Dynamics in the Ultrastructure of Asymmetric Axospinous Synapses in the Frontal Cortex of Hibernating European Ground Squirrels (*Spermophilus citellus*)

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KEY WORDS  cortical synapses; layer 2; hypothermia; synaptic plasticity

ABSTRACT  Recent theories on the function of arousals from torpor in hibernating mammals focus on the repair of the central nervous system from damage accumulating during prolonged hypothermia. In this framework, we investigated the synaptic ultrastructure in Layer 2 of the frontal cortex from hibernating European ground squirrels (*Spermophilus citellus*) sacrificed at four different phases in the torpor-arousal cycle. Using electron microscopy, we quantified synapse number and morphometric data on asymmetric axospinous synapses. Length, width, and surface area of postsynaptic densities (PSDs), and the synaptic apposition length of the analyzed synapse were measured. Five groups of animals were compared during entrance into torpor (Torpor Early, TE, *n* = 6), late torpor (Torpor Late, TL, *n* = 5), beginning of euthermic arousal episodes (Arousal Early, AE, *n* = 5), late in the euthermic arousal episode (Arousal Late, AL, *n* = 5), and during continuous euthermia in spring (EU, *n* = 6). The results showed that during torpor and at the beginning of arousals the PSD length and synaptic apposition length are significantly increased compared to synapses during late arousal and in spring conditions. In contrast, the width and surface area of the PSDs are decreased in torpor. At the beginning of an arousal the width of the PSD increases and gains maximum value in late arousals (AL), returning to spring (EU) values. No differences were found in total number of synapses during the torpor-arousal cycle. The results indicate reversible changes in ultrastructure of (asymmetric axospinous) synapses in the frontal cortex, which may be critical for the maintenance of cortical neuronal networks and for protection against potential deleterious effects of prolonged hypothermic phases of hibernation. *Synapse* 61:343–352, 2007.

INTRODUCTION  Hibernation is a behavioral and physiological adaptation of several endothermic animal species, enhancing survival during extended seasonal periods of low ambient temperature and reduced food supply (Lyman et al., 1981). In the state of torpor, the energy expenditure and metabolic rate of hibernating mammals are reduced considerably, and their body temperature drops almost to the level of the surrounding temperature (Buck and Barnes, 2000; Hut et al., 2002a; Strijkstra and Daan, 1997b; Wang, 1979). Metabolic (Kilduff et al., 1990) and electrical activity of the brain are markedly reduced and cortical electrical activity is virtually absent for many days (Krilowicz et al., 1988). Since electrical activity of the brain plays an important role in the dynamic stabilization of synapses and neuronal circuits (Hebb, 1949; Kavanau, 1997), hibernating animals may be a useful model to study the capacity of nerve cells to maintain synaptic efficacy in the absence of electrical activity.

Contract grant sponsor: The 6th European Framework Project EUCLOCK; Contract grant number: No. 018741; Contract grant sponsors: University of Groningen, The Netherlands Science Foundation, NWO, #016.021.017 and #863.02.002.

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Received 11 June 2006; Accepted 28 November 2006
DOI 10.1002/syn.20380
Published online in Wiley InterScience (www.interscience.wiley.com).
Nearly all hibernators periodically interrupt the state of torpor by euthemic episodes or arousals. These arousals are expensive in terms of energy expenditure (Humphries et al., 2003; Kenagy, 1989; Wang and Lee, 2000) and are responsible for up to 90% of the energy consumed during hibernation (Lyman et al., 1982). The physiological process that triggers the periodic arousals during hibernation is largely unknown, but several data indicate the involvement of external cues (e.g., ambient temperature; Heller and Ruby, 2004) and a novel, circannually regulated hormonal signaling pathway (i.e., hibernation-specific protein complex – HP; Kondo et al., 2006). There is a close association between frequency of arousal, body temperature, metabolic rate, and sleep homeostasis (Larkin and Heller, 1996; Lyman et al., 1982). Sleep during arousal from torpor bears a striking resemblance to recovery sleep in euthemic animals following extended periods of sleep deprivation (Daan et al., 1991; Trachsel et al., 1991). This sleep is characterized by high initial slow-wave activity (SWA), which declines during the subsequent hours of sleep. This resemblance has led to the theory that high SWA following arousal from hibernation reflects the neuronal recovery from sleep debt accumulated during the previous torpor bout, and that this recovery is the primary function of the arousals (Daan et al., 1991, Trachsel et al., 1991). This hypothesis is supported by the positive correlation between initial arousal SWA and preceding torpor bout duration (Strijkstra and Daan, 1997a). However, the negative correlation between SWA during the arousal episodes and the brain temperature during the preceding torpor bout does not explain why hibernators spontaneously reduce torpor duration at higher temperatures (Larkin and Heller, 1996; Strijkstra and Daan, 1997b).

High SWA has been associated with periods of intense synaptogenesis (Arendt et al., 2003; Popov et al., 1992; Strijkstra et al., 2003). The SWA peak following arousal from torpor corresponds to the time during which there is massive regrowth of dendrites and synaptogenesis (Popov et al., 1992). Popov and Bocharova (1992) suggest that the low electrical activity during torpor is responsible for the loss of dendrites and synapses observed. Torpor at lower temperatures is characterized by lower levels of electrical brain activity and higher subsequent SWA peaks during arousal (Larkin and Heller, 1998). Torpor at high brain temperatures (above 17–21°C) does not result in elevation of SWA during the subsequent arousal (Larkin and Heller, 1996, 1998; Strijkstra and Daan, 1997a, 1998). Synaptic properties, that may very well depend on torpor brain temperature, are likely to affect SWA in the subsequent arousal. It will therefore be important to quantify the dynamics of these synaptic properties to elucidate relationships between torpor bout duration, torpor brain temperature, and SWA in the subsequent arousal.

The synaptic plasticity related to the torpor-arousal cycle as described by Popov and Bocharova (1992) refers to the giant synapses between mossy fibers and hippocampal pyramidal cells in the Arctic ground squirrel, Spermophilus undulatus. The presynaptic boutons of the mossy fibers are large and normally have contact with 4–5 spines. The spines are also very large and often branched. During torpor bouts the number of synapses, number of spines per presynaptic bouton, size of the spines, and size of the postsynaptic densities (PSD) per profile is greatly reduced (Popov and Bocharova, 1992; Popov et al., 2005). All these hippocampal morphological changes are consistent with reduced synaptic efficacy.

Studies of synaptic plasticity in different regions of the brain (e.g., occipital cortex, hippocampus, hypothalamus, and spinal cord) indicate that morphological changes of dendritic spines may vary not only with the experimental procedure and species studied, but to some extent also with the brain area involved (Malinský and Poláč, 1985; Popov et al., 1992, but see Von der Ohe et al., 2006). To further elucidate torpor-related changes in neuronal connectivity, we used brain material collected at specific phases of the torpor-arousal cycle in hibernating European ground squirrels and during spring euthermia (Arendt et al., 2003; Strijkstra et al., 2003). We studied the effect of the torpor-arousal cycle on synaptic morphology in Layer 2 of the frontal cortex just above the pyramidal cell bodies. This region contains the apical dendrites of Layer 2, 3, and 5 pyramidal cells, as well as dendritic profiles of nonpyramidal cells. The frontal cortex was chosen for the present investigation because of its multitude of direct and indirect connections with many other brain areas and its important role in the complex integration of sensory and visceral activities. As such, it is a suitable region to study hibernation-related structural changes in the adult brain (Malinský and Poláč, 1985; Popov and Bocharova, 1992; Popov et al., 1992; Von der Ohe et al., 2006). Structural changes at the level of spines and dendrites seem to be similar for different brain regions (Von der Ohe et al., 2006), but this does not exclude differences at the ultrastructural level. We therefore examined whether the cortex undergoes ultrastructural changes related to hibernation, such as the one reported for the hippocampus. To begin to answer this question, we studied the synaptic ultrastructural morphology of frontal cortex Layer 2 during hibernation with focus on changes between early and late torpid state, and early and late aroused state.

MATERIALS AND METHODS

The European ground squirrels (Spermophilus citellus) used in this study were either captured near
Vienna (Millesi et al., 2001), or born to captive females released in outside enclosures in Haren, The Netherlands (Hut et al., 1999, 2002a). The animals were kept individually in Lucite cages (1 x w x h = 48 x 28 x 50 cm³) with a nest box attached (1 x w x h = 15 x 15 x 15 cm³). Wood shavings were used as bedding material, food (rabbit breeding chow, Teurlings, Waalwijk, The Netherlands) and water were supplied ad libitum. The animals were kept in a climate-controlled room (relative humidity = ad libitum. The animals were kept in a climate-controlled room (relative humidity = 60%) throughout the experiment (Hut et al., 2002b). Hibernation was made permissive by gradually lowering ambient temperature from 20 to 7°C and changing light conditions from a 12 h light/dark cycle to continuous dim red light (<1 lux) in autumn. Individual torpor-arousal patterns were assessed by measuring nest box temperatures every minute with a computer-based recording system and by recording locomotor activity in the main cage (Hut et al., 2002b; Oklejewicz et al., 2001). These registrations of torpor-arousal patterns have previously been validated by using customized temperature loggers (Tidbit; Onset; Hut et al., 2002a,b) that registered body temperature every 48 min. The study was approved by the Animal Experiments Committee of the University of Groningen (BG02198) in compliance with Dutch law and international regulations.

In total 27 animals were studied. Except for six individuals in spring conditions (EU, see below), the animals were killed in four different stages within the torpor and arousal episodes during hibernation: torpor early (TE; n = 6), torpor late (TL; n = 5), arousal early (AE; n = 5), and arousal late (AL; n = 5). The spontaneous torpor bout duration lasted 17.6 ± 1.34, 20.0 ± 3.4, 27.6 ± 4.3, and 18.3 ± 3.3, respectively days for the AS, AL, TS, and TL group, and the spontaneous arousal duration lasted 8.4 ± 0.9, 6.3 ± 1.0, 6.5 ± 0.7, and 7.3 ± 0.6 h, respectively for the AS, AL, TS, and TL group. For the AE and AL animals, the last arousal from the torpor state was induced by handling at room temperature (20 ± 1°C) until rectal temperature was found to be between 20°C and 30°C. This procedure took about 30–60 min, after which the animals were returned to their nest boxes. The last arousal in these animals was induced after they had spent in torpor 51% (±1.1) of their previous spontaneous torpor bout duration. A fifth group of nonhibernating, euthermic animals (EU; n = 6) was sacrificed 6–7 days after cessation of hibernation, initiated by an increase in ambient temperature from 7 to 25°C in early spring (Hut et al., 2002b). Body temperature of these animals at the start of perfusion was 36.5 ± 0.4°C.

All animals were sacrificed by an overdose of pentobarbital (500 mg/kg; intra peritoneal) under dim red light conditions (<1 lux). When the animals failed to show reflexes in response to pinching one of the hind paws (3–6 min in euthermic animals; 14–22 min in torpid animals) they were brought to the perfusion set where the head was covered with ice to stabilize brain temperatures during transcardiac perfusion. The thoracal aorta was clamped to keep other organs from being perfused, since these were used in different studies. After 6 min of ice-cold heparinized saline perfusion the animals were subsequently perfused with 300 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. Thereafter, the brain was removed and the left frontal part containing a bulbus olfactorius and a frontal cortex was stored in 2% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4°C.

These parts of the brains were transported to the Institute of Anatomy in Jena in the above mentioned solution at 4°C. Vibratome sections (70 μm) were made (Vibratome series 1000) for electron microscopy. Sections were rinsed in cacodylate buffer (pH 7.2) and subsequently post fixed for 2 h at room temperature with 1% OsO₄ in 0.1 M cacodylic buffer (pH 7.2). Sections were then rinsed three times in aqua bidest until the solution remained clear, followed by dehydration in a graded ethanol series. Sections were finally embedded in Epon 812 following routine procedures. Ultrathin sections were cut on a Reichert Ultracut S and contrasted for 20 min with 5% uranyl acetate in aqua bidest and 2 min in Reynolds lead citrate.

Layer 2 of the frontal cortex was investigated by electron microscopy (Transmission Electron Microscope Zeiss-EM 900). Photomicrographs were taken using a nonbiased approach of randomly selected areas of 100 μm² in Layer 2 in the region of apical dendrites above the pyramidal cell bodies. The following features of synapses were assessed from the photomicrographs as indicated in Figure 1: length of a postsynaptic density (PSD length, parameter A in Figure 1; in case of complex (perforated) synapses we took the sum of the separated fragments of the PSDs), the width of a PSD (PSD width, parameter B in Figure 1), and the length of the presynaptic zone (synapse length or synaptic apposition length, parameter C in Figure 1; the contact zone between a postsynaptic membrane and the presynaptic membrane of an axon terminal). The PSD surface area was approximated by multiplying length and width of a PSD.
For statistical evaluation using SPSS, either one-way parametric ANOVA or nonparametric (Kruskal-Wallis) ANOVA was applied to assess variation among multiple groups, least significant difference (LSD) post-hoc test was used to define homogenous groups (Fig. 4). All averages are given with SEM.

RESULTS

Electron photomicrographs of the dendritic area of Layer 2 in the frontal cortex of different individuals of the five experimental groups of ground squirrels are shown in Figure 2. In general, the basic ultrastructure of Layer 2 of all animals was intact irrespective of the phase in the torpor-arousal cycle; dendritic spines and presynaptic elements with numerous vesicles and mitochondria could be encountered in all individuals.

The total number of synapses per 100 μm² revealed no differences between the five groups (P > 0.07; ANOVA; Fig. 3, top panel). However, the number of perforated synapses revealed significant alterations (Fig. 3, lower panel). The number was highest in the AE group and significantly so compared to TL and EU groups (P < 0.05; ANOVA). During early arousal, the proportion of perforated synapses was 23% in AE animals, whereas it was 12% and 14%, respectively in TL and EU animals. Surprisingly, the number of complex synapses during spring euthermia was closer to the hypothermic late torpor state than to the late euthermic state.

The morphology of the synapses was further investigated in order to determine whether morphological changes occur at this level during hibernation. Eighteen to 30 synapses per individual were studied for synapse length, PSD length and PSD width. PSD surface area was approximated by multiplying PSD length and PSD width. The group averages of the individual values are shown in Figure 4. Statistical analysis (one way ANOVA) revealed significant variation between the five groups concerning synaptic length (in view of the unequal variances tested by nonparametric Kruskal-Wallis ANOVA), and PSD length, PSD width, and PSD surface area. The synapse length (the contact zone between a postsynaptic membrane and the presynaptic membrane of an axon terminal) was significantly longer in torpor conditions (TE and TL) in comparison to nontorpid animals (P < 0.0001; Fig. 4), except for the AE animals, which resembled the torpid animals for this parameter. Quantification of the PSD length and PSD width revealed that torpid animals have thin but long PSDs as compared with the PSDs of euthermic animals (TE and TL vs. AL and EU; LSD: P < 0.0001; Fig. 4). AE animals seem to reflect an intermediate state with a PSD width comparable to that of AL and EU animals, but a PSD length comparable to that of torpid (TE and TL) animals. As a consequence, the PSD surface area was significantly reduced under torpor conditions (LSD: P = 0.0001; Fig. 4), as compared to euthermic conditions.

The relative frequency distributions for synapse length, PSD length, and PSD width are shown in Figure 5. The frequency distribution of the PSD width is shifted to smaller values in torpid animals (TE and TL; no significant differences were found between 2.3 and 7.1 days of torpor) as compared with euthermic hibernating and spring animals (AE, AL, and EU). This corresponds with the observation that the thick PSDs, as observed in the AL and EU animals, were no longer encountered in the torpid state. The PSD width of the AE group is in between the value for the torpid state and the euthermic states (AL and EU animals), suggesting that the PSD width is in a transition phase with increasing thickness 1.5 h after the induction of an arousal. 8.3 h after this induction, PSD ultrastructural characteristic were indistinguishable from those of euthermic spring animals.

DISCUSSION

The morphological changes which occur in the PSDs in Layer 2 of the frontal cortex are the main finding of this study. In the euthermic spring and arousal late animals, clear PSDs were present throughout this layer. Such PSDs were only rarely encountered in squirrels in early and late torpor. Synapse length was significantly longer in the torpid than in the euthermic state. In summary, during torpor, synaptic contacts in Layer 2 of the frontal cortex are characterized by being relatively large with a long but thin PSD. Since most, if not all, synapses studied are
dendritic spine synapses (asymmetric axospinous synapses), it can be concluded that during torpor large spine heads with long and thin PSDs predominate. In the course of arousal and subsequent euthermy, these synapses return to normal conditions (smaller synapse length, with smaller and thicker PSDs), which may be necessary for periodic stabilization of the neuronal cortical network(s) during hibernation in order to

Fig. 2. Electron photomicrographs of synapses in Layer 2 of the frontal cortex of ground squirrels in euthermic spring conditions (euthermic (EU; a–c), torpid conditions (torpor early (TE; d–f) and torpor late (TL; g–i), and euthermic torpor conditions (arousal early (AE; j–l) and arousal late (AL; m–o]). Scale bar indicates 400 nm.
prevent loss of stored information in these neuronal assemblies.

A decrease in cortical spine density has been reported in hibernating mammals (hedgehog: Malinský and Poláčk, 1985; golden-mantled ground squirrel: Von der Ohe et al., 2006). This could result in a decrease in number of synapses, although Layer 2 of the cortex was not examined in the above studies. We did not find evidence for a significant reduction in total number of synapses in the hibernating ground squirrel, but did find a significant loss of synapses with perforated PSDs. This suggests that this type of synapse is lost during torpor, and rearranged into synapses with nonperforated, long and thin PSDs. However, such conclusions need to be considered with caution, because we were not able to track individual synapses during the torpor-arousal cycle or to make 3D reconstructions of the PSDs (which requires serial ultrathin sections). It is interesting to note that the number of perforated synapses increases in the arousal early group. This is in line with findings of the proportion of perforated PSDs in hippocampus CA3 mossy fiber synapses, which was highest 2 h after arousal, and significantly higher compared with euthemic spring animals (Popow and Bocharova, 1992). Apparently, early arousal goes along with remodeling of PSDs in favor of perforated synapses, which are considered to have a higher efficacy as non-perforated ones. Comparison of the number of perforated synapses during the torpor-arousal cycle with euthemic spring animals may suggest that perforated synapses are a specific target of synaptic remodeling and plasticity, because numbers in the torpor long group do not differ from those of the euthemic spring animals. Usually perforated synapses are found in the larger spines with a relatively broad stem. Free polyribosomes are likely to be involved in synaptic renewal, and are known to be present in such spines. Cytosol-soluble PSD proteins could be rapidly synthesized at this location (Marrs et al., 2001).

**Temporal dynamics of the ultrastructural changes**

The torpor-arousal cycle and the experimental design with four groups at different phases of this cycle (arousal early and late (AE and AL), torpor early and late (TE and TL)) allows the examination of the temporal dynamics in which the changes occur. Of the parameters examined, only the PSD width in arousal early animals is intermediate between the torpor late and arousal late condition (PSD length and synapse length in the arousal early group resembles that of the torpor late group). On the basis of the observation it can be concluded that the PSD width is the first property that changes as a ground squirrel enters arousal, subsequently followed by decreases in PSD length and synapse length in the course of an arousal. No ultrastructural differences were found between torpor early and torpor late, which clearly indicates that the transition to torpid conditions in PSD morphology and synapse length takes at most 2.3 days of torpor to be induced and developed fully. The spring conditions in the respective ultrastructural features are realized in full within 8.3 h of euthermia during the torpor-arousal cycle. Taken together, reversible transformations in synaptic ultrastructure in relation to the phase of the torpor-arousal cycle also occur in the frontal cortex, which is in line with the observations of Von der Ohe et al. (2006) at the light microscopic level.

**Underlying molecular mechanisms**

At present we can only speculate about the process(es) underlying the observed ultrastructural changes. Since we did not observe a change in total number of synapses, it is most likely that we study a reorganization of existing synapses, but a certain degree of synapse turnover cannot be excluded. The first changes are realized within the first 1.3 h of arousal, in which a torpid animal gradually warms up to euthermic conditions. This limited time makes it unlikely that new synapses are formed if it depends...
on de novo protein synthesis, which is greatly reduced during torpor (Frerichs et al., 1998) and needs time to be restarted. However, if new synapses can be formed from existing proteins previously located elsewhere in the cell, then the observed ultrastructural changes could reflect the formation of new synapses. Moreover, it is possible that during arousal cytosol-soluble PSD proteins are rapidly synthesized at this

Synapse DOI 10.1002/syn
location (Marrs et al., 2001). The changes in PSD width and length are more robust than those seen for the synapse length. During torpor, nearly all PSDs thicker than 50 nm disappeared and higher numbers of thin PSDs are found instead (Fig. 3). This indicates that existing PSDs are being remodeled; proteins present in or attached at the PSD under euthermic conditions may be retracted to the dendrites or even to the nucleus, as observed in the same ground squirrels for the γ-isooform of protein kinase C (Van der Zee et al., 2004). PSDs are re-established and thickened during arousal. Obviously, the change in synapse length and size of PSDs may not be independent events. The process underlying the lengthening of the synapse may cause PSDs to become thinner and longer, or vice versa, the decline of the PSD size may cause a larger synapse.

Under euthermic conditions, the width of a PSD is primarily determined by the amount of CaMKII incorporated, which is mainly induced by synaptic activity (Dosemeci et al., 2001; Rostas et al., 1991). Since PSD width is the first feature to be changed during an arousal, CaMKII activation during entrance of an arousal may be critical in this respect.

**Functional consequences of the ultrastructural changes**

What functional consequences may the ultrastructural changes have? The obtained results (increased length of the PSD and increased synaptic apposition length during torpor) are surprising and may argue at first sight for a strengthening of the synapse, and not for reduction of its efficacy during torpor. However, electrical activity is nearly absent during torpor bouts (Daan et al., 1991; Krilowicz et al., 1988; Trachsel et al., 1991; Walker et al., 1977) and changes in the ionic microenvironement during entrance into torpor result in depression of synaptic transmission (Igelmund, 1995). In general, deep torpor bouts represent states of cortical inactivation without any significant electrical activity (Kavanau, 1997). That makes activity-dependent increase in synaptic size during torpor very unlikely.

More important from a functional point of view, is the width of the PSD. A thick PSD indicates a large protein concentration in the postsynaptic element responsible for processing incoming signals. For example, the number of receptors (e.g., the ratio of AMPA to NMDA receptors; Matsuzaki et al., 2001; Takumi et al., 1999) per synapse is proportional to the PSD surface area and spine volume (Ganeshina et al., 2004; Nusser et al., 1998; Racca et al., 2000; Takumi et al., 1999). More receptors, second messenger related proteins and effector proteins will be present in a thick PSD, and the efficacy of such a synapse will be higher than in the case of a thin, almost non-existing PSD. Hence, the ultrastructural changes observed in torpor are more consistent with a reduction of synaptic neurotransmission.

Retaining cortical networks is important, for example in relation to long-term memory storage. Dendritic spines, the postsynaptic sites of excitatory synapses, are believed to provide the structural basis for synaptic plasticity, learning and memory (Greenough and Chang, 1988; Hayashi and Shirao, 1999; Yuste and Bonhoeffer, 2001). Changes in number, shape, and composition of dendritic spines (with emphasis on the postsynaptic density and number and type of incorporated receptors) may play a crucial role in synaptic plasticity, efficacy and maintenance of networks (Calverley and Jones, 1990; Geinisman et al., 2001; Grutzendler et al., 2002; Yuste and Bonhoeffer, 2001). It should be noted that hippocampal-dependent memory acquired before going into hibernation is lost after the hibernation season, but not some forms of social memory, which is more cortical dependent (Mateo and Johnston, 2000; Millesi et al., 2001). In this respect it is of interest to note that hippocampal changes in synaptic ultrastructure (Popov and Bocharova, 1992) differ from those seen in the frontal cortex, and to realize that in general the hippocampus is a temporal (recent) memory store whereas the cortex serves as a permanent (remote) memory store (see for review Frankland and Bontempi, 2005).

In conclusion, Layer 2 of the frontal cortex undergoes major ultrastructural changes at the synaptic level, which seem to be only partly similar to changes in hippocampal mossy fiber synapses. Periodic activation of dendritic spine synapses during arousal periods possibly represents a mechanism that protects against structural deterioration of neuronal cortical networks which potentially could occur if a torpor state at low temperature would last several weeks or months. Our findings are consistent with the hypothesis of Strijkstra and Daan (1998) that hibernators interrupt the torpor states regularly to protect the brain against these deleterious effects of prolonged hypothermia (Strijkstra, 2006). However, the ultrastructural features of the frontal cortex synapses are not the limiting factor for time spent in torpor, since no difference were observed between early and late torpor. This does not exclude the possibility that it is a limiting factor in other brain regions, such as the thermoregulatory areas. These results therefore suggest that it is important to study other brain regions at the EM level in relation to synaptic plasticity during hibernation.

**ACKNOWLEDGMENTS**

The authors thank Prof. Dr. G. Jirikowski, Institute of Anatomy, Friedrich-Schiller-University, for his
ideas and expertise, and Mrs. C. Kaemnitz and colleagues, Institute of Pathology, Friedrich-Schiller-University, for their technical assistance and the provision of the electron microscope.

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