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Effects of bacterial melanin on motor recovery and regeneration after unilateral destruction of Substantia Nigra pars compacta in rats

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ABSTRACT

We examined the potential neuroprotective action of bacterial melanin (BM) in rats after unilateral destruction of Substantia Nigra pars compacta (SNc) dopaminergic neurons. 24 rats were initially trained to an instrumental conditioned reflex (ICR) and then subjected to unilateral electrolytic destruction of SNc. Unilateral deficit in balancing hindlimb movements was observed in all rats after the destruction. On the next day after the destruction part of the animals ($n = 12$) was intramuscularly injected with BM solution at the concentration 6 mg/ml (0.17 g/kg). The other 12 operated rats served as a control group. On the second day after the operation the testing of instrumental conditioned reflex was resumed in both groups.

Comparison of recovery periods for the ICR in both groups showed that recovery of the reflex and balancing hindlimb movements in melanin treated rats took place in three postoperative testing days, whereas in control group the recovery was not complete after 23 testing days. Electrophysiological study was conducted in 12 intact rats to show the effects of BM on the activity of SNc neurons. The firing rate of neurons was significantly increased by the BM injection.

Morpho-histochemical study of brain sections was conducted after the completion of behavioral experiments. In melanin injected rats the study revealed absence of destruction or electrode trace in Substantia Nigra pars compacta of melanin injected rats. BM stimulates regeneration and microcirculation in SNc. Increased electrical activity of SN neurons and regenerative efforts induced by BM accelerate motor recovery after unilateral SNc destruction.

1. Introduction

Research publications of key researchers in the field of neurobiology consider in detail the mechanisms of axon regeneration in mammalian central nervous system (Brosamle and Schwab, 1996), regeneration in the spinal cord (Bregman, 1998), formation of glial cicatrix (Fawcett and Asher, 1999), neuroglia activation in the damaged brain (Raivich et al., 1996), strategies to assist and maintain axonal regeneration (McKerracher, 2001). These reviews also dwell upon the possibilities of application of physiologically active compounds regulating cascade of processes involved in nervous tissue regeneration and enhancement of this process.

Number of neurodegenerative disorders, including Parkinson's disease, are due to lesions of brain nigrostriatal system that takes part in the regulation and control of motor functions (Gevorkyan et al., 2007b; Sarkissian et al., 2007). Brain dopamine is produced in several areas of the brain, including the Substantia Nigra (Eisenhofer et al., 2004). It is produced in dopaminergic neurons of Substantia Nigra pars compacta (SNc). Lesions of this system result in decrease or insufficiency of this substance, which is also typical of neurodegenerative disorders, including Parkinson's disease (Fanardjian et al., 2001, Khudoerkov et al., 2007). Vast numbers of morphofunctional connections have been revealed, that link Substantia Nigra with various structures of central nervous system (CNS), including the striatum and sensorimotor cortex (Gevorkyan et al., 2007b). Substantia Nigra functionally not only controls the movements, but also regulates motor functions in higher levels of brain integration, that are responsible for adaptation of animals in changeable environmental conditions (Proctor, 1989). The activity of midbrain dopaminergic (DA) neurons is modified by Locus Coeruleus (LC) (Raewskij, 1998). Loss of LC neurons results in altered activity of dopaminergic neurons (Guiard et al., 2008; Wang et al., 2010).

In the present study neuroprotective action of bacterial melanin is tested after SNc lesion. The most prominent function of the pars compacta is motor control (Hodge and Butcher, 1980). Pars

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compacta is heavily involved in learned responses to stimuli. In primates, dopaminergic neuron activity increases in the nigrostriatal pathway when a new stimulus is presented, but it decreases with repeated stimulus presentation (Ljungberg et al., 1992). However, behaviourally significant stimulus presentation (such as classical conditioning) continues to activate the dopaminergic neurons. In addition, the pars compacta is important in spatial learning, the observations about one's environment and location in space. Lesions in the pars compacta lead to learning deficits in repeating identical movements (Da Cunha et al., 2006). The pars compacta is activated during time reproduction and lesions in the pars compacta lead to temporal deficits (Matell and Meck, 2000).

For the last decade the efforts of researchers to manage the neurodegenerative disorders have targeted the problem of activity regulation in dopaminergic neurons. The goal has been not only the identification of underlying mechanisms of altered activity in such neurons, but also the routes of activity regulation and methods, used to prevent changes in neuronal activity of patients with neurodegeneration.

Treatment options for the neurodegenerative disorders have provided clinicians with a number of neuroprotective agents, with different structure and different mechanism of action. Many of the offered options are aimed to support the cell survival, accelerate posttraumatic recovery of CNS functions.

Numbers of studies have shown neuroprotective action of melanocyte-stimulating-hormone on locomotor recovery following CNS lesion (Aronsson et al., 2006; Chen et al., 2008; Bharne et al., 2011). Currently melanins of various origins are being actively studied and applied as medicinal and cosmetic preparations. Melanins are multicoloured pigments of polymer structure. They are unique transmitters of energy with the properties of amorphous semiconductor. They can absorb the energy and convert it into various types of energy (Proctor, 1989; Sarna, 1992; Zecca et al., 2001). Melanins break free radical chain reactions and accomplish antioxidant protection. These unique abilities of melanin explain its presence in tissues and organs connected with energy transmission, such as skin, retina, inner ear and nervous system. Melanin metabolism disorders can be involved in the etiology of such diseases as Parkinsonism, senile macular degeneration, and senile deafness (Proctor, 1989; Zecca, 2002). This pigment is also relevant to the well-known association between pigmentary abnormalities and deafness (Warrensburg's and Usher's syndromes). The Alzheimer disease and down syndrome were observed to be also accompanied with pathological disorders in melanin metabolism (Mann et al., 1984). The majority of synthetic and natural melanins are insoluble in water that significantly complicates preparation of pharmacological and cosmetic preparations based thereon. Obtaining of low-cost soluble natural melanin can essentially stimulate and speed up application of melanin in medicine, cosmetology and other fields. For the first time melanin-synthesizing strain with high level of pigment synthesis – *Bacillus thuringiensis* was obtained. The ecologically safe technology of biosynthesis, isolation and purification of the bacterial melanin (BM) has been elaborated. High biological activity of melanin was shown both on animals and plants (Fanardjian et al., 2001; Popov, 2003; Azaryan et al., 2004; Gevorkyan et al., 2007a,b; Sarkissian et al., 2007). BM and its metabolites cross the blood–brain barrier. BM shows higher C_{max} after intramuscular (i/m) injection, while a long retention was registered after intraperitoneal (i/p) injection.

In the experiments on laboratory animals (white rats) with brain surgical trauma it was revealed that BM facilitated the recovery of instrumental conditioned reflexes after unilateral ablation of sensorimotor cortex that had caused paresis of limbs. Low doses of BM accelerated the recovery of physiological functions lost because of nervous tissue damage (Gevorkyan et al., 2007b).

The purpose of the present study is to analyze effects of water soluble bacterial melanin on recovery processes of initially trained instrumental conditioned reflex and paralyzed limb movements after unilateral electrolytic destruction of Substantia Nigra pars compacta, and subsequent intramuscular administration of BM solution on the next day following the operation. Various rodent models have been used to study therapeutic effects of neuroprotective agents (Reglödi et al., 2006; Wang et al., 2008; Rampersaud et al., 2012). In all experimental series of our project (destruction of cerebellar nuclei, Substantia Nigra pars compacta, etc.) we have used electrolytic destruction. The same method was used also for the present study. Excitotoxic lesions potentially have advantages over the applied electrolytic destruction, except for the possible chemical interaction and neuropeptide changes. Presented study is part of the experimental research, conducted to show the neuroprotective action of BM after lesions of different CNS structures (sensorimotor cortex, lateral cerebellar nucleus, pyramidal tract, rubrospinal tract) (Meliksetyan, 2007; Petrosyan et al. 2009; Manvelyan et al., 2010; Petrosyanm et al., 2012).

The selected concentration (6 mg/ml, calculated as 170 mg/kg) has been successfully applied in previous series of experiments, to induce fast recovery of hampered motor functions in rats after destruction of various CNS structures, responsible for motor behavior (Meliksetyan, 2007; Manvelyan et al., 2010; Petrosyanm et al., 2012). Application of bacterial melanin in the animal model with destruction of SNc is of greater interest, as SN dopaminergic neurons are known to contain high levels of melanin.

2. Materials and methods

2.1. Animals

Experimentally-naive 36 mongrel male rats, bred and housed in the Institute of Physiology NAS, were used for the study. Rats were 3–6 months of age, weighing 180–250 g of age at the start of experiment. Rats were housed with their littermates in plastic boxes covered by a wire lid. Animals were maintained on a standard light–dark cycle with food and water available ad libitum. Animals were maintained and handled in accordance with institutional guidelines and national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 86-23, 1985).

Rats were divided into three groups ($n = 12$). One group of intact rats was used for electrophysiological experiments, to study changes of neuronal activity in SNc after BM application. The other two groups were trained to the instrumental conditioned reflex and then subjected to unilateral destruction of SNc. After the destruction one group of rats was injected with BM solution and the other 12 animals served as a control group. All efforts were made to minimize the number of animals used in this study and their suffering.

2.2. Elaboration of instrumental conditioned reflex

The instrumental conditioned reflex (ICR) was developed as follows: rats were trained to balance on a slowly rotating (9 r/min) horizontal bar of diameter 2 cm and length 30 cm, located at a height of 90 cm above a soft pillow. Training to the balancing reflex was assessed in terms of the time spent by the animal on the rotating bar, on which the animal balanced exclusively using the hindpaws, which they alternated. Trials were repeated 10 times daily, with intertrial intervals of 60 s.

The criterion for performance of the reflex was a time spent balancing on the rotating bar for at least 250s. (Kennedy, 1990; Fanardzhyan, 2001).

2.3. Electrophysiological study

Experiments were conducted in 12 mongrel male rats (200–300 gr). Spiking activity of SNc neurons was registered in response to high frequency stimulation (HFS) of caudate-putamen before and after the injection of BM solution (6 mg/ml, 170 mg/kg). Overall 152 neurons were registered, including 90 neurons before the BM injection and 62 after the injection.

Under urethane anesthesia (1.1 gr/kg) all rats were immobilized with intraperitoneal injection of 1% Dithilinum solution (25 mg/kg, i/p), and were kept on mechanical ventilation. Spinal transection was performed on the T2 level using an ultrasonic knife to isolate brain activity. Head of the animal was fixed in stereotaxic apparatus and laminectomy was performed. A steretaxically oriented glass electrode with tip diameter 1–2 μm and filled with 2 M NaCl solution was inserted into SN region to register the firing rate of SNc neurons, in response to single and tetanic stimulation of ipsilateral caudate-putamen (stimulation with rectangular current pulses of 0.5 ms and with frequency 50 Hz for 1 s). The stimulating and registering electrodes were inserted according to stereotaxic atlas coordinates (Gambaryan et al., 1981): SNc (AP − 5, L ± 2, DV + 7.5–8.0 мм); Caudate Putamen (NC) (AP + 1.7, L ± 2–3, DV + 4–5).

Tetanic potentiation (TP) or tetanic depression with post-tetanic effects (post-tetanic potentiation and/or depression of different latency and duration) were registered in response to caudate-putamen stimulation. These effects were evoked in 20–30 subsequent testings of a single neuron.

90 neurons of intact rats were tested using the same parameters of stimulation, and registering spiking activity in response to tetanic stimulation. Sustained monitoring (for several hours) of post-effects was conducted, registering duration, intensity and dynamics of post-stimulation effects, to confirm the unidirectionality of responses. Response properties of neurons were also studied before the BM injection to ensure the validity of testing results.

The online registration and analysis of spiking activity was conducted using a special program (Chavushyan et al., 2013) to select spikes, based on amplitude discrimination, evaluating tetanic and posttetanic activity. Peri-stimulation cumulative histograms were built programmatically (PETH – Peri-Event Time Histogram). Data processing was carried out according to a special algorithm for the analysis of PETH intervals. Analysis of the data obtained in response to tetanic stimulation was based on sliding frequency charts. The firing rate of neurons for 200 ms was estimated, with a shift of 50 ms. The same approach was used to calculate background activity rate.

2.4. Surgery

The animals were anesthetized with Nembutal (40 mg/kg, intraperitoneal). Before starting the operation, an intramuscular injection with 2% Novocainum solution was performed at the area of intervention. Unilateral destruction of Substantia Nigra pars compacta was performed stereotaxically according to coordinates from stereotaxic atlas (Paxinos and Watson, 2005) – $P = 5$ mm, $L = 2$ mm и $V = 8$ mm. In all animals destruction was performed on the left side, with the same parameters of current. Intensity of current was 2 μA, and the destruction time – 20 s.

2.5. Administration of bacterial melanin

Animals of the experimental group were administered with bacterial melanin solution at the concentration of 6 mg/ml (170 mg/kg). Intramuscular injection of BM solution was performed in femoral region on the second day after the operation. In electrophysiological experiments the BM solution was injected intraperitoneally 15 min after the immobilization. The i/p injection was used because of the convenience and ease of performance. The residence time of the substance is significantly longer after i/p injection.

2.6. Morphohistochemical study

After the completion experiments, all experimental animals were anesthetized with Nembutal (45–50 mg/kg). Animals were decapitated under deep anaesthesia, brains were removed and were then fixed in 5% Neutral Buffered Formalin (phosphate buffer (pH = 7.4)). Sections, 50–60 μm thick, were obtained for microscopy. The morphofunctional state of cellular structures in midbrain was assessed by performing histochemical and histoangiological studies. A histoangiological method was used to identify the microcirculatory bed (Chilingaryan, 1986) and a modified histochemical method was used to identify acid phosphatase activity (Meliksetyan, 2007), providing not only a clear morphological picture, but also an assessment of the functional state of the structures.

2.7. Statistical analysis

The significance of differences on recovery periods of the operant conditioned reflex and morphometric data was assessed using Student's t test (Ivanov and Pogorelyuk, 1990).

Stabilized firing rate histograms were compared for data obtained before and after the injection using data processing program developed by V.S. Kamenetsky Student's t-criterion was used for statistic evaluation of differences in peristimulus interspike intervals.

3. Results

3.1. Behavioral experiments

Hemiparesis was observed in control rats after unilateral electrolytic destruction of Substantia Nigra pars compacta. Animals of control group were constantly walking in circles, continually expanding the diameter of circles. After 2–3 laps some rats started spinning in place, while others continued walking in circles. When walking, the rats were putting right paw on the floor with fingers spread, and were raising it higher than the left paw. Tail of the operated rats was not touching the floor, like in normal rats, but stayed well above their body with a curved tip.

In both groups testing of the ICR was resumed on the next day after the destruction. When positioned on the rotating bar, control group rats were not able to keep balance for the whole testing period (250 s), sometimes balancing only with their left hind paw, and not always succeeding. Usually, 20–30 s after being positioned on the bar, the right hindlimb slipped and hanged from it, in front of the bar or behind it.

After 4–5 experiments, the rats began to turn their heads to the right and sat on the bar along its length, clinging onto it by their legs. In 10 experimental days, the hyperkinesias in rats became more expressed, but the animals continued to balance on the bar for the whole testing period, did not sit quietly all the time and periodically changed their position. Unlike normal rats, who kept balance on the bar using their hind limbs, alternating them on the bar while their free front paws hanged down from it, the operated rats were constantly moving their front paws in the air. Sometimes these movements were reminiscent of human movements,

as if trying to pull something with their hands. The most common behavior of rats resembled the movements of a man, sawing wood, as the rats were continually flexing and then extending their forelimbs. After three weeks, during which the specific picture of hyperkinesia was observed, behavior of animals became calm. Starting from 21st postoperative testing day of the study (46 days after surgery), rats were already balancing like intact animals (Fig. 1B).

A completely different picture of the balancing movements and ICR recovery was observed in rats, injected intramuscularly with bacterial melanin solution (6 mg/kg) on the next day after the unilateral destruction of SNc.

In these rats ICR fully recovered in two experimental days, and balancing movements of the right hindlimb fully recovered within 1–3 days after the testing was resumed. The ICR recovered completely (250 s or 100%), and the level of task completion was not changed till the end of the testing period (Fig. 1A).

Interestingly, the movements of rats injected with bacterial melanin, both during the ICR testing, and when moving freely almost did not differ from intact animals. Hyperkinesia observed in control group animals, was not revealed in rats injected with BM solution. Over the entire period of experimentation, starting from the third day, the task (ICR) completion for this group was at one hundred percent, and the balancing movement of the right hindlimb was fully recovered on the average in 2.7 ± 0.3 days. The period of ICR recovery was more than 6 times longer in control rats ($P < 0.01$).

3.2. Electrophysiological study

Analysis of obtained data was carried out taking into consideration the background spike activity of neurons. Common patterns were registered in post-stimulation effects before the BM injection in SNc neurons, depending on the type of neuron and/or level of electrode. Typical, repeated and significantly reproducible effects in a population of similar neurons were observed. Correspondingly, depending on the initial pattern of spike activity, variety of

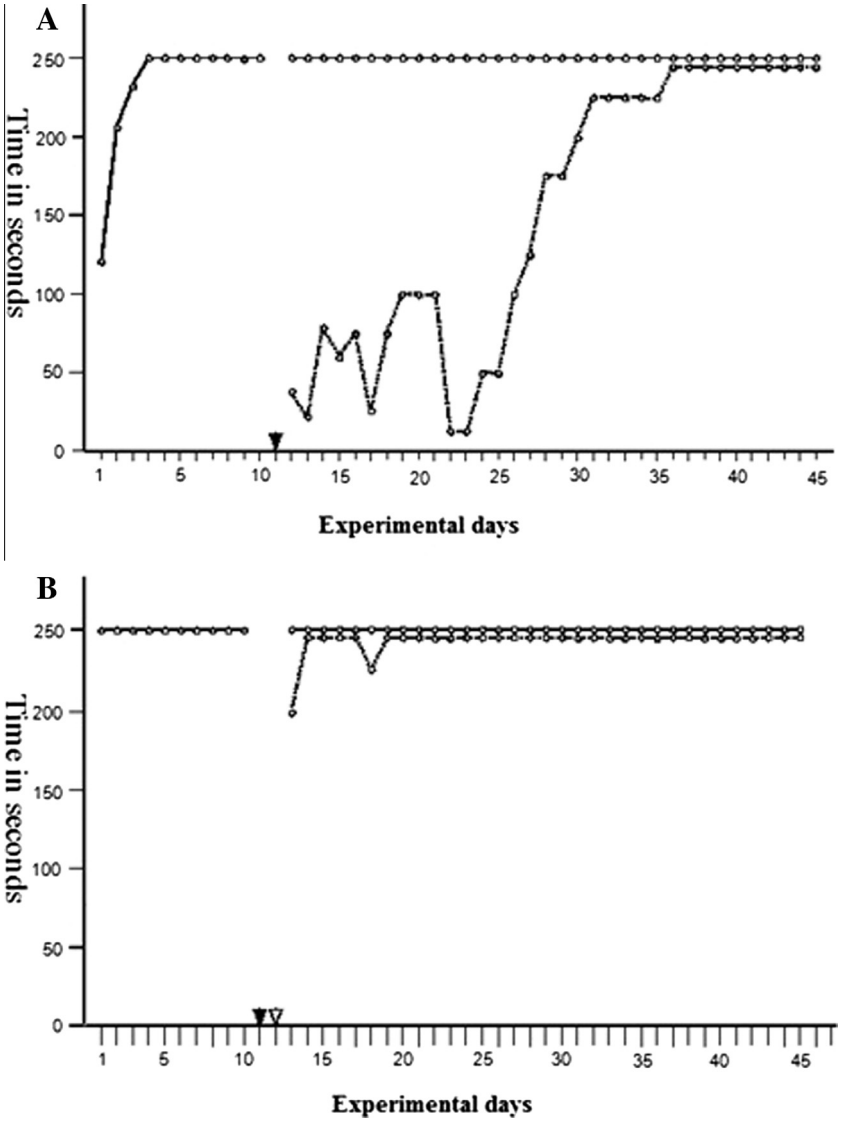

Fig. 1. Effects of electrolytic destruction of Substantia Nigra pars compacta after initial elaboration of ICR in control rats (A) and in experimental rats (B), injected intramuscularly with bacterial melanin solution on the next day following the destruction (6 mg/ml, and the volume of solution was determined by calculation from the optimally tolerated dose of 170 mg/kg). Horizontal axis – experimental days, vertical axis – average time (in seconds) spent by the animal on the rotating bar (testing was repeated 10 times daily). The dotted line (250 s) – is the criterion for reflex completion. Black triangle on the horizontal axis shows the SNc destruction day, and the white triangle indicates – the day of intramuscular injection of BM. The figure demonstrates experimental procedure with average time intervals for the control and bacterial melanin injected groups.

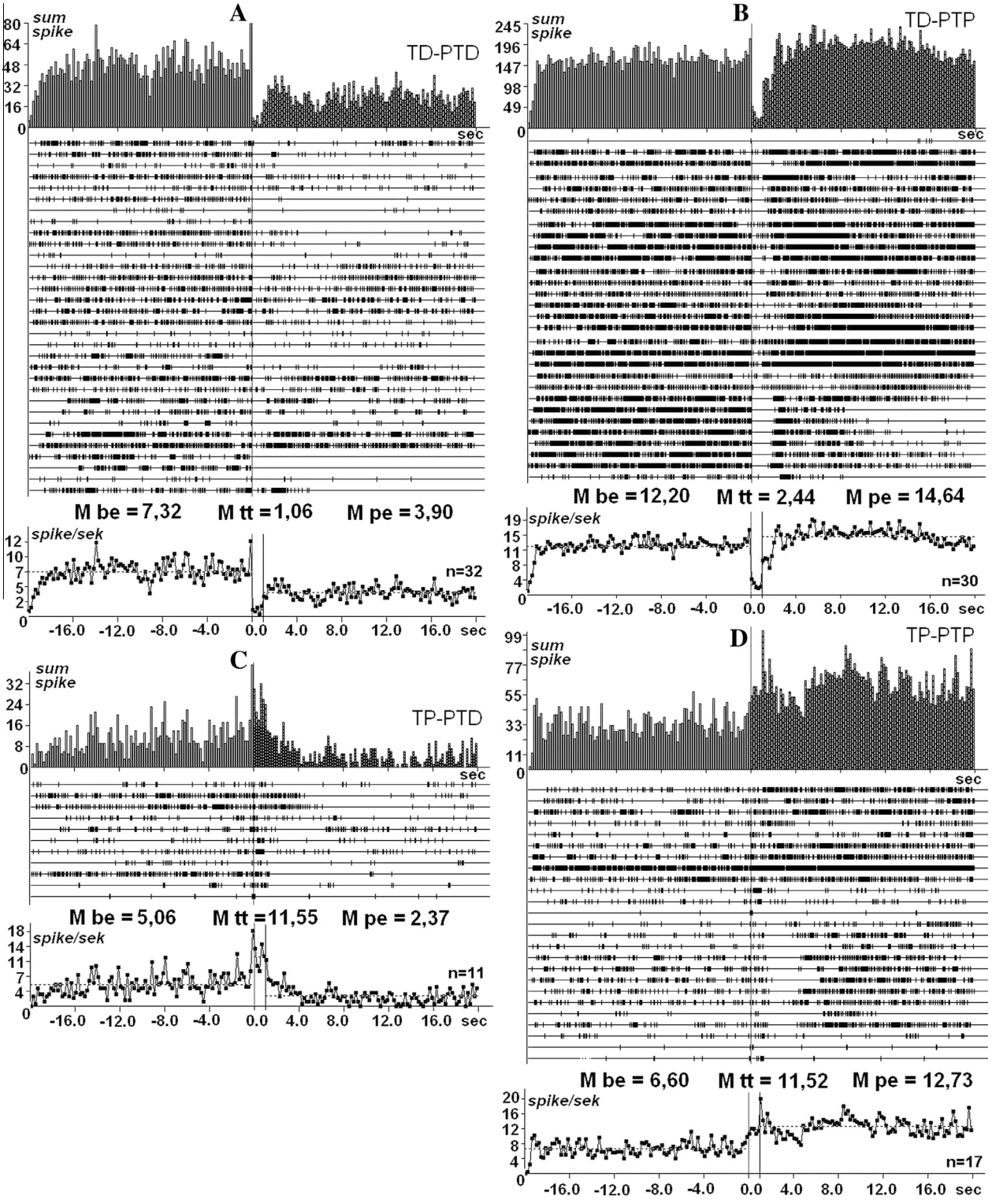

Fig. 2. Peri-stimulation histograms for the sum of spikes based on the analysis of spike activity of Substantia Nigra neurons in the form of inhibitory (A), TD + PTP (B) and TP + PTD (C) or excitatory responses (D), in response to HFS of Caudate putamen before the injection of BM. Diagrams of average spike frequency below the histograms are presented with specified mean values of frequency (spike/s) for each type of neuronal response in real time 20 s before HFS (M_{BE}), 20 s after HFS (M_{PE}), during the HFS for 1 s (M_{TT}); *n* – number of neurons used to calculate the averaged frequency of spiking activity for the diagram.

responses (by intensity and length), including TP, TD, PTP and PTD were registered after BM injection, but with a clearly identifiable directivity pattern of the same reaction type.

Histograms of post-stimulation activity of SN neurons were obtained programmatically for 60 (and longer) minutes in response to stimulation of ipsilateral nucleus caudatus (NCi) after intraperitoneal injection of BM, before the initial firing activity was restored. The BM solution was injected intraperitoneally 15 min after the immobilization.

In neurons of SN, in response to HFS of caudate-putamen, before the injection expressiveness of inhibitory responses increased 6.9 (7.32:1.06 spike/s., Fig. *"SN norm"* A) and 5 times

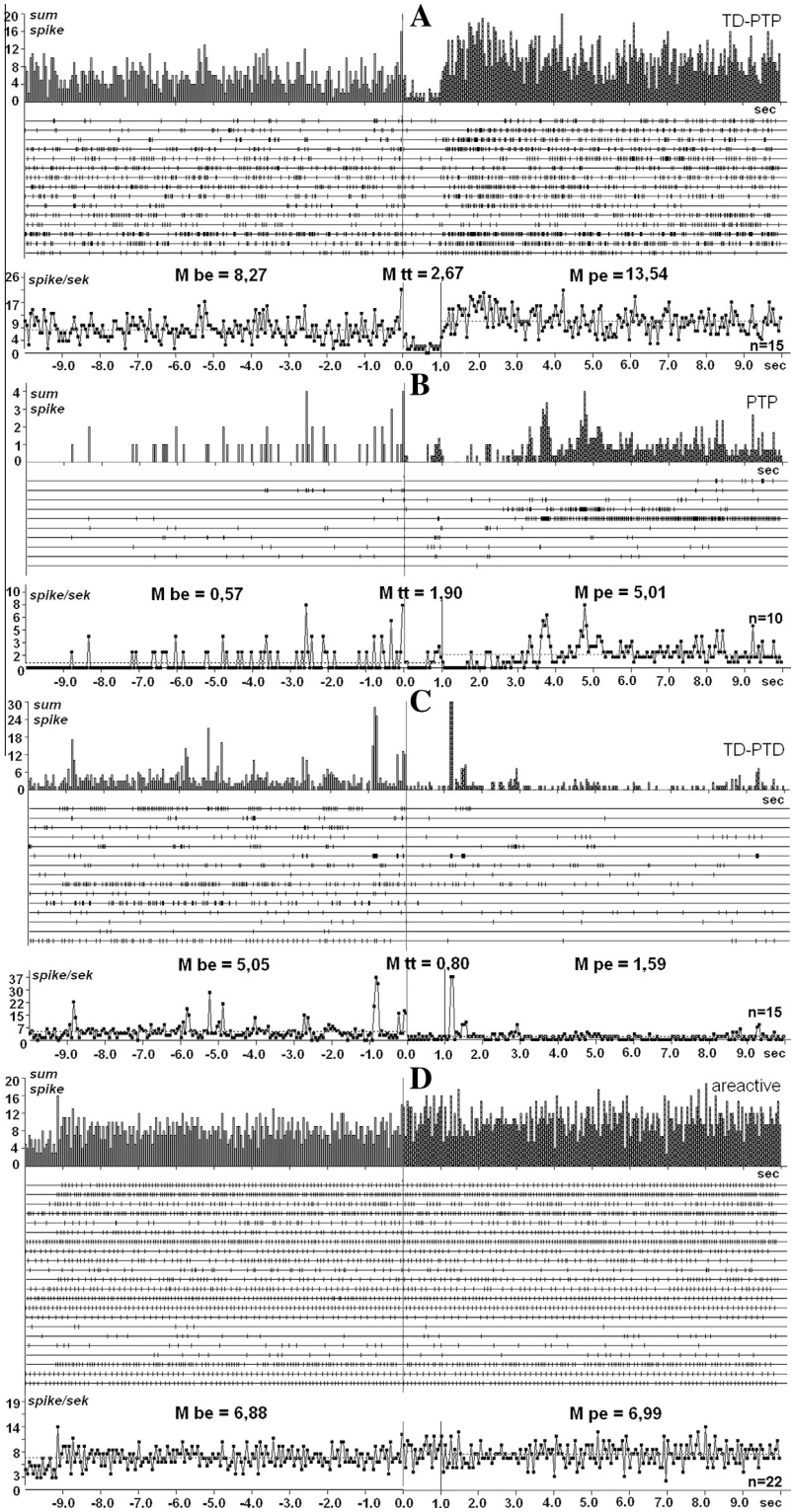

Fig. 3. Peri-stimulation histograms for the sum of spikes based on the analysis of spike activity of Substantia Nigra neurons in the form of TD + PTP (A), excitatory (B), inhibitory (C) responses and areactivity (D), registered in response to HFS of Nucleus Caudatus after the injection of BM. Diagrams of average spike frequency below the histograms are presented with specified mean values of frequency (spike/s.) for each type of neuronal response in real time 10 s before HFS (M_{BE}), 10 s after HFS (M_{PE}) and during the HFS for 1 s (M_{TT}); *n* – number of neurons used to calculate the averaged frequency of spiking activity for the diagram.

(12.20:2.44 spike/s., Fig. 2B) correspondingly. In post-stimulation period the expressiveness of inhibitory effects increased 1.88 (7.32:3.90 spike/s. Fig. 2A) and 2.14 times (5.06:2.37 spike/s. Fig. 2C). During the HFS of Caudate-putamen excitotary effects were increased for 2.28 (11.55:5.06 spike/s., Fig. 2C) and 1.75 times, respectively (11.52:6.60 spike/s., Fig. 2D). For the post-stimulation time period excitatory effects were increased for 1.2 (14.64:12.20 spike/s., Fig. 2B) and 1.93 times, respectively (12.73:6.60 spike/s., Fig. 2D). The proportions of SN neurons with TD-PTD (30 from 90) and TD-PTP (28 from 90) responses is

Table 1
Expressiveness of inhibitory and excitatory effects registerd in response to HFS before and after the BM injection.

	M_{BE}/M_{TT} before the injection	M_{BE}/M_{TT} after the injection
Inhibitory (TD-PTD)	6.9	5.67*
Inhibitory (TD-PTP)	5.0	3.09*
Excitatory (TP-PTD)	2.28	0.97*
Excitatory (TP-PTP)	1.75	0.30*
For the post-effects		
Inhibitory (TD-PTD)	1.88	3.17*
Inhibitory (TP-PTD)	2.14	1.63*
Excitatory (TD-PTP)	1.2	0.61*
Excitatory (TP-PTP)	1.93	0.11*

* $P < 0.01$.

Table 2
Proportions of effects before and after bacterial melanin administration.

Response type	Before the injection (%)	After the injection (%)
TP-PTP	16.70	17.40
TD-PTD	33.30	24.40
TD-PTP	31.10	42.60
TP-PTD	10.30	6.80
Areactivity	8.60	8.80

practically identical (33.30% and 31.10%, respectively). TP-PTD effects were registered in 10.30% (9 from 90) of testings, whereas TP-PTP effects were expressed in 16.70% (15–90) of testings. Areactivity was registered in 8 testings (8.60%) (Fig. 2A–D).

In responses obtained after the injection of BM, increased proportion of tetanic potentiation was registered, compared to the effects registered before the BM application (Fig. 3). The overall percentage of PTP post effects was increased. The following responses were registered in neurons after BM injection: TD-PTP (26 from 62 neurons – 42.60%); TP-PTP (11 from 62 neurons – 17.40%); TD + PTD (15 from 62 neurons – 24.40%), TP-PTD (4 from 62 neurons – 6.80%) and also areactivity (5 from 62 neurons – 8.80%). Frequency of excitatory responses in post stimulation period was increased 1.6 (13.54:8.27) and 8.8 (5.01:0.57) times correspondingly (Fig. 3A and B). TD frequency was increased for 3 times (8.27:2.67) in population of neurons with TD-PTP responses and 6.3 times (5.05:0.80) in neurons with TD-PTD responses (Fig. 3A and C). In areactive neurons unaltered firing rate was registered with maintained level of pre- (M_{be} = 6.88 spike/s) and post stimulation (M_{pe} = 6.99 spike/s) activity (Fig. 3D).

After BM injection expressiveness of inhibitory effects (M_{BE}/M_{TT}) was diminished, whereas expressiveness of the excitatory effects was significantly increased (Table 1). Comparison of mean values for M_{BE}/M_{TT} and M_{BE}/M_{PE} showed increase in the expressiveness of effects for all types of excitatory responses after bacterial melanin injection ($P < 0.05$). Areacvtive neurons were found (see Fig. 3D) in destruction area. Number of areactive responses was not changed after BM injection. Overall proportion of excitatory effects increased after BM injection, whereas the proportion of inhibitory effects was decreased (Table 2).

3.3. Morphohistochemical study

After completion of behavioral experiments a morphohistochemical study of SNc region was conducted in all animals.

The morphohistochemical study of the Substantia Nigra destruction area was performed in obtained midbrain sections of experimental animals. In sections the SNc area was identified between the loop layer and the base, starting from the level of the lower hills, and was characterized by the abundance of pigment in its cells (Fig. 4A).

The cells of this structure have different shapes – triangular, elongated, and polygonal (Fig. 4B). The precipitate of lead phosphate in the form of granules was clearly seen in the cytoplasm and processes of cells. In these structures, particularly in the axons, alternating equidistant bright and dark areas were revealed, making a picture of cross-striation. Presumably, these dark spots correspond to areas of high phosphatase activity. In general, the granularity of sediment in the neurons of the Substantia Nigra is well expressed. In addition, in slices of melanin treated rats, this area is penetrated by blood vessels, indicating expressed vascularization. On the walls of blood vessels dark, homogenously stained pericytes were identified, which contribute to the different aspects of angiogenesis (Ribatti et al., 2011).

In sections of the control animals at the site of the SNc destruction reactive proliferation of glial cell nuclei was observed, with the formation of a dense scar barrier, blocking the course of axons (Fig. 4H). Response or reaction of neurons was not revealed on both sides of the scar.

In the destruction area of midbrain sections obtained from animals, injected with bacterial melanin, the glial scar was not formed (Fig. 4A–D and Fig. 5A, C, E). Moreover, in most cases, the trace of destruction electrode was not identified and the scar was not revealed (Fig. 4D). Nerve cells were revealed at the site of injury (Fig. 4C and D, Fig. 5C and E).

Any specific structural differences compared with intact animals in the nature of the neuronal response were not revealed in sections of melanin injected rats (Fig. 5D and E). In the lesion area chromatolysis of neurons was not observed, and only a slight shortening of processes was revealed. In majority of sections enlarged nuclei of neuroglia were found, surrounding intensely stained neurons with high phosphatase activity.

The increased vascularization was another favorable factor for regeneration. Separate branches of blood vessels of the microcirculatory bed were identified in sections. Conducted morphometry showed 12% increase in capillary diameter ($P < 0.01$).

4. Discussion

Analysis of the study results showed that in control rats after unilateral destruction of the SNc, the recovery of the paralyzed hindlimb movements occurred three weeks later, after ten-fold daily testing of the balancing instrumental reflex.

The injection of bacterial melanin on the next day after surgery was an attempt to eliminate the deficit of the melanin in rats, and restore motor functions. After the injection of melanin solution at the concentration of 6 mg/ml, the operated animals, from the first postoperative day, were able to keep balance on the rotating bar for the whole testing period. Balancing movements of the paralyzed hindlimb after the surgery recovered completely within 1–3 post-operative testing days.

Results obtained earlier in acute experiments have shown the favorable effect of the selected concentration of bacterial melanin solution used in the present study (Petrosyanm et al., 2012).

Studies have confirmed (Mauro et al., 2006) that in patients with Parkinson's disease intensive degeneration (70%) of melanin – containing neurons takes place in Substantia Nigra pars compacta. Oxidative stress and high level of metals lead to the death of neurons in SNc, as the dopaminergic neurons of this structure have a peculiarity to accumulate different metal ions, especially iron (Proctor and Reynolds, 1984). Experimental research showed that the main cause of Parkinson's disease is the loss of nigrostrial dopamine containing neurons, which are located in the compact part of Substantia Nigra and their nerve endings positioned in

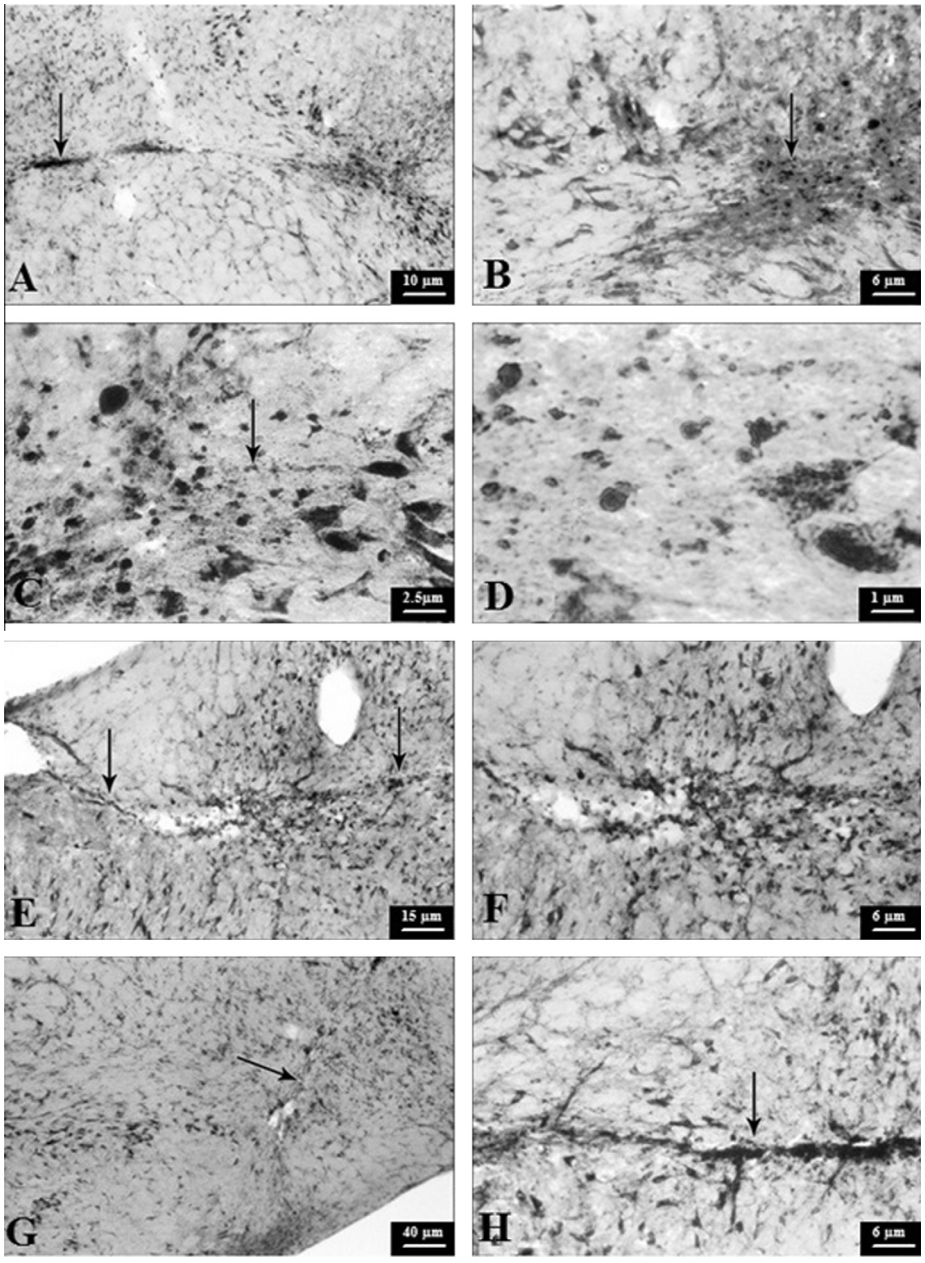

Fig. 4. Frontal sections of rat's midbrain at the level of lower colliculi after destruction of Substantia Nigra (melanin injected rats: A–D) and subsequent injection of bacterial melanin (control group sections: E–H). Arrows indicate destruction area: A-melanin injected rat, H-control group rat. In figure D the trace of electrode was not revealed. In sections of melanin injected rats (C and D) preserved nerve cells are identified.

the striatum (Hirsch et al., 1988). It is proved that inflammatory factors may lead to the death of DA neurons in SNc. Bacterial melanin supports the survival of neurons in SNc after induced destruction and preserves dopaminergic cell bodies. The substance promotes also sprouting of nerve fibers, as it has been shown in series of experiments conducted in rats (Petrosyanm et al., 2012).

Studies have shown also that neuromelanin containing dopaminergic neurons of the SNc are more subject to degeneration in patients with Parkinson's disease than dopaminergic neurons that do not contain melanin (Faucheux et al., 2003). The authors have shown that the free extracellular neuromelanin and microgliosis are the main causes of Parkinson's disease (Wei et al., 2011). The latest data show that the human extracellular neuromelanin in the absence of microglia itself is not toxic for neurons. But release of neuromelanin from destructed neurons causes the activation of microglia and subsequent neurodegeneration, proving that melanin containing neurons of Substantia Nigra are targeted in Parkinson's disease.

Different concentrations of bacterial melanin have been tested by us in previous series of experiments. The concentration 6 mg/ml calculated as 170 mg/kg showed to be the most effective (Gevorkyan et al., 2007b). It had more favorable and expressed effects on nervous tissue regeneration, axonal sprouting, and functional recovery than the higher doses of BM. It does not produce any toxic effects in the organism. Topical application of BM was used in electrophysiological experiments to study its effects on sensorimotor cortex activity (Pogosyan and Hovsepyan, 2008; Petrosyan, 2013) and the substance did not show toxic action. The physicochemical properties of bacterial melanin are not

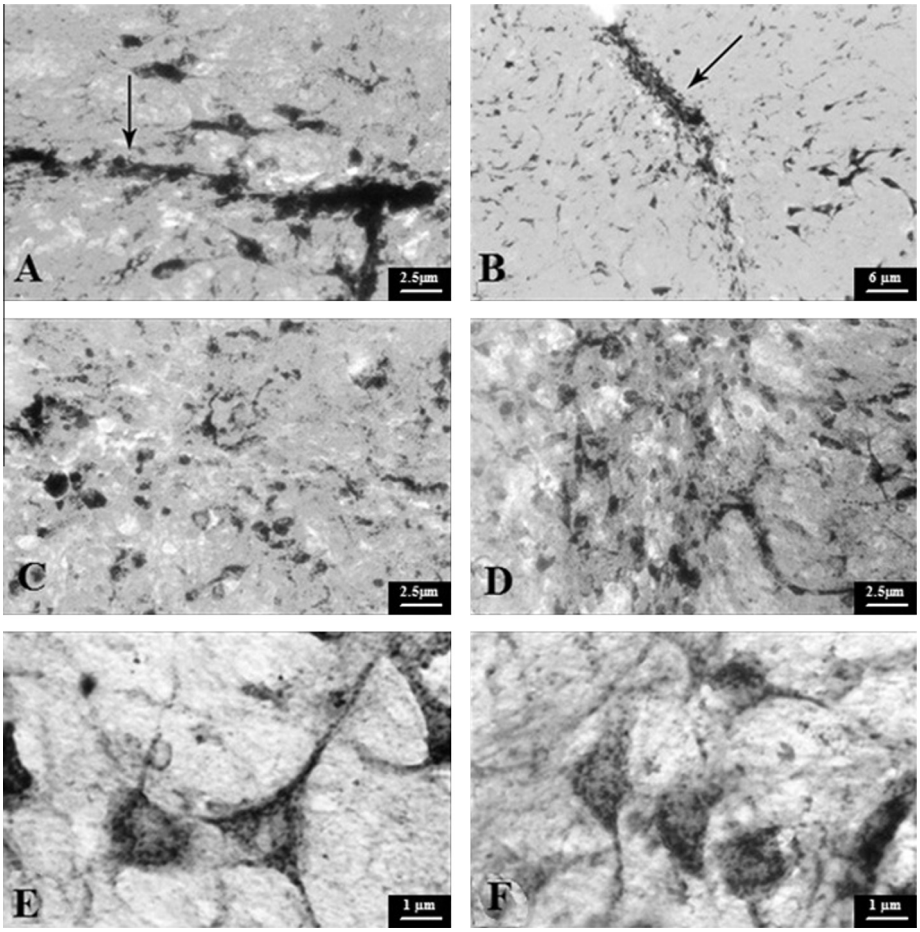

Fig. 5. Frontal sections of rat' midbrain at the level of lower colliculi after destruction of Substantia Nigra (control group sections: B, D, F) and subsequent injection of bacterial melanin (melanin injected rats: A, C, E) Arrows indicate destruction area: A-melanin injected rat, B-control group rat. Expressed gliosis in destruction area of control animal (D). Nerve cells of regular shape were revealed in destruction area of melanin injected rats (E). Fig. 5F is the section of intact rat to compare with 5E (section of melanin injected rat).

absolutely identical with neuromelanin, and the metabolites differ in structure (Popov, 2003). BM was tested in sensorimotor cortex and cerebellum after neuronal destruction and the substance has stimulating effect on regeneration and recovery, prevents secondary inflammation and destruction of neurons (Gevorkyan et al., 2007, Manvelyan et al., 2010).

Experimental data obtained in the present study have shown that in rats treated with bacterial melanin solution, deficiency of this substance is compensated, and presumably the balance of the substance is restored in the brain, providing fast recovery of locomotion and the initially elaborated ICR. The recovery of balancing instrumental conditioned reflex after the destruction of SNc was completed in a very short period of time in rats injected with BM solution, suggesting not only the neuroprotective action of BM, but also possible compensating role of BM in the process of motor control and recovery. The preserved dopaminergic cell bodies and modulating role of melanin contribute to the increased electrical activity of SNc neurons. Electrical activity of Substantia Nigra pars compacta neurons is critical for associative learning. The regulatory mechanisms of SNc activity are not completely studied. Such a high frequency activity is not induced or regulated by somatic input. Studies have confirmed that activation of dendritic NMDA receptors or GPCR-mediated reduction of action potential after activation of cation channels underlie such an activity (Sarah et al., 2007). Electrophysiological data showed increase in post stimulation potentiation effects – $M_{BE}/M_{TT} < 1$. Decrease in post stimulation inhibitory effects with higher amplitude of spiking was registered after tetanic potentiation and the amplitude of spiking is decreased after tetanic depression. After BM injection the overall pattern of posttetanic spiking activity is preserved but all post-effects became more expressed.

Taken together, these results indicate that bacterial melanin treatment enhances neuronal activity in SNc neurons. The increased regeneration effects and improved electrical activity of SN neurons support motor recovery after unilateral SNc lesion.

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