Evolution of Electroencephalographic and Protein-synthesizing Activities of the Neocortex and Hippocampus during Rewarming after Hypothermia in Hibernating (Ground Squirrel) and Nonhibernating (Rat) Animals

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Abstract—In ground squirrels arousing from hibernation and in rats rewarming after artificial hyperthermia, the EEG frequency spectrum returned to normal following a similar course. The low-frequency components were the first to reappear. The EEG amplitude at θ- and α-frequencies started to rise later, at the cold shivering stage, when breathing became faster. The protein-synthesizing activity in hypothermic animals of both species was restituted to euthermic levels at body temperatures of 21–22°C. In the ground squirrel, protein synthesis was slower in the neocortex than in the CA1 and CA3 fields of the hippocampus. By contrast, in the rat the capacity for protein synthesis was higher in the neocortex than in CA3.

Key words: hibernation, artificial hyperthermia, neocortex, EEG, protein synthesis

INTRODUCTION

Winter dormancy in hibernating mammals is an adaptation allowing them to survive a seasonal shortage of food and low environmental temperatures by minimizing the physiological functions of the body. For example, in the state of deep dormancy (torpor), ground squirrels allow their body temperature to fall nearly to 0°C. Their heart, breathing, and metabolic rates drop dramatically, by tens and hundreds of times [1–3]. Hibernation proceeds in cycles (bouts), each 2–3 weeks in duration, which alternate with short periods of wakefulness. When an animal goes down into or arouses from torpor, changes in its physiological functions are under the control of the central nervous system [4–6]. Neurons in the ground squirrel brain are adapted to low temperatures and resume full activity upon rewarming. Nonhibernating endotherms are little studied in this respect. What is known is that hypothermia can be helpful in protecting nerve cells of nonhibernating species against damage caused by various environmental stress factors [7].

Hypothermia first of all depresses the activity of the neocortex and then the activity of subcortical structures. It seems biologically appropriate that different divisions of the central nervous system are not switched off simultaneously; rather, adaptation to lowering body temperature proceeds as a transition to poikilothermia, which is a more ancient stage of phylogeny [8]. In hibernators, the neocortex is inhibited first, with the brain temperature declining below 20°C, and recovered last upon rewarming during arousal. In phylogenetically more ancient formations like the hippocampus, bioelectric activity remains detectable even in deep torpor [4–6].

The indices most often used to assess the state of brain function recovery after exposure to hypothermia, ischemia, and some other detrimental factors is the bioelectric activity of neurons and the rate of protein synthesis [9]. However, it remains unknown whether their dynamics during rewarming in hibernators is similar to that in nonhibernators or not. Such comparative data might be useful in developing the techniques for producing states of artificial hypobiosis based on natural physiological mechanisms.
The purpose of this study was to compare the rewarming-associated changes in the EEG power spectrum and the capacity for protein synthesis in neocortical neurons in hibernating ground squirrels and rats exposed to artificial hyperthermia.

EXPERIMENTAL

Ground squirrels (*Citellus undulatus*) were caught in summer in Sakha and kept singly in a vivarium. Animals used in the experiments weighed 500–800 g. Before hibernation, animals were placed in a dark room maintained at 1–3°C. Two weeks prior to the experiment, animals were anesthetized intraperitoneally with 50 mg/kg sodium pentobarbital, and stainless steel recording electrodes were implanted in the sensorimotor cortex. The electrode placement was as follows (stereotactic coordinates are given in millimeters): $F = 10$, $L = 2$, and $H = 1.5$, epidurally. The indifferent electrode was positioned in the nasal bone. During the experiment, a cage with a dormant animal in it was placed in a soundproof chamber maintained at 2–4°C (temperature optimum for hibernation). Brain temperature was recorded with an electrothermometer sensor specially constructed to insert it into the external auditory meatus of the ground squirrel (temperature at the tympanic membrane coincides with the brain temperature within 0.5°C). The measurement error was 0.2°C. Insertion of the sensor into the ear provoked arousal. The EEG was recorded with a UBF4-03 potential amplifier (Russia). On-line spectral analysis of the EEG was performed in the $\delta$ (0.5–3.6 Hz), $\theta$ (3.6–8.0 Hz), $\alpha$ (8.0–14 Hz), and $\beta$ (14–26 Hz) frequency bands using the amplitude–interval algorithm described previously [10]. Briefly, each band was divided into 20 subbands further referred to by their center frequencies. For each subband, we performed amplitude summation. Thereafter, we calculated the ratio of each particular sum to the total sum taken over all frequencies in the EEG segment being analyzed. During arousal, spectrograms were obtained for every 20-s epoch of the EEG and then averaged over every 5 min. The averaged EEG spectra were compared with the baseline spectra, that is, spectra of 30-min EEG recordings from the same animals in the active state between hibernation bouts. The significance of differences between the spectra was assessed using the Wilcoxon test.

The animals were sacrificed by decapitation in the middle of a bout (body temperature of 6–7°C), during arousal (body temperature of 16–21°C), and in the active state between two bouts (36°C).

Two weeks prior to the experiment, male Wistar rats (body weight, 200–250 g) were anesthetized intraperitoneally with 60 mg/kg sodium pentobarbital and implanted with recording electrodes in the sensorimotor cortex. The electrode placement was as follows (stereotactic coordinates are given according to the atlas [11] in millimeters): $AP = -7$, $L = 3$, epidurally. Electrodes were also placed in the CA1 field of the hippocampus: $AP = -2.5$, $L = 2.5$, and $H = 3.5$ [11]. The biopotentials were recorded monopolarly; the indifferent electrode was implanted into the nasal bone.

The rats were cooled using a "closed vessel" technique [12, 13]. Specifically, they were kept at 2–4°C for 4 h in a closed 5.7-l vessel. So, during cooling, they experienced progressively increasing hypoxia and hypercapnia induced by their breathing. By the end of the 4-h cooling period, their temperature was 17–18°C. After being transferred into normal gas conditions, the animals spontaneously recovered from hypothermia over a period of 3–4 h. During rewarming, the EEG was recorded and analyzed for its frequency spectrum as described above for the ground squirrel. The state of the rats was monitored by their body temperature and heart rate. Colonic temperature measurements were taken with a TEMP60 electrothermometer (measurement error, 0.2°C). Its sensor was inserted 6 cm deep. Heart rate was recorded with an EEG-4-02 instrument; the recording electrodes were placed subcutaneously on the left paw and over the animal shoulder.

The rats were sacrificed by decapitation at body temperatures of 17°C, 22°C, and 37°C.

After decapitation, the rat and ground squirrel brains were processed similarly. Brain specimens for fluorescence studies were fixed in Carnoy's fluid; for ultrastructural analysis, in 2.5% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer.

The protein-synthesizing capacity of neurons was assessed using fluorescence microscopy [14] (from the intensity of staining with acridine orange) or electron microscopy (from the morphology of the Golgi apparatus, endoplasmic reticulum, and polysomal complexes). For fluorescence microscopy, 6-μm-
thick paraffin sections were prepared and stained with acridine orange. Bound to single-stranded ribonucleic acid, acridine orange fluoresces at 640 nm ($I_{640}$); bound to double-stranded ribonucleic acid, at 530 nm ($I_{530}$). The $I_{640}/I_{530}$ ratio ($K_a$) is an index of the state of rRNA in ribosomes and correlates with the number of ribosomes in the polysomal complexes (that is, with the fraction of ribosomes involved in protein synthesis) [14]. Thus, determining $K_a$, one can assess how the capacity for protein synthesis varies with the state of the animal. The stained sections were analyzed using a DMF-2 microfluorimeter [15]. Each $K_a$ value is the mean over 300–700 cells. The significance of differences between the means was estimated with the Student’s $t$-test.

Ultrathin sections for electron microscopy were stained with uranyl acetate and lead citrate in a standard way. The magnification factor was determined using a calibration grating replica (2160 lines per mm).

**RESULTS**

Figure 1a shows how the EEG from the sensorimotor cortex varies with time in a ground squirrel arousing from hibernation. At a brain temperature of 7°C, no regular rhythmic activity was discernible in the EEG; segments of electric "silence" were interspersed with low-amplitude (about 50 μV) patterns synchronous with heart beat and breathing activity. With increasing brain temperature, cardiac and respiratory components faded and eventually vanished. At 14–18°C, slow-wave δ-activity was dominating; against this background, there were random bursts of spiking activity up to 200 μV in amplitude. Further warming was associated with the appearance of more complicated EEG patterns. At brain temperatures of 20–29°C, the EEG contained segments of regular θ rhythm and occasional spindles. When motor activity started, more high-frequency components were added,
Fig. 2. Evolution of the power spectrum of the EEG recorded from the ground squirrel sensorimotor cortex during arousal from hibernation. The results are means over six animals. Spectrograms were obtained for every 20-s epoch of the EEG and then averaged over every 5 min. From top to bottom, the lines correspond to consecutive 5-min EEG segments. For each frequency bin, the power was calculated as the percentage of the EEG power in that bin obtained for active awake animals and is shown as their difference. Gray coloring indicates significant ($p < 0.05$) increments; black coloring, significant decrements.
Fig. 3. Evolution of the power spectrum of the EEG recorded from the rat sensorimotor cortex during rewarming after hypothermia. The results are means over six animals. Spectrograms were obtained for every 20-s epoch of the EEG and then averaged over every 5 min. From top to bottom, the lines correspond to consecutive 5-min EEG segments. For each frequency bin, the power was calculated as the percentage of the EEG power in that bin obtained for normothermic animals and is shown as their difference. Gray coloring indicates significant ($p < 0.05$) increments; black coloring, significant decrements.

along with sharp-apex discharges that occurred repeatedly throughout the rest of the arousal period.

Figure 2 shows the evolution of the EEG power spectrum of ground squirrels arousing from hibernation expressed as a percentage of the EEG spectrum of active awake animals. During torpor, EEG activity was profoundly depressed in most frequency bands (from 3 to 26 Hz), and slow waves dominated the EEG. At the start of arousal, there was an increase in power in the narrow $\theta$-band (6–7 Hz). Further rewarming raised the power of higher-frequency components. The stage of enhanced thermogenesis was characterized by a sharp rise in activity in a broad range of $\delta$- and $\theta$-frequencies. At brain temperatures of 32–34°C, arousing animals opened their eyes. In the EEG, $\alpha$- and $\beta$-activities were restored to the normal levels, and $\theta$-activity was even higher than normal.
The EEG of rats recovering from hypothermia was analyzed similarly. A representative tracing showing the evolution of the EEG recorded from the rat sensorimotor cortex during rewarming is given in Fig. 1b. Compared with the ground squirrel brain, the rat brain tolerates hypothermia to a much lesser extent. Its EEG activity nearly disappears at temperatures reduced to only 19-20°C. Deep hypothermia resulted in electrical silence on the electrocorticogram; only occasional slow waves up to 300 µV in amplitude and brief episodes of low-amplitude (<30 µV) high-frequency activity could be observed. Cold shivering in hypothermic animals began when their rectal temperature rose to 22°C and was associated with the appearance of high-frequency components in the EEG. After reaching 28°C, rewarming was greatly accelerated, and a sharp rise in the β-activity was observed. Thereupon, α- and β-activities started to increase and...
Protein-synthesizing activity of hippocampal CA1 and CA3 neurons assessed as the $K_{\alpha}^{\text{exp}}/K_{\alpha}^{\text{contr}}$ ratio in ground squirrels and rats during rewarming.

<table>
<thead>
<tr>
<th>$t$, °C</th>
<th>Heart rate, bpm</th>
<th>Hippocampus</th>
<th>Sensorimotor cortex</th>
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<td>CA1</td>
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<td>Ground squirrels</td>
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<td>7</td>
<td>5–8</td>
<td>55 ± 3**</td>
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<td>16</td>
<td>170–200</td>
<td>57 ± 3**</td>
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<td>21</td>
<td>270–300</td>
<td>115 ± 9</td>
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<td>Rats</td>
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<td>17.5</td>
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<td>22</td>
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Note: (*) Significant differences from the normothermic level ($p < 0.05$ and $p < 0.01$, respectively; Student's $t$-test). Heart rate in ground squirrels during normothermic arousals between hibernation bouts is 120–180 beats per min (bpm); in normothermic rats, 340–360 bpm.

sharp-apex discharges appeared and recurred from time to time. The evolution of the EEG spectrum during rewarming averaged over six animals is qualitatively summarized in Fig. 3.

The rats recovering from hypothermia were also studied for the electric activity of their hippocampus. Spectral analysis of the electrohippocampogram (EHG) did not reveal any regular evolution pattern (Fig. 4). The contribution of $\theta$-, $\alpha$-, and $\beta$-frequencies to the EHG spectrum was higher in the state of cold anesthesia than in the normothermic state. During rewarming, their contribution declined until the cold shivering stage (zone of enhanced thermogenesis). Thereafter, it began to rise again.

To assess how the capacity for protein synthesis varies with the state of the animal, we determined the coefficient $K_{\alpha}$ for hippocampal CA1 and CA3 neurons in ground squirrels arousing from hibernation and in rats rewarming after artificial hyperthermia (table). In torpid ground squirrels (7°C), decreased $K_{\alpha}$ values were observed in all the structures studied. The decrease was less pronounced in the cortex. Electron microscopy revealed profound alterations in the state of the components of the protein-synthesizing machinery in neurons. The percentage of translating ribosomes fell to 26% (compared with 80% in the active state [14]). The number of ribosomes associated with endoplasmic reticulum diminished, possibly because the latter disintegrated into separate cisternae. The Golgi apparatus nearly disappeared, leading to enhanced vacuolization of the cytoplasm. Cortical neurons showed similar, albeit milder, changes. Polyribosomes were only slightly less abundant, and the structure of the Golgi apparatus and endoplasmic reticulum remained largely spared (Figs. 5, 6).

At the start of arousal, as long as the brain temperature went up slowly to 14–16°C, only a moderate increment in $K_{\alpha}$ was observed. In the zone of enhanced thermogenesis (25°C), its values began to rapidly increase, concurrently with the heart rate, $O_2$ consumption, and, hence, the metabolic rate. Ultrastructural analysis of hippocampal neurons showed that, at this stage, the integrity of endoplasmic reticulum and the Golgi apparatus was restored at a high rate, and polyribosomes grew in number. At 22°C, the ultrastructure of neurons was close to that of their counterparts in active animals, suggesting that the protein-synthesizing machinery was fully functional.

In rats cooled to 17°C, the greatest $K_{\alpha}$ decrements were found in neocortical cells and hippocampal CA1 neurons (table). Ultrastructural examination revealed dissociation of polyribosomes, swelling and disintegration of endoplasmic reticulum, detachment of ribosomes, and partial or complete reduction of the Golgi apparatus (Figs. 5, 6). The changes in CA3 neurons were much less severe, supporting the view that the CA1 field of the hippocampus and the cortex are more vulnerable to various factors than the CA3 field [16].

Upon cessation of rat exposure to cold, hypoxia, and hypercapnia, the protein-synthesizing system in neurons started to recover. The $K_{\alpha}$ became fairly large at 22°C, and its increase was correlated with the increase in the number of polysomal ribosomes and the restoration of the integrity of the Golgi apparatus and endoplasmic reticulum.
Fig. 5. Ultrastructure of the cytoplasm of hippocampal neurons: (a) active ground squirrel, (b) torpid ground squirrel, and (c) rat cooled to 17°C in a closed vessel (hypothermia + hypoxia + hypercapnia). Designations: ER, endoplasmic reticulum; Mt, mitochondria; P, polyribosomes; M, monosomes; GA, Golgi apparatus.

Fig. 6. Ultrastructure of the cytoplasm of pyramidal neurons from the sensorimotor cortex: (a) active ground squirrel, (b) torpid ground squirrel, and (c) rat cooled to 17°C in a closed vessel (hypothermia + hypoxia + hypercapnia). See Fig. 5 for designations.
DISCUSSION

The generalization of cardiac and respiratory rhythms in the EEG of torpid hibernating animals was reported in the literature previously [14]. This phenomenon was suggested to be due to proprioceptive afferentation that converges in the reticular formation, is amplified in other subcortical divisions, and spreads over the cerebral cortex [4]. In the state of torpor, the capacity for protein synthesis was reduced in the neocortex to a lesser extent than in the hippocampus, despite the fact that the electric activity of the neocortex is known to be the first to switch off during entry into hibernation and the last to reappear during subsequent arousal [4–6]. However, even without stimulatory influences from subcortical nuclei at low temperatures, the ground squirrel neocortex did not lose function completely, retaining the ability to respond to afferent input. In contrast, the ground squirrel hippocampus produced relatively regular electric activity even during deep torpor [4–6], but its capacity for protein synthesis was found to be depressed to a greater extent than in the cortex. This difference may be related to their roles in the mechanisms of hibernation. The hippocampus is a kind of a watch station in the central nervous system responsible for the control over the course of hibernation [5, 6]. Therefore, its energy stores—limited under low-temperature conditions—are directed first and foremost toward producing the electric activity necessary for keeping connections with other structures. Interestingly, compared with cortical neurons, hippocampal neurons restore their capacity for protein synthesis during arousal more rapidly. The role of the neocortex in the mechanisms of hibernation is less important, and its electric activity is depressed upon entry into hibernation. However, the neocortex retains much of its capacity for protein synthesis, which facilitates its functional restitution during arousal from hibernation.

The limbic system controls the degree of central inhibition, which increases during entry into hibernation. The hippocampus and reticular formation mutually inhibit each other. Switching between the states of the animal depends on their mutual inhibition [5, 6]. During entry into hibernation, the temperature set point of the hypothalamus is lowered. A shift to a lower set point reduces the stimulatory influence from the reticular formation, suppressing the activity of θ-generating medial septum neurons and thereby attenuating the inhibition of the hippocampus. Activity of inhibitory θ-interneurons in the hippocampus declines, whereas the activity of projection pyramidal cells increases, thereby enhancing the inhibitory influence on the reticular formation and further reducing its activity. This positive-feedback mechanism accelerates the onset of hibernation. During arousal, the order of events is reversed.

Our results do not contradict this view. At the start of arousal from hibernation, we observed enhancement of a narrow θ-band (6–7 Hz) in the EEG power spectrum. At low brain temperatures, there are no patterns of bioelectric activity specific to particular brain structures; rather, they are generalized across all structures [4]. During this period, θ-activity of the neocortex reflects influences irradiating from the limbic structures to the cortex. At brain temperatures of 20–22°C, stimulatory influences of the reticular formation on the cortex are restored, and, from this point, the brain is likely to behave as a united whole [4]. Our data demonstrate that, at this stage of rewarming, the capacity for protein synthesis is regained, and the EEG displays enhanced power in the broad θ-frequency range. Thereafter, desynchronization takes place, and an increase in the α- and β-activities is observed, which are characteristic of wakefulness (in rodents, high θ-activity and a shift to higher frequencies are correlated with an increase in their brain activity [17]).

Interestingly, in ground squirrels arousing from hibernation and in rats rewarming after artificial hyperthermia, the EEG frequency spectrum returned to normal following a similar course. In both rats and ground squirrels, the contribution of the low-frequency (6–7 Hz) components into the EEG power spectrum increased to nearly the normothermic level at the very start of rewarming, when the θ- and α-frequencies were still depressed. The EEG amplitude at θ-frequencies started to rise later, at the cold shivering stage. The contribution of the broad θ-range first increased to the normothermic level and then exceeded it. In ground squirrels, the dynamics of this process was more clearly outlined.

In rats, the contribution of θ-, α-, and β-frequencies to the EHG spectrum was higher and that to the EEG spectrum was lower in the state of cold anesthesia than in the normothermic state. During rewarming, the contribution of these frequencies to the EHG spectrum declined until the cold shivering stage (enhanced thermogenesis), at which spinal
motoneurons innervating the respiratory musculature began to receive increased input from the respiratory center of the bulbar reticular formation via descending pathways [8]. Influences from the respiratory center are likely to also encompass the overlying structures. Stimulatory input from the medullar reticular formation to the neocortex reestablishes, and the broad θ-range in the EEG spectrum first regains and then exceeds the normothermic level. The enhancement of θ-rhythm in the EEG resulting from the restoration of stimulatory influences from the medullar reticular formation to the neocortex reestablishes, which in hibernators facilitates their transitions between different states [5, 6], also plays a role in recovery of nonhibernating mammals from artificial hypothermia.

Closed-vessel cooling of rats adds hypoxia and hypercapnia to hypothermia and thereby sets in action some mechanisms that can be used to develop techniques for producing states of artificial hypobiosis. Ground squirrels hibernate in burrows with a closed entrance. At the start of the hibernation season, O2 in the burrow is reduced to about 10% and CO2 is slightly elevated (9–12%) [18, 19]. Hypoxia and hypercapnia inhibit the thermoregulatory center in the ground squirrel hypothalamus and decrease the heart rate, facilitating the entry into hibernation [3]. When animals become dormant, their metabolic rate drops dramatically, and the gas conditions in the burrow return to normal. If a nonhibernator is being cooled experiencing hypoxia and hypercapnia, the activity of its thermoregulatory center is lowered and its body produces less heat [20]. Hypoxia and hypercapnia are associated with accumulation of γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the central nervous system. GABA accumulation slows down synthesis of the excitatory neurotransmitter glutamate [21, 22]. As a result, activity of neurons diminishes, improving their tolerance to hypoxia [23]. Hypoxia-induced enhancement of inhibitory processes results in the appearance of slow waves in the EEG. Another cause for domination of hypersynchronized low-frequency activity is a reduction in the input from subcortical centers. In ground squirrels and hedgehogs, the reticular formation becomes inactive at brain temperatures of 19–21°C [4]. In rats, its activity is lost at temperatures below 25°C [8]. The relationship between brain temperature and EEG frequencies resembles the temperature dependence of the Na+/K+-pump activity, suggesting that inhibition of transmembrane ion currents in neurons at low temperatures accounts for the depression of high frequencies in the EEG [24], which also spares cell function under conditions of hypoxia and low temperatures. Cooling increases CO2 consumption in blood [25]. As a result, tissue pH in hibernators becomes more acidic (ΔpH = 0.2–0.4). This acidic shift inhibits tissue metabolism, maintaining the state of hypothermia [26, 27]. At low temperatures, the Ca2+ pump works slowly, and intracellular Ca2+ rises, causing damage to cells [28, 29]. An acidic shift in tissues inhibits the inward Ca2+ current [30], allowing cells to survive at low temperatures.

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