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PROTECTIVE EFFECT OF NAJA NAJA OXIANA COBRA VENOM IN ROTENONE-INDUCED MODEL OF PARKINSON'S DISEASE: ELECTROPHYSIOLOGICAL AND HISTOCHEMICAL ANALYSIS

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Abstract

Parkinson's disease (PD) is the most common movement disorder in the broad spectrum of neurodegenerative diseases frequently associated with gradual decline of the higher mental functions. Great number of PD treatment options has been accumulated to date, but research of novel effective drugs is still necessary. Small doses of different snake venoms possess immunomodulatory-neuroprotective properties and could be beneficial. Our objective was the morphofunctional study of protective action of small doses of Central Asia cobra *Naja naja oxiana* (NOX) venom in rotenone-induced model of PD. Three experimental series (n =22 rats) were carried out with sham-control, placebo control (unilateral medial forebrain bundle injection of rotenone with further i/m administration of saline solution) and treatment (rotenone intoxication with further i/m administration of small doses of NOX venom every other day during 3 weeks). Acute experiments of electrophysiological recording of impulse activity flow frequency changes of *substantia nigra compacta* (SN) single neurons (n = 209), evoked on high frequency stimulation of *nucleus caudatus*, were carried out on postoperative survival of 33-90 days. Activity was recorded as tetanic (TP) and post-tetanic potentiation (PTP) and depression of diverse intensity and duration. Subsequent histochemical investigation of SN frozen sections for detection of Ca^{2+}-dependent acid phosphatase activity was performed. Rotenone induced significant neurodegeneration on SN slices in placebo-control with absence of TP and PTP effects or occasional weak effect in the first trial, disappearing in the subsequent trials. Regular administration of NOX venom led to restoration of morphological picture with intensified vascularisation. Approximation of electrophysiological parameters to normal level was revealed under NOX treatment. The obtained findings suggest that small doses of NOX venom act as a neuroprotective agent, and further research should be carried out to reveal the mechanisms of action, and to propose it as a potential drug for PD treatment.

Keywords: neuronal evoked spike activity, substantia nigra, histochemistry, Parkinson's disease, cobra venom, neuroprotection

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Introduction

Parkinson's disease (PD) affects one in every 100 persons above the age of 65 years, making it the second most common neurodegenerative disease after Alzheimer's disease (*Singh N. et al., 2007*). Movement syndromes in PD are the result of progressive and selective lesion mainly of dopaminergic (DA) neuromelanin-containing neurons of *substantia nigra compacta* (SN), and to a lesser extent of catecholaminergic neurons (*Brooks D., 2004; Fahn S., Sulzer D., 2004*). The "convergent model" of cognitive dysfunction in PD is related to the critical synaptic balance of dopamine and acetylcholine (ACh) ratio (*Calabresi P. et al., 2006*). Motor symptoms are sequent of failure of this balance, accompanied with decrease of DA activation and domination of that cholinergic. The forms of long synaptic potentiation and depression provide the associated activation of DA and ACh receptors. Abnormalities in their interaction cause cognitive disorders, violating the physiological induction of synaptic plasticity (*Calabresi P. et al., 2006; Picconi B. et al., 2005*). Recently Roach (*Roach E., 2007*) has published a detailed review of pre- and post-synaptic dysfunctions in PD. Two arguments are presented in favour of involvement of the decrease of DA neurons and postsynaptic shifts in different stages of PD based on the secondarity of motor complications pathophysiology (*Linazasoro G., 2007*) and their presynaptic mechanisms (*de la Fuente-Fernandez R., 2004, 2007*). Specific processes, including oxidative stress sources (dopamine hyperturnover, lowered levels of reduced glutathione, raised level of iron, presence of neuromelanin, as well as dyscrasia of calcium and "excitotoxicity"), trigger acceleration of death of DA neurons of SN (*Lang A., 2007*). The combined therapy aimed to both deceleration of DA neurons death and exclusion of non-DA symptoms progressing (developing in terminal stages of PD) could be effective (*Lang A., 2007*). Hence, longterm utilization of symptomatic means causes development of complications (*Lebel M. et al., 2007*). Moreover, Lebel M. et al. (2007) have revealed recently neuronal toxicity of elevated synaptic levels of DA-receptors, which are responsible for prolonged neuronal dysfunction and degenerative processes.

The leading molecular pathways and pathogenetic mechanisms of sporadic and family forms of PD are the deficit of mitochondrial function, oxidative stress, accumulation and dysfunction of aberrant proteins (*Gandhi S., Wood N., 2005; Moore D. et al., 2005*).

Neuroinflammatory cascade and gliosis play significant role in PD pathogenesis (*Fahn S., Sulzer D., 2004; Mosley R. et al., 2006*). Microglia serves as innate immune system of CNS and produces neuroinflammatory mediators (*Mosley R. et al., 2006; Streit W. et al., 2004*). It has neurotoxic potential and promotes neuronal injury and death especially in SN, which is comparatively rich in microglia (*Whitton P., 2007*). Croisier E. et al. (2005) have confirmed responsibility of microglia for α-synuclein deposition in Levy's corpuscles and dystrophic neuritis, over-accumulation of which can cause development of PD (*Fortin D., et al. 2005*). However, they excluded the role of α-synuclein in neuronal decrease in PD (*Croisier E. et al., 2005*). At the same time, over-expressed α-synuclein is wedge in important phases of neuronal membranous transport and selectively blocks the transport from endoplasmic reticulum to Golgi apparatus (*Chua C., Tang B., 2006*) causing progressive degeneration. Moreover, α-synuclein accumulates everywhere in CNS causing other behavioral disorders and dementia in addition to Parkinsonism (*Lee V., Trojanowski I., 2006*). Finally, astrocytes play a vital part in synaptic plasticity maintenance by means of prevention of glutamate toxicity and providing compensation of dopamine deficit in striate neurons (*Dervan A. et al., 2004*). Recent investigations focus upon contribution of non-genetic or ecological factors to the development of sporadic form of PD. Among these factors, pesticides' impact strongly correlates with increase of probability of Parkinsonism: e.g., chronic administration of herbicide Rotenone in rats induces Parkinson's like disease (*Hanan M. et al., 2004*).

Our research group has previously obtained encouraging data on neuroprotective effects of small doses of different snakes' venoms (Central Asia cobra - *Naja naja oxiana*; Armenian viper *Vipera Raddei*; gjurza - *Vipera lebetina obtusa*) in models of central and peripheral non-specific neurodegeneration (*Abrahamyan D., 2005; 2006; Abrahamyan S. et al., 2007; Chavushyan V., 2006; Chavushyan V. et al., 2006; Sarkissian J. et al., 2006a; b*). This allowed us to test their action in models of specific neurodegeneration (Alzheimer's disease, PD).

The present study is an attempt of morpho-functional investigation of protective action of small doses of Central Asia cobra *Naja naja oxiana* (NOX) venom in Rotenone-induced model of PD.

Materials and Methods

Experimental series: Experiments were carried out in three series in 22 adult Albino rats (200-250*g*):

I. sham-operated (sham control), 16 rats injected with sterile distilled water in combination with i/m administration of saline solution;

II. unilateral injection of Rotenone (placebo control, 12μg in 0.5μl Dimexide, speed 0.1μl/*min;* 4 rats) into the medial forebrain bundle as per coordinates (AP+0.2, L±1.8, DV 8*mm*) of stereotaxic atlas (*Paxinos G., Watson C., 2005*) with i/m administration of saline solution every other day during 3 weeks;

III. unilateral injection of Rotenone in combination with *i/m* administration of small doses of NOX venom (5% of LD_{50}= 1*mg/kg*) every other day during 3 weeks (2 rats). The surgery was performed under pentobarbital narcosis (40*mg/kg i/p*). Animals were kept in similar conditions during the entire postoperative period before acute (electrophysiological) experiment.

Electrophysiological recording: Electrophysiological investigation (acute experiment) was carried out on isolated brain preparations in paralyzed (ditillin, 25*mg/kg i/p*) rats after spinal cord transection by ophthalmic knife at T_2-T_3 level under novocain. After affixing the skull in the stereotaxic apparatus the cranial bones were removed from bregma to lambda and *dura mater* was separated. The electrical spike activity of SN neurons was recorded in coordinates of the same atlas (SNc AP-5.0, L±2.0 and DV+7.5-8.0 *mm*, SNr AP-5.2, L±2.0-3.0 and DV+7.5-8.5 *mm*, SNl AP-5.0, L±3.0 and DV+7.0 *mm*) by means of glass microelectrodes (tip Ø =1-2 μm), filled with 2*M* saline solution. Ipsilateral *nucleus caudatus* (NC, *caudate putamen*) was stimulated with the tungsten bipolar electrodes (coordinates AP+1.7, L±2.0 and DV+4.0 *mm*) by single rectangular impulses (current duration 0.5 *ms*, frequency 50 and 100 *Hz* for 1 *sec*). The activity was expressed as tetanic potentiation (TP) with subsequent post-tetanic manifestations in the form of post-tetanic potentiation (PTP) and depression (PTD). Multiple trials at maximal frequency of NC stimulation (100 *Hz*, 1 *sec*) were performed in order to detect the dynamics of early manifestations of neuromediation deficiency and to estimate the protective action of NOX venom in restoration of the normal level of neuronal TP, PTP and PTD. The software analysis developed by our laboratory (Laboratory of CNS Functions' Compensation Physiology) enabled the extraction of artifacts during TP, which let mainly consider it as a strongly constant activation parameter in contrast to less stable PTP and PTD effects (even in norm). The analysis of the obtained data under high frequency stimulation was made based on perievent time histograms (PETH) of spike flow in real-time. The recording was carried out with special software ("Spike-Registrator v. 2.0") supporting the "on-line" selection of spikes by means of amplitude discrimination. Averaged PETH, cumulative and frequency histograms were constructed as well. On average, up to 10-15 post-stimulus trials were conducted during one registration. The statistical analysis of the data was performed according to the algorithm, which was specially developed to assess the value of PETH sections.

Histochemical investigation: Corresponding brain sections were fixed in 5% neutral formalin solution prepared on phosphate buffer for 2-3 days.

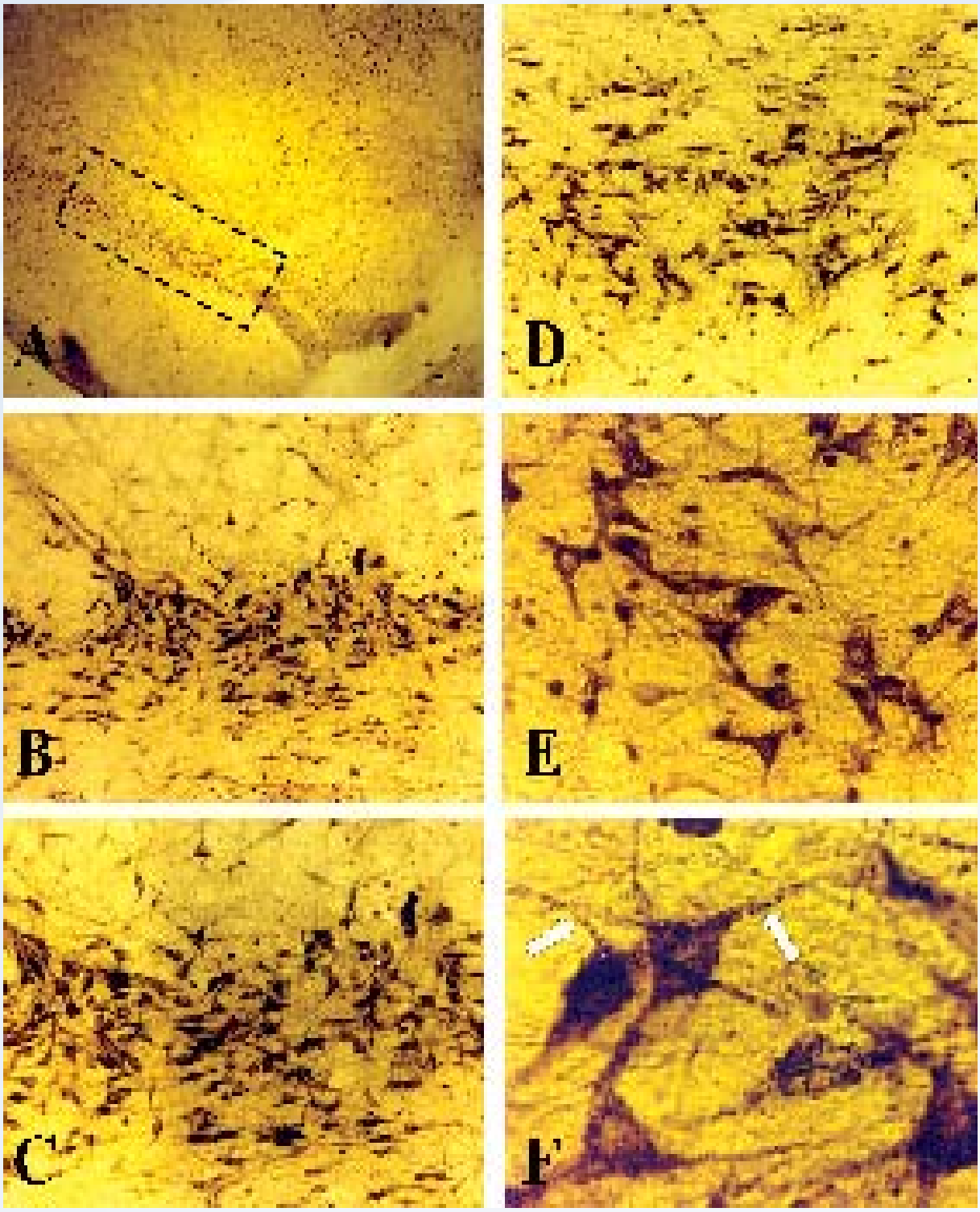


Fig. 1. *Frontal section of the midbrain: detection of Ca^{2+}-dependent acid phosphatase in substantia nigra (SN) of sham-operated rats. A - SN (rectangle); B-F - fragments of A (rectangle) in various magnifications. F - grain of lead deposition in processes (arrows). Oc. x10 (A-F); Ob. x2.5 (A), x10 (B), x16 (C, D), x40 (E), x100 (F).*

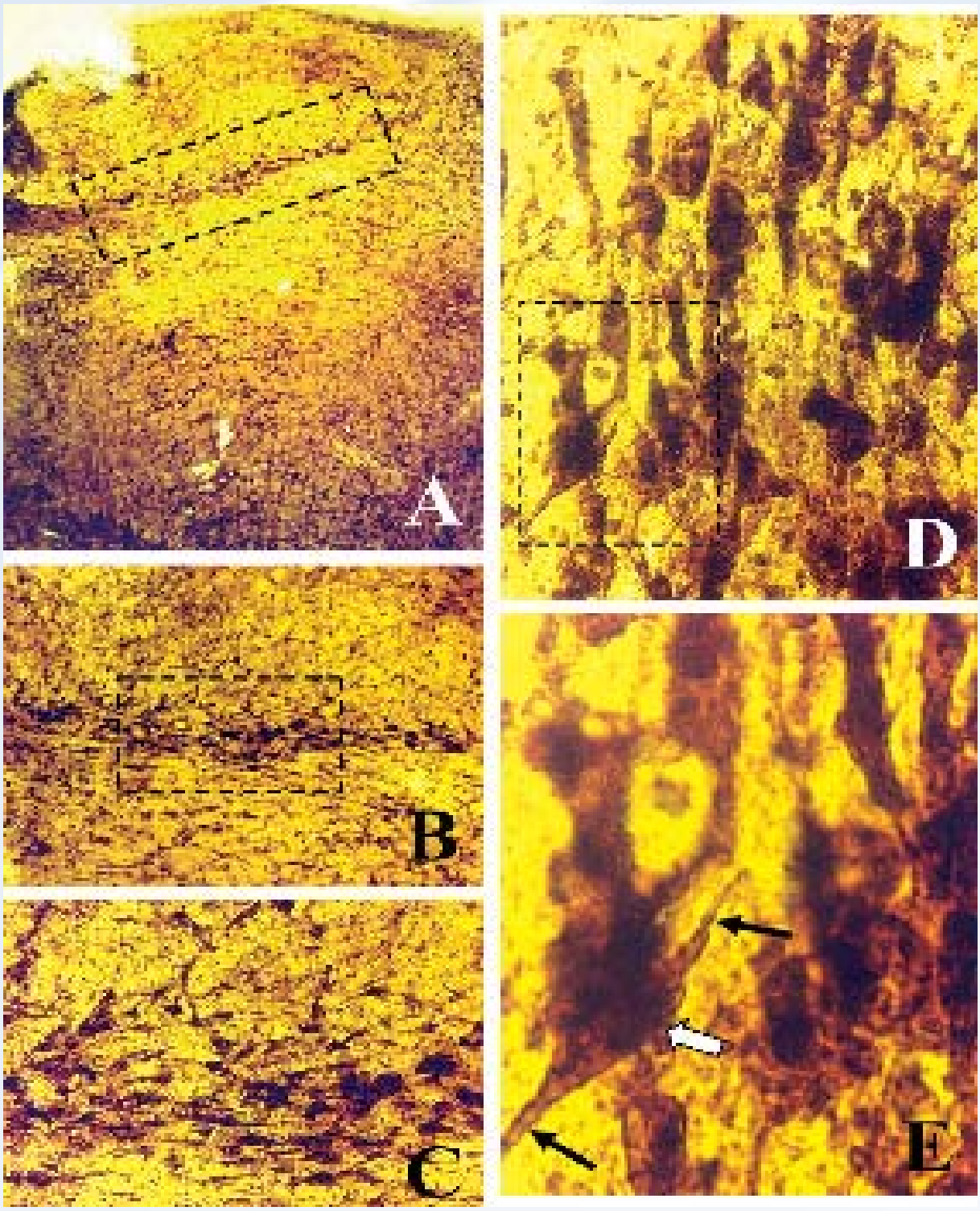


Fig. 2. *Detection of Ca^{2+}-dependent acid phosphatase in SN in conditions of Rotenone intoxication (placebo-control). A, B - SN in different magnifications (rectangle); C-E - fragments of B; E - Neuronal soma (big arrow), processes (small arrows); E - fragment of D (rectangle). Oc. x10 (A-E); Ob. x2.5 (A), x6.3 (B), x10 (C), x40 (D), x100 (E).*

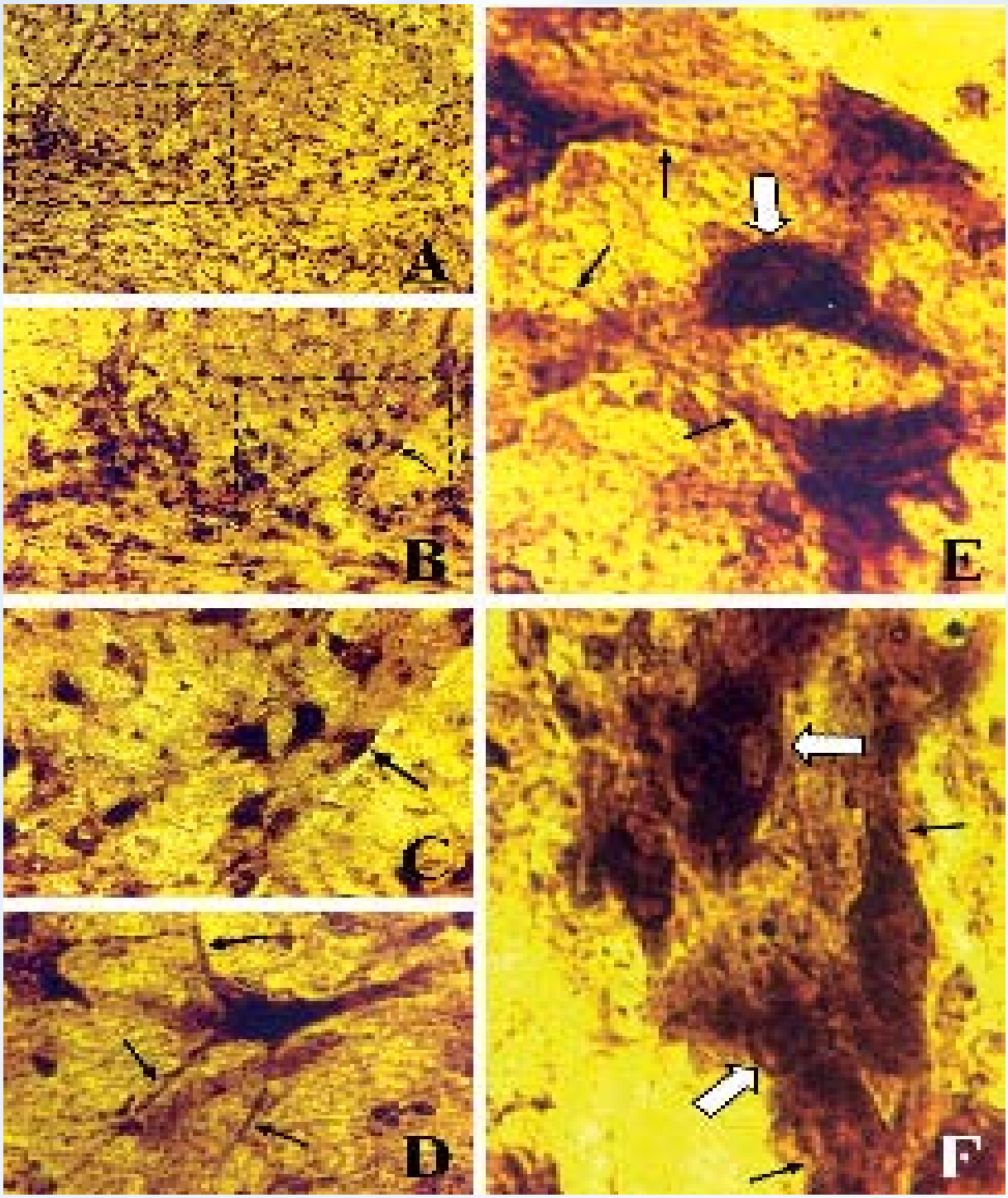


Fig. 3. *Ca^{2+}- dependent acid phosphatase activity in SN in conditions of Rotenone intoxication and regular administration of small doses of NOX venom during 3 weeks. A - SN (rectangle); B - fragment of A: vessel (rectangle), which is presented on C in higher magnification (arrow indicates a pericyte); D-F - darkened nerve cells in the phase of recovery in different magnifications; D, E - neuronal soma (big arrow), processes (small arrows); F - nucleus (big arrows), thickened dendrite (small arrows). Oc. x10 (A-F); Ob. x6.3 (A), x10 (B), x40 (C, D), x100 (E, F)*

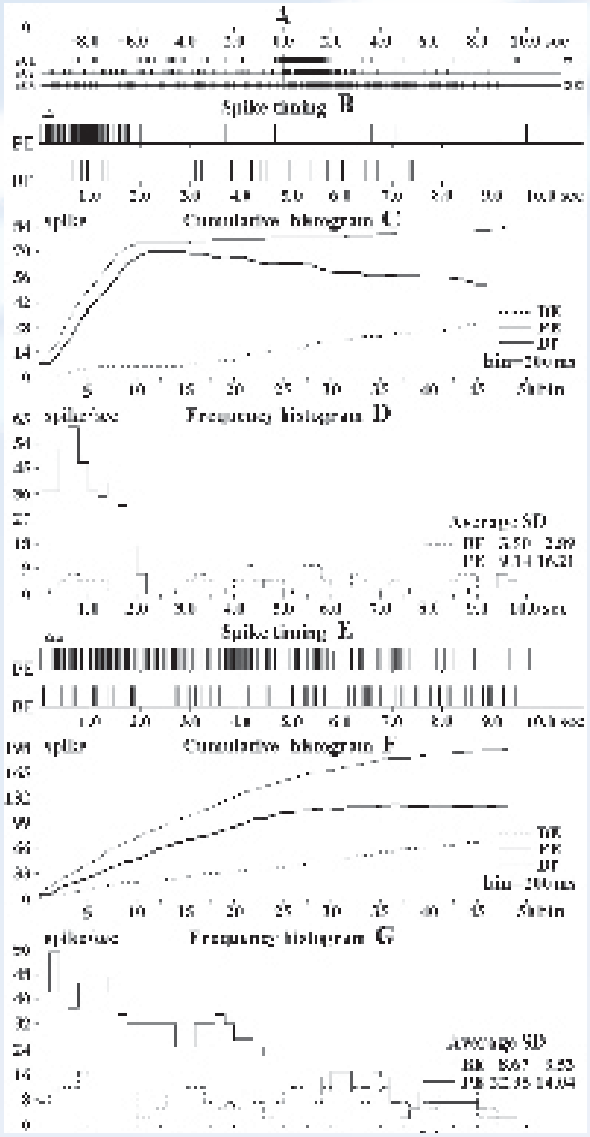


Fig. 4. *Spike activity of a single SN neuron evoked on high-frequency (50Hz - B-D and 100Hz - E-G) stimulation of nucleus caudatus (NC) in norm. A - raster of activity in repetitive trials (n01-n03). Here and on the following figures 5-8: Spike timing full-scaled picture of spikes' real-time distribution before (BE) and post- (PE) stimulation-event; PETH - peri-event time histogram: ordinate - spikes' number, abscissa - time (bins); Cumulative histogram - cumulative histogram of spikes' number before and after stimulation with a curve of difference (Df): ordinate - spikes' number, abscissa - time (bins); Frequency histogram: ordinate spikes' frequency (Hz) before and after stimulation with estimation of average frequency (M ± SD), abscissa - time (sec). Other notation on figure.*

Then frozen frontal SN sections (40-50μm) were treated by the method of detection of Ca^{2+}-dependent acid phosphatase activity developed by I. Meliksetyan (*Meliksetyan I., 2004; 2006*). After rinsing, the slices were further developed in 3% sodium sulphite solution and embedded in Canada balsam.

Results

The morphological investigation by the method for detection of Ca^{2+}-dependent acid phosphatase activity has revealed the following findings.

On cerebral slices of sham-operated rats (I series), the neurons of SN were similar to those in intact rats (Fig. 1). Despite the astonishing variety, the SN cells are mainly of polygonal shape, and located within the bounds of three cellular groups. The cellular nuclei are weakly stained and have stretched shape. The neurons are closely located to each other and intensively stained. In vast majority of neurons the nuclei seem brighter. Characteristic property is the deposition of coarse-grained lead [$Pb_3(PO_4)_2$] sediment in cytoplasm and processes. Meantime, dark zones of neuronal processes evenly alternate with bright ones, creating the pattern of "cross striation" (Fig. 1F). The deposit of $Pb_3(PO_4)_2$ is located in the zone surrounding the nucleus and along processes, which trace at a rather long distance from the soma.

There was significant neurodegeneration on brain slices of animals receiving Rotenone injection and placebo (II series; Fig. 2). The polygonal shape of cells is broken, the clear delimitation of cellular groups is absent (Fig. 2D). The boundary between the soma and processes, as well as between the nucleus and cytoplasm is missing (Fig. 2E). Cut thickenings are seen in place of dendrites, which correspond to the site of their origin from soma. Nuclei of glial cells react ubiquitously. Short, thicken process origins from neuronal somas in certain cases. In such neurons a clumpy deposit of $Pb_3(PO_4)_2$ is irregularly distributed in soma, indicating possible full decay. However, there are cells, which have weakly expressed processes, shape and sheath

with amplified staining intensity. Neurons with homogenous distribution of lead deposit often encounter. Such cells are lacking processes or the processes are thickened, stretched along with somas, giving the impression of thick rough stumps (Fig. 2D). Neurons with individual processes stand out against a background of such degenerating cells, but their nuclei are also intensively stained and at least one of their processes is considerably thickened (Fig. 2E). On cerebral slices of animals receiving Rotenone injection and small doses of NOX venom the polygonal shape of cells was not mainly restored (III series; Fig. 3). Brightly stained nuclei with occasionally visualized thin process are observed in some cells. Processes, cellular membrane, and ectopic nuclei with nucleoli are observed in a number of cases (Fig. 3E). In vast majority of neurons, a thin neurite stretches from soma on rather long distance (Fig. 3D). There are cells with very weak activity of Ca^{2+}-dependent acid phosphatase; still synaptosomes around the sheath and nuclei are noticed (Fig. 3D). The number of cells with accompanying fibres increases. Fine rounded cells begin reacting among large neurons and intensification of vascularisation is evident (Fig. 3C). Dilated vessels strongly react penetrating SN (Fig. 3B, C). Dark pericytes are clearly and intensively stained on vascular walls, which envelope the vessel with their well-detectable processes (Fig. 3B, C). Thus, evident improvement of vascularisation takes place under administration of small doses of NOX venom.

We performed the electrophysiological recording of responses from 209 neurons: I series - 85 cells, II series - 74 cells {20 and 15 cells from injured and uninjured sides of the

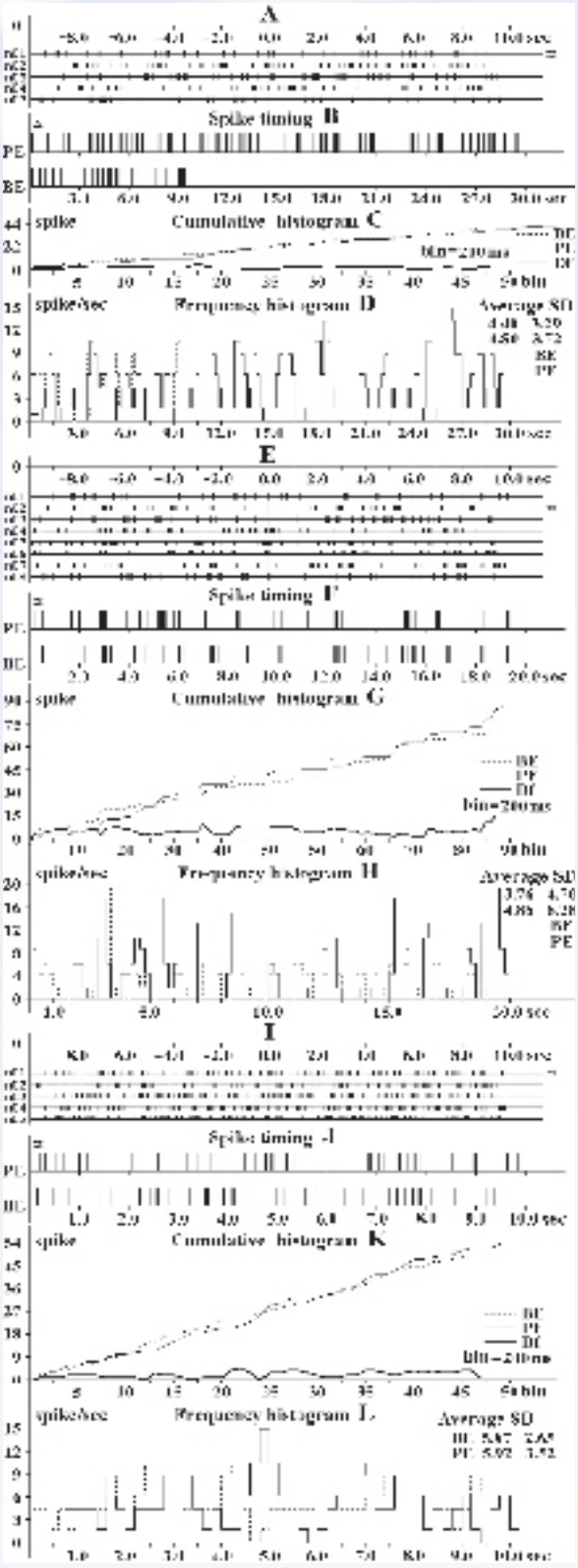
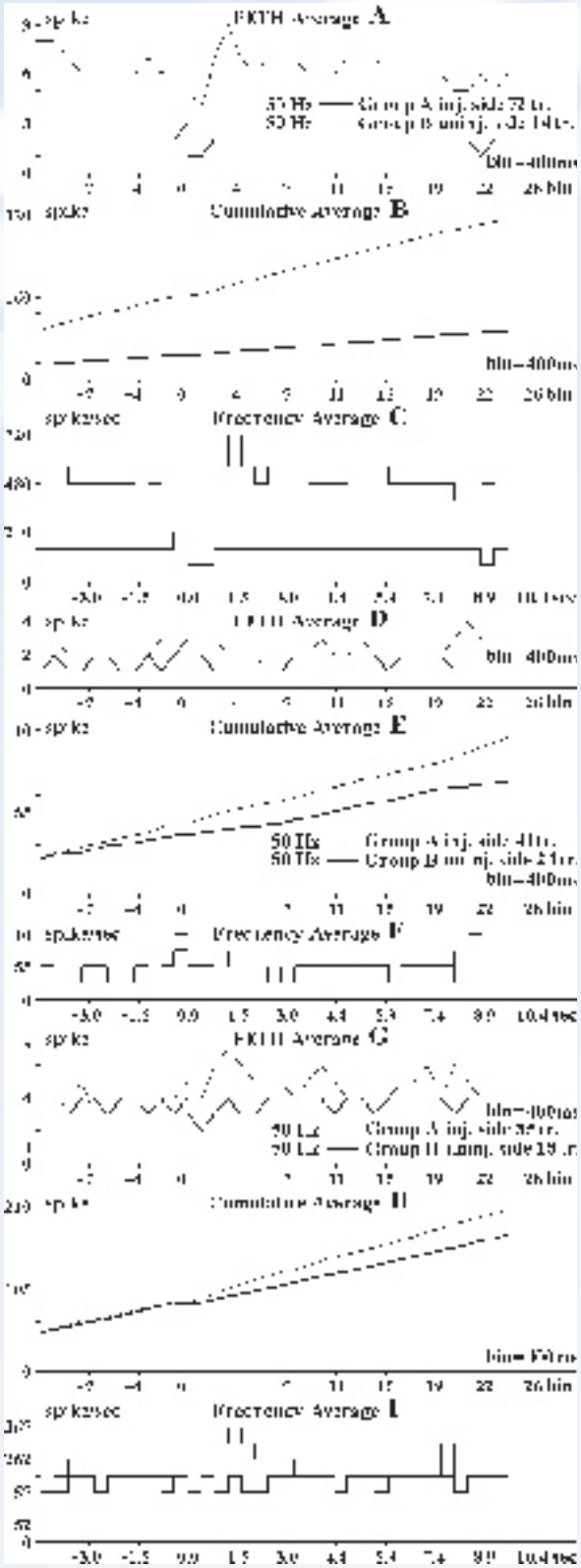


Fig. 5. *Spike activity of three single background-active SN neurons from injured side evoked on high-frequency (50Hz) stimulation of NC in placebo-control on postoperative survival days 33 (A-D), 60 (E-H) and 90 (I-L), with activity raster in repetitive trials (A, E and I, respectively).* ✡*-trials, which are selected from raster for detailed analysis. Other notation on figure*



brain, respectively - 33-34 days of postoperative survival; 10 and 15 cells - 60 days of postoperative survival; 9 and 5 cells - 90 days of postoperative survival}; III series - 50 cells {27 and 23 cells from injured and uninjured sides of the brain, respectively 40-41 days of postoperative survival}. Totally in II and III series we recorded 66 and 58 cells from injured and uninjured cerebral sides, respectively.

Electrophysiological investigation of activation frequency shifts evoked by NC tetanic stimulation in SN neurons of intact rats revealed expressed TP and early PTP on 50 *Hz* stimulation. The 100 *Hz* stimulation evoked intensive cascade of excitatory activity phenomena, including TP and both early and late PTP up to 7-8 *sec* of post-stimulus recording time (Fig. 4). This is evident on averaged histograms of activation parameters of SN neurons in same frequencies of stimulation (Fig. 8D-F).

Electrophysiological investigation of activation frequency shifts evoked by NC tetanic stimulation under conditions of Rotenone intoxication without treatment revealed absence of TP and PTP effects or occasional weak effect in the first trial, which disappears in the subsequent trials. Figure 5 reflects such effects by the example of three SN neurons from injured side in case of 50 *Hz* stimulation on postoperative days 33 (Fig. 5A-D), 60 (Fig. 5E-H), and 90 (I-L). Average meanings of activation parameters from all recorded neurons in identical conditions from injured and uninjured sides confirm the obtained results (Fig. 6).

Under low doses of NOX venom in Rotenone induced model of PD, the electrophysiological data approximate to normal level, i.e. to regular multiple revealing TP of analogous intensity. Fig. 7 demonstrates such approxi-

Fig. 6. Average meanings of activation parameters of recorded neurons from injured (inj.) and uninjured (uninj.) sides of the brain in placebo-control on postoperative survival days 33 (A-C), 60 (D-F), and 90 (G-I). Here and on Fig. 8: PETH Average - averaged perievent time histogram (A, D, G); Cumulative Average - averaged cumulative histogram (B, E, H); Frequency Average - averaged frequency histogram (C, F, I). Other notation on figure.

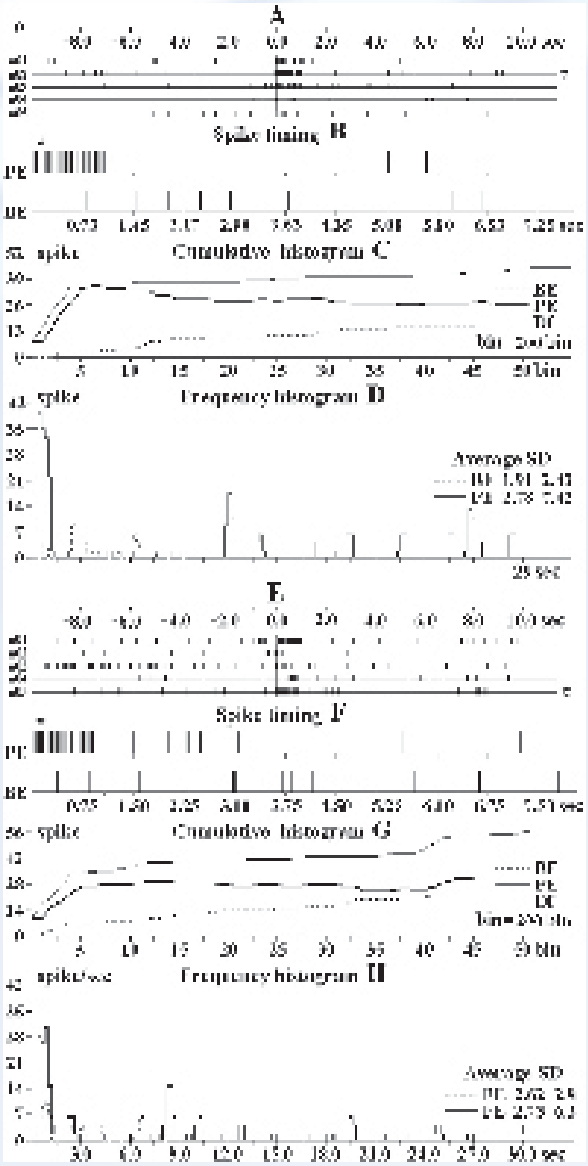


Fig. 7. *Spike activity of two single background-active SN neurons (postoperative survival day 40) from injured side evoked on high-frequency (50Hz) stimulation of NC in conditions of Rotenone intoxication and regular administration of small doses of NOX venom during 3 weeks (A-D and E-H, respectively). A and E- raster of activity of the same neurons in repetitive trials. Other notation on figure*

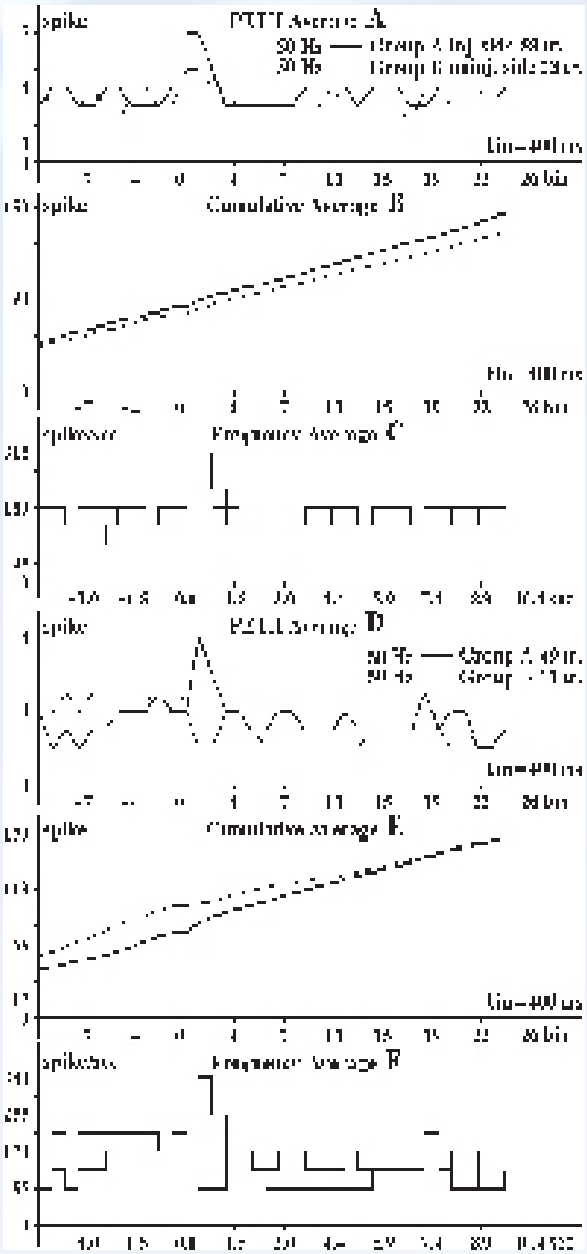


Fig. 8. *Average spike activity of single SN neurons (postoperative survival days 40-41) from injured (Group A inj.) and uninjured (Group B uninj.) sides evoked on high-frequency (50Hz) stimulation of NC in conditions of Rotenone intoxication and regular administration of small doses of NOX venom during 3 weeks (A-C). D-F the same in intact rat on high-frequency (50Hz Group A, 100Hz Group B) stimulation of NC. Other notation on figure.*

mation by the example of two SN neurons, which is confirmed by averaging different activation parameters (Fig. 8 A-C). Thus, the obtained data suggest that small doses of NOX venom act as a protective agent and further research should be carried out to reveal the mechanisms of action and to propose it as a potential drug for the treatment of PD.

Discussion

Analysis of the recent studies on neuroprotective agents, antioxidants, stem cells, vaccines, and different neurosurgical techniques (including high-frequency stimulation of deep cerebral structures) concludes that novel effective therapeutic options are necessary for PD treatment (*Benazzouz A. et al., 1995; Beurrier C. et al., 2001; Garcia L. et al., 2005; Lee K. et al., 2004; Lozano A. et al., 2002; Magarinos-Ascone C. et al., 2002; Singh N. et al., 2007*). Promising strategies are the restoration of MHC class II antigen complex, which is altered by mircoglial expression; down-regulation of oxidative stress; reduction of aggregation of α-synuclein with enhancement of its degradation (*Dawson T., Dawson V., 2005; Fahn S., Sulzer D., 2004*); removal of glutamate-excitotoxicity; application of trophic factors, counteraction to inflammation, and inhibition of apoptosis (*Fahn S., Sulzer D., 2004*). Uncovering pathogenetic mechanisms of neuro-degeneration in PD for the last 6 years has outlined the necessity of combination of the following therapeutic options: MAO inhibition enhancement of mitochondrial, antiapoptotic, antiinflammatory and neurotrophic activities, and inhibition of protein aggregation (*Bonuccelli U., Del Dotto P., 2006*). Another target is the prevention of non-aggregated α-synuclein accumulation due to the age-related decrease of activity of tyrosine hydroxylase (a DA production limiting enzyme) (*Chu Y., Kordower I., 2007*). The novel strategy of pharmacological intervention envisages application of non-DA means, including NMDA- or AMPA-antagonists or means, acting on 5-OHDA (5-hydroxydopamine) receptors α-adrenergic, A2A-adenosine and cannabinoid (*Bonuccelli U., Del Dotto P., 2006; Kalda A. et al., 2006; Lastres-Becker I., Fernandez-Ruiz I., 2006*). Future strategies will also focus on pre- and post-synaptic components, which regulate basal ganglia neurons discharge pattern; proteins of synaptic vesicles; means of influence on signal transduction systems, modulating NMDA-receptors phosphorylation (*Bonuccelli U., Del Dotto P., 2006; Konitsiotis S., 2005*).

We believe that the therapeutic approach proposed by us, used separately or combined with other drugs, may provide long-term and stable action, as different components (toxins, enzymes, etc.) of snake venoms possess high selective specificity and irreversibility of effects.

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