UDC 612.08

T. R. PETROSYAN,[1] O. V. GEVORGYAN,[2] A. S. HOVSEPYAN,[3] and A. S. TER-MARKOSYAN[1]

EFFECTS OF BACTERIAL MELANIN ON NEURONAL SPIKE ACTIVITY IN THE RAT SENSORIMOTOR CORTEX

Received May 23, 2014

We examined post-stimulation changes in the electrical activity of neurons of the rat sensorimotor cortex after intapraperitoneal injections or direct applications of bacterial melanin (a strict analog of neuromelanin). Activation of cortical neurons was evoked by high-frequency stimulation of the hindlimb peripheral nerves. The patterns of within-stimulation responses and long-lasting post-stimulation effects were rather similar in both subgroups (with systemic introductions or direct applications of melanin). Comparison of the results of previous electrophysiological experiments, where the effects of melanin on electrical activity generated by neurons of the *substantia nigra pars compacta* were studied, showed close similarities of the effects of this agent (mostly activating influence of bacterial melanin with the predominance of excitatory/facilitatory post-stimulation modifications of spike activity). The effects of bacterial melanin can contribute to the recovery processes in neurodegenerative diseases.

Keywords: melanin, sensorimotor cortex neurons, evoked spike activity, potentiation, depression.

INTRODUCTION

Many types of neural cells contain neuromelanins (NMs); the level of the latter is especially high in neurons of the *substantia nigra* (NS). As was reported, NMs are natural antioxidants capable of considerably suppressing lipid peroxidase [1]. The oxidative pathway of dopamine metabolism in the human brain leads to the formation of NM deposits in the cytoplasm of nigro-striatal dopaminergic neurons. There are data of that NM significantly contributes to the neurodegenerative processes underlying Parkinson's disease (PD) but the respective information is still controversial. Effects of melanin-concentrating hormone (MCH) and their correlation with memory retention following amnestic effects induced by NG-nitro-L-arginine (L-NOARG) have been studied; it was demonstrated that L- NOARG does not block potentiation effects induced by the above peptide [2]. An increase in the level of redox-active iron (free Fe^{2+} form) in association with NM has been revealed in the SN of patients with PD [3]. According to the cited authors, most extensive degeneration of dopaminergic neurons in patients with PD was observed in a subpopulation of neuro melanin-(NM-)containing neurons of the *pars compacta* (pc) of the SN. The redox activity of NM aggregates was studied in a group of patients with PD. A statistically significant reduction (–70%) in the number of NM-containing neurons and an increased content of non-heme iron (Fe^{3+}), which contribute to oxidative stress and intraneuronal damage, were found in these subjects. Samples from the lateral hypothalamic area (LH) showed that MCH inhibits synaptic activity of both glutamatergic and GABAergic neurons [4].

A melanin-synthesizing strain of *Bacillus thuringiensis* with a high intensity of pigment synthesis was obtained in the Armenian Institute of Biotechnology [5]. This allowed researchers to test the effects of BM, i.e., an agent nearly completely similar to NM, in experiments on CNS neurons. Bacterial melanin has been tested in a few series of experiments as a neuroprotector capable of enhancing motor recovery after CNS lesions [6]. Effects of BM on dopaminergic neurons in the SNpc were also tested; high-frequency stimulation of the caudate-putamen was used in these experiments [7]. The results

[1] Yerevan State Medical University, Republic of Armenia.
[2] Institute of Physiology of the NAS of Armenia, Yerevan, Republic of Armenia.
[3] SPC "Armbiotechnology" of the NAS of Armenia, Yerevan, Republic of Armenia.
Correspondence should be addressed to T. R. Petrosyan (tigpetrosyan@mail.ru).

indicated that BM applications modulate electrical activity of SNpc dopaminergic neurons, and excitatory post-stimulation effects prevail over the inhibitory responses.

In the current study, we examined the influences of BM on spike activity of neurons of the sensorimotor cortex (SMC); the effects of systemic (intraperitoneal, i.p.) injections of BM and its direct applications to the cortex were compared.

METHODS

Experiments were carried out on 12 mongrel albino male rats (200–300 g). They were housed in wire mesh cages in a room lighted at the 12/12 h light/dark cycle with the temperature set at 22°C. Testing was done during a light portion of the cycle.

During the experiments, rats were immobilized with 1% dithylinum solution (25 mg/kg; i.p.); mechanical ventilation was applied. The head of the experimental animal was fixed in a stereotaxic apparatus, and the SMC region was opened (3 mm lateral from the midline and 3 mm posterior from the bregma). The region corresponded to the representation of the contralateral hindlimb [8]. A stereotaxically oriented glass microelectrode (tip diameter 1-2 μm) filled with 2 M NaCl solution was inserted for a depth of 250 to 1800 μm; spike activities of single pyramidal neurons or cortical interneurons were recorded. Single or high-frequency (HF) stimulation (duration 1.0 sec, frequency 100 sec^{-1}) was applied to peripheral nerves of the hindlimb (mixed, *n. ischiadicus*, I; flexor, *n. peroneus comm.*, P, or extensor, *n. gastrocnemius*, G). Modifications of spike activity in the form of post-tetanic potentiation (PTP) and depression (PTD) after HF stimulation were examined; the latency, expressiveness, and duration of these modifications were measured using a computer. Extracellular neuronal activity was recorded for rather long periods (2–3 h).

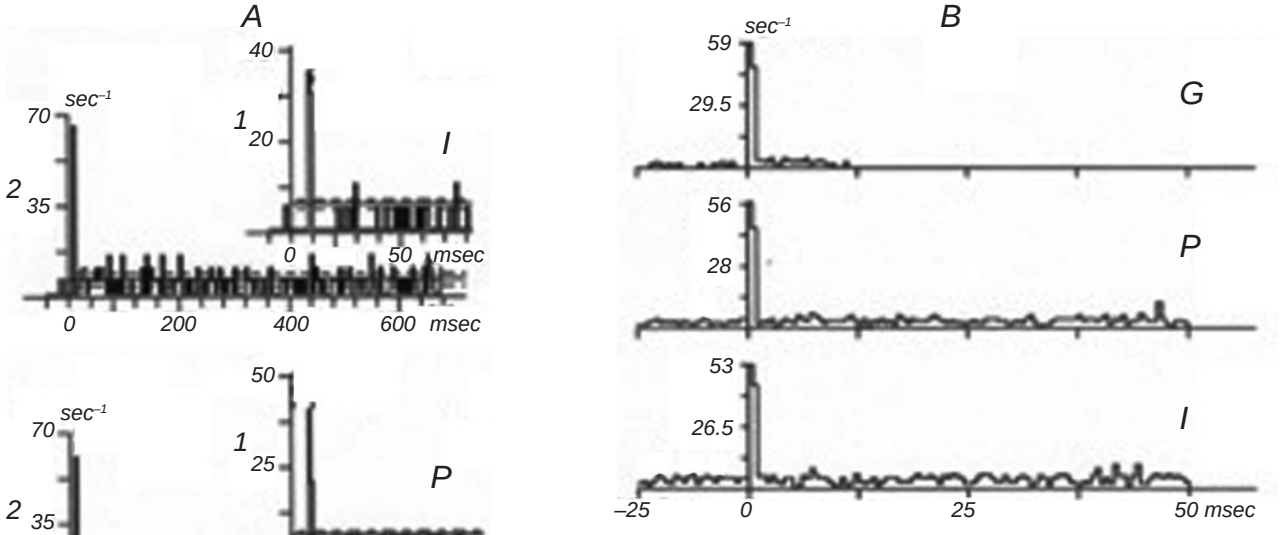
Data Analysis. A special computer software developed by V. Kamenetsky was used. It provided on-line selection of spikes based on amplitude discrimination. The program displayed peri-stimulation time histograms (PSTHs) of interspike intervals (ISIs). Parts of the PSTHs exceeding the $M \pm 2$ s.d. limits were calculated. This procedure was repeated until the M and s.d. values were stabilized (differences became smaller than 2%). The final sampling was considered a tonic post-stimulation activity of the examined neuron. The conformity of distributions to the normal, Poisson, or uniform law was estimated. Depending on the distribution type, the significance of differences (exceeding the $M \pm 2$ s.d. limits in the PSTHs) was estimated using the adequate criteria. The significance (P) of the difference between background and tonic post-stimulation activities was estimated by the two-side Student's t- test. Cumulative curves were plotted for the background and tonic activity. The least-square method was used to determine the slope of lines in curve plotting [9].

Animals of one subgroup ($n = 6$) were subjected to direct BM application to the cortex. Continuous irrigation of the cortical surface within the SMC registration area was performed with a special device with a tube attached to a vial with BM solution (0.6 mg/ml) to maintain a constant BM level within the recording area. In another animal subgroup ($n = 6$), the BM solution was systemically (i.p.) injected at a rate 6 mg/ml (170 mg/kg) 15 min after immobilization.

RESULTS

In most examined SMC neurons, primary (early) responses to single stimulations of the hindlimb peripheral nerves looked as a single spike or a short burst of spikes. As such primary responses, we considered significant changes in the background spiking of SMC neurons observed within 9-msec-long time intervals after peripheral nerve stimulations (Fig. 1A). More rarely, primary responses to single stimulations were poorly manifested at the stimulation intensities used.

At 1-sec-long HF stimulation of the peripheral nerves, typical changes in spiking of SMC neurons looked as significant increases in the discharge frequency within the period of nerve stimulation (tetanic potentiation, TP) followed by later long-lasting facilitation of spike activity (long-lasting posttetanic potentiation, PTP) (Fig. 1). In more rare cases, changes in spike activity of cortical neurons within the period of repetitive HF (100 sec^{-1}) stimulations of the nerves looked as suppression of background spiking (tetanic depression, TD) followed by later long-lasting post-stimulation modifications, PTP or posttetanic depression (PTD). Thus, in general, modifications in spike activity of SMC neurons related to HF stimulations of the hindlimb nerves could be classified as four combinations of the effects, purely facilitatory (TP + PTP), purely inhibitory (TD + PTD),



F i g. 1. Peristimulus histograms of responses of two sensorimotor cortex neurons (A, B) to single (A, 1) and repetitive high-frequency (A, 2; B) stimulations of the peripheral hindlimb nerves observed in the control (with no application/injection of bacterial melanin). I, P, and G are *nn. ishiadicus*, *peroneus comm.*, and *gastrocnemius*, respectively. High-frequency stimulation, 1.0-sec-long trains, 100 sec^{-1}. Bin width: 1 msec (A, 1), 4 msec (A, 2), and 500 msec (B). Abscissa) Time, msec in A and sec in B; ordinate) spike frequency in a bin, sec^{-1}.

Р и с. 1. Перистимульні гістограми відповідей двох нейронів сенсомоторної кори (*A*, *B*) на поодиноку (*A*, *1*) та серійну високочастотну (*A*, *2; B*) стимуляцію периферичних нервів задньої кінцівки в контролі, без дії бактеріального меланіну.

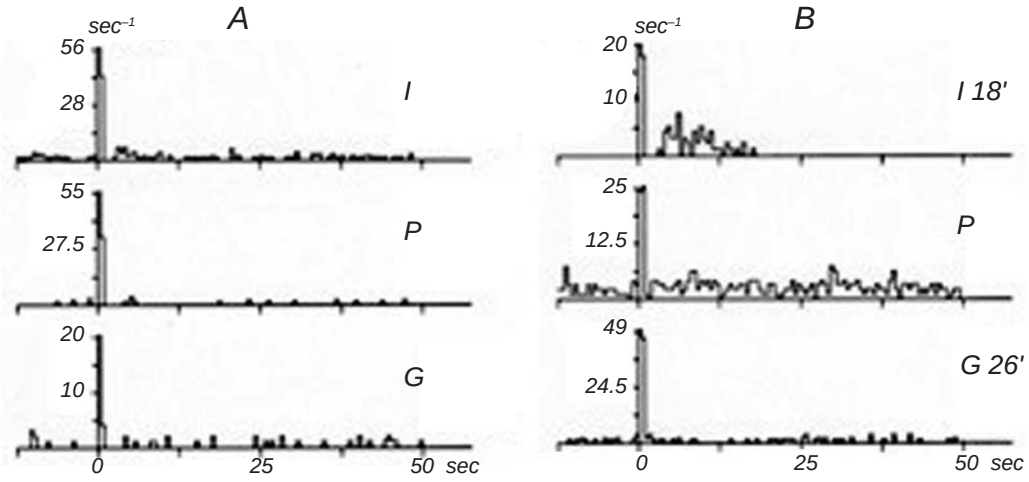
or mixed (TP + PTD or TD + PTP).

It should be emphasized that long-lasting facilitation of spiking of SMC neurons was much more clearly manifested in the cases of stimulations of either a common nerve trunk (I) or its branching supplying the flexor hindlimb muscles (P). Under conditions of HF stimulation of the extensor muscle nerve (G), PTP was either much less intense (shorter and weaker) or practically absent at all, and only short-lasting TP was observed in such cases (Fig. 1B).

Both direct application of BM on the SMC surface and systemic (i.v.) introduction of this agent resulted in sustained increases in impulse activity of a great majority of the examined cortical neurons. Tetanic stimulation-related modifications of spiking of these neurons were analyzed in detail in 109 units under conditions of i.v. injections of BM (Fig. 3) and in 106 cells under conditions of direct application of the latter (Fig. 2).

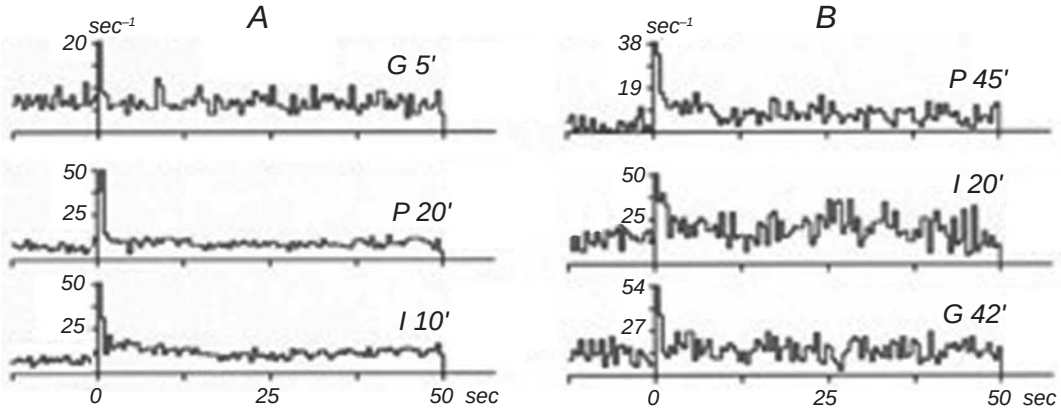
The patterns of HF nerve stimulation-induced modifications of spike activity of SMC neurons surface and of i.v. injections of BM are illustrated by Table 1.

As can be easily seen, both modes of PM application (systemic and direct) provided clear increases in the proportions of facilitatory modifications of spike activity of SMC neurons related to HF stimulations of the hindlimb nerves. Under the action of BM, the number of pure inhibitory stimulation-related modifications (TD + PTD) decreased considerably. In the case of i.p. BM injections, a drop was nearly twofold. At the same time, the proportions of pure excitatory effects (TP + PTP), which were observed before BM applications in less than one third of the neurons, tended to reach half of the total number of examined SMC neurons under conditions of action of this agent.



F i g. 2. Spike activity of two neurons (A, B) of the sensorimotor cortex; responses to high-frequency stimulation of the hindlimb nerves and long-lasting modifications of the activity under conditions of direct application of bacterial melanin to the cortical surface. Time after beginning of application, min, is shown above the peristimulus histograms. Other designations are similar to those in Fig. 1.

Р и с. 2. Імпульсна активність двох нейронів сенсомоторної кори (*A*, *B*): відповіді на високочастотну стимуляцію нервів задньої кінцівки та тривалі модифікації активності в умовах прямої аплікації бактеріального меланіну на поверхню кори.



F i g. 3. Spike activity of two neurons of the sensorimotor cortex (A, B): responses to high-frequency stimulation of the hindlimb nerves and long-lasting modifications of the activity under conditions of systemic (i.v.) injections of bacterial melanin. Designations are similar to those in Fig. 2.

Р и с. 3. Імпульсна активність двох нейронів сенсомоторної кори (*A*, *B*): відповіді на високочастотну стимуляцію нервів задньої кінцівки та тривалі модифікації активності в умовах системного (внутрішньовенного) введення бактеріального меланіну.

DISCUSSION

One of the unique properties of melanins is their ability to successfully cross the blood–brain barrier (BBB) [10]. The mechanism of this aspect of permeability of the BBB is not known. This property facilitates the transport of (NM and also of BM) from the blood into the brain parenchyma. We tested different concentrations of BM in our previous experiments, and the pharmacokinetic profile of BM has been studied. Bacterial melanin is enzymatically stable in the blood and brain parenchyma. It demonstrates the saturable transport across the BBB and selectively reaches certain targets in the CNS. Circulating BM enters all regions of the CNS, but its uptake is higher in the lumbar spinal cord, thalamus, hypothalamus, and SN.

The primary goal of our study was to compare the obtained results with the data from an electrophysiological study where effects of BM on SNpc dopaminergic neurons were examined. The effects of BM on these neurons were found to be predominantly excitatory. Such activating effect

T a b l e 1. Number of neurons with different types of modifications of spike activity induced by high-frequency stimulation of hindlimb peripheral nerves before applications/injections of bacterial melanin and under the action of the latter

Т а б л и ц я 1. Кількість нейронів з різними типами модифікацій імпульсної активності, індукованих високочастотною стимуляцією периферичних нервів задньої кінцівки, перед аплікаціями/ін'єкціями бактеріального меланіну та в умовах його дії

Type of the effects	Groups			
	i.p. injection		direct application	
	pre-inject.	post-inject.	pre-appl.	post-appl.
Inhibitory, TD + PTD	21 (19.3%)	11 (10.1%)	16 (15.1%)	10 (9.4%)
Mixed, TD + PTP	34 (31.2%)	19 (17.4%)	29 (27.4%)	21 (19.8%)
Mixed, TP + PTD	23 (21.1%)	32 (29.4%)	29 (27.4%)	27 (25.5%)
Facilitatory, TP + PTP	32 (29.4%)	47 (43.1%)	32 (30.2%)	48 (45.3%)
Total number of examined neurons	109	109	106	106

Footnotes: normalized numbers, %, of neurons with one type of HF stimulation-related modifications or another are shown in parentheses; TD and TP are tetanic depression and potentiation; PTD and PTP are long-lasting post-tetanic depression and potentiation, respectively.

could be a contributing factor in the process of motor recovery after destruction of dopaminergic neurons. The advantage of the present study was that the effects of direct application of BM on superficial cortical neurons could be observed.

In experiments of SNpc neurons, we used only systemic (i.p.) injections of BM because its direct application was impossible in such *in vivo* study. It was also interesting to compare the respective modifications of the responses in SNpc and cortical (SMC) neurons. For i.p. injections, the concentration (6 mg/ml, 170 mg/kg) similar to that in the above-mentioned experiments was selected. This concentration was used in all previous studies with BM and has been proven to be rather effective and, at the same time, nontoxic. Selection of the BM concentration for direct application was based on the pharmacokinetic profile of BM [11]. The tenfold-diluted concentration of BM was chosen for direct application.

LTP was shown to be a specific phenomenon not only in the hippocampus where it has been studied thoroughly. As is supposed, this modification is involved directly in the process of formation of short-term memory. It is also manifested in other brain structures (in the visual striatal cortex [12] and in synapses formed by the *inferior colliculi brachia* neurons in the medial geniculate body [13]). Studies of induced spike activity in SMC neurons showed that induction of long-time depression (LTD) is possible in this case. This phenomenon might be associated with decrease in the dendritic length and density in layers III and V of the SMC [14-16]. The same authors have also studied skilled learning-induced potentiation in the rat sensorimotor cortex as an example of the transient form of behavioral LTP [17]. Long-term depression and depotentiation in the SMC of freely moving rats was registered by Frog et al. [18]. Recording of electrical activity from superficial cortical neurons is a route to examine an immediate action of the substance (BM), and such technique is advantageous over registration of activity from deeply located neurons. In responses obtained after both i.p. injection or direct application of BM, an increased proportion of neurons with TP was observed, compared to the effects registered before the BM action. The overall percentage of PTP-like post-effects also increased. Thus higher proportions of facilitatory effects (TD-PTP and TP-PTP) were found after direct applications of BM.

Data of this and previous studies correlate well. Such a correlation confirms the potentiating action of BM on central neurons (at least, on their significant part).

Increased electrical activity of neurons can support the process of motor recovery after injuring influences on these neurons. In behavioral experiments with operantly conditioned rats, enhanced recovery of the limb movements and of the operant conditioned reflex was observed after SMC ablations in animals treated with rather small BM amounts. Intraperitoneal injection proved to be sufficiently effective; the respective effects were induced very rapidly and persisted for a longer period (2-3 h) than those after direct applications; it is important that all such effects demonstrated an identical pattern.

Bacterial melanin has a clear neuroregenerative action; it stimulates axonal sprouting and supports neuronal viability. Probably, these effects of BM provide the facilitation of motor recovery in rats

after SMC damage (the effect mentioned above). Augmentation of neuronal activity in the cortex is, supposedly, another supporting factor that enhances motor recovery.

Animals were maintained and handled in accordance with the 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).

The authors, T. R. Petrosyan, O. V. Gevorgyan, A. S. Hovsepyan, and A. S. Ter-Markosyan, confirm that they have no conflict of interest with any organization or person that may be related to this study; there was also no conflict of interest in interrelations between the authors.

Т. Р. Петросян[1]. О. В. Геворгян[2], А. С. Овсепян[3], А. С. Тер-Маркосян[1]

ВПЛИВИ БАКТЕРІАЛЬНОГО МЕЛАНІНУ НА АКТИВНІСТЬ НЕЙРОНІВ У СЕНСОМОТОРНІЙ КОРІ ЩУРА

[1] Єреванський державний медичний університет (Республіка Вірменія).

[2] Інститут фізіології Національної Академії наук Вірменії, Єреван (Республіка Вірменія).

[3] СПЦ «Армбіотехнологія» Національної Академії наук Вірменії, Єреван (Республіка Вірменія).

Р е з ю м е

Ми досліджували постстимуляційні зміни імпульсної активності нейронів сенсомоторної кори щура після внутрішньоочеревинних ін'єкцій або прямих аплікацій бактеріального меланіну (близького аналога нейромеланіну). Активація кортикальних нейронів викликалася високочастотною стимуляцією периферичних нервів задньої кінцівки. Патерни відповідей у перебігу стимуляції та тривалих постстимуляційних ефектів в обох підгрупах (із системним уведенням або прямою аплікацією меланіну) виявилися дуже близькими. Порівняння з результатами попередніх електрофізіологічних експериментів, в яких досліджували впливи меланіну на електричну активність нейронів *substantia nigra pars compacta*, показало, що спостережувані ефекти (в основному активаційний вплив бактеріального меланіну з домінуванням збуджувальних/полегшувальних постстимуляційних модифікацій імпульсної активності) були дуже близькими. Ефекти бактеріального меланіну, ймовірно, можуть бути використані для інтенсифікації процесів відновлення в разі нейродегенеративних захворювань.

REFERENCES

1. T. Wilczok, K. Stepien, A. Dzierzega-Lecznar, et al., "Model neuromelanins as antioxidative agents during lipid peroxidation," *Neurotox. Res.*, **1**, No. 2, 141-147 (1999).
2. M. Varas, M. Perez, M.E. Mouzon, and S. R. de Barioglio, "Melanin concentrating hormone, hippocampal nitric oxide levels and memory retention," *Peptides*, **23**, No. 12, 2213-2221 (2002).
3. B. A. Faucheux, M. E. Martin, C. Beaumont, et al., "Neuromelanin associated redox-active iron is increased in the *substantia nigra* of patients with Parkinson's disease," *J. Neurochem*., **86**, No. 5, 1142-1148 (2003).
4. A. A. Galoyan, J. S. Sarkissian, T. K. Kipriyan, et al., "Protective effect of a new hypothalamic peptide against cobra venom and trauma induced neuronal injury," *Neurochem. Res*., **26**, 1023-1038 (2001).
5. A. E. Aghajanyan, A. A. Hambardzumyan, A. S. Hovsepyan, et al., "Isolation, purification and physicochemical characterization of water-soluble Bacillus thuringiensis melani," *Pigment Cell Res*., **18**, No. 2, 130-135 (2005).
6. M. H. Monfils and G. C. Teskey, "Skilled-learning - induced potentiation in rat sensorimotor cortex: a transient form of behavioral long-term potentiation," *Neuroscience*, **125**, No. 2, 329-336 (2004).
7. T. R Petrosyan, V. A. Chavushyan, and A. S. Hovsepyan, "Bacterial melanin increases electrical activity of neurons in substantia nigra pars compacta," *J. Neural Transm*., **121**, 259-265 (2014).
8. Y. Komatsu, K. Toyama, J. Maeda, and H. Sakaguchi, "Long term potentiation investigated in a slice preparation of striate cortex of young kittens," *Neurosci. Lett*., **26**, 269-274 (1981).
9. R. M. Sulkhanyan and T. S. Khachatryan, "Comparison of the protection against neuronal injury by hypothalamic peptides and by dexamethasone," *Neurochem. Res*., **25**, 1567-1578 (2000).
10. D. L. Berliner, R. L. Erwin, and D. R. McGee, "*Methods of treating Parkinson's disease using melanin*," US Patent 5, 210, 076 A (1993).
11. T. R. Petrosyan and A. S. Hovsepyan, "Bacterial melanin crosses the blood–brain barrier in rat experimental model," *Fluids Barriers CNS*, **11**, No. 20, 1-7 (2014).
12. D. J. Froc, C. A. Capman, C. Trepel, and R. J. Racine, "Long-term depression and depotentiation in the sensorimotor cortex of freely moving rat," *J. Neurosci*., **20**, No. 1, 438-445 (2000).
13. R. A. Gerren and N. M. Weinberger, "Long term potentiation in the magnocellular medial geniculate nucleus of anesthetized cat," *Brain Res*., **265**, 138-142 (1983).
14. O.V. Gevorkyan, "Neuronal activity of sensorimotor cortex on the mesemcephalic reticular formation stimulation," *Biol. J. Armenia*, **40**, No. 12, 993-997 (1987).
15. O. V. Gevorkyan, I. B. Meliksetyan, A. S. Ovsepyan, and A. S. Sagiyan, "Effects of BT-melanin on recovery of operant conditioned reflexes in rats after ablation of the sensorimotor cortex," *Neurosci. Behav. Physiol*., **37**, No. 5, 471-476 (2007).
16. S. P. Hicks and C. I. D'Amato, "Locating corticospinal neurons by retrograde axonal transport of horseradish peroxidase," *Exp. Neurol*., **56**, 410-420 (1977).

17. A. A. Galoyan, J. S. Sarkissian, T. K. Kipriyan, et al., “Melanin concentrating hormone depresses synaptic activity of glutamate and GABA neurons from rat lateral hypothalamus,” *J. Physiol.*, **15**, No. 533, 237-252 (2001).

18. M. H. Monfils and G. C. Teskey, “Induction of long-term depression is associated with decreased dendritic length and spine density in layers III and V of sensoromotor neocortex,” *Synapse*, **53**, No. 2, 114-121 (2004).