Arch. Biol. Sci., Belgrade, 57 (3), 181-187, 2005.

MODIFICATION OF THE ACETYLCHOLINE-INDUCED CURRENT OF THE SNAIL *HELIX POMATIA* L. BY FAST TEMPERATURE CHANGES

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Abstract - Using the single electrode voltage clamp method, we found that acetylcholine (ACh) induces transient inward dose-dependent current on the membrane of the identified *Helix pomatia* Br neuron. We analyzed the effects of fast cooling and heating as well as thermal acclimation on the ACh inward current. The experiments were conducted on active and dormant snails acclimated to either 20 or 7^{0}C for at least four weeks. The Hill coefficient remained approximately 1 in all cases, which means that there is a single ACh binding site on the membrane. Fast temperature alternations induce binding affinity changes. In the work presented, we analyzed the effects of cooling on the ACh-induced inward current. The amplitude of ACh-induced inward current was markedly reduced after cooling, and the speed of decay of the ACh response was lower.

Key words: Acetylcholine, fast cooling, identified neuron, inward current, temperature, *Helix pomatia*

UDC 612:59(479.11-14)

INTRODUCTION

The ambient temperature is, along with humidity and the light-dark cycle, one of the most important abiotic parameters in the environment of the pulmonate snails. The nervous system plays an important role in the process of adaptation to different temperatures (Lagerspetz, 1974). Molluscan neurons proved to be a useful model for investigation of the cellular basis of neurophysiological processes, given the potential for identification of individual neurons from one animal to another. They have been used for studies of responses to temperature change for some time. Such studies investigations of the temperature dependence of sodium-potassium permeability ratio (Johnston, 1980) and the effect of the sodium pump on the resting potential (Airapetyan, 1969). The effect of temperature on the resting membrane potential has been demonstrated in numerous molluscan neurons (Kerkut and Ridge, 1962; Carpenter, 1967, 1970; Marchiafava, 1970). Adding to the relevance of this experimental model, there is evidence that thermosensitivity of firing patterns in molluscan neurons has behavioral consequences (Moffett and Wachtel, 1976).

The most notable feature of the Br neuron, located in the right parietal ganglion of *Helix pomatia*, is its bursting pacemaker activity pattern, which persists in the absence of any inputs to the cell and consists of bursts of action potentials separated by interburst intervals. The *Aplysia* homolog of the Br neuron, called R15 (Frazier *et al.* 1967), has been extensively studied in the past. This neurosecretory cell (Weiss *et al.* 1989) innervates the digestive gland sheet, pericardium, heart, and ganglionic artery (Rittenhouse and Price, 1985). The R15 neuron is important for regulation of osmotic balance (Jahan-Parwar *et al*. 1969), and it integrates various aspects of egg laying behavior (Alevizos, 1991). Moreover, it has been demonstrated that changes in activity of R15 affect hemolymph concentration of ions and metabolites (Bablanian and Treistman, 1985). As a neuron involved in various homeostatic mechanisms and reproductive behavior, it is a particularly interesting model for studying effects of temperature change on neuronal function.

Temperature increase causes a regular and reproducible increase in the frequency of the generation of pacemaker potential in most *Aplysia* and *Helix* neurons (Zečević and Pašić, 1981; Fletcher and Ram, 1990). Others have shown on *Aplysia* R15 (Wilson and Wachtel, 1974) and in our lab on the *Helix* Br neuron,

that its bursting pattern is temperature-sensitive and that the bursting stops with cooling to 10°C (Zečević and Pašić, 1981). The Br neuron shows its characteristic bursting activity only in the temperature range between 12 and 30°C. Outside this range, the burst pattern disappears and the action potentials become regular. As all of the above-described studies were performed on isolated and de-sheathed ganglionic complexes, it is particularly important that a reversible decrease in the frequency of bursts in response to transient change in ambient temperature has been shown for this neuron in the intact *Aplysia* animal as well (Treistman and Bablanian, 1985).

This neuron receives a large cholinergic excitatory input from the right visceropleural connective (Woodson *et al*. 1974) and, in addition, it is postsynaptic to cholinergic cardiovascular command interneuron L10 (Koestler *et al*. 1974). Also, it has been known for a long time that its bursting pattern is modified by acetylcholine, along with serotonin and dopamine (Frazier *et al.* 1967). We wondered whether cholinergic response of the Br neuron is affected by transient cooling and so possibly represents one of the mechanisms of fine-tuning of its firing pattern in response to change in temperature. After measuring ACh-induced inward current on the Br neuron at 20°C and 7°C, we found that amplitude of the response is reduced and decay of current slowed down with cooling.

MATERIALS AND METHODS

The experiments were carried out on the isolated circumesophageal ganglia of *Helix pomatia* acclimated at 20°C. The snails were collected locally, placed in a chamber at 20°C, and kept in an active state for at least 4 weeks before the experiment.

The ganglionic complex was isolated and de-sheathed according to the usual dissecting technique (Zečević and Levitan, 1980), installed in a temperature-controlled recording chamber, and continuously perfused with the snail solution. The snail solution consisted of the following (in mM): NaCl–80; KCl–4; $CaCl_2$–10; $MgCl_2$–5 and TRIS (hydroxymethylaminomethane hydrochloride) – 5; buffered to pH 7.8 (Kerkut *et al*. 1975). Antagonists were added hyperosmotically to the snail solution used for perfusion. Antagonists were obtained from: Sigma (atropine), Boehringer-Mannheim (pirenzepine), and Abbot Laboratories (tubocurarine). The recording pipettes, made from a glass capillary and filled with 1 M potassium citrate, were of low resistance (1-5 MΩ). A single electrode voltage clamp (SEC-2M, laboratory-designed at the Jozsef Attila University, Szeged, Hungary) was used for current and voltage measurements, together with a Tektronix 5113 oscilloscope and a I-V Plotter (Hewlett Packard, 7005B).

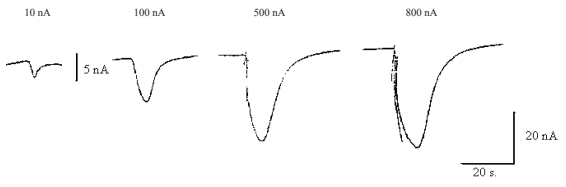

Fig 1a. Voltage clamp recordings of inward current induced by increasing ACh dose. Note the different scale for the response to the lowest dose

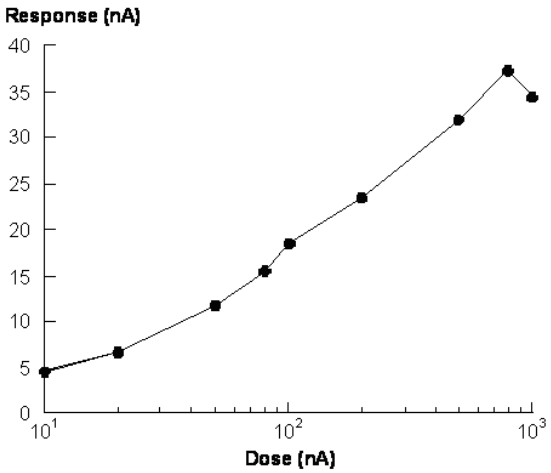

Fig. 1b. representative example of the amplitude of inward current as a function of the iontophoretic pulse strength used for application of ACh on the Br neuron. Points are connected with straight lines as a visual guide only. All recordings were made at a holding potential of –65 mV.

The Br neuron in *Helix pomatia* is analogous to R15 in the *Aplysia* abdominal ganglion (Frazier *et al*. 1967) and to cell A in *Helix aspersa* (Kerkut and Meech, 1967). To elicit a response to iontophoretic application of ACh, the neuron was carefully and completely freed of connective tissue. An extracellular electrode filled with 1 M ACh (160 WPI microiontophoresis programmer) was used to apply acetylcholine (ACh) iontophoretically. Positive current pulses of 3-s duration and various strengths (10-1000 nA) were used for neurotransmitter ejection. The tip of ACh-filled electrode was placed immediately above the cell body. A negative retaining current (10 nA)

was passed continuously through the iontophoretic pipette in order to prevent spontaneous diffusion of ACh from it.

To test the effect of temperature on ACh response, the dose-response curve was first established at the temperature of acclimation (20 °C). After establishing the dose-response curve for ACh induced current at 20 °C, the preparation was rapidly cooled to 7 °C and the effect of ACh was tested again. Cooling was completed in 7-10 minutes, the rate of temperature change being 1.3-1.8 °C/min.

The sample was washed with snail solution for five minutes between two doses of ACh. After the maximal dose (1000 nA), the sample was washed for another five minutes, and the minimal dose of ACh (10 nA) was applied again. In all of the experiments included in this study, responses of the Br neuron to the minimal dose of ACh at the beginning and at the end of experiment were equal, meaning that under all experimental conditions ACh-induced current was fully recovered from desensitization. The second control response to the minimal dose of ACh was diminished only on rare occasions when the neuron became leaky, as monitored by a decrease in membrane resistance (those experiments were discarded).

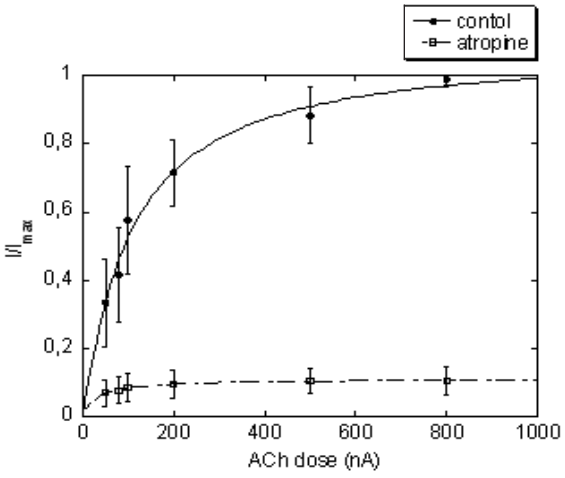

Fig. 2a. Effect of ACh antagonists on ACh-induced current at -65 mV. ACh dose-response curves in 0.1 mM atropine (n=4)

Hill parameters (n_h-Hill coefficient; K_d-dissociation constant) were obtained from dose-response curves by fitting them with $I/I_{max} = I_{max} [S]^n_h / (K_d{}^n_h + [S]^n_h)$, where I is the measured amplitude of current, I_{max} is maximal amplitude of current during the maximal response, and [S] is the concentration of ACh expressed as the dose (nA). Time constants of current decay were obtained by fitting the current traces with $f(t) = \Sigma I_i e^{-t/\tau i} + C$. Antagonist potencies were derived from dose-response curves using the formula $pA_2 = -\log K_d$. The term pA_2 is defined as the negative logarithm to base 10 of the antagonist concentration (expressed in molar units) corresponding to a dose ratio of 2 (i.e., the concentration that produces a twofold shift in the antagonist concentration-response curve).

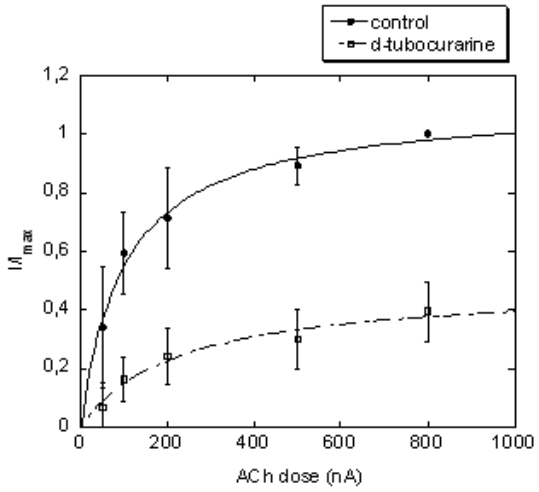

Fig. 2b. 0.1 mM d-tubocurarine (n=3)

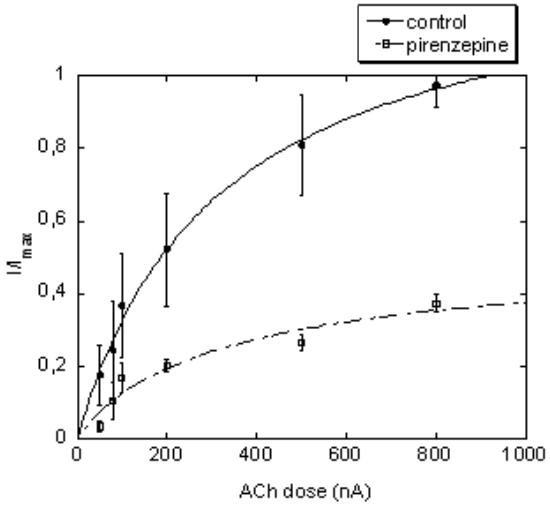

Fig. 2c. 0.1 mM pirenzepine (n=4) as a function of the iontophoretic pulse strength used for application of ACh. Data are normalized to maximal response without antagonist and presented as mean value ± SD. Control without antagonist (filled circles) and treatment with respective antagonist applied in the bath (squares). Lines represent the best fit as indicated by the data.

Graph Pad Prism software was used for statistical analysis. Two-way ANOVA with repeated measurements

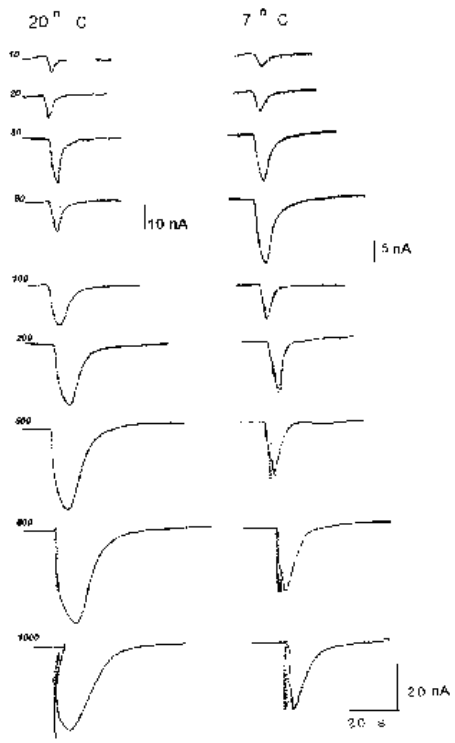

Fig. 3. Typical example of the effect of rapid temperature changes on the inward current induced by ACh. Holding potential – 65 mV. Doses of applied ACh are in nA. Note different amplitude calibration bars at 20°C and 7°C for low ACh doses.

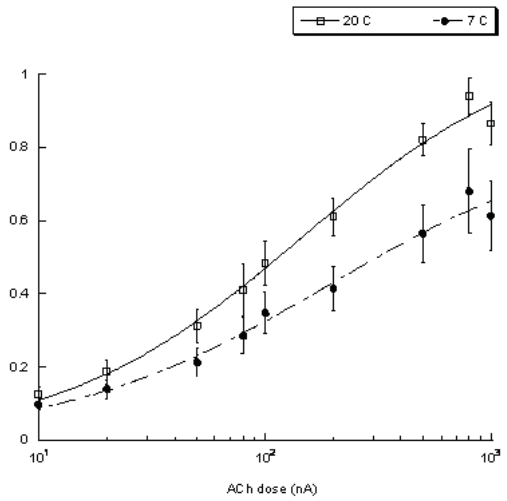

Fig. 4. Current amplitude normalized to maximal value of current at 20 °C as a function of the iontophoretic pulse strength used for application of ACh on the Br neuron (n=10). Data are presented as mean value ± SE. Lines represent best fits. Upper dose-response curve (filled squares, 20°C) before and lower (filled circles, 7°C) after cooling

was used for statistical evalution of the obtained data. All p values lower than 0.05 were considered to be significant.

RESULTS

A typical response to iontophoretically applied ACh recorded from the Br neuron is shown in Fig. 1a. Generally, the maximal response was obtained with 800-nA pulses, which elicited inward current with an average amplitude of 37.25 ± 6.8 nA (n=6) at -65 mV (Fig. 1b).

The effect of some ACh antagonists on the ACh dose-response curve is shown in Fig. 2. Atropine (2a), d-tubocurarine (2b), and pirenzepine (2c) all reduced size of the ACh response of the Br neuron. Neither of the tested antagonists abolished the response. Antagonist potencies expressed as pA2 values are given in Table 1. Atropine was more potent than d-tubocurarine, a specific nicotinic antagonist; pirenzepine, a specific antagonist of m1 receptors, was less potent then atropine, a general muscarinic antagonist.

The effect of cooling on ACh-induced current is shown in Fig. 3. To test the effect of temperature on ACh, the response was first established at the acclimation temperature (20°C). The preparation was then cooled to 7°C and the response to ACh was tested again. Cooling significantly decreased the ACh-induced inward current of the Br neuron ($p<0.001$). The response recovered upon rewarming (data not shown). The mean dose-response curves obtained at 20°C and after rapid cooling to 7°C are shown in Fig. 4. Data normalized to the maximal response at 20°C were subjected to nonlinear regression and comparison between models (a sigmoidal dose-response curve with variable slope and a polynomial of the first order) was performed.The sigmoidal dose-response curve was the preferred model, with p= 0,0011 (20°C) and p=0,0327 (7°C).

Comparison of dose-response curves at both temperatures indicates reduced reactivity of the Br neuron to ACh at low temperature (7°C). Neither the slope of the Hill curve fits of dose-response nor the apparent affinity of the receptor for ACh were affected by cooling, because the differences between parameters were not statistically significant ($p = 0.81$) (Table 2). The estimate of the Hill coefficient remained close to 1.

Next, we analyzed the effect of temperature on the

kinetics of decay of current activated by ACh. All currents were adequately fitted with a single exponential, as increasing the number of exponentials did not significantly improve the quality of the fit. As it can be seen (Table 3), increase of the iontophoretic pulse (from 100 to 800 nA) prolonged the decay by about 40%. Cooling the neuron increased the decay time constant (prolonged the decay) of the ACh response (also by approximately 40%), in the cases of both the 100- and the 800-nA iontophoretic pulses.

DISCUSSION

Our study demonstrates the effect of cooling on ACh-induced inward current of the Br neuron from warm (20°C) acclimated *Helix pomatia* animals. The electrophysiological and some pharmacological properties of the *Helix* Br neuron were described previously (Zečević *et al.* 1973).The data presented here show that changes in the amplitude of ACh-induced current are not induced by altered apparent affinity of the receptor for the antagonist, as shown by Kd (Table 2).The Hill coefficient nh is in both conditions around 1, and slopes of the dose-response curves at 20°C and at 7°C are not statistically different, suggesting that there is no change of cooperativity with cooling. However, we found that cooling slows the decay of ACh-induced current.

Previous experiments conducted in our lab have shown that cooling causes little or no change in membrane potential of the Br neuron of *Helix pomatia* (Zečević and Pašić, 1983). Data from the literature also suggest that cooling does not influence significantly the reversal potential of the ACh response in molluscan neurons (Katchman *et al.* 1980; Ger *et al.* 1979). We therefore focused on analyzing receptor properties in attempting to explain the observed effect of cooling on the ACh-induced current. However, we cannot exclude the possibility that some small change in the reversal potential of ACh-induced current might contribute to the observed effect of cooling on the amplitude of this current.

Similar observations before the effect of temperature reduction on ligand-gated currents have been reported before in the literature. For instance, in frog sensory neurons, GABA-induced current is temperature-dependent in the range of 5-35 °C (Ffrench–Mullen *et al.* 1988). This reduction of current with cooling cannot be attributed to changes in antagonist binding properties, but rather to subsequent channel activation. Temperature sensitivity of the decay of end-plate currents in frog skeletal muscle is well established (Kordas and Zorec, 1984; Peper *et al.* 1982). In that system, decreased activity of cholinesterase is proposed as a factor that contributes to prolongation of current decay with cooling. Although it is possible that reduction of cholinesterase activity with cooling plays a role in temperature modulation of synaptic transmission in intact snails, it cannot explain the observed effect of cooling on the current amplitude in our experiments: we see a reduction of current amplitude, whereas the expected effect of reduced cholinesterase activity is potentiation.

Evans and Carpenter (1989) and Salanky *et al.* (1989) described temperature sensitivity of ACh-induced currents in some *Aplysia* neurons.The responses they described had two components, whereas the ACh-induced current we recorded from the Br neuron is well fitted with a single exponential and has a speed of current decay intermediate between the currents described in *Aplysia*. Amplitude of the *Aplysia* ACh-induced current was practically not temperature-dependent in contrast to the significant reduction of current on the *Helix* Br neuron described in our study. Also, temperature sensitivity of the time constant of current decay of the "fast" *Aplysia* current was weak, whereas the time constant of current decay in our experiments increased with cooling, similar to that of the "slow" *Aplysia* ACh-current. It is worth noting that ACh-induced current on the *Helix* Br neuron and the "slow" component of ACh-induced current in *Aplysia* share another similar property: for both, the time constant of decay increased with an increasing dose of ACh.

Qualitative differences between the findings reported in the indicated studies on *Aplysia* neurons and ours can be attributed to differences between channels underlying the currents described in *Aplysia* and those in the *Helix* Br neuron. The ACh responses of molluscan neurons are classified into three basic types: slow, potassium-dependent; fast, chloride dependent; and fast, sodium dependent (Kehoe, 1972), but numerous pharmacological differences were reported between different species or even between responses on different identified neurons in the same species. Our experiments testing the effect of ACh antagonists, d-tubocurarine, and atropine showed that both antagonists lowered the amplitude of ACh-induced current, indicating the presence of a mixture of nicotinic and muscarinic properties of ACh receptor(s) on the Br membrane. At this point, we cannot be sure whether there are actually two types of ACh receptors (nicotinic and

muscarinic) on the Br neuron, or one type that is excitatory but shows "mixed" pharmacology. However, the effect of atropine was more pronounced suggesting that muscarinic pharmacology dominated. The response to ACh we recorded from the Br neuron is mediated mostly by sodium and to some extent by potassium (unpublished observations), while the ACh-induced currents described by Salanki *et al.* (1989) and Evans and Carpenter (1989) in *Aplysia* are chloride-dependent ("fast") and potassium-dependent ("slow") and are completely abolished by d-tubocurarine.

Cooling of the Br neuron reduces its spontaneous parabolic bursting activity, increases the threshold for action potential firing, and broadens the action potential, suggesting that some voltage-gated conductances or intrinsic membrane properties are affected by cooling (Zečević and Pašić, 1983). Our data demonstrate that cooling reduces the amplitude of ACh-induced current on the Br neuron of *Helix* pomatia by a mechanism that does not involve change in the apparent receptor affinity, while increasing the time constant of current decay.

Both physiological functions known to be influenced by Br neuron activity pattern, reproductive behavior and homeostatic mechanisms are affected by lowering of ambient temperature. In the present study, we examined the effect of cooling on one of the three known transmitter responses relevant for the modulation of the Br activity pattern (ACh, serotonin, and dopamine). Whether cooling affects the amount or the time profile of synaptically released ACh on the Br neuron is, to the best of our knowledge, unknown. Also, it is probable that Br is not the only neuron in the two circuitries (for reproductive behavior and homeostatic regulation) whose activity is modulated by cold temperature.

Finally, it is not known whether changes of the ACh response with cooling actually produce a physiological effect in the intact snail or whether they are compensated in some way. Until more data are gathered on these questions, it will be hard to understand the physiological mechanism acting on the neuronal level to allow the snail to adjust its reproductive behavior and homeostasis to changes in ambient temperature.

Acknowledgements: The present work was supported by the Ministry of Science of the Republic of Serbia, Grant No. 1636. The animals used for procedures were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985) and with the current laws in our country.

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МОДИФИКАЦИЈА ACh-ИНДУКОВАНЕ СТРУЈЕ БРЗИМ ПРОМЕНАМА ТЕМПЕРАТУРЕ КОД ПУЖА *HELIX POMATIA* L.

М. НЕДЕЉКОВИЋ, ГОРДАНА КАРТЕЛИЈА и ЛИДИЈА РАДЕНОВИЋ

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Употребом методе наметнуте волтаже установили смо да ацетилхолин (ACh) изазива дозно-зависну улазну струју на мембрани идентификованог Br неурона пужа *Helix pomatia.* Анализирали смо ефекте брзог хлађења и загревања као и термалне аклимације на ACh улазну струју. Експерименти су рађени на пужевима аклимираним на 20 и 7°C у току четири недеље. Hill-ов коефицијент је увек остајао око 1, што значи да постоји само једно место за везивање ACh на мембрани неурона. Аклимација на 7°C није мењала афинитет за везивање ACh, за разлику од аклимације на 20°C где је установљено повећање афинитета за везивање ACh на мембрани идентификованог Br неурона пужа *Helix pomatia.*