Changes in Synapses and Axons Demonstrated by Synaptophysin Immunohistochemistry Following Spinal Cord Compression Trauma in the Rat and Mouse[1]

GUI-LIN LI[#,*,2], MOHAMMAD FAROOQUE[*], JONAS ISAKSSON[*], AND YNGVE OLSSON[*]

[#]Beijing Neurosurgical Institute, Beijing 100050, China; [*]Research Group of Neuropathology, Department of Genetics and Pathology, University Hospital, Uppsala, Sweden, S-75185

Objective and methods To evaluate synaptic changes using synaptophysin immunohistochemstry in rat and mouse, which spinal cords were subjected to graded compression trauma at the level of Th8-9. **Results** Normal animals showed numerous fine dots of synaptophysin immunoreactivity in the gray matter. An increase in synaptophysin immunoreactivity was observed in the neuropil and synapses at the surface of motor neurons of the anterior horns in the Th8-9 segments lost immunoreactivity at 4-hour point after trauma. The immunoreactive synapses reappeared around motor neurons at 9-day point. Unexpected accumulation of synaptophysin immunoreactivity occurred in injured axons of the white matter of the compressed spinal cord. **Conclusion** Synaptic changes were important components of secondary injuries in spinal cord trauma. Loss of synapses on motor neurons may be one of the factors causing motor dysfunction of hind limbs and formation of new synapses may play an important role in recovery of motor function. Synaptophysin immunohistochemistry is also a good tool for studies of axonal swellings in spinal cord injuries.

Key words: Synaptophysin; Synapses; Axons; Rat; Spinal cord; Trauma

INTRODUCTION

Trauma to the spinal cord results in pathological changes in various constituents, including primary and secondary injuries of nerve cell bodies, axons, dendrites, synapses and glial cells. There have been a number of reports on the general pathology[1-5] and on alterations of axons[2,3,6-11] and dendrites[12-14] of the traumatized spinal cord. However, synaptic changes after spinal cord trauma have rarely been reported[15,16].

Synaptophysin (also termed P38) is the most abundant integral membrane protein of synaptic vesicles[17,18] and can be used as a marker protein of synaptic vesicles[19,20]. It is present in such vesicles of the central and peripheral nervous systems as well as in vesicles of some neuroendocrine cells[19,21,22]. Synaptophysin immunohistochemistry has therefore been used as a reliable tool in studies of synaptic pathology of some nervous system disorders[23-26] and in the diagnosis of certain neuronal and neuroendocrine tumors[21,27].

[1]This study was approved by the Uppsala Ethical Committee for Animal Research. It was supported by grants from the Swedish Association of Neurologically Disabled, the Swedish Society for Medical Research (project no. 950006) and the National Natural Science Foundation of China (project no. 30370543).

[2]Correspondence should be addressed to Gui-Lin LI, Tel: 86-10-67016611-2658. E-mail: liguilin40@hotmail.com

Biographical note of the first author: Gui-Lin LI, female, born in 1961, research fellow, majoring in spinal cord injury and repairment.

删除的内容:

删除的内容:

0895-3988/2004
CN 11-2816

However, only a few publications are available on the use of synaptophysin for studying synaptic changes in traumatized spinal cord. One study demonstrated that rat spinal cord hemisection resulted in an increased expression of synaptophysin in the gray matter caudal to the injury at day 7 after trauma, and this was thought to imply formation of new synapses[15,28]. Another study showed that spinal cord hemisection in the rat resulted in a remarkable decrease in synaptophysin immunoreactivity at the surface of motor neurons caudal to the injury at days 3 and 10 after trauma[16].

It is not known whether synaptophysin expression changes in other structures apart from synapses following spinal cord trauma. Synaptophysin is transported anterogradely and retrogradely by fast axonal flow[29,30]. Disturbances in axonal transport and consequent axonal swelling are the most common alterations of axons after trauma[31,32]. Certain proteins that are transported anterogradely and retrogradely are accumulated in the axonal swellings as a result of arrested axonal flow[33,34]. β-Amyloid precursor protein (APP) is one of these proteins and has been widely used in studies of axonal changes following brain and spinal cord trauma[6,9,35-38]. There are no previous reports describing accumulation of synaptophysin in axons of injured spinal cords.

We have now investigated changes in synaptophysin in two models of spinal cord compression trauma. To that end we have used immunohistochemistry and focused our attention not only on synaptic changes but also on accumulation of this compound in axons of the white matter.

MATERIALS AND METHODS

In the present study we selected tissue sections from our previous experiments on spinal cord compression trauma in rat and mouse models[9,39]. Details of the experimental procedures have been described elsewhere[9,39].

Animals

Forty male rats (Sprague-Dawley, mean weight 370 g) and 16 female mice (B6CBAF1 hybrids, mean weight 25 g) were used. Food and water were provided ad libitum before and after the experiments. The rats were kept at a temperature of 20℃ by thermostatic control and exposed to alternate light and dark periods of 12 h.

Spinal Cord Compression Trauma

The spinal cord compression trauma was produced by a similar procedure in rats and mice[9,39]. The animals were anaesthetized and laminectomized at vertebrae Th7-8 in rats and at vertebra Th8 in mice. They were then placed in a stereotaxic apparatus with two adjustable forceps applied to the spinous processes of vertebrae cranial and caudal to the laminectomy in order to stabilize the spinal cord. A predetermined weight was applied extradurally to the spinal cord for 5 min.

Rats After surgery the rats were randomized into eight groups with four rats in each according to the loads applied on the exposed dura (35 g causing a moderate injury and 50 g resulting in a severe injury) and the periods for which the rats were allowed to survive (4 h, 1, 4, or 9 d). Four normal and four laminectomized rats without compression served as controls. The neurological function was tested by the inclined plane technique[40] before and each day after injury. Moderate injury induced transient paraparesis and severe injury caused paraplegia of the hind limbs.

Mice Two groups of four mice in each were subjected to moderate and severe spinal cord trauma, achieved by 5 g/mm^2 and 10 g/mm^2 compression, respectively. After compression they were allowed to survive for 14 d. Four normal and four laminectomized mice without compression served as controls.

Samples and Staining

The animals were sedated and perfused through the heart with a phosphate buffer solution (PBS, pH 7.4) followed by a 4% formaldehyde solution in the same buffer. Relevant segments of spinal cords were excised and placed in the same fixative for 24 h. They were then dehydrated in ethanol and embedded in paraffin. Sections 5 µm thick were cut. Haematoxylin and eosin staining was used to evaluate the general morphological changes.

In the present study transverse sections were collected from segments Th7, Th8-9 (the compressed site), and Th10 of the rat spinal cord and from compressed segments proximal and distal to the trauma in the mouse spinal cord.

For synaptophysin immunohistochemistry the sections were boiled in a microwave oven for 10 min in citrate buffer (pH 6.0) to retrieve the antigen. To improve the background quality, the sections were treated in 1% hydrogen peroxide in PBS for 30 min and in 1% bovine serum albumin in PBS buffer for 30 min. The sections were then incubated overnight with rabbit serum anti-synaptophysin (code no. A 0010, DAKO, Glostrup, Denmark) at a dilution of 1:50. A swine anti-rabbit IgG was applied to the sections for 30 min. The reaction product was visualized by the avidin-biotin-peroxidase complex method, using ethylcarbazol as the chromogen. Haematoxylin was used to counterstain cell nuclei. For control purposes the primary antibody was omitted and thereafter the sections were treated as those in which the rabbit serum anti-synaptophysin had been applied. Synaptophysin immunohistochemistry was carried out on all sections at the same time and blindly.

RESULTS

Rats-general Morphological Changes

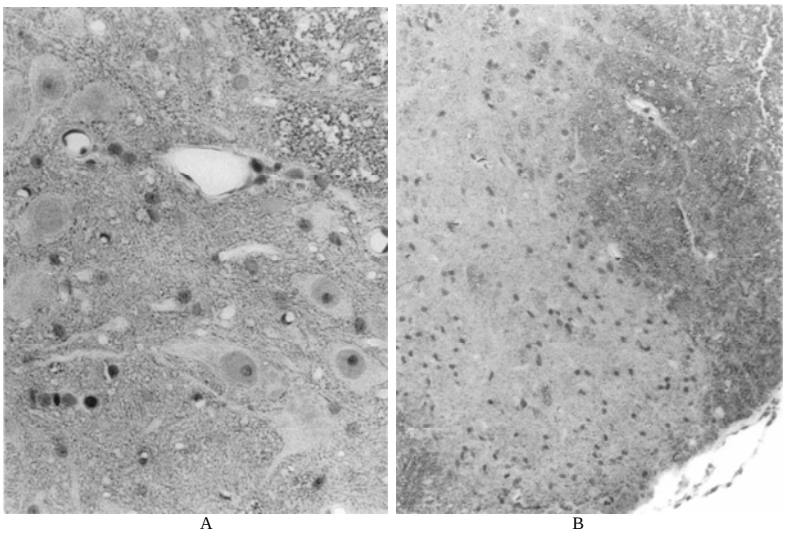
Moderate compression resulted in multiple small bleedings and condensation or loss of nerve cell bodies in the gray matter of the injured Th8-9 segment at 4 h and day 1. Expanded axons and vacuoles were seen in the white matter, particularly 1-9 d after injury. The subpial regions of the white matter were well preserved. The cranial Th7 and, especially, the caudal Th10 segments showed some condensed neurons and vacuolation of the white matter.

Severe compression resulted in pronounced edema of the Th8-9 segment and many bleedings in the gray matter. At day 1 after compression, there was a large area of necrosis, which occupied almost the entire cross-section of the spinal cord. Reactive gliosis was seen in necrotic areas at day 9. The caudal Th10 segment displayed small bleedings, condensed neurons and vacuolization of the white matter. Condensation of neurons and vacuolization of the white matter were also observed in the cranial Th7 segment.

Rats-synaptophysin Immunohistochemistry

Normal controls In the spinal cord of both normal and laminectomized rats, synaptophysin immunoreactivity appeared as numerous diffusely distributed fine dots in the neuropil of the gray matter. The dots were more marked and more intensely stained along the surface of motor neurons and their proximal dendrites, and delineated their polygonal contours (Fig. 1A). The most frequent immunoreactive dots were observed in the substantia

gelatinosa and neighbouring white matter (Fig. 1B).

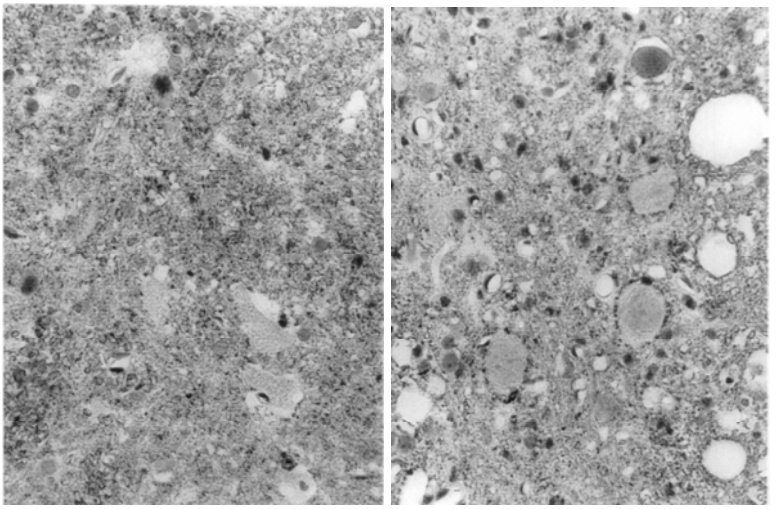


A B

FIG. 1. Normal distribution of synaptophysin immunoreactivity in rat spinal cord. A. An anterior horn. Immunoreactive profiles appear as fine dots diffusely distributed in the gray matter neuropil. The dots are more dense along the surface of motor neurons and their proximal dendrites and delineate their polygonal contours. B. A dorsal horn. The most frequent dots are present in the substantia gelatinosa.

Moderate trauma Moderate compression of the spinal cord induced rapid and pronounced changes in synaptophysin immunoreactivity. Four hours after trauma, the synaptophysin immunoreactivity in the gray matter neuropil was strikingly increased. The immunoreactive profiles appeared as coarse granules of different sizes. Remaining motor neurons of the anterior horns had lost immunoreactive dots on their surface (Fig. 2A). Many slightly expanded axons with intense synaptophysin immunoreactivity were seen throughout the white matter (Fig. 4A), including the corticospinal tracts, in the Th8-9 segments. One and four days after injury, the immunoreactive axons were more expanded and had increased in number (Figs. 4B and C). The immunoreactive intensity varied considerably between the enlarged axons. Immunoreactive materials were also present in the debris of necrotic areas. Nine days after compression, coarse granules with intense immunoreactivity were scattered in the gray matter and were arranged along the surface of remaining motor neurons of the anterior horns (Fig. 2B). Many immunoreactive axons were still present in the white matter.

Conspicuous changes in synaptophysin immunoreactivity also occurred in the cranial Th7 segment. Four hours after injury, increased immunoreactivity was seen in the gray matter of this segment, especially at the surface of motor neurons in the anterior horns (Fig. 3). The caudal Th10 segment did not show visible changes in synaptophysin immunoreactivity. Nine days after trauma, the immunoreactivity was normal in the Th7 and Th10 segments.



A B

FIG. 2. Changes in synaptophysin immunoreactivity in rat spinal cord after trauma. A. An anterior horn. Four hours after moderate trauma there was an increase in synaptophysin immunoreactivity in the gray matter neuropil of the injured Th8-9 segments. Motor neurons lost immunoreactive dots on their surface. B. At day 9 following moderate trauma, intensely immunoreactive profiles had reoccurred on the surface of motor neurons in the anterior horn of the Th8-9 segments.

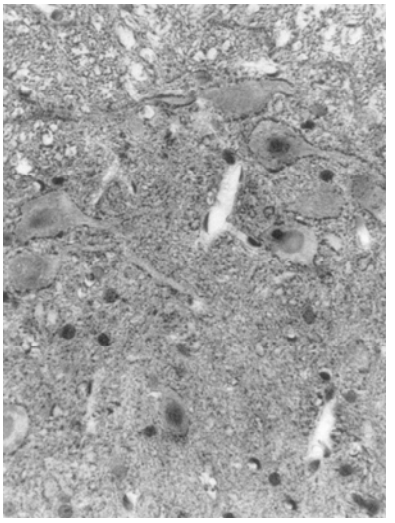


FIG. 3. An anterior horn. Markedly intense immunoreactivity was seen around the surface of motor neurons in the Th7 segment.

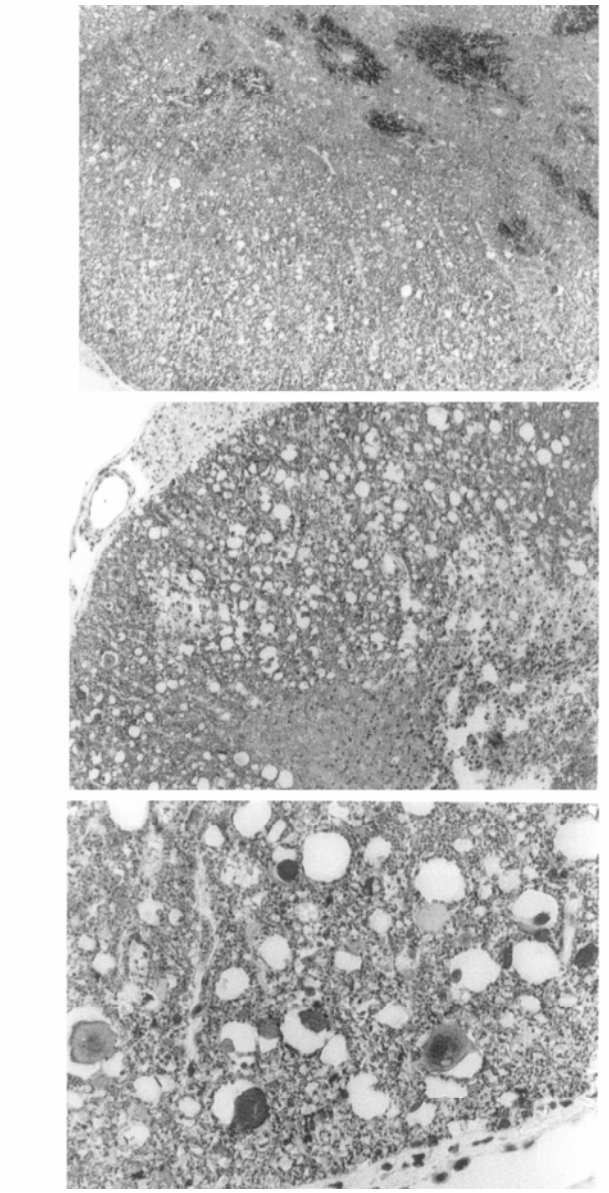


FIG. 4. A. Accumulation of synaptophysin immunoreactivity in axonal swellings (arrows) in the white matter of Th8-9 segments 4 h after compression. B. Axonal swellings became numerous at day 1 after injury. C. A higher magnification of Fig. 4B.

Severe trauma Severe compression resulted in a large area of necrosis occupying almost the entire cross-section of the Th8-9 segment at 4 h and day 1. Reactive gliosis was seen in the lesion at days 4 and 9. In some of the rats the subpial part of the dorsal horns was preserved and showed increased immunoreactivity. Otherwise, synaptophysin immunoreactivity was lacking in the gray matter. A number of enlarged axons with intense synaptophysin immunoreactivity were present in the white matter from 4 h to 9 d after trauma.

The cranial Th7 and caudal Th10 segments displayed expanded axons with synaptophysin immunoreactivity, but they were much less abundant than in the Th8-9 segment. The changes in immunoreactivity in the gray matter of the Th7 and Th10 segments were similar to those in rats with moderate injury.

Mice-general Morphological Changes

Atrophy of mouse spinal cords was noted 14 d after both moderate and severe injury. The architecture of the spinal cords was distorted. After moderate compression, the central area of the spinal cord exhibited gliosis, but the subpial regions of the dorsal horns and white matter were preserved. The area of gliosis occupied almost the whole cross-section of the spinal cord subjected to severe trauma.

Mice-synaptophysin Immunohistochemistry

As in the rats, both normal and laminectomized mice showed synaptophysin immunoreactivity in the spinal cord. Fourteen days after moderate compression, the immunoreactivity was increased in the gray matter, especially around motor neurons. Expanded axons with intense synaptophysin immunoreactivity were present in the white matter. In mice subjected to severe injury such axons were only seen in the subpial regions. Preserved dorsal horns showed increased immunoreactivity.

DISCUSSION

In the present study synaptophysin immunohistochemistry demonstrated that compression trauma induced striking synaptic changes in rat and mouse spinal cords. The synaptic changes were observed as early as 4 h after compression and were noted at all survival periods in the rats. Such changes were not confined to the injured segment, but extended into the neighbouring segments. The synaptic alterations thus seem to be important components of the secondary injuries in spinal cord trauma.

Synaptophysin immunoreactivity was markedly increased in the gray matter of the Th8-9 segments in the rats 4 h after moderate trauma. Synaptophysin is the major protein of the synaptic membrane[17,18] and may play an important role as a channel in synaptic vesicle exocytosis, e.g. in neurotransmitter release[41-47]. Thus, the early increase in synaptophysin immunoreactivity may reflect upregulation of synaptic functions and may be related to release of neurotransmitters, especially excitatory amino acids, which have been intensely studied[31,32].

Nine days after moderate injury in the rats, coarse granules with intense immunoreactivity were scattered in the gray matter with a distribution pattern different from that in normal rats. The immunoreactive profiles reappeared around the surface of remaining motor neurons. These changes may indicate formation of new synapses.

The changes in synaptophysin immunoreactivity around motor neurons in the anterior horns varied at different survival periods and in different segments. The injured Th8-9 segments showed a loss of immunoreactive dots on the surface of motor neurons at an early

stage (4 h) of injury. This may be one of the mechanisms causing motor dysfunction of hind limbs[9]. The motor neurons regained the immunoreactive profiles, then showing more intense immunoreactivity than that in normal rats. This restoration may play an important role in the recovery of motor function as tested by behaviour assessments[9]. In the cranial Th7 segment increased immunoreactive dots surrounded motor neurons, but the caudal Th10 segment did not show any visible changes in immunoreactivity. The reason for this difference is not clear.

One interesting finding in both the rat and mouse is the accumulation of synaptophysin immunoreactivity in injured axons after compression trauma to the spinal cord. Such accumulation occurred soon after the injury and lasted for a long period. Although there are several markers available for axonal swellings[6,9,35-38], we did not find any reports on accumulation of synaptophysin immunoreactivity in injured axons of the spinal cord. The present study introduced synaptophysin immunohistochemistry as a good tool for demonstration of axonal swellings in rat and mouse models of spinal cord compression trauma.

In summary, compression trauma induced remarkable alterations of synapses in injured Th8-9 segments as well as in neighbouring segments. The accumulation of synaptophysin immunoreactivity in injured axons occurred soon after spinal cord injury and lasted for a long period of time.

ACKNOWLEDGEMENTS

The authors would like to thank Madeleine JARILD and Gunilla TIBBLING for their technical help.

REFERENCES

1. Anderson, D. K. and Hall, E. D. (1993). Pathophysiology of spinal cord trauma. *Ann. Emerg. Med.* **22**, 987-992.
2. Bunge, R. P., Puckett, W. R., Becerra, J. L., Marcillo, A., and Quencer, R. M. (1993). Observations on the pathology of human spinal cord injury. *Adv. Neurol.* **59**, 75-89.
3. Hughes, J. T. (1992). Disorders of the spine and spinal cord. In: Adams JH , Duchen LW (eds) Greenfield´s neuropathology, Fifth edn. Edward Arnold, London, pp. 1083-1089.
4. Jellinger, K. (1976). Neuropathology of cord injuries. In: Vinken, P. J., Bruyn, G. W., Braakman, R. (eds) Handbook of clinical neurology. Injuries of the spine and spinal cord, Elsevier/North-Holland, Amsterdam, pp. 43-121.
5. Kakulas, B. A. and Taylor, J. R. (1992). Pathology of injuries of the vertebral column and spinal cord. In: Frankel HL (eds) Handbook of clinical neurology, Elsevier Science Publishers, Amsterdam, pp. 21-51.
6. Ahlgren, S., Li, G. L., and Olsson, Y. (1996). Accumulation of ß-amyloid precursor protein and ubiquitin in axons after spinal cord trauma in humans: Immunohistochemical observations on autopsy material. *Acta Neuropathol.* **92**, 49-55.
7. Balentine, J. D. (1978). Pathology of experimental spinal cord trauma. II. Ultrastructure of axons and myelin. *Lab. Invest* **39**, 254-265.
8. Li, G. L. and Farooque, M. (1996). Expression of ubquitin-like immunoreactivity in axons after compression trauma to rat spinal cord. *Acta Neuropathol.* **91**, 155-160.
9. Li, G. L., Farooque, M., Holtz, A., and Olsson, Y. (1995). Changes of β-amyloid precursor protein after compression trauma to the spinal cord: An experimental study in the rat using immunohistochemistry. *J. Neurotrauma* **12**, 269-277.
10. Li, G. L., Farooque, M., Holtz, A., and Olsson, Y. (1996). Increased expression of growth-associated protein 43 immunoreactivity in axons following compression trauma to rat spinal cord. *Acta Neuropathol.* **92**, 19-26.
11. Martin, J. E., Mather, K. S., Swash, M., Garofalo, O., Dale, G. E., Leigh, P. N., and Anderton, B. H. (1990). Spinal cord trauma in man: studies of phosphorylated neurofilament and ubiquitin expression. *Brain* **113**, 1553-1562.
12. Farooque, M., Isaksson, J., Jackson, D. M., and Olsson, Y. (1999). Clomethiazole (ZENDRA, CMZ) improves

hind limb motor function and reduces neuronal damage after severe spinal cord injury in rat. *Acta Neuropathol.* **98**, 22-30.
13. Li, G. L., Farooque, M., Holtz, A., and Olsson, Y. (1995). Microtubule-associated protein 2 as a sensitive marker for dendritic lesion after spinal cord trauma: An immunohistochemical study in the rat. *Restor. Neurol. Neurosci.* **8**, 189-197.
14. Li, G. L., Farooque, M., Holtz, A., and Olsson, Y. (1997). Effects of a-phenyl-N-tert-butyl nitrone (PBN) on compression injury of rat spinal cord. *Free Radical Res.* **27**, 187-196.
15. Krassioukov, A. V. and Weaver L. C. (1996) Morphological changes in sympathetic preganglionic neurons after spinal cord injury in rats. *Neurosci.* **70**, 211-225.
16. Nacimiento, W., Sappok, T., Brook, G. A., Toth, L., Schoen, S. W., Noth, J., and Kreutzberg, G. W. (1995). Structural changes of anterior horn neurons and their synaptic input caudal to a low thoracic spinal cord hemisection in the adult rat: a light and electron microscopic study. *Acta Neuropathol.* **90**, 552-564.
17. Jahn, R., Schiebler, W., Ouimet, C., and Greengard, P. (1985). A 38 000 dalton membrane protein (P38) present in synaptic vesicles. *Proc Natl. Acad. Sci. USA* **82**, 4137-4141.
18. Wiedenmann, B. and Franke, W. W. (1985). Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. *Cell* **41**, 1017-1028.
19. Navone, F., Jahn, R., Gioia, G. D., Stukenbrok, H., Greengard, P., and Camilli, P. D. (1986). Protein P38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J. Cell Biol.* **103**, 2511-2517.
20. Thiel, G. (1993). Synapsin I, Synapsin II, and synaptophysin: marker proteins of synapses vesicles. *Brain Pathol.* **3**, 87-95.
21. Gould, V. E., Lee, I., Wiedenmann, B., Moll, R., Chejfec, G., and Franke, W. W. (1986). Synaptophysin: a novel marker for neurons, certain neuroendocrine cells, and their neoplasms. *Hum. Pathol.* **17**, 979-983
22. Wiedenmann, B., Franke, W. W., Kuhn, C., Moll, R., and Gould, V. E. (1986). Synaptophysin: A marker protein for neuroendocrine cells and neoplasms. *Proc. Natl. Acad. Sci. USA* **83**, 3500-3504.
23. Ikemoto, A., Kawanami, T., Llena, J. F., and Hirano, A. (1994). Immunohistochemical studies on synaptophysin in the anterior horn of lower motor neuron disease. *J. Neuropathol. Exp. Neurol.* **53**, 196-201.
24. Masliah, E. and Terry, R. (1993). The role of synaptic proteins in the pathogenesis of disorders of the central nervous system. *Brain Pathol.* **3**, 77-85.
25. Matsumoto, S., Goto, S., Kusaka, H., Ito, H., and Imai, T. (1994). Synaptic pathology of spinal anterior horn cells in amytrophic lateral sclerosis: an immunohistochemical study. *J. Neurol. Sci.* **125**, 180-185.
26. Sasaki, S. and Maruyama, S. (1994). Decreased synaptophysin immunoreactivity of the anterior horns in motor neuron diseases. *Acta Neuropathol.* **87**, 125-128.
27. Wiedenmann, B. (1991). Synaptophysin: A widespread constituent of small neuroendocrine vesicles and a new tool in tumor diagnosis. *Acta. Oncol.* **30**, 435-440.
28. Krassioukov, A. V. and Weaver, L. C. (1995). Reflex and morphological changes in spinal preganglionic neurons after cord injury in rats. *Clin. Exp. Hypertens* **17**, 361-373.
29. Dahlstrom, A. B. and Böölj, S. (1988). Rapid axonal transport as a chromatographic process: the use of immunocytochemistry of ligated nerves to investigate the biochemistry of anterogradely versus retrogradely transported organelles. *Cell Motil Cytoskeleton* **10**, 309-320.
30. Dahlstrom, A. B. and Li, J. Y. (1994). Fast and slow axonal transport - different methodological approaches give complementary information: contributions of the stop-flow/crush approach. *Neurochem Res.* **19**, 1413-1419.
31. Tator, C. and Fehlings, M. (1991). Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanism. *J. Neurosurg* **75**, 15-26.
32. Tator, C. H. (1995). Update on the pathophysiology and pathology of acute spinal cord injury. *Brain Pathol.* **5**, 407-413.
33. Griffin, J. W., Price, D. L., Engel, W. K., and Drachman, D. B. (1977). The pathogenesis of reactive axonal swellings: role of axonal transport. *J. Neuropathol. Exp. Neurol.* **36**, 214-227.
34. Povlishock, J. T. (1993). Traumatic brain injury. The pathobiology of injury and repair. In: Gorio A (eds) Neuroregeneration, Raven Press, Ltd, New York, pp. 185-216.
35. Gentleman, S. M., Nash, M. J., Sweeting, C. J., Graham, D. I., and Roberts, G. W. (1993). ß-Amyloid precursor protein (ßAPP) as a marker for axonal injury after head injury. *Neurosci. Lett.* **160**, 139-144.
36. Lewen, A., Li, G. L., Nilsson, P., Olsson, Y., and Hillered, L. (1995). Traumatic brain injury in rat produces changes of ß-amyloid precursor protein immunoreactivity. *NeuroReport* **6**, 357-360.
37. Sherriff, F. E., Bridges, L. R., Gentleman, S. M., Sivaloganathan, S., and Wilson, S. (1994). Markers of axonal injury in post mortem human brain. *Acta Neuropathol.* **88**, 433-439.
38. Sherriff, F. E., Bridges, L. R., and Sivaloganathan, S. (1994). Early detection of axonal injury after human head trauma using immunocytochemistry for ß-amyloid precursor protein. *Acta Neuropathol.* **87**, 55-62.
39. Farooque M. (2000). Spinal cord compression injury in the mouse: presentation of a model including assessment of motor dysfunction. *Acta Neuropathol. (Berl).* **100**, 13-22.
40. Rivlin, A. and Tator, C. (1977). Objective clinical assessment of motor function after experimental spinal cord

injury in the rat. *J. Neurosurg* **47**, 577-581.
41. Alder, J., Kanki, H., Valtorta, F., Greengard, P., and Poo, M. M. (1995). Overexpression of synaptophysin enhances neurotransmitter secretion at Xenopus neuromuscular synapses. *J. Neurosci.* **15**, 511-519.
42. Alder, J., Lu, B., Valtorta, F., Greengard, P., and Poo, M. M. (1992). Calcium-dependent transmitter secretion reconstituted in Xenopus oocytes: requirement for synaptophysin. *Science* **257**, 657-661.
43. Alder, J., Xie, Z. P., Valtorta, F., Greengard, P., and Poo, M. M. (1992) Antibodies to synaptophysin interfere with transmitter secretion at neuromuscular synapses. *Neuron*. **9**, 759-768.
44. Johnston, P. A., Cameron, P. L., Stukenbrok, H., Jahn, R., Camilli, P. D., and Sudhof, T. C. (1989). Synaptophysin is targeted to similar microvesicles in CHO and PC12 cells. *E. M. B. O. J.* **8**, 2863-2872.
45. Leube, R. E., Wiedenmann, B., and Franke, W. W. (1989). Topogenesis and sorting of synaptophysin: synthesis of a synaptic vesicle protein from a gene transfected into nonneuroendocrine cells. *Cell* **59**, 433-446.
46. Rehm, H., Wiedenmann, B., and Betz, H. (1986). Molecular characterization of synaptophysin, a major calcium-binding protein of the synaptic vesicle membrane. *E. M. B. O. J.* **5**, 535-541.
47. Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E., Franke, W. W., and Betz, H. (1988). Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. *Science* **242**, 1050-1053.

(*Received December* 2, 2003 *Accepted April* 28, 2004)