Anatomical mechanism of spontaneous recovery in regions caudal to thoracic spinal cord injury lesions in rats

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Background: The nerve fibre circuits around a lesion play a major role in the spontaneous recovery process after spinal cord hemisection in rats. The aim of the present study is: in the re–control process, do all spinal cord nerves below the lesion site participate , or do the spinal cord nerves of only one vertebral segment have a role in repair? **Methods:** First we made a T7 spinal cord hemisection in 50 rats. Eight weeks later, they were divided into 3 groups based on distinct second operations at T7: ipsilateral hemisection operation, contralateral hemisection, or transection. We then tested recovery of hindlimbs for another 8 weeks. The first step was to confirm the lesion had role or not in the spontaneous recovery process. Secondly, we performed T7 spinal cord hemisections in 125 rats. Eight weeks later, we performed a second single hemisection on the ipsilateral side at T8-T12 and then tested hindlimb recovery for another 6 weeks. **Results:** In the first part, the Basso, Beattie, Bresnahan (BBB) scores and the electrophysiology tests of both hindlimbs weren't significantly different after the second hemisection of the ipsilateral side. In the second part, the closer the second hemisection was to T12, the more substantial the resulting impairment in BBB score tests and prolonged latency periods. **Conclusions:** The nerve regeneration from the lesion area after hemisection has no effect on spontaneous recovery of the spinal cord. Repair is carried out by all vertebrae caudal and ipsilateral to the lesion, with T12 being most important.

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Abstract

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Conclusions: The nerve regeneration from the lesion area after hemisection has no effect on spontaneous recovery of the spinal cord. Repair is carried out by all vertebrae caudal and ipsilateral to the lesion, with T12 being most important.

Keywords: spinal cord injury, hemisection, transection, recovery, electrophysiology, nerve

1 Introduction

Brain is plastic and mammals are capable of spontaneous recovery after spinal cord injury. The mechanisms underlying this process are not yet clear and are disputed. After brain or spinal cord injury, many researchers have found that transplantation of multifunctional three-dimensional scaffolds and stem cells treatment with neurotrophic factors, administration of small molecules, or genetic modifications in the lesion area promote neuronal regeneration in the lesion and improve motor function recovery (Estrada et al. 2014; Fan et al. 2010; Jee et al. 2012; McCall et al. 2012; Shi et al. 2015; Tan et al. 2016; Wright et al. 2011). In addition, several groups have shown that, after hemisection of the thoracic spinal cord, both hindlimbs show significant improvement 3-5 weeks later. Moreover, if a second hemisection on the side contralateral to the lesion was performed, rats showed complete paralysis of both hind limbs with no signs of recovery of locomotor function over 4 weeks (Courtine et al. 2008). This observation demonstrates that nerve fibers around the lesion participate in repair. These fibers must originate rostral to the lesion on the ipsilateral side, then cross the midline to the contralateral side, travel down the spinal cord, and re-cross the midline caudal to the lesion (Ballermann & Fouad 2006; Courtine et al. 2008; Etlin et al. 2010; Reed et al. 2008). These two different repair mechanisms are in conflict with each other and further research is needed to confirm and explain the repair process. If the mechanisms of repair in both contexts were resolved it would inform novel treatments to promote recovery and further improve limb function in patients with spinal cord injury.

Among the questions that remain to be answered is whether the nerve fiber circuits that control the ipsilateral hindlimb after injury are comprised of spinal cord nerves in a single vertebra or spinal cord nerves in multiple vertebrae contribute to these nerve fiber circuits and

participate in the recovery process. Accordingly, we designed a study to resolve these two possibilities. We first carried out a hemisection of thoracic vertebrae 7 (T7) on the left side of spinal cord, then 8 weeks later performed a second hemisection of thoracic vertebrae 8-12 (T8-T12) on the ipsilateral side. We then compared the extent of recovery of hindlimb function acrosss each hemisection group.

2 Materials and Methods

2.1 Animals

In this study, we used adult Sprague–Dawley rats (200–220 g). All rats were allowed to acclimate to the new environment for 7 days before the start of any experimental procedures. The rats were housed on a 12 h light-dark cycle with food and water provided ad libitum. All rats were anesthetized before any procedure that would cause pain. The rats in the study were obtained from the Vital River Company. Ethical approval was obtained from the Beijing Neurosurgical Institute Laboratory Animals Ethics Committee in China.

2.2 Groups

In the first part of the study, we investigated if regenerated axons coursed ipsilaterally or contralaterally. All rats underwent two operations on T7 spinal cord and were divided into 5 groups (Fig. 1A-E). The first group was a sham operation group (N=10) in which both operations were sham. Groups 2-5 all first received a hemisection of the left side of spinal cord at T7, but had different second operations. The second group was the control group (N=6), in which the second operation was sham, consisted a midline cut in the spinal cord at T7. The third group was the ipsilateral experimental group (N=8), in which a second hemisection was conducted on the

ipsilateral side. The fourth group was the contralateral group (N=8), in which a second hemisection was made on the contralateral side. The fifth was the transection group (N=8), which received a full transection at T7 in the second operation.

In the second part of the study, we investigated the innervation of vertebrae downstream of the lesion site to determine if regenerated axons target single or multiple vertebrae. These experiments comprised 3 main groups, each of which were divided into 5 smaller sub-groups defined according to the site of the second operation: T8-T12. The first group was the operation group (N=37, Fig. 2A1-A2), in which all rats underwent 2 hemisection operations. The first operation was at T7 on the left side; 8 weeks later the second operation was carried out ipsilaterally at a single vertebra from T8-T12 depending on sub-group. The second group was the control group (N=37, Fig. 2B1-B5). All received a hemisection at T7 on the left side, and 8 weeks later underwent a sham operation at a single vertebra at T8-T12 depending on sub-group. The third was the sham operation group (N=35, Fig. 2C1-C5), in which rats in all sub-groups received a sham operation at both time points.

2.3 Hemisection operation

2.3.1 The first hemisection operation

We generated the first hemisection operation according to previously published methods (Arvanian et al. 2009). Before normal bladder control returned, we manually expressed the bladder of each rat once per day.

2.3.2 The second hemisection operation

The second hemisection operation was performed 8 weeks after the first hemisection operation. Except for the vertebra receiving a laminectomy, other operation procedures were the same as in

111 the first hemisection operation.

112

113 **2.4 Basso, Beattie, Bresnahan (BBB) score tests in both hindlimbs**

114 Motor performance was scored were performed according to the well-known Open Field BBB
115 locomotor scale (Basso et al. 1995). It was given each week after each hemisection operation
116 until the 8th week.

117 **2.5 Electrophysiological examinations：**

118 Before beginning electrophysiological examinations each rat was anesthetized. An
119 electrophysiological examination was given before and after each hemisection operation.

120 **2.5.1 Motor-evoked potential (MEP) studies on the body surface**

121 MEP examination and analyses were performed mainly according to previously published
122 methods (Shen et al. 2016; Yin et al. 2015; Ziegler et al. 2011). Before the examination, the rat
123 was in a relaxed state. Intramuscular electrode needles were implanted in the anterior tibial
124 muscle (TA) and little toe abductor muscle (LTA) on both sides. There were 5 wires used for
125 MEP examination, and each wire was connected to a stainless steel pin. The rostral-caudal
126 locations of the wires were as follows: the first needle was in the midline 5 mm from the nose;
127 the second needle was located subcutaneously in the midline of the head; the third needle was
128 located subcutaneously in the mid-belly; the fourth needle was located in the middle of the
129 muscle being tests; and the fifth needle was located in the tail 3 cm from the root. Stimulations of
130 10 mA at 1 Hz were administered once per time point: for 1 ms per stimulus. Each muscle
131 received 3 standard stimuli, and the interval time was 30 s.

132 **2.5.2 MEP on the spinal cord**

133 The purpose of the test in spinal cord was to observe the change of conduction from T7-T8 after

the operation. Eelectrophysiological examinations involved stimulating microelectrodes and recording microelectrodes (Fig. 3F) (Arvanian et al. 2009; Schnell et al. 2011). The responses evoked by stimulating the ventral horn from the rostral end of T7 to the caudal end of T8 on the ipsilateral side were recorded on the same side or on the contralateral side. The stimulation electrode was positioned approximately 0.7 mm from midline, with a depth of 1.3 mm, and an angle of 25-30° from the vertical sagittal plane. The recording electrodes were positioned approximately 0.7 mm from middle line, with a depth of 1.3 mm and an angle of 15-20° from the vertical sagittal plane. We used the average of two recordings for each side. There was a 30 s interval between the two stimuli. The ventral horn stimulus had a duration of 0.01 ms and a current of 0.5 mA and was delivered at 1 Hz.

2.6 Criteria for excluding animals

Rats were excluded from the research according to the following criteria: (1) death during or after the operation; (2) signs of autophagia and/or a serious skin infection; (3) an edematous hindlimb that would affect the BBB score test; (4) death during the electrophysiological recordings.

2.7 Statistics

The statistical analysis was performed using SPSS database (version 19.0; SPSS Inc., Chicago, IL, USA). The BBB scores and electrophysiological examination data are shown as means ± SEM. When the data agreed with the Bartley Ball Test, the repeated measures general linear model test was used to determine the overall differences in the different test times after the operation (1 week to 6, 8, or 16 weeks), followed by LSD (least significant difference) tests to

157 make comparisons among groups. P values less than 0.05 were considered statistically
158 significant.
159
160 **3 Results**
161 **3.1 Part 1: Determination of an ipsilateral versus contralateral course**
162 **3.1.1 BBB score s after the first hemisection operation at T7**
163 After the first operation, none of the rats could move their left hindlimb or perform weight-
164 bearing movements, while the right hindlimb could move slightly. The most significant and rapid
165 improvements in BBB scores of both hindlimbs occurred over the first 2 weeks. BBB scores
166 continued to increase through the 3rd week after the operation and then reached a plateau phase
167 in the 4th week that persisted through the end of the evaluation period at week 8 (Fig. 4A).
168 **3.1.2 BBB scores after a second hemisection operation at T7**
169 A second hemisection at T7 had little effect on movement of either hindlimb in the ipsilateral
170 group. In 7 of 8 rats, BBB scores recovered to the pre-second operation level on the 3rd day and
171 the BBB scores of all rats recovered to the pre-second operation level by the end of the first week
172 following the second operation (Fig. 4D). No significant differences in the BBB scores between
173 the ipsilateral group and the control group were observed after the operation ($p>0.05$, Fig. 4C
174 and D).
175 After the second hemisection at T7 on the contralateral side, movement in both hindlimbs
176 was instantly obstructed and then started to recover 2 weeks later. By the 4th week, recovery
177 entered a plateau phase. Compared to the rats in transection group, there were no significant
178 differences in BBB scores of the ipsilateral group after the operation ($p>0.05$, Fig. 4E and F).
179 However, there were significant differences in BBB scores between the ipsilateral group and the

contralateral group ($p<0.05$, Fig. 4D and E).

3.1.3 Electrophysiological examinations in part 1 of the study

After the second hemisection at T7, the latency periods in both TA muscles and both LTA muscles between rats in the ipsilateral group and control group were not significantly different ($p>0.05$, Fig. 5A-D). However, they were longer in the contralateral group than in the control group ($p<0.05$). There were no significant differences between the contralateral group and the transection group ($p>0.05$). All experimental groups had significantly longer latency periods than the sham group ($p<0.05$).

There were not significant differences in latency periods in the spinal cord between the sham, control, and ipsilateral groups ($p>0.05$, Fig. 6A and B, and Fig. 3A-C). However, the latency period and wave amplitude disappeared in the contralateral and transection groups after the second hemisection ($p>0.05$, Fig. 6A and B, and Fig. 3D1, D2, E1, E2).

3.2 Part 2: Determination of the involvement of vertebrae T8-T12

3.2.1 BBB scores after a second hemisection operation at T8-T12

In the T8 second hemisection group, BBB scores for the left hindlimb decreased slightly, while BBB scores of the right hindlimb were barely affected. Approximately 3 weeks later, BBB scores of both hindlimbs recovered to the level before the operation ($p>0.05$， Fig. 7A1). In comparisons of BBB scores 6 weeks after the second hemisection, the left hindlimbs in T8-T12 sub-groups displayed poorer and poorer improvement ($p<0.05$， Fig. 7A1-A5). Seven of 8 rats in T12 operation sub-groups exhibited no left hindlimb movement 6 weeks after the operation.

Compared to the operation group, BBB scores for both hindlimbs in the T8-T12 sub-groups in the control group decreased significantly after the second hemisection ($p<0.05$, Fig. 7A1-A5,

B1-B5).

BBB scores of both hindlimbs in all T8-T11 sub-groups in the sham operation group recovered completely by the second week after the second hemisection ($p>0.05$, Fig. 7 C1-C4). Compared to the T12 sub-group, the T8-T11 sub-groups displayed better improvement ($p<0.05$, Fig. 7 C1-C5).

3.2.2 Electrophysiological examinations in part 2 of the study

After the second hemisection in the operation group, the latency period in left TA muscles and left LTA muscles became progressively longer from the T8 sub-group to T12 sub-group ($p<0.05$, Fig. 8A, C).

4 Discussion

4.1 Effects of a second hemisection operation at T7

Many previous animal experiments have shown that after a hemisection operation of the spinal cord, the transplantation of stem cells or various engineered tissue materials in the injury region can improve movement of both hindlimbs. Immunohistochemical examinations have also shown that the nerve fibers in regions rostral to the injury site increased and deeply innervated the lesion site. This indicates that nerves fibers penetrating into the area of injury probably play important roles in recovery after spinal cord injury (Estrada et al. 2014; Fan et al. 2010; Jee et al. 2012; McCall et al. 2012; Wright et al. 2011).

However, some studies using animal models have reached conflicting conclusions. An important example is the work of Courtine et al (Courtine et al. 2008), in which they carried out two successive lesions of the rat spinal cord and showed that the contralateral but not ipsilateral side was essential for recovery.

However, Courtine et al did not performed a second hemisection at the injury region, so whether nerve regeneration within the injury region was attributable to hindlimb movements recovery could not be ruled out (Courtine et al. 2008). Accordingly, in this study, we added an additional experimental group that received a second hemisection in the region of injury.

BBB scores for both hindlimbs decreased significantly after the first hemisection at T7, and 4 weeks later, the improvement reached a plateau. When the second hemisection operation was performed on the injury region, it had almost no effect on movement of either hindlimb ($p>0.05$, Fig. 4C and D, Fig. 3B1, B2, C1, C2). The MEP results from body surface and spinal cord also displayed no significant differences ($p>0.05$, Fig. 5A-D, Fig. 6A and B, Fig. 3C1, C2, D1, D2). However, in the contralateral and transection groups, hindlimb movement on both sides disappeared after the second operation and there were no significant difference between groups at 8 weeks post-injury ($p>0.05$, Fig. 4E and F). Moreover, MEP in the spinal cord disappeared after the second operation in both groups (Fig. 6A, B and Fig. 3D1, D2, E1, E2). By contrast, we found that, after the spinal cord transection operation MEP activities were still observed at the body surface. For this reason, MEP examination of the spinal cord is a more accurate indicator of injury and recovery than MEP at the body surface.

We thus conclude that the nerve repair in the injury region has no effect after the hemisection operation at T7 on the ipsilateral side, and reparative responses involved nerve fibers on the contralateral side.

4.2 Effects of a second hemisection operation at T8-T12 on the ipsilateral side

Results of hemisection at a single vertebra from T8-T12 after an initial hemisection at T7 impaired hindlimb movement recovery in all instances, with the most pronounced effects

249 occurring at T11-T12. Therefore, we conclude based on the data in part 2 of this study that, when
250 caudal to the injury region area, spinal cord segments underlying the T8-T12 vertebrae on the
251 ipsilateral side all participated in the spontaneous recovery process after a hemisection operation
252 at T7. In this case, the T12 vertebral area appeared to be the most important for nerve repair.
253

254 **4.3 Repair processes occurring rostral to the lesion**

255 Many findings have shown that, although the direct conduction pathway was destroyed in rats
256 with a spinal cord injury, commands from brain conducted by the corticospinal tract (CST) can
257 still be transmitted to the lumbar spinal cord below the lesion on the ipsilateral side (Bareyre et al.
258 2004; Courtine et al. 2008; Jankowska & Edgley 2006; Kerschensteiner et al. 2004; van den
259 Brand et al. 2012). After injury, an important mechanism of the spontaneous recovery process is
260 thus that the structure and course of nerve fibers in the CST are remodeled such that they make
261 contact with propriospinal neurons that form detour pathways bypassing the lesion (Nishimura &
262 Isa 2012; Pierrot-Deseilligny 2002; Rosenzweig et al. 2010; Zaaimi et al. 2012). However, the
263 CST is not the only descending tract that affects movement and is not be the only projection
264 system that conveys functional recovery (Han et al. 2013; Hurd et al. 2013). Spared
265 reticulospinal fibers play an important role in the recovery process through spontaneous
266 compensatory sprouting and increases in density after injury; they may also operate caudal to the
267 lesion by enhancing indirect access to reticulospinal commands (Ballermann & Fouad 2006;
268 Zorner et al. 2014). Therefore, we hypothesize that the nerve fiber circuit underlying repair in
269 this study was composed of CST and reticulospinal fibers and propriospinal neurons. We
270 speculate that around the T12 vertebra, which was the most important for nerve repair, there
271 were more nerve fibers relative to the other vertebrae that crossed the midline from the

contralateral side to the injury side. However, more research, particularly nerve fiber tracing experiments, is needed to confirm this.

4.4 Repair processes occurring caudal to the lesion

Tillakaratne et al. showed that rats exhibited spontaneous recovery via a step-wise process after a complete transection of the mid-thoracic spinal cord, even though the region caudal to the spinal cord lesion did not make any connections to the brain and in absence of descending tracts passing through the lesion (Tillakaratne et al. 2010). Thus, roles of activity of a locally acting central pattern generator (CPG), which is present in many species, are important to consider (Deliagina et al. 1999; Ekeberg & Pearson 2005; Grillner 1985). While, our research argues for the presence of significant spontaneous locomotor recovery resulting from new forms of dynamic control in the spinal CPG from newly generated or remodeled descending tracts, the local CPG still may play the primary role in recovery following spinal cord injury (Rossignol et al. 2007).

The CPG of the spinal cord is located in the lumbar enlargement at about the T10-12 segments (Magnuson et al. 1999). The second part of this study showed that areas closer to T12 vertebra are more important for nerve repair. Therefore, we could conceivably use various treatments to reinforce the role of CPG and the nerve fibers that connect to it to promote recovery. However, given that a second hemisection operation at T9 on the ipsilateral side could also impair hindlimb movements, which could not recover to pre-operation levels, the CPG is likely not the only factor playing a role in the recovery process.

4.5 Study Limitations

These studies assessed spontaneous recovery from spinal cord injury, but not the impact of any treatment. Further research is needed to confirm if similar results are observed in the case of using a therapeutic intervention within the same experimental injury paradigm; potential therapies include targeting neuroinflammation, transplantation of engineered tissue materials, stem cells, neurotrophic factors, or genetic modifications. We also did not directly assess the path of newly projected nerve fibers to more conclusively and precisely define their course.

5 Conclusions

Our study demonstrates an anatomical mechanism for spontaneous repair processes caudal to spinal cord injury sites in which regenerative fibers cross to the contralateral side, course around the lesion, and then re-cross the midline innervating all caudal, ipsilateral vertebrae with T12 being most important. Further studies should investigate therapeutic approaches that enhance this process and identify the molecular mechanisms that control it.

6 Disclosures

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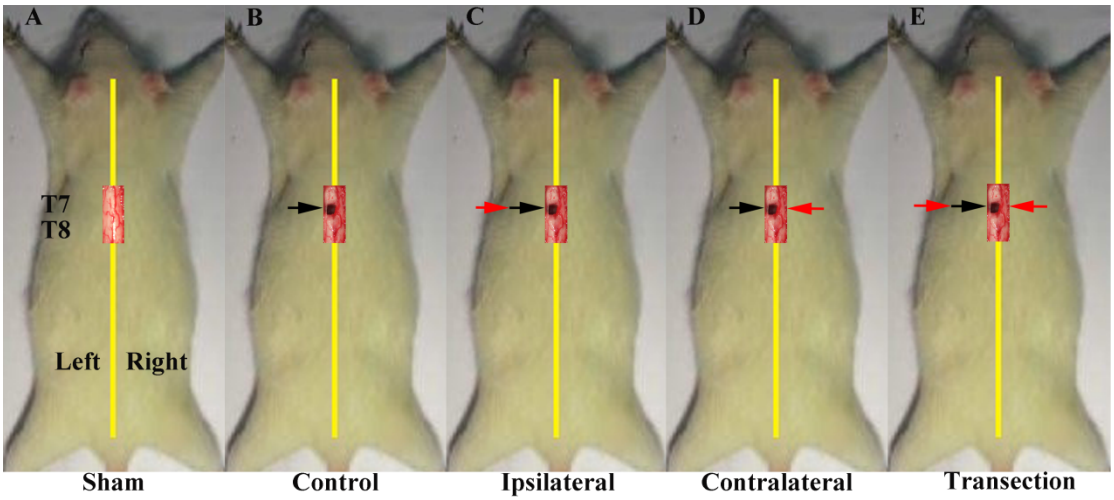
411

412 **8 Figure Legends**

413



414

415 **Fig 1**. Demonstrations of the operations for each group in first part of this study. All the
416 operations were made at T7. A black arrow indicates the level of the first hemisection operation;
417 a red arrow indicates the level of the second hemisection operation. (A) The sham group
418 underwent two sham operations. (B) The control group first underwent a hemisection and a
419 subsequent sham operation. (C) The ipsilateral group first underwent a hemisection and then a
420 second hemisection operation on the ipsilateral side. (D) The contralateral group first underwent
421 a hemisection operation and then a second hemisection operation on the contralateral side. (E)
422 The transection group first underwent hemisection operation, which was followed by a
423 transection operation.

424

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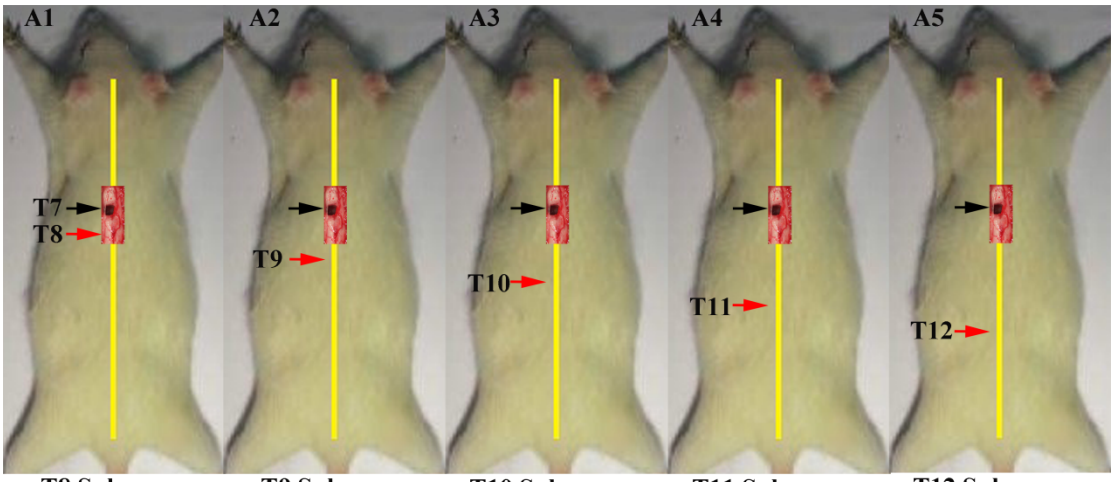
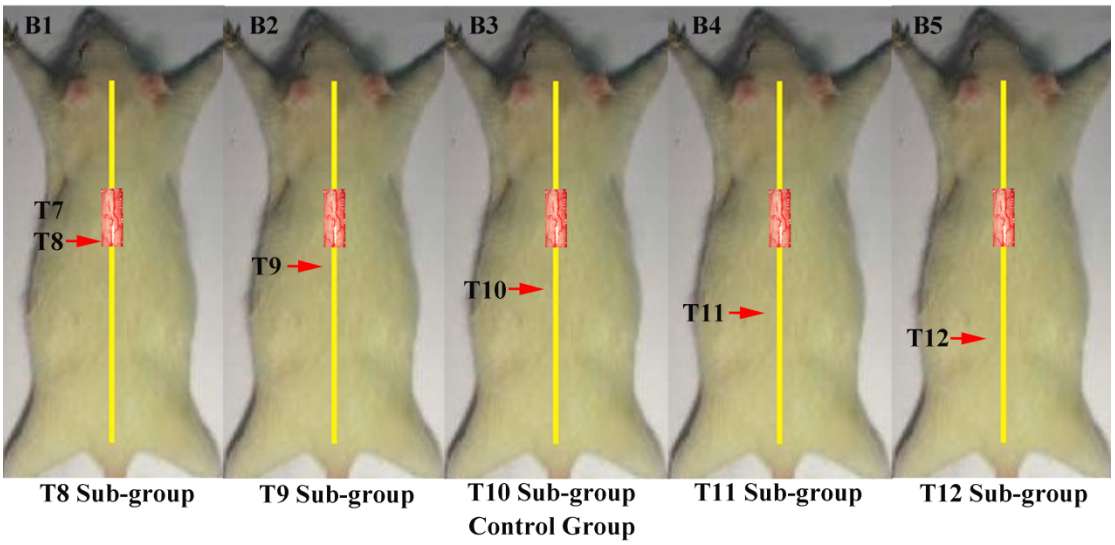
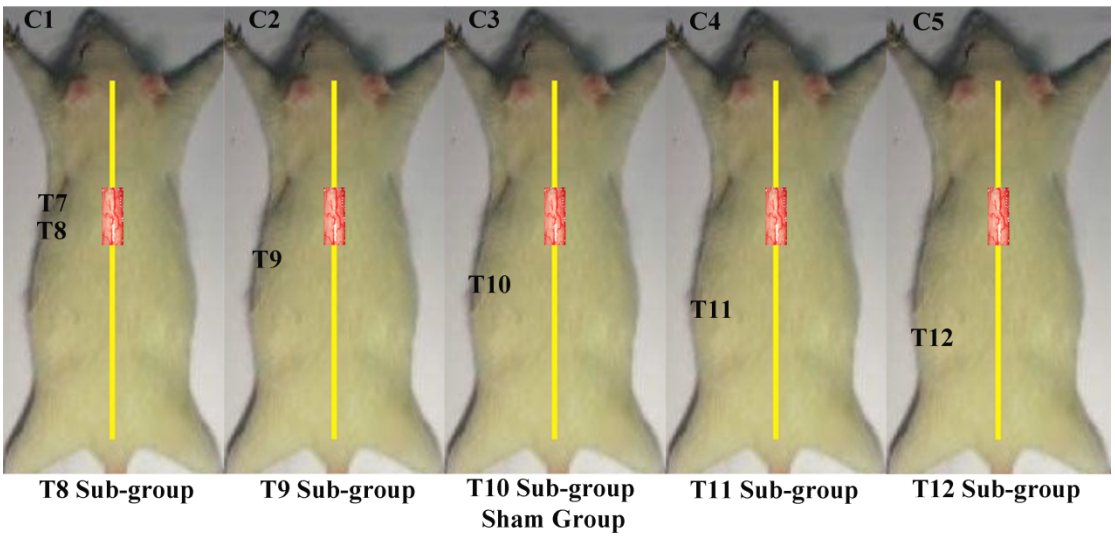

A1
A2
A3
A4
A5
T7
T8
T9
T10
T11
T12
T8 Sub-group
T9 Sub-group
T10 Sub-group
T11 Sub-group
T12 Sub-group
Operation Group


B1
B2
B3
B4
B5
T7
T8
T9
T10
T11
T12
T8 Sub-group
T9 Sub-group
T10 Sub-group
T11 Sub-group
T12 Sub-group
Control Group


C1
C2
C3
C4
C5
T7
T8
T9
T10
T11
T12
T8 Sub-group
T9 Sub-group
T10 Sub-group
T11 Sub-group
T12 Sub-group
Sham Group

427 **Fig 2.** Demonstrations of the operations for each group in second part of this study. A black
428 arrow indicates the level of the first hemisection operation; a red arrow indicates the level of the
429 second hemisection operation. (A1-A5) T8-T12 sub-groups in the operation group. Each group
430 underwent the same first hemisection operation at T7 and a second hemisection at a single
431 vertebra from T8-12. (B1-B5) T8-T12 sub-groups in the control group. Each group underwent
432 the same first sham operation at T7 and a second hemisection operation at a single vertebra from
433 T8-T12. (C1-C5) T8-T12 sub-groups in the sham group. Each group had the same first sham
434 operation at T7 and a second sham operation at a single vertebra from T8-T12.
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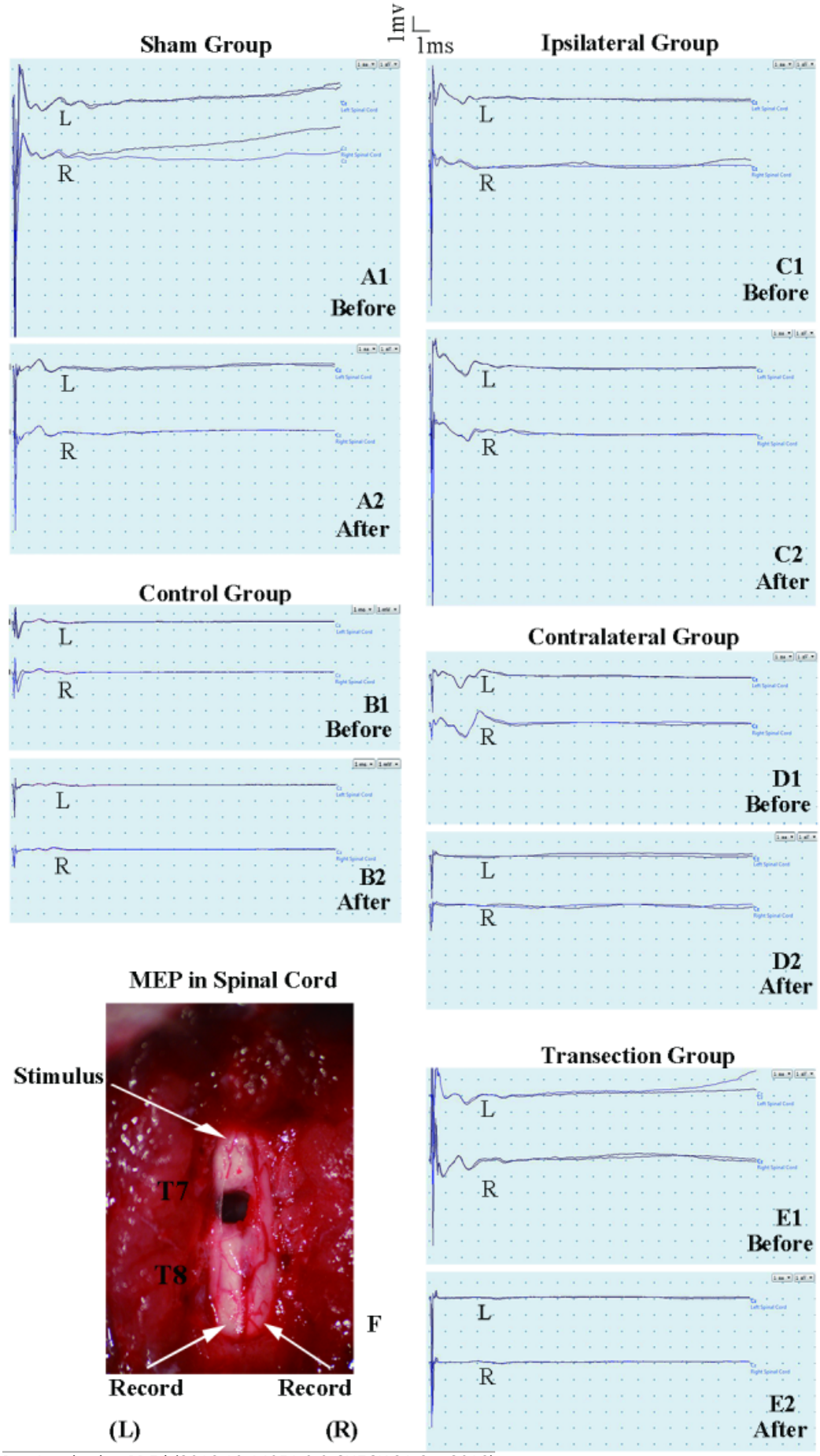
436


1mv
1ms
Sham Group
L
R
A1
Before
L
R
A2
After
Control Group
L
R
B1
Before
L
R
B2
After
MEP in Spinal Cord
Stimulus
T7
T8
Record
Record
(L)
(R)
F
Ipsilateral Group
L
R
C1
Before
L
R
C2
After
Contralateral Group
L
R
D1
Before
L
R
D2
After
Transection Group
L
R
E1
Before
L
R
E2
After

438 **Fig 3**. Electrophysiological examinations of the spinal cord in first part of this study before and
439 after the second hemisection operation at T7: (A) Sham group, (B) Control group, (C) Ipsilateral
440 group, (D) Contralateral group, (E) Transection group.

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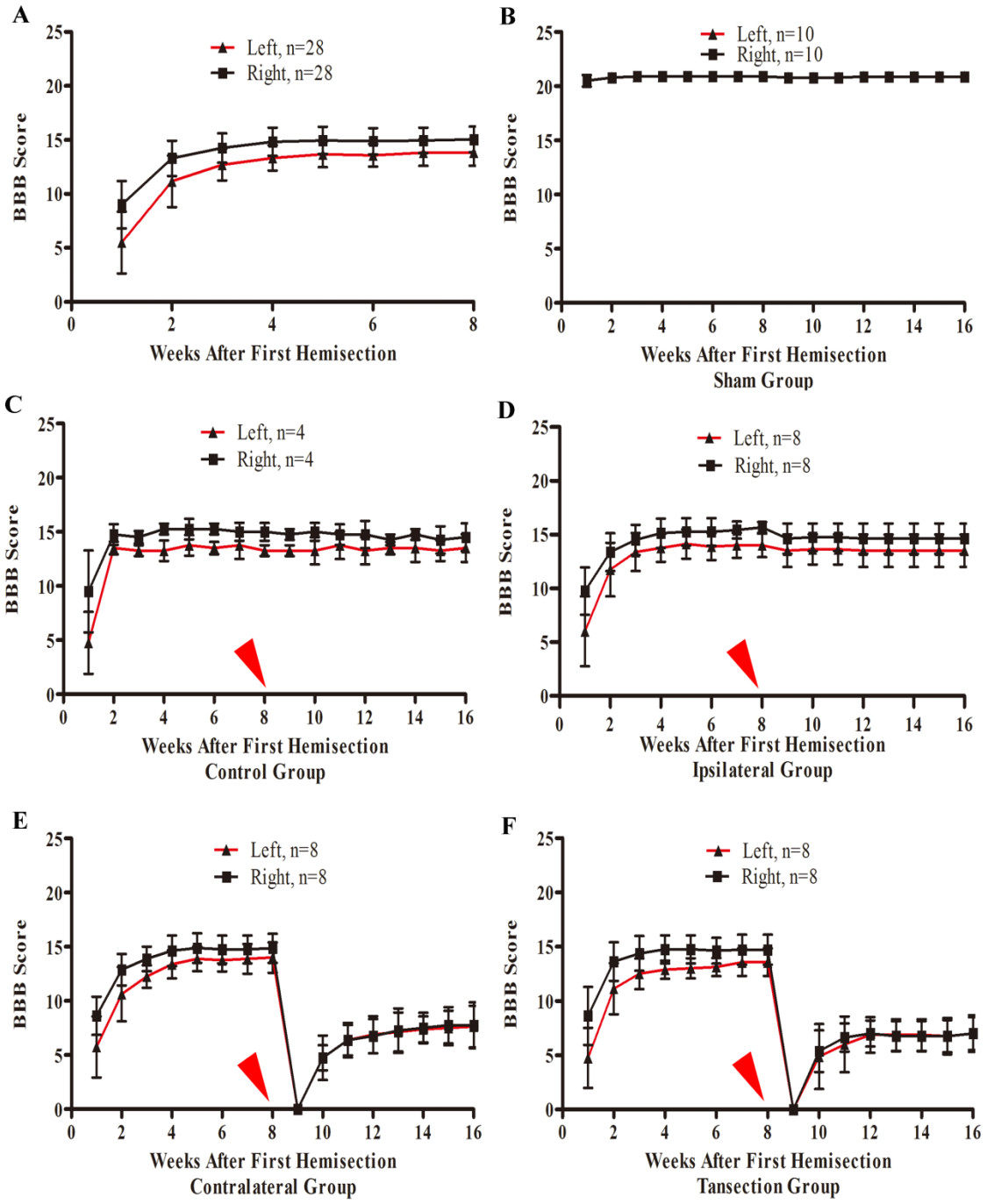
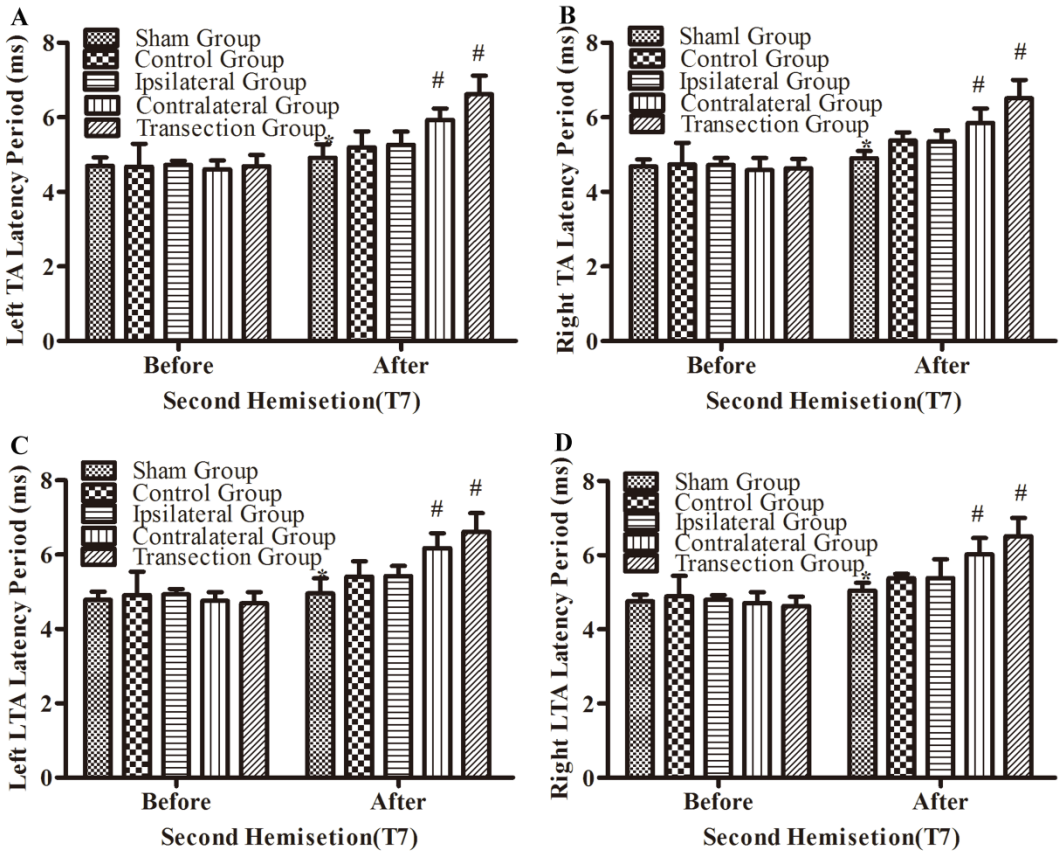


Fig 4. The BBB scores for part 1 of the study in which two hemisection operations were performed at T7. A red arrow indicates the time point for the second hemisection. (**A**) The trend in BBB scores all rates of the first 8 weeks, before the second hemisection operation at T7. (B-E) Trends in BBB scores for each group after the first and second hemisection operations:(B) Sham

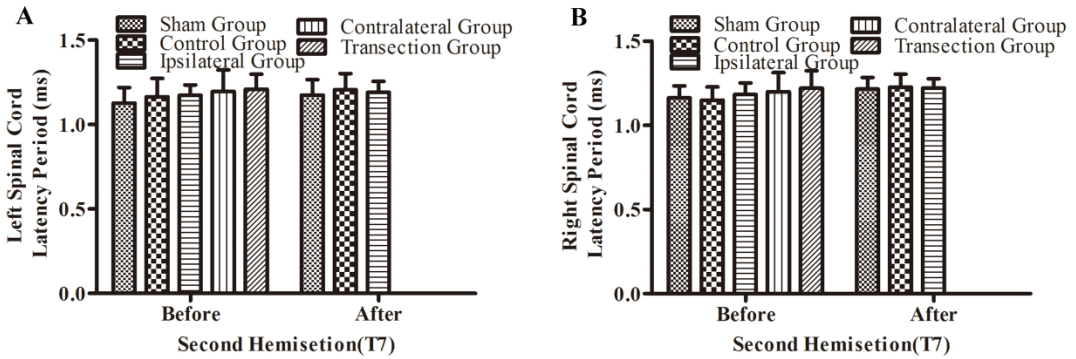
449 group, (C) Control group, (D) Ipsilateral group, (E) Contralateral group, (F) Transection group.
450 Data are presented as mean ± SEM.

451
452



453
454 **Fig 5**. Electrophysiological examinations (MEP, latency periods) in body surface in part 1 part of
455 this study before and after the second hemisection operation at T7. (A-D) The latency periods in
456 both TA muscles and both LTA muscles. *, P<0.05, compared to the control group. #, P<0.05,
457 compared to the other 4 groups. Data are presented as mean ± SEM.

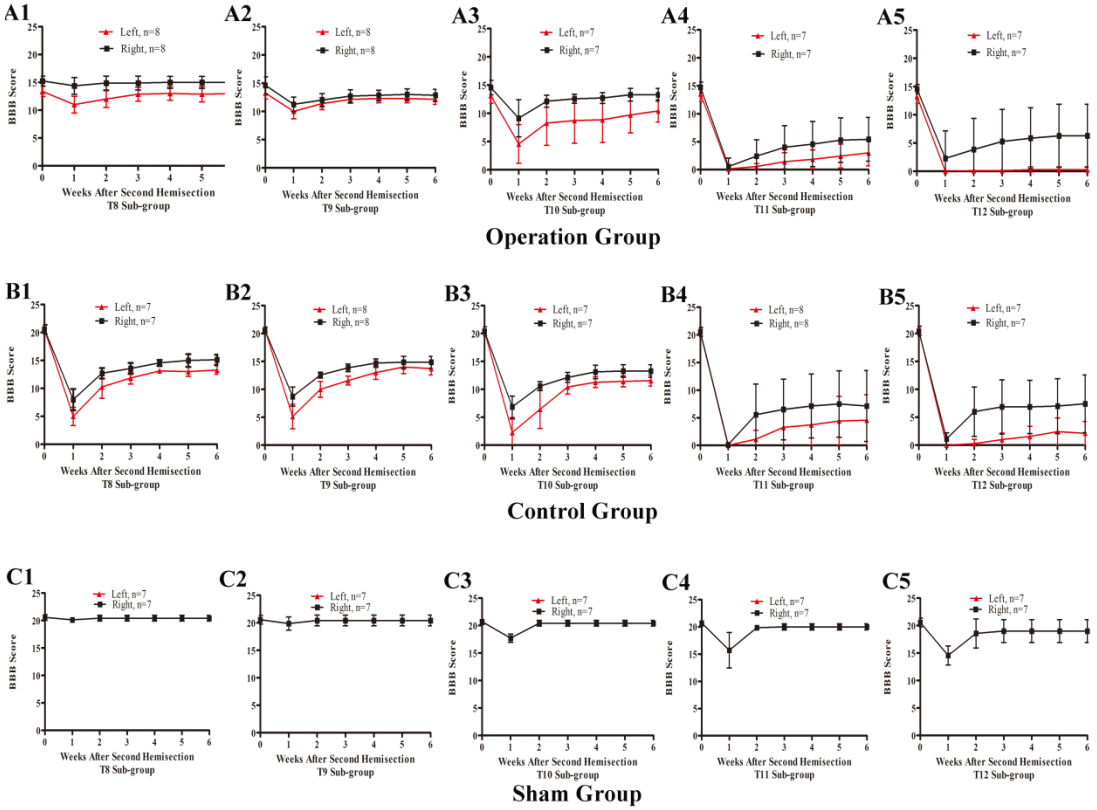
458
459



460 **Fig 6.**

461 Electrophysiological examinations (MEP, latency periods) of the spinal cord in part 1 of this
462 study, before and after the second hemisection operation at T7. (A-B) The latency periods on
463 both sides of the spinal cord before and after the second hemisection operation. Data are
464 presented as mean ± SEM.

465
466

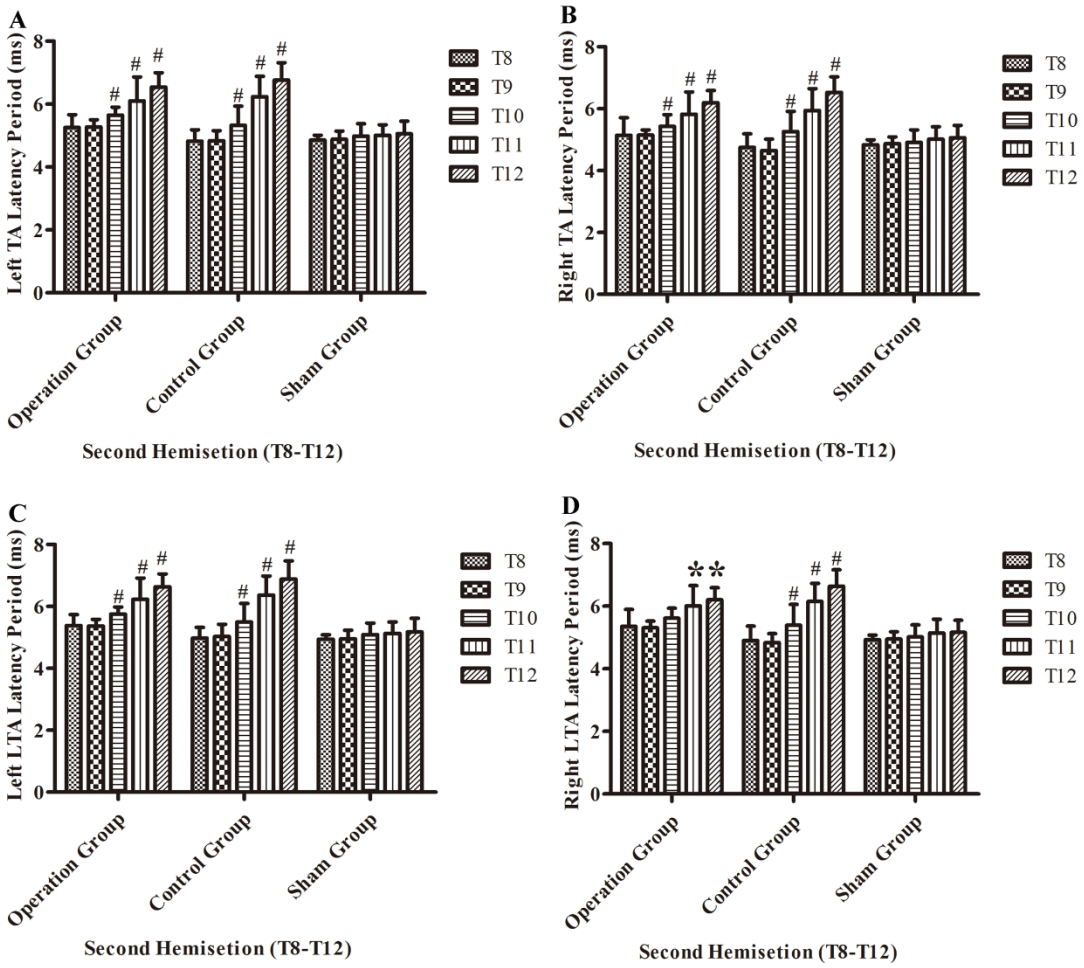


467 **Fig 7.**

468 Trends in BBB scores tendency for the T8 to T12 sub-groups in part 2 of the study: (A1-A5)

469 Operation group, (B1-B5) Control group, (C1-C5) Sham group. Data are presented as mean ±
470 SEM.

471



472

473 **Fig 8**. Electrophysiological examinations (MEP, latency periods) of the T8-T12 sub-groups at
474 the body surface in part 2 of this study after the second hemisection operation at the (A) left TA
475 muscle, (B) right TA muscle, (C) left LTA muscle, (D) right LTA muscle. *, P<0.05, compared
476 to T8 sub-group in each main group. #, P<0.05, compared to the other 4 sub-groups. Data are
477 presented as mean ± SEM.

478

479