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Changes in choline acetyltranferase distribution in the cervical spinal cord after reversible cervical spinal cord injury

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Reversible spinal cord injury (SCI) at C6 level in rats, produced by the weight-placed method, resulted in a severe motor functional deficit initially, followed by a gradual recovery. During the recovery, choline acetyltransferase (CAT) distribution in the cervical spinal cord was investigated at 2, 4, 7, 14 and 28 days after the injury by quantitative immunohistochemistry with a fluorescence microphotometry system. At C6 level, the flourescence intensity of the ventrolateral anterior horn (VLAH), which reflected the concentration of CAT, decreased to approximately 50% of that of the sham-operated group at 2 days. It then recovered to 60% at 4 days after the injury, and remained unchanged thereafter. Fluorescence intensities in VLAH at C4–5 and C7–8 levels decreased to approximately 60–70% at 2 days after the injury, but it recovered and increased to 110–130% thereafter.

Keywords: reversible cervical spinal cord injury in rats; choline acetyltransferase; fluorescence microphotometry; quantitative immunohistochemistry.

Introduction

The introduction of the weight-drop technique by Allen[1] in 1911 inaugurated the modern era of spinal cord trauma research, and compression injuries have since been studied using the balloon compression technique,[2–4] the clip compression model,[5] and the method used in this study, the weight-placed technique.[6] These techniques all allow the creation of reproducible and graded SCI. Reversible SCI, produced by the placement of a 20 g weight for 5 min on the cord, was characterised by an initially severe motor functional deficit followed later by recovery of motor function. However, it remains an unsettled question whether spinal motor neuron function really recovers completely from such a trauma. Choline acetyltransferase (CAT), the final enzyme in acetylcholine synthesis, is known as the most specific marker for cholinergic neurons and its expression sensitively reflects spinal motor neuron function.[7] In this study, changes in CAT distribution in rat spinal cord after reversible SCI were investigated by quantitating indirect CAT immunofluorescence of spinal cord sections using a microphotometry system.

Materials and method

Spinal cord injury model

Reversible SCI was produced by the modified weight-placed method of Croft *et al*.[6] Forty Wistar rats weighing about 250 g were anaesthetised with pentobarbital (40 mg/kg, i.p.) and laminectomy was performed at C5 and C6 under a microscope. The cervical spinal column was stabilised by fixing the skull and a spinous process of T2. In twenty rats, a 20 g weight was then placed on the exposed dura at C6 level for 5 min (compression group). In the remaining 20 rats, only laminectomy was performed at C5 and C6 (sham-op group). In another five rats, no operation was performed (non-op group). Rat motor function was then evaluated by the inclined plane method of Rivlin *et al*.[8] According to the original method, the maximum inclination of the plane at which a rat could maintain itself for 5 s was recorded, which was used as the rat's motor functional ability.

Immunohistochemistry
Four rats each (compression group and sham-op group) were perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at 2, 4, 7, 14 and 28 days after the operation. The spinal cord was then removed and postfixed in the same solution for 2 h and rinsed in 5% buffered sucrose at 4 °C for 12 h. The spinal cord was then divided into three blocks under the microscope: the compressed portion (C6 level); that rostral to it (C4–5 level); and that caudal to it (C7–8 level). Each tissue block was frozen on dry ice and sectioned at 10 μm on a cryostat. The sections of non-op group were made by the same procedures above.

The immunohistochemical procedures were performed according to Sutoo's method.[9] Ten sections from each block were reacted with normal goat serum (Chemicon S-20 (Chemicon Inc, Temecula, CA, USA) diluted 1:20 with PBS) at room temperature for 1 h and then reacted with anti-CAT rabbit serum (Chemicon AB-143, diluted 1:100 with PBS) at 4 °C for 12 h. After being washed with three changes of PBS for a total 3 h, they were reacted with FITC-labelled, anti-rabbit IgG goat serum (TAGO 4320 (Tago Inc, Burlingame, CA, USA) diluted 1:20 with PBS) at room temperature for 3 h. The stained sections were embedded in 20% glycerin-PBS.

In the control experiment, the sections of non-op group were processed as above except that the primary antibody, which was made by Bruce,[10,11] was replaced with normal rat serum.

Analysis of CAT distribution
CAT immunofluorescence intensity in the spinal cord was measured at several regions using a microphotometry system which was developed by Sutoo *et al*.[12]

This system consists of a fluorescence microscope, a photomultiplier tube, an auto-scanning stage, and a computer. This system can measure the fluorescence intensity in a small spinal region through a measuring spot. The measuring spot can be selected freely. The spinal section is moved in the X- and Y-directions by means of a high-precision step-motor scanning stage under the objective lens of the fluorescence microscope, and the fluorescence intensity of the entire surface of the section is analysed without the fading of the fluorescence intensity. Fluorescence intensity and its position are transferred to the computer and displayed in two or three dimensions. The background fluorescence intensity can be suppressed with an interference filter which is inserted into the photomultiplier tube.

The microphotometry system was used under the following conditions: excitation range, 420–490 nm; interference filter, 530 nm; photomultiplier voltage, 850 V; objective lens, 20×/0.75 (magnification/numerical aperture); and measuring spots, 40 μm. The fluorescence intensity of uran glass was used as the fluorescence intensity standard, the value of which was 64.[12]

Histopathology
After the measurement of fluorescence intensities, the slices were stained with hematoxylin-eosin (HE) and luxol fast blue(LFB) for histological examination.

Results

Evaluation of motor functional deficit
In the sham-op group, there were no motor functional deficits throughout the experiment. In the compression group, motor functional deficits were most prominent at 2 days after the injury (inclined plane angle: 60°), when the motor dysfunction was dominant in the upper limbs, but the lower limbs were not so seriously affected and the animals could stand on their lower limbs. Motor function gradually improved after 2 days, and it recovered to almost the same level as that of the sham-op group by the end of the experiment (inclined plane angle: 82.5°). Injured animals were able to run normally 3 weeks after injury (Fig 1).

Histopathological findings
There were no differences histologically between the sham-op group and the non-op group. In the compression group, haemorrhagic lesions were present in the central grey matter at C6 level 2 days after the

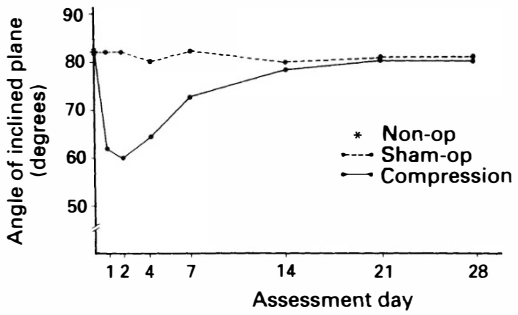

Figure 1 Inclined plane method.

injury (Fig 2a, b). The number of anterior horn cells at C6 level decreased gradually until 4 weeks after injury. According to the decrease, glial cell numbers were increased (Fig 2c, d). No changes in numbers were observed in the anterior horn cells at C4–5 and C7–8 level. The axon and myelin sheaths in the white matter of the spinal cord remained normal except for the posterior column at C6 level (Fig 3a, b).

Immunohistochemical findings

In both the non-op and the sham-op groups, large-sized cells, which appeared to be the motor neurons of the spinal cord, were present in large numbers in the ventrolateral area of the anterior horn (VLAH) (Fig 4). In the control experiment, no CAT immunoreactive neurons were observed in the spinal cord. Fluorescence intensities were uniformly high in VLAH, implying the high concentration of CAT (Fig 5). The spinal cord was divided into seven functional regions (Fig 6) and average values of fluorescence intensity at the measured points were analysed in each region. There was no difference of the average values of VLAH between the non-op group and the sham-op group statistically (Table I). In the sham-op group, time-correlated changes of the average values in the VLAH were not found throughout the experiment (Table II).

In the compression group, CAT immunoreactive neurons at C6 level were still maintained at 2 days after the injury (Fig 7a), but

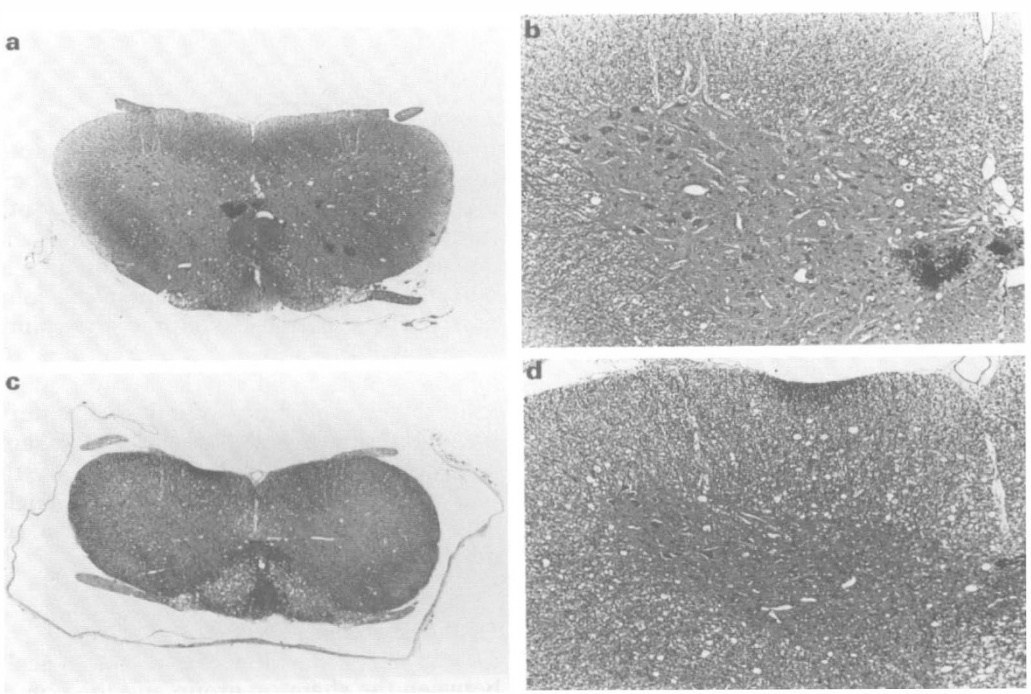

Figure 2 Photomicrograph of the spinal cord at C6 level (the compressed portion). Post injury 2 days (a) 8×, (b) 20×; post injury 4 weeks (c) 8×, (d) 20× (HE + LFB staining).

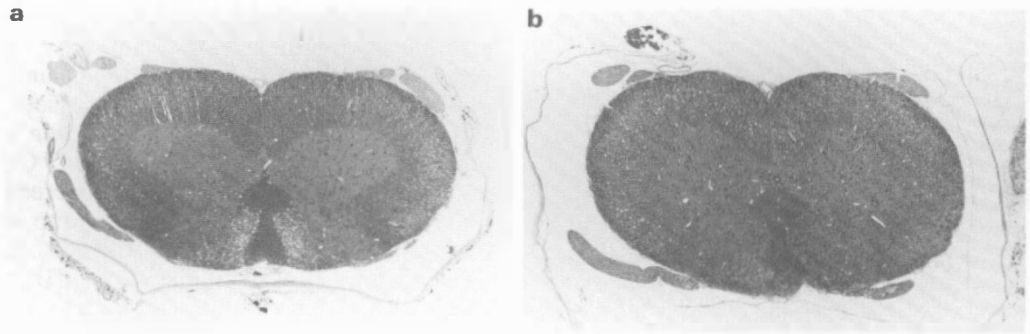

Figure 3 Photomicrograph of the spinal cord at (a) C4-5 level, (b) C7-8 level (HE + LBF staining, 8×).

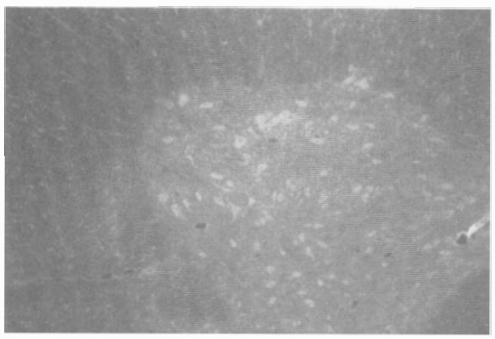

Figure 4 Fluorescence microphotograph of the anterior horn in sham-op group at C6 level (B-excitation, 40×).

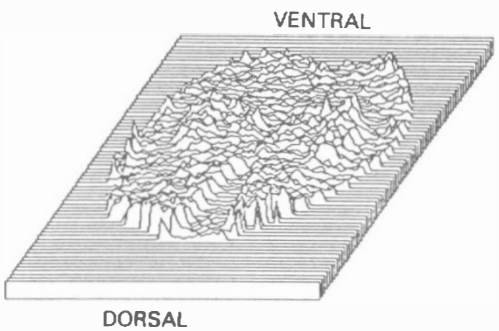

Figure 5 Quantitative distribution of CAT immunofluorescence intensity (three dimensional display).

the number of these decreased gradually until 4 weeks after injury (Fig 7b). On the other hand, both at the C4–5 and C7–8 levels, CAT immunoreactive neurons remained normal at 4 weeks following the injury (Fig 8a, b).

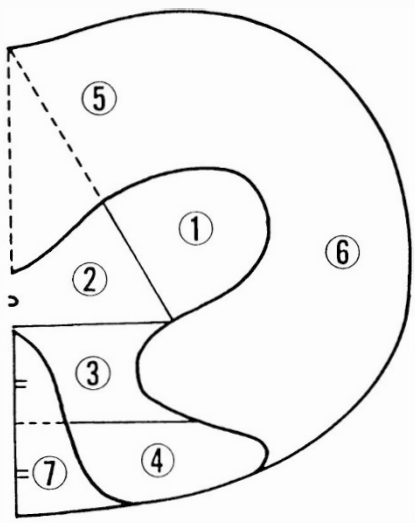

Figure 6 The spinal cord was divided into seven functional regions.

Table I Fluorescence intensity of each region (C6 level)

No. of region	Non-op group ($n = 5$)	Sham-op group ($n = 20$)
1	87.6 ± 1.4	88.2 ± 1.3
2	69.3 ± 1.9	66.8 ± 3.8
3	65.2 ± 2.5	64.3 ± 4.2
4	74.2 ± 2.0	72.9 ± 2.1
5	34.1 ± 0.9	33.1 ± 1.2
6	34.2 ± 1.6	32.7 ± 1.1
7	5.4 ± 0.5	5.3 ± 0.7

Each value represents the mean ± SE

Table II Sham-op group: changes of the average values in the ventrolateral anterior horn (VLAH)

	Fluorescence intensity
2 days post op	87.9 ± 1.5 (n = 4)
4 days post op	90.2 ± 0.7 (n = 4)
7 days post op	88.3 ± 1.2 (n = 4)
14 days post op	86.9 ± 2.4 (n = 4)
28 days post op	87.2 ± 2.2 (n = 4)

Each value represents the mean ± SE

Changes in CAT immunofluorescence intensity in VLAH after reversible spinal cord injury

At C6 level, CAT immunofluorescence intensities in VLAH decreased to approximately 50% of those in the sham-op group at 2 days but increased to approximately 60% at 4 days after the injury. However, no further recovery occurred.

On the other hand, at the C4–5 level, CAT immunofluorescence intensity decreased to 66% of those of the sham-op group at 2 days, but then increased to 115–120% at 1 week or more after the injury. Similarly, at the C7–8 level, CAT immunofluorescence intensities decreased to 77% of those of the sham-op group at 2 days and then increased to 110–130% at 1 week or more after the injury (Table III).

Discussion

The fluorescence microphotometry system used in this study is superior to a conventional television image analyser for quantitation. Though it cannot measure the absolute quantity of CAT, it is excellent for the examination of the relative changes of CAT among different groups of animals.[9]

It is interesting and important biochemi-

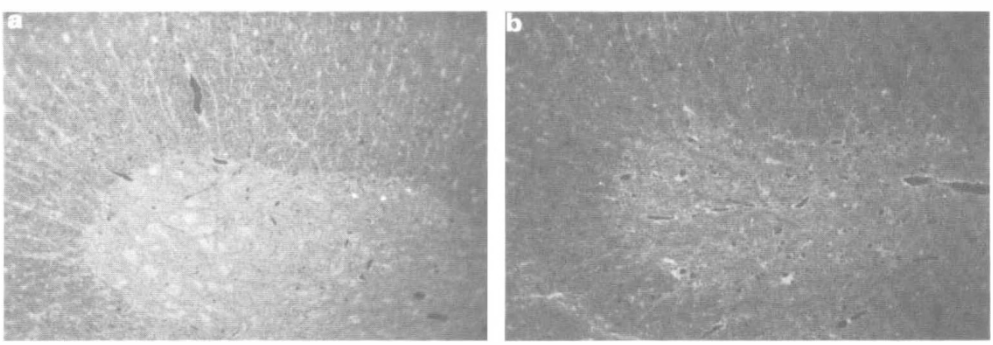

Figure 7 Fluorescence microphotograph of the anterior horn in compression group at C6 level (a) post injury 2 days, (b) post injury 28 days (B-excitation, 40×).

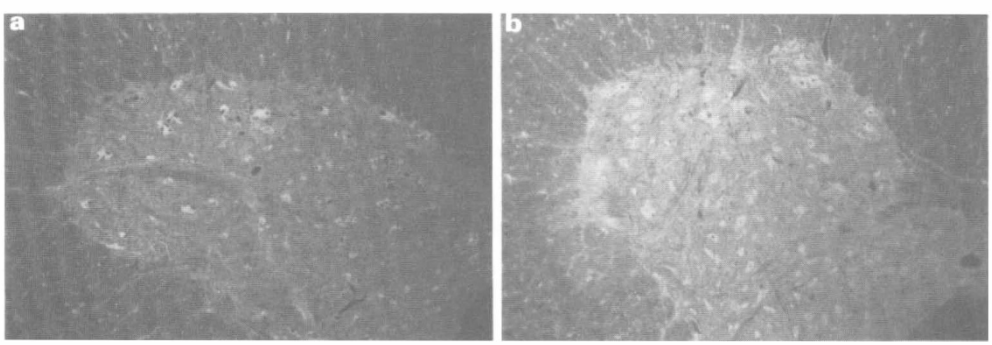

Figure 8 Fluorescence microphotograph of the anterior horn in compression group at 28 days after the injury (a) C4–5 level, (b) C7–8 level (B-excitation, 40×).

Table III Changes in fluorescence intensities of the VLAH

	C4–5 level	C6 level	C7–8 level
Sham-op group ($n = 20$)	82.1 ± 2.2	88.2 ± 1.3	90.6 ± 3.8
Post injury 2 days ($n = 4$)	54.2 ± 4.5	46.0 ± 3.6	70.0 ± 3.4
Post injury 4 days ($n = 4$)	70.7 ± 4.3	52.0 ± 3.3	72.3 ± 7.4
Post injury 1 week ($n = 4$)	102.9 ± 4.7	51.7 ± 5.1	111.3 ± 2.7
Post injury 2 weeks ($n = 4$)	105.6 ± 5.2	56.0 ± 3.6	117.2 ± 7.9
Post injury 4 weeks ($n = 4$)	98.1 ± 3.4	50.2 ± 2.7	99.9 ± 9.3

Each value represents the mean ± SE. The fluorescence intensity of uran glass was used as the fluorescence intensity standard, the value of which was 64 measured under the following condition: the excitation range, 420–490 nm; interference filter, 530 nm; photomultiplier voltage, 850 V; objective lens 20×/0.75 (magnification/numerical aperture); and measuring spot, 40 $\mu m\phi$.

cally that the changes of CAT immunofluorescence intensity in VLAH, reflecting changes of CAT concentration in spinal motor neurons, is correlated to the histopathological and neurological findings in this model. Reversible SCI in the rat results in an initial profound functional deficit, followed by a gradual recovery over a period of a few weeks after the injury. However, this recovery of function has not yet been fully explained. Our findings were that CAT immunofluorescence intensities in VLAH were significantly decreased in parts of the cord distant from the site of trauma at 2 days after the injury, which suggests that some of the functional deficit at this time may reflect low levels of this key enzyme in the synthesis of acetylcholine. Moreover, the persistent low CAT immunofluorescence intensities in VLAH at the site of compression at 4 weeks after the injury suggested that the dysfunction of the motor neurons at the site of compression were irreversible. A strong correlation was found between the late excessive recovery of CAT immunofluorescence intensity in VLAH both rostral and caudal to the injury and the motor functional recovery.

In conclusion, in spite of the fact that irreversible changes occurred at the site of compression, motor function completely recovered, which was due to the recovery and compensatory overexpression of CAT in neighbouring portions of the injured part.

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