Marija STANOJEVIĆ[*], Srdjan LOPICIC[*], Branko DJUROVIC[**], Ivan MIHALJEV[*], Svetolik SPASIC[*], Isidora ALEKSIC[*], Vladimir NEDELJKOV[*], Milica PROSTRAN[***]

AN ANIMAL MODEL OF EXPERIMENTAL EPILEPSY RESEARCH AT THE CELLULAR LEVEL

Abstract: The aim of this study is to present Retzius neurons of the leech Haemopis sanguisuga as a valuable animal model system for investigation of the cellular basis of epileptogenesis. We examined the effects of transitional metal nickel on intracellularly recorded membrane potential and electrical activity of these cells. Superfusion with Ringer solution containing $Ni2^+$ ions (3 mmol/l $NiCl_2$) disturbed spontaneous activity of Retzius cells, inducing epileptiform activity which consisted of rhythmic membrane potential oscillations in a form of repetitive paroxysmal depolarization shifts (PDSs). The induced epileptiform activity is considered essentially independent of chemical synaptic transmission, as it develops due to $Ca2^+$ channel blockade with $Ni2^+$. Several PDS parameters were measured to describe the induced epileptiform activity: PDS frequency, PDS duration and PDS amplitude. Possible cellular mechanisms underlying generation and termination of the nonsynaptic $Ni2^+$ – induced epileptiform activity of leech Retzius neurons are discussed.

Key words: *experimental epileptiform activity, leech Retzius neuron, nickel*

INTRODUCTION

Human epilepsy encompasses a wide variety of clinical manifestations, electrographic features and treatment responses. Long history of epilepsy research has brought forward a similarly wide array of experimental animal models, both in terms of species and experimental protocols used for induction of acute or chronic epileptiform activity. Although no single animal model can fully represent the condition given its specific diversity, different animal models of experimental ep-

[*] Institute for Pathological Physiology, School of Medicine, University of Belgrade, Belgrade, Serbia;

[**] Clinic for Neurosurgery, Clinical Center of Serbia, School of Medicine, University of Belgrade, Belgrade, Serbia;

[***] Institute for Pharmacology, Clinical Pharmacology and Toxicology, School of Medicine, University of Belgrade, Belgrade, Serbia

ilepsy can be chosen to match the adventages of the model with the particular research questions posed.

Retzius neurons of the leech are serotonergic neurons which show pacemaker activity in isolated leech ganglia (Rose et al. 2006). Although they are not intrinsically bursting, after exposure to different convulsive pharmacological agents they change their low-frequent spontaneous firing of single action potentials (APs) to generate paroxysmal depolarization shifts (PDSs). The PDSs are prolonged, large amplitude depolarizations of membrane potential superimposed by high-frequent bursts of APs, which represent the cellular basis of epileptic seizure activity (Goldensohn and Purpura, 1963; Prichard, 1972; Prince 1978). Generation of PDSs can readily be provoked in leech Retzius neurons by several exogenous agents: penicillin, bemegride and phenobarbital (Prichard 1972), neutral red (Lent & Frazer 1977), pyrethroids (Leake 1982), and FMRF-amide (Sahley et al. 1993). Rhythmical bursting activity can also be induced in these cells by low Ca^{2+} or by replacement of external Ca^{2+} by inorganic Ca^{2+} channel blockers, such are transitional metal ions: $Ni^{2+,}$ $Co^{2+,}$ $Mn^{2+,}$ $Cd^{2+,}$ Zn^{2+} and La^{3+} (Yang & Lent 1983; Dean & Leake 1988; Angstadt & Friesen 1991; Angstadt et al. 1998), as well as by low Cl^- saline (Beck et al. 2001). Therefore, epileptogenic conditions transform Retzius neurons into bursting cell oscillators that allow intracellular recording of acute epileptiform activity for several hours, thus enabling the study of cellular and ionic basis of epileptogenesis (Altrup, 2004).

MATERIAL AND METHODS

Preparation

The experiments were performed at room temperature (22–25 °C) on Retzius neurons in the isolated segmental ganglia of the ventral nerve cord of the horse leech *Haemopis sanguisuga*. The method of dissection has been previously described (Beleslin 1971), and complies with institutional research council guidelines. Dissected segments of 3–5 ganglia were immediately transferred to a 2.5 ml plastic chamber with leech Ringer solution and fixed by means of fine steel clips. The experimental chamber was then placed in a grounded Faraday's cage mounted on a fixed table in a manner that prevents vibrations. Identification and penetration of the cells were performed in the cage under a stereomicroscope. Retzius neurons were identified by their position on the ventral surface of the ganglion, their size and bioelectrical properties. To change the solution, the chamber was flushed with a volume of fluid at least 5 times greater than its own. The superfusion was usually completed in 10–15 s.

Electrophysiological recordings

The membrane potential was recorded using standard single-barrel glass microelectrodes. Micropipettes were pulled from glass capillaries with internal filament (Harvard Apparatus GC 150 F-10, UK) on a vertical puller (Narishige, Japan) and filled with 3 mol/l KCl. The microelectrode resistance was 20–35 MΩ. The recordings were amplified using a high input impedance amplifier (model 1090, Winston

Electronics, CA, USA; R=10^9 Ω). Microelectrodes were connected to the amplifier via a Ag-AgCl wire. The ground electrode was a Ag-AgCl wire in a separate chamber filled with Ringer solution connected to the experimental chamber by a 3 mol/l KCl – 3% agar bridge. The recordings were displayed on a two channel oscilloscope (Hameg, Germany) and permanently recorded on a pen recorder (Linseis, Germany), and a thermal graphic printer (Hameg, Germany).

Solutions

The standard Ringer solution (Ri) used in these experiments had the following composition (in mmol/l): NaCl 115.5, KCl 4, $CaCl_2$2, Na_2HPO_4 1.2, NaH_2PO_4 0.3 (pH=7.2). In the Ni^{2+} – containing solution (Ni^{2+} Ri) 3 mmol/l $NiCl_2$ was added.

Data analysis

All results are expressed as means±SEM, with *n* indicating the number of experiments. Comparisons between mean values were made using Student's *t*-test; *p* values of less than 0.05 were considered significant.

RESULTS

Epileptiform activity induction by nickel

In standard Ri solution leech Retzius neurons had a resting membrane potential (RMP) of –43.86±2.03 mV (n=6) and spontaneously fired single APs at a low rate of 0.34±0.08 Hz (Fig. 1A and 1B to the left). Superfusion of isolated ganglia with Ri solution containing Ca^{2+} channel blocker Ni^{2+} (3 mmol/l $NiCl_2$) induced a small transient membrane depolarization and the development of epileptiform activity. This activity was oscillatory and rhythmic, and characterized by repetitive generation of PDSs – large and prolonged plateau waves of depolarization superimposed by bursts of APs. Each PDS comprised several phases: depolarization, plateau and repolarization phase. The PDSs were followed by inter-PDS intervals which consisted of slowly depolarizing ramp potentials (Fig. 1A and 1B to the right). The PDSs developed gradually increasing in frequency, duration and amplitude, eventually to reach final level of stabilized epileptiform activity, which was sustained all throughout Ni^{2+} exposure. Nickel-induced PDSs had an average frequency of 5.32±0.42 min^{-1}, average duration of 4.84±0.18 s and average amplitude of 13.00±0.50 mV. Induced epileptiform activity was fully reversible, as regular spontaneous activity was restored upon superfusion with standard Ri saline.

DISCUSSION

It is generally considered that essential mechanisms of epileptic seizures are identical wherever they originate in human or animal nervous system. Major cellular event underlying the process of epileptogenesis is an increased neuronal excitability manifested by rhythmical PDS generation. Nevertheless, basic mechanisms underlying PDS generation are still incompletely understood. In most experimen-

tal models described so far Ca^{2+} is essential for rhythmicity, as generation of plateau depolarizations requires Ca^{2+} influx through voltage-gated Ca^{2+} channels. However, an increasing number of studies report of induction of epileptiform activity as a Ca^{2+} – independent and synaptically independent process, both on invertebrate and vertebrate neuron model systems. For example, a single mechanically isolated B 3 neuron of snail bucal ganglia generates PDSs when treated with an epileptogenic drug (Speckmann and Caspers 1973). Also, in single isolated Retzius neurons of *H. medicinalis* Ca^{2+} channel blockers induce Na^+ -dependent plateau potentials (Angstadt and Choo, 1996). Similarly, in rat hippocampal slices bursting discharges develop in low or zero Ca^{2+} solutions (Wang et al. 2004).

In the presence of Ni^{2+} in the perfusing saline Retzius neurons of the leech *H. sanguisuga* spontaneously develop endogenous oscillatory bursting activity (Pathak et al., 2009; Pathak et al. 2010). This epileptiform activity is an essentially nonsynaptic process, as it is induced under conditions of suppression of chemical synaptic transmission. The model thus represents an experimental model of the cell's own bursting rhythm. Nickel-induced PDSs were found to be Na^+ -dependent as they are completely eliminated in Na^+ –free saline and recover upon Na^+ readdition.

The oscillatory pattern of this PDS activity is probably maintained by the activation of the depolarizing ionic currents responsible of oscillatory cycle induction on one side, and the activation of repolarizing ionic currents responsible of cycle termination, on another, which alternate in a synchronized manner to establish a stable and regular nerve cell oscillator. Tonically active in Retzius cells are the two voltage-dependent inward ionic currents: a persistent Na^+ current (I_{NaP}) and a hyperpolarization-activated cation current (I_h) (Angstadt and Choo, 1996; Angstadt, 1999). Competing their effects is the major outward current in leech Retzius cells: Ca^{2+} -dependent K^+ current ($I_{K\ (Ca)}$), an important regulator of cell membrane excitability and activity pattern (Dean and Leake 1988; Stewart et al. 1989; Kleinhaus and Angstadt, 1995). Calcium channel blockade by Ni^{2+} eliminates tonic $I_{K\ (Ca)}$, thus causing membrane potential depolarization. This unmasks the effect of the opposing I_{NaP} current, leading to PDS initiation – the depolarizing and bursting plateau phase.

The mechanisms responsible of the PDS repolarizing phase are, however, less well examined. A conceptual model was proposed by Angstadt and Friesen (1991) suggesting that an increase in intracellular Na^+ concentration after a prolonged Na^+ influx consequently activates the Na^+/K^+ pump current ($I_{Na/K}$) that terminates the PDSs. In Retzius neurons of *Hirudo* ouabain in low concentrations decreases the frequency of this epileptiform activity, while in high concentrations it completely disrupts it (Angstadt and Friesen, 1991). However, it has been shown that in leech Retzius cells ouabain induces an increase in intracellular Ca^{2+} concentration due to the activation of voltage-dependent Ca^{2+} channels (Hochstrate et Schlue 2001) and cell swelling due to the uptake of NaCl (Dierkes et al. 2006). This prevents the use of this agent as a specific inhibitor of the Na^+/K^+ pump in the study of the mechanism underlying PDS termination. The reduced K^+ saline prolongs the induced PDSs, slowing the repolarization (Angstadt, 1998). Since in our model Ca^{2+} was not

ommited from the perfusing saline, nor was Ca^{2+} release from the internal stores blocked, it can be considered that $I_{K\,(Ca)}$ could still be involved to modulate the activity. Further investigation is required to enlighten the problem. The I_h is considered to contribute to the generation of rhythmic oscillatory activity by driving the slowly depolarizing ramp potential of the inter-PDS intervals.

To conclude, the mechanism of Na^+ -dependent Ni^{2+} -induced epileptiform activity in our model is probably closely related to the suppression of tonic $I_{K\,(Ca)}$ and a consequential prevalence of the I_{NaP}. We assume that it is probably driven by I_{NaP} and I_h, linked to the opposing currents: $I_{K\,(Ca)}$ and $I_{Na/K}$. The involvement of other outward K^+ currents in the PDS repolarization phase is also possible. A PDS-generating Retzius neuron of the leech provides a valuable tool for epilepsy research. Major advantage of the described animal model system is enabling the investigation of basic mechanisms of pathogenesis of acute epileptic seizures at a cellular level. Epilepsy research on this experimental model may help to clarify the intrinsic neuronal epileptogenic factors in general, and also facilite the testing of new potential antiepileptic drugs.

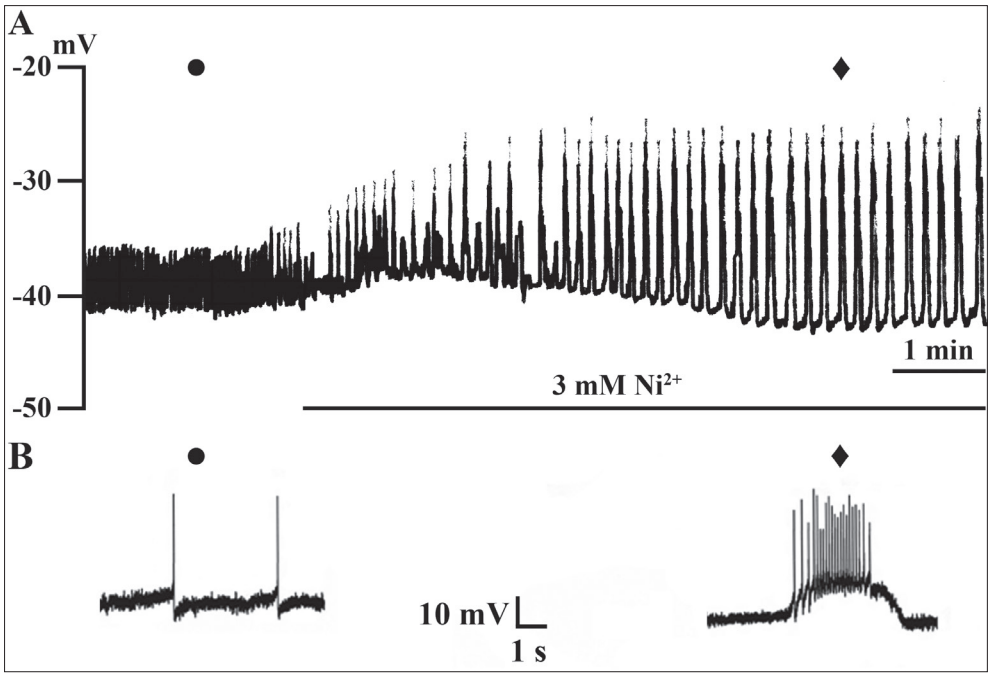

Figure 1. Induction of epileptiform activity in Retzius neurons of the leech H. sanguisuga upon superfusion with 3 mmol/L NiCl 2. A – pen recorder trace, B – oscilloscope printer recordings of representative points. In standard Ringer saline Retzius neuron fires single action potentials (•). In the presence of Ni 2+ cell develops epileptiform activity (♦) consisting of rhythmic and repetitive generation of paroxysmal depolarization shifts (PDSs). Because of the smaller timescale, each „spike" of the induced epileptiform activity on the pen recorder trace actually represents a single PDS.

ACKNOWLEDGEMENT

This work was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia, grant number 175023.

REFERENCES

[1] Altrup U. 2004. Epileptogenicity and epileptic activity: mechanisms in an invertebrate model nervous system. Curr. Drug Targets. **5(5)**: 473–484.
[2] Angstadt, J. D. 1999. Persistent inward currents in cultured Retzius cells of the medicinal leech. J. Comp. Physiol. A **184**: 49–61.
[3] Angstadt, J. D. and Choo, J. J. 1996. Sodium-dependent plateau potentials in cultured Retzius cells of the medicinal leech. J. Neurophysiol. **76**: 1491–1502.
[4] Angstadt J. D., Choo J. J., Saran A. M. 1998. Effects of transition metal ions on spontaneous electrical activity and chemical synaptic transmission of neurons in the medicinal leech. J. Comp. Physiol. A. **182**: 389–401.
[5] Angstadt J. D., Friesen O. W. 1991. Synchronized oscillatory activity in leech neurons induced by calcium channel blockers. J. Neurophysiol. **66**: 1858–1873.
[6] Beck A., Lohr C., Nett W., Deitmer J. W. 2001. Bursting activity in leech Retzius neurons induced by low external chloride. Pflügers Arch. **442(2)**: 263–272.
[7] Beleslin B. 1971. Effects of different external media on the leech ganglion cells interaction. Period. Biol., **73**: 63–67.
[8] Dean J. A., Leake L. D. 1988. Pharmacological control of the pattern of activity in leech Retzius neurones. Comp. Biochem. Physiol. C. **89(1)**: 31–38.
[9] Dierkes, P. W., Wüsten, H. J., Klees, G., Müller, A., Hochstrate, P. 2006. Ionic mechanism of ouabain-induced swelling of leech Retzius neurons. Pflügers Arch. **452(1)**: 25–35.
[10] Goldensohn, E. S. and Purpura, D. P. 1963. Intracellular potentials of cortical neurons during focal epileptogenic discharges. Sciences. **139**: 840–842.
[11] Hochstrate, P., Schlue, W. R. 2001. The ouabain-induced $[Ca^{2+}]_i$ increase in leech Retzius neurones is mediated by voltage-dependent Ca^{2+} channels. Brain Res. **892**: 248–254.
[12] Kleinhaus, A. L. and Angstadt, J. D. 1995. Diversity and modulation of ionic conductances in leech neurons. J Neurobiol. **27(3)**: 419–433.
[13] Leake L. D. 1982. Do pyrethroids activate neurotransmitter receptors? Comp. Biochem. Physiol. C. **72**: 317–323.
[14] Lent C. M., Frazer B. M. 1977. Connectivity of the monoamine-containing neurons in central nervous system of the leech. Nature **266**: 844–847.
[15] Pathak, D., Lopicic, S., Bratic – Stanojevic, M., Pavlovic, D., Andjus, P. R., Nedeljkov, V. 2010. Modulation of nickel – induced bursting with 4-aminopyridine in leech Retzius nerve cells. Arch. Biol. Sci. **62(4)**: 1035–1045.
[16] Pathak D., Lopicic S., Stanojevic M., Nedeljkov A., Pavlovic D., Cemerikic D., Nedeljkov V. 2009. Ethanol and magnesium suppress nickel-induced bursting activity in leech Retzius nerve cells. Gen. Physiol. Biophys. **28**: 9–17.
[17] Prichard J. W. 1972. Events in leech Retzius neurons resembling the „paroxysmal depolarizing shift". Neurology **22**: 439.
[18] Prince D. A. 1978. Neurophysiology of epilepsy. Ann. Rev. Neurosci. **1**: 395–415.
[19] Rose, T., Gras, H., Hörner, M. 2006. Activity-dependent suppression of spontaneous spike generation in the Retzius neurons of the leech Hirudo medicinalis L. Invert. Neurosci. **6(4)**: 169–176.

[20] Sahley C. L., Strong, J. A., Kleinhaus, A. L. 1993. FMRF-amide modulates the electrical activity of the leech Retzius cell. Neurosci. Lett. **164**: 37–40.
[21] Speckmann EJ, Caspers H. 1973. Paroxysmal depolarization and changes in action potentials induced by pentylenetetrazol in isolated neurons of Helix pomatia. Epilepsia. **14(4)**: 397–408.
[22] Stewart, R. R., Nicholls, J. G., Adams, W. B. 1989. $Na^{+,}$ K^{+} and Ca^{2+} currents in identified leech neurones in culture. J. Exp. Biol. **141**: 1–20.
[23] Yang J., Lent C. M. 1983. Calcium depletion produces Na-dependent, sustained depolarizations of Retzius cell membranes in leech CNS. J. Comp. Physiol. **150**: 499–507.
[24] Wang, T., Wang, J., Cottrell, J. E., Kass, I. S. 2004. Small physiologic changes in calcium and magnesium alter excitability and burst firing CA 1 pyramidal cells in rat hipppocampal slices. J. Neurosurg. Anesthesiol. **16**: 201–209.