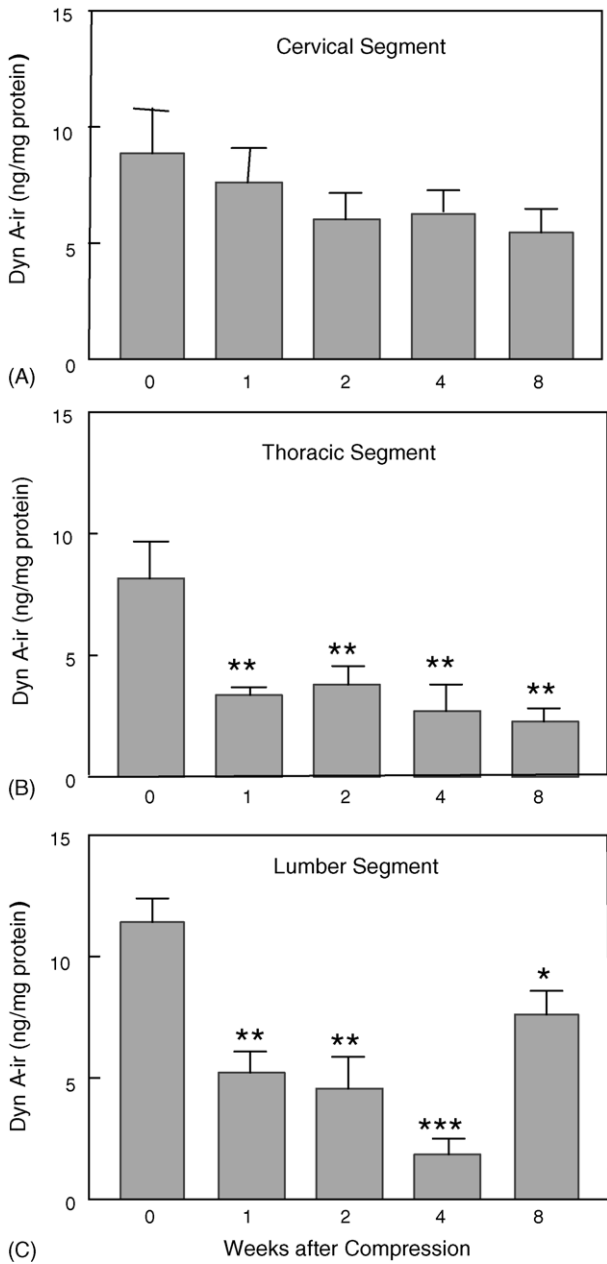


Fig. 4. Decreased dynorphin A (1–17)-ir in the spinal cord of spastic animal. Dynorphin A (1–17)-ir was detected at the different segments of spinal cord on the 7th, 14th, 28th and 56th day after the surgery. Control is the animal with sham surgery. (A) Dynorphin A (1–17)-ir in cervical segment. (B) Dynorphin A (1–17)-ir in thoracic segment. (C) Dynorphin A (1–17)-ir in lumbar segment. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with the control group, ANOVA.

two groups on the 4th day after surgery. TENS of 100 Hz was applied for 30 min once every other day, for a total of seven sessions. The control group was administrated skin electrodes without electric delivery (Mock TENS). Ashworth score was measured before and immediately after the stimulation. The results were shown in Fig. 6. From sessions 1 to 3, no significant difference can be seen in the three groups (mock group, after stimulation; TENS group, before and after

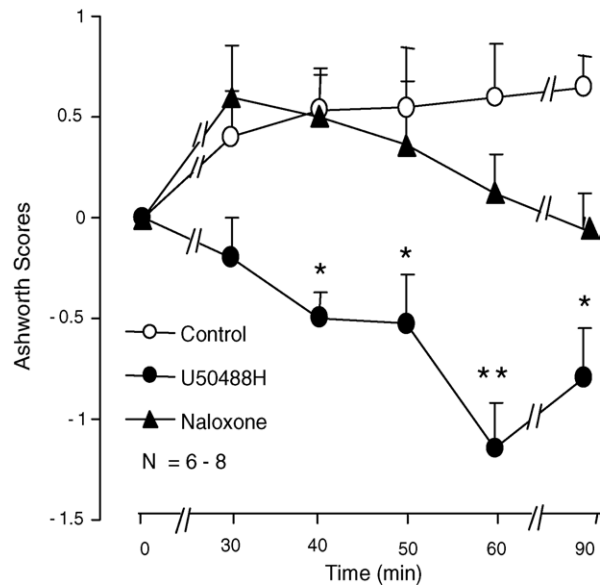


Fig. 5. The effect of κ receptor agonist U50,488H and antagonist naloxone on spinal spasticity. U50,488H (10 mg/kg), naloxone (20 mg/kg) and equal volume of normal saline (5 mg/kg) were injected intraperitoneally in the 14th day after surgery. The relative Ashworth score means the difference of Ashworth score measured before and after drug injection. * $p < 0.05$ and ** $p < 0.01$ when compared with normal saline group.

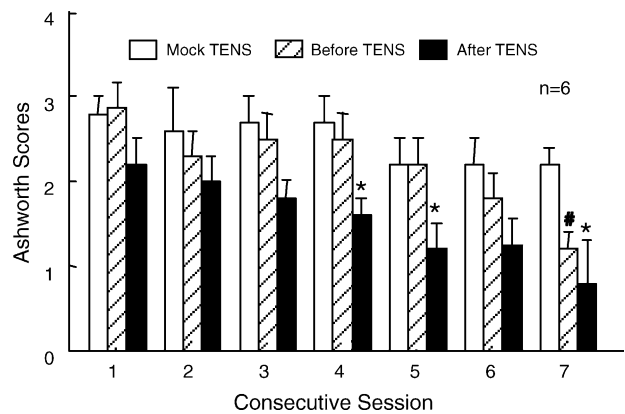


Fig. 6. The influence of repeated 100 Hz TENS on spinal spasticity. Mock TENS group served as the control group, in which electrodes were placed in situ without electric current delivery. Ashworth score was measured before and after every stimulation session and expressed as before TENS and after TENS. #* $p < 0.05$ when compared with Mock group, Mann–Whitney test.

TENS administration). However, by the 4th consecutive session, the Ashworth scores decreased immediately after the stimulation. By the 7th session, the Ashworth scores that were measured before the stimulation also showed a significant reduction ($p < 0.05$ versus control group, Mann–Whitney test).

4. Discussion

In the present study, we demonstrated that spinal spasticity in the rat could be induced by compressing the spinal cord

at C3–C4 with a wax ball, with symptoms lasting for at least 8 weeks. Along with the development of spasticity there is a corresponding reduction of the endogenous dynorphin A (1–17)-ir in spinal cord, especially in the thoracic and lumbar segments of the cord.

If the reduction of dynorphin A in the spinal cord is one of the reasons to induce the spinal spasticity, administration of dynorphin A targeting receptor (κ opioid receptor) agonist might relieve the muscle spasm. To test this hypothesis, we used the κ opioid agonist U50,488H to mimic the effect of dynorphin A [20]. Results shown in Fig. 5 indicate that U50,488H produced a significant reduction of the spasticity, which lasted for 60 min. This result is in line with that reported by Tortella et al. who found an anticonvulsive effect of U50,488H in the rat [25]. Also, Yuan et al. reported that 66A-078, a dynorphin (1–8) analog functioning as a long acting κ receptor agonist, injected intrathecally reduced the muscle spasm of the rabbit produced by cervical spinal compression and this effect could be reversed by the selective κ antagonist nor-BNI [28]. However, when we used a large dose of naloxone (20 mg/kg) to block the κ opioid receptors to see if it could potentiate muscle spasm. Results showed (Fig. 5) that naloxone produced no further exacerbation of the spasticity. Considering the fact that in the present study the dynorphin A content in the spinal cord has already kept at a very low level, there was no room for naloxone to show its efficacy of reversing the κ opioid function.

If the spinal spasticity is indeed due to the deficiency of dynorphin A in the cord, one should be able to relieve the spasticity by increasing the release of endogenous dynorphin A in the spinal cord. It has been repeatedly shown that 100 Hz peripheral electrical stimulation can accelerate dynorphin A release in the spinal cord of the rat and humans [3,8,11,12,13,17]. We therefore employed TENS at high frequency (100 Hz), 30 min per session, once every other day for seven consecutive sessions. The results shown in Fig. 6 indicated that in the first week (session 1–3), there seemed to have a tendency of reducing the spasticity, but the change was not statistically significant. Starting from the second week (4th session) there was a significant reduction of muscle spasm immediately after the TENS stimulation. This effect became stronger in the second week. Besides the changes in the amplitude, there was a prolongation of the therapeutic effect, such that the after effect of the 6th session of TENS lasted for as long as 48 h (shown in Fig. 6 as a significant lowering of the spasticity assessed before the 7th session of TENS treatment). This effect bears an extreme similarity with the observation made in a human study, where patients with spinal spasticity were treated with TENS of 2 or 100 Hz, respectively, five times per week for a total of 4 weeks. An improvement of muscle spasm was observed only in the 100 Hz group but not in 2 Hz group. Within the 100 Hz group, a marked therapeutic effect started to emerge only from the second week, and then remained effective in the rest of the observation period [26].

Interestingly, dynorphin A-ir was shown the reduction only in the segments below the injury site. Although there

is a decreasing tendency in the injured cervical segments, the difference is not statistically significant. Considering the early results reported by Faden et al. that a significant elevation of dynorphin A-ir after the traumatic cord injury was only found at the injury site but not distant from the lesion [7], we assume that the possible decreased dynorphin A in the cervical segments might be marked by the elevation of dynorphin A resulting from the compressive injury.

Comparing the two animal models for spinal cord injury-induced muscle spasticity [28], one can find that in the rabbit model the compression was processed slowly by the driving of a screw progressively toward the cervical cord (C3/C4) in a period of several weeks, muscle spasm started to develop until the screw compressed the cord up to 1/2 or 2/3 of the diameter in the X-ray image. In the rat model used in the current study, however, the procedure was completed by a single surgical operation of inserting a wax ball into the cervical canal. Muscle spasm developed in the 4th day and remained stable for at least 8 weeks. The later seems to be a much easier model for investigating the mechanisms of spinal spasticity.

In summary, results from our experiments suggested that the decrease of dynorphin A (1–17) in the spinal cord may play a critical role in the pathogenesis of spinally induced muscle spasticity and replenishment of its shortage may relieve the spasticity. The mechanism of the therapeutic effect of TENS needs a further investigation.

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