ELECTROPHYSIOLOGICAL AND MORPHOHISTOCHEMICAL STUDY ON DYNAMICS OF DEGENERATIVE AND REGENERATIVE PROCESSES IN FLEXOR AND EXTENSOR BRANCHES OF SCIATIC NERVE AFTER THE CRUSH

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Abstract
The comparative morphohistochemical study of the sciatic nerve was performed in rats by the method of detecting Ca2+-dependent acid phosphatase activity at different periods after the crush. It was shown that in 2 hours the activity abruptly decreased at the site of injury, but in 5 days there was a recovery from the proximal area, although a tendency to degeneration of the nerve distal segment was outlined. Moderate proliferation of Schwann cells and endoneurium cells were observed since day 13 after crush; in 35 days the restoration of enzyme activity only in the extensor bundles of fibers (n. peroneus communis) was registered. In 70 days after Schwann cells crushing, a connective tissue layer between the nerve fibre bundles and cross-collaterals of nerve fibre winding the adjacent muscles was detected. To confirm the results of morphohistochemical study, the electrophysiological investigation was carried out to record the spinal cord single motoneurons activity to stimulation of extensor and flexor (n. gastrocnemius) branches of Schwann cells. Software analysis revealed a progressive increase of excitatory poststimulus reactions not reaching the normal level for flexor in contrast to the extensor nerve. Depressor effects, almost twice exceeding the normal levels and more pronounced for the extensor nerve, suggest the possible impact of the protective purpose. The use of pharmacological protection in order to accelerate the rehabilitation of the damaged nerve might be required.

Keywords: nerve crushing, extensor and flexor branches of sciatic nerve, spike activity of the spinal cord single neurons, Deiters’ nucleus, red nucleus.

INTRODUCTION
Crush or compression of peripheral nerve (PN) is investigated extensively and thoroughly. Under this state a number of mechanisms, which facilitate the objective assessment of the changes taking place not only at the source of compression, but also far beyond its borders, are activated. In particular, it is revealed that chronic compression of PN evokes competitive apoptosis and proliferation of Schwann cells (SCs), i.e. their turnover with minimal damage and axon progressive demyelination [Gupta R., Steward O., 2003]. As known, the distal stump of the nerve during crush undergoes Wallerian’s degeneration, during which the microenvironment is generated promoting ingrowth of fibers from the proximal stump. SCs at the same time respond by down-regulation of myelin genes, dedifferentiation, proliferation and through lining tubules (Bungner’s bands) express the surface molecules that conduct regenerating fibers. Simultaneously in the distal stump molecular changes occur, including up-regulation of neurotrophins, cytokines, adhesion of nerve cell’s molecules, etc. Meanwhile, nerve regeneration occurs in the range of 3-4 mm/day and might be fed up pharmacologically [Stoll G., Müller H., 1999]. In addition, the crushing gave rise to a temporary but complete loss of function, which was restored to the control level closer to 4 weeks [Hare G. et al., 1997].

In this work after crushing the sciatic nerve (SN), the morphohistochemical and electrophysiological study was carried out on restoring dynamics of the
flexor (n. gastrocnemius - G) and extensor (n. peroneus communis - Pi) nerves of the hind limbs. Recently data on application of parathyroid hormone during SN crush was published [Minasyan A. et al., 2011].

**MATERIALS AND METHODS**

Experiments were performed in male Albino rats (250 ± 30 g): intact rats (n = 3) and rats subjected to unilateral compression of SN (control; n = 10). All procedures were performed according to “Rules for the care of laboratory animals” (NIH publication № 85-23 revised in 1985), as well as specific guidance provided by Committee of the National Health Service and Health for animals care. SN crushing was inflicted in the upper third of the femur (4 mm above the trifurcation) under Nembutal anesthesia (40 mg/kg i/p). In morphohistochemical series of experiments, the nerve was taken under deep-drugged sleep in 2 hours and 5-70 days after crushing and fixed 3-7 days in 10% neutral formalin. Long-term induration of material in the fixative provides better results. Frozen longitudinal sections of 30-40 microns were transferred into the freshly prepared mixture for identification of the activity in Ca²⁺-dependent acid phosphatase (AP) considering the mechanisms of the concentration relationships [Meliksetyan I., 2007].

To detect enzyme activity, we recommend incubation mixture of the following composition:

- 20 mL of 0.40% solution of lead acetate;
- 5 mL of 1 M pH 5.6 acetate buffer;
- 5 mL of 1% solution of β-glycerophosphate sodium.

The given mixture was brought to 100 mL using 3% solution of calcium chloride. Incubation was carried out in an oven at 37°C for 3-4 hours. After washing, the sections were developed in 3% solution of sodium sulfide and embedded in balsam.

The comparative analysis of sensory (reflex test of recording - RTR) and motor (sciatic static index - SSI) indices of functional recovery after crushing was conducted. In electrophysiological experiments, at days 5, 13, 16, 21, 25, 32, 35 and 70 after SC crushing and fixation in the stereotaxic apparatus under Nembutal anesthesia craniotomy, dorsal laminectomy of the lumbosacral spinal cord and distal osteoporosis of the SC squeezed flexor and extensor branches were produced. Then the animals were immobilized by 1% ditillinium (25 mg/kg i/p) and transferred to artificial respiration. For extracellular recording of spike activity glass microelectrodes with a tip diameter of 1 μm filled with 2M solution of NaCl were inserted into the ventral horns of gray matter lumbar segments (L4-L5) in the region of spinal cord motoneurons (MN) (Rexed VIII-IX plates). In intact animals high-frequency stimulation (HFS) (0.05 ms, 0.10-0.16 mA, 50 Hz for 1 sec) of hind limb extensor (n. peroneus communis - Pi) and flexor (n. gastrocnemius - Gi) nerves and at the ipsilateral side relative to the damage - i, was performed by bipolar silver electrodes. For identification of MN the structures of central control [Minasyan A. et al., 2009], such as giant-cell red nucleus (RN - AP-6, L ± 0.8, DV +7.7 mm) and lateral vestibular nucleus (LVN - AP-11.5, L ± 2.5, DV +7 mm) were stimulated by stereotactic oriented according to the brain atlas [Paxinos G., Watson C., 2005] cylindrical bipolar electrodes (current parameters - 0.05 ms, 0.08 mA, 50 Hz for 1 sec). Moreover, the paired reciprocity of effects of central structures stimulation, which manage the facilitation of flexion and inhibition of extension, and vice versa, and/or the peripheral structures with certain orientation was used [Minasyan A. et al., 2009].

To determine the statistical significance of differences in duration of inter-spike intervals before and after the stimulus nonparametric criterion for testing homogeneity of two independent samples - Two Sample Wilcoxon-Mann-Whitney’ criterion (Wilcoxon-Mann-Whitney test) was used. Since the number of recorded spikes was large enough (up to several hundreds of spikes in 10-second interval after the stimulus), the variety of this test, namely: z-test was performed, taking into account its asymptotic normality. The comparison of critical values with tabulated values of the normal distribution at a significance level of 0.05, 0.01 and 0.001 (for different trials) showed that as a result of HFS for most samples of neuronal activity spiking had a statistically significant change with a minimum significance level of 0.05.

**RESULTS**

The longitudinally cut SN of intact rats has wavy layered shape accentuated by elongated nuclei of the SC (Figure 1 A). In 2 hours after the SN crushing it is without characteristic waviness, but a modest delamination of nerve fibers (NF) and decreased activity of AP may be observed (Figure 1B). After 5 days the activity of AP at the site of injury dramatically decreases (Figure 1B). Frequently in the course of thin nerve fibers the transverse branches are...
Figure 1. Photomicrographs of intact rats longitudinal sections of the sciatic nerve (A - wavy laminated Figure of SN accented by elongated nuclei of SC); after 2 hours (B - no waves, no AP activity in the form of light stripes on the borders of distal d - and proximal p - portions shown by triangles); 5 days (c - a sharp fall in AP activity at the site of injury and C - crosscut branches), 15 days (D - mixed winding hollow tubes); 25 days (d - the reaction of blood vessels and p - originating from the proximal part of the isolated NF with low phosphatase activity), 35 days (F - restoration of AP activity in extensor bundle) and 70 days (G-I) after crushing: (place of injury covered by the dotted bracket; D - distal, P - proximal parts of crushed nerve, white arrows - hollow Schwann tubules; arrow with double head - transverse branches of NF; black arrows - separate NF with low AP activity; circle - a layer of connective tissue; M - muscle fibers). Magnification: oc. 10, ob. 2.5 (A, B, e, g, i); 6.3 (c, d); 10 (F); 16 (G, I); 40 (E, H); 100 (C, D).
Figure 2. Complex Average Peri-Event Time (PETH Average), cumulative (Cumulative Average) histograms and histogram of frequency (Frequency Average) of excitatory (A, B) and depressor (C, D) poststimulus manifestations of activity of the spinal cord motoneurons to HFS of Gi (A, C) and Pi (B, D) nerves 5-70 days after the SN crush in the control and norm.
observed (Figure 1 B). Since day 13 after crushing, a weak proliferation of SC nuclei and endoneurium cells is detected. In the proximal and distal divisions the hollow winding of different caliber tubules are visible (Figure 1 D). The course of thin nerve fibers is also traced, though being intermittent here and there. From the proximal part originate few NF, which are characterized by waviness. Under these terms, there is already a tendency to degeneration of the distal nerve. In areas distant from the injury, the wavy lamination characteristic for the nerve is preserved. However, the foliated nerve fibers are found everywhere. In 25 days after SN crushing the blood vessels are traced (Figure 1 D). From the proximal part, isolated NF originates with low AP activity and there is a tendency to form bundles (Figure 1 D). In the course of each NF in the given section there exists poorly marked convolution. It is noteworthy that the high activity of Ca\(^{2+}\)-dependent AP is also observed in the distal nerve, although at transition to the injured area the enzyme activity abruptly decreases and this area looks bright in preparations. Often in the course of the rectified fibers there is observed its refraction in the plane of the section; therefore, along with the process of continuous thin fibers the Figure of intermittent linear circuit can be seen. AP reactivation 35 days after nerve crushing occurs in the extensor bundles of fibers (Figure 1 E). Empty Schwann tubes are not visible in these terms, and thin myelinated fibers with weak AP activity are traced to connect the distal and proximal parts of injury. There is a moderate proliferation of the endoneurium cell nuclei and SC providing conditions for NF restoration. In 70 days after crushing there is morphologically different pattern. Only in one group of animals the extensor bundle has properties of the reconstructed nerve (Figure 1 F; I). However, by this time the flexor and extensor bundles are already separated by a broad layer of connective tissue. In the other group of animals AP activity restored throughout the nerve, but in contrast to the intact nerve, marked stratification between the bundles is often recorded and the rolled up NF found. In some cases NF change the longitudinal position and go into neighboring muscles and entangle them, which is highly undesirable and may cause severe pain (Figure 1 B).

In a series of electrophysiological studies in the Control at days 5, 13 16, 21, 25, 32, 35 and 70 after the SC crush in intact animals (57 neurons, 168 tests) and from the affected side (118 neurons, 446 tests) to HFS of Gi and Pi there have been recorded excitatory (TP) and depressor (TD) tetanic post stimulus effects in spinal cord motoneurons. To HFS of Gi at days 5, 13 and 35 compared with prestimulus level almost 2-fold increase of activity was noted and at the 21st day it showed 2.77-fold excess. Lowest level of activity excess to 1.23-fold was observed at day 25 and the highest – at the 32nd (4.54 times) and 70th (4.07 times) days, whereas excess in norm was much greater: up to 12-fold (Figure 2 A). To HFS of Pi the lowest (about 2 times) TP was observed at the beginning of the test: at day 5 (1.33 times), in 21, 25 and 35 days after the SN crush, with subsequent about 4-fold progression of the excess at days 13 and 32, with the highest levels of 5-fold exceeding at the 70th day, whereas the level of 1P in the norm was twice higher. It is of interest that along with transition tendency of changes for recovery, at HFS of both Gi and Pi, by the 32nd day the posttetanic effect reached the maximum and decreased by half at the 35th day, then at day 70 it increased again: 2-fold or above (Figure 2 B). However, actually, compared to norm in both cases, there was no recovery of activity up to norm until the 70th day, having a twofold deviation. As to depressor manifestations of motoneurons poststimulus activity, in case of Gi HFS deepening of depression also grew abruptly (at days 5, 25, 32 and 35 from 1.3 to 1.48-fold activity underestimation, and at days 13 and 21 from 1.8 to 2.44 times) until the last day of test, at the 70th day reaching higher than 10-fold pre- stimulus activity underestimation which, in comparison with the norm, calculated as about 3 times activity underestimation (only 3.21 times) is three-fold deeper (Figure 2 D).

Finally, during HFS of Pi in initial period at days 5 and 13 the activity reached 1.5-fold underestimation prestimulus level, then almost up to 2-fold (from 1.74 to 1.97) at days 21, 32 and 35, reaching 4.5-fold at the 70th day, but with a maximum of almost three times higher (11.32 times) than the normal level (3.2 times) - at the 21st day of trial. Moreover, unlike the norm, TP to HFS of Gi and Pi in pathology was accompanied by posttetanic potentiation (PTP). That is, in both nerves deepening of depression more than three times exceeds the level of the norm, which in view of its protective effect indicate the active reaction to neurodegeneration, even allowing that excitation level up to the 70th day reached only 3 times below the level of norm. This demonstrates deepen-
ing of depression in pathology with the extension of the rehabilitation terms, which in addition is relatively more expressed for extensor nerve, and, in contrast to posttetanic depression in norm, is accompanied by PTP. In conclusion, poststimulus excitatory and depressive effects are progressively increased with trials time prolongation in disease, but without actually successful regeneration, which confirms data of morphological and histochemical studies, foreseeing the need for pharmacological intervention.

Figure 3 demonstrates in real-time the sum peri-
stimulus histograms and diagrams of the frequency averaged values of excitatory (A, B, D, F) and inhibitory (B, D, E, H) tetanic manifestations of spike activity accompanied by posttetanic excitatory (A, B, D, E) and inhibitory (F, H) effects in spinal cord MN to HFS of Gi (A, B, D, E) and Pi (B, F, G, H) nerves in norm (A-E) and at the 70th day after the SN crush (A-D). The ratio of pre- and poststimulus levels of spike activity frequency to HFS of Gi nerve on the 70th day of SN crush revealed TP within a 4.1-fold excess of the initial level and TD - 10.4-fold underestimation, which in comparison to 5.8-fold 1P and 7.2-fold 1D in norm indicates the absence of approaching the normal level of 1P in pathology and in case of 1D - its excess up to 1.4 times. At the 70th day after the SN crush in response to HFS of Pi nerve there occurred TP with about 4.9-fold increase in the spike activity frequency and TD – with its 4.5-fold decrease, compared with the norm within a 4.9-fold increase and 4.7-fold of its underestimation, respectively. In other words, there was an actual restoration of the level of excitatory and inhibitory processes in the respective MN of the spinal cord to the activation of extensor nerve. Finally, Figure 4 represents the mentioned changes in the average frequency of MN’s activity to HFS of G (A, C) and P (B, D), excitatory (A, B) and depressive (C, D) origin in a comparative angle on multiple (how many times the levels of prestimulus and tetanic frequencies differ) and percentage for the norm and in 5-70 days after the SC crush

**DISCUSSION**

For a successful recovery of the nerve the earlier proliferation of SC is required and differentiations are critically needed for successful nerve regeneration [Macica C. et al., 2006]. De Ruiter and co-authors have shown that during the crush of peripheral nerve more distant from the damaged part the number of myelinated axons greatly increases, and, the change of direction of the regenerating motor axons serves as a serious factor counteracting to its optimum recovery [de Ruiter G. et al., 2008]. According to the results of this study, in confirmation to data of mentioned authors from the proximal and distal parts of the damaged PN, the thin myelinated fibers can be traced since day 13. As for the transverse nerve fibers, they are detected at the 5th and 13th days; at the 25th day only in few sites of the distal portion the empty-crossed Schwann tubes are observed. In addition, to this term in the median section of injury there is a weak proliferation of SC and endoneurium. Up to the 35th day after surgery we observed only the thin myelinated fibers. At the same time, in such conditions other authors showed an increase in number and diameter of large myelinated fibers (up to 80% of control level) [Fugleholm K. et al., 2000]. In turn, along with long-term re-enervation at the 9th month after the PN section in mature cats a significantly increased proportion of slow muscle fibers was discovered as compared with the fast ones [Foehring R. et al., 1986]. Comparative histomorphometric evaluation of the restoration of mice damaged PN at 2-4 and 6th week after the operation led to the conclusion on significant normalization of the nerve length at the 14th day and full recovery in 28 days [George L. et al., 2003]. According to other authors, despite the complete paralysis of the nerve within a day after the operation, on day 14 the nerve becomes completely anomalous and histologically only limited features of Wallerian’s degeneration is identified [Omura T. et al., 2004].

In the present study at day 35, the recovery of enzyme activity was determined only in the extensor bundle. It should be anticipated that the complete restoration of flexor bundle requires a longer period. However, our data indicate that at the 70th day after crushing the flexor and extensor bundles are already separated by a broad layer of connective tissue. Probably, not the last role belongs to proliferation of endo- and perineurium elements that promote the growth of collagen fibers; as a result, nerve trunks can turn into a bundle of connective tissue fibers.

The results of this study agree with the need to use protectors in order to achieve accelerated and successful recovery [Macica C. et al., 2006]. Therefore, we should agree with G. Stoll and H. Müller that a full recovery requires longer periods with pharmacological intervention [Stoll G., Müller H., 1999].

It is important to note that in a few animals in 70 days after the compression lateral collaterals of nerve fibers were not identified at the site of injury, and there were observed only signs of restored nerve. Data suggest greater probability of rapid recovery, depending on the least deviation of the fibers. In conclusion, it is interesting that at the 7-144th day after transection of the SN levels of brain derived neurotrophic factor (BDNF) mRNA sharply elevated, in contrast to other types of injuries, including crushing, at which the changes were weaker [Omura T. et al., 2004].
et al., 2004]. Nevertheless, a comparison of weekly electrophysiological and functional data for 100 or 150 days after the crush or section of SN showed that recovery was faster and more complete in nerves regenerating after the crush, but not the section [Wolthers M. et al., 2005]. Moreover, through the control cat tibial nerve regeneration, particularly after the crush (up to 140 days), data was received in favor of the almost identical levels of rapid growth and maturation during regeneration of both motor and sensory myelinated fibers [Moldovan M. et al., 2006].

Only in presence of sensory input there was revealed the formation of regenerated axons distal to crush and an increase in number of motor axons innervating extrafusal fibers (extensor digitorum longus) proximal to the fracture. In other words, the ability of sensory synaptic input to motor neurons was demonstrated to affect the intermuscular sprouting [Cuppini R. et al., 2002].

Overall, the results of electrophysiological studies confirmed the morphohistochemical data. It was shown that there is a progressive increase of excitatory poststimulus reactions not reaching the norm for the flexor nerve, in contrast to the extensor, and in relation to depressive effects, almost twice elevating the normal levels and more pronounced for the extensor nerve. Deepening the depressor responses is apparently a consequence of their nomination as a protector, as evidenced by published data. It is of interest to estimate correlations of expressiveness and dynamics of the growth time of the aforementioned depressive and excitatory responses in terms of the successful protective effect. As known, in some brain structures during development of the nervous system GABA acts as a trophic factor that influences proliferation, migration, differentiation, maturation of

**Figure 4.** Comparative disc diagrams of differences for the excitatory (TP - A, B) and depressor (TD - C, D) reactions in MN’s of spinal cord to HFS of Gl (A, C) and Pi (B, D) for multiple and percentage rate of averaged prestimulus frequency level of activity (BE), followed by poststimulus tetanic effect (TT) in the norm and 5 -70 days after crushing of SN (AD). The remaining notations are in the Figure.
synapses, cell death and receptor expression of GABA (A) [Owens D., Kriegstein A., 2002]. Modern studies on the cellular and network levels show that synaptic inhibition cannot be assessed only as opposing to synaptic excitation and additionally serves as highly specific function in the nervous system of mammals [Birke G., Draguhn A., 2010]. According to our data, depressor responses in various brain regions are more intensely involved in both non-specific (peripheral and central) and specific neurodegenerations [Sarkissian J. et al., 2007; Galoyan A. et al., 2008; 2010]. Thus, if we consider depression as supporting the protective burden, according to our and published data, despite the slightly better performance in the rehabilitation of the damaged extensor nerve, at the 35th day depression becomes weaken in both nerves to values below those at norm and at the 70th day higher than those of norm. In other words, the nerves exposed to crushing are unable to recover without pharmacological intervention.

Thus, our data suggest that successful and timely proliferation of SC requires pharmacological protection at the initial stage of axon regeneration that might facilitate neuroregeneration of peripheral nerve.

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