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EXPERIMENTAL ARTICLES

Seasonal Changes in Actin and Cdk5 Expression in Different Brain Regions of the Yakut Ground Squirrel (*Spermophilus undulatus*)

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Abstract—In this study we described the seasonal profile of the actin and Cdk5 levels in different brain structures of Yakut long-tailed ground squirrels (*Spermophilus undulatus*) during their annual cycle. Experiments were performed with adult Yakut ground squirrels, both male and female, weighing 600–800 g ($n = 35$) at different stages of their annual cycle, viz., in the summer, the period of the maximum activity of the animals; in the autumn, during preparation for hibernation; in the winter, during hibernation; in the spring, at the exit of the animals from the hibernating state. Our results indicate that actin mRNA increased by 1.9 times ($p = 0.0001$) in the frontal cortex, the hippocampus, and the caudal brainstem during hibernation. In the brainstem, a significant increase in actin mRNA started to develop in autumn, in normothermic animals at the stage of their preparation for hibernation ($p = 0.0078$). At the exit of animals from the torpid state, the level of hippocampal expression decreased significantly by 4.5 times; in the cortex and brainstem it decreased to the level of summer animals. In contrast, the dynamics in the cerebellum had opposite direction: actin mRNA level decreased significantly during the preparation for hibernation ($p = 0.037$), remained low in torpid animals ($p = 0.051$), and increased after awakening. The changes in the total protein level were observed only in the hippocampus, along with increased expression of Cdk5 mRNA during hibernation ($p = 0.003$) and at the exit from it ($p = 0.001$). Detected differences in the seasonal metabolic profile of cytoskeleton proteins in the hippocampus of Yakut long-tailed ground squirrels support a substantial structural plasticity of this brain structure during the hibernation cycle that was described previously in morphological and biochemical studies.

Keywords: hibernation, Yakut long-tailed ground squirrels, seasonal changes, actin mRNA expression, Cdk5 mRNA expression, total protein level

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INTRODUCTION

Hibernation is accompanied by a drastic lowering of the metabolism and is a form of adaptation to unfavorable environmental conditions [1]. In particular, during the hibernation the blood flow in the brain dramatically decreases, and EEG activity virtually disappears from most structures [2–4]. In addition, hibernating animals demonstrate plastic changes of neurons: dendritic arborization simplifies with decreasing body temperature, and quickly recovers when it increases [5–8]. The changes in the synaptic apparatus during hibernation resemble those that develop after sensory deprivation in neonatal rat pups. However, hibernation reduces the number of already developed and functioning synapses. The main feature of these changes is their complete reversibility and multiple repetitions during hibernation period. Changes that occur during hibernation are characterized by a high rate of development [6]. Changes in dendrites of pyramidal neurons in the hippocampal CA3 area of ground squirrels during hibernation are more pronounced than the changes in rats during pathology [9]. It has been shown that microstructural changes that have been revealed in the hippocampus are not specific for this area of the brain only and are part of a global phenomenon [10, 11]. A comparison of the changes that develop in hibernating animals in cell bodies and dendritic branches, and the density of spines in different areas of the brain (in the fourth layer of the somatosensory cortex, somatosensory relay neurons of the thalamus, and pyramidal neurons of CA3 hippocampal area) showed that all three cell types demonstrated similar statistically significant changes in their parameters in torpid animals. It has been found that the area of cell bodies significantly decreased by 35–40% ($p < 0.001$), the branching of basal and apical dendrites decreased by 20–25% ($p < 0.014$), and the density of the spines by 20–30% ($p < 0.001$) compared to euthermic animals [10]. The same

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authors have found that cytoskeleton rearrangement, leading to a reduction in the number of synapses and postsynaptic density, was accompanied by a decreased clustering of synaptic proteins. Researchers have suggested that hibernating animals develop a pool of synaptic proteins that can be quickly mobilized and used to build spines and synapses de novo [12]. During hibernation, a significant decrease has been shown in the level of protein synthesis in the CNS [13–15]. In active ground squirrels the level of protein biosynthesis varies between different parts of the brain: the highest levels of synthesis were detected in the hippocampus and hypothalamus, while the lowest levels were found in the spinal cord, cerebellum, cerebral cortex, and reticular formation [13, 16]. In hibernating ground squirrels, low-power methods (inclusion of L-[1-C^{14}] leucine into the frontal sections of the brain) failed to even detect protein biosynthesis. A more sensitive method (inclusion of L-[1-C^{14}] leucine in brain homogenate) detected the traces of protein biosynthesis: 0.04% of the protein synthesis rate in the brain tissue of active animals [13]. Elongation of proteins in the brain occurs slowly during hibernation [13]. Inhibition of elongation that occurs in the brain of ground squirrels during hibernation is probably associated with regulation of protein degradation.

Actin is a major structural protein of the cell. Analysis of its expression is informative, since it reflects the total structural changes that occur in the CNS cells. Cdk5 protein in the nervous system regulates the activity of the proteins specifically associated with neuronal morphology, cytoskeleton, cell motility, and plays an essential role in the neurodegenerative process [17, 18].

The problem of hibernation is relevant not only from a fundamental, but also from a practical point of view: the results of research in this field can be used to develop the methods of naturally regulated hibernation [19–21]. These methods appear to be extremely clinically important, especially for patients who are maintained in a state of reduced physiological functions for a certain period of time, e.g., in the treatment of stroke, epilepsy, or neonatal asphyxia by cooling the injured parts of the brain [22–30]. Furthermore, the hibernation of mammals can serve as a model of the hypoxic resistance of nervous tissue, as well as a neuroplasticity and neuroprotection model in cerebral pathologies [13, 25, 31–33]. Moreover, it has been found that the infusion of the blood-plasma albumin fraction of hibernating marmots to mice after cerebral ischemia that was induced by occlusion of the middle cerebral artery exerts a neuroprotective effect [34].

In this regard, the aim of our study was to describe the seasonal characteristics of the expression of actin and Cdk5 protein in several brain structures of the long-tailed ground squirrel (*Spermophilus undulatus*) during its annual life cycle.

METHODS

Experimental animals. The experiments were performed with adult Yakut long-tailed ground squirrels (*Spermophilus undulatus*), both male and female, that weighed 600–800 g ($n = 35$) at different periods of the annual cycle. The animals were caught in Yakutia and then kept in individual 35 × 40 × 20 cm cages. Dry food, fresh vegetables, grass, and water were available ad libitum. In mid-October the animals were placed into a special chamber, where they entered hibernation at an air temperature of approximately 0°C and remained dormant until complete awakening in mid-April. At the time of the experiments the animals were transferred to a laboratory room with a constant temperature of 20°C.

The experiments were carried out with four groups of animals that were taken at different phases of their annual cycle:

Group 1 was summer active animals ($n = 8$, $t = 37$–38°C, from mid-June to the beginning of July);

Group 2 was autumn animals that were preparing for hibernation ($n = 7$, $t = 36$–37°C, October to the beginning of November);

Group 3 was winter animals taken on the 6th to 7th day of hibernation ($n = 10$, $t = 1$–2°C, the end of January to the beginning of February);

Group 4 was spring animals that were taken at the spring awakening on the 6th to 7th day after hibernation ended ($n = 10$, $t = 31$–32°C, March).

The experiments were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Strasbourg, March 18, 1986.

Tissue pretreatment. Immediately following decapitation, the brains were removed, cooled in ice-cold isotonic NaCl solution, and the frontal cortex, cerebellum, hippocampus, and brainstem were isolated on ice according to the coordinates that were given in [35]. The samples were frozen and stored in liquid nitrogen. The tissue was homogenized at 4°C in a Potter homogenizer at 1500 rev/min for 15–20 s in HEPES buffer (20 mM, pH 7.4) containing 5 μg/mL of aprotinin, pepstatin, leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA, and 1 mM dithiothreitol. Homogenates were then centrifuged for 30 min at 13000 rev./min and 4°C (Biofuge 15R centrifuge, Heraeus, Germany). The supernatant was transferred to another tube and used to determine the protein expression. Precipitates were mixed with Trizol at 1 mL for the cerebral cortex and 0.5 mL for other parts of the brain, and kept frozen at –40°C.

Determination of the total protein concentration. The total protein concentration was determined using the Bradford method [36]. Ten microliters of the supernatant at an optimal dilution were mixed with 500 μL of 0.01% solution of Coomassie bright blue G-250 dye and incubated for 15 min at room tempera-

ture. Thereafter, the absorbance at 595 nm was measured. Bovine serum albumin in the concentration range of 0.1–0.8 mg/mL was used as a calibration standard.

Western blot analysis of the Cdk5 level. Proteins from the supernatants of different brain structures were subjected to electrophoretic separation under denaturing conditions on a 4–10% SDS-polyacrylamide gel using a Mini-Protean II apparatus (Bio-Rad, United States). After separation, the proteins were transferred to a PVDF membrane using Immun-Blot transfer chamber (BioRad, United States). Non-specific binding sites of membranes were blocked with a 5% solution of non-fat milk in a buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween-20. The membranes were incubated with primary polyclonal antibodies against Cdk5 (C-8, Santa-Sruz Biotechnology, United States) diluted at 1 : 1000 ratio in blocking solution at 4°C overnight. After washing, the membranes were incubated with secondary goat anti-rabbit IgG conjugated with horseradish peroxidase (BioRad, United States). Binding of the secondary antibodies was visualized on X-ray films using the ECL chemiluminescent system (Thermo Scientific, United States) and densitometric analysis of detected protein bands was performed.

Analysis of Cdk5 mRNA expression. The precipitates that were obtained after centrifugation of homogenates were used for isolation of the total RNA. For this purpose, pellets were resuspended with Trizol; each sample was divided into two halves and then processed in duplicate. The parallel samples were adjusted to 1 mL of volume with Trizol, carefully resuspended, mixed with 200 μL of chloroform, and stirred for 10–20 seconds. The samples were then incubated for 15 min at room temperature and centrifuged (using a Biofuge 15R centrifuge, Heraeus, Germany) for 15 minutes at 12000 rpm and 4°C. The resulting aqueous fraction was collected from each sample and transferred to a clean tube. An equal volume of isopropanol (500–700 μL) was then added, the samples were stirred, incubated for 10 min at room temperature, centrifuged for 10 minutes at 12000 rpm at 4°C, and the supernatant was completely removed. To wash the resulting precipitate of total RNA, we added 900 μL of 75% ethanol and mixed (top–bottom) and then the solution was centrifuged for 5–10 minutes at 12000 rpm to precipitate RNA. We pipetted off the entire volume of ethanol and centrifuged the tube again for 1–2 minutes at 5000–8000 rpm to collect the remaining ethanol from the bottom and wall of the tube for its subsequent complete removal.

To clear RNA the precipitate pellet was dissolved in 80 μL of sterile water, mixed with 40 μL of 7.5 M LiCl, incubated for 15–30 min at 0°C, and centrifuged for 15 minutes at 12000 rpm 4°C. After removing the supernatant, the RNA precipitate was washed first with 75% and then with 96% ethanol as described above. Finally, RNA was dissolved in 30 μL of sterile RNAse-free water.

The concentration and purity of extracted RNA was determined by measuring the absorbance at 260 nm and 280 nm on a spectrophotometer (Ultrospec II, LKB, Sweden). The average concentration of the isolated RNA was 1.6 mg/mL, and the average purity was 1.6. The RNA samples were frozen and stored at –80°C.

The reverse transcription reaction was performed using set of reagents for complementary DNA synthesis with Moloney murine leukemia virus (M-MLV) reverse transcriptase and random hexameric primers according to the manufacturer's instructions (Silex, Russia).

Primers for the polymerase chain reaction (PCR) were chosen in order to correspond to the parts of the proteins that are most conserved among mammalian species using the rat, mouse and human nucleotide sequences of these proteins [37], since the genome of *Spermophilus undulatus* has not been sequenced completely as yet. The primers were obtained from the Synto company (Moscow, Russia). The nucleotide sequences of the primers used are presented below:

Actin F: gag-gag-cac-ccy-gtg-ctg-cts-acc-gag-gcc-cc

Actin R: agg-tcc-cgg-cca-gcc-agg-tcc-aga-cgc-agg-atg-gc

Cdk5 F: act-gtg-ttc-aag-gct-aaa-aac-c

Cdk5 R: caa-ttt-caa-ctc-ccc-att-cc

PCR was performed in a volume of 25 μL using reagent kits from the Silex and Synthol companies (Russia). PCR products were introduced into an agarose gel containing ethidium bromide; they underwent electrophoresis at 100 V for 30–40 min. To evaluate the intensity of band luminescence in the gel after electrophoresis, it was photographed and digitized using TotalLab software.

Statistical data processing. Statistical analysis was performed using Excel 2003 and Statistica 8.0 software. Correlations were calculated by Spearman method. The results were presented in graphic form using Sigma Plot 11.0 software.

RESULTS

Actin. Actin is a major structural protein of the cell. Analysis of its expression is informative, since it reflects the total structural changes that occur in the cells of the brain. Throughout the entire annual cycle of the Yakut ground squirrels, the level of actin mRNA expression was significantly higher in the hippocampus than in the other investigated parts of the brain. Thus, in active animals its value in the hippocampus exceeded that of the frontal part of the neocortex and brainstem by a factor of 5 ($p = 0.002$) and in the cerebellum by a factor of 2.3 ($p = 0.0008$). Figure 1 demonstrates that the level of actin mRNA expression in the

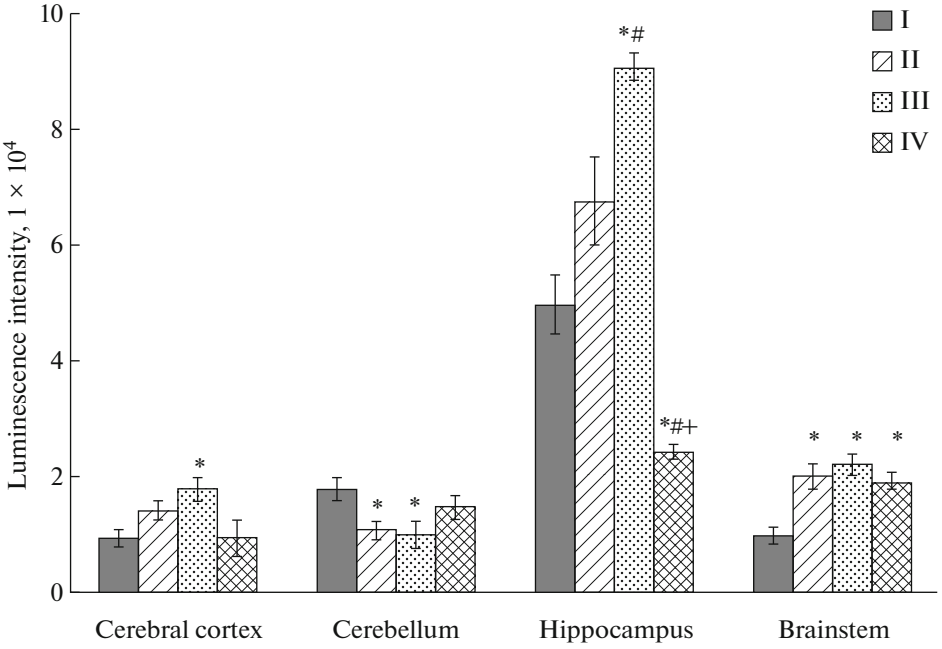

Fig. 1. The seasonal changes in actin-mRNA expression in different brain regions of Yakut ground squirrels. Experimental groups: I, group 1 (summer, $n = 8$); II, group 2 (autumn, $n = 7$); III, group 3 (winter, $n = 10$); IV, group 4 (spring, $n = 10$). *Difference from group 1; # difference from group 2; + difference from group 3 (Explained in the text).

hippocampus began to increase already in normothermic animals at the stage of preparation to hibernation and reached its maximum during hibernation. In torpid animals the level of actin mRNA expression was 1.9 times higher than in active animals ($p = 0.001$). In the spring, during the exit of ground squirrels from hibernation, the expression of actin mRNA dramatically decreased by 4.5 times compared with the torpid state ($p = 0.001$), and its level was significantly lower ($p = 0.001$) than in active summer animals (Fig. 1).

Changes of actin mRNA expression in the frontal part of the neocortex and the caudal brainstem resemble those in the hippocampus. In the cortex its level increased during hibernation by 1.9-fold ($p = 0.046$), while during the exit from hibernation it decreased to the level of summer animals.

In the brainstem, a significant increase in actin mRNA expression level was observed not only in winter torpid animals ($p = 0.0014$), but also in autumn normothermic animals during their preparation for hibernation ($p = 0.0078$). In contrast to these structures, the mRNA actin expression in the cerebellum changed in the opposite manner: its level decreased significantly during preparation for hibernation ($p = 0.037$), remained low in torpid animals ($p = 0.051$), and increased slightly during awakening.

Thus, the analysis of seasonal changes of actin mRNA expression in various brain regions of hibernating animals showed that its level is the highest in the hippocampus throughout the entire hibernation cycle as compared to other brain structures. However, the extent of changes of this indicator during hibernation in comparison to the summer period was comparable in magnitude in the cortex, brainstem, and hippocampus (Fig. 1).

The total protein content. The temporal profile of annual changes of the total protein level was similar in the hippocampus and the frontal cortex of hibernating animals (Fig. 2). In the autumn, during the preparation for hibernation, an increase in total protein levels was observed in normothermic animals in these structures, which was significant in the hippocampus ($p = 0.011$) and present as a trend in the cerebral cortex. A further increase in the total protein level in the hippocampus was observed when the body temperature decreased to 10°C but even more during the hibernation at a body temperature of 1–2°C, in comparison with the summer period ($p = 0.001$). In the frontal neocortex of ground squirrels the changes in total protein concentration during the annual cycle had a similar, although less pronounced pattern, and were present only as a trend.

At the entrance of animals into the hibernation state, during a body temperature decrease to 10°C, a significant decrease in the total protein level in the cerebellum ($p = 0.028$) and brainstem ($p = 0.021$) was revealed compared with the summer period (data not shown). In the winter, during hibernation, reduction in the total protein concentration in the brainstem remained significant ($p < 0.0099$) in comparison with

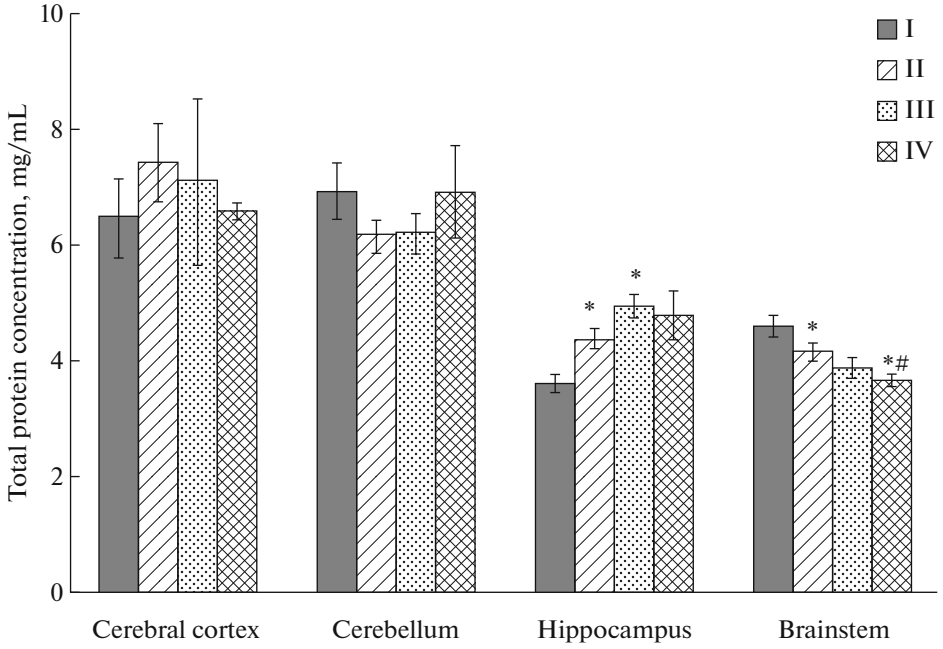

Fig. 2. Seasonal changes in the total protein level in different brain structures of Yakut ground squirrels. The designations are the same as in Fig. 1. *Difference from group 1; # difference from group 2 (Explained in the text).

the active summer period (Fig. 2). A similar temporal profile of the total protein level was demonstrated in the cerebellum; however, it was expressed there only as a trend. During the exit of the animals from the torpid state in the spring, the total protein level remained low in the brainstem, while in the cerebellum it tended to rise.

Thus, our data suggest that in Yakut ground squirrels the hippocampus is the only structure where a significant increase in the total protein concentration was revealed during the hibernation.

Cdk5. The highest level of Cdk5 mRNA in the brain of ground squirrels was measured in the frontal part of the neocortex and the caudal part of the brainstem; an intermediate level was found in the cerebellum, and the lowest level was found in the hippocampus (Fig. 3). If calculated without correction to actin, the Cdk5 mRNA expression was also low in these structures.

The seasonal changes in Cdk5 mRNA expression affected all parts of the brain. In the cerebellum they were manifested as a significant decrease at the stage of preparation for hibernation ($p = 0.028$), during hibernation ($p = 0.041$), and at awakening ($p = 0.021$). A similar seasonal profile of Cdk5 mRNA expression was observed in the frontal cortex and the brainstem; however, in these structures it was less evident, only in the form of a trend. An opposite profile of changes was found in the hippocampus: a significant increase in cdk5 mRNA during hibernation ($p = 0.003$) and awakening of animals ($p = 0.001$).

The changes in the levels of Cdk5 protein and mRNA differed both in their seasonal dynamics and in the ratio in the analyzed brain regions. A decrease in Cdk5 mRNA expression ($p < 0.021$) was accompanied by increased Cdk5 protein level in the cerebellum at the stage of spring awakening (Fig. 4).

A comparison of the data that are presented in Figs. 3 and 4 demonstrates that Cdk5 mRNA was higher in the frontal cortex, cerebellum and caudal brainstem than in the hippocampus but Cdk5 protein level in these regions was, in contrast, lower than in the hippocampus.

DISCUSSION

Our results indicate the presence of the profound changes in the expression of actin and Cdk5 protein in different brain regions of Yakut long-tailed ground squirrels (*Spermophilus undulatus*) during their annual lifecycle. We found seasonal and structure-specific changes in activity of these proteins. The expression of Cdk5 and actin in the hippocampus was significantly different from other brain regions. These differences confirm the pronounced structural plasticity of the hippocampus in the hibernation cycle, which was reported previously in morphological and biochemical studies [6, 7, 37–41]. On the other hand, Cdk5, and particularly its complex with p39 or p35 activators, is involved in the regulation of the actin cytoskeleton dynamics in neurons and the formation of functionally active synapses.

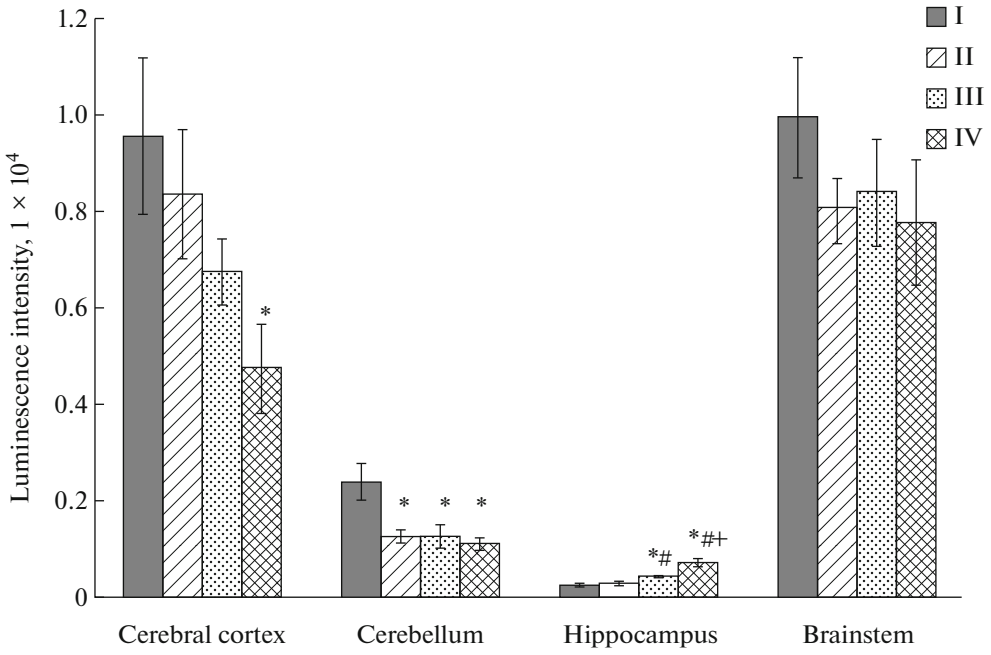

Fig. 3. Seasonal changes in the Cdk5 mRNA level in different brain structures of Yakut ground squirrels. The designations are the same as in Fig. 1. *Difference from group 1; # difference from group 2; + difference from group 3 (Explained in the text).

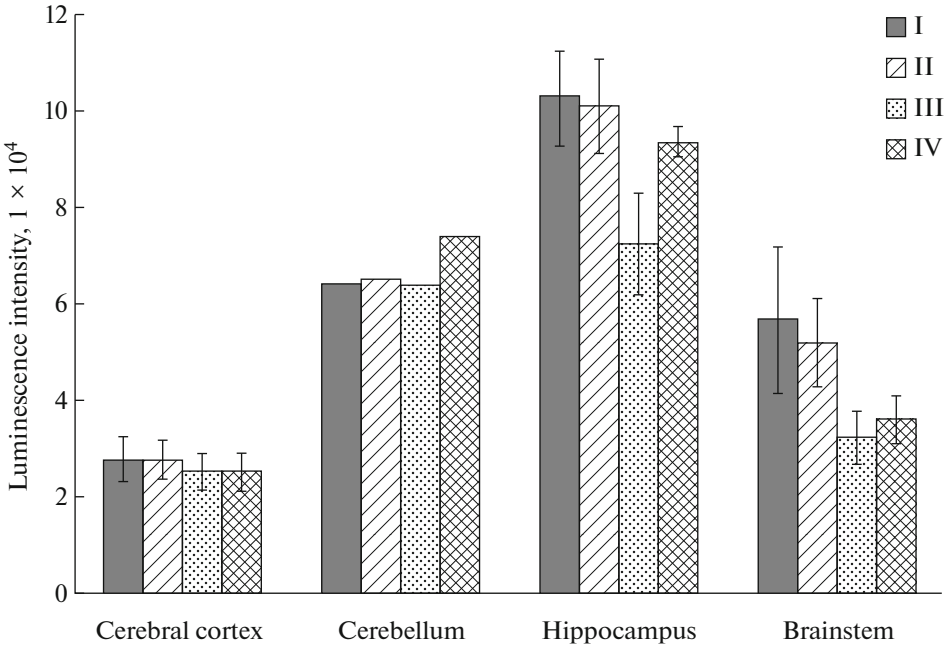

Fig. 4. Seasonal changes in the Cdk5 protein level in different brain structures of Yakut ground squirrels. The designations are the same as in Fig. 1.

Actin is a major structural protein of the cell. Analysis of actin expression is informative since it reflects the global structural changes in the brain. We have found that actin mRNA expression was the highest in the hippocampus, being 3–4 times higher than in other analyzed brain regions, viz., the frontal cortex, the cerebellum, and the caudal brainstem. All this indicates the pronounced reactivity and the special role of the hippocampus in the processes that occur during hibernation and is consistent with the literature data concerning the extreme abilities of the hippocampus for plastic modifications in different situations

[42, 43]. Perhaps, the special role of the hippocampus during hibernation consists in the fact that, despite structural changes that were similar to those that have been detected in other brain regions [10–12], the hippocampus nevertheless retains EEG activity and regulates the process of hibernation [1, 3, 4, 43].

The peculiar seasonal changes in the expression of actin mRNA in the hippocampus of hibernating animals is consistent with the observation that in homeothermic animals this structure can undergo plastic changes under various pathological conditions, such as ischemia [13, 14, 24, 26–33, 44] and epilepsy [45, 46]. Our findings are also consistent with the results of morphological studies that have shown that during hibernation a strong reduction of the dendritic system, which is accompanied by a reorganization of the cytoskeleton, occurs in the hippocampus [5, 6, 8, 10].

Increased hippocampal expression of mRNA of such a widely represented protein as actin at the onset of hibernation does not influence the total protein level, which may imply that synthesis of new actin molecules is accompanied by degradation of existing fibrils. These changes may underlie the cytoskeleton reorganization that has been reported by other researchers [5, 6]. A decreased level of actin mRNA at late awakening stages may be determined by the depletion of the mRNA pool as a result of intensified mRNA degradation due to enhanced translation of actin.

All this indicates the pronounced reactivity and the special role of the hippocampus in the processes that occur during hibernation. The results of our study suggest that the hippocampus has an substantial ability for making plastic changes. The concept of the high plasticity of this brain structure has been confirmed by numerous studies [13, 23–27, 31–33, 45, 46].

A peak of actin mRNA expression was detected in the frontal neocortex during hibernation as compared to the expression level that was recorded in the summer in active animals ($p = 0.046$). The high level of actin mRNA in the cortex during hibernation may be associated with strongly increased mRNA stability at this time [47, 48].

The profile of the total protein-level changes in different brain regions is completely consistent with the existing literature data that indicate that in winter during hibernation animals have sharply reduced metabolic and physiological activity of most of the parts of the brain [1–3, 24, 25, 49].

Thus, seasonal changes in actin and Cdk5 mRNA expression and total protein level were observed in all of the studied brain structures of ground squirrels; however, these changes were most pronounced in the hippocampus. Despite the initially high level of actin mRNA expression in the hippocampus, the degree of the change in this level during the hibernation cycle was comparable to the cerebral cortex and brainstem. However, the high and seasonally stable level of Cdk5 protein expression in this structure was accompanied by low levels of Cdk5 mRNA, which increases during hibernation and awakening of animals. In the hippocampus, unlike other brain structures of the ground squirrels, the total protein biosynthesis was enhanced from the period of preparation for hibernation and during the torpid state. The most-pronounced changes in all of the parameters were observed in the hippocampus of hibernating animals, indicating a pronounced ability of this brain region for making plastic changes, as identified in morphologic [6, 8, 10] and biochemical [12, 25, 39, 41, 44] studies. We believe that these changes determine the changes in the functional state of the central nervous system [1–4] and underlie the formation of the adaptive behavior of hibernating animals [50–53] under extreme environmental conditions.

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