SYNCHRONOUS AND PERIODIC CALCIUM OSCILLATIONS IN NEURONAL NETWORKS FORMED BY SENSORY NEURONS IN PRIMARY CULTURE[#]

BEATRICE MIHAELA RADU[*], M. RADU[**], D.D. BANCIU[*]

[*]Department of Animal Physiology and Biophysics, Center of Neurobiology and Molecular Physiology, Faculty of Biology, University of Bucharest, 91–95, Splaiul Independentei, Bucharest, 050095, Romania, beatrice_macri@yahoo.com
[**]Department of Health and Environmental Physics, "Horia Hulubei" National Institute for Physics and Nuclear Engineering, Atomiştilor, 407, Măgurele, 077125, Romania

Abstract. Neurite outgrowth was observed for the neuronal networks formed in primary neuronal cultures from dorsal root ganglia. Their activity was analyzed by the fluorescence microscopy technique in order to evidence the level of calcium ions (calcium imaging) in cytosol of the neurons. Cells were loaded with calcium fluorescent dye Calcium Green-I. Using this technique, we have evidenced the spontaneous oscillations of the cytosolic calcium concentration in neurons coupled in a network. We have proved the synchronization and periodicity of these oscillations. The potassium chloride addition stimulates the neurons by producing a large and transient increase of the cytosolic calcium that proves the functionality of the neuronal network.

Key words: Neurite outgrowth, sensory neurons, calcium imaging, oscillations.

INTRODUCTION

Sensory input from the periphery to the CNS is critically dependent on the strength of synaptic transmission at the first sensory synapse formed between primary afferent dorsal root ganglion (DRG) and superficial dorsal horn neurons of the spinal cord.

Several experimental strategies for enhancing outcome after human spinal cord injury have advanced to human application in recent years. These include steroids, anti-oxidants, GM_1 ganglioside, fetal cell grafting, Rho kinase inhibition (Cethrin), nogo antibody infusion (Novartis), activated macrophage injection into the lesioned spinal cord, olfactory ensheathing cell transplantation, body cooling, minocycline, riluzole and others [2, 6, 7, 11, 13, 14, 23, 25, 27].

[#]This study was presented as a poster at the *National Conference of Biophysics*, Cluj-Napoca, 2009, in the section "Biophysical and biochemical investigations in cells and tissue cultures".

Received July 2009.

ROMANIAN J. BIOPHYS., Vol. 19, No. 4, P. 227–237, BUCHAREST, 2009

Neurite outgrowth in cultured neurons is considered one indication of neuroregenerative potential [10, 17, 19]. Assays designed to measure parameters of neurite outgrowth can be helpful in the elucidation of mechanisms inhibiting axon repair and in the development of strategies to overcome repair limitations. Neurons from multiple sources capable of extending neurites can be utilized for *in vitro* assays and changes in neurite outgrowth can be assessed while screening molecules that promote, inhibit, or overcome inhibition of neurite outgrowth. In fact, many variations of neurite outgrowth assays have been developed using both primary neuron cultures, such as dorsal root ganglia, cortical neurons, cerebellar granule neurons (CGNs), and established cell lines, such as NG108-15, SH-SY5Y and PC12 [8, 16, 18, 24].

Calcium ions are crucial messengers in the regulation of synaptic efficacy. In the postsynaptic neuron, this is exemplified by the tight temporal and spatial co-segregation of calcium ions with calcium-dependent signal transduction protein complexes in dendritic spines. Over the last several years optical imaging, physiological, structural, and biological studies have clarified the molecular mechanisms underlying differential calcium signaling within the spine [1].

In our study we have used the calcium imaging technique in order to evidence the functionality of neuronal networks formed spontaneously in primary cell culture by sensory neurons from dorsal root ganglia.

MATERIALS AND METHODS

SOLUTIONS

The IncMix solution for DRG incubation contained (in mM): NaCl 155, K_2HPO_4 1.5, HEPES 5.6, NaHEPES 4.8, glucose 5. The antibiotic gentamicin was added to 50 μg / mL. The standard extracellular solution used in all experiments contained (in mM): NaCl 140; KCl 4; $CaCl_2$ 2; $MgCl_2$ 1; Hepes 10; NaOH 4.55; glucose 5 (pH 7.4 at 25 °C). Stock solutions were prepared: Calcium Green-I – AM (Molecular Probes) was solubilised in dimethyl sulfoxide at a concentration of 4 mM and pluronic acid (Molecular Probes, F-127) was solubilised in dimethyl sulfoxide at a concentration of 10 mM. Pluronic acid has the role to facilitate the penetration of the esterified dye molecules inside the neurons.

SENSORY NEURONS IN PRIMARY CULTURE

Adult rats (150–200 g) were killed by inhalation of 100% CO_2, followed by decapitation. DRG neurons were cultured as described elsewhere [20]. DRGs were removed and incubated in 1 mg / mL collagenase (type XI from Sigma) and 3 mg / mL Dispase (nonspecific protease, Sigma) for 1 h at 37 °C in IncMix

solution (see Solutions). After trituration the dissociated cells were plated onto borosilicate glass coverslips (0.17 mm thick, 24 mm diameter) which had been treated with poly D-lysine (0.1 mg/mL for 30 min), and cultured (37 °C, 5% CO_2 in air) in a 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 10% horse serum and 50 μg/mL gentamicin.

RECORDING CALCIUM CONCENTRATION VARIATIONS BY FLUORESCENCE TECHNIQUE

In order to record the calcium concentration variations inside the sensory neurons, we have used the fluorescent dye Calcium Green-I – AM (Molecular Probes). The culture medium was removed and cells were rinsed 3 times with extracellular Ringer solution. 1 mL Ringer solution was mixed with 0.5 μL Calcium Green stock solution and 0.5 μL pluronic acid stock solution. Cells were incubated with that mixture for 1 h at 37 °C. The solution containing the dye was removed and cells were rinsed 3 times in extracellular Ringer solution. Afterwards, cells were incubated for another 0.5 h at 37 °C in order to allow the cytosolic esterases to act and to release the active form of the dye.

Sensory neurons were observed with a wide field epifluorescence microscope (Olympus IX71) equipped with a filter set U-MWB2 (λ_{ex} = 460–490 nm, λ_{em} > 520 nm, dichroic filter λ > 500 nm). This filter was suitable for the observation of the Calcium Green fluorescence emission (λ_{ex} = 505 nm; λ_{em} = 532 nm).

IMAGE ACQUISITION AND ANALYSIS

The image acquisition was performed with the CCD camera (iXon DU897, Andor) and with the software package iQ (Andor). Fluorescence images are captured with a frequency of 3 frames/s with an exposure time of 10 ms/frame (the CCD camera was cooled at –80 °C). In order to analyse the fluctuations of the cytosolic calcium, we have recorded series of images up to 240 s. In each neuron, the intensity variation was followed by choosing a corresponding region of interest and by measuring the medium emission intensity in this region. Images were analysed with the software package ImageJ [22].

RESULTS AND DISCUSSION

SPONTANEOUS NEURITE OUTGROWTH

A neurite refers to any process (either an axon or a dendrite) from the cell body of a neuron. The term is frequently used when speaking of immature or developing neurons, especially of cells in culture, because it can be difficult to

distinguish axons from dendrites before differentiation is complete. Neurites are often packed with microtubule bundles, the growth of which is stimulated by Nerve Growth Factor (NGF), as well as tau proteins, MAP1, and MAP2 [5].

In our experiments sensory neurons were maintained in culture up to 10 days. They were plated at a relatively low density, without any inhibitor of glial cell growth. If cells are plated to a higher density, the neurite outgrowth is accelerated and the neuronal network formation is very fast. This fact is mainly due to the NGF release in the culture medium. Cells were inspected every day on an inverted microscope and the medium was changed every 3 days. From the 2nd day of culture (Fig. 1A), one can see the neurite formation from the growth cone. If we follow the process, we can observe the evolution of these neurites to a complete axon formation (in the 5th day of culture; Fig. 1B). We presume to be an axon because of its particular shape, and the branching process at 90 °C that is typical.

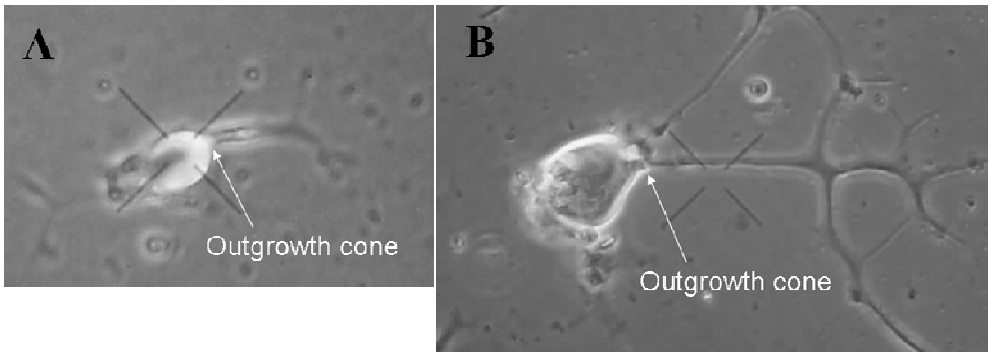

Fig. 1. Neurite formation from the growth cone of a sensory neuron, in the 2nd day of culture (A), or in the 5th day of culture (B). Images are obtained on an inverted Olympus microscope, 40× air objective (phase contrast imaging).

NEURON-NEURON SYNAPSE

In vivo, once neurons begin to aggregate into recognizable structures, and sometimes even before, they begin to extend neurites. These neurites grow toward other regions of the nervous system or other structures on which the neurons will eventually form synapses, such as glands, muscle, etc; these tissues are usually referred to as the 'targets' of the neurons. The proper functioning of the nervous system depends on the formation of proper connections. It appears that neurons from different parts of the nervous system grow more or less directly toward their normal targets, and ignore or bypass other potential synaptic sites. First, growing axons interact directly with molecules on the surfaces of cells or in the extracellular matrix of the tissues through which they grow. These physical interactions attract the axons to grow in certain directions and to avoid growing in others [5].

Growing neurites are directed through the embryo to their targets by a variety of complex direct molecular interactions with each other, with cells over which they pass, and with the extracellular matrix. These interactions can be attractant or repellant to the growing neurite, and are mediated by the leading edge of the neurite, a highly motile and specialized structure called the growth cone [5].

In Fig. 2 is presented an example of a pair of coupled neurons (cells A and B) in primary DRG neuronal culture. In this case, the attractive condition was achieved and these two neurons have coupled in a synapse (see arrow). The presence of the neuronal connexion is more obvious in transmitted light rather than in fluorescence. This fact is determined by the high number of calcium ions confined into the soma of the neurons, and their weak presence in neurites.

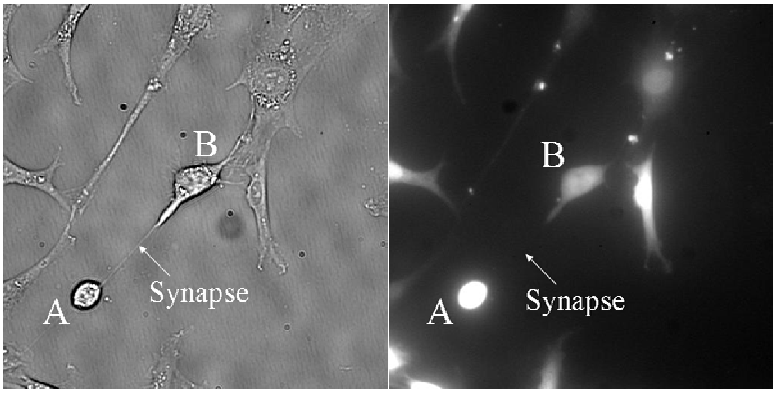

Fig. 2. Example of two neurons (A and B) coupled in a synapse. (left) transmission image; (right) fluorescence image (40x air objective).

NEURAL NETWORKS

During development, neurons become assembled into functional networks by neurites which connect synaptically to other neurons. Axon branching enables a single neuron to connect with multiple targets and is therefore essential for the assembly of complex neural circuits [5]. Given the fundamental importance of axon branching for establishing synaptic connectivity, surprisingly little is known about how branching is regulated.

In Fig. 3 is presented a complex network formed by neuronal and glial cells, either coupled in neuron-neuron synapses or in neuron-glia synapses. We have noticed (unpublished results) that the use of glial cell growth inhibitors is unappropriate for the development of that spontaneous neuronal network. It seems that the presence of glial cells is very important for the formation of neuron-neuron synapses and they are also implicated in the formation of neuron-glia synapse.

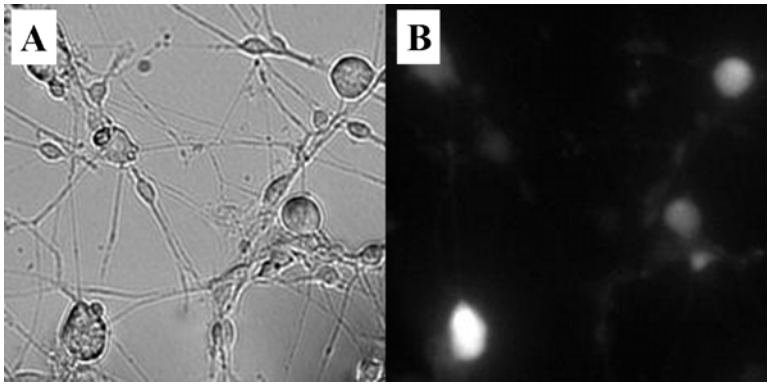

Fig. 3. Primary neuronal culture in the 7th day. Multiple connexions between neurons and glia are present. (A) Transmitted light image. (B) Fluorescence image (40x air objective).

CALCIUM OSCILLATIONS IN NEURONAL NETWORK

Changes in intracellular free calcium concentration $[Ca^{2+}]_i$ regulate many diverse cellular functions. Neurons possess complex calcium homeostatic mechanisms to control $[Ca^{2+}]_i$, two main pathways being involved: the inositol 1,4,5 trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR) [3]. In the rat neurons of dorsal root ganglia two types of Ca^{2+}-dependent currents have been described; a Ca^{2+}-activated chloride current ($I_{Cl(Ca)}$) and a Ca^{2+}-activated nonselective cation current (I_{CAN}) [3].

In our experiments, we have adjusted the protocol of image acquisition in order to eliminate the dye photobleaching effect, in conditions of constant calcium concentration. We have increased the CCD camera sensitivity and reduced the excitation light intensity until the fluorescence emission was time-stable. This fact can be onserved in Fig. 4 for the six selected cells during an acquisition interval of 240 s.

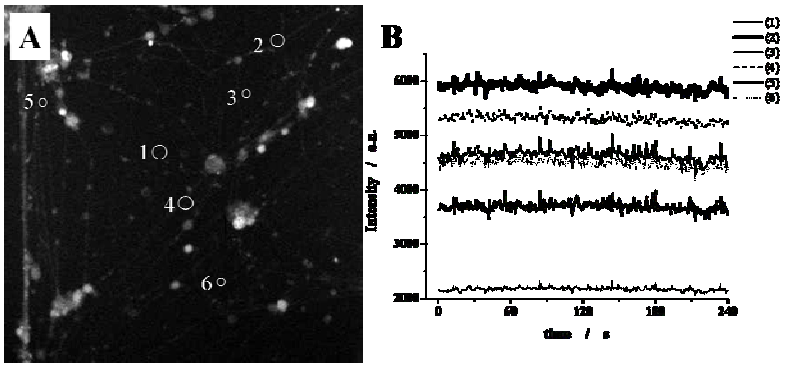

Fig. 4. Fluorescence intensity of sensory neurons in the absence of any stimulus. A. Fluorescence image (six cells are marked; 20x air objective). B. Fluorescence emission intensity for the six analysed cells.

In the same figure, we can observe the sponataneous $[Ca^{2+}]_i$ fluctuations of the analysed neurons, this fact being a proof of the interconnected neurons functionality [26]. In Fig. 5A, it is shown a detail of the trace recorded for cell 2. The represented duration is approximately 60 s and the oscillation period is 2–5 s similar to the data reported in hippocampus neurons [26]. Furthermore, in Fig. 4B one can follow the calcium oscillations for the six recorded cells and observe their synchronicity. A better view of the synchronous calcium waves was illustrated in Fig. 5B for relative fluorescence intensity (fluorescence intensity in each cell is normalized to the medium intensity) in cells 1, 2 and 3 recorded for a period of 60 s.

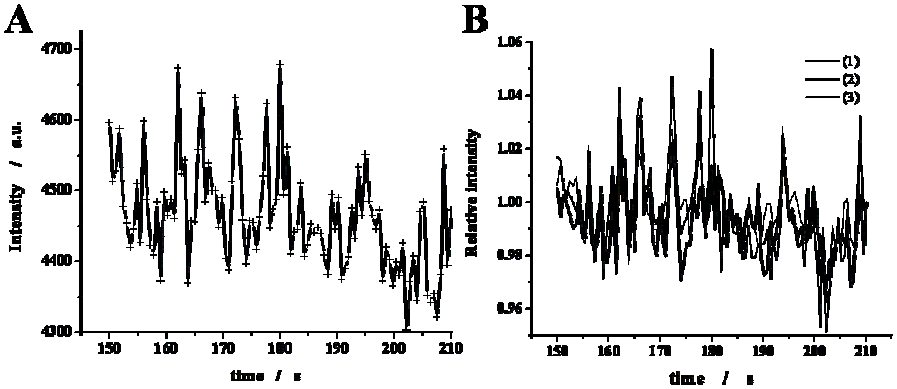

Fig. 5. A. Spontaneous oscillations in calcium concentration (A) Fluorescence emission intensity in cell 2 shown in Fig. 4, for a period of 60 s. (B) Relative fluorescence intensity in cells 1, 2 and 3 from Fig. 4, for a period of 60 s.

MODULATION OF THE CALCIUM OSCILLATIONS IN NEURONAL NETWORK

Intracellular calcium waves can be modulated by various agents, such as a protein based sperm factor (SF) extracted from mammalian sperm, heat treatment, replacement of Ca^{2+} ions with Ba^{2+} ions, intracellular application of cyclic ADP-ribose [3], tetrodotoxin (TTX) [26]. In our study, the modulation of the neuronal cytosolic calcium oscillations was done by the extracellular perfusion of potassium chloride (40 mM). The plasma membrane depolarisation is induced by K^+ ions outside increase. The direct consequence of this depolarisation is the transient release of calcium from the intracellular reservoirs to the cytosol [9]. In Fig. 6A are presented six cells stimulated by potassium ions. In Fig. 6B, we have analysed the synchronous increase of the fluorescence intensity in the neurons upon potassium chloride superfusion and their synchronous recovery.

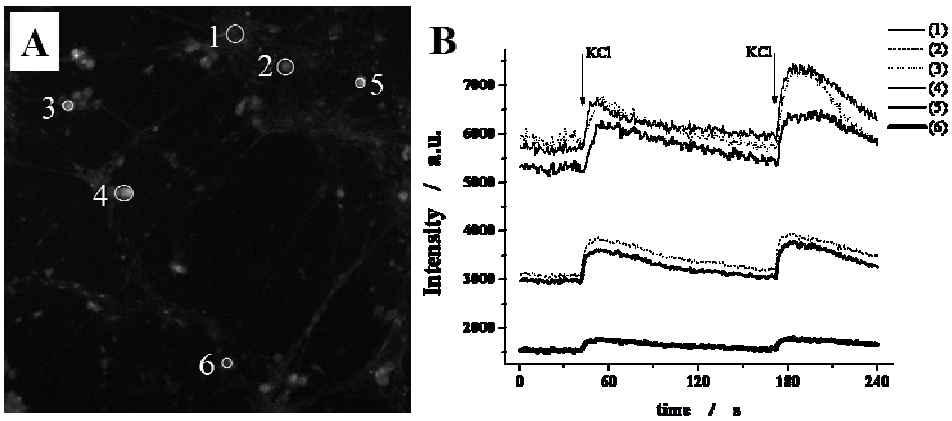

Fig. 6. Fluorescence intensity in sensory neurons stimulated with potassium chloride (40 mM). A. Fluorescence image (six neurons are marked by circles and are numbered; 20× air objective). B. Fluorescence intensity in the six analysed cells; the addition of potassium chloride is marked by arrows.

Synchronisation can be observed in Fig. 7, where the same traces as in Fig. 6B are represented after that fluorescence emission intensity values were normalized for each cell to the medium value acquired during the first 50 s of unstimulated recording. It is obvious the good synchronisation of the neurons response. In addition, it is observed a variability in neurons response, as it concerns their oscillation amplitude and the relaxation time. These results are in agreement with the data reported for the calcium oscillations in hippocampus [26].

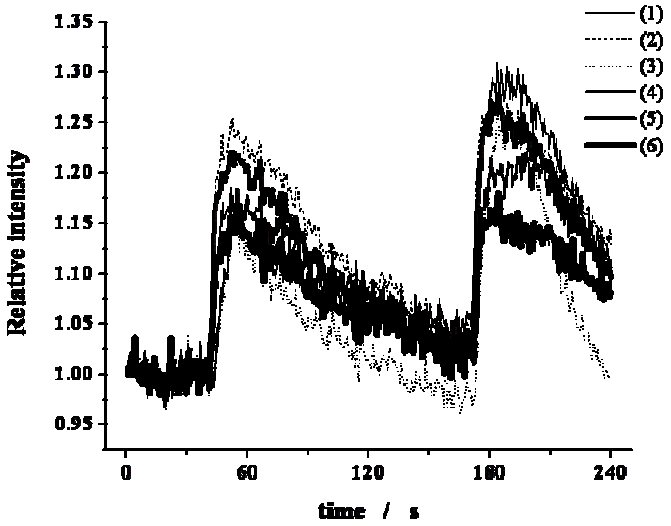

Fig. 7. Relative fluorescence emission intensity in neurons from Fig. 6 in the presence of potassium chloride.

CONCLUSIONS

Intracellular free calcium ions act as important second messengers that regulate signal transduction processes that control nerve growth, including neurite outgrowth and axonal pathfinding [12]. Previous investigations have revealed disrupted Ca^{2+} signaling in primary sensory neurons after injury [21]. An easy method to evidence the functionality of neuronal growth is to follow the calcium concentration variations due to depolarization induced by potassium ions. An optimal range of $[Ca^{2+}]_i$ in growth cones is required for proper neurite outgrowth [4]. Therefore, the amplitude of the $[Ca^{2+}]_i$ is considered to be an important factor in calcium signaling. Sensory neurons proved to be viable from the beginning of the experiment (1^{st} and 2^{nd} days in primary culture) until the 10^{th} days in cultures. This was proved by the incorporation of Calcium Green-I – AM into the neurons and by their esterases activity that released the fluorescent form of Calcium Green. In addition, the neurite growth is visualized in transmitted light. These neurons are able to form neuronal networks, in which are implicated both other neurons and also the glial cells. A good proof that these networks are constituted and functional is to follow the calcium oscillations in the connected neurons. A synchronous activity can be visualized in these cells, and the potassium ions are able to modulate these oscillations.

Neurite extension, or neurite outgrowth, plays a fundamental role in embryonic development, neuronal differentiation, and nervous system function. Neurite outgrowth is also critical in some neuropathological disorders as well as neuronal injury and regeneration [5]. Neurons in the adult mammalian central nervous system do not spontaneously regenerate their axons after injury [15]. In our experiments, sensory neurons from the peripheral nervous system proved the ability to form spontaneous neurites that interconnected. Further investigations are necessary to certify the functionality of these connections and if they are made according to the anatomical architecture. Consequently, our preliminary study might have a clinical importance in understanding the mechanisms of recuperation upon peripheral nerve injury in adults.

Acknowledgment. This study was financed by the national research grant PNII 61-011/2007.

REFERENCES

1. BLACKSTONE, C., M. SHENG., Postsynaptic calcium signaling microdomains in neurons, *Front Biosci*., 2002, **7**, d872–885.
2. CAPPUCCINO, A., L.J. BISON, Hypothermia for spinal cord injury, *Spine J*., 2009, **9**(7), 615–616
3. CURRIE, K.P.M., K. SWANN, A. GALIONE, R.H. SCOTT, Activation of Ca^{2+}-dependent currents in cultured rat dorsal root ganglion neurones by a sperm factor and cyclic ADP-ribose, *Mol. Biol. Cell*, 1992, **3**, 1415–1425.

4. DAVIS, L., P. DOU, M. DEWIT, S.B. KATER, Protein synthesis within neuronal growth cones, *J. Neurosci*., 1992, **12**, 4867–4877.
5. DE CURTIS, I., *Intracellular Mechanisms for Neuritogenesis*, Springer, New York, USA, 2007.
6. DERGHAM, P., B. ELLEZAM, C. ESSAGIAN, H. AVEDISSIAN, W.D. LUBELL, L. MCKERRACHER, Rho signaling pathway targeted to promote spinal cord repair, *J. Neurosci.*, 2002, **22**, 6570–6577.
7. DOBKIN, B.H., A. CURT, J. GUEST, Cellular transplants in China: observational study from the largest human experiment in chronic spinal cord injury, *Neurorehabil. Neural Repair*, 2006, **20**, 5–13.
8. ENCINAS, M., M. IGLESIAS, Y. LIU, H. WANG, A. MUHAISEN, V. CENA, C. GALLEGO, J.X. COMELLA, Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor dependent, human neuron-like cells, *J. Neurochem*., 2000, **75**, 991–1003.
9. EVANS, R.M., K.N. WEASE, C.J. MACDONALD, H.A. KHAIRY, R.A. ROSS, R.H. SCOTT, Modulation of sensory neuron potassium conductances by anandamide indicates roles for metabolites, *British J. Pharmacol*., 2008, **154**, 480–492.
10. FOURNIER, A.E., B.T. TAKIZAWA, S.M. STRITTMATTER, Rho kinase inhibition enhances axonal regeneration in the injured CNS, *J. Neurosci*., 2003, **23**, 1416–1423.
11. GEISLER, F.H., F.C. DORSEY, W.P. COLEMAN, Recovery of motor function after spinal cord injury – a randomized, placebo-controlled trial with GM-1 ganglioside, *N. Engl. J. Med*., 1991, **324**, 1829–1838.
12. HENLEY, J., M.M. POO, Guiding neuronal growth cone using Ca^{2+} signals, *Trends Cell. Biol*., 2004, **14**, 320–330.
13. http://clinicaltrialsfeeds.org/clinical-trials/show/NCT00406016.
14. HUANG, H., L. CHEN, H. WANG, B. XIU, B. LI, R. WANG, J. ZHANG, F. ZHANG, Z. GU, Y. LI, Y. SONG, W. HAO, S. PANG, J. SUN, Influence of patients' age on functional recovery after transplantation of olfactory ensheathing cells into injured spinal cord injury, *Chin. Med. J.* (Engl.), 2003, **116**, 1488–1491.
15. KIM, H.S., M. SONG, E. KIM, S.H. RYU, P.G. SUH, Dexamethasone differentiates NG108-15 cells through cyclooxygenase 1 induction, *Exp. Mol. Med*., 2003, **35**, 203–210.
16. KIM, J., J. SCHAFER, G.L. MING, New directions in neuroregeneration, *Expert Opin. Biol. Ther*., 2006, **6**(8), 735–738.
17. LEHMANN, M., A. FOURNIER, I. SELLES-NAVARRO, P. DERGHAM, A. SEBOK, N. LECLERC, G. TIGYI, L. MCKERRACHER, Inactivation of Rho signaling pathway promotes CNS axon regeneration, *J. Neurosci*., 1999, **19**, 7537–7547.
18. LOZANO, A.M., M. SCHMIDT, A. ROACH, A convenient in vitro assay for the inhibition of neurite outgrowth by adult mammalian CNS myelin using immortalized neuronal cells, *J. Neurosci. Methods*, 1995, **63**, 23–28.
19. NG, W.P., N. CARTEL, J. RODER, A. ROACH, A. LOZANO, Human central nervous system myelin inhibits neurite outgrowth, *Brain Res*., 1996, **720**, 17–24.
20. Reid, G., M. Flonta, Cold transduction by inhibition of a background potassium conductance in rat primary sensory neurons, *Neurosci. Lett.*, 2001, **297**(3), 171-174.
21. RIGAUD, M., G. GEMES, P.D. WEYKER, J.M. CRUIKSHANK, T. KAWANO, H.E. WU, Q.H. HOGAN, Axotomy depletes intracellular calcium stores in primary sensory neurons, *Anesthesiology*, 2009, **111**(2), 381-392.
22. rsb.info.nih.gov/ij/.
23. SCHWARTZ, G., M.G. FEHLINGS, Evaluation of the neuroprotective effects of sodium channel blockers after spinal cord injury: improved behavioral and neuroanatomical recovery with riluzole, *J. Neurosurg*., 2001, **94**, 245–256.

24. SIMPSON, P.B., J.I. BACHA, E.L. PALFREYMAN, A.J. WOOLLACOTT, R.M. MCKERNAN, J. KERBY, Retinoic acid evoked-differentiation of neuroblastoma cells predominates over growth factor stimulation: an automated image capture and quantitation approach to neuritogenesis, *Anal. Biochem*., 2001, **298**, 163–169.
25. STIRLING, D.P., K. KHODARAHMI, J. LIU, L.T. MCPHAIL, C.B. MCBRIDE, J.D. STEEVES, M.S. RAMER, W. TETZLAFF, Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury, *J. Neurosci*., 2004, **24**, 2182–2190.
26. TAFOYA, L.C., C.W. SHUTTLEWORTH, Y. YANAGAWA, K. OBATA, M.C. WILSON, The role of the t-SNARE SNAP-25 in action potential-dependent calcium signaling and expression in GABAergic and glutamatergic neurons, *B.M.C. Neurosci*., 2008, **9**, 105–122.
27. WIRTH, E.D., P.J. REIER, R.G. FESSLER, F.J. THOMPSON, B. UTHMAN, A. BEHRMAN, J. BEARD, C.J. VIERCK, D.K. ANDERSON, Feasibility and safety of neural tissue transplantation in patients with syringomyelia, *J. Neurotrauma*, 2001, **18**, 911–929.