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·Original Article·

# Contrasting neuropathology and functional recovery after spinal cord injury in developing and adult rats

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## ABSTRACT

Conflicting findings exist regarding the link between functional recovery and the regrowth of spinal tracts across the lesion leading to the restoration of functional contacts. In the present study, we investigated whether functional locomotor recovery was attributable to anatomical regeneration at postnatal day 1 (PN1), PN7, PN14 and in adult rats two months after transection injury at the tenth thoracic segment of the spinal cord. The Basso, Beattie, and Bresnahan scores showed that transection led to a failure of hindlimb locomotor function in PN14 and adult rats. However, PN1 and PN7 rats showed a significant level of stepping function after complete spinal cord transection. Unexpectedly, unlike the transected PN14 and adult rats in which the spinal cord underwent limited secondary degeneration and showed a scar at the lesion site, the rats transected at PN1 and PN7 showed massive secondary degeneration both anterograde and retrograde, leaving a >5-mm gap between the two stumps. Furthermore, retrograde tracing with fluorogold (FG) also showed that FG did not cross the transection site in PN1 and PN7 rats as in PN14 and adult rats, and re-transection of the cord caused no apparent loss in locomotor performance in the rats transected at PN1. Thus, these three lines of evidence strongly indicated that the functional recovery after transection in neonatal rats is independent of regrowth of spinal tracts across the lesion site. Our results support the notion that the recovery of locomotor function in developing rats may be due to intrinsic adaptations in the spinal circuitry below the lesion that control hindlimb locomotor activity rather than the regrowth of spinal tracts across the lesion. The difference in secondary degeneration between neonatal and adult rats remains to be explored.

**Keywords:** neonatal; spinal cord injury; regeneration; functional recovery; rat

## INTRODUCTION

It is widely accepted that neonatal rats receiving a complete spinal cord transection spontaneously recover a significant level of locomotor performance[1-4], whereas minimal recovery is attained in transected adult rats[5-8]. The mechanisms involved are controversial. Many lines

of evidence suggest that recovery of locomotor function in neonatal rats receiving a complete transection may be due to spontaneous regeneration through the lesion site[4, 9-12]. For example, Wakabayashi *et al.*[4] reported that neonatal rats undergoing a complete spinal cord transection regained some hindlimb function, which was attributable to the regeneration of some descending tracts based on retrograde labeling. Moreover, they also found a positive correlation between the number of labeled neurons in each of the supraspinal nuclei and the locomotor performance of the rats[4]. In contrast, other investigators have suggested that the recovery of locomotor function in neonatal rats receiving a complete transection may be due to changes in the lumbosacral neuronal circuitry rather than the regeneration of axons across the lesion[3,13-15], based on their finding that no signs of regeneration occurred through the transection site by either anterograde or retrograde tracing[3,13-15]. Fundamental evidence for the latter suggestion comes from a recent study showing that re-transection of the cord causes no apparent loss in locomotor performance in neonatally-transected rats[3]. In this study, we re-evaluated the link between functional recovery and regrowth of spinal tracts across the lesion leading to the restoration of functional contacts in postnatal day 1 (PN1), PN7, PN14 and adult rats by using extensive anatomical evidence through morphological analysis around the lesion site, retrograde labeling, and re-transection experiments.

## MATERIALS AND METHODS

### Animals

Data were collected from 50 Sprague-Dawley PN1, PN7, PN14 and adult rats ($n$ = 10 for each age). All surgical interventions and subsequent care and treatment were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong.

### Surgical Procedures

As described previously[16, 17], animals were anesthetized under deep hypothermia (for PN1 and PN7 rats) or with ketamine (80 mg/kg) and xylazine (8 mg/kg) (for PN14 and adult rats). A posterior mid-line incision was made to expose the thoracic spine. Laminectomy was then performed to expose the spinal cord. After opening the dura matter, a complete transection of the cord was performed at T10 using a pair of spring scissors. A fine glass probe was gently passed through the transection site and the cut ends of the cord were then lifted to verify that the transection was complete. After the operation, the superficial wound was closed with 5-0 sutures and surgical wound clips. The postoperative survival period was 2 months.

### Quantitative Assessment of Locomotor Performance

To assess locomotor performance two months after surgery, we used the BBB open-field locomotor scale developed by Basso, Beattie and Bresnahan[18]. Rats were allowed to move individually for 5 min in an open field, which was a wooden square enclosure with a smooth, non-slip floor (150 cm each side, 25 cm wall height). Locomotor performance was videotaped and given a score ranging from 0 to 21 based on the hindlimbs. Values for the left and right extremities were averaged.

### Retrograde Labeling with Fluorogold (FG)

Four days before the end of the study, the spinal cord of some rats in the PN1, PN7, PN14 and adult groups ($n$ = 5) were re-transected two segments below the initial transection site. Then, FG-soaked gelfoam was inserted at the new transection site to retrogradely label spinal pathways reaching specific regions of the brain. Intact young adult rats were also used for FG retrograde labeling.

### Perfusion and Tissue Processing

At the end of the postoperative survival period, the rats were deeply anesthetized with 20% dorminal and perfused intracardially with normal saline followed by 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4). The brain and the spinal cord around the lesion site were dissected out, immersion-fixed in the same fixative overnight, and then placed in 30% sucrose in 0.1 mol/L PBS overnight. Transverse, 30-μm serial sections of the brain and cord were cut on a Leica cryotome (Ontario, Canada).

### Immunohistochemistry

Neurofilament immunohistochemistry was performed to study the secondary damage around the lesion site after transection using a rabbit polyclonal antibody against neurofilament 200 (NF200) (1:3 000, Chemicon International, Temecula, CA). Following incubation with the primary antibody, the sections were reacted with their

corresponding Alexa 488-conjugated secondary antibody (1:400, Molecular Probes, New York, NY) and then visualized under a Zeiss fluorescent microscope (Berlin, Germany).

### Statistical Analysis

The data on the BBB scale are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA using GraphPad Prism (Version 4.0, San Diego, CA). Statistical significance was set at $P$ <0.05.

## RESULTS

### Age-dependent Hindlimb Locomotor Performance

Similar to intact rats, those transected at PN1 and PN7 showed plantar placement that their paws rested flat on the ground, but had no weight support (Fig. 1A–C). However, rats transected at PN14 and as adults never showed paw placement with weight support; the dorsal paw surface faced the ground (Fig. 1D, E). The BBB scores of the rats transected at PN1 and PN7 ranged from 8 to 10 (Fig. 1F). These rats were able to move their hindlimbs to some extent, and stepped with or without weight support (Supplemental Video 1), although their locomotor performance did not approximate that of intact rats, which showed consistent plantar stepping, a coordinated gait and consistent trunk stability (Supplemental Video 2). However, the BBB scores of the rats transected at PN14 and as adults were from 0 to 1. Most of these rats had complete paralysis and could not move their hindlimbs. Some of them showed slight movement of one or two hindlimb joints, but never showed weight-supported steps (Fig. 1F, Supplemental Video 3).

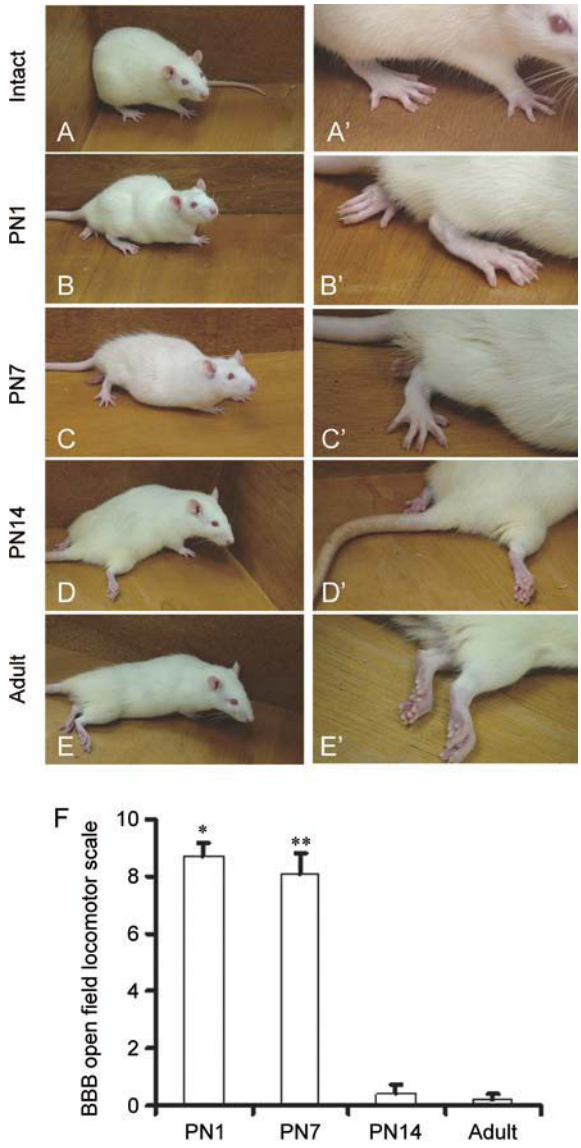


**Fig. 1. Examples of intact young adult rats (A and A'), along with those transected at postnatal day 1 (PN1) (B and B'), PN7 (C and C'), PN14 (D and D') and as adults (E and E') two months post-injury. F: Comparison of the BBB scores among the four age groups. *$P$ < 0.05, **$P$ <0.01 compared with the PN14 and adult groups.**

### Age-dependent Secondary Degeneration in Spinal Cord around the Lesion Site

At the end of the 2-month survival time, the spinal cords of all animals were collected for gross examination. In the intact group, the surface of the cord was smooth. In the PN14 and adult groups, scar tissue was observed at the transection site. However, in the PN1 and PN7 rats, an opaque white tissue was found; this was repaired dura matter and connective tissue between the spinal stumps. Significant atrophy of the spinal cord stumps around the lesion site was present in animals transected at PN1 and PN7. To further investigate the secondary degeneration around the lesion site, NF200 immunohistochemistry was performed[19]. In rats transected at PN14 and as adults, a large number of fibers was found around the lesion site from 5.0 mm rostrally to 5.0 mm caudally, including the lesion site although they were fewer than those in intact

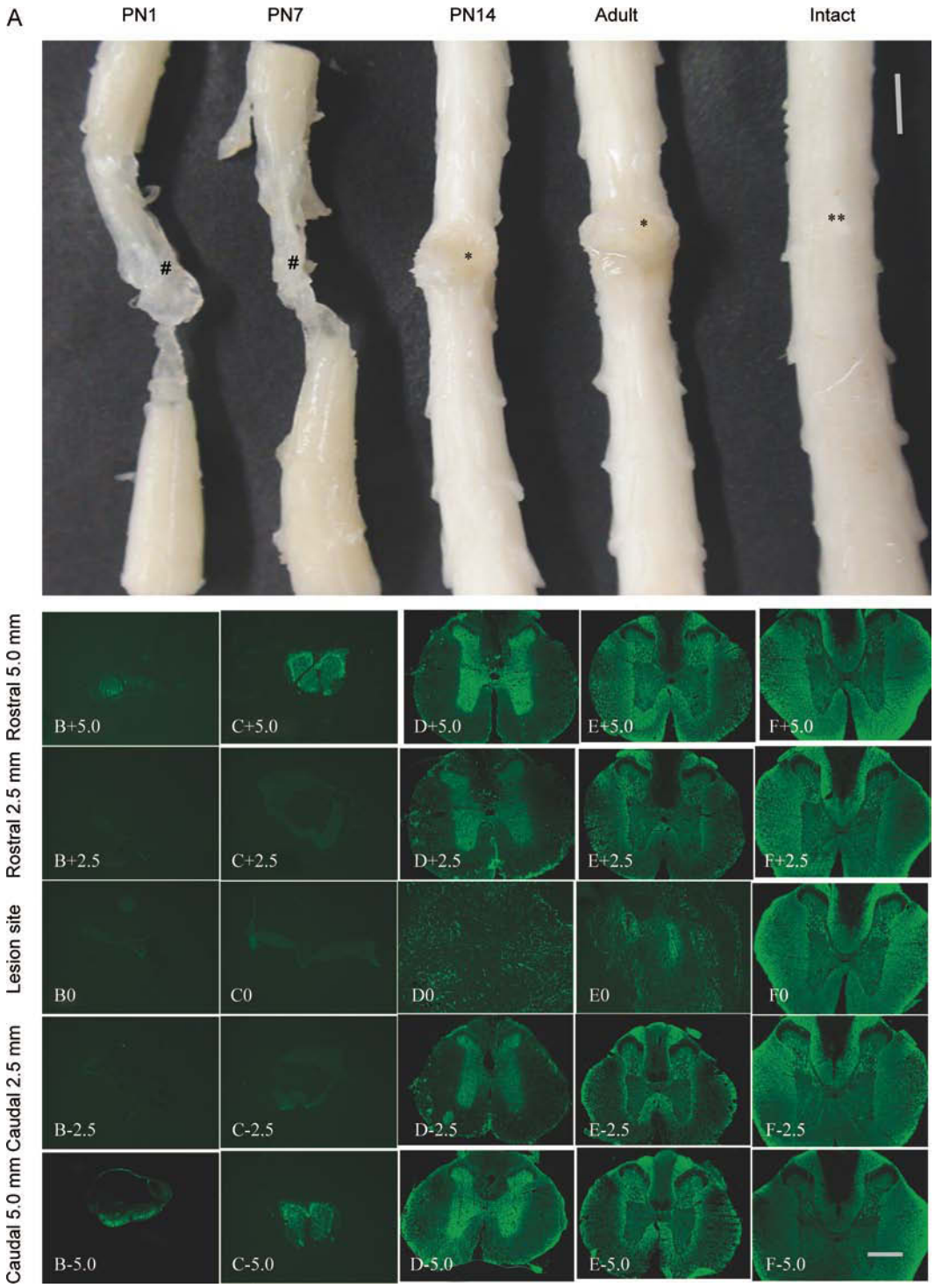


**Fig. 2. Upper panels: Representative examples of the spinal cord lesion after two months. **Normal spinal cord; *scar tissue at the transection site in PN14 and adult; #atrophy around the transection site in P1 and P7. B–E: Immunostaining for neurofilaments around the lesion site in rats transected at PN1 (B+5.0 to B-5.0), PN7 (C+5.0 to C-5.0), PN14 (D+5.0 to D-5.0), and as adults (E+5.0 to E-5.0). F: Staining in intact rats (F+5.0 to F-5.0). Scale bar for A, 3 mm, for B–E, 300 μm.**

rats. In contrast, the neurofilament staining showed no fibers around the lesion site from 2.5 mm rostrally to 2.5 mm caudally in rats transected at PN1 and PN7, although the neurofilament-immunoreactivity was seen at 5 mm rostrally and caudally (Fig. 2B–F). The quantitative data also showed a >5-mm gap at the lesion site (7.5 ± 0.9 mm for PN1; 6.0 ± 0.5 mm for PN7), and white/gray matter was formed at both stumps two months after complete transection at PN1 and PN7.

**FluoroGold Did Not Cross the Transection Site to Reach Specific Regions of the Brain in Any Age Group**

Fluorescence imaging of the brain for FG revealed robust staining of neurons in the sensorimotor cortex of the hindlimb area and red nucleus in the intact rats (Fig. 3A, B). In contrast, the same brain regions in the rats transected at PN1, PN7, PN14, and as adults showed no FG staining, indicating that no reconnection occurred across the initial transection site at any age.

**Re-transection Caudal to the Original Transection Site Causes No Apparent Loss in Locomotor Performance in Rats Transected at PN1**

Before re-transection, rats transected at PN1 showed plantar placement that the paws rested flat on the ground with weight support (Fig. 4A, A'). After re-transection, the rats also showed plantar placement and weight support (Fig. 4B, B'). The BBB scores before and after re-transection of PN1 rats were both from 8 to 10, with no significant difference (Fig. 4C). Besides, the re-transection caused no apparent loss in the locomotor performance of rats transected on PN1 (Supplemental Videos 1 and 4).

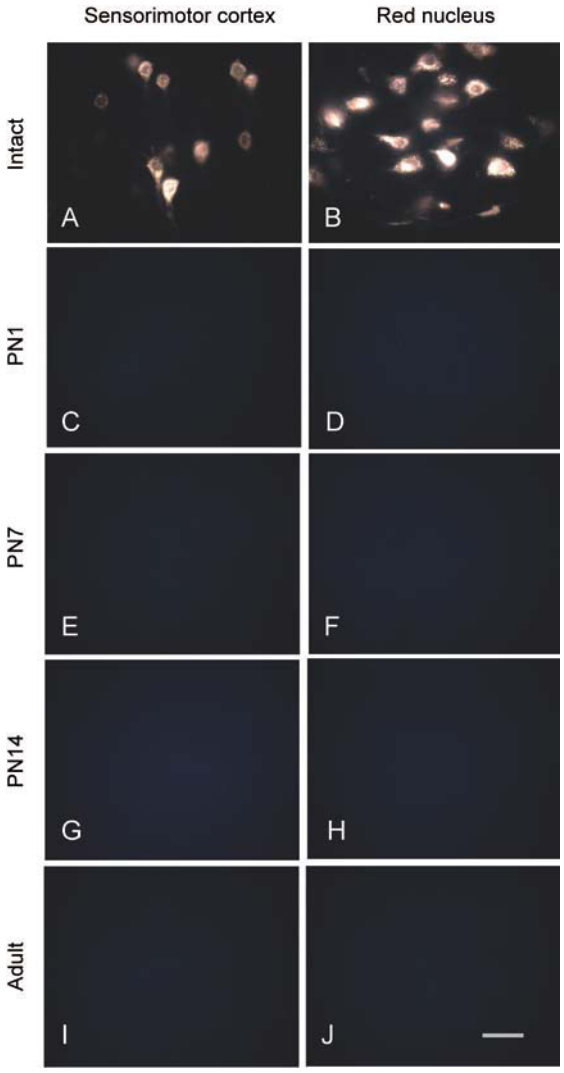


**Fig. 3. Coronal sections of the brain from representative intact, and transected PN1, PN7, PN14 and adult rats. Fluorogold was present in the sensorimotor cortex (A) and red nucleus (B) of intact rats, but not in transected rats (C–J). Scale bar, 50 μm.**

## DISCUSSION

In this study, we investigated whether there is a link between the recovery of locomotor function and regeneration after complete spinal cord transection at PN1, PN7, PN14 and in adult rats. In good agreement with previous studies[1-4], the rats transected at PN1 and PN7 showed a significant level of functional locomotor recovery on the BBB scale, in contrast to those transected at PN14 and in adulthood, which failed to recover. However, thorough anatomical investigations showed no signs of regeneration through the lesion site in PN1 and PN7 rats. First, our histological studies of the retrograde transport of FG showed no signs of regeneration in the transected PN1 and PN7, just as in the PN14 and adult rats. Second, the PN1 rats showed similar locomotor functions before and after spinal re-transection. And third, we unexpectedly found age-dependent secondary degeneration around the lesion site. Unlike that of the rats transected at PN14 and in adulthood, the spinal cord of PN1 and PN7 rats underwent massive secondary degeneration around the lesion site,

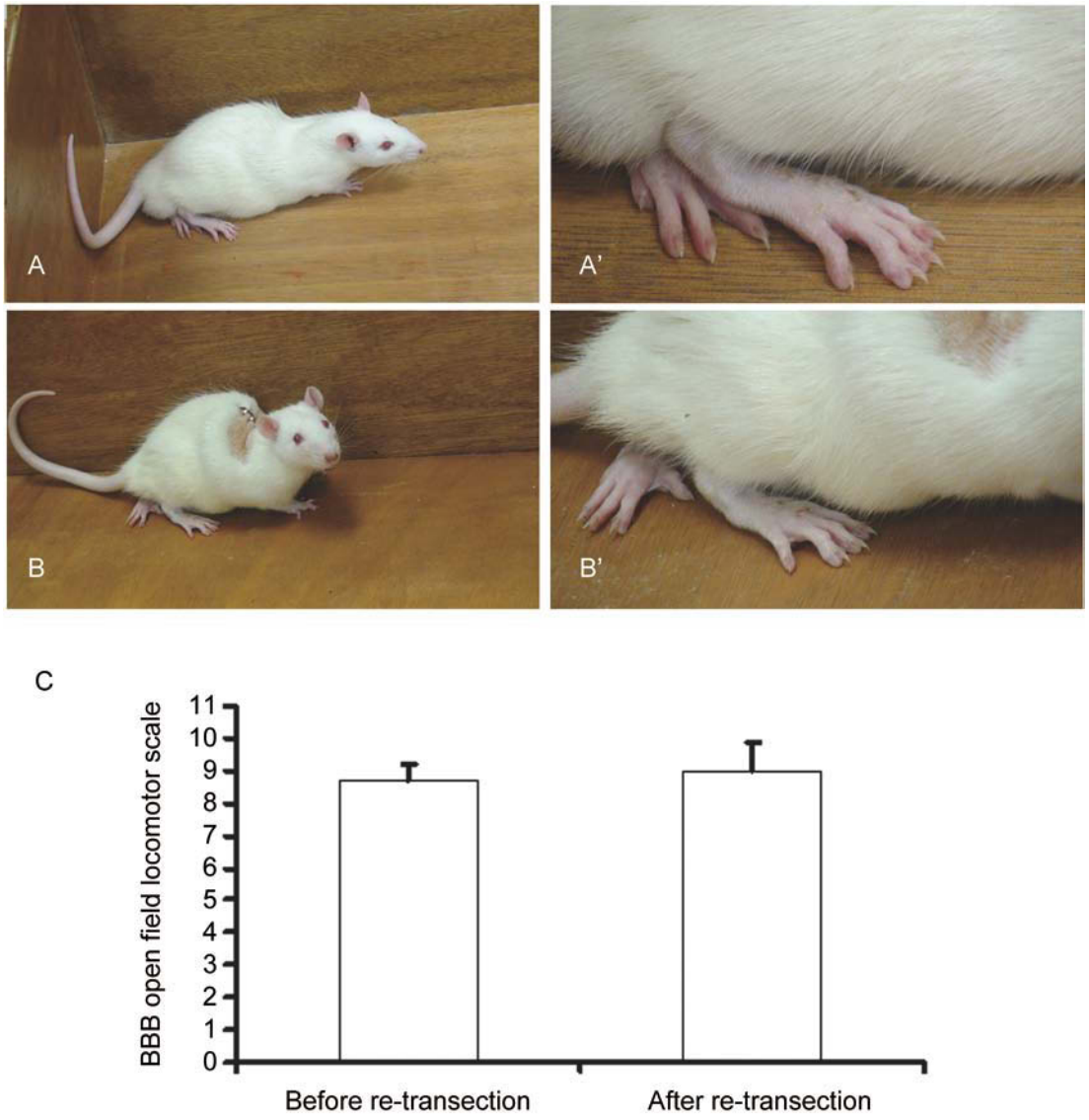


**Fig. 4. Examples of PN1 animals before (A and A') and after (B and B') re-transection. A' and B' are close-ups of A and B. C: BBB scores before and after re-transection.**

leaving a >5-mm gap between the two stumps, where no fibers were revealed by neurofilament immunostaining. The secondary degeneration following spinal cord injury (SCI) was age-dependent. It is known that SCI results in immediate damage followed by a secondary phase of tissue damage. The secondary injury mechanisms include, but are not limited to, glutamate excitotoxicity, ischemia, inflammation, free radical-induced cell death, and the induction of extrinsic and intrinsic apoptotic pathways[20]. We suggest that the massive secondary degeneration in the rats transected at PN1 and PN7 may be due to the vulnerability of the injured immature spinal cord to the above factors. Studies involving traumatic brain injury in developing rats lend support to this suggestion[21, 22]. Bittigau *et al.* found that the severity of trauma-triggered damage in the rat brain is age-dependent, the immature brain being exceedingly vulnerable to excitotoxicity[21].

Taken together, these three lines of evidence clearly demonstrated that the improvements in locomotor performance of rats transected at PN1 and PN7 were

not attributable to supraspinal input. Our results strongly support the hypothesis that the recovery of locomotor function in developing rats depends only on the lumbosacral circuitry below the lesion rather than the regrowth of spinal tracts across the lesion site. The fact that the spontaneous recovery of motor function in the developing rat was not due to the re-establishment of brain-spinal cord connectivity is in conflict with the conventional concept that one necessary element of the recovery of locomotor function in post-traumatic spinal cord is long-tract axonal regeneration [reviewed by[23]]. The present data together with previous studies[3,15] provide a new insight into the basic mechanisms of the neural control of locomotion. In fact, conventional locomotor network theory in which locomotor function needs supraspinal input has been challenged in human studies, which showed that adequate electrical stimulation of the lumbar segments can induce locomotor movements in complete SCI[24]. In order to identify specific areas that are responsible for the recovery of locomotor function in cases of traumatic lesions to the spinal cord, it is necessary to gain further insights into this issue in animal models. The transected spinal cord of neonatal rats may provide an excellent model in which to identify the locomotor networks.

## CONCLUSION

It is widely accepted that neonatal rats receiving a complete spinal cord transection spontaneously recover a significant level of locomotor performance[1, 3, 4]. However, no general agreement has been reached on whether this recovery is due to regeneration across the lesion site of transected axons. Our data provide new solid evidence of the occurrence of massive secondary degeneration around the lesion site in the developing rat, together with anatomical evidence from retrograde labeling and re-transection experiments, and support the notion that the spontaneous recovery of locomotor function in neonates is not necessarily attributable to regeneration.

## ACKNOWLEDGMENTS

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## SUPPLEMENTAL DATA

Supplemental data include four representative recordings of locomotor performance of transected, intact and re-transected rats, and can be found online at http://www.neurosci.cn/epData.asp?id=105.

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