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Original Article

Time Course of Axotomy-induced Changes in Synaptophysin Pattern and Synaptic Reaction of Spinal Motoneurons in Adult Rat

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ABSTRACT

Background and Objective: **Evaluation of degenerative changes of motoneurons and their related synapses can be useful in understanding the mechanisms of neurodegenerative diseases and their potential treatment. The present electron microscopic and immunohistochemical study investigates the axotomy-induced changes of adult spinal motoneurons up to 3 months following sciatic nerve transection.**

Materials and Methods: **Following unilateral mid-thigh sciatic transection in adult rats, the synaptophysin expression and ultrastructure of synapses in ventral horn of related spinal cord segments were studied 1 day, 1 week, 1 month, and 3 months postoperatively. In all groups the unaxotomized side of spinal cord was considered as control. The pattern of synaptophysin immunolabeling was classified into intact, partial, diffused, and negative types.**

Results: **In 1 week and 1 month groups, the intact pattern decreased and the other 3 patterns increased, whereas 3 months postoperatively the patterns changed vice versa, i.e. intact pattern increased and the other 3 decreased. Electron microscopic studies indicated consistent ultrastructural changes such as synaptic vesicle displacement, synaptic membrane irregularity and synaptic stripping, which were most prominent after 1 month and declined in 3 month group.**

Conclusion: **The present data indicate that following axon injury in adult motoneurons, synapses undergo obvious changes in ultrastructure and synaptophysin distribution, which increase up to 1 month postoperatively, and if the cell survives the insult the changes will attenuate and return to normal conditions thereafter.**

Key words*:* Motor Neurons, Synapse, Synaptophysin, Axotomy

Introduction

Axotomy has been reported to cause synaptic degeneration (1) and detachment (2), central synaptic stripping (3), and reduction in the synapse number (4). Electron microscopy and immunohistochemistry are two routine ways to investigate synapses. Synaptophysin, a presynaptic vesicle protein (molecular weight 38000) found in all nerve terminals is readily detectable by immunohistochemistry and is useful in the identification of synapses. Levels of synaptophysin and several other vesicle proteins within the nerve terminals are believed to remain constant in the

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absence of nerve injury owing to the recycling of vesicle material, and can be used as a marker to quantify the number of terminals (5). There are many reports using the synaptophysin immunoreactivity to investigate the changes of synapses; Svensson and Aldskogius reported a reduction in the synaptophysin immunoreactivity following hypoglossal nerve transaction (6), and many other investigators evaluated the pattern of synaptophysin reactivity in Amyotrophic Lateral Sclerosis (7-9).

Therefore, the purpose of this study was to evaluate the time course of changes in the pattern of synaptophysin immunoreactivity as well as synaptic morphological changes in the spinal motoneurons of adult rats following transection of sciatic nerve.

Materials and methods

The animal care and all experimental procedures were carried out according to ethical guidelines established by the Shahed University. Twenty young adult Sprague-Dawley rats (100-150 g) obtained from Razi Institute (Karaj, Iran) were housed under a 12 hour light/dark cycle with free access to food and water. Under general anesthesia with 35 mg/Kg intraperitoneal injection of Nesdonal, the left sciatic nerve was transected at the mid-thigh level under the long head of biceps femoris, and to hinder innervation a 5 mm piece of the nerve was extracted. The animals were divided into four groups and sacrificed 1 day, 1 week, 1 month, and 3 months postoperatively by an overdose of pentobarbital and transcardially perfused with heparin containing normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and the L4-L6 spinal cord segments were removed. In each group the samples of 3 animals were used for immunohistochemistry and 2 animals for electron microscopy.

For immunohistochemical studies, after fixation of samples in buffered formalin and preparing of paraffin blocks, 8 µm transverse sections were mounted on glass slides and processed for synaptophysin immunolabeling. In each group, the ventral horn of unaxotomized side was considered as controls. The sections were preincubated in 3% H_2O_2 in 0.01 M phosphate-buffered saline (PBS) to block endogenous peroxidase activity followed by incubation in 5% normal goat serum. Then, the sections were incubated overnight with monoclonal mouse anti-synaptophysin antibody (1:1000, Serotec) as primary antibody followed by peroxidase-conjugated goat anti-mouse immunoglobulins (1:100, Dako) for 30 minutes at room temperature as secondary antibody in a moist chamber. After 10-min incubation in a solution containing 0.05% diaminobenzidine + 0.003% H_2O_2 the sections were dehydrated, cleared, and covered with coverslips. Omission of primary antibody was used as negative control.

For electron microscopic studies, nearly 1 mm3 pieces from both ventral horns of mid-L5 segment were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer at 37 ºC for 1 hour, dehydrated in acetone and embedded in resin. After trimming, the blocks were sectioned and 50-70 nm ultrathin sections were collected on copper grids and stained with uranyl acetate and lead citrate and examined in a ZEISS EM 900 electron microscope. In each group, samples obtained from right ventral horn were considered as controls.

Results

The immunolabeled motoneurons in the ventral horn of spinal cord were classified into four types according to the pattern of synaptophysin immunoreactivity: type1- intact pattern (IP), with a nearly complete subplasmalemal labeling; type2- partial pattern (PP), with a dicontinuous labeling covering less than 2/3 of plasmalema, type3- diffused pattern (DP), with a scattered cytoplasmic labeling, and type4- negative pattern (NP), with no remarkable synaptophysin labeling (Fig. 1).

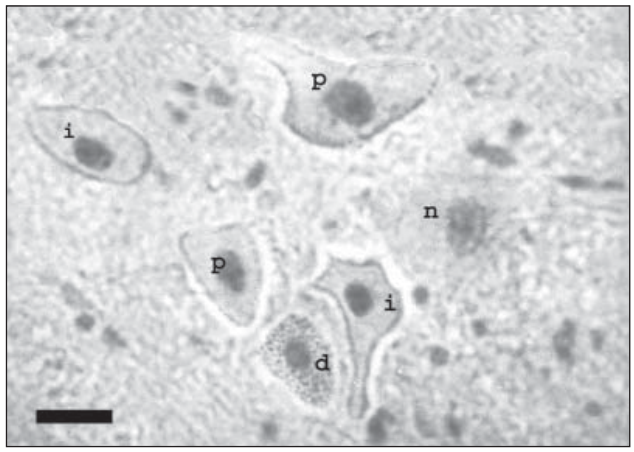

Figure 1. Photomicrograph of anti-synaptophysin immunoperoxidase labeling of axotomized spinal motoneurons 1 week postoperatively, indicating different patterns of synaptophysin immunolabeling, i: intact; p: partial; d: diffused, and n: negative pattern; (counterstained with Haematoxylin, scale bar = 25 µm).

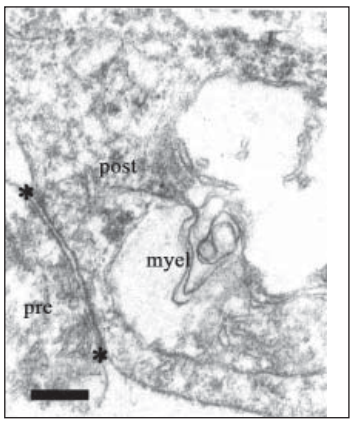

Figure 2. Electron micrograph of the axotomized ventral horn neuropil 1 month postoperatively, indicating synaptic degenerative changes such as irregularity in the presymaptic density and synaptic cleft with dissociation of synaptic vesicles, and a myelin formation in the postsynaptic component. Pre: presynaptic, post: postsynaptic, myel: myelin formation, asterisks denote the boundaries of the synapse (scale bar = 0.23 µm).

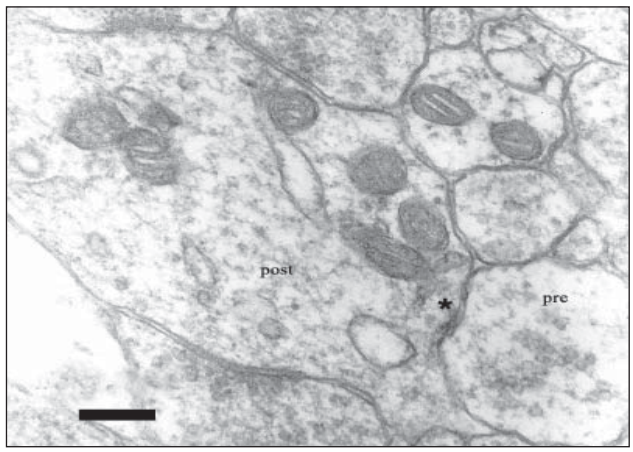

Figure 3. Electron micrograph of the axotomized ventral horn neuropil 1 week postoperatively, indicating a degenerative synapse undergoing collapse of the synaptic cleft and irregular detachment of pre- and postsynaptic membranes. Pre: presynaptic, Post: postsynaptic, asterisk denotes the synapse (scale bar = 0.23 µm).

In all groups, the percentage of each of the four patterns were calculated and statistically compared by Analysis of Variance (ANOVA) and Tukey's test. All above mentioned patterns can be seen in Figure 1. In unaxotomized side of all groups, the prominent pattern is IP. The study of axotomized side showed no significant alterations in percentage of different synaptophysin patterns compared to unaxotomized side 1 day postoperatively, but after 1 week the IP decreased and the other 3 patterns increased significantly. The changes visible in 1 week group continue to become more conspicuous up to 1 month, where the minimum percentage of IP and the maximum percentage of DP and NP could be seen, though the highest percentage of PP took place 1 week postoperatively and did decrease in 1 month group. In 3-months group the changes had been significantly reversed, namely there occurred an increase in IP and a decrease in other 3 patterns. Also in all time points the percentage of intact pattern reduction (PIR) was calculated, indicating the highest value 1 month postoperatively (Table 1). In each pattern, the differences between percentages found in all time points were statistically significant.

Electron microscopic results showed degenerative ultrastructural changes in synapses of axotomized samples which were apparent 1 week postoperatively, became more obvious in 1 month group, and attenuated after 3 months. These changes included myelin formation in the postsynaptic region as a sign of synapse degenerative changes, irregular distribution and dissociation of synaptic vesicles from active zone of synapse, irregularity and collapse of synaptic cleft, loss of pre and/or postsynaptic density, complete or partial detachment of pre and postsynaptic membranes, and ensheathment of degenerative syna-pses by

Table 1. The corrected percentages of different synaptophysin immunolabeling patterns at the axotomized ventral horn at different time points

Group	IP	PP	DP	NP	PIR
1 Day	84.1 ± 4.7	11.5 ± 2.9	1.44 ± 1.44	0	13.76 ± 2.6
1 Week	51.8 ± 4.1	27.3 ± 2.1	12.3 ± 2.1	4.04 ± 2.6	45.6 ± 4.2
1 month	42.4 ± 7.4	25.2 ± 1.7	15.7 ± 2.23	7.9 ± 2.5	53.32 ± 2.1
3 months	54.7 ± 3.3	18.7 ± 1.8	10.9 ± 1.9	1.5 ± 1.5	41.42 ± 3.4

IP: intact pattern; PP: partial pattern; DP: diffused pattern; NP: negative pattern; PIR: percentage of intact pattern reduction. In every pattern as well as PIR column the statistical difference between all time points is significant ($p<0.05$).

astrocytic processes and lamellae (Fig. 2- 4).

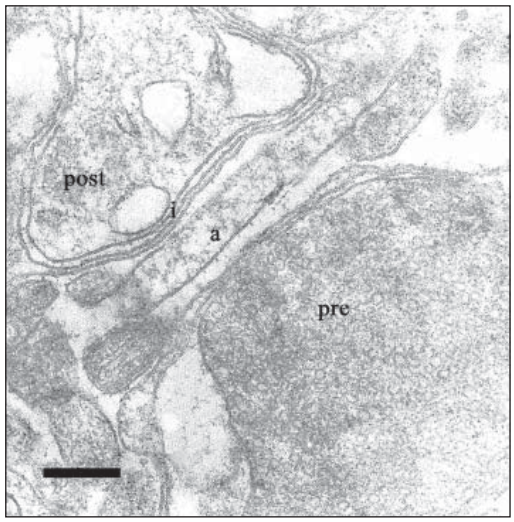

Figure 4. Electron micrograph of the axotomized ventral horn neuropil 1 week postoperatively, indicating an astrocytic process (a) and many astrocytic lamellae (l) separating the pre- and post-synaptic membranes and occupying the interval between them. The pre-synaptic button is laden with abundant numbers of synaptic vesicles (scale bar = 0.23 µm).

Discussion

A prominent feature of axotomy-induced degeneration of neurons is an extensive stripping of synapses from the cell membrane which is more pronounced on the soma than on the dendrites (4). If target reinnervation does not occur synaptic stripping may become permanent, whereas in the case of reinnervation the synaptic inputs will be gradually restored suggesting a role for target-derived neurotrophic factors in the regulation of synaptic plasticity (10, 11).

Degenerative changes of synapses of motoneurons can be studied by means of electron microscope and immunohistochemistry using synaptic proteins such as synaptophysin. The classification of synaptophysin immunolabeling pattern was introduced by Cruz-Sanchez et al who explained different synaptophysin patterns in spinal motoneurons of human amyotrophic lateral sclerosis (ALS) patients: a- well preserved synaptophysin reactivity around the soma and the proximal dendrites of histologically normal neurons; b- dot-like presynaptic terminals around the cell body of chromatolytic neurons in a fused pattern; c- intense, diffuse and homogeneous reactivity of some neurons; d-chromatolytic neurons showing complete absence of synaptophysin reactivity. Attenuation of synaptophysin reactivity in the ventral horn neuropil to its complete loss was observed in all ALS cases (12). Above mentioned changes in the pattern of synaptophysin immunoreactivity in cell bodies may represent synaptic plasticity and/or degeneration. Other investigators used synaptophysin to study synapse alterations following transection of sciatic (13), facial (14), and hypoglossal (6) nerves. Alvarez et al reported a decrease in synaptophysin immunoreactivity 2 weeks following axotomy (13), which is nearly consistent with our findings where the highest NP was noticed at 1 month and the highest PP could be apparent at 1 week postoperatively, which has been reported to be a critical time for medical interference for surgical anastomosis of sectioned facial nerve (15). So the PP and DP may be supposed as a reversible neuronal injury correlative with chromatolysis, whereas NP may be a sign of irreversible injury where the synaptic losses can not be replaced. These findings are consistent with the well approved statement that following axon injury the adult motoneurons undergo chromatolytic response which may be reversible, and if the insult were intense enough the cell will enter an irreversible process leading to apoptotic cell death.

Electron microscopically, the detachment of synaptic membranes has been reported in ALS (16), human facial nerve lesion (17), and peripheral nerve injury in experimental animals (2, 18). Chen reported synaptic detachment associated with disappearance of both pre- and postsynaptic membrane densities (16), Sasaki and Iwata reported presynaptic alterations associated with motor neuron disease (19), and Marsala et al indicated irregularity in the arrangement of synaptic vesicles (20). All of these reports are consistent with our electron microscopic findings indicating the same ultrastructural changes, which in the adult neurons are usually reversible.

Virtually every lesion to the nervous system results in rearrangement of the synaptic connections and retraction of nerve terminals, which occur in the early stages of the nerve injury and may persist for a variable period (4). In addition to pre- and postsynaptic neurons the glial cells are key players in this process (21), and the displacement of presynaptic terminals from the postsynaptic membrane is accompanied by the projections of thin, sheet like astrocytic processes (22); this feature has been found in our electron microscopic study (Fig. 4). This astroglial activation

and ensheathing of injured motoneuron soma and dendrites appears to be related to early response to injury and is influenced by a shift in the physiological state of alpha-motoneurons from a transmitting to a survival and regeneration state (23, 24). So the synaptic stripping should be beneficial for the survival and restorement of function of injured neurons (24). Linda et al reported that following a proximal axotomy of spinal motoneurons the removed synapses were mainly glutamatergic than glycinergic and GABAergic (25). This was interpreted as a reaction favoring the removal of potential excitotoxic influences, while at the same time the conserved inhibitory terminals would set the injured neurons into a less active state which may be more proper for regeneration.

Conclusion

These results indicate that after axon injury in the adult motoneurons, the neuron undergoes a series of usually reversible synaptic changes to lessen its connections and providing the chance to compensate the injury; these changes may be continued up to nearly 1 month postoperatively and thereafter they will be reversed and the cell will reestablish its synaptic connections once more. If the cells are not able to survive the insult, the synapses will not be restored and the cell will undergo apoptotic cell death.

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