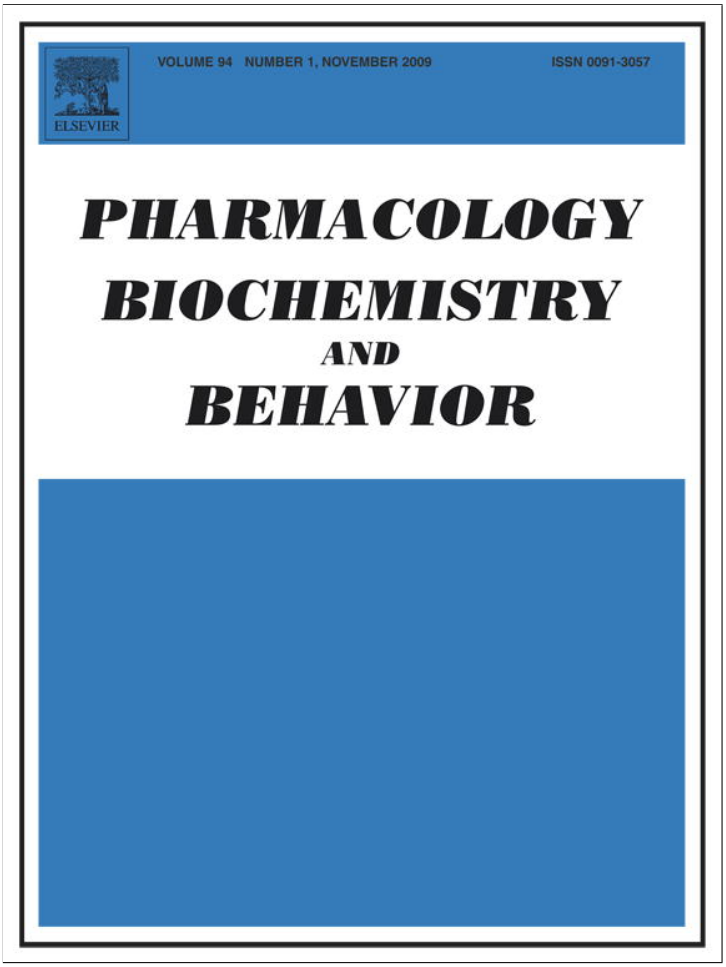

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Antibodies to calcium-binding S100B protein block the conditioning of long-term sensitization in the terrestrial snail

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ABSTRACT

The effects of antibodies to calcium-binding S100B protein diluted to 10^{-12} (LAS100B) on the long-term sensitization in the *Helix lucorum* snail (neurobiological model of the anxious-depressive state) were evaluated. The administration of LAS100B prior to conditioning of long-term sensitization in the terrestrial snail 10 min prior to the first electric stimulus) prevents strengthening of the defensive reaction of withdrawing the ommatophores (eye tentacles) and the defensive reaction of closing the pneumostome. This effect is termed "protective", as it prevents the conditioning of long-term sensitization. At the same time, snails given an injection of saline developed long-term sensitization with a significant strengthening of the defensive reactions of withdrawing the ommatophores and closing the pneumostome.

When LAS100B was administered before long-term sensitization in advance, the membrane and threshold potentials of premotor interneurons, which regulate defensive behaviour, decreased to a significantly lesser extent compared to the long-term sensitization arm. It is possible that the "protective" effect is linked to the mechanisms of maintaining the membrane potential and changes in extra- and intracellular balance of calcium-binding S100B protein.

1. Introduction

Calcium ions are involved in the regulation of various neuronal processes, which is due to their specific physicochemical characteristics that make them perfectly universal intracellular mediators responsible for the link between electrical events unfolding in the surface membrane, on the one hand, and the reactions developing inside the nerve cell, on the other hand. The exceptionally high capability of the intracellular environment to bind calcium ions is due to the presence of effective buffer systems in it, these systems being predominantly composed of calcium-binding proteins (Brini and Carafoli, 2000; Verkhratsky, 2005).

S100 is a superfamily of proteins characterized by the presence of calcium-binding domains and such fundamental properties as tissue (brain) specificity, evolutionary stability, and the capability to bind calcium ions by interacting with them. The available results imply that a physiological level of S100B protein expression inside the cell is a prerequisite for normal neuronal function, whereas increased or decreased concentrations of this protein will impair normal regulation of cell functions and the interaction with intracellular signaling systems (Donato, 2003; Gainutdinov et al., 2006; Santamaria-Kisiel et al., 2006; Scotto et al., 1998).

Studies of S100 protein's functions that employed antibodies to it (AS100) started very soon after it was first identified (Hyden and Lange, 1970; Karpiak et al., 1976; Shtark et al., 1981). It has been demonstrated that the results of AS100's action include the alteration of electrical characteristics of the neuron: inhibition of action potential generation accompanied by depolarization of the membrane and impaired function of calcium channels (Gainutdinov et al., 1996; Jankovic, 1985; Kubista et al., 1999; Nikitin et al., 2002; Shtark et al., 1981; 1987; Solnzeva, 1988). It has also been demonstrated that AS100 blocks the conditioning of long-term post-tetanic potentiation in the hippocampal slice (Epstein et al., 2003; Lewis and Tayler, 1986; Rebaudo et al., 2000).

The role of calcium ions is particularly important in view of their participation in the training and memory mechanisms (Hawkins et al., 2006), and therefore, our study included an attempt to modulate the development of certain behavioural alterations using antibodies to calcium-binding S100B protein with the aim to search for (identify) their possible "behavioural" effect. We chose a model of long-term sensitization in a higher invertebrate, the *Helix lucorum* terrestrial snail, which (i.e. the neurobiological model) can imitate (depending on impact parameters, i.e. electric stimuli in this case) non-species-specific signs of a stable pathological phenomenon that is homologous

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to chronic stress, depression, and anxiety disorders (Antzoulatos et al., 2006; Balaban and Bravarenko, 1993; Frost et al., 1985; Gainutdinov and Beregovoy, 1995; Kandel, 1976, 2006).

The reported study is a continuation of our long-standing efforts aimed to evaluate the role of neurospecific proteins, in particular S100B protein, in the function of neuronal systems of various organization complexity levels. Monospecific and monoclonal antibodies to S100 proteins have been and still are the major "instrument" of these studies (Epstein et al., 2003, 2006; Gainutdinov et al., 1996, 2006; Jankovic, 1985; Lewis and Teyler, 1986; Rebaudo et al., 2000; Shtark et al., 1981, 1987; Starostina et al., 1981, 1993), but they are now used according to the pre-conditioning paradigm that is new to us (Epstein et al., 2003; Jonas et al., 2001; Soriano et al., 2006).

2. Materials and methods

2.1. Conditioning of long-term sensitization

The experiments employed adult *H. lucorum* snails that were identical in body weight and size, and had been in an active state for at least 2 weeks before the experiment (Balaban and Bravarenko, 1993). The terrestrial snails had been anaesthetized before the preparation procedure by placing them in cold water with ice for a period of 20 to 30 min. Experimental procedures are in compliance with the Guide for the care and use of Laboratory animals published by the National Institutes of Health (USA).

The defensive reflex long-term sensitization was conditioned in accordance with the regimen used for *Aplysia* (Frost et al., 1985), the one we had used for the terrestrial snail (Gainutdinov and Beregovoy, 1995). The animals received an electric stimulus – rectangular current impulses with an amplitude of 6 to 8 mA, a duration of 10 ms, and a frequency of 50 Hz – to the head, 4 times per day, over 4 days, with an interval of 1.5 to 2 h. The duration of each impulse was half a second. During electric stimulation, the animals were placed on a copper electrode plate covered with a layer of paper moistened in water. The second electrode presented a metal rod applied to the snail's head. Control group animals underwent the same procedures as those in the main group, without electric stimulation. The long-term sensitization conditioning criterion was a statistically significant increment in the period of pneumostome closure as a response to the presentation of the test stimulation, as compared to the baseline reaction. Tests were carried out daily in both groups, before the series of electric stimuli were first delivered.

2.2. Experimental series and antibodies

Two series of experiments were conducted: before the series of electric shocks was started, half of the snails received injections (daily, into the sinus node) of antibodies to calcium-binding S100B protein in volume 0.1 mL; control group animals were administered with saline (in the same regimen and volume). During the long-term sensitization conditioning and for several days after it, the pneumostome closure and ommatophore withdrawal defensive reactions to the test stimulus were quantitatively evaluated. All behavioural reactions and locomotion velocity were analyzed after recording all reactions of the animals on video.

We used antibodies to calcium-binding S100B protein diluted to 10^{-12} (LAS100B), which corresponds to the concentration of 6×10^{-11} mg/mL. This dilution was performed in pharmaceutical company "Materia Medica Holding". Antibodies to calcium-binding S100B protein (AS100B) were provided by Dr S.M. Sviridov from the Institute of Cytology and Genetics of Siberian Branch of Russian Academy of Sciences (Novosibirsk). He with his collaborators in the 70s received "S100 protein from bovine brain tissue by means of thermal denaturation, ammonium sulphate fractionation, chromatography on DEAE-Sephadex A-50 and gel filtration through Sephadex G-100" (Maletskaja et al., 1976; Starostina et al., 1981). They purified the fraction of S100B protein which was the main part of received protein and produced monospecific immune serum by immunization of rabbits by S100B protein conjugated with bovine serum albumin. The specificity of the antibodies and their production was earlier described by the authors (Maletskaja et al., 1976; Starostina et al., 1981, 1993). We (laboratory of Prof. M.B. Shtark in Novosibirsk) began to use these antibodies in our investigations also in the 70s. AS100B were characterized in relation to the nerve tissue of snail (Shtark et al., 1981). By the indirect immunofluorescence technique (Dr V.I. Khichenko) it was found that "isolated neurons incubated in AS100B had a bright specific fluorescence which, in most neurons, was ring-like". By the method of rocket immunoelectrophoreses (Dr M.V. Starostina) it was shown that "the aqueous solution of snail nervous system did not form any precipitation peak, while the pentanol and Triton extracts (membrane fraction) reacted positively with the anti-S-100" (Shtark et al., 1981). Later Professor A. Hermann with collaborators examined the distribution of S-100-immunoreactive material in the central nervous system of snail, *Helix pomatia* (Kubista et al., 1996; 1999). They found that "S-100-like protein exclusively presented in neurons of the *Helix* central nervous system was correlated with spontaneous discharge activity of these cells and Western blots showed a single S-100-immunoreactive band at 12–14 kDa".

2.3. Testing

To measure the pneumostome closure reaction, a testing séance was performed, which consisted of several tactile stimuli of the same intensity delivered to the "parapneumostomal" region. The procedure took place in a dedicated installation that consisted of a water reservoir with a light polyethylene ball floating in it and a support with a bracket, with the mollusc shell fixed on it permanently (Balaban and Bravarenko, 1993). Snails moved around the ball freely thus rotating it, which allowed the objective registration of motions of the pneumostome and the ommatophores, as well as stimulation of any area of the animal's body. Duration of the pneumostome closure state following stimulation of the mantle cushion region with a brush hair was registered; such reaction is the initial component of defensive behaviour, and also allows to achieve its objective registration (Balaban, 1993).

Another parameter characteristic of animal defensive behaviour is the ommatophore withdrawal reaction; in this experiment, amplitude of withdrawal in response to tactile stimulation (touching one of the ommatophores with a brush hair) was measured, and the eye tentacle contraction was visually assessed and expressed as a percentage: the maximum length of the ommatophores corresponded to 100%, and the withdrawal amplitude was characterized (by 0%, 25%, 50%, 75%, or 100%) (Balaban and Bravarenko, 1993). By processing the video recordings, duration of the pneumostome closure and ommatophores withdrawal amplitude was measured with more precision. Finally, one-minute run velocity was measured in molluscs moving along the vertical wall of the rectangular glass terrarium: the start and the end of one-minute straight-line movement were marked on the glass using a marker for each study animal, and these distances were then measured (Pavlova, 2001). Besides, the locomotion velocity was analyzed after recording all reactions of the animals on video.

2.4. Preparation and electrophysiology

After the behavioural experiments were finished, the electrical characteristics of the LPa3, PPa3, LPa2, and PPa2 premotor interneurons responsible for defensive behaviour were registered (Balaban, 1993). To do that, the preparations obtained from intact, control (saline injected before long-term sensitization) and main (LAS100B injected before long-term sensitization) animals were employed. The analyses of electrical characteristics were carried out on an isolated preparation of the subesophageal ganglion complex. The terrestrial snails had been anaesthetized before the preparation procedure by

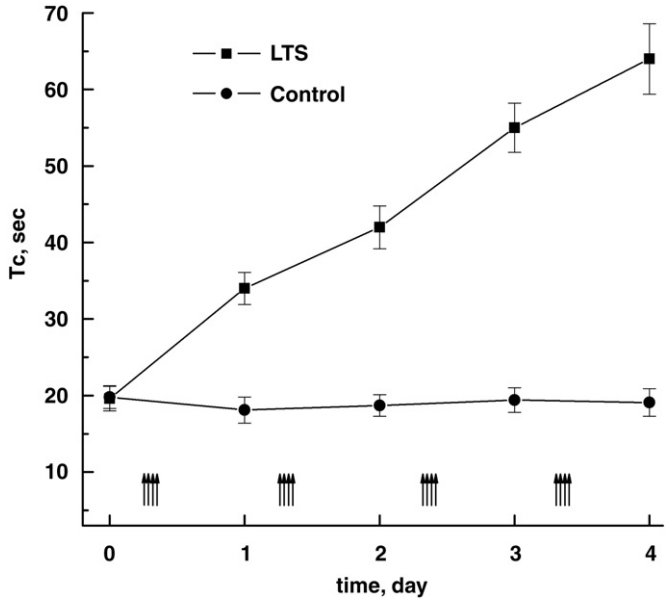

Fig. 1. Snail pneumostome behaviour changes during sensitization conditioning. LTS — sensitization group, Control — control group. Axis of ordinates — duration of pneumostome closure in response to tactile stimulation of the mantle (*Tc*, seconds); axis of abscissas — experiment's stages where 0 is the pre-long-term sensitization test, and 1 (2, …) are tests conducted at 1 (2, …) days after the start of long-term sensitization, respectively.

placing them in cold water with ice for a period of 20 to 30 min. These procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985), the UK Animals Scientific Procedures Act 1986 or the European Communities Council Directive of 24 November 1986 (86/609/EEC). Electrophysiological measurements were carried out at room temperature (20–22 °C), with an intracellular glass microelectrodes with a resistance of 5 to 40 MΩ and filled with 2.5 M potassium chloride solution. The experimental technique has been described in earlier publications (Gainutdinov and Beregovoy, 1995). Saline administered to the terrestrial snail contained the following substances (mmol/L): NaCl — 80, KCl — 4, $CaCl_2$ — 10, $MgCl_2$ — 5, and $NaHCO_3$ — 5; pH 7.6–7.8. In the course of the experiment, the membrane resting potential (Vm) and the action potential generation threshold (Vt) were registered.

The results were analyzed statistically: the mean values of measured parameters and standard error of the means ($M \pm$ SEM) were presented. One-way analysis of variance (ANOVA) and independent *t*-tests were used to make specific group comparisons. $p<0.05$ was established as the level of significance.

3. Results

3.1. Conditioning of long-term sensitization

To obtain a clearer picture of how the long-term sensitization conditioning procedure affects the behaviour of the animals, several parameters were examined simultaneously at the initial stage of the experiments. The animal behaviour observations carried out during the long-term sensitization conditioning demonstrate that snails become restless, move more, and eat less in the first two days, while in the subsequent two-day period their motor activity becomes significantly limited; spontaneous pneumostome closures are observed, but the pneumostome remains open most of the time, and this was the time when the testing séance was conducted.

The defensive behaviour testing carried out in the studied animal group during the long-term sensitization conditioning procedure demonstrated a gradual increment in the time of pneumostome closure in response to tactile stimulation (Fig. 1) ($t(48)=3.40$, $p<0.01$). Intact snails tested alongside the experimental group exhibited no behavioural changes. Checking the time of pneumostome closure changes revealed that the mean time of pneumostome closure began to differ from that of the control group with a high level of statistical significance as soon as after the first day of sensitization ($p<0.01$) ($t(48)=2.52$, $p<0.05$).

3.2. Effects of antibodies to calcium-binding S100B protein on the conditioning of long-term sensitization

The analysis of the pneumostome closure reaction period in response to tactile stimulation demonstrated that this parameter value increased with statistical significance upon long-term sensitization conditioning, as compared to the baseline level and the control group (Fig. 2A) ($t(34)=28.01$, $p<0.01$). The period of ommatophore retraction in response to tactile stimulation increased significantly as compared to the control group and the baseline level as well (from 15 s to 30 s) (Fig. 2B) ($t(28)=5.65$; $p<0.01$). The locomotion velocity decreased at the very beginning of the long-term sensitization

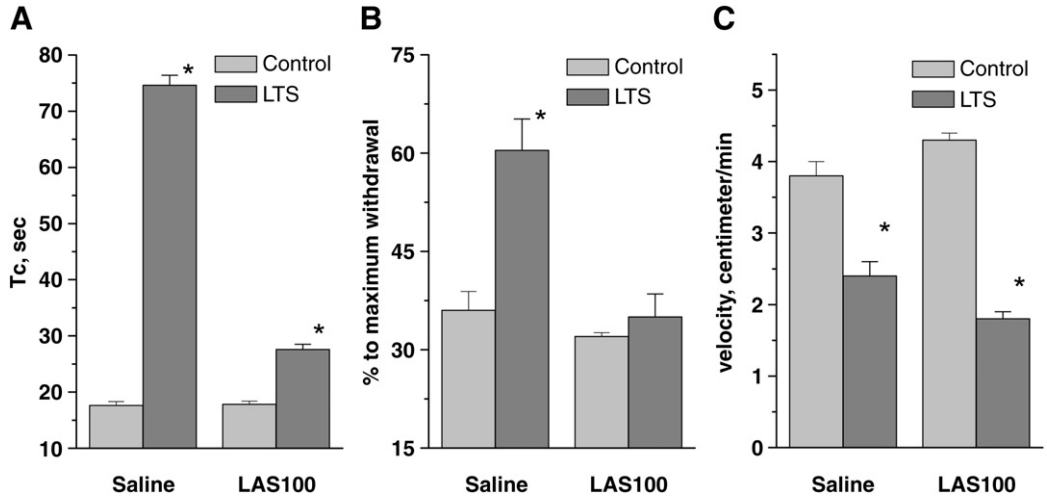

Fig. 2. Changes of the pneumostome defensive reaction (closure period) in response to tactile test stimulation, in seconds (A), of the ommatophore (eye tentacle) withdrawal defensive reaction in response to the test touch, in percentage of the maximum ommatophore retraction (B), of the locomotion velocity (for vertical locomotion along the terrarium wall), in seconds (C): saline — snails administered with 0.1 mL of snail saline, LAS100B — snails administered with antibodies to S100B diluted to 10^{-12} (in volume 0.1 mL of saline).

conditioning (a 1.3-fold reduction on the average) (Fig. 2C) ($t(24) = 2.65$; $p<0.05$). A saline injection given in advance, before the electric shock series, did not change the course of the long-term sensitization conditioning or the behavioural pattern of snails.

Preliminary administration of AS100 diluted to 10^{-12} to the terrestrial snail before the start of the long-term sensitization conditioning (10 min prior to the first electric stimulus) prevents the formation of the defensive reaction of withdrawing the ommatophores (eye tentacles) (Fig. 2B) ($t(22) = 1.22$; $p<0.05$) and significantly decreases the growth of the pneumostome closure defensive reaction as compared to control group snails (Fig. 2A) ($t(22) = 19.35$; $p<0.05$).

3.3. Electrical characteristics of premotor interneurons subject to long-term sensitization

The analysis of electrical characteristics of the LPa2, RPa2, LPa3, and RPa3 premotor interneurons (silent under normal circumstances), which are responsible for the defensive reaction, was carried out on the day following the day of the long-term sensitization conditioning. A typical response of these cells to the intracellular stimulation is presented in Fig. 3. Sensitized snails were demonstrated to be significantly more likely to generate action potentials in these cells as compared to control group animals; measurements of electrical parameters of premotor interneuron membranes demonstrate that the membrane resting potential and the threshold potential decrease by 5 to 8 mV in sensitized animals, as compared to control group animals (Fig. 4) ($t(48) = 3.58$, $p<0.01$ and $t(42) = 3.11$, $p<0.01$, for the membrane resting potential and the threshold potential, respectively).

3.4. Effects of antibodies to calcium-binding S100B protein on the electrical characteristics of premotor interneurons in long-term sensitization

Electrophysiological measurements demonstrate that the administration of saline before the series of electric shocks does not influence the long-term sensitization conditioning pattern: similarly to long-term sensitization, a depolarization shift of the membrane resting potential and a reduction of the threshold potential of premotor interneurons (by 5 to 8 mV in comparison with intact animals) were observed ($F1,52 = 16.93$; $p<0.01$ and $F1,52 = 10.65$; $p<0.01$, for the membrane resting potential and the threshold potential, respectively). When LAS100B was administered in advance before the long-term sensitization conditioning, the membrane and threshold potentials of the defensive behaviour-regulating premotor interneurons decreased to a significantly lesser extent as compared to the long-term sensitization (Fig. 5) ($F1,56 = 9.75$; $p<0.01$ and $F1,52 = 7.8$; $p<0.01$, for the membrane resting potential and the threshold potential, respectively). Therefore, the effect of antibodies to calcium-binding S100B protein on the long-term sensitization conditioning is accompanied by a partial recovery of the magnitudes of the membrane and threshold potential. It has been an important finding that control group animals administered with the LAS100B before hand had membrane and threshold potential values of premotor interneurons at the baseline control group level.

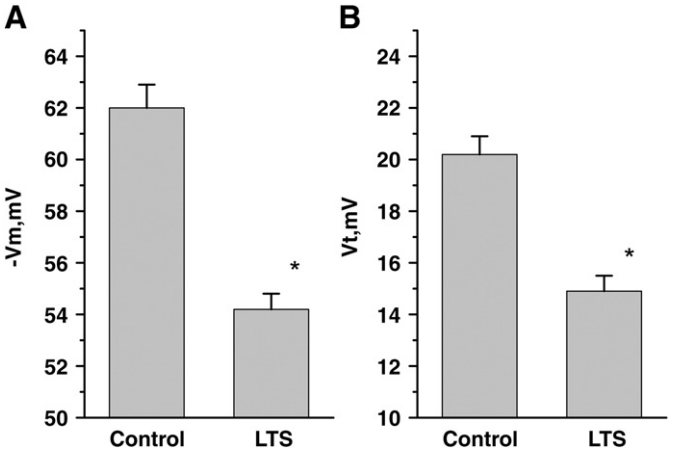

Fig. 4. Premotor interneuron membrane parameters: resting membrane potential (Vm) and action potential generation threshold (Vt). LTS — sensitization group, Control — control group. Axis of ordinates — potential, mV. *Statistically significant difference ($p<0.01$).

4. Discussion

So, antibodies to calcium-binding S100B protein exert a protective effect in the long-term sensitization conditioning process unfolding in the terrestrial snail, i.e. they prevent this process. In practical terms, the administration of LAS100B before long-term sensitization prevented the growth of the ommatophore (eye tentacle) withdrawal defensive reaction and significantly inhibited the conditioning of the pneumostome closure defensive reaction as compared to those snails that were administered with saline. The choice of the long-term sensitization phenomenon as a test model was due to a considerable experience available from the use of this anxiety and depressive state model, the presence of identified premotor interneurons that "manage" the defensive behaviour components, and the opportunities to conduct

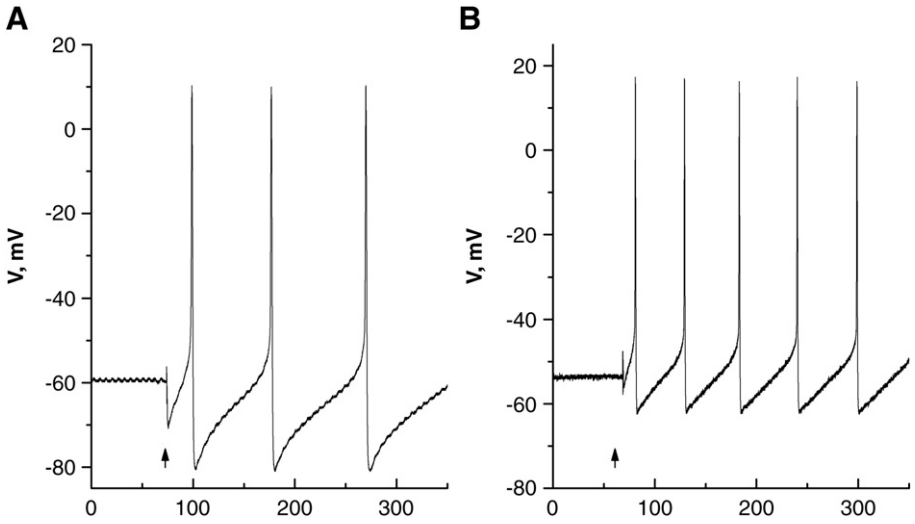

Fig. 3. Example of defensive reflex LPa2 premotor interneuron electrical activity. LTS — sensitization group, Control — control group. Axis of ordinates — potential, mV.

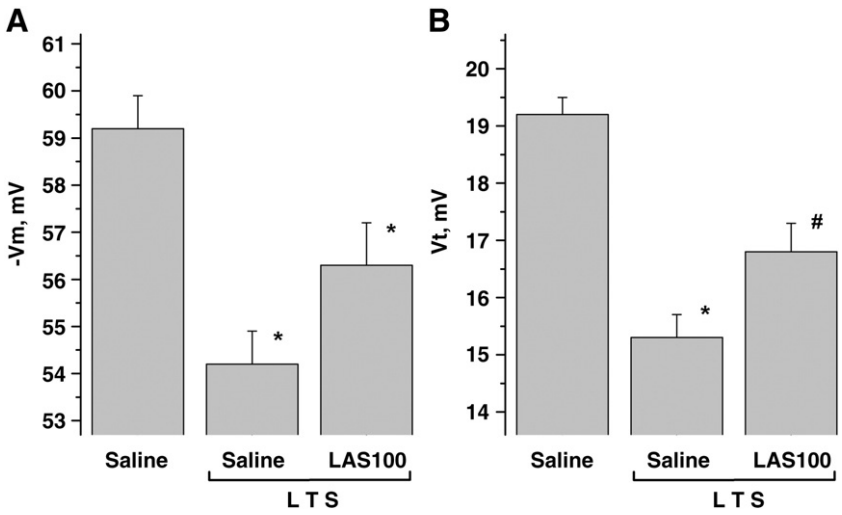

Fig. 5. Premotor interneuron membrane parameters: action potential generation threshold (Vt) and resting membrane potential (Vm). LTS — sensitization group, Saline — snails administered with 0.1 mL of snail saline, LAS100B — snails administered with antibodies to S100B diluted to 10^{-12} (in volume 0.1 mL of saline). Axis of ordinates — potential, mV. *Statistically significant difference ($p<0.01$).

rather accurate measurements of reactions from the efferent organs, i.e. the pneumostome and the eye tentacles. All of the above allow to reconstruct an almost perfect scenario of a stable "pathological" disorder of defensive behaviour and to assess the neuronal network that forms the basis of it (Antzoulatos et al., 2006; Frost et al., 1985; Gainutdinov and Beregovoy, 1995; Kandel, 1976).

Here are the following membrane mechanisms underlying long-term sensitization: 1. synaptic transmission efficacy changes (Dale et al., 1987; Frost et al., 1985; Walters, 1987); 2. changes in cAMP and K-current levels (Scholz and Byrne, 1988); 3. transcription-dependent augmentation in phosphorylation of some proteins (Sweatt and Kandel, 1989); and 4. depression of the action potential generation threshold in premotor interneurons and motor cells (Gainutdinov and Beregovoy, 1995). The analysis of volt-ampere characteristics of the premotor interneurons, produced by Dr. P.D. Lisachev (Beregovoi et al., 1992), has allowed to presume that the excitability of these network elements also grows as a result of faster expenditure of intracellular calcium buffer systems and increased low-threshold calcium conduction, the "target" of the preventive effect of antibodies to S100B.

It was predominantly the results of numerous experiments that demonstrated that LAS100B could exert anxiolytic and antidepressant effects in conflict situation experiments in rats (Loskutova et al., 2003). Other publications that have become available lately confirm this information (Castagne et al., 2008; Kheifets et al., 2007); both antihypoxic (survivability in hypoxia) and differentiating (neuritis-producing) effects of LAS100B have been discovered in C-1300 neuroblastoma culture tests (Pankova et al., 2007). These studies were the driving-force behind the attempt to use antibodies to S100B protein to modulate the development of certain behavioural changes that unfold during long-term sensitization, in order to search for (identify) the mechanism of the possible preventive effect (Gainutdinov et al., 2006). The search for preventive (protective) effects of various drugs in the pre-conditioning paradigm is in fact a commonplace now, with the glutamate and NMDA receptor literature prevailing in this discussion (Jonas et al., 2001; Soriano et al., 2006).

The reported results demonstrate the role played by membrane structures in the development of the effect of antibodies to S100B during the long-term sensitization conditioning. This conclusion is also supported by the data obtained earlier concerning the participation of S100B in the functioning of calcium-dependent potassium channels (Epstein et al., 2006; Kubista et al., 1999), the direct effect on calcium channels (Gainutdinov et al., 1996; Solnzeva, 1988) and the AS100-promoted calcium ion concentration changes (Shtark et al., 1981). Therefore, the improper balance of S100B protein caused by AS100B may result in an inhibition or alteration of the key mechanisms developing along with the formation of plastic readjustments in the body. The membrane and threshold potential reductions in premotor interneurons mentioned above, which are a reflection of the neurons' hyperexcitability, fit well in the conventional model. It makes sense to presume that the different types of test reactions of these elements during the long-term sensitization conditioning are a result not only of the state of the neuronal membrane of cell elements responsible for defensive behaviour but also of the specifics of the function of intracellular signaling systems (Scholz and Byrne, 1988; Sweatt and Kandel, 1989; Song et al., 2006). The activation of secondary messengers and the subsequent expression of genetic material result in alteration of the behavioural responses (anxiety and depression), which are the characteristics of long-term sensitization. For instance, only recently we discovered a role of the calcium-dependent MAP-kinase system-mediated signaling pathway in the manifestations of the effects of the long-term action of LAS100B (Epstein et al., 2007).

It is important to underline that S100B protein takes part not only in the functioning of intracellular signaling systems, but it can also act as a neurohormonal and neurotrophic factor, i.e. it can exhibit extracellular effects. An assumption was made that nanomolar concentrations of brain-specific extracellular S100B can exert regulatory influences, whereas the micromolar level of this protein is characterized by a destructive effect (Donato, 2003; Sen and Belli, 2007). Consequently, the preventive effect seen in a wide range of observations, including our own experiments, seems absolutely explainable as one which regulates the physiological range of S100B concentrations. It is just this sequence of extracellular and intracellular events underlying long-term sensitization that can be used to interpret the nature of anxiolytic and antidepressant effects of antibodies to S100B protein (Castagne et al., 2008; Kheifets et al., 2007; Loskutova et al., 2003), as well as their antihypoxic action (Pankova et al., 2007).

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