The brain at low temperature:

neuronal and behavioural dynamics in mammalian hibernation and torpor

Ate Sake Boerema

The PhD research presented in this thesis was carried out in the departments of Chronobiology and Molecular Neurobiology of the University of Groningen. This thesis would not have existed without the work of many collaborators from other departments inside and outside the University of Groningen.

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Hope

Hope is a state of mind, not of the world.
Either we have hope or we don't; it is a dimension of the soul, and it's not essentially dependent on some particular observation of the world or estimate of the situation.

Hope is not prognostication.
It is an orientation of the spirit, and orientation of the heart;
it transcends the world that is immediately experienced, and is anchored somewhere beyond its horizons... Hope, in this deep and powerful sense, is not the same as joy that things are going well, or willingness to invest in enterprises that are obviously heading for success, but rather an ability to work for something because it is good, not just because it stands a chance to succeed.

The more propitious the situation in which we demonstrate hope, the deeper the hope is.
Hope is definitely not the same thing as optimism. It is not the conviction that something will turn out well, but the certainty that something makes sense,
regardless of how it turns out.

Václav Havel *(5 Oct 1936 – 18 Dec 2011)*

Contents

Chapter 1

Introduction

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Chapter 1

Hibernation is a strategy evolved in many animals to see them through seasonally returning episodes of food shortage, usually the winter. In hibernation animals lower their metabolism to save energy. As a consequence, hibernating animals also cool down and enter a state of torpor. Mammalian hibernators, such as the European ground squirrel and the Syrian hamster, cool down to a body temperature near the ambient temperature as a result of their decreased metabolism. One might expect that hibernating mammals maintain this low metabolic state throughout the hibernation season to maximize energy savings, but this is rarely the case. Most hibernators intersperse long (multiple days) phases of torpor with short (10 - 24 hours) warm episodes throughout hibernation. In these periodic "arousals", in which they fully rewarm to the euthermic level, hibernators such as Ground squirrels, spend approximately 86 - 88% (Wang 1979; Strijkstra 1999 p.50) of the total energy used during the entire hibernation period. This represents most of the fat stores laid down during the summer months. In European ground squirrels the amount of energy spent during hibernation appears linked to mating success (Millesi et al. 1998) and litter size (Millesi et al. 1999b), and as such may have direct consequences on the fitness of the animals.

Ever since Dubois, (Dubois 1896), biologists have wondered about the function of these 'arousals', and many biological functions have been attributed to them. For hibernating bats, for instance, (Daan 1973a) found that the arousals allow the animals to periodically adjust the optimal location in the caves where they hibernate to the changing microclimate throughout the winter. Most other hibernators mainly stay in the same underground burrow and do not have the opportunity to change location, which brings more internal physiological functions to mind. Alternative hypotheses speculate, for example, on a function of periodic euthermy in clearing metabolic waste products (Fisher 1964; Németh et al. 2010), preventing muscular atrophy (Wickler et al. 1987, 1991), or maintaining immune function (Prendergast et al. 2002). A general functional meaning of arousals has not been unequivocally established (Willis 1982) until this day.

Hibernation and the brain

Hibernation research at the University of Groningen has focused on hypotheses around the need for brain maintenance processes in hibernating animals during the euthermic intervals in hibernation. This direction was triggered when Daan et al (1991) found that hibernating Arctic ground squirrels spend most time in the periodic euthermic phases during hibernation sleeping. This was simultaneously observed in Golden mantled ground squirrels by (Trachsel et al. 1991) at Stanford University. Both ground squirrel species sleep most of the time during arousals, and arousals started of sleeping with initially high cortical EEG slow wave activity (SWA), an indicator of deep non-REM sleep (Rechtschaffen and Kales 1968; Borbély 1982a). This indicates that hibernators sleep intensively at the beginning of an arousal, and less deeply later. The prevalence of sleep during arousals makes the term 'arousal' a misnomer. We shall henceforth speak about periodic rewarming or periodic euthermy during hibernation.

Non-REM sleep, or sleep in general, cannot be detected in the cortical EEG's of torpid hibernators such as European ground squirrels (Strijkstra et al. 1999; Strijkstra 2006). In Golden mantled ground squirrels firing rates of single neurons do not resemble NREM sleep patterns below brain temperatures of 14 °C (Krilowicz et al. 1988). In normal sleep, Non-REM sleep SWA is homeostatically controlled, i.e., it increases following sleep deprivation as described by the two process model of sleep regulation (Borbély et al. 1981; Borbély 1982b; Daan et al. 1984). Observing the initial high SWA after torpor, both (Daan et al. 1991) and (Trachsel et al. 1991) proposed that ground squirrels needed to

warm up from torpor to be able to sleep, to reduce a sleep debt accumulated during the previous torpor bout.

Independent experiments by the labs in Stanford and Groningen subsequently showed that the decay of Non-REM sleep EEG SWA over a periodic euthermic episode in hibernation is also present when European ground squirrels or Golden mantled ground squirrels are deprived of the expression of the initial high Non-REM sleep SWA (Larkin and Heller 1998; Strijkstra and Daan 1998). This argues against the hypothesis that the observed high SWA is associated with homeostatic sleep regulation. The result does not rule out the possibility that there is a need for sleep in general during the periodic euthermic phases. For example, European ground squirrels extended their euthermic phase duration after the initial loss of SWA during a euthermic phase (Strijkstra and Daan 1998). During this extension, animals still slept most of the time, suggesting that whatever restorative process takes place in periodic euthermy, may need or prefer the sleep state.

Extensive investigations on daily torpor in Djungarian hamsters have clearly demonstrated that, contrary to deep torpor, the state of daily torpor does act as a kind of sleep deprivation. Daily torpor is followed immediately by high SWA Non-REM sleep (Deboer and Tobler 1994; Deboer et al. 1994), and this SWA cannot be suppressed by sleep deprivation without a regulatory response with high SWA Non-REM sleep afterwards. It remains an attractive hypothesis that the restoration of synaptic connectivity during euthermy following torpor is intimately connected with the basic brain restoration function of sleep (see (Tononi and Cirelli 2006)). This view is further supported by the resemblance of the effects of sleep deprivation in Fruit flies (Bushey et al. 2011) and effects of torpor in European ground squirrels and Arctic ground squirrels on the shape of synaptic contacts, as investigated with electron microscopy (Rüdiger et al. 2007; Popov et al. 2007).

EEG slow wave activity is a manifestation of the electrical activity of neurons in the cortex in “burst-firing mode”. In burst-firing mode the action potentials are synchronized, leading to low frequency high power signals in the EEG. The cortex typically enters burst-firing mode if it is deprived of input signals. This is the case in deep non-REM sleep when, for instance, the thalamus assists in actively blocking most of the input signals to the cortex (Villablanca and Salinas-Zeballos 1972; Amzica and Steriade 2000). The fact that there was high Non-REM sleep EEG SWA present in the beginning of euthermic phases following torpor, which appeared not associated with homeostatic sleep regulation, suggested that the input signals to the cortex may be attenuated or blocked by another process. This led to the hypothesis that during torpor some kind of accumulating ‘damage’ occurs in neurons, affecting the input signals to the cortex. Periodic rewarming might be necessary to recover and restore proper neuronal function (Strijkstra 1999 p.150).

This research direction led to the discovery of a cyclic pattern of Synaptohysin, Map-2 and PSA-NCAM amounts, as indicators of neuronal connectivity in European ground squirrel brains over the hibernation cycle (Strijkstra 1999 p.147; Strijkstra et al. 2003; Arendt et al. 2003). For instance: the amount of Synaptophysin, a protein found in synaptic vesicles, decreases during torpor in the stratum lucidum of the hippocampal CA3 area. The amount of Synaptophysin is completely restored to summer levels during the euthermic intervals (Strijkstra et al. 2003). These effects were confirmed and expanded to other connectivity markers in European hamsters (Magariños et al. 2006) and Golden mantled ground squirrels (von der Ohe et al. 2006, 2007). In the cortex of European ground squirrels (Rüdiger et al. 2007) observed a high degree of synaptic plasticity over the torpor-rewarming cycle.

The brains of European ground squirrels also showed hyperphosphorylation of the neuronal skeletal protein tau (see box 1: tau hyperphosphorylation) during torpor: this phosphorylated state of the tau protein was fully reversed during the periodic euthermic intervals following torpor (Arendt et al. 2003). In humans hyperphosphorylation of the tau protein may lead to formation of paired helical filaments and tau deposits, which eventually may result in so called neurofibrillar tangles (Iqbal et al. 1989; Goedert et al. 1989a, 1992), resulting in neuronal cell death. Tangle formation is one of the two major hallmarks on which the diagnosis of Alzheimer's disease (AD) is based in humans (Grundke-Iqbal et al. 1986; Braak and Braak 1994). Cognitive decline correlates well with the degree of tau phosphorylation observed in the brain of AD patients (Arriagada et al. 1992).

On the behavioural level (Millesi et al. 2001) found that hibernation affects aspects of memory function of European ground squirrels: acquired spatial memory and operational conditioning memory were lost after hibernation when torpor was allowed. The observed hyperphosphorylation of the tau protein in Ground squirrels, together with synaptic plasticity changes and memory loss, but also reduced circadian rhythmicity (Hut et al. 2001, 2002b), after hibernation have a remarkable resemblance with the characteristics of AD. The fact that the tau hyperphosphorylation was fully reversible upon return to euthermia would make it worthwhile to study phosphorylation dynamics and regulation under natural circumstances, *in vivo*, in hibernators. This provides a natural animal model system for the study of Paired Helical Filament like (PHF-like; see box 2) tau phosphorylation dynamics.

Accumulating tau hyperphosphorylation is potentially detrimental to the brains of hibernators. Periodic return to euthermy may provide the opportunity to dephosphorylate the tau protein, preventing serious brain damage resulting from prolonged exposure of neurons to hyperphosphorylated tau protein. This may in turn give novel insight into the problem why hibernators have generally evolved periodic rewarming from torpor.

Aims and outline of this thesis

Until 2005, all results regarding synaptic plasticity dynamics and tau hyperphosphorylation dynamics in hibernation were obtained in one species of hibernator, the European ground squirrel (*Spermophilus citellus*). To elucidate the relevance of the observed brain plasticity changes for periodic euthermy during hibernation in general, it was necessary to see if the observed plasticity dynamics are a general feature of hibernators, or specific to European ground squirrels.

The goals of the project were (1) Replication of the ground squirrel findings in a more easily accessible species that have torpor in their natural behavioural pattern. As a deep hibernator, the Syrian hamster (*Mesocricetus auratus*) was used. This species is a standard laboratory animal, easily bred and maintained in the lab, and also readily brought into hibernation because of its strong photoperiodic response (Oklejewicz et al. 2001a). (2) The analysis of neuronal plasticity dynamics, with a focus on tau-protein hyperphosphorylation, along the brain-temperature trajectory observed during cooling and rewarming to and from torpor in Syrian hamsters. (3) Investigation of functional consequences of brain plasticity during hibernation, by testing memory performance before and after hibernation in Syrian Hamsters.

The results of these endeavours are described in part I "*Hibernation*", of this thesis. Chapter 2 describes neurotransmission capacity changes by comparing synaptophysin levels in the stratum lucidum of the Hippocampus of Syrian hamsters in torpor, during arousal and in summer euthermy. In

chapter 3, data are presented about tau protein hyperphosphorylation in torpor, after rewarming from torpor and in summer euthermic Syrian hamsters. Torpor induced tau hyperphosphorylation is compared with aspects of tau hyperphosphorylation observed in Alzheimer's disease. Chapter 4 studies tau hyperphosphorylation dynamics in Syrian hamsters in more detail. Data are presented about the body- and brain- temperature during cooling to and rewarming from torpor together with the associated hyperphosphorylation of the tau protein in the brain, with a focus on the cortex and hippocampal areas. In Chapter 5 the data of an experiment testing spatial memory in Syrian hamsters before and after hibernation are presented, in an effort to find possible functional consequences that hibernation related brain plasticity may have for the behaviour of the animals after hibernation.

In part II "*Natural and induced daily torpor*", I took a comparative approach, aiming to establish whether hyperphosphorylation of the tau protein also occurs naturally in species with less extreme torpor, such as daily torpor in Djungarian hamsters (chapter 6) or in species even better suited for molecular studies: house mice. Chapter 7 demonstrates the incidence of daily torpor in mice that are faced with the task to work for their daily food supply, and the remarkable effects on the circadian organization of mouse behavioural patterns in this experimental paradigm. In Chapter 8 the mechanism behind the induction of torpor and diurnal activity in mice is further elucidated and data on tau hyperphosphorylation during torpor in 'hard-working' mice is presented. Finally, in chapter 9 we investigated the pharmacological induction of torpor-like hypothermia in mice and studied the associated tau protein hyperphosphorylation. In chapter 10 the results are discussed in the context of the role of brain plasticity dynamics in our understanding of the evolutionary significance of periodic euthermy in hibernation, as well as the potential biomedical importance of hibernation and torpor.

Box 1: Hibernation and torpor in mammals

Hibernation and torpor: saving energy

Hibernation is a set of adaptive behaviours allowing animals to survive the winter by minimizing their exposure to harsh winter conditions (*hibernare*: 'to winter', in Latin). Animals that hibernate usually retract to a safe place (a cave, den or a burrow), a "hibernaculum". Hibernation in mammals contains periods during which animals have a very low metabolic rate. This physiological state is called torpor. It is good to realize that hibernation and torpor are not limited to the mammalian kingdom. Many species of insects, amphibians and reptiles also hibernate (overwintering behaviour and reduced metabolism). In homeothermic animals, torpor is not only observed in mammals (Heldmaier et al. 2004), but also in several species of birds (Geiser et al. 2006) e.g. hummingbirds (Hainsworth and Wolf 1970) and night jars (Dawson and Fisher 1969; Brigham et al. 2000).

For homoeothermic animals, the main benefit of hibernation is in saving energy. Food is hard to come by in the winter and as such, in order to survive, it pays off to spend less. Energy is saved by reducing metabolism (Heldmaier and Steinlechner 1981; Snapp and Heller 1981; Geiser and Ruf 1995). In torpor, metabolic rate is actively suppressed and body temperature goes down by passive cooling, effectively rendering the animal inactive and low responsive. This enhances survival.

Hibernation appears to prolong life. In European ground squirrels in the field, more than 80% of the animals survive hibernation, whereas less than 40% of the animals survive the active season, mainly due to predation (Millesi et al. 1999a). Life-span is also influenced by hibernation. Turkish hamsters that (spontaneously) hibernated relatively much of their lifetime lived longer than individuals that did not hibernate, or hibernated less (Lyman et al. 1981). European ground squirrels, not allowed to hibernate, or not given the proper conditions to hibernate, lived 1-2 years shorter than animals that did hibernate (Zivadinovic and Andjus 1996).

Many mammalian species show some form of overwintering behaviour with torpor (Heldmaier et al. 2004). Torpor is not only used in hibernation. The same strategy may be used to overcome the dry periods in summer (Brigham et al. 2000; Dausmann et al. 2005; Geiser et al. 2007) or periods of low food availability (Schubert et al. 2010).

In coping with harsh conditions, different species face different problems, based on their own life history. Therefore different torpor patterns are employed by mammals Three common forms of torpor patterns can be discriminated: A) hibernation with continuous torpor, B) hibernation with interrupted torpor, long (multi-day) bouts of torpor (deep torpor) are interspersed with short euthermic periods, and C) Daily torpor, short (multi-hour) bouts of torpor, organized within the circadian organization of behaviour.

A. Hibernation with continuous torpor

Hibernation with continuous torpor is the rarest form of hibernation in mammals. Bears hibernate in this way. They typically retract into a den in which they spent the entire hibernation period (several months), refraining from outside activity. They are continuously torpid, i.e. in a state of reduced metabolism, in this period. Their large body size and good insulation, together with large fat stores cause them to maintain a relatively high body temperature, between 30 °C and 36 °C during torpor, even at a metabolic rate of approximately 25% of their normal basal metabolic rate (Nelson 1973; Watts et al. 1981; Tøien et al. 2011).

Another species known to be able to hibernate with continuous torpor is the fat-tailed dwarf lemur (*Cheirogaleus medius*). These animals hibernate to pass the dry season. Hibernation occurs at relatively high ambient temperatures of on average 22 °C, with daily maxima of up to 34 °C, and nightly minima down to 10 °C. If the lemur hibernates in a poorly buffered environment exposing it to the full outside temperature fluctuation, it hibernates with continuous torpor. The exposure to the ambient temperature cycle results in passive periodic warming of the body temperature to values above 30 °C. If the lemur hibernates in a well-buffered environment, limiting exposure to the outside environment, limiting exposure to the outside temperature and resulting in a fairly constant ambient temperature of approximately 22 °C, the lemur hibernates like a deep hibernator, showing periods of multi-day torpor, interspersed with spontaneous autonomous rewarming to body temperatures > 30 °C (Dausmann et al. 2004, 2005).

B. Hibernation with interrupted torpor

Hibernation with deep torpor (sometimes also called deep hibernation) is a form of hibernation performed by many small rodents, such as several species of ground squirrels (Barnes 1989; Hut et al. 2002a), but also dormice (Mrosovsky 1977; Wilz and Heldmaier 2000) and Syrian hamsters (Lyman 1948). Deep torpor is also observed in bats (Hock 1951) and in Lemurs (Dausmann et al. 2004).In deep hibernation, long multi-day(up to 30 days) torpor bouts are interspersed with short periods during which the animal fully rewarms to normal euthermic body temperatures (see figure 1a for an example of body temperature during deep hibernation in the Syrian hamster). During torpor, deep hibernators can reduce their metabolic rate to a stunning 1-2% of their basal metabolic rate (Lyman 1948; Hock 1951; Heldmaier and Ruf 1992). As a consequence of the reduced metabolism, body temperature drops to about one or two °C above ambient temperature (Lyman and O'Brien 1974; Heldmaier and Ruf 1992; Geiser and Ruf 1995; Heldmaier et al. 2004). In mammals, body temperatures below 0°C have been measured in Arctic ground squirrels (Barnes 1989) and in European ground squirrels (Strijkstra 1999 p.102). Thorax and head temperatures do not show sub-zero temperatures. Although body temperatures may drop to very low levels, this does not mean that there is no regulation of body temperature present in torpor. Deep hibernators actively defend body temperatures, by increasing their metabolic rate for heat production when ambient temperature drops below the freezing point (Kayser 1950; Eisentraut 1956; Heller and Colliver 1974; Buck and Barnes 2000).

Most likely hibernators do not tolerate brain temperatures lower than 0 °C, since the central nervous system has a crucial role in thermoregulation during hibernation (Chatfield et al. 1951; Kayser and Malan 1963; Heller and Hammel 1971, 1972; Lyman and O'Brien 1972; Heller and Colliver 1974). Several areas important for thermoregulation in the brain are known to (minimal) activity, also during torpor (Heller 1979a; Drew et al. 2007; Osborne and Hashimoto 2008; Epperson et al. 2010). Maintaining at least a very minimal level of brain activity, including an intact thermoregulatory system, makes perfect sense, since all hibernators have to be able to initiate rewarming from torpor at least once.

C. Daily torpor

Some small mammals, for instance the Djungarian hamster (*Phodopus sungorus*) (Heldmaier and Steinlechner 1981) are not capable of storing enough food, or mounting the fat reserves necessary to survive deep hibernation during the whole hibernation season. These animals still have to show surface activity to acquire food. In order to minimize energy expenditure as much as possible during the winter period they employ daily torpor. Animals using daily torpor, show short (usually <12 hours) periods of torpor on a daily basis. During torpor the metabolic rate is strongly reduced and as a consequence body temperature drops as well. Daily torpor animals seem to defend a lower body temperature of approximately 12 - 15 °C, even in colder environments (Song et al. 1995; Geiser and Ruf 1995). Animals using daily torpor, also maintain their circadian organisation of behaviour. In fact, the torpor bouts occur in the inactive phase of the day. Many deep hibernators start hibernation by a few daily torpor bouts, sometimes referred to as"test-drops".

Some species such as edible dormice (*Glis glis*)(Wilz and Heldmaier 2000), or the Syrian hamster are capable of both deep hibernation and daily torpor. An example of apparent daily torpor in the Syrian hamster is shown in figure 1. Figure 1a shows a body temperature trace of a Syrian hamster in deep hibernation. Figure 1b shows an example of the body temperature of a Syrian hamster employing regular daily torpor.

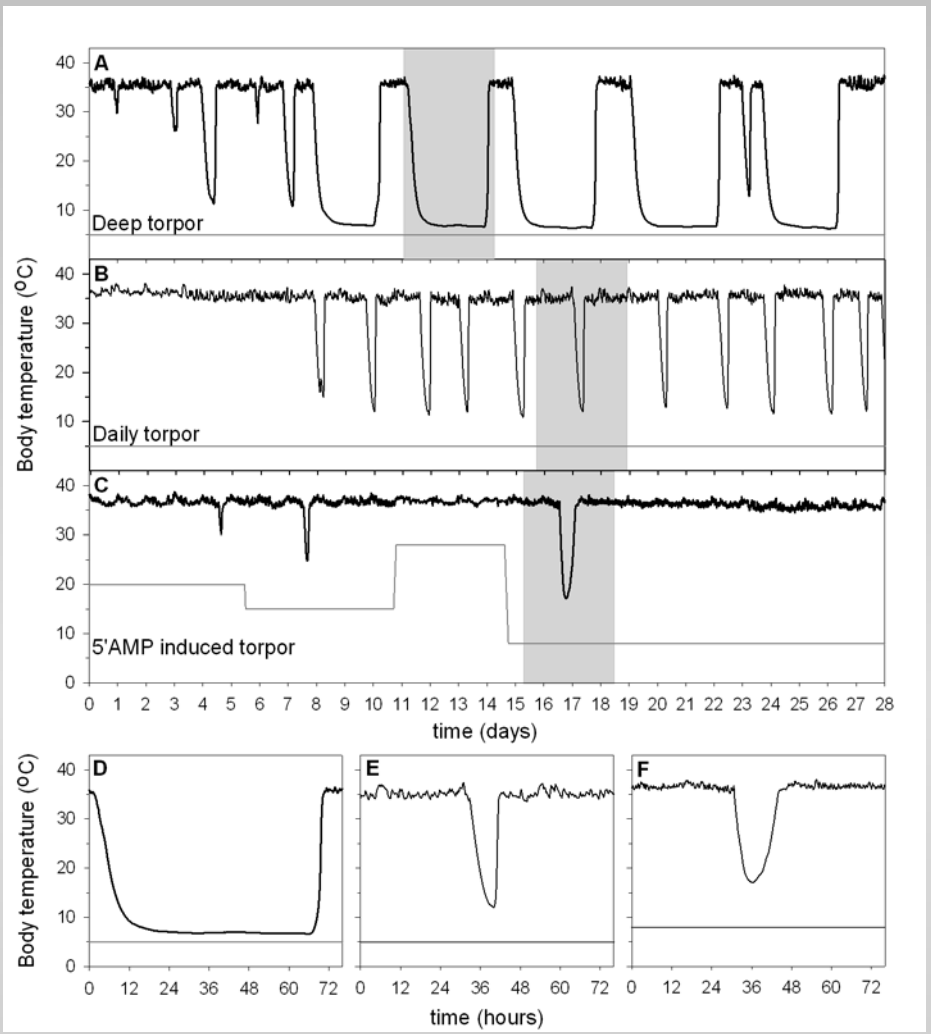

Figure 1 *Body temperature (black lines) and ambient temperature (Dark grey lines) of three Syrian hamsters showing deep hibernation (A), Daily torpor (B) and 5'AMP induced torpor at different ambient temperatures (C). The grey shaded areas are shown in more detail in panels D-F on the right. Body temperatures were recorded using I-button temperature loggers implanted in the abdominal cavity. Hamsters in panel A and B were housed in continuous dim red light at an ambient temperature of 5 °C (+/- 1 °C). The hamster in panel C was housed at an LD-Cycle of 8 hours light and16 hours darkness and varying ambient temperatures. Torpor was induced by i.p. injections of 3.0 µmol / g. body mass 5'AMP at days 4, 7, 13 and 16.*

Induced torpor

Torpor, or a torpor-like hypo metabolic state, can also be induced artificially in many animal species. For instance in house mice it is possible to induce daily torpor by food deprivation. This is has been demonstrated in several strains of laboratory mice (Hudson and Scott 1979; Webb et al. 1982; Koizumi et al. 1992; Rikke et al. 2003; Dikic 2008). Also, when mice have to work for their food, this can induce torpor. Under these hard-working conditions, torpor can even develop in to a daily torpor-like pattern (Schubert et al. 2010; Hut et al. 2011).

Recently pharmacological ways have been discovered to induce a torpor-like hypo metabolic state associated with lowered body temperature in mammals. It is possible to induce a torpor-like hypo metabolic state in mice by exposing them to H2S gas in relatively high concentrations (Blackstone et al. 2005). A more practical way is to inject mice with 5'-adenosine-monophosphate (5'AMP) (but also ADP and ATP) which suppresses metabolism and leads to a reduction in body temperature in mice (Zhang et al. 2006; Swoap et al. 2007; Swoap 2008), and also in dogs and pigs. This approach is also successful in Syrian hamsters. Figure 1c shows an example of several torpor bouts induced by an intra-peritoneal injection with 3 µmol/g body mass 5'AMP at different ambient temperatures. The panel on the right (figure 1f) shows the last torpor bout in more detail. This torpor bout was induced at an ambient temperature of 8 °C. It is still under debate if torpor induced in this way is really torpor (Swoap et al. 2007), and whether 5'AMP is involved in the mechanism of natural torpor entrance (Swoap 2008). It appears that both cooling and rewarming rates are different between daily torpor and pharmacologically induced torpor-like states (see figure 1e & 1f) in Syrian hamsters, indicating that the physiological mechanisms behind natural and induced torpor may be different.

Even though induced torpor may not be the same as natural occurring torpor, studying induced torpor might yield valuable insights in the physiological consequences of metabolic depression and/or temperature reduction for the body and the brain.

Box 2: The microtubule-associated protein tau.

Microtubules are an essential part of the cytoskeleton of cells, and certainly in neurons with their axon and dendrite structures. The cytoskeleton provides the cell with shape and structure. Microtubules are cylinders consisting of alpha- and beta-tubulin polymers and several microtubule-associated proteins. The microtubules are involved in many key processes of the cell, such as cell division, transport of organelles and transport of vesicles. The latter is of particularly importance for the function of neurons because neuronal activity relies on transport of neurotransmitter containing vesicles.

Physiological role of tau

The tau protein is an example of a Microtubule-Associated Protein (MAP). It was discovered and first described in the 1970's in the lab of Marc Kirschner (Weingarten et al. 1975; Cleveland et al. 1977). Tau is a member of a family of MAP's. The other described MAP's are MAP1, MAP2, and MAP3/4 (Murphy et al. 1977; Vallee 1980, 1990; Olmsted 1991). All of these proteins seem to be involved in the regulation of the cytoskeleton physiology of neurons. This physiology is largely achieved by post translational regulation mechanisms, such as phosphorylation and de-phosphorylation (Jameson et al. 1980; Vallee 1980).

Since microtubule proliferation is very important for growth and remodelling of neuronal structures such as axons, dendrites and spines, the MAP's play an important role in synaptic plasticity, providing the basis for neuronal development and memory processes (see (Arendt 2003) for an extensive review on the interaction of the tau protein and synaptic plasticity).

Tau is primarily present in the neurons of the central nervous system. Contrary to MAP2, the other major neuronal MAP which is primarily found in dendrites (Huber and Matus 1984), tau is located in the axons of neuronal cells (Binder et al. 1985). The only established function of the tau protein is promoting the assembly of tubulin into the microtubules, (Weingarten et al. 1975) and the stabilization of microtubule structure (Drubin and Kirschner, 1986). Tau has further been hypothesized to be involved in the regulation of axonal transport (Ebneth et al. 1998; Dixit et al. 2008).

The tau protein is found in six isoforms in the adult human brain (Goedert et al. 1989b). All isoforms are generated by alternative splicing of mRNA from a single gene (Himmler et al. 1989). The six different isoforms differ in their amount of microtubule binding repeats and N-terminal amino inserts.

This influences the binding capacity of the isoform to the microtubules, more binding repeats and/or N-terminal inserts result in enhanced capacity of the tau protein isoform for promoting microtubule assembly (Alonso et al. 2001, 2004). The binding of tau to microtubules is also affected by its phosphorylation state (Wagner et al. 1996; Sontag et al. 1996). The amount of tau bound to the microtubules and the phosphorylation state of the tau protein play a major role in microtubule proliferation (Lindwall and Cole 1984; Mandelkow et al. 1996).

Tau pathology

In a range of neurodegenerative diseases, often referred to as “tauopathies”, abnormal phosphorylation of the tau protein is observed, which can result in neurodegeneration, which in turn is associated with dementia. The abnormal phosphorylation is usually referred to as hyperphosphorylation. A major example of a tauopathy is Alzheimer’s disease (AD), but other examples include Pick’s disease and corticobasal degeneration (see (Iqbal et al. 2010) for a review).

When the tau protein gets hyperphosphorylated it tends to aggregate and assemble in so called Paired Helical Filaments (PHF’s) (Grundke-Iqbal et al. 1986). Hyperphosphorylated tau can stimulate self-aggregation in to PHF’s (Du et al. 2007). Tau deposits aggregated in PHF’s can eventually form NeuroFibrillar Tangles (NFT’s), and cause neuronal cell death (Grundke-Iqbal et al. 1986; Alonso et al. 1994). The neurofibrillar tangles are one of the hallmarks of AD, on which the medical diagnosis of the disease is based (Grundke-Iqbal et al. 1986; Braak and Braak 1991b, 1994).

PHF formation and NFT’s are preceded by hyperphosphorylation. The tau protein can be phosphorylated at many sites (Goedert et al. 1994; Morishima-Kawashima et al. 1995; Billingsley and Kincaid 1997; Stieler et al. 2011), but phosphorylation on specific locations (such as the serine 202 & threonine 205 sites recognized by the AT8 antibody (Biernat et al. 1992; Mercken et al. 1992; Goedert et al. 1995) or the serine214 & threonine 212 Sites recognized by the AT100 anti-body (Matsuo et al. 1994; Mailliot et al. 1998)), are indicated to be involved in the switch from physiological phosphorylation to the early stages of pathological PHF-like phosphorylation of the tau protein.

Part I

Hibernation

Chapter 2

Synaptophysin immunoreactivity in the hippocampus of Syrian hamsters is not affected by natural hypothermia.

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Abstract

The hibernating brain is affected by torpor. In Ground squirrels, an obligate hibernator with long torpor bouts, neuronal connectivity is affected. We investigated if in Syrian hamster hibernation, torpor has similar effects. We measured immunoreactivity (IR) of the synaptic vesicle marker synaptophysin in Syrian hamster hippocampi at different time points in the cycle of torpor and euthermic phases during hibernation. Hamsters were sacrificed in early (2.5 hours after arousal onset) and late (8.5 hours) stages of euthermic phases, and early (24.1 hours after torpor onset) and late (94.7 hours after torpor onset) stages of torpid phases. Synaptophysin IR did not show significant differences between these groups or with continuously euthermic Syrian hamsters in long day (summer) conditions. These findings contrast those of hibernating European ground squirrels, where a decrease in IR of these markers was found in torpid conditions. It is concluded that torpor in Syrian hamsters does not affect hippocampal neurotransmission capacity. The findings are discussed in the framework of regulation of torpor bout duration.

Introduction

In mammalian hibernators which allow body temperature to drop below 12°C, torpor bouts always alternate with spontaneous euthermic phases. Apparently, low body temperature does not allow maintenance of the hypometabolic state over the entire winter. All hypotheses on the function of euthermic phases during hibernation suggest that regular repair of some kind is needed. This repair may be brain related (Strijkstra & Daan 1998, Arendt et al. 2003).

The brain in hibernators appears affected by torpor: major changes in cortical neuronal connectivity and CA3 pyramidal cells have been observed (Popov and Bocharova 1992; Popov et al. 1992; von der Ohe et al. 2006; Magariños et al. 2006; Rüdiger et al. 2007). Also neurotransmission capacity in the hippocampus appears reduced in ground squirrels (Strijkstra et al. 2003).

Neurophysiological effects of torpor have been mainly investigated in ground squirrels and European hamsters. Ground squirrels hibernate for over six months without surfacing, show little activity, ingest very little food or water (if any) and tolerate very low (even sub-zero) body temperatures (Barnes 1989; Strijkstra and Daan 1997b). The observed neurophysiological changes may be specific for this extreme type of hibernation.

We investigated effects of torpor on neurophysiology in an opportunistic hibernator, the Syrian hamster (*Mesocricetus auratus*). Syrian hamsters hibernate under short photoperiod and low ambient temperature and have long euthermic phases with movement and feeding activity. To investigate neurotransmission capacity in Syrian hamsters, we focussed on the neurotransmitter-vesicle bound protein synaptophysin. We show that synaptophysin immunoreactivity in the stratum lucidum of the hippocampus does not significantly change in the course of torpid and euthermic phases in hibernating Syrian hamsters, contrasting the profound changes observed in hibernating European ground squirrels.

Materials and methods

Animals

Thirty-five male Syrian hamsters (*Mesocricetus auratus*) between 100-153 days old were derived from our local breeding colony. A summer control group of seven animals was maintained at a photoperiod of 14 hours light and 10 hours darkness and a temperature of 21 (+/-1) °C until sacrifice. Twenty-eight animals were transferred to a separate climate controlled room. Hibernation conditions were applied following the protocol of (Oklejewicz et al. 2001a). After nine weeks of acclimatization, the photoperiod was changed from 14 hours light and 10 hours darkness to a short-day photoperiod of 8 hours light and 16 hours darkness. Temperature was maintained at 21 (+/-1) °C. After 5 weeks, ambient temperature was reduced to 5 (+/-1) °C and lighting was changed to continuous dim red light (< 0.5 Lux). These conditions triggered the majority of the animals to enter hibernation and were maintained until sacrifice.

General movement activity of all animals was continuously monitored with passive infrared detectors. Activity was accumulated in 2 min time bins using a PC-based recording system. Activity patterns were used for the discrimination of torpid and euthermic phases. Periods with > 24 hours of inactivity were considered to be torpid phases. Entry of the first bout of torpor defined onset of hibernation. The period between the change in ambient temperature from 21°C to 5°C and the onset of the first torpor bout defined hibernation latency. Periods containing activity after the onset of hibernation defined euthermic phases. Torpor bout duration was defined as the difference between the first and last 2-min interval without activity in a torpor phase. Euthermic phase duration was defined as the time between two consecutive torpor phases.

Four groups of seven animals were sacrificed at different times in the torpid-euthermic cycle during hibernation: early hypothermic (HE), late hypothermic (HL), early euthermic (EE), late euthermic (EL), the summer control group was sacrificed during continuous euthermia (EU) in long day summer conditions during the inactive phase of day.

For obtaining late (EL) and early (EE) euthermic animal material, arousal was artificially induced after a standardized duration of torpor: for EE animals 74.7 (SEM 0.85) hours, for EL animals 75.6 (SEM 1.22) hours. Arousal was induced by handling, accompanied by measurement of rectal body-temperature (T_b) and mouth-temperature (T_m). EE animals were sacrificed 2.5 (SEM 0.04) hours after initiation of arousal. This timing of sacrifice can be considered very early because mouth temperature (32.1 (SEM 0.8) °C, range 29.9-35.3°C) was still considerably higher than body temperature (21.9 (SEM 2.8) °C, range 14.9-30.1 °C). EL animals were sacrificed 8.5 (SEM 0.09) hours after initiation of arousal. At this time, mouth temperature was 34.7 (SEM 0.6) °C, ranging between 33.5-36.5C and close to the corresponding body temperatures of on average 31.7 (SEM 0.5) °C, ranging between (29.9-32.5°C).

Torpid animals were sacrificed after torpor bouts of different durations: HE animals were sacrificed after 24.1 (SEM 0.51) hours and HL animals after 94.7 (SEM 2.93) hours of inactivity. Torpid mouth-temperatures before arousal induction for EE animals were 6.1 (SEM 0.12) °C and for EL animals 6.7 (SEM 0.54) °C. Torpid mouth-temperatures before sacrifice for HE animals were 6.6 (SEM 0.18) °C, and for HL animals 6.8 (SEM 0.47) °C. These temperatures confirm the hypothermic state of the animals before manipulation.

EU animals were sacrificed in the middle of the resting phase. Mouth temperature just before sacrifice in EU animals was 35.4 (SEM 0.39) °C. Of each group, five animals were transcardially perfused for immunocytochemistry, brains of two animals were frozen and stored at -80°C for western blotting. All animals were sacrificed by i.p. injection of 60mg/kg Pentobarbital 10 min before perfusion or decapitation. The experiment was approved by the Animal Experiments Committee of the University of Groningen under license number DEC-2954.

Immunocytochemistry

Five animals of all experimental groups were transcardially perfused with 100 ml phosphate-buffered saline (PBS) followed by 300 ml 4% paraformaldehyde in 0.1 M PBS. Brains were dissected, post-fixated for 24 hours in 4% paraformaldehyde in 0.1 M PBS at 6 °C, and stored in PBS with 1% sodium-azide at 6 °C. Before cutting, brains were cryo-protected by submergence in 30% sucrose and 1% sodium-azide in 0.1 M PBS at 6 °C for 48 hours of. Five brains from each group were cut coronally on a cryostat in 30 µm sections.

Free-floating sections were treated with 1% H2O2 (Merck, Germany) in tris-buffered saline (TBS) for 30 min to eliminate endogenous peroxidase activity, after which the sections were thoroughly rinsed with TBS. Non-specific binding sites were blocked with 5% normal goat serum (NGS) in TBS, containing 0.3% Triton X-100 (T) (Sigma, Germany) for 1 hour. Free-floating sections were incubated for 16 hours at room temperature with the polyclonal synaptophysin antibody (rabbit; 1:6000; Dako, Germany). The sections were again thoroughly rinsed in TBS and treated with byotinylated Goat anti-mouse IgG and preformed streptavidin/byotinyl-peroxidase complexes, followed by a nickel-enhanced diaminobenzidine reaction. Sections were stained in a solution consisting of 0.05 M Tris Buffer (pH 8.0) containing 0.4% ammonium nickel sulfate hexahydrate (Riedel de Haën, Germany), 0.02% diaminobenzidine tetrahydrochloride (Sigma, Germany) and 0.015% H2O2 (Merck, Germany). The staining reaction was performed for a standard duration, guided by visual assessment. Following the staining procedure, the thoroughly washed sections were mounted on slides, air-dried, cover-slipped and sealed in Entellan in Toluene (Merck, Germany). Histochemical controls were performed by the omission of the synaptophysin antibody, resulting in the absence of any visible cellular staining

An image analysis system (Quantimet, Leica, The Netherlands) was used to determine optical density (OD) of synaptophysin immunoreactivity (IR). OD was measured in the stratum lucidum of the hippocampus at the CA3 region and expressed in grey levels on a scale from 0 to 255. OD's were corrected for background staining by subtracting OD measured in the corpus callosum, which has low synaptophysin IR. No difference in background staining between the experimental groups was found. OD values were averaged per experimental group and statistically analysed using non-parametric Kruskal-Wallis One-Way Analysis of Variance.

Western blotting

Two animals of every group were decapitated. Brains were dissected and frozen on dry-ice within 2 minutes and stored for further processing at -80 °C. Afterwards, sub-samples of the cortex were dissected from the frozen brains. These were homogenized in ice-cold lysis buffer (20 mM Tris (HCl), pH 7.2, 2 mM MgCl2, 100 mM NaCl, 5 mM NaF, 1 mM Na3VO4, 0.5% NP-40, 1 mM DTT, 100 mg/ml PMSF, 2 mg/ml Leupeptin, 2 mg/ml Pepstatin A, 600 nM okadaic acid (Sigma, Deisenhofen, Germany); ratio of tissue to buffer: 1:5). After spinning (5000 x g, 30 min, 4 °C), lysates were filled up with glycerol to a concentration of 50% (v/v). Protein content was determined by Bradford assay. Proteins were

separated on 10% sodium dodecylsulfate polyacrylamide gels using 30 µg total protein per well and transferred to a polyvinylidene difluoride (PVDF) transfer membrane (PolyScreen; Perkin-Elmer Life Sciences, Boston, MA). Membranes were washed in PBS, blocked in PBS containing 2% BSA (w/v) and probed with a primary antibody against synaptophysin (rabbit; 1:5000; Dako, Germany). Detection of bound primary antibodies was performed with biotinylated secondary antibodies (1:2000; Amersham Pharmacia, Freiburg, Germany) and the Extravidin Peroxidase conjugate (1:5000; Sigma, Germany). Blots were developed with a Super Signal West Pico ECL-System (Pierce, Rockford, IL).

Results

Average body weight, age and hibernation timing variables of the different experimental groups are presented in Table 1. For all above mentioned variables no significant differences between the experimental groups were found (Kruskal Wallis non-parametric ANOVA: $p>0.05$; see Table 1).

Within the stratum lucidum of the hippocampus, synaptophysin is a marker for neurotransmission capacity because this region only contains synaptic vesicles in axon terminals and lacks cell-bodies. Average OD of the synaptophysin staining in the stratum lucidum is shown in figure 1b. OD of hibernating hamsters is plotted in the left panel, OD of continuously euthermic summer animals in the right panel. No statistically significant differences were found between groups (Kruskal-Wallis One-Way AOV: $F = 3.6019$ $n = 25$ $p = 0.4626$).

Examples of synaptophysin western blots of cortex protein extracts are presented in figure 1c. In all samples a prominent band at the molecular weight of ~38 kDa was present. Together with the absence of other prominent bands in the blots this indicates that the applied antibody indeed selectively recognized synaptophysin in Syrian hamsters.

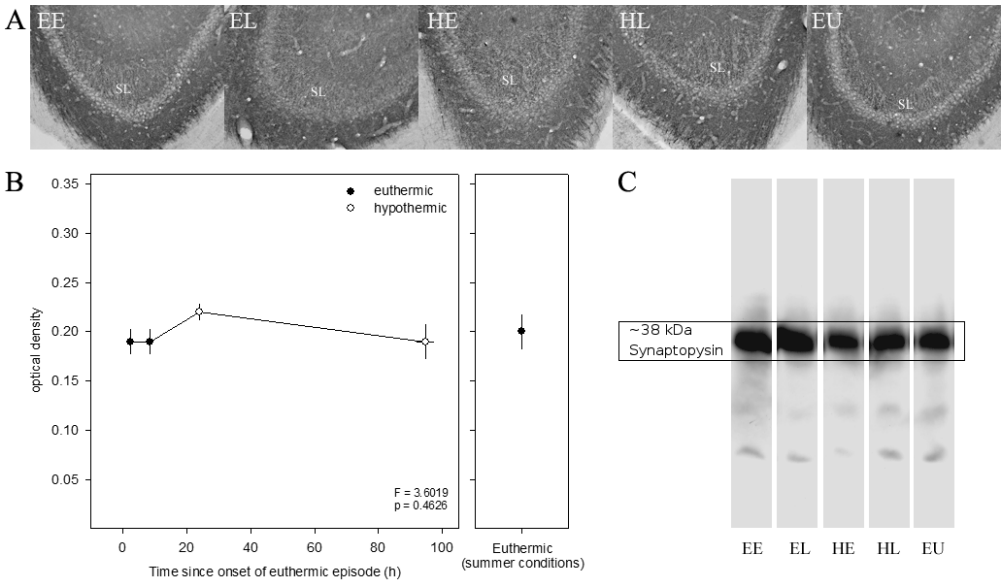

Figure 1 *Synaptophysin immunoreactivity of Syrian hamsters sacrificed in different phases of the torpor-euthermic phase cycle during hibernation and in summer control conditions. Figure 1A shows examples of the CA3 region of the hippocampus, immunostained for synaptophysin in a hibernating hamster sacrificed 2.48 hours after arousal (EE), 8.50 hours after arousal (EL), 23.80 hours in torpor (HE), and 95.08 hours in torpor (HL), and of a continuous euthermic hamster in long day summer conditions (EU). There is variation suggesting slightly lower immunostaining in hypothermic conditions, but this did not survive statistical evaluation. In the stratum lucidum (SL), no clear differences in synaptophysin immunoreactivity are apparent. Figure 1B (left panel) shows the average optical density (OD) (+/- SEM) of synaptophysin immunoreactivity for the different groups of hibernating animals. Firgure 1B (right panel) shows OD for the summer controls. Open circles indicate torpid animal material, closed circles indicate euthermic animals material. No significant differences were detected between groups (Kruskal-Wallis one-Way ANOVA. F=3.6019, n=25, p=0.4626). Figure 1C shows synaptophysin immunoreactivity on a western blot of cortex tissue protein extracts of hamsters from all 5 experimental groups. Synaptophysin immunoreactivity band showed up consistently at ~38 kDa, no other bands were detected.*

Table 1 *Hibernation characteristics, ages and body weights for Syrian hamsters sacrificed in different phases of the torpor bout / euthermic phase cycle during hibernation and in summer control conditions. Timing of sacrifice: EE: ~2.5 hours after arousal to periodic euthermy, EL: ~8.5 hours after arousal to periodic euthermy, HE: ~24 hours in periodic hypothermia, HL: >84 hours after periodic hypothermia, EU: during long day (summer) conditions. Age, torpor bout durations, and euthermic period duration do not differ between the experimental groups. The body weight of the animals in long day summer conditions tend to be slightly higher, but not significantly so. P-values indicate significance levels obtained using a Kruskal-Wallis One-Way ANOVA on all experimental groups.*

	ES		EL		HS		HL		EU		n	p
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM		
weight at hibernation induction (g)	108.3	4.51	98.2	5.85	100.8	5.93	100.9	5.65	113.1	9.97	30	*0.5299*
weight at perfusion (g)	87.6	7.19	83.0	6.95	81.5	6.80	80.8	5.59	110.2	11.71	29	*0.4532*
Age at perfusion (days)	292.5	11.16	300.0	13.13	308.8	9.91	304.8	9.04	308.7	9.74	30	*0.8594*
hibernation latency (days)	16.2	3.10	14.6	4.75	13.8	4.41	16.1	4.15	-	-	24	*0.9575*
total time in torpor (h)	857.1	34.51	883.0	102.3	888.9	53.00	861.5	60.41	-	-	22	*0.8125*
average torpor bout duration (h)	71.9	3.67	74.0	6.09	73.2	3.00	83.9	3.62	-	-	24	*0.1929*
max. torpor bout duration(h)	130.0	7.16	127.1	7.52	121.2	5.15	125.7	4.79	-	-	24	*0.9732*
Last natural torpor bout duration (h)	105.6	7.88	103.7	7.94	104.0	8.48	106.9	4.97	-	-	24	*0.9828*
total time in euthermia (h)	267.1	21.50	225.0	40.99	258.9	14.30	191.5	18.61	-	-	22	*0.0539*
average euthermic phase duration (h)	24.1	1.26	24.7	2.27	23.4	1.41	20.6	1.05	-	-	24	*0.2245*
Last natural euthermic phase dur. (h)	13.0	1.30	18.0	1.54	16.2	3.27	16.7	1.18	-	-	24	*0.1511*

Discussion

The hamsters readily hibernated using the applied protocol, and showed the expected pattern of activity (euthermic phases) and inactivity (torpor) of deep hibernators, resembling the patterns found previously (Oklejewicz et al. 2001a). The possibility to induce hibernation by changes in photoperiod and ambient temperature makes Syrian hamster hibernation an attractive model system for studying (neuro)biology of the natural hypothermic state.

In ground squirrels, several neurobiological changes during the torpid state have been described. Reduced dendritic branching in the mossy fibre system and reduced numbers of synaptic boutons on CA3 pyramidal cells in the hippocampus have been found in torpid Arctic ground squirrels (Popov and Bocharova 1992)). Furthermore, synaptic connectivity dynamics were shown with electronmicroscopic 3D reconstruction of CA3 neurons (Popov et al. 2007). Synaptophysin immunoreactivity was reduced in torpid European ground squirrels, indicating reduced neurotransmission capacity in torpor (Strijkstra et al. 2003). This suggests that ground squirrels do not, and perhaps cannot, maintain neuronal connectivity during torpor (Strijkstra and Daan 1998).

The current experiment addressed the question if neuronal connectivity is affected similarly in the Syrian hamster, a species with a different pattern of deep hibernation. In view of the similarly low body temperature reached in torpor, neuronal connectivity dynamics of hibernating Syrian hamsters may be expected to mimic those of ground squirrels. However, this appeared not to be the case: synaptophysin immunoreactivity in the stratum lucidum of the hippocampus did not show a decrease in torpor. This indicates firstly that maintenance of neurotransmission capacity in the hippocampus is possible at low temperature. This could occur either by arrest of synaptic vesicle mediated neurotransmission, or by replenishment of synaptic vesicles in torpor. The latter explanation is unlikely since at temperatures of <18 °C both DNA read-off and protein production is greatly impaired (Carey et al. 2003).

Functionally, this lack of effect may be understood as beneficial for maintaining behavioural function in hamsters. In ground squirrels, torpor leads to spatial and operational memory impairments (Millesi et al. 2001). Hamsters may have more need for accurate memory function during hibernation because they feed during hibernation.

The decreased neurotransmission capacity in ground squirrels may be associated with the long torpor duration in this species: at 5C ground squirrels may have torpor bouts as long as 8-20 days (personal observations Strijkstra), whereas Syrian hamsters rarely have torpor bouts longer than 4-5 days (Oklejewicz et al. 2001a). The hippocampus is involved in the regulation of torpor bout duration via an inhibitory effect on the brainstem reticular formation (Heller 1979a). Activity of the hippocampus may thus suspend arousal in ground squirrels, but deplete local neurotransmission capacity.

In conclusion, the lack of change in neuronal connectivity markers in torpid hamsters does not support the proposition that restoring hippocampal neuronal connectivity is the prime function of periodic euthermic phases in all hibernators. However, it may still be possible that restoring neuronal connectivity is necessary in other brain areas. Prime targets for future research would be thermoregulatory centres in the hypothalamus and the brainstem, because of their crucial role in rewarming and regulation of other vital functions.

Chapter 3

Hibernation model of tau phosphorylation in hamsters: selective vulnerability of cholinergic basal forebrain neurons – implications for Alzheimer's disease

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Abstract

Neurofibrillar tangles made up of 'paired helical filaments' (PHFs) consisting of hyperphosphorylated microtubule-associated protein tau are major hallmarks of Alzheimer's disease (AD). Tangle formation selectively affects certain neuronal types and systematically progresses throughout numerous brain areas, which reflects a hierarchy of neuronal vulnerability and provides the basis for the neuropathological staging of disease severity. Mechanisms underlying this selective neuronal vulnerability are unknown. We showed previously that reversible PHF-like phosphorylation of tau occurs during obligate hibernation. Here we extend these findings to facultative hibernators such as hibernating Syrian hamsters (Mesocricetus auratus). In this model, we showed in the basal forebrain projection system that cholinergic neurons are selectively affected by PHF-like phosphorylated tau, while c-aminobutyric acid (GABA)ergic neurons are largely spared, which shows strong parallels to the situation in AD. Formation of PHF-tau in these neurons apparently does not affect their function as pacemaker for terminating hibernation. We conclude that although formation of PHF-like phosphorylated tau in the mammalian brain follows a certain hierarchy, affecting some neurons more frequently than others, it is not necessarily associated with impaired neuronal function and viability. This indicates a more general link between PHF-like phosphorylation of tau and the adaptation of neurons under conditions of a 'vita minima'.

Introduction

Alzheimer's disease (AD) is a dementia neurodegenerative disorder characterized by extracellular neuritic plaques, composed of b-amyloid peptides (Selkoe 2001) and intracellular neurofibrillar tangles made up of 'paired helical filaments' (PHFs) consisting of hyperphosphorylated microtubule-associated protein tau (Goedert et al. 1989a; Lee et al. 2001). Neurofibrillar degeneration, i.e. neuronal death associated with PHF-tau pathology, typically affects long-axon projection neurons of cortical input systems arising in the basal forebrain and intracortical circuitry, which are both involved in the cortical processing of memory function (Morrison and Hof 1997).

Degeneration at very early and even pre-symptomatic stages of AD starts at circumscribed sites and progresses in an orderly fashion throughout numerous cortical and subcortical brain areas. This systematic progression of neurofibrillar pathology reflecting a hierarchy of neuronal vulnerability provides the basis for the commonly accepted neuropathological staging of disease severity (Braak and Braak 1991a). The cholinergic basal forebrain projection neurons giving rise to the cholinergic innervation of the entire cortical mantle (Wenk et al. 1980; Bigl et al. 1982; Butcher and Woolf 2004; Semba 2004) is one of those areas where neurofibrillar degeneration occurs very early on in AD(Arendt et al. 1983; Morrison and Hof 1997).

Cholinergic projection neurons in the basal forebrain were classified according to their mainly cortical projection targets as Ch1–Ch4 regions in the rat (Mesulam et al. 1983). The medial septum / diagonal band (MSDB) complex (also termed Ch1–Ch2) projects to the hippocampus and contains a cholinergic and a c-aminobutyric acid (GABA)ergic component (Köhler et al. 1984; Freund and Antal 1988; Záborszky et al. 1999; Sarter and Bruno 2002), as well as numerous glutamatergic neurons(Gritti et al. 2003; Colom 2006). Loss of cholinergic basal forebrain neurons causes cholinergic deafferentation of both the hippocampus and neocortex, and is considered as an early pathological event in AD (Davies and Maloney 1976; Whitehouse et al. 1981; Arendt et al. 1983; McGeer et al. 1984; Geula et al. 1998; Härtig et al. 2002), which very likely contributes to cognitive dysfunction (Kása et al. 1997; Geula and Mesulam 1999). Both neuronal loss and tangle load in the basal forebrain inversely correlate with antemortem neuropsychological assessments of cognitive function in patients with AD (Samuel et al. 1994; Iraizoz et al. 1999).

Other components of basal forebrain corticopetal projections, with GABAergic neurons being the most prominent (Fisher et al. 1988), are largely spared in AD (Sarter and Bruno 2002), indicating a selective neuronal vulnerability of cholinergic basal forebrain projection neurons. In a previous study, we have described the reversible formation of PHF-like phosphorylated tau during hibernation in the European ground squirrel (Arendt et al. 2003).

As hibernation in mammals is associated with a high degree of structural neuronal plasticity (Popov and Bocharova 1992; Popov et al. 1992; Arendt et al. 2003) it might be a model suitable to analyse cellular aspects of selective neuronal vulnerability potentially linked to plastic neuronal mechanisms. Therefore, the present study was focused on the tau phosphorylation in cholinergic and other neuronal subpopulations in the basal forebrain of hibernating Syrian hamsters.

Materials and methods

Hibernating animals

This study was performed with 53 wild-type Syrian hamsters (*Mesocricetus auratus*). Forty animals were derived from the breeding colony in Haren, University of Groningen, and 13 hamsters were from the colony in the Paul Flechsig Institute, University of Leipzig. Twelve animals were maintained at a constant temperature of 21 °C and a daily photoperiod of 14 h light and 10 h darkness. Forty-one animals were subjected to hibernation conditions according to (Oklejewicz et al. 2001a). Torpor was induced by exposing the animals to several weeks of short photoperiod as follows: 4 weeks of 'short day' (8 h light and 16 h darkness) at room temperature (20–25 °C) were followed by continuous dim red light conditions (< 0.5 Lux, 25 W red light bulb) and a reduction of the ambient temperature to 5–7 °C. General locomotor activity was monitored with passive infrared detectors allowing the discrimination between torpor (periods with > 24 h of inactivity) and euthermic phases (Oklejewicz et al. 2001a). Upon death the state of the animals was confirmed by measurements of rectal body and mouth temperatures, ranging between ~7 °C for torpid animals and ~34 °C for euthermic hamsters. According to their hibernation state the following animal groups were distinguished: euthermy (EU), torpor early (TE), torpor late (TL), arousal early (AE) and arousal late (AL). Depending on their time of inactivity after an euthermic episode hamsters were designated as TE (24 h of inactivity) or TL (4 days of inactivity), while animals that were sampled 2.5 h and 8.5 h after arousal induction represented the groups AE and AL, respectively. All animals were about 11 months old when they were killed by means of an intraperitoneal injection of 60 mg / kg pentobarbital, preceding perfusion or decapitation. The experiments were approved by the Animal Experiments Committee of the University of Groningen (license number DEC-2954), and the Animal Care and Use Committee of the University of Leipzig (T74 / 05). They conformed to the European Communities Council Directive (86 / 609 / EEC).

Tissue preparation

For immunohistochemistry, six animals each of all experimental groups were perfused with 4% paraformaldehyde in 0.1 m phosphatebuffed saline, pH 7.4 (PBS) followed by post-fixation overnight at 6 °C. After equilibration and cryoprotection with 30% sucrose in PBS, 30-lm-thick coronal sections from forebrains were cut using a freezing microtome. Sections were collected in 0.1 m Tris-buffered saline, pH 7.4 (TBS) containing sodium azide. For Western blot analysis, 23 animals of all experimental groups were decapitated. The brains were removed from the skulls, dissected and frozen on dry-ice within less than 2 min and then stored until further processing at -80 °C.

Western blotting

For the detection of PHF-like phosphorylated tau, samples containing the MSDB, the neostriatum, the neocortex or the hippocampus were dissected according to the atlas of (Morin and Wood 2001). They were homogenized in ninefold volumes of cold protein extraction buffer [in mm: Tris, 20, pH 7.2; NaCl, 150; MgCl2, 2; 1% nonylphenylpolyethylenglycol, 40; dithiothreitol, 1; Na3VO4, 1; NaF, 5; phenylmethylsulphonylfluoride, 1; 1 lg / mL leupeptin and complete proteinase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany)] using an Ultra Turrax T8 (IKA Labortechnik, Staufen, Germany). Homogenates were centrifuged for 30 min at 40 000 g and 4 °C. Clear supernatants were removed and their protein concentration was determined by Bradford assay. Samples were adjusted

to an equal protein concentration and applied to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using 10% gels. Following SDS–PAGE, separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Polyscreen, Perkin-Elmer Life Sciences, Boston, MA, USA). Blots were then treated with blocking buffer (TBS containing 2% bovine serum albumin and 0.05% Tween 20) and probed overnight with the primary antibody AT8 (1 : 1000; Perbio, Bonn, Germany) recognizing phosphorylated serine 202 and threonine 205 from tau (Goedert et al., 1995), affinitypurified goat anti-choline acetyltransferase (ChAT) (1 : 500) or rabbit anti-vesicular acetylcholine transporter (VAChT) (1 : 1000, see also Table 1), which were all diluted in blocking buffer. Following several rinses with PBS, the blots were processed with peroxidase-conjugates of goat anti-mouse IgG (1 : 10 000 in blocking buffer; Pierce, Rockford, IL, USA), donkey anti-goat IgG (1 : 5000; Dianova, Hamburg, Germany, supplier for Jackson ImmunoResearch, West Grove, PA, USA) or swine anti-rabbit IgG (1 : 5000, Dakocytomation, Hamburg, Germany) and rinsed again with PBS. Immunoreactivities were then detected by enhanced chemiluminescence (0.23 mg / mL Luminol, 0.1 mg / mL p-coumaric acid and 0.27 lL / mL H2O2 in 0.1 m Tris solution, pH 8.6) and visualized applying a KODAK Image Station 2000R (Tokyo, Japan).

Table 1 *Antibodies applied for immunofluorescence labeling of neuronal subpopulations.*

Antigen	Host	Source	Designation	Dilution for Cy2/Cy5 labelling
VACht	Rabbit	Synaptic Systems, Göttingen, Germany	I39003	1 : 50
ChAT	Goat	Chemicon, Temcula, CA, USA	AB144P	1 : 25
$P75^{NTR}$	Rabbit	Advanced Targeting Systems, San Diego, CA, USA	AB-01 AP	1 : 300
VGLUT2	Rabbit	Synaptic Systems	135102	1 : 250
VGAT	Rabbit	Synaptic Systems	131002	1 : 250
Parvalbumin	Rabbit	Swant, Bellinzona, Switzerland	PV28	1 : 200
Calbindin	Rabbit	Chemicon	AB 1778	1 : 200
Calretinin	Rabbit	Swant	7699/4	1 : 300

Immunohistochemistry

Free-floating sections were applied to immunoperoxidase staining of phospho-tau with AT8 (1:2000) and a streptavidin / biotinyl-peroxidase method using nickel-enhanced diaminobenzidine as chromogen, as previously described (Ha¨rtig et al., 1995, 2005). Double- and triple-fluorescence labelling with AT8 in combination with various markers was started by blocking of sections with 5% normal donkey serum in TBS containing 0.3% Triton X-100 for 1 h. Subsequently, AT8 was used at a dilution of 1 : 300 in the blocking solution at room temperature combined with neuronal markers as listed in Table 1. For double-immunofluorescence labelling, AT8 was concomitantly used with primary rabbit antibodies directed against the neuronal markers VAChT, low-affinity neurotrophin receptor p75 ($p75^{NTR}$), vesicular glutamate transporter 2 (VGLUT2), vesicular GABA transporter (VGAT), parvalbumin, calbindin and calretinin. Immunoreactivities were revealed with a cocktail of carbocyanine (Cy)3-tagged donkey anti-mouse IgG and donkey anti-rabbit IgG conjugated to Cy2. Both fluorochromated antibodies were obtained as all other secondary immunoreagents from Dianova and applied at 20 lg / mL in TBS containing 2% bovine serum albumin for 1 h. Combined immunofluorescence staining of phospho-tau and ChAT was achieved by applying mixtures of AT8 and goat anti-ChAT, which were visualized by Cy3-tagged donkey anti-mouse IgG and donkey antigoat IgG

coupled to Cy2. In parallel, this double-labelling was combined with the application of rabbit anti-parvalbumin and Cy5-conjugated donkey anti-rabbit IgG. For the triple-immunofluorescence labeling of phospho-tau, $p75^{NTR}$ and ChAT, the latter was visualized by Cy5-donkey anti-goat IgG, whereas the other markers were stained as described above. Controls were performed by omitting primary antibodies. In additional control experiments, the fluorophores related to the relevant markers were switched. After fluorescence labelling, all sections were treated with SudanBlack B to quench their autofluorescence, as described by (Schnell et al. 1999). Finally, the sections were mounted onto fluorescence free glass slides and coverslipped with glycerol/gelatin (Sigma, Taufkirchen, Germany).

Microscopy

The analysis of immunolabelling was based on an atlas for the hamster brain (Morin and Wood 2001) and the previous detailed description of cholinergic neurons in the hamster basal forebrain by Brauer and co-workers (Schober et al. 1989). Tissue sections were inspected with a Zeiss Axioplan microscope equipped with the filters no. 9 and 15 for the visualization of Cy2 and Cy3, respectively. For the concomitant detection of both carbocyanines, the 'double' filters 24 and 25 were used. In addition, sections were examined with a confocal laser-scanning microscope (LSM 510 Meta, Zeiss) equipped with an argon laser exciting Cy2 (at 488 nm) and two helium-neon lasers (543 nm for Cy3 and 633 nm for Cy5). Possibly interfering red fluorescence within the green spectrum in sections stained both for Cy2 and Cy3 was prevented by applying a band-pass filter (530–550 nm). In triplestained sections, Cy5-labelled structures were colour-coded in blue. In general, original pictures were slightly altered in brightness, contrast and sharpness using Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA, USA).

Results

Immunohistochemical preparations with AT8 of the basal forebrain, hippocampus, cerebral cortex and ventral striatum (figures 2 and 3) also displayed a strong immunoreactivity during torpor, which declined again after arousal confirming the reversible tau phosphorylation obtained on Western blots.

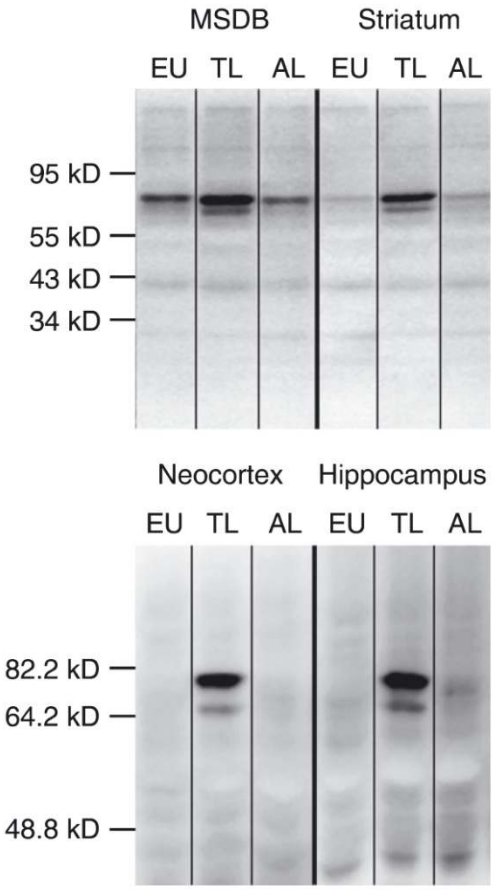

Figure 1 *Western blot analysis of PHF-like protein tau with the monoclonal mouse antibody AT8 in the medial septum / diagonal band (MSDB) complex, striatum, neocortex and hippocampus during different stages of hibernation. Comparison of representative samples obtained from euthermic animals (EU) and hamsters in torpor late (TL) and arousal late (AL) applied to SDS–PAGE and blotted to PVDF membranes. Immunodetection with peroxidase-conjugated goat anti-swine IgG and a chemiluminescence detection system. Notice the double band with apparent molecular weights between 50 and 70 kDa predominantly in torpor, largely disappearing during arousal and hardly visible in EU, except in the MSDB.*

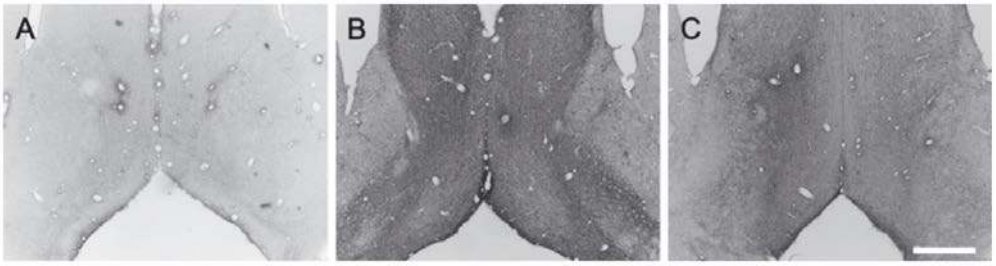

Figure 2 *Detection of reversible tau phosphorylation during hibernation of hamsters in the MSDB complex. AT8-staining with a streptavidin/biotin immunoperoxidase method and nickel-enhanced diaminobenzidine. (A) Euthermy. (B) Torpor late. (C) Arousal early. Already visible at lower magnification, AT8-immunoreactivity is apparently absent under euthermic conditions, strong in torpor and diminished during arousal. Scale bar: 400 µm.*

These data exemplify similar findings, e.g. in the dorsal striatum as well as in the motor and entorhinal cortex (not shown). Most cells containing PHF-like phosphorylated tau in the medial septum and diagonal band area were identified as cholinergic neurons, based on double-immunofluorescence for AT8 with cholinergic markers such as VAChT or the neurotransmitter-synthesizing enzyme ChAT (Figure 4). As shown in Fig. 4, both neuronal somata and axon terminals of cholinergic neurons, immunoreactive for VAChT, contained PHF-like phosphorylated tau. The specific involvement of cholinergic neurons in the MSDB area was further verified by tripleimmunofluorescence for the simultaneous detection of PHF-like tau (AT8), ChAT and $p75^{NTR}$. The majority of AT8-immunopositive structures were found to co-express ChAT in conjunction with $p75^{NTR}$. In addition, numerous ChAT-expressing cells of the striatum displayed immunopositivity for AT8, as exemplified in figure 5. Despite the formation of PHF-like phosphorylated tau in cholinergic neurons of the MSDB, no obvious alterations in the expression of ChAT and VAChT were observed in Western blots of samples from the MSDB and the striatum during the hibernation cycle (figure 6).

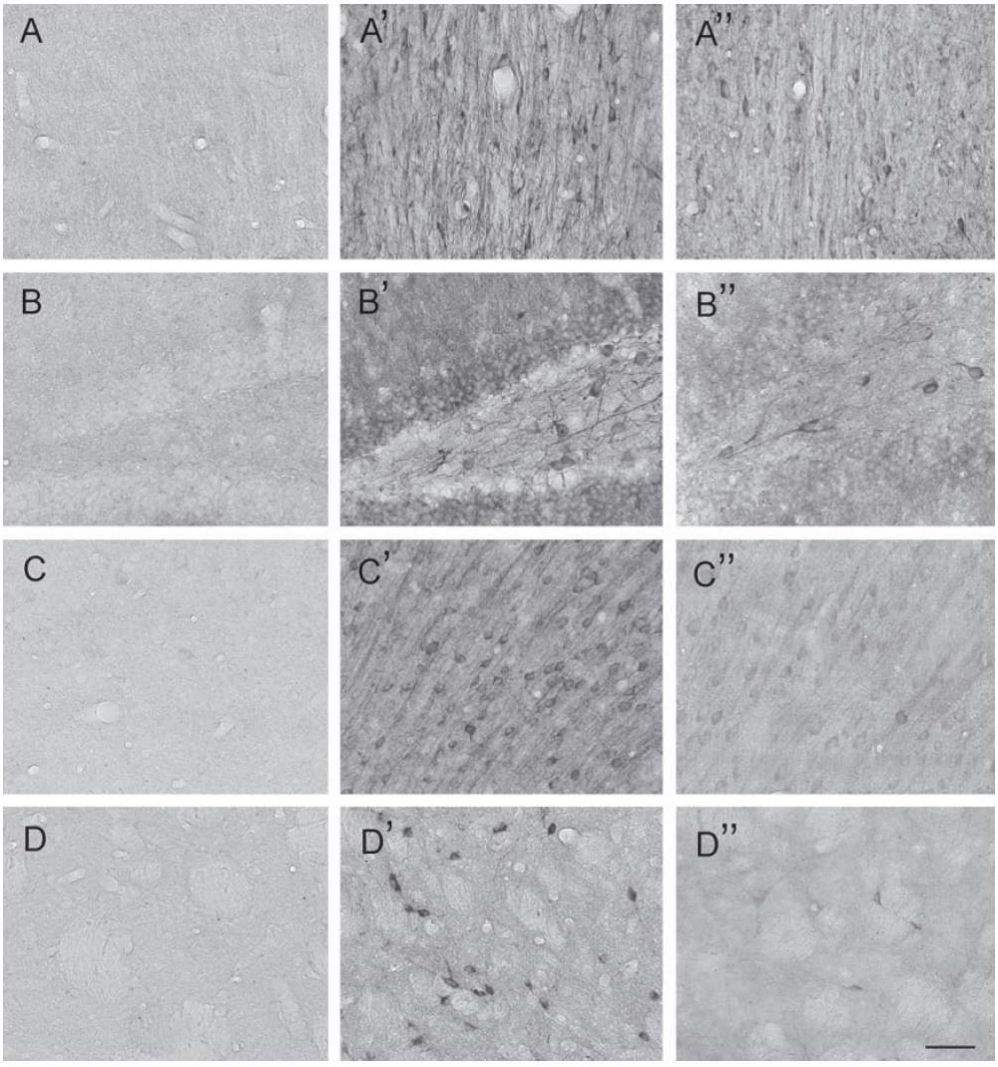

Figure 3 *Reversible tau phosphorylation during hibernation of hamsters in representative regions of the forebrain at a cellular level in (A, A', A'') the medial septum, (B, B', B'') the hippocampal gyrus dentatus, (C, C', C'') the cingulate cortex and (D, D', D'') the ventral striatum. AT8-staining with a streptavidin/biotin immunoperoxidase method and nickel-enhanced diaminobenzidine. (A–D) Euthermy. (A'–D') Torpor late. (A''–D'') Arousal early. AT8-immunoreactivity is apparently absent under euthermic conditions, strong in torpor and considerably diminished during arousal. Scale bar: 50 µm (in D'', valid for all parts of the plate).*

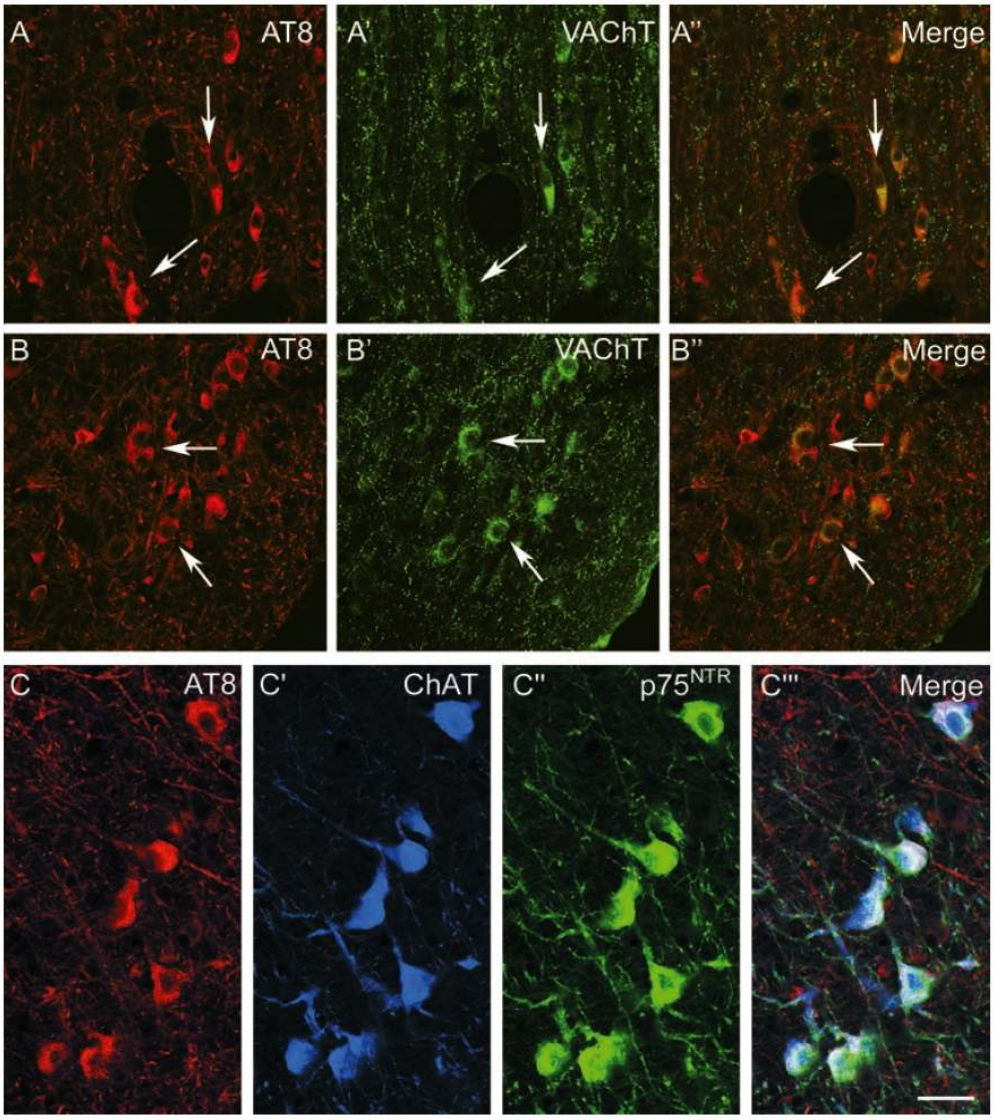

Figure 4 *PHF-like protein tau in cholinergic neurons in the basal forebrain of torpid hamsters revealed by multiple indirect immunofluorescence labelling and confocal laser-scanning microscopy. Combined immunodetection of AT8 and the vesicular acetylcholine transporter (VAChT) in the medial septum (A, A', A'') and in the diagonal band (B, B', B''). (A and B) Cy3-staining of AT8. (A' and B') Cy2-immunodecoration of VAChT. (A'' and B'') Merge. (C–C''') Multiple immunofluorescence labelling of AT8, choline acetyltransferase (ChAT) and the low-affinity neurotrophin receptor p75 (p75NTR) in the diagonal bond. (C) Cy3-staining of AT8. (C') ChAT-detection with Cy5. (C'') p75NTR-labelling with Cy2. (C''') Merge. Co-expression of AT8 and ChAT-ir resulted in purple staining, whereas structures containing all three stained markers appeared white. Arrows indicate AT8-ir neurons co-expressing the cholinergic markers. Scale bar: 100 µm.*

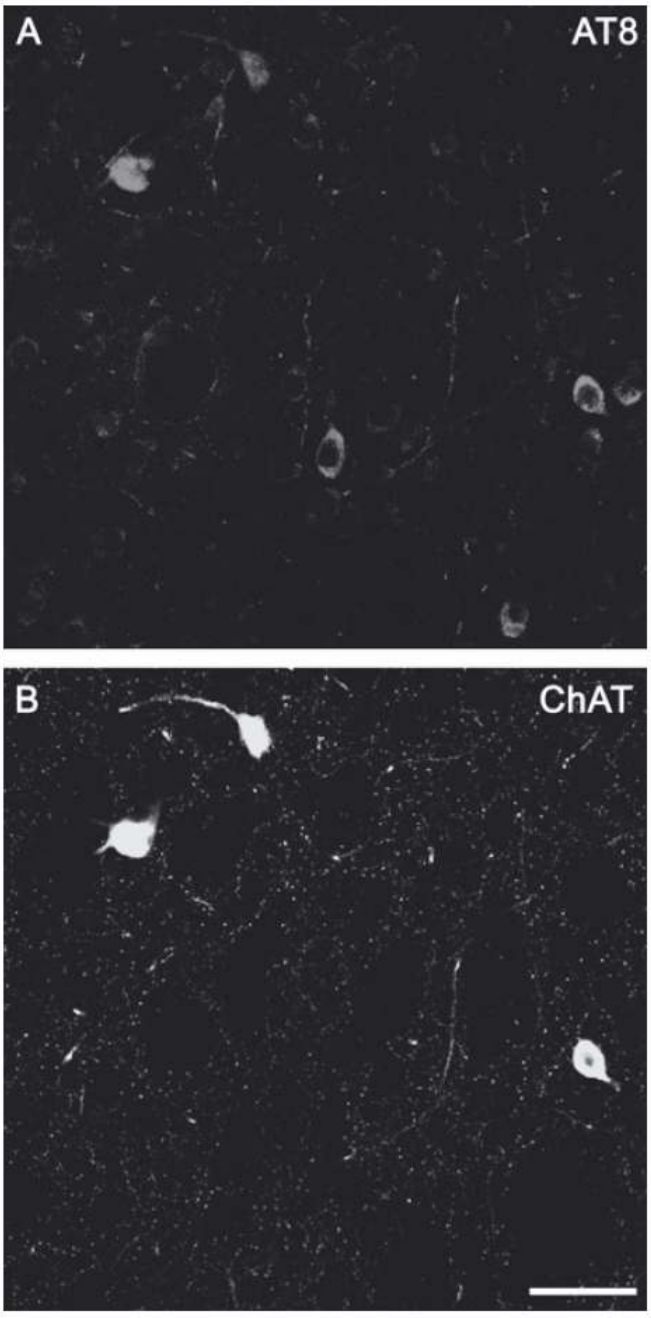

Figure 5 *PHF-like protein tau in cholinergic striatal neurons of torpid hamsters revealed by double-fluorescence labelling and confocal laser-scanning microscopy. (A) Cy3-staining of phospho-tau with AT8. (B) Cy2-immunolabelling of choline acetyltransferase (ChAT)-ir. Notice the co-expression of both markers in several neurons. Scale bar: 100 µm.*

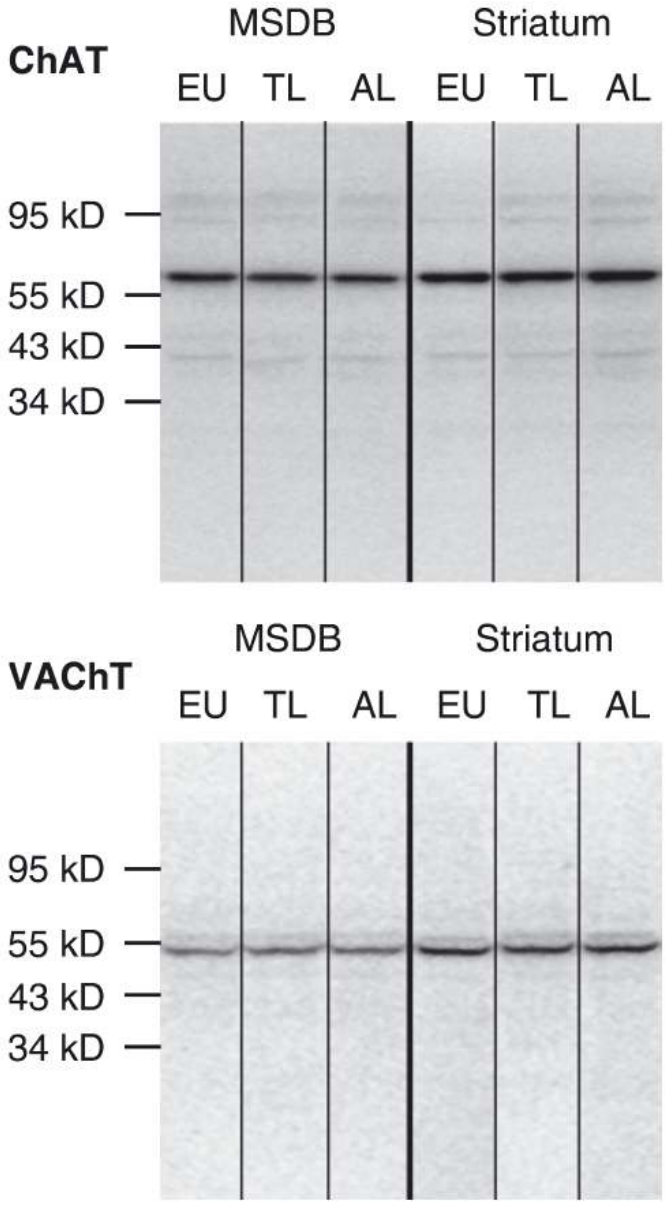

Figure 6 *Western blot analysis of the cholinergic markers choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) in the medial septum/diagonal band (MSDB) complex and the striatum during different stages of hibernation. No alterations of these proteins were revealed. Comparison of representative samples obtained from euthermic animals (EU), hamsters in torpor late (TL) and arousal late (AL), and applied to SDS–PAGE and blotting to PVDF membranes. immunolabelling with rabbit anti-VAChT and goat-anti-VAChT followed by the peroxidase-conjugates of swine anti-rabbit or donkey anti-goat and a chemiluminescence detection system.*

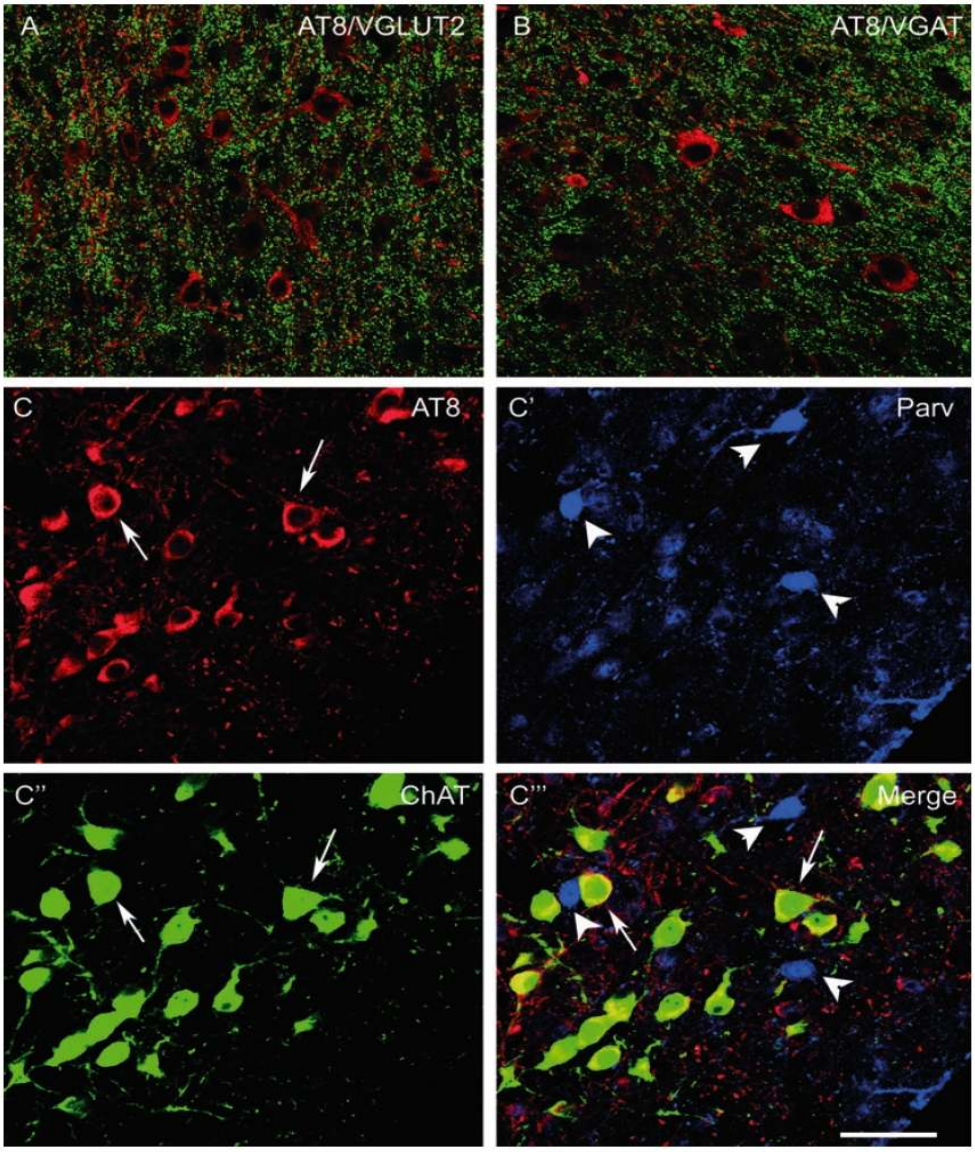

Figure 7 *Absent co-expression of PHF-like tau in vesicular glutamate transporter (VGLUT)2- and vesicular GABA transporter (VGAT)-immunopositive axonal endings and parvalbumin (Parv)-expressing somata in the diagonal band of torpid hamsters in contrast to cholinergic neurons containing phospho-tau. (A) Concomitant indirect immunofluorescence labelling of AT8 (Cy3, red) and VGLUT2 (Cy2, green). (B) Simultaneous indirect immunodetection of AT8 (Cy3, red) and VGAT (Cy2, green). (C–C''') Multiple immunofluorescence labelling of PHF-like protein tau, Parv and choline acetyltransferase (ChAT). (C) Cy3-staining of AT8. (C') ChAT-detection with Cy2. (C'') Parv-labelling with Cy2. (C''') Merge. Arrows in (C, C'', C''') indicate cholinergic neurons containing phospho-tau, whereas arrowheads in (C', C''') depict parvalbumin-containing neurons devoid of AT8-immunoreactivity. Scale bar: 100 µm.*

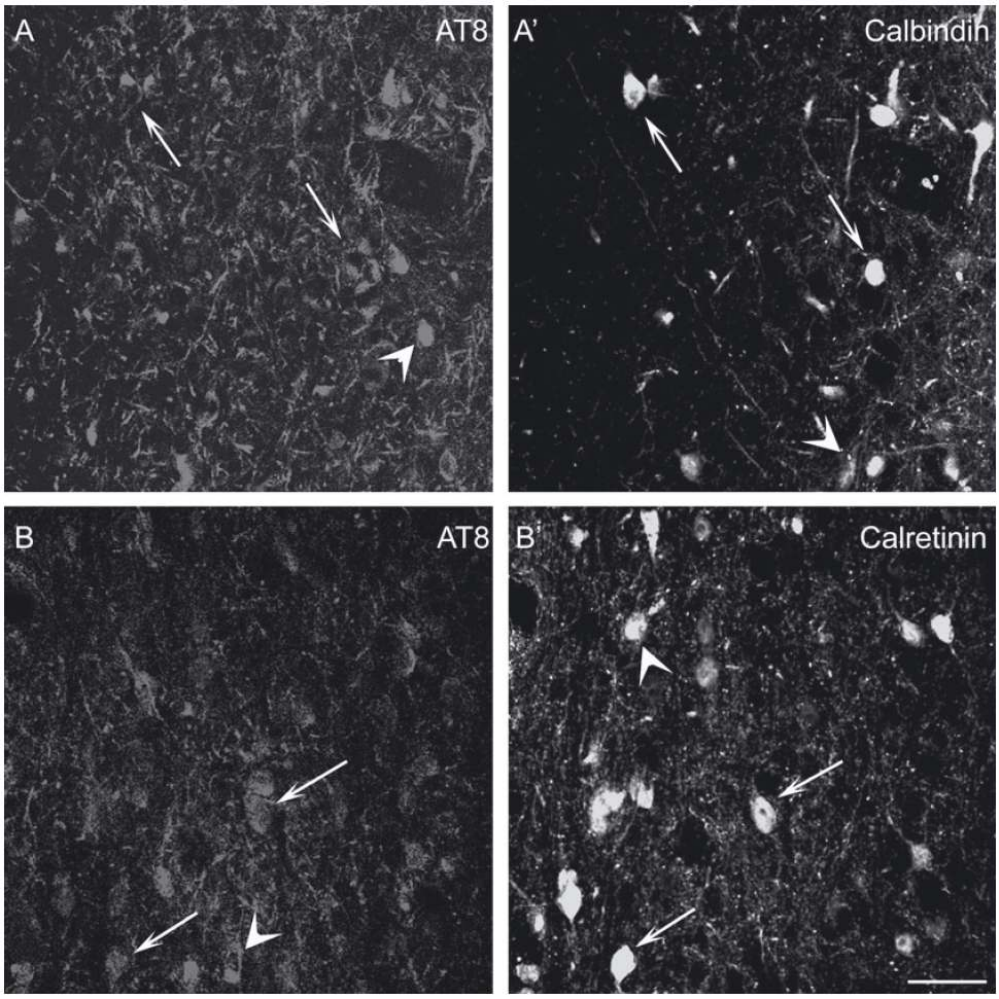

Figure 8 *AT8-immunopositive, highly phosphorylated tau in a subset of calbindin- and calretinin-ir neurons in the medial septum of torpid hamsters. (A and B) Red fluorescent Cy3-immunolabelling of highly phosphorylated tau using AT8 combined with green fluorescent Cy2-immunolabelling of calbindin (A') and calretinin (B'). Arrows exemplify cells co-expressing two stained markers, whereas the arrowheads indicate AT8-ir cells devoid of calcium-binding proteins (in A and B) or neurons containing calcium-binding proteins, but no AT8-ir (in A' and B'). Scale bar: 100 µm.*

While cholinergic basal projection neurons were constantly affected by reversible tau phosphorylation during hibernation, non-cholinergic components of the basal forebrain corticopetal projection system were either not involved at all or at least less frequently affected. It is noteworthy that glutamatergic and GABAergic axon terminals could be identified by applying antibodies raised against peptide sequences corresponding to amino acid sequences of rat VGLUT2 and VGAT, respectively. Contrary to cholinergic axon terminals (see figure 4), none of the glutamatergic or GABAergic terminals contained PHF-like phosphorylated tau (figure 7A and B). Furthermore, parvalbumin containing neurons, which in

the basal forebrain basically represent GABAergic neurons (Freund & Antal, 1988; Freund, 1989; Brauer et al., 1991) (Freund and Antal 1988; Freund 1989; Brauer et al. 1991), were devoid of PHF-like tau (figures 7C). Neurons of the MSDB area immunoreactive for calbindin (figure 8A) or calretinin (figure 8B), which most likely represent the populations of glutamatergic neurons (Gritti et al. 2003), only occasionally contained PHF-like tau (figure 8A' and B'). In control experiments, the omission of primary antibodies resulted in the expected absence of any staining. The switch of the fluorophores related to the relevant markers as additional control led to unaltered staining patterns. The treatment of all fluorescently labelled sections with Sudan Black B quenching the fluorescence of age-dependently occurring lipofuscin was essential due to some autofluorescence in untreated tissues interfering with the specific immunosignals in parallel experiments (not shown).

Discussion

The present study demonstrates reversible PHF-like phosphorylation of the microtubule-associated protein tau during the hibernation cycle of Syrian hamsters, facultative hibernators forced into hibernation. Formation of PHF-like phosphorylated tau was found both at cortical and subcortical areas such as the basal forebrain.

Selective neuronal vulnerability against PHF-like phosphorylation of tau.

To define why neurofibrillar degeneration selectively affects special types of neurons and brain areas while others are spared, i.e. to unravel the mechanism behind selective neuronal vulnerability, is a key issue to understand the process of neurodegeneration. More than 100 years ago, Paul Flechsig (1896) had advanced the idea that variations in vulnerability of different groups of neurons are to be traced back in large part to developmental conditions, a concept, later defined by Cecile and Oscar Vogt as the 'principle of pathoclisis' (Vogt and Vogt 1951). Brain areas and neuronal types highly vulnerable against neurofibrillar degeneration in AD are indeed different to the rest of the brain at least with respect to two other aspects, which might be the key to understanding underlaying mechanisms: they mature rather late during ontogenic development (Braak and Braak 1996); and they exhibit a particularly high degree of synaptic plasticity and probably synaptic turnover (for review, see Arendt 2003). The present study shows within the MSDB complex a rather selective neuronal vulnerability with respect to formation of highly phosphorylated tau. Cholinergic neurons were constantly affected. Almost all neurons, expressing ChAT and VAChT, also contained PHF-like phosphorylated tau. Moreover, axon terminals of cholinergic neurons, identified by VAChT-immunolabelling, were typically filled with phosphorylated tau. This indicates that PHF-like phosphorylated tau is highly abundant not only in the soma but also in the presynaptic compartment of cholinergic neurons.

Numerous AT8-positive neurons were shown to be co-immunopositive for $p75^{NTR}$ that in the basal forebrain of various mammalian species is exclusively expressed by cholinergic projection neurons (Springer et al. 1987; Tremere et al. 2000). The susceptibility of cholinergic neurons to tau hyperphosphorylation, however, was not restricted to the basal forebrain projection nuclei affected by neurofibrillar degeneration early in the course of AD (Morrison and Hof 1997), but also found in cholinergic neurons of the ventral striatum that are similarly known to be involved in AD (Lehéricy et

al. 1989). PHF-like phosphorylation of tau in hamsters was fully reversible, very quickly after arousing from torpor. Moreover, no quantitative changes in the expression of ChAT or VAChT were observed in the MSDB area during the hibernation cycle. This indicates at least that formation of PHF-like phosphorylated tau in these neurons does not have any major effect on protein synthesis, and very unlikely affects neurotransmitter synthesis or even viability of neurons. A major finding of this study is the absence of PHF-like phosphorylated tau in GABAergic basal forebrain neurons, identified by co-expression with parvalbumin (Freund 1989; Brauer et al. 1991). These neurons are also spared in human disorders associated with neurofibrillar degeneration, such as AD (Rossor et al. 1982; Sarter and Bruno 2002). Quite similar, parvalbumin-containing basal forebrain neurons are less vulnerable than cholinergic septohippocampal projection neurons after experimental insults in rat such as intraseptal injection of b-amyloid peptide$_{1\text{-}42}$ (Harkany et al. 1995). Neurons expressing calbindin or calretinin were also affected to some extent by formation of PHF-like phosphorylated tau, but much less frequent than cholinergic neurons. Gritti et al. (2003) suggested that significant portions of both calbindin- and calretinin-containing neurons are likely to be glutamatergic. Calretinin-immunopositive cells might predominantly comprise caudally or locally projecting glutamatergic neurons, while calbindin-containing neurons might represent cortically projecting glutamatergic basal forebrain neurons (Gritti et al. 2003). Still, their neurotransmitter remains to be elucidated, and a GABAergic phenotype cannot be ruled out (Manns et al. 2001).

Basal forebrain projection neurons and the control of cortical activity

Cholinergic projection neurons of the mammalian basal forebrain have been shown to control the excitability of neurons in large portions of the CNS including the cerebral cortex, hippocampus and thalamus (Xiang et al. 1998; McCormick 1999; Rasmusson 2000) Anatomical, electrophysiological and pharmacological properties of these neurons and their effects on cortical neuronal excitability indicate that this system is utilized for setting the tone of cortical processing (Steriade and Buzsáki 1990) and, thus, can be viewed as the most rostral extension of the ascending activation system. Degeneration of these ascending cholinergic projections in AD might result in disturbances of cortical activation mechanisms as a prerequisite for efficient processing and transformation of afferent information, which clinically appears as cognitive dysfunction. To some extent, this situation of depressed cortical activation is mimicked by the process of hibernation, which presumably begins with a reduction of the excitatory influence of the ascending activation system (Heller 1979a). In particular, it had been the septo-hippocampal projection that was proposed to act as a ‘sentry post’ controlling depression of brain activity during torpor and triggering activation of the forebrain, which leads to arousal of hibernating animals (Belousov 1993; Popova and Kokoz 2004). As this pacemaker function that controls termination of torpor is essential for the survival of hibernating animals, it requires protective mechanisms that guarantee their functional integrity even under conditions of metabolic suppression. It is therefore unlikely that formation of PHF-like phosphorylated tau in these neurons impairs their viability, and might rather reflect the induction of an active neuroprotective program against effects of reduced energy supply. This assumption is in agreement with a previous study, where after inhibition of protein phosphatase 2A in vivo through intrathecal application of okadaic acid, we observed a reduced rate of apoptosis in neurons containing hyperphosphorylated tau (Arendt et al. 1998).

Hibernation as a model for hypometabolism, an early feature of neurodegenerative disorders

Neurodegeneration in AD and related disorders is associated with a slowly progressing deterioration of cell function, which for an unknown period of time represents a cellular 'vita minima' before cell death occurs. It might be assumed that this transient condition results in a hypometabolic state of the cell that confines their energy expenditure to functions essential for survival. Hypometabolic states and deficiencies in brain energy metabolism, however, have alternatively been proposed to be primary events in a pathogenetic chain eventually leading also to a hyperphosphorylated form of tau (Schubert et al. 2003, 2004) and the whole spectrum of AD pathology (Frölich et al. 1998; Hoyer 1998; Gasparini and Xu 2003; Haley et al. 2006). As shown by functional brain imaging, this hypometabolic state occurs very early during the course of AD, even in presymptomatic stages, is a predictor of cognitive decline (de Leon et al. 2001; Devous 2002; Kim et al. 2005) and might, thus, be an attractive therapeutic target (Swaab et al. 2003). A potential pathogenetic role of hypometabolic conditions for AD is supported by recent data on thyroid disease as a potential risk factor for AD (Kalmijn et al. 2000) as well as by the promoting effects of starvation on tau phosphorylation (Yanagisawa et al. 1999; Planel et al. 2001). However, the link between hypometabolic states and tau phosphorylation and the question whether tau phosphorylation might be cause or consequence of a reduced cell metabolism has not been analysed in vivo, as appropriate models have been lacking so far.

PHF-like phosphorylation of tau during obligatory and facultative hibernation

Hibernation is known as a hypometabolic state of lethargy with torpor, e.g. a subeuthermic temperature, shown by various mammalian species during winter time (Carey et al. 2003; Storey and Storey 2004; Heldmaier et al. 2004), and may serve as a model for neuroprotection (Zhou et al. 2001). Daily torpor and hibernation are considered as the most powerful measures of endotherms to reduce their energy expenditure. Small mammals use these strategies not only in a cold environment, but also under tropical conditions during timely limited 'bottlenecks of energy supply' (Heldmaier et al. 2004). Characteristics of torpid animals are the suppression of protein synthesis (Frerichs et al. 1998), controlled by protein phosphorylation and prolonged protein life time favouring adjusted metabolism and the protection of cell functions for long-term survival (Storey 2003; Storey and Storey 2004). We showed previously that reversible PHF-like phosphorylation of tau occurs in European ground squirrels, which are obligate hibernators (Arendt et al. 2003). Here we confirm and extend these findings, and demonstrate that similar changes can be induced in facultative hibernators such as Syrian hamsters forced into hibernation. These results point towards the following. (i) A more general link between PHF-like phosphorylation of tau and the adaptation of neurons under conditions of a 'vita minima'. They indicate, furthermore, that (ii) formation of PHF-like phosphorylated tau in mammalian brain follows a certain hierarchy, affecting some neuronal types more frequently than others. Moreover, (iii) formation of PHF-like phosphorylated tau is not necessarily associated with impaired neuronal function and viability.

Syrian hamsters can much more easily be kept under animal house conditions than European ground squirrels and, thus, provide a comfortable physiological animal model suitable to analyse the mechanisms of PHF-like phosphorylation of tau and its cellular sequelae.

Acknowledgements

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Abbreviations

AD, Alzheimer's disease; AE, arousal early; AL, arousal late; ChAT, choline acetyltransferase; Cy, carbocyanine; EU, euthermy; GABA, c-aminobutyric acid; MSDB, medial septum / diagonal band; $p75^{NTR}$, low-affinity neurotrophin receptor p75; PBS, phosphate-buffered saline; PHF, paired helical filament; PVDF, polyvinylidene fluoride; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TE, torpor early; TL, torpor late; VAChT, vesicular acetylcholine transporter; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter.

Chapter 4

The cooling and rewarming hamster brain: temperature effects on tau hyperphosphorylation in the cortex and the hippocampus during hibernation.

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Unpublished manuscript

Abstract

Hibernating animals such as Syrian hamsters and European ground squirrels are affected by tau protein hyperphosphorylation during torpor. This process is fully reversed upon rewarming to euthermy. During torpor entry and rewarming from torpor hibernators are exposed to huge changes in metabolic rate and body temperature in only several hours. During rewarming large differences between body and brain temperature have been reported. Not much is known about the hyperphosphorylation dynamics during cooling and rewarming to and from torpor. In this study we addressed the question what the influence of brain temperature is on tau hyperphosphorylation during hibernation in Syrian hamsters. We measured the relation between body and brain temperature during hibernation and found that brain temperature increases before body temperature during arousal from torpor. This results in peak differences of on average 18.4 °C (SD 4.2), 139 min after the induction of arousal. We found a negative relation between brain temperature and the amount of tau hyperphosphorylation in the cortex and the hippocampus. This relation was not linear: the fastest changes in the hyperphosphorylation state of the tau protein occurred around brain temperatures of 27 - 29 °C. This suggests that tau hyperphosphorylation is not only passively connected with brain temperature, but might be actively regulated during torpor entry and arousal from torpor in hibernating Syrian hamsters.

Introduction

Hibernation is an overwintering behaviour observed in many mammalian species (Heldmaier et al. 2004). In hibernation, animals decrease their metabolic rate to save energy. As a consequence of their reduced metabolism, animals cool down and enter the physiological state of torpor. In many hibernators, phases of low metabolism and body temperature are interspersed with short periods during which metabolism and body temperature are restored to normal values (Heldmaier et al. 2004).

Syrian hamsters (*Mesocricetus auratus*) are commonly used in a laboratory setting, already for a long time (Hoffman et al. 1968). In Syrian hamsters, deep hibernation was already described by (Lyman 1948). The Syrian hamster is not only triggered to hibernate by an endogenous yearly rhythm, similar to Dormice or Ground squirrels (Mrosovsky 1977; Barnes 1989; Körtner and Geiser 2000; Hut et al. 2002a). In contrast to these species, the incidence of hibernation in Syrian hamsters is under strong photoperiodic control. If Syrian hamsters are exposed to winter photoperiod (less than 10 hours light per day) for several weeks followed by continuous darkness and winter temperatures, hamsters can enter deep hibernation independent of the actual time of year (Lyman and Chatfield 1955; Oklejewicz et al. 2001a).

At an ambient temperature of 5 °C, Syrian hamsters typically show torpor bouts of 3-6 days. Torpor bout duration initially increases with time in hibernation, but decreases abruptly at the end of the hibernation (Oklejewicz et al. 2001a; Boerema et al. 2008b). Torpor is interspersed with euthermic phases of on average 1 day, although the length of these periods can be quite variable (Oklejewicz et al. 2001a; Boerema et al. 2008b). In general, Syrian hamsters are relatively easy to use in the study of hibernation and torpor associated physiological changes in the body and the brain.

One of the major changes observed in the brains of hibernating animals is the hyperphosphorylation of the neuronal microtubule associated tau protein. So far, tau hyperphosphorylation during torpor has been reported for European ground squirrels (*Spermophilus citellus*) (Arendt et al. 2003), Arctic ground squirrels (*Spermophilus parryii*) (Stieler et al. 2008; Su et al. 2008; Stieler et al. 2009, 2011), Syrian hamsters (Härtig et al. 2007; Stieler et al. 2008, 2011), American black bears (*Ursus americanus*) (Stieler et al. 2011) and Djungarian hamsters (*Phodopus sungorus*) (Boerema et al. 2008a). Outside the scope of natural hibernation and torpor, tau protein hyperphosphorylation is well known for its occurrence in a range of neurodegenerative pathologies, of which Alzheimer's disease (AD) is the most prominent example. Under normal physiological conditions the tau protein is stabilizing the microtubules and promoting microtubule assembly (Weingarten et al. 1975; Cleveland et al. 1977), mainly in the axons of neurons (Binder et al. 1985). Under pathological conditions, i.e. in deposits of highly phosphorylated tau protein, aggregated paired helical filaments (PHF's) can be found in the brain. Neurofibrillar tangles (NFT's) can result from that, and NFT's are one of the major hallmarks of AD (Goedert et al. 1989a; Braak and Braak 1994).

Although it is clear that tau hyperphosphorylation is associated with the PHF's and NFT's observed in neurodegenerative disorders, it is still under debate whether tau hyperphosphorylation in itself is a bad thing, or alternatively may be a neuroprotective response to pathological conditions which may become pathological after prolonged duration (Lee et al. 2005). Hibernation presents a natural physiological model system to study tau phosphorylation dynamics *in vivo* (Arendt et al. 2003; Härtig et al. 2007). Apart from this apparent medical relevance, hyperphosphorylation may also be functionally relevant for hibernation brain physiology as well.

So far, the study of tau hyperphosphorylation during hibernation has focused on the differences between summer euthermic, periodic euthermic (warm) animals and torpid (cold) animals, showing tau hyperphosphorylation in torpor, which apparently must be reversed after return to euthermia. The change from euthermy to torpor and vice versa implies a massive change in metabolic rate and a temperature difference of > 30 °C in only a few hours. Somewhere along this temperature trajectory the tau protein is phosphorylated and de-phosphorylated. The temperature at which the phosphorylation takes place may give new insights in the underlying mechanisms.

In the present study we address the question what the influence of brain temperature is on the hyperphosphorylation of the tau protein during natural hibernation in Syrian hamsters. We sampled brain material of hibernating Syrian hamsters at different time points and temperatures during cooling, during torpor, and during rewarming from torpor and measured tau hyperphosphorylation. Because differences between posterior (brain) and anterior (core) body temperature have been reported during arousal and torpor (Lyman 1948; Barnes 1989), we carefully described the hibernation patterns and associated body and brain temperatures, prior to sampling.

Materials and methods

Animals and housing

A total of 111 Syrian hamsters (*Mesocricetus auratus*) were used in this study. The hamsters were bred and raised in our local breeding colony (Zoological laboratory, Haren, the Netherlands) under summer conditions (photoperiod: 14 hours light, 10 hours darkness; temperature 21 °C +/- 1). Hamsters were housed in Macrolon type 3 cages on sawdust bedding, with hay as nesting material. Water and food (RMHB 2141, Arie Blok, Woerden, the Netherlands) were available *ad libitum* throughout the experiment.

At age 8 months, 13 hamsters were individually housed under the same summer conditions, to be sacrificed later as summer euthermic controls. The other 98 hamsters were transferred to a climate, by housing the animals at winter photoperiod conditions (8 hours light and 16 hours darkness) at 21 °C +/- 1). After six to eight weeks the temperature was reduced to 5 °C (+/- 1) and lighting was changed to continuous dim red light (< 0.5 Lux). These conditions triggered the majority of the animals to enter hibernation and were maintained until sacrifice.

Activity registration and behavioural definition of hibernation patterns

General activity of all animals was continuously monitored with passive infrared detectors. Activity was accumulated in 2 min time bins using a PC-based event recording system (ERS). Activity patterns were visualized using a custom built PHP/MySQL based web interface, allowing for remote observation of activity patterns. The activity patterns were used for the discrimination of torpor and euthermic phases. Episodes with > 24 hours of inactivity were considered to represent torpor. Entry of the first bout of torpor defined onset of hibernation. The period between the change in ambient temperature from 21°C to 5°C and the onset of the first torpor bout defined hibernation latency. Periods containing activity after the onset of hibernation defined euthermic phases. Torpor bout duration was defined as the difference between the first and last 2-min interval without activity in a

torpor phase. Euthermic phase duration was defined as the time between two consecutive torpor phases. This method slightly over-estimates the torpor bout duration (Oklejewicz et al. 2001a), because the hamsters tend to start to move around only after their return to euthermia. Nevertheless it is a convenient and reliable non-invasive measure and the estimation differences are similar between different animals.

Oxygen consumption

Two hamsters were transferred during hibernation to a climate controlled room equipped with an open-flow respirometry system as described previously (Oklejewicz et al. 1997; Schubert et al. 2008). The hamsters were housed in cylindrical PVC chambers (diameter: 20 cm, height: 30 cm). Food was available *ad libitum*, water was provided as a gel pack. Saw-dust was provided as bedding material, no nesting material was provided. Hamsters were housed at a slightly higher ambient temperature of 8 °C +/-1 and in continuous dim red light (< 0.5 lux). An eight-channel system was used which sampled each hamster over a one minute interval once every three minutes and recorded differentials in gas concentrations between dried excurrent chamber air and dried reference air (drier: 3 Å molecular sieve drying beads, Merck, Darmstadt, Germany). O_2 consumption (VO_2; ml h^{-1}; Servomex Xentra 4100 paramagnetic analyzer, Crowborough, UK) and CO_2 production (VCO_2; ml h^{-1}; Servomex 1440 infrared analyzer) were measured simultaneously. Inlet airflow was set at 60 l h^{-1} (Brooks Type 5850 mass flow controller, Rijswijk, The Netherlands).

Body temperature registrations

For the general description of body temperature during hibernation, a subset of 25 animals was implanted with unmodified and intact Thermochron I-button® temperature loggers. I-buttons (type DS1922L, Maxim, Sunnyvale, CA, U.S.) were sealed in a 80%/20%, paraffin/Elvax (DuPont chemicals) (Lovegrove 2008)(Lovegrove, 2008)(Lovegrove 2008)(Lovegrove, 2008) coating to make them waterproof (Lovegrove 2008). The sealed I-buttons were sterilized by overnight incubation in a 2.5% glutaraldehyde solution and subsequently stored in sterile saline until implantation. The I-buttons were implanted in the abdominal cavity under 2.5%/97.5% isoflurane/O_2 anesthesia, one to two weeks before hibernation induction. I-buttons were preprogrammed to start recording 1 week after implantation at a resolution of one sample each 15 min at 0.0625°C resolution. This allowed for sampling during ~42 days, which was sufficient to cover the onset of hibernation, including several torpor bouts and euthermic phases in most hamsters.

Temperature during entry into and arousal from torpor

To accurately sample hamsters during torpor entry and exit precise and instant information on the state of the animal is needed. Although activity patterns yield an accurate description of the hibernation behaviour in general they are not usable to sample animals accurately during torpor entry and torpor exit, especially high on the cooling and the rewarming curves. The I-button temperature loggers only provide temperature data after explantation and are therefore also not usable for this purpose. To provide live temperature information during torpor entry we used telemetry. The transmitters we had available were usable until body temperatures as low as ~14 °C, allowing us to sample hamsters high on the cooling curve. Hamsters lower on the cooling curve were sampled based on a prediction of euthermic phase duration and absence of activity in the actogram.

Large differences between posterior (rectal) body temperature and anterior (head) temperature have been reported in animals arousing from torpor. Cheek pouch temperature in hamsters increases much earlier and may be up to 20 °C higher at some phases of the arousal than rectal body temperatures in Syrian hamsters (Lyman 1948; Lyman and Chatfield 1950). In the heart of 13-lined ground squirrels temperature starts to increase prior to rectal body temperature (Lyman and O'Brien 1961). In Arctic ground squirrels, body and brain temperature may differ considerably during torpor. During arousal from torpor these differences increase, which has been shown by thermal imaging. The anterior parts of the body (head and thorax) rewarm before the posterior part of the body (Drew and Strijkstra, personal communication). This means that both locomotor activity and core body temperature are probably not good enough as indices for determining when to sample hamsters on the rewarming curve. We therefore measured combinations of brain temperature, skull temperature and rectal body temperature with thermocouples in a subset of arousing hamsters to quantify the differences between brain vs. skull temperature and anterior (brain & skull) vs. posterior (rectal) body temperature during arousal. We subsequently used brain and skull temperature during arousal to determine the moment of sacrifice on the rewarming curve. The recordings of anterior temperature were always combined with instantaneous recordings of mouth and rectal body temperature upon sacrifice of the hamsters.

Telemetry

To record body temperature during cooling TA-F20 transmitters (Data Sciences International, St. Paul, USA) were surgically implanted in the abdominal cavity under 2.5%/97.5% isoflurane/O_2 anesthesia one - two weeks before hibernation induction. Temperature signals were acquired in 1 minute intervals and transmitter based activity recordings were summed in 1 minute bins, using Dataquest 4 software (Data Sciences International, St. Paul, USA). The on-screen temperature values were used to determine the correct sampling time during torpor entrance.

Thermocouples

To record body, brain and skull temperature during rewarming from torpor, a thermocouple setup was used. Rectal body temperature was measured using a PFTE insulated, bare tip, chromel-alumel (Type K) thermocouple (Omega engineering, Stamford, Connecticut, U.S.A.) with a wire diameter of 1 mm (18G). Brain and skull temperature were measured using PFA insulated, bare tip, copper-constantan (Type T) thermocouples (Omega engineering, Stamford, Connecticut, U.S.A.) with a wire diameter of 0.08 mm (40G). All thermocouples were connected to an 8 channel TC-08 Thermocouple Data Logger (Pico technology, Cambridgeshire, U.K.), which was connected via USB to a data acquisition pc. Temperature was recorded with version 5 of the PicoLog®, Data logging software (Pico technology, Cambridgeshire, U.K.), using a sampling interval of 30 seconds and as many readings as possible per sample. All thermocouples were calibrated, prior to use, in a water bath between 0 °C (melting ice) and 50 °C.

Brain temperature and cannula placement

Brain temperature (Tbrain) was measured by placing a thermocouple in the cortex of hibernating hamsters at arousal induction, using a previously placed cannula. Cannulae were constructed from 18G injection needles with a luer connection (B. Braun Medical B.V., Melsungen, Germany) by cutting the needle to a length of 2.8 mm. The cannulae were cleaned and sterilized by overnight incubation in a 2.5 % glutaraldehyde solution and subsequently stored in sterile saline. Caps to seal of the cannulae were constructed from the tips of Omnifix® 2 ml luer lock syringes (B. Braun Medical B.V., Oss, the Netherlands). The thermocouples were also fitted in a 2ml luer-lock syringe tip, in such a way that

when screwed to the cannula the bare tip of the thermocouple would just protrude outside of the cannula, preventing metal contact between cannula and thermocouple. The luer-lock syringes system allows needles to be screwed to the syringes. We utilized this system to screw the thermocouples (or closing caps) to the cannula. This screw system also made sure that thermocouples were always inserted precisely to a final depth of 1.2 mm in to the cortex of the hamsters.

Cannulae were surgically mounted on the skull between four and two weeks prior to hibernation induction under 2.5%/97.5%, isoflurane/O2 anesthesia. A skin incision was made on top of the skull, around bregma. Subsequently the skull was anesthetized locally by applying a drop of a lidocaïne solution directly on the bone. The surface of the skull was carefully cleaned, first with cotton and sterile saline and then with a 1% H2O2 solution. The surface of the skull was then made rough by scratching it with a scalpel. At bregma, 1 mm right or left of the midline of the brain (to avoid the sinus), a 1.6 mm hole was drilled through the skull using a spherical shaped dental drill, mounted to a battery powered Dremel Stylus® precision drill (Dremel Benelux, Breda, the Netherlands). Three smaller (1.0 mm) holes were then drilled in a triangular layout, with the cannula hole in the middle. One hole was placed five mm posterior of the middle hole and two holes were placed next to each other five mm anterior of the cannula hole. In the small holes 1.0 mm diameter and 4.0 mm length stainless steel screws (King microscrews, Boxtel, the Netherlands) were fitted. These screws were used as anchors for the cannula. The dura mater was then carefully punctured by inserting a sharp needle in the middle hole. Subsequently the cannula was inserted in the middle hole and glued to the skull under a slight backwards angle, using a small drop of industrial Pattex® super glue (Henkel, Nieuwegein, the Netherlands). The cannula and screws were then surrounded with dental cement (Durelon™, 3M ESPE dental products, Seefeld, Germany), securing and anchoring the cannula to the screws and the skull. After letting the cement dry, the skin was pulled over the dental cement foundation, around the cannula and stitched together. A cap was screwed on the cannula closing the opening and preventing infection of the brain. Analgesia was provided by a subcutaneous injection of 4.0 mg/kg Fynadine® (Schering-Plough, Oss, the Netherlands) during the surgery which was repeated 12 hours after the surgery.

Skull temperature

Skull temperature (T_{skull}) was measured by placing a thermocouple under the skin, on the skull at arousal induction. The thermocouple was inserted from behind in a 18G x 40mm injection needle (B. Braun Medical B.V., Melsungen, Germany). The needle was injected in the neck of the hamsters and moved forward underneath the skin until the point of the needle reached bregma. The needle was then pulled back over the thermocouple wire, leaving the thermocouple wire in place. This approach allowed measurements of skull temperature until the moment the animals start to move around. At this point the thermocouple slid out due to the movements of the animal.

Rectal temperature

Rectal temperature (T_{core}) temperature was measured by inserting a rectal thermocouple approximately 2 cm in the gut of the hamsters. When animals were fully rewarmed, or upon sacrifice, the thermocouple was removed.

Instantaneous temperature measurements at sacrifice

Instantaneous rectal (T_{core}) and mouth (T_{mouth}) temperatures were measured upon sacrifice of the hamsters, using a Voltcraft K102 (Conrad, the Netherlands) thermometer, equipped with a PFTE insulated 1mm bare tip copper constantan (Type K) thermocouple.

Sacrifice

Hamsters were sacrificed at different times throughout the torpor-arousal cycle and in summer euthermic conditions. Figure 1 presents an overview of all 111 animals that were sampled (white circles). All points are depicted on a schematic curve of mouth temperature during a torpor-arousal cycle. The sampled animals were divided in experimental groups, visualized by the background colours and the letters A-H in figure1.

Summer euthermic animals (group A; n = 13) were sampled at euthermic body temperatures in summer in the middle of the resting phase. Groups B-H represent animals sampled during hibernation. The animals in groups B (n=6) and C (n=22) were sampled during entry into torpor. Animals in group B had a T_{mouth} > 30 °C, animals in group C had a T_{mouth} < 30 °C. The animals in groups D and E were sampled in steady state torpor, after 24-30 hours since entry into torpor (group D; n = 3) and after >84 hours since torpor entry, Group E (n=15). The animals in groups F and G were sampled during rewarming from toror. Animals in group F (n=23) had a T_{mouth} < 30 °C, animals in group G (n=11) had a T_{mouth} > 30 °C. Finally the animals in Group H (n=18) were sampled with euthermic body temperatures > 8.5 hours following induction of arousal.

Upon sampling the hamsters were euthanized with an overdose of i.p. injected 6% Sodium Pentobarbital. T_{core}, T_{mouth}

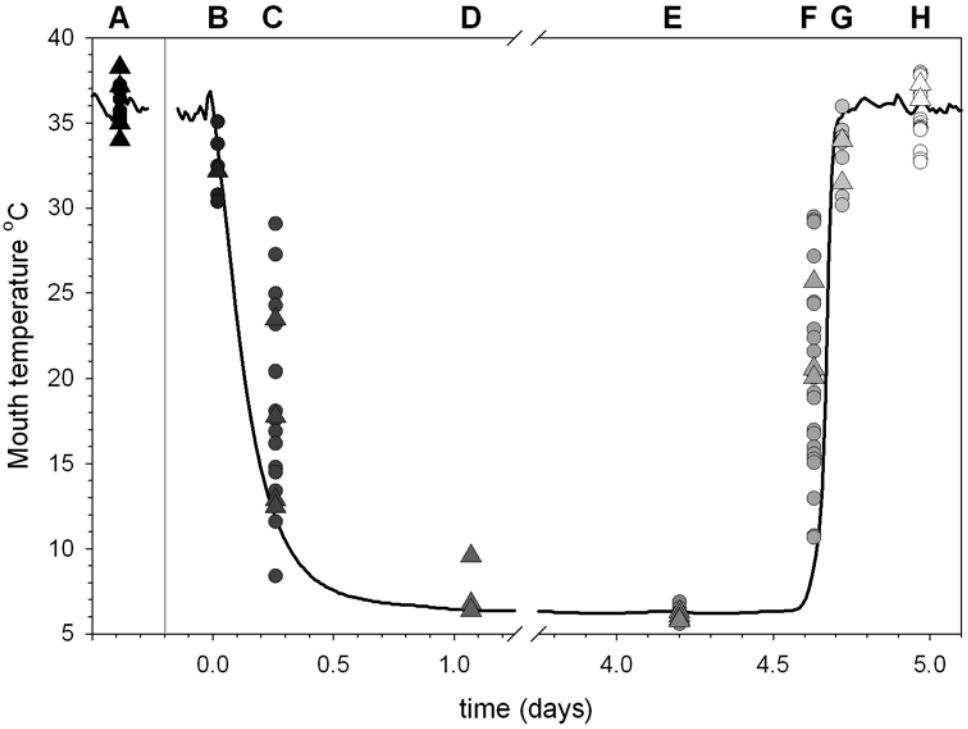

Figure 1 *Overview of the hamsters sampled on a schematic brain temperature curve over the hibernation cycle. Circles represent animals sampled for biochemical or EM analysis. Triangles represent animals sampled for ICC analysis and included in this study, letters and symbol colours correspond to the different experimental groups, as listed in table 1.*

Immunocytochemistry (ICC)

ICC was performed on 23 of the brains, as summarized in table 1. The fixated brains were cryo-protected with 30% sucrose and 1% sodium-azide in 0.01 M PBS at 6°C. Subsequently brains were frozen and cut coronally into 25 µm sections on a freezing microtome. Sections were applied to a free-floating immunoperoxidase labeling. To block endogenous peroxidase reactivity, all sections were exposed to 0.3% hydrogenperoxide in 0.01 M Tris buffered saline (TBS), pH 7.4, for 30 min. Sections were pre-incubated for two hours in 0.5% Triton-X-100 and 1.0% Bovine Albumin Serum (BSA) in TBS to permeabilize membranes and to block background epitopes. Subsequently sections were incubated with the primary antibody AT8 (MN1020, PerBio, Etten-Leur, the Netherlands) diluted 1:100 for approximately 60 hours at 6 °C in a solution of Triton X-100 (0.5%) and BSA (1.0%) in TBS (pH 7.4). Thereafter, sections were incubated for two hours with goat anti-mouse IgG Biotin-SP (1:500; Jackson, West Grove, USA) as second antibody. Negative controls were performed by omitting primary and secondary antibodies. The immunostaining was visualized using the avidin–biotin system (Vectastain, Vector Laboratories, Burlingame, USA) and diaminobenzidine-NaAC–H_2O_2 (DAB Kit: Sigma-Fast 3,3'Diaminobenzidine Tablets; Sigma, St Louis, USA).

The intensity of the AT8 immunoreactivity was measured in the hippocampus and in layers I-VI of the motor cortex (CO). These brain areas are known to be affected during torpor and daily torpor (Arendt et al. 2003; Härtig et al. 2007; Boerema et al. 2008a). The hippocampus is also thought to be involved in the regulation of torpor bout duration (Heller 1979a; Sallmen et al. 2003b). OD was measured by placing a region of interest (ROI) on the layer. Each OD was corrected for the non-specific background staining, by measuring an area in the Corpus Callosum in the same slice and subtracting the OD from this area from the ROI. Images were captured using a Leica DFC350 FX camera attached to a Leica DM 1RB microscope. The Leica Qwin software (Leica, Cambrigde, UK) was used to analyse the OD in the regions of interest. OD was measured in the cortex (CO) and areas in the hippocampus: the stratum oriens (SO), stratum pyramidale (PY) and stratum radiatum (SR) of the CA1 & CA3 regions, the stratum lucidum (SL) of the CA3 region and the Molecualar (MO), Granular cell layer (GR) and Polymorph (PM) cell layers of the DG. AT8 staining intensity was determined, using an analysis macro specifically detecting lineated structures. In this procedure both the coverage area and the OD contain relevant information; therefore the AT8 staining levels were expressed using Integrated Optical Density (IOD). The IOD was multiplied with the percentage coverage of the ROI detected by the macro to get an IOD measure corrected for the size of the complete ROI. This area corrected IOD was used in the further analysis. In one of the hamsters of group F the OD could not reliably be measured in the CA3 area of the hippocampus, resulting in n=2 for the F group and total n= 22 for the CA3 IOD analysis. In one animal of Group D the IOD in the CA1 PY could not reliably be determined, resulting in n=3 for this group for the analysis.

Statistical analysis

The relation between mouth temperature of the hamsters and the intensity of the AT8 signal (IOD) was analysed by Continuous two phase regression. This procedure is able to statistically detect physiological thresholds, or stepwise changes in physiological parameters (Nickerson et al. 1989). A regression model (see equation 1) based on the model by Nickerson et al and previously applied by (Dietz and Drent 1997; Strijkstra 1999 p.29) was used.

$$LOG(IOD) = (A+B1*Tm-R*(B1-B2)*LOG(1+EXP((Tm-C1)/R))) \quad \text{(equation 1)}$$

The parameters in the model (offset, (A), slope1 (B1), slope2 (B2) and breakpoint (C1) were iteratively solved utilizing the algorithm described by (Dennis et al. 1981) and implemented in the NonLin program (Sherrod n.d.). The Constant R was set to 0.05 as suggested by (Koops and Grossman 1993). The initial values of the parameters in model at the start of the iteration were set to A = 4, B1 = 0, B2 = -1 and C1 = 30. IOD data were log transformed prior to regression analysis. None of the estimated slopes for the left part of the fitted curves (B1) deviated significantly from zero. We therefore simplified the model and assumed 0 for B1, yielding the following model:

$$LOG(IOD) = (A+R*B2)*LOG(1+EXP((Tm-C1)/R))) \quad \text{(equation 2)}$$

This model was used to estimate the breakpoint (C1) and the slope of the second curve (B2). The relation between Tm and IOD was subsequently tested using a non-parametric Spearman rank correlation, if the model did not yield a significant fit, or if the determined break point was not significant. This was the case for the CA1 PY, CA3 PY & DG GR areas.

Table 1 *Overview of all the animals sampled for ICC analysis. The different experimental groups are colour-coded and denoted with a letter (A-H) the colours and letters are used consistently throughout the rest of this manuscript to refer to the experimental groups. The asterisk (*) indicates a value that was known at the time of sampling, but lost due to a computer crash, corrupting the data table holding the values for these animals, at a later time.*

	Animal	Condition	T_{core} (°C)	T_{mouth} (°C)	T_{skull} (°C)	body mass (g)	Time since cooling onset (h)	Time since rewarming onset (h)
A	540b	Euthermic in summer	34	34.8	-	107	-	-
A	538e	Euthermic in summer	35	36.3	-	140	-	-
A	287	Euthermic in summer	36.8	37.2	-	136.7	-	-
A	291	Euthermic in summer	37.9	38.3	-	149.1	-	-
B	289	Cooling: T_m > 30 °C	32.4	32.2	-	141.4	1.5	-
C	351	Cooling: T_m < 30 °C	12.5	12.5	-	119.9	*	-
C	352	Cooling: T_m < 30 °C	12.7	12.9	-	104.5	6.5	-
C	299	Cooling: T_m < 30 °C	18	17.8	-	116.4	4.6	-
C	365	Cooling: T_m < 30 °C	23.5	23.5	-	104.6	2.0	-
D	331	Torpor early (24 - 30 h)	6.5	6.4	-	111.5	26.0	-
D	340	Torpor early (24 - 30 h)	6.8	6.8	-	102.7	26.0	-
D	556f	Torpor early (24 - 30 h)	9.4	9.6	-	66	25.0	-
E	325	Torpor late (> 84 h)	5.8	5.8	-	112.2	118.7	-
E	296	Torpor late (> 84 h)	5.9	5.9	-	98.9	105.6	-
E	311	Torpor late (> 84 h)	6.1	6.1	-	127.1	110.6	-
E	554e	Torpor late (> 84 h)	6.9	6.3	-	73.5	85.5	-
F	367	Rewarming: T_m > 30 °C	12.1	17	17.3	109.2	-	1.40
F	366	Rewarming: T_m > 30 °C	16.6	20.6	-	97.5	-	1.52
F	348	Rewarming: T_m > 30 °C	14.1	25.7	24.7	92.9	-	1.67
G	358	Rewarming: T_m < 30 °C	23.1	31.5	31.4	121.4	-	1.92
G	356	Rewarming: T_m < 30 °C	26.3	34	33.8	112.1	-	1.77
H	342	Arousal late (euthermic > 8.5 h)	35.4	36.4	-	108.5	-	11.37
H	369	Arousal late (euthermic > 8.5 h)	37.2	37.3	-	103.5	-	12.35

Inclusion of animals in this study

In this study data of all animals, if available, is used for the analysis of body, brain and skull temperature. The biochemical data analysis with regard to tau hyperphosphorylation is not yet complete and will be included later. The results of EM analysis will be reported elsewhere. Therefore, the focus in this study will be on the data of the AT8 ICC staining. An overview of the 23 hamsters used for ICC analysis can be found in table 1 and in figure 1 the included animals are marked with a white triangle as symbol.

The experiment was approved by the Animal Experiments Committee of the University of Groningen under license numbers DEC-2954 and DEC-4746a.

Results

Core body temperature, activity and metabolism during hibernation

Winter photoperiod followed by a decrease in ambient temperature, induced hibernation in Syrian hamsters. Figure 2A shows core body temperature (T_{core}), as measured by abdominally implanted temperature loggers, of three representative individuals during the last 4 days of winter photoperiod and the first 4 weeks of hibernation conditions. The hamsters started to hibernate within 2 weeks following the onset of hibernation conditions.

All three animals displayed at least one shallow (daily) torpor episode prior to the first deep torpor episode. During deep torpor animals cooled down to near ambient temperature levels. In the first part of the hibernation, as shown here, the duration of torpor was approximately 3 days. As hibernation progressed torpor bout duration increased to 4-7 days in most hamsters. Torpor is interspersed with periodic euthermic phases. The duration of these episodes was quite variable ranging between 1-3 days. Figure 2B shows single plotted actograms of the movement activity of the same 3 animals. In the background T_{core}, is plotted in colour. During cooling to torpor no activity is observed. During arousal from torpor movement was observed as early as T_{core} = 25 °C, but was sometimes observed not before several hours in euthermy.

Figure 3A shows an example of the oxygen consumption of one individual during torpor and during arousal from torpor at an ambient temperature of 8 °C. The oxygen consumption is markedly decreased during torpor to levels as low as 0.1 ml $O_2 * h^{-1} * g^{-1}$, which is approximately 5.5% of the level observed during inactivity in periodic euthermy (1.8 ml $O_2 * h^{-1} * g^{-1}$). During arousal from torpor, oxygen consumption peaks at nearly 6 ml $O_2 * h^{-1} * g^{-1}$, more than three times the level observed during inactivity in periodic euthermy.

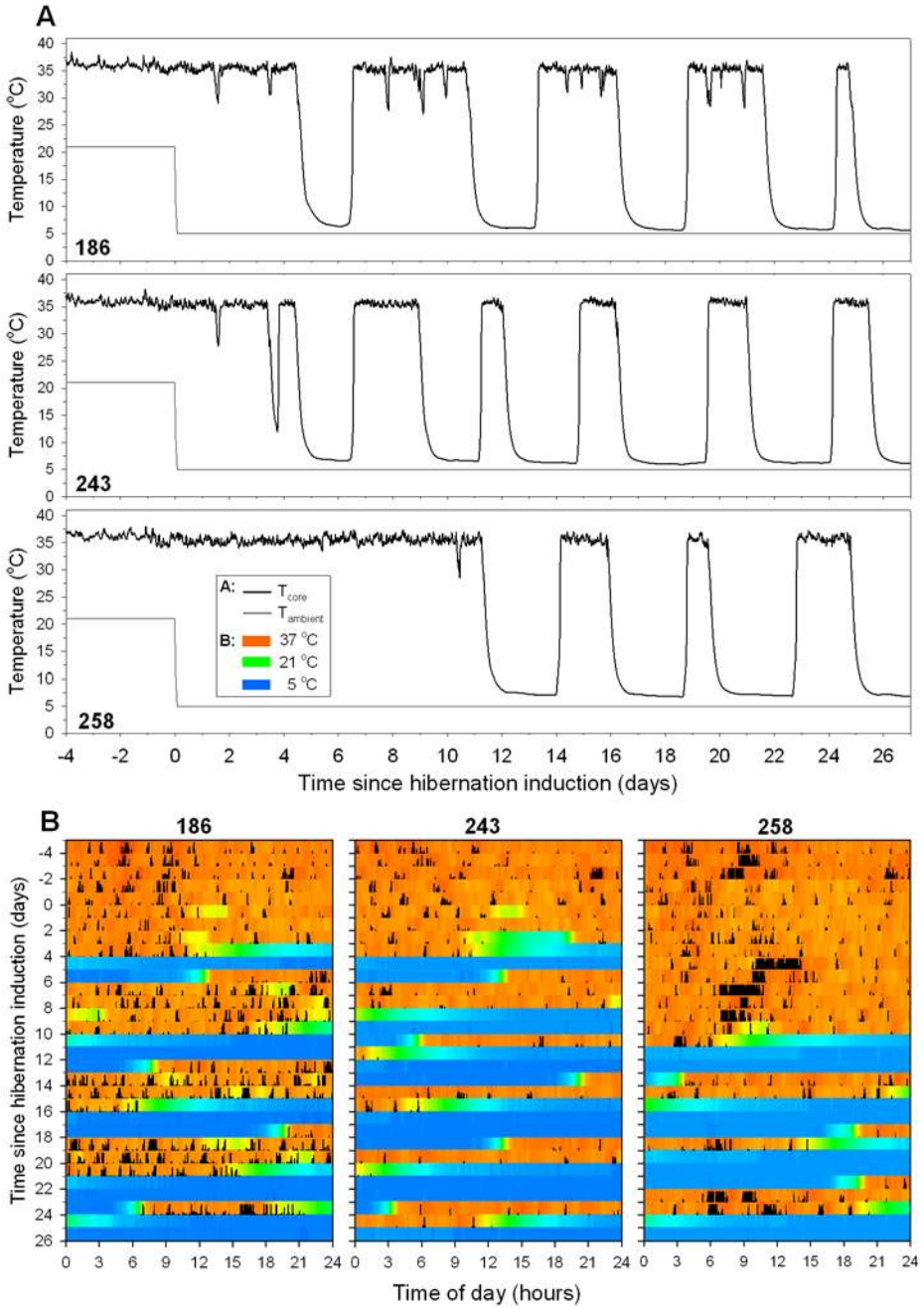

Figure 2 *Panel A, core body temperature curves (black lines) and ambient temperature (dark-grey lines) of three representative hamsters (186, 243 & 258) during hibernation onset. Panel B shows a single plotted actogram of the activity of the same animals in the same time period. The background colour represents core body temperature (red is warmer, blue is colder).*

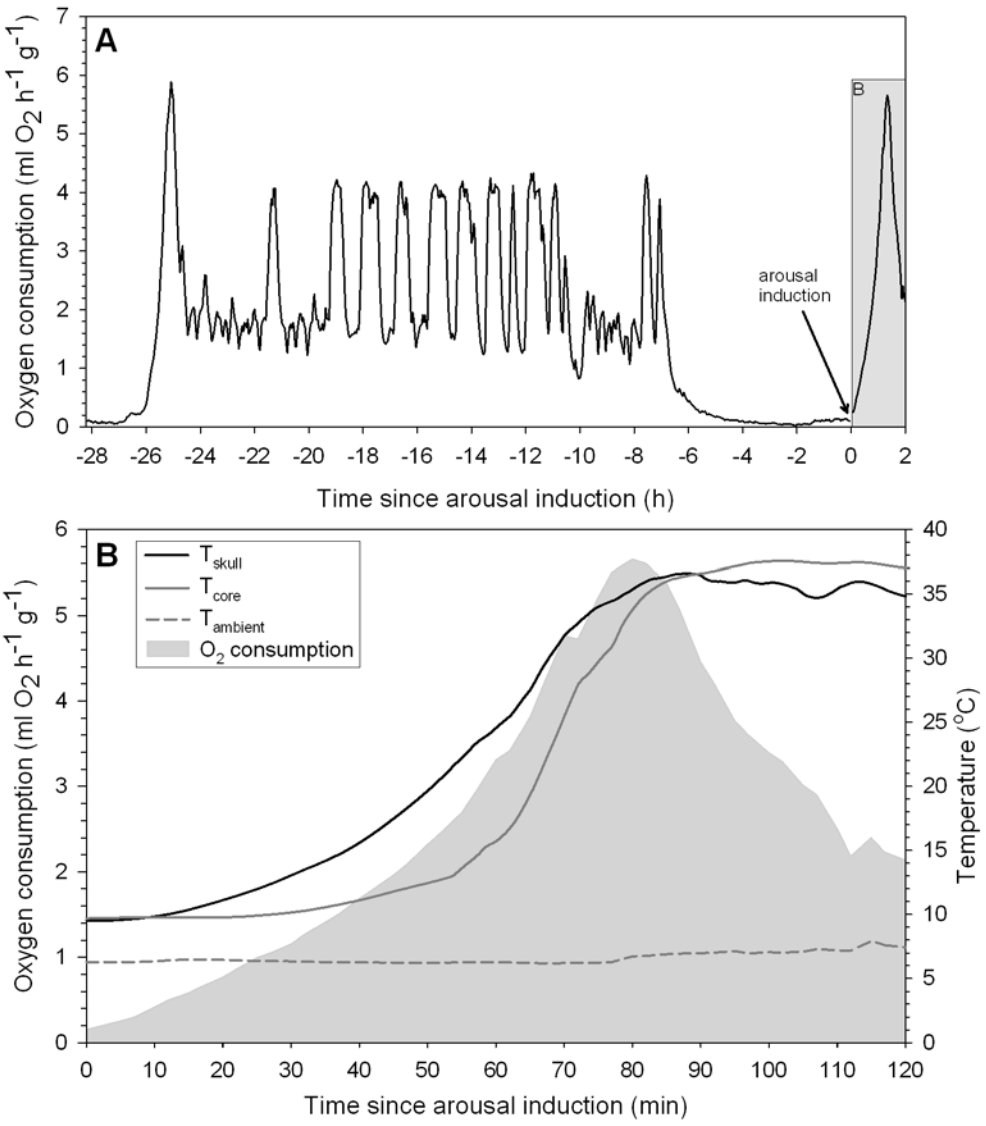

Figure 3 *Example of the oxygen consumption of a Syrian hamster during torpor and arousal from torpor (panel A). The second arousal is shown in detail, together with thermocouple recordings of skull temperature (Tskull; black line), rectal body temperature (Tcore; dark gray line) and ambient temperature (Tambient; dashed line) in panel B. The filled grey area is the oxygen consumption. Body temperature curves were smoothed by a running mean with a window of 10 min prior to plotting.*

Differences in rewarming rate between anterior and posterior temperature

In figure 3B a detailed view of T_{core}, skull temperature (T_{skull}) and oxygen consumption during arousal from torpor is presented. The oxygen consumption starts to increase before the onset of the increase in T_{skull}, peaks prior to the full return to euthermia and decreases again after rewarming is complete. T_{skull} starts to increase before T_{core}. As soon as the brain temperature (indicated by T_{skull}) is above approximately 15 °C, the animal is capable of generating heat by shivering, on top of non-shivering thermogenesis from the brown fat. At that point core body temperature also starts to rise and increases even faster than T_{skull}.

In figure 4, individual sets of temperature curves of 28 hamsters during arousal from torpor are presented. Not all arousals were completely recorded for all animals due to sampling or methodological problems. Every individual is represented in at least two of the three panels. There was a substantial variation in the rewarming rate, even when excluding the single individual (269, red curve), which appeared to be rewarming from shallow torpor. The differences in timing of rewarming between animals may be as large as 1 hour. This means that timing based on onset of arousal is an imprecise estimator of the state of the brain during rewarming. Apart from the variation in timing, large differences within individuals were observed in posterior (T_{core}) body temperature and anterior (T_{brain} & T_{skull}) during arousal from torpor. These differences are summarized in figure 5. The difference between T_{brain} and T_{skull} was calculated within individuals. The average difference with standard deviation around the mean was plotted (blue line). It appeared that there only small differences occur between skull temperature and cortical temperature during arousal from torpor, making T_{skull} a good substitute for brain temperature. In red the average difference between head temperature (i.e. T_{brain} or T_{skull}) and core temperature (T_{core}) is plotted. During the first 30 min of arousal posterior and anterior temperature appeared similar. After 30 minutes, the difference between anterior and posterior temperature increases rapidly peaking after 139 minutes, where anterior temperature is on average 18.4 °C (SD 4.3) higher than T_{core}. Apparently, core body temperature is not a good estimate for brain temperature during arousal from torpor.

Immunocytochemistry

Hyperphosphorylation of the tau protein was assessed by immunocytochemical staining with the AT8 antibody. Representative images of the AT8 stained brain slices during different phases in the hibernation cycle (see table 1 for description), are presented for different brain areas in figure 6 (cortex (CO)), figure 8 (hippocampus CA1 (CA1)), figure 10 (hippocampus CA3 (CA3)) and figure 12 (Dentate Gyrus of the hippocampus (DG)).

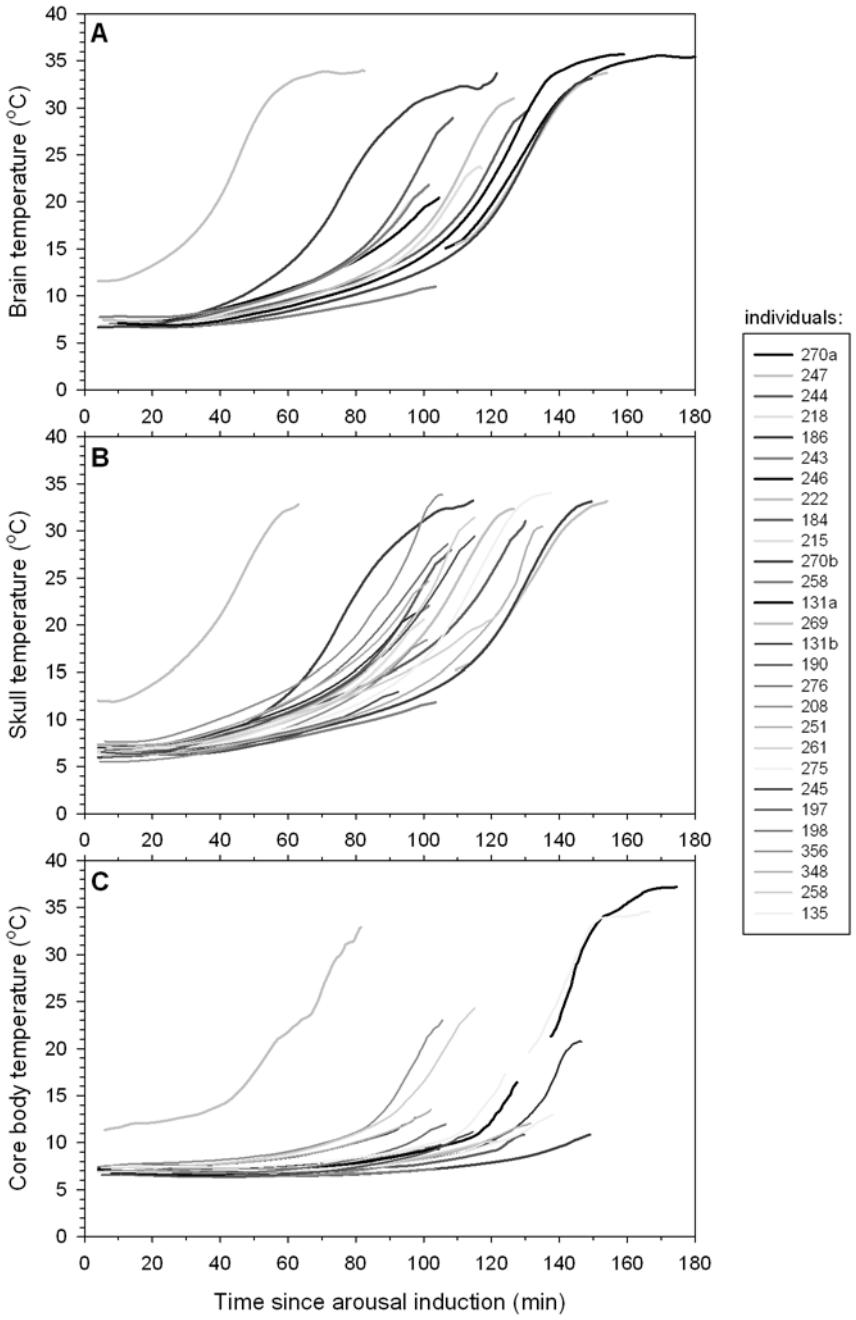

Figure 4 *Individual recordings of brain temperature (panel A), skull temperature (panel B) and rectal body temperatures during arousal from torpor. Temperature was recorded with thermocouples placed at arousal induction. The temperature curves were smoothed by a running mean with a window of 10 min prior to plotting.*

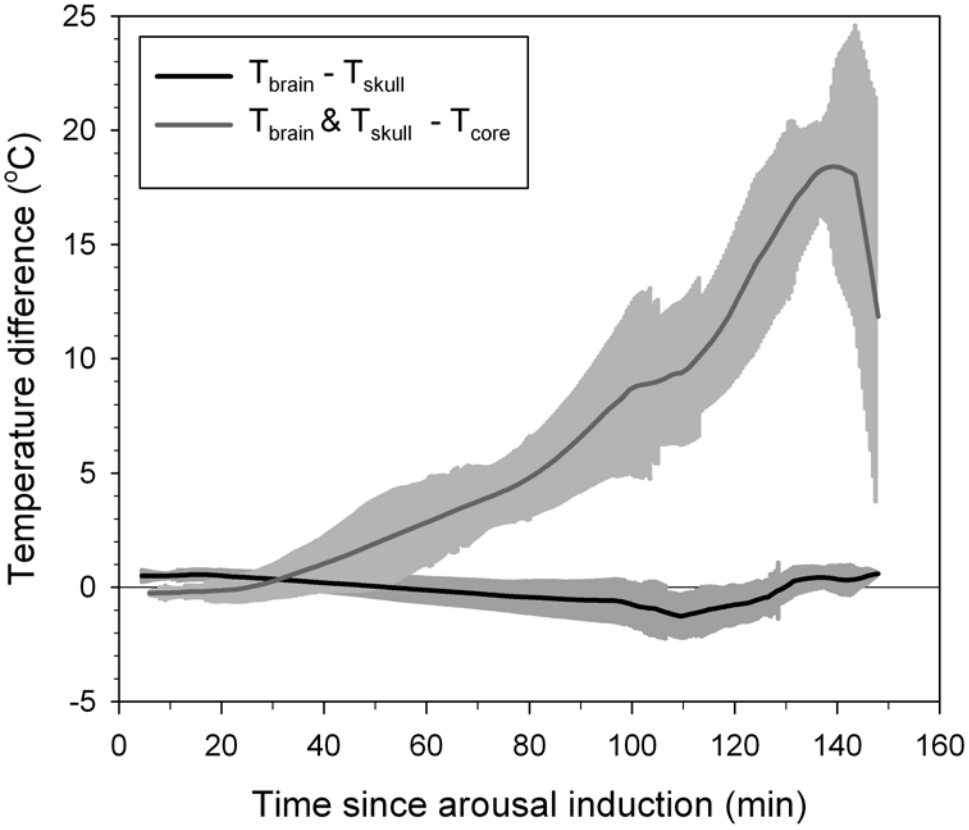

Figure 5 *Average temperature difference since induction of arousal, between cortical temperature (T_{brain}) and skull temperature (T_{skull})(blue line) and between anterior (T_{brain} / T_{skull}) and posterior (T_{core}) temperature (red line). Grey shaded areas indicate standard deviations of the mean. The average temperature curves were smoothed by a running mean with a window of 10 min prior to plotting.*

In general, little hyperphosphorylation was detected in summer euthermic hamsters (A) and Arousal late hamsters (H). In euthermic animals some staining was visible around the nucleus. This staining location was not observed during torpor, cooling or arousal and may be a side effect of the relatively high first antibody concentration. During torpor (D&E), a clear pattern of AT8 positive neurons emerged in the hippocampus and the cortex, including staining in cell bodies and apical dendrites of pyramidal cells in the cortex (figure 6 E,D). The intensity of the staining progressed with decreasing temperature during cooling (figures 6,8,19 &12 B,C). Fibers were increasingly stained as a result of the progression from eutermy, via cooling, into torpor. During rewarming from torpor the reverse process takes place (figures 6,8,19 &12 F, G). Fiber staining disappeared first and cell bodies were increasingly lighter stained with progression in to arousal towards euthermy.

AT8 optical density analysis

Cortex

Tau hyperphosphorylation was systematically analyzed by comparing the intensity of the staining in the cortex (figure 7) and several regions in the hippocampus figures 9, 11 & 13). The average IOD of fibers measured in the cortex (layers I-VI) over the hibernation cycle is shown in the left panel of figure 7. The bars are colour-coded for hibernation stage as described in table 1. Fibers in the cortex showed a relatively high background staining when compared to the hippocampus.

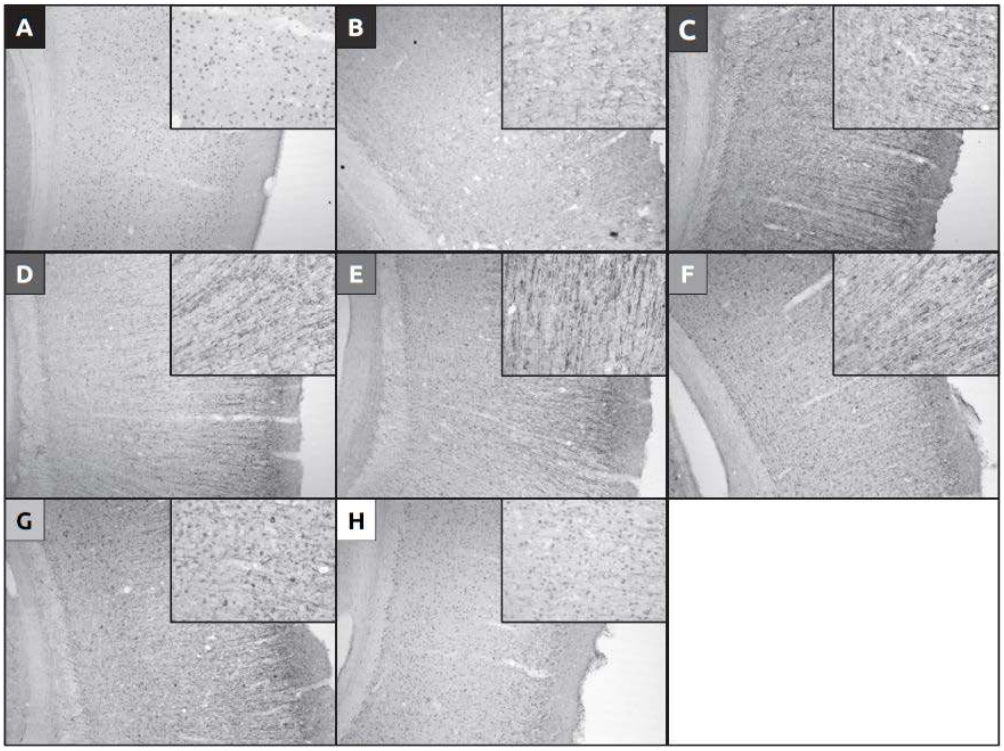

Figure 6 *Overview of the AT8 immunoreactivity in the cortex of Syrian hamsters over the hibernation cycle, at 50x magnification. The different panels show a representative example of the different experimental groups. Animas during : euthermy in summer (A); high on the cooling curve with Tmouth > 30°C (B); low on the cooling curve with Tmouth <30°C (C); short torpor 24-30 hr (D); long torpor (> 82 hr (E); low on the rewarming curve with Tmouth <30°C (F); high on the rewarming curve with Tmouth > 30°C (G) and late arousal: 8.5 hours euthermic after torpor (H). The insert panels show a more detailed view of the cortex at 200x magnification.*

The IOD increased nevertheless 20-30 times in animals with mouth temperatures (T_{mouth}) < 30 °C during cooling (C) torpor (D,E) and rewarming (F). The cooling and rewarming animals with T_{mouth} > 30 °C showed intermediate IOD levels. Together this results in a clear cyclic pattern over the torpor arousal cycle. The right panel shows the correlation of AT8 IOD with T_{mouth}, the circle symbols represent the values of individual animals. The symbols are also colour-coded for hibernation stage as

described in table 1. Continuous non-linear two phase regression analysis between T_{mouth} and IOD explained 81 % of the observed variance ($F_{2,20}$ = 41.91; p < 0.001). The analysis also revealed a breakpoint at T_{mouth} = 26.9 °C (t=8.93; p < 0.001; see also table 2), indicating that the fastest changes in hyperphosphorylation state occur around this temperature.

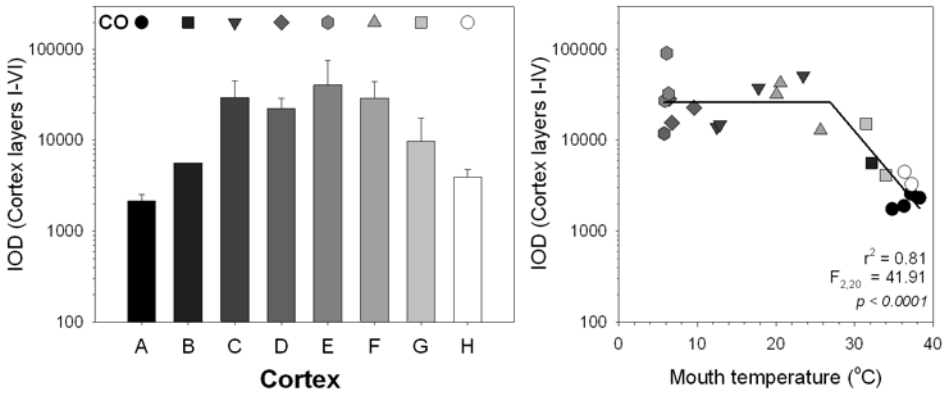

Figure 7 *IOD measurements and their relation with mouth temperature in the layers I-VI of the cortex. The left panel shows an overview of the average IOD for each experimental group with the standard deviation of the mean. Bars are colour-coded according to the list presented in table 1. The right panel shows the measured IOD for each individual, plotted against mouth temperature during sampling. The fitted curve is the result of the regression model.*

The IOD increased nevertheless 20-30 times in animals with mouth temperatures (T_{mouth}) < 30 °C during cooling (C) torpor (D,E) and rewarming (F). The cooling and rewarming animals with T_{mouth} > 30 °C showed intermediate IOD levels. Together this results in a clear cyclic pattern over the torpor arousal cycle. The right panel shows the correlation of AT8 IOD with T_{mouth}, the circle symbols represent the values of individual animals. The symbols are also colour-coded for hibernation stage as described in table 1. Continuous non-linear two phase regression analysis between T_{mouth} and IOD explained 81 % of the observed variance ($F_{2,20}$ = 41.91; p < 0.001). The analysis also revealed a breakpoint at T_{mouth} = 26.9 °C (t=8.93; p < 0.001; see also table 2), indicating that the fastest changes in hyperphosphorylation state occur around this temperature.

Hippocampus

With the exception of the Pyramidal cell layer (PY) in the hippocampal CA1 area, a similar hyperphosphorylation pattern as in the cortex is observed in the hippocampus. A cyclic pattern in the AT8 immunoreactivity is observed in the stratum oriens (SO) and stratum radiatum (SR) of the CA1 area of the hippocampus (figure 9), the SO, PY, SR and stratum lucidum (SL) of the CA3 area of the hippocampus (figure 11) and in the Molecular cell layer (MO), the Granular cell layer (GR) and the Polymorph cell layer (PM) of the Dentate Gyrus of the hippocampus (figure 13). Continuous non-linear two phase regression analysis or Spearman rank correlations between T_{mouth} and IOD show a negative relationship between T_{mouth} and the intensity of the AT8 staining in these areas. With the exception of the CA1 PY, CA3 PY and DG GR significant breakpoints (see table 2 for an overview), indicating changes in tau phosphorylation were found at temperatures between T_{mouth} = 22.5 – 29.1 °C (see

table2 and figure 14). In the Pyramidal cell layer of the CA1 area and the Granular cell layer of the Dentate Gyrus the iterative regression analysis did not converge to a solution, indicating a difficulty to show a stepwise change in phosphorylation in this area. Non-parametric Spearman rank correlation confirmed the existence of negative relations between T_{mouth} and IOD in these areas (CA3 PY: r = -0.51, p < 0.015, n = 22; DG GR: r = -0.48, p = 0.021, n= 23). In the pyramidal cell layer of the CA1 area no significant relation between temperature and IOD could be found (regression: $F_{2,20}$ = 0.17; p = 0.842; Spearman rank correlation: r = -0.04, p < 0.84, n = 23), indicating an absence of differences in hyperphosphorylation over the hibernation cycle in this area.

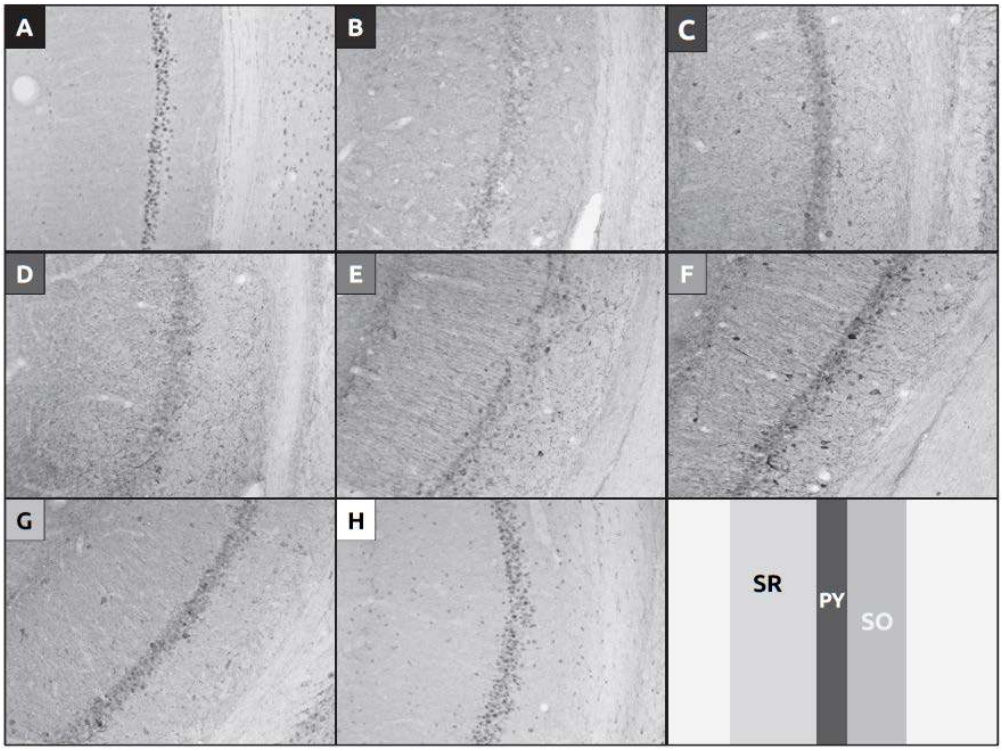

Figure 8: *Overview of the AT8 immunoreactivity in the CA1 area of the hippocampus of Syrian hamsters over the hibernation cycle, at 100x magnification. The different panels show a representative example of the different experimental groups. Animas during : euthermy in summer (A); high on the cooling curve with T_{mouth} > 30°C (B); low on the cooling curve with T_{mouth} <30°C (C); short torpor 24-30 hr (D); long torpor (> 82 hr (E); low on the rewarming curve with T_{mouth} <30°C (F); high on the rewarming curve with T_{mouth} > 30°C (G) and late arousal: 8.5 hours euthermic after torpor (H). The bottom right panel shows a schematic representation of the location of the areas where OD was measured (SO = stratum oriens, PY = stratum pyramidale, SR = stratum radiatum.*

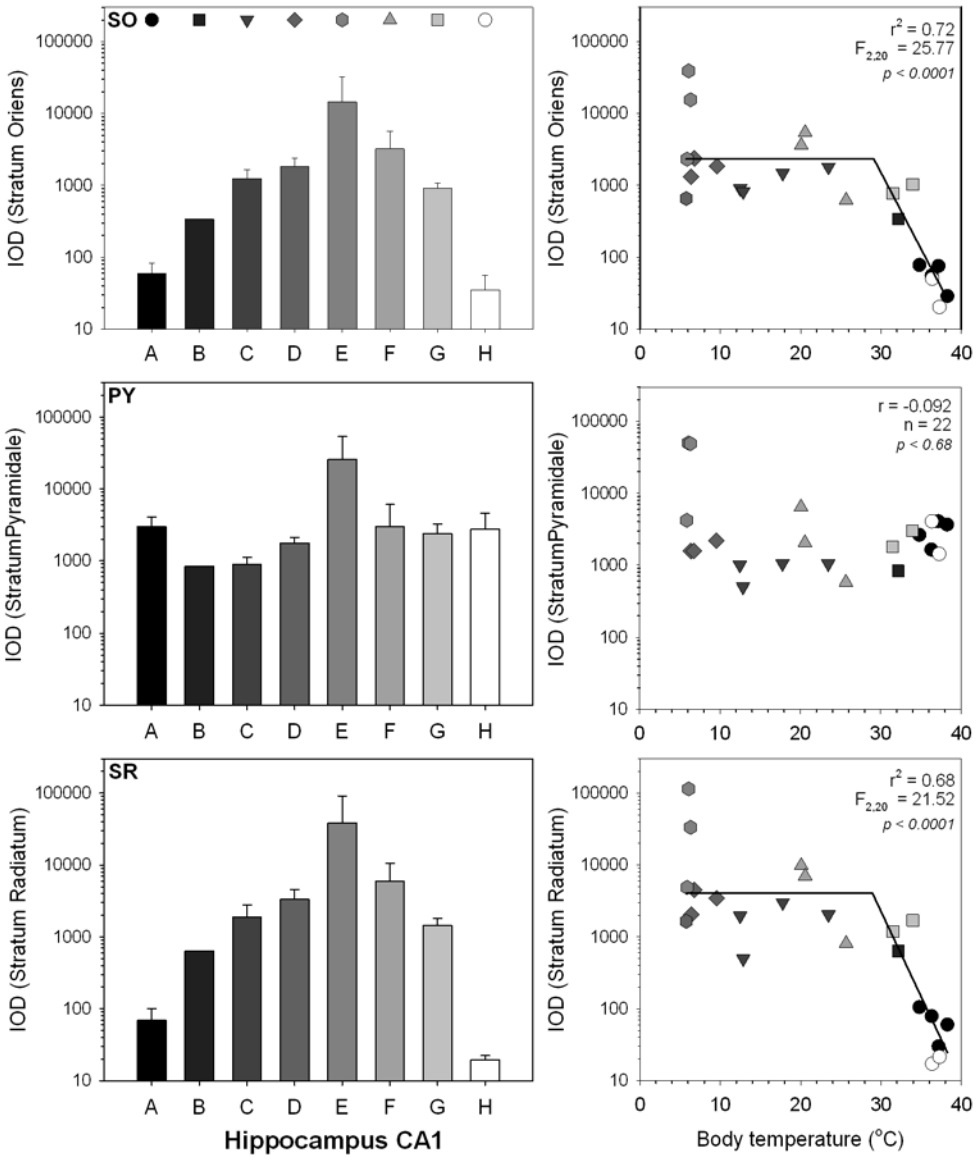

Figure 9 *IOD measurements and their relation with mouth temperature in the CA1 area of the hippocampus. The left panels show an overview of the average IOD for each experimental group with the standard deviation of the mean per CA1 sub-area. Bars are colour-coded according to the list presented in table 1. The right panels show the IOD for each individual, plotted against mouth temperature during sampling. The fitted curves are the result of the regression model.*

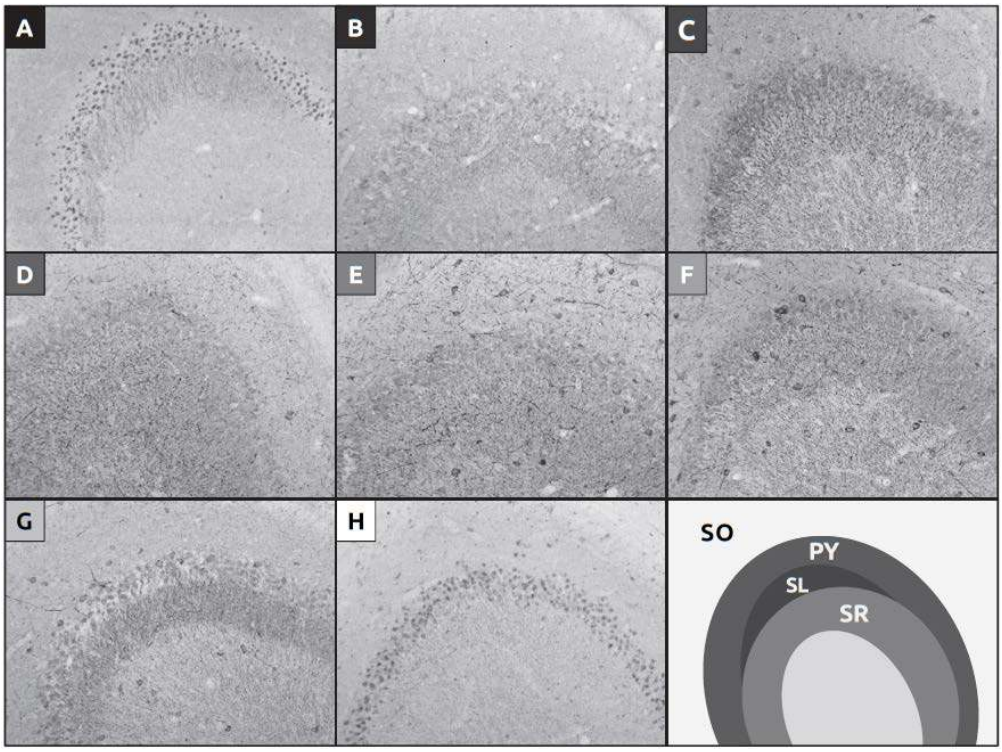

Figure 10 *Overview of the AT8 immunoreactivity in the CA3 area of the hippocampus of Syrian hamsters over the hibernation cycle, at 100x magnification. The different panels show a representative example of the different experimental groups. Animas during : euthermy in summer (A); high on the cooling curve with T_{mouth} > 30°C (B); low on the cooling curve with T_{mouth} <30°C (C); short torpor 24-30 hr (D); long torpor (> 82 hr (E); low on the rewarming curve with T_{mouth} <30°C (F); high on the rewarming curve with T_{mouth} > 30°C (G) and late arousal: 8.5 hours euthermic after torpor (H). The bottom right panel shows a schematic representation of the location of the areas where OD was measured (SO = stratum oriens, PY = stratum pyramidale, SL= stratum lucidum, SR = stratum radiatum.*

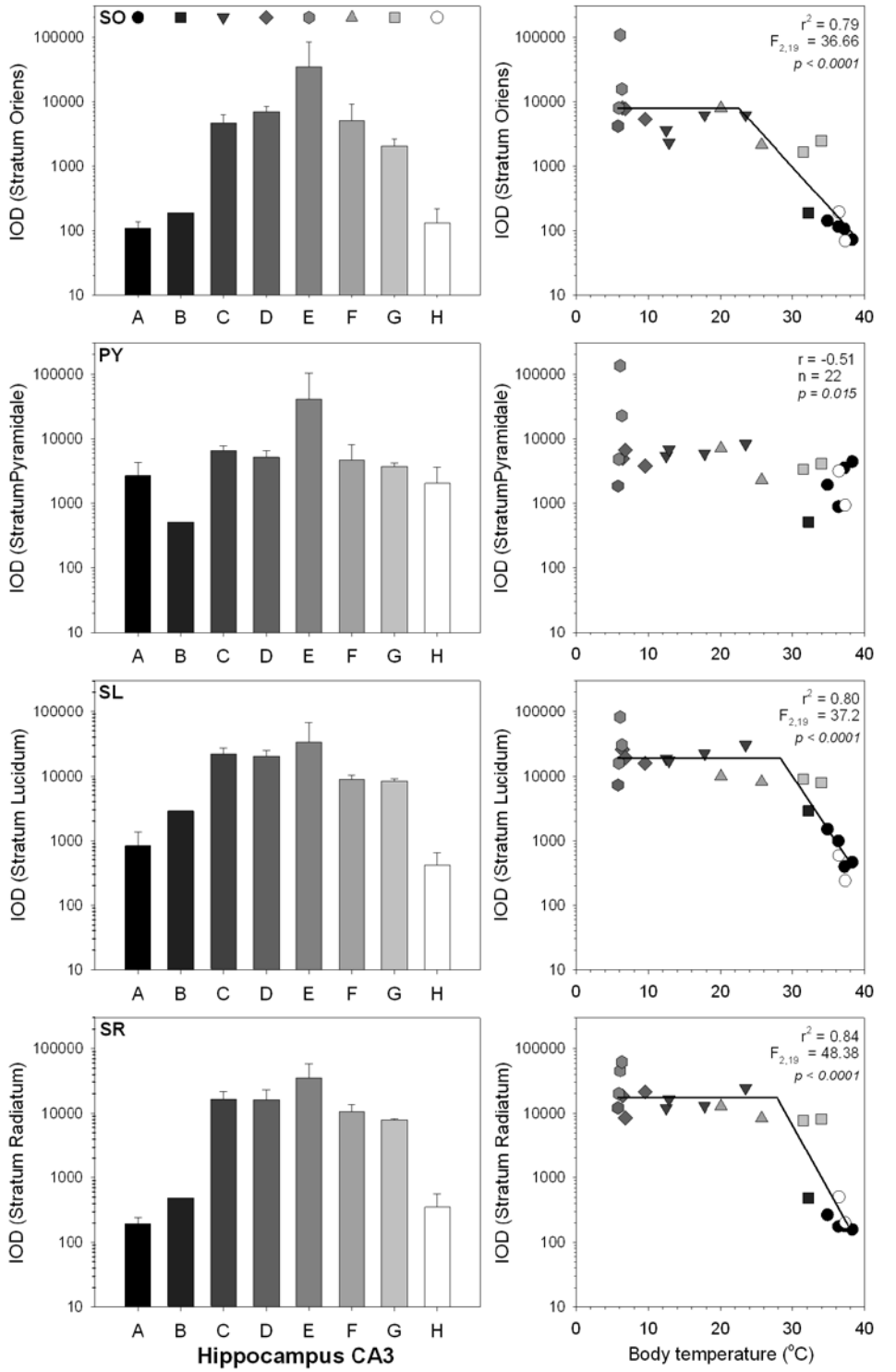
SO
IOD (Stratum Oriens)
r² = 0.79
F2,19 = 36.66
p < 0.0001
PY
IOD (StratumPyramidale)
r = -0.51
n = 22
p = 0.015
SL
IOD (Stratum Lucidum)
r² = 0.80
F2,19 = 37.2
p < 0.0001
SR
IOD (Stratum Radiatum)
r² = 0.84
F2,19 = 48.38
p < 0.0001
100000
10000
1000
100
10
A B C D E F G H
Hippocampus CA3
0 10 20 30 40
Body temperature (°C)

Figure 11 *IOD measurements and their relation with mouth temperature in the CA3 area of the hippocampus. The left panels show an overview of the average IOD for each experimental group with the standard deviation of the mean per CA3 sub-area. Bars are colour-coded according to the list presented in table 1. The right panels show the IOD for each individual, plotted against mouth temperature during sampling. The fitted curves are the result of the regression model*

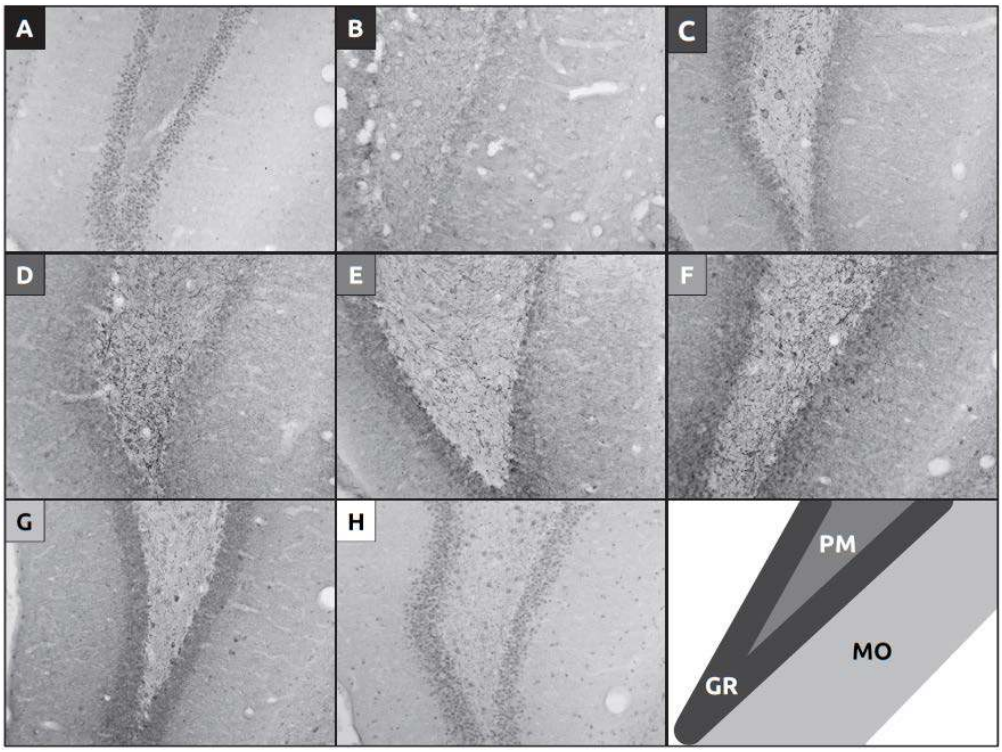

Figure 12 *Overview of the AT8 immunoreactivity in the Dentate Gyrus of the hippocampus of Syrian hamsters over the hibernation cycle, at 100x magnification. The different panels show a representative example of the different experimental groups. Animas during : euthermy in summer (A); high on the cooling curve with T_{mouth} > 30°C (B); low on the cooling curve with T_{mouth} <30°C (C); short torpor 24-30 hr (D); long torpor (> 82 hr (E); low on the rewarming curve with T_{mouth} <30°C (F); high on the rewarming curve with T_{mouth} > 30°C (G) and late arousal: 8.5 hours euthermic after torpor (H). The bottom right panel shows a schematic representation of the location of the areas where OD was measured (MO = Molecular cell layer, GR = Granular cell layer, PM = Polymorph cell layer.*

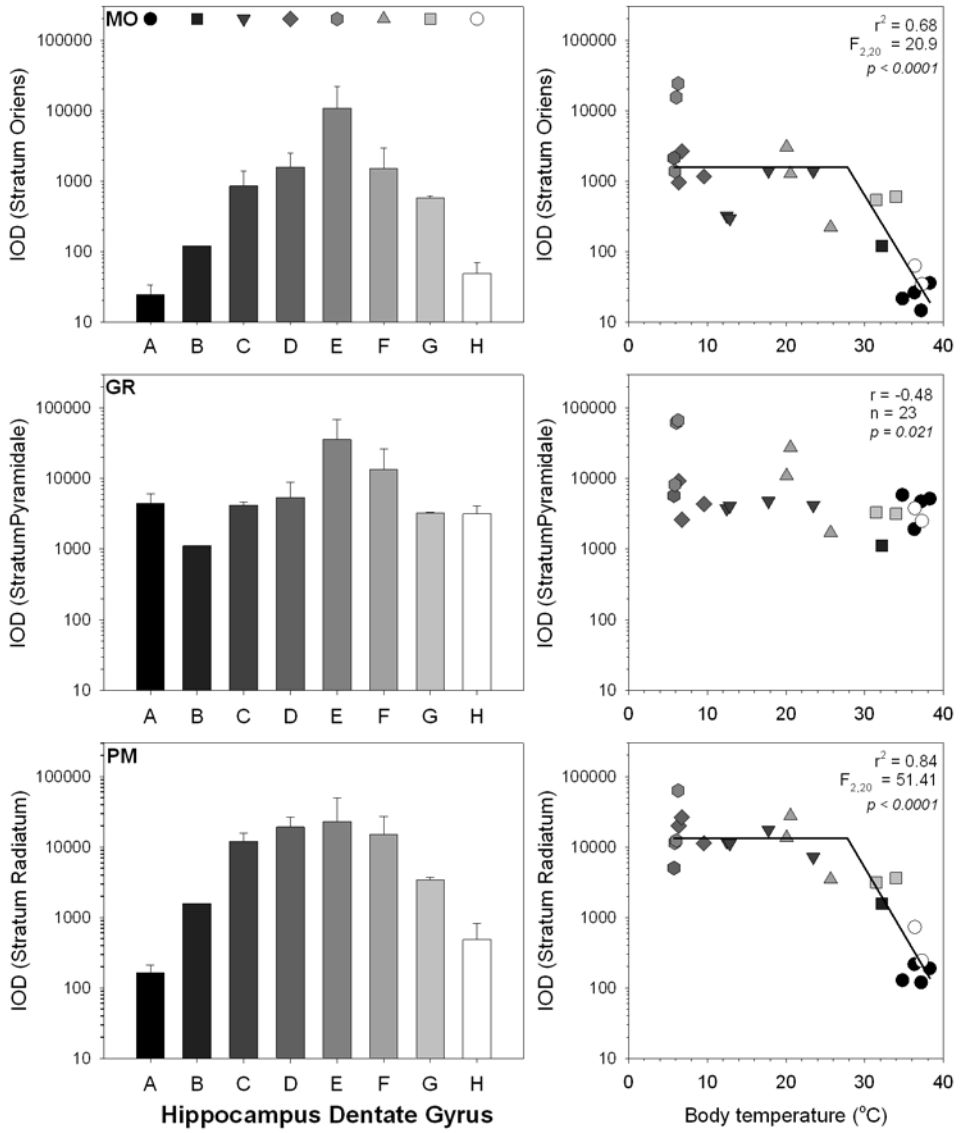

Figure 13 *IOD measurements and their relation with mouth temperature in the Dentate Gyrus of the hippocampus. The left panels show an overview of the average IOD for each experimental group with the standard deviation of the mean per sub-area of the Dentate Gyrus. Bars are colour-coded according to the list presented in table 1. The right panels show the IOD for each individual, plotted against mouth temperature during sampling. The fitted curves are the outcome of the regression model.*

Table2 *Overview of outcome of the parameters estimated by the regression analysis and their t, F and p values.*

	Continuous two phase regression model												Spearman rank correlation		
Area	A0	t	p	B2	t	p	C1	t	p	r^2	dg	F	p	r	p	n
Cortex	4.42	71.2	< 0.001*	-0.10	-2.95	0.008*	26.9	8.93	< 0.001*	0.81	2,20	41.91	< 0.001*			
CA1 SO	3.36	26.58	< 0.001*	-0.21	-3.01	0.007*	29.1	12.87	< 0.001*	0.72	2,20	25.77	< 0.001*			
CA1 PY										*<0.01*	*2,19*	*0.04*	*0.966*	*-0.09*	*0.68*	*22*
CA1 SR	3.61	22.8	< 0.001*	-0.24	-2.68	0.014*	28.9	11.11	< 0.001*	0.68	2,20	21.52	< 0.001*			
CA3 SO	3.90	30.35	< 0.001*	-0.12	-4.43	< 0.001*	22.5	7.86	< 0.001*	0.79	2.19	36.66	< 0.001*			
CA3 PY														-0.51	0.015*	22
CA3 SL	4.28	45.18	< 0.001*	-0.17	-3.31	0.004*	28.3	12.42	< 0.001*	0.80	2.19	37.20	< 0.001*			
CA3 SR	4.24	40.32	< 0.001*	-0.20	-3.56	0.002*	27.8	12.35	< 0.001*	0.84	2.19	48.38	< 0.001*			
DG MO	3.19	22.46	< 0.001*	-0.18	-2.31	< 0.001*	27.8	8.02	< 0.001*	0.68	2,20	20.90	< 0.001*			
DG GR														-0.48	0.021*	23
DG PM	4.13	43.81	< 0.001*	-0.19	-3.60	< 0.001*	27.7	12.44	< 0.001*	0.84	2,20	51.41	< 0.001*			

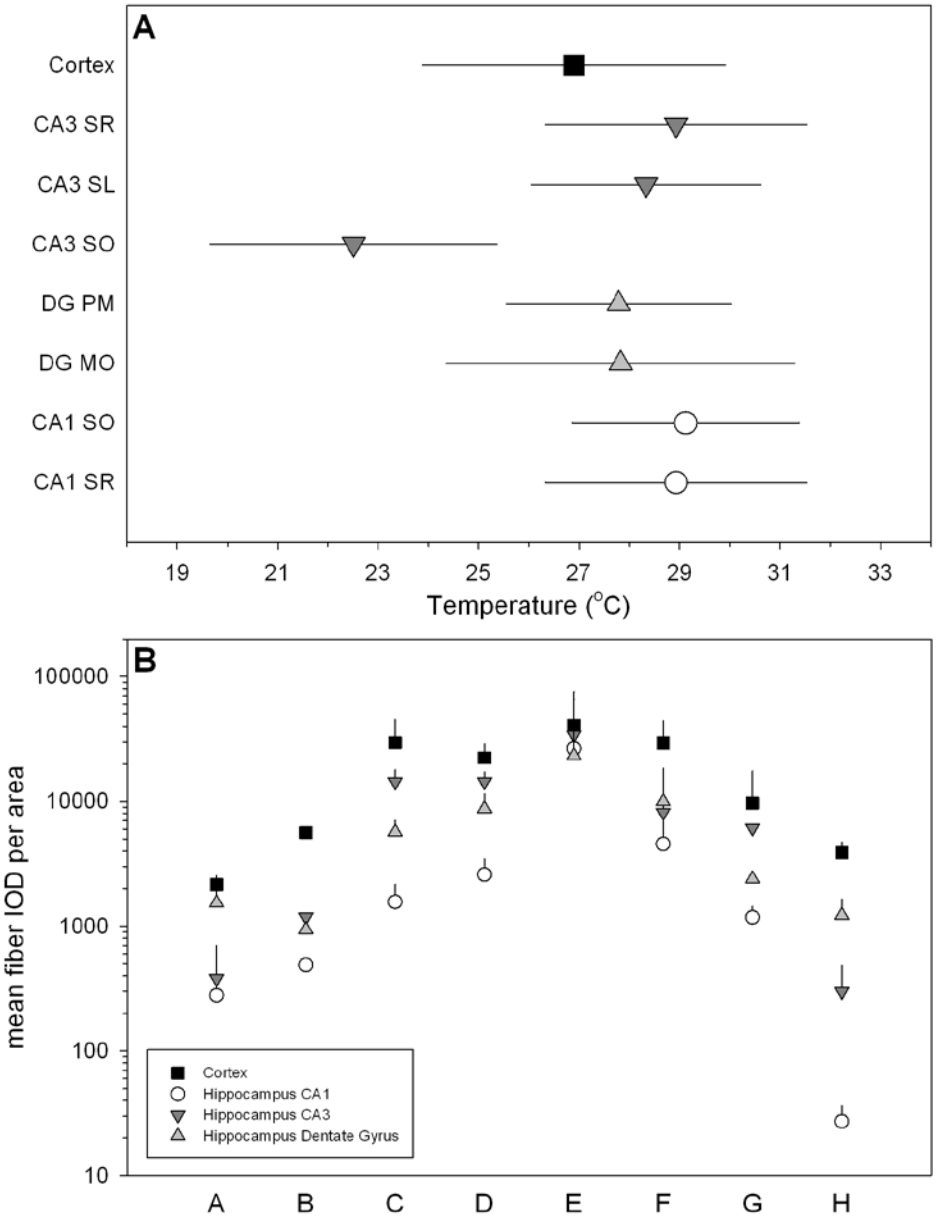

Figure 14 *Panel A: The relation between mouth temperature detected breakpoints in different brain areas. The plotted break points are the significant breakpoints (C0), indicating the fastest changes in tau hyperphosphorylation state, as determined by the regression model, with their standard error around the mean. Panel B: Comparison of the average AT8 staining intensity averaged per measured brain area, with the standard deviation around the mean, for the different experimental groups (A-H)*

Differences in regulation of tau hyperphosphorylation

The continuous non-linear two phase regression analysis revealed significant break points in the fitted curve in most measured brain areas. An overview of all estimated parameters by the model (offset (A), slope of the second part of the curve (B2) and the breakpoint (C1), is presented in table 2. The significant break points with their standard error estimate around the mean are plotted per brain area in figure 14A. The IOD data suggest that the fastest changes in tau hyperphosphorylation occur between 26 °C and 30 °C brain temperature. Only the stratum oriens of the CA3 area appears to differ from the general picture with a breakpoint at 22.5 °C. In general changes in tau hyperphosphorylation seem to occur at a slightly higher temperature in the hippocampus than in the cortex. Figure 14B shows a comparison of the average AT8 IOD levels in the cortex (CO) and the hippocampus Apart from torpor late (group E) the CA1 areas of the hippocampus appears to be generally less affected by hyperphosphorylation than the other measured brain areas.

Discussion

Body and brain temperature during hibernation.

In this study we examined the effect of brain temperature on the degree of tau hyperphosphorylation during hibernation in Syrian hamsters, with a focus on cooling to torpor and rewarming from torpor. In order to asses a correct relation between temperature and tau hyperphosphorylation in these relatively rapid changes, it is crucial to have a good estimate of timing and brain temperature. We therefore investigated differences in body temperature, between body (rectal temperature, abdominally implanted temperature loggers and transmitters) and head temperature (cortical temperature, skull temperature and mouth temperature).

During cooling and steady state torpor or euthermy, anterior and posterior measures of body temperature correlated well, with only a small temperature difference. During arousal from torpor large differences between posterior and anterior temperature were found. This is consistent with previous reports (Lyman 1948; Lyman and O'Brien 1961). The rewarming rate during arousal from torpor was variable between animals, to the degree that an hour difference may occur, obviously associated with a large temperature difference. This means that the predictive value of both time since arousal onset and core body temperature are insufficient to indicate a state related to brain temperature. We therefore sampled brain material during arousal from torpor based on a continuous measurement of skull temperature and/or cortical temperature and always measured mouth temperature during sacrifice as a standardized measure of anterior body temperature.

Results from previously reported measures of Syrian hamsters sampled early in arousal in (Härtig et al. 2007; Boerema et al. 2008b; Stieler et al. 2011) might be subject to variability resulting from these differences, because the definition of arousal early was defined as 2.5 hours after the induction of arousal.

Tau hyperphosphorylation dynamics

We confirmed reversible hyperphosphorylation of the tau protein over the torpor arousal cycle in Syrian hamsters. This is in line with results from previous studies in species performing deep hibernation and daily torpor (Arendt et al. 2003; Härtig et al. 2007; Boerema et al. 2008a; Stieler et al. 2011). We found a cyclic pattern over the torpor arousal cycle in the amount of tau hyperphosphorylation present in the cortex and most hippocampal areas. A study in hypothermic anesthetized mice (Planel et al. 2007) found a strong increase in tau hyperphosphorylation with decreasing body temperature. These authors hypothesized that hyperphosphorylation of the tau protein as observed in hypothermic animals (Planel et al. 2001, 2004, 2007; Okawa et al. 2003; Run et al. 2009) was passively mediated by the inhibition of Protein Phosphatase 2A (PPT2A), the major phosphatase responsible for de-phosphorylating the tau protein at lower temperatures. In our study we did not find a linear relation between mouth temperature, an estimate of brain temperature, and the amount of tau protein hyperphosphorylation. Instead, we found evidence for a "stepwise" change in the tau protein hyperphosphorylation levels during cooling and rewarming. Between 26 °C and 34 °C most areas in the hippocampus and the cortex show fastest changes in tau hyperphosphorylation state. Stieler et al (2011) reported an increased phosphate incorporation speed into the tau protein in cortex tissue harvested from hibernating Arctic ground squirrels (*Spermophilus parryii*). They also reported an increased tau phosphate turnover rate in incubation of cortex tissue from torpid animals

at temperatures of 25 & 30 °C, as compared to incubation at lower and higher temperatures (Stieler et al. 2011). These results together with the results from this study, suggest active and not only passive regulation of tau hyperphosphorylation during cooling in torpor and rewarming from torpor in hibernation.

We also observed differences in the threshold temperature for the change in hyperphosphorylation of the tau protein. The hippocampus appears to change its phosphorylation state at a slightly higher (around 28-30 °C) temperature than the cortex (around 27 °C). Within the hippocampus the CA1 area appears affected at the highest temperature, followed by the CA3 area and the DG, but the observed differences are small. The exception is the relatively low breakpoint temperature observed in the stratum oriens of the CA3 area. This might be an artefact of the relatively low sample size in this study, especially since the first run of the regression model, without a fixed B1 parameter estimated the breakpoint at 28.5 °C ($F_{3.19} = 25.11$, $p < 0.001$; $C1 = 28.51$, $t = 6.94$, $p < 0.001$).

The observed cortical tau hyperphoshorylation breakpoint temperature is consistent with the temperature where cortical EEG activity appears and disappears during arousal from torpor in Syrian hamsters (Chatfield et al. 1951). Spontaneous bursts of subcortical electrical activity originating in the hippocampus were recorded only below a cerebral temperature of ~29 °C in hamsters (Chatfield and Lyman 1954), matching the breakpoint temperature. The finding appears also in line with the disappearance of EEG theta activity and REM sleep from the cortical EEG at temperatures around 25 °C in Ground squirrels and Djungarian hamsters (Deboer and Tobler 1995; Strijkstra et al. 2000; Strijkstra 2006, 2009 p.1831).

The tau protein is thought to be involved in axonal transport (Ebneth et al. 1998; Zhang et al. 2004; Dixit et al. 2008) and hence in the ability of neurons to perform neurotransmission. Active regulation of the phosphorylation of the tau protein and perhaps also other MAP's during torpor entry might affect axonal vesicle transport and thus impair neurotransmission, thereby facilitating the decrease in metabolic demand of the brain in torpor. We observed an increase in tau hyperphosphorylation in the granule cells of the DG during torpor. This differs from the situation in European ground squirrels where the Granular cells appear unaffected by tau hyperphosphorylation (Arendt et al. 2003). If tau hyperphosphorylation impairs neuronal activity, this might suggest that ground squirrels maintain a more active hippocampal system then hamsters. This fits the differences observed in torpor bout duration and synaptophysin immunoreactivity in the stratum lucidum in European ground squirrels and Syrian hamsters (Strijkstra et al. 2003; Boerema et al. 2008b) and the role of the hippocampus in the regulation of the duration of torpor bouts (Heller 1979b; Sallmen et al. 2003a, 2003b).

In summary we observed a large variation in rewarming rates between animals and large differences between brain temperature and core body temperature during arousal from torpor in Syrian hamsters. There appeared to be a stepwise change in the hyperphosphorylation state of the tau protein during cooling and rewarming: The fastest changes in hyperphosphorylation of the tau protein occur between brain temperatures of 26 - 34 °C in the hippocampus and the cortex. This suggests active regulation of the tau hyperphosphorylation during torpor entry and rewarming from torpor in hibernating Syrian hamsters.

Chapter 5

Effects of hibernation on spatial memory in Syrian hamsters.

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Unpublished manuscript

Abstract

Hibernation is an energy saving behavioural strategy used by many animals. In Syrian hamsters and other mammals hibernation is associated with dynamic changes at the cellular level in the brain, such as hyperphosphorylation of the microtubule associated neuronal tau protein. These changes may have repercussions for the behaviour after hibernation. Memory has shown to be suppressed by hibernation in European ground squirrels, but not in Alpine marmots and greater mouse eared bats. The variation in memory performance after hibernation between species may reflect differences in life history. In this study we tested the hypothesis that spatial memory is retained over hibernation in a food hoarding deep hibernator: the Syrian hamster. We trained Syrian hamsters in a functionally relevant spatial memory task and examined the effect of hibernation on the acquired spatial memory. The hamsters successfully learned the location of a food reward in a maze, prior to hibernation. Subsequently one group of hamsters entered hibernation at 5 °C. A control group of hamsters was kept at 21°C, preventing the occurrence of hibernation. After 3 months spatial memory was re-tested. We found that both groups of animals remembered the location of the food in the spatial maze task. We conclude that hibernation in Syrian hamsters does not affect long term spatial memory. This corresponds with the life history of the hamster which includes feeding on food stores throughout the winter.

Introduction

Nature exposes animals to both predictable and unpredictable variation in energy resources. Species have evolved different strategies to cope with low food supply in winter. Some migrate to escape the harsh conditions. Others store energy supplies in the form of food hoards (Van der Wall 1990). Another strategy minimizes the energy spent during winter. Mammalian hibernators achieve this by decreasing metabolic rate and entering a state of torpor (Heldmaier et al. 2004). Some hamsters use a combination of strategies (Lyman 1954). The Syrian hamster hoards food (Phillips et al. 1989; Buckley and Schneider 2003) and also uses deep hibernation to minimize energy expenditure (Lyman 1948). Deep hibernation in mammals is characterized by periods of decreased metabolism (torpor) interspersed with short periods of normal metabolic rate called arousals (Heldmaier and Ruf 1992). European ground squirrels and Syrian hamsters cool down to near environmental temperatures during torpor, and restore metabolism and body temperature to normal euthermic values during arousals (Lyman 1948; Hut et al. 2002a). Next to direct sensory cues, food hoarding behaviour also relies on spatial memory, or the recognition of cues (Pravosudov and Smulders 2010; Smulders et al. 2010), since animals have to find back their cached food stores. Combining memory retention with deep torpor is a potentially interesting challenge (Roth et al. 2010). Torpor has considerable effects, on sleep and sleep regulation (Deboer et al. 1994; Strijkstra and Daan 1997a, 1997b, 1998; Strijkstra et al. 1999; Strijkstra 2006) and is associated with changes in neuronal organization (Popov and Bocharova 1992; Popov et al. 1992; Popov 2003; von der Ohe et al. 2006; Magariños et al. 2006; Rüdiger et al. 2007). Both sleep and neuronal plasticity are important processes for memory function.

Several brain areas, e.g., basal forebrain, cortex and hippocampus, are important in the formation, storage and retrieval of memory, such as novelty recognition in rats and mice (reviewed in (Dere et al. 2007)). The hippocampus and cortex are also important for formation, storage and retrieval of spatial memory (Wang et al. 2006; Gilbert and Brushfield 2009). Synaptic plasticity, such as remodelling of dendritic spines (Kasai et al. 2010) is necessary for memory function (Kavanau 1997; Martin et al. 2000). The changes in brain organisation and neuronal plasticity occurring in torpor may well lead to deficits in memory performance. Tau protein hyperphosphorylation negatively affects memory performance in tau transgenic mice (Jeugd et al. 2010). Since tau hyperphosphorylation and reduced synaptic efficacy are hallmarks of hibernation and torpor (Strijkstra et al. 2003; Arendt et al. 2003; von der Ohe et al. 2006; Magariños et al. 2006; Härtig et al. 2007)we may expect resulting memory deficits. The literature on effects of hibernation and torpor on memory is controversial. There are indications that ground squirrels learn more easily during the arousals in hibernation (Mihailović et al. 1968; Weltzin et al. 2006). Millesi and colleagues have shown that European ground squirrels forget spatial information and operant conditioning during hibernation, but retain memory of familiar individuals (Millesi et al. 2001). Beldings ground squirrels remember kin, but do not remember familiar individuals after hibernation (Mateo and Johnston 2000). In contrast, Alpine marmots retain operant condition memory over hibernation (Clemens et al. 2009) and greater moused eared bats are capable of remembering spatial information over hibernation (Ruczynski and Siemers 2010).

The Syrian hamster is a facultative deep hibernator that hoards food in its burrow. The burrow consists of tunnel networks that can be over 9 m long, with some tunnels having specific purposes, such as urine deposit, or food cache (Gattermann et al. 2001). During the winter Syrian hamsters retreat in to their burrow systems to hibernate. Unlike European ground squirrels hamsters eat during the periodic euthermic intervals in hibernation (Lyman and Chatfield 1955). Spatial memory may well assist them in

finding their food stores to survive the winter. We therefore addressed the question whether Syrian hamsters loose spatial memory over hibernation as was found in the European ground squirrel (Millesi et al. 2001). We trained Syrian hamsters in a functionally relevant spatial memory task and tested whether the acquired spatial memory is retained over the hibernation period.

Materials and methods

Hamsters and housing

Adult Syrian hamsters (age 11 months; n = 20) were derived from our local breeding colony. They were housed in macrolon type III cages provided with sawdust as bedding material. Hay was provided as nesting material. Water was available *ad libitum* throughout the experiment. Food (RMHB 2141-10mm, Arie Blok, Woerden, the Netherlands) was available *ad libitum* throughout the rest of the experiment but was restricted in the context of the spatial memory test. Hamsters were divided into two groups. In the experimental (H; hibernation) group (n=8; four males and four females) the LD cycle was changed to continuous dim red light and ambient temperature was dropped from 21°C to 5°C to induce hibernation. The control (C) group (n=8; five males and 3 females) remained at 21°C which prevented hibernation.

General activity of hamsters was continuously monitored with passive infrared detectors. Activity counts were accumulated in two minute time bins using a PC-based event recording system (ERS). Activity patterns were used to discriminate between torpor and euthermic phases. Inactivity episodes longer than 24 h were considered to be torpor (see (Oklejewicz et al. 2001a; Boerema et al. 2008b)). The start of the first torpor bout defined onset of hibernation. The time from the change in ambient temperature from 21°C to 5°C to the onset of the first torpor bout defined hibernation latency. Episodes with activity after the onset of hibernation were defined as euthermic phases. Torpor bout duration was defined as the time from the first to the last 2-min interval without activity. Euthermic phase duration was defined as the time between two consecutive torpor phases. The experiment was approved by the Animal Experiments Committee of the University of Groningen under license number DEC-5249.

Time course and design of the experiment

Figure 3C (bottom) shows an overview of the experimental design. All hamsters were kept for 43 days at summer photoperiod conditions (LD 14:10, ambient temperature (T_a) of 21 °C +/-1), followed by 36 days of winter photoperiod (LD 8:16, T_a 21 °C +/-1). Subsequently hibernation was induced in the H group as previously described (Oklejewicz et al. 2001a; Boerema et al. 2008b), by transferring these hamsters to a climate room with constant dim (<1 Lux) red light at an ambient temperature of 5 °C +/-1. The C group was kept at LD 8:16, T_a 21 °C +/-1 for 87 days. The high ambient temperature prevented hibernation in these hamsters. After 87 days, hibernation was artificially terminated in the H group and the hamsters were transferred back to the same room where the control hamsters were housed, and summer photoperiod (LD 14:10, T_a 21 °C +/-1) was reinstated.

To assess spatial memory performance, before and after hibernation, a hippocampus-dependent spatial memory test was used. Memory training occurred on different days, in sequences of five days for the spatial memory task. Hamsters were initially trained for four weeks, from day 6-34, until they significantly improved their performance in the spatial memory task. On the first day of each training sequence hamsters were deprived of food, but not tested. On the following four days hamsters were trained in the spatial memory task for two sessions/hamster/day on days 9-12, 15-18, 22-25 and three sessions/hamster/day on day 30-34. The performance of the hamsters on days 30-34 was used as the performance measure in summer photoperiod, prior to hibernation.

To maintain memory performance of the hamsters, memory was also trained during the 36 days of short photoperiod prior to hibernation induction in the H group, but in a less intensive schedule. The spatial memory task was trained on days 48-52 and 64-68, for three sessions/hamster/day. No memory training in either group was performed in the period during which the H group hibernated (day 87-166). Five days after the end of hibernation in the experimental group, performance of the hamsters in the memory task was measured. All hamsters were deprived of food on day 169. The spatial test was performed in 3 trials/hamster/day on days 171-174 for all hamsters in both groups. The performance of the hamsters on these days was compared to the performance of the hamsters on days 30-34 pre-hibernation to assess the differences before and after hibernation.

Spatial memory task

Spatial memory was tested using a custom built complex maze (figure 1A) in which the hamsters had to learn the location of a food reward. The maze consisted of 13 cylindrical PVC units (20 cm in diameter and 12 cm high) connected with each other via PVC tubes (7.5 cm in diameter). All arms were baited with inaccessible food to prevent odour bias of the test. A food reward (rodent chow, Ab diets 2141 AM-II 10 mm) was randomly assigned to one of the outer positions (position 1-8) for each hamster and remained at the same position throughout all trials for each individual. A transparent acrylic plate covered the maze on top. Trials were recorded with a 420TVL night vision camera (figure 1B).

During a trial a hamster had to find the food reward in the maze. At the start of each trial the home cage of a hamster was connected with a 50 cm PVC tube attached to the central unit of the maze. The start time of a trial was defined by the first entrance of the hamster in the central unit. The latency to maze entrance, the number of errors and the time needed to find the food were recorded for each trial as performance indicators. The time it took a hamster from the moment the connection between the cage and the maze opened, until entering the central unit of the maze was defined as maze entrance latency time. Trials ended when the hamster found the food, or five minutes after trial start. Each turn at a crossing not leading to the food reward via the shortest route, as counted from the actual position of the hamster, was counted as an error. The same maze was used for all hamsters. The maze was not cleaned between trials. By doing so, the maze would be rapidly saturated with a variety of different odours from different hamsters. A pilot study confirmed that hamsters did not follow each other's scent trails. Analysis of recorded trials was performed after all sessions were completed.

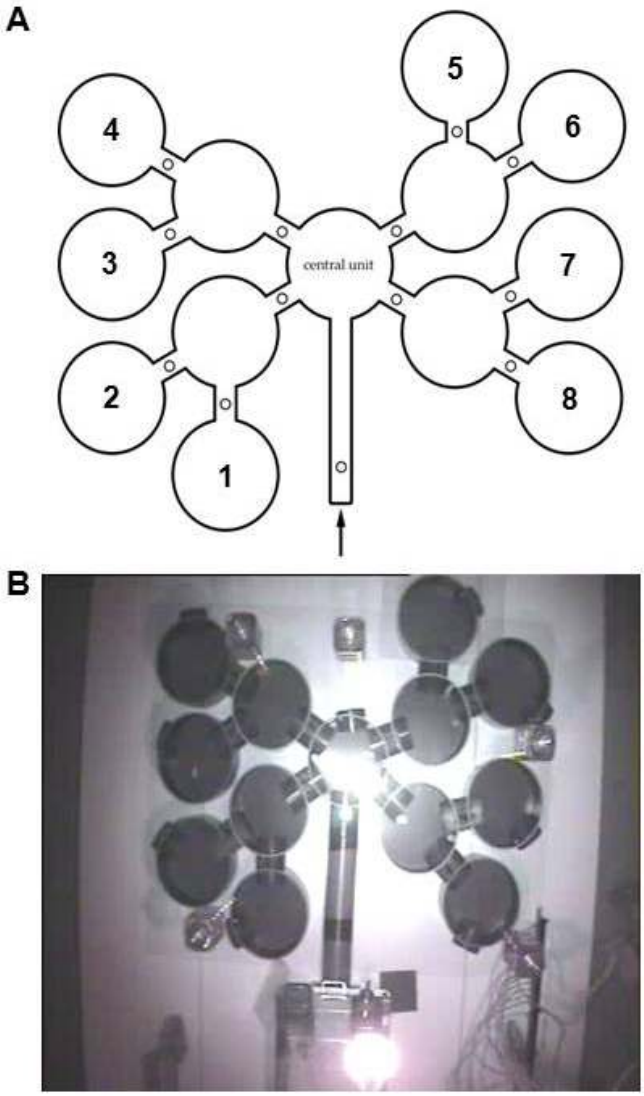

Figure 1 *Panel A: Schematic drawin. Panel B: video image of the spatial memory setup showing the layout of the maze. Hamsters entered the maze via a PVC tube connected to the central unit. All outer units (1-8) were baited with inaccessible food. A food reward was randomly assigned to one of the outer units for each individual. In each trial, hamsters had 5 minutes time to find the food reward.*

A pilot study showed that hamsters were not sufficiently interested in the food reward located inside the maze when they were fed *ad libitum.* This made it impossible to reliably quantify the time needed to find the food reward. Hamsters were therefore deprived of food 24 hours prior to the start of the spatial memory task trials. On the four days of the spatial memory task, food was not available *ad libitum* in the cage. Instead 75% of the daily food ration was equally divided over the food rewards in

each trial. Hamsters could earn their food by finding it in the maze. If a hamster did not find a food reward within the five minutes of the trial the food was given in the cage after the last trial of the day. The daily food reward was individually tuned and was based on the average food intake of each individual during the first week of the experiment (on average 7.54 g. (SEM 0.18, n = 16) per day). The body mass of all hamsters was carefully monitored and if necessary additional food was given to prevent large decreases in body mass. The food deprivation was not intended to starve the hamsters, but was necessary to motivate the hamsters to find the food in the maze. To assess whether animals had learned the spatial task, the average number of errors, latency and time to the food reward on days 9-12 for each animal (n = 16) were compared to the performance of the hamsters on days 31-34. Wilcoxon signed rank tests were used to test for statistical significant differences between the two periods. To assess differences in performance between the C and H group after hibernation, the average performance for each animal on days 171-174 was calculated. The average performances for the C and the H group were compared using t-tests (n = 8 per group).

Results

Hibernation characteristics

All hamsters in the H group eventually entered hibernation, after a latency of on average 35.9 days (SD 13.4). The total time spent in hibernation was on average 50.3 days (SEM 4.7; n = 8). The number of torpor bouts varied between animals from 7 – 14, with an average of 10.5 (SEM 0.9; n = 8) and an average duration of 91.8 hours (SEM 2.3; n = 8; range 31.0 - 135.1 h) per bout. Hibernation was terminated artificially for all animals after 87 days, at the moment that the first hamsters approached the expected end of their hibernation. During this period hamsters spent on average 966.8 hours (SEM 93.2; n = 8) in torpor. The maximum individual torpor bout duration observed was 5.6 days.

The total time spent in periodic euthermic intervals (excluding the hibernation latency) was on average 253.9 hours (SEM 43.6; n = 8; range 9.7 – 200.2 h) resulting in an average arousal duration of 23.2 hours (SEM 3.4; n=8). Figure 2 shows the average torpor bout duration for all hamsters in the H group, plotted against the torpor bout sequence (upper panel and the number of animals still present in the average torpor bout durations plotted (lower panel). Torpor bout durations were short early in hibernation, on average 2.2 days (SD 0.38; n = 8) and increased with time spent in hibernation, reaching a maximum of on average 4.9 days (SD 0.4; n = 6) in the 9^{th} torpor bout. This pattern did not differ between animals with few (<= 7) and many (> 7) torpor bouts. Visual observations and activity records confirmed that no torpor occurred in hamsters in the C group.

Spatial memory

All hamsters were eager to enter the maze as it was presented to them. The first time the hamsters were confronted with the maze (day 9) it took the C-group on average 189.5 seconds (SEM 66.5) and the H-group 450.9 seconds (SEM 170.2) to enter the maze. This latency rapidly decreased within the first days to 11.6 seconds (SEM 2.2) for the C-group and 12.4 seconds (SEM 2.9) for the H-group (figure 3A). Upon first entrance to the maze, animals carefully explored the whole maze, occasionally even

ignoring the food reward. This behaviour rapidly changed in the subsequent trials. The hamsters were moving faster through the maze. They also became more determined in their movements, eating or hoarding the food reward when they found it. During the training phase in summer photoperiod (day 0-43) the hamsters steadily increased their performance. The time it took the animals to find the reward and the number of errors made are shown in figure 3 B and C, respectively. In the last trial series during long photoperiod hamsters had learned the location of the food reward.

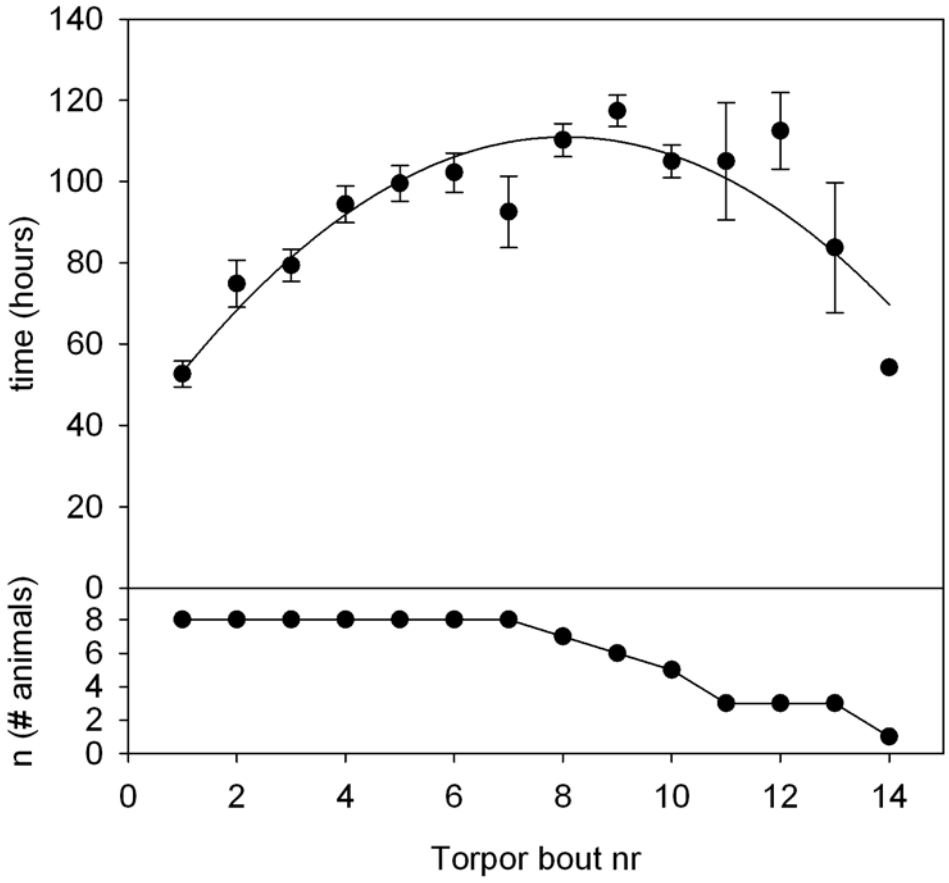

Figure 2 *Average Torpor bout duration for all animals (upper panel) and the number of animals present in the average (lower panel).*

The maze entrance latency was significantly decreased from on average 65.1 seconds (SEM 12.9) for day 9-12 to 4.3 seconds (SEM 0.4) on day 31-34. ($p < 0.001$, $W = -136$, $n = 16$). The time spent to find the reward decreased from 154 seconds (SEM 35) to 12.1 seconds (SEM 5.7) ($p < 0.001$, $W = -136$, $n = 16$). The number of errors made before finding the food decreased from 8.1 (SEM 0.8) to 3.9 (SEM 0.5) ($p < 0.001$, $W = -134$, $n = 16$) over the same time period (see figure 4 A, B and C).

A learning curve can also be observed within each series of sequential spatial maze trials, in the time needed to find the reward and the errors made (figure 3B and C). When animals had not been tested for several days it took them initially a few more errors and a little longer before reaching the reward. This learning curve within a test series was present throughout the experiment, in long photoperiod, short photoperiod and after hibernation. Training of the maze task continued in short photoperiod. The hamsters did not further increase performance during these sessions but performed stably at the level of the last test series in long day photoperiod (figure 3A, B and C).

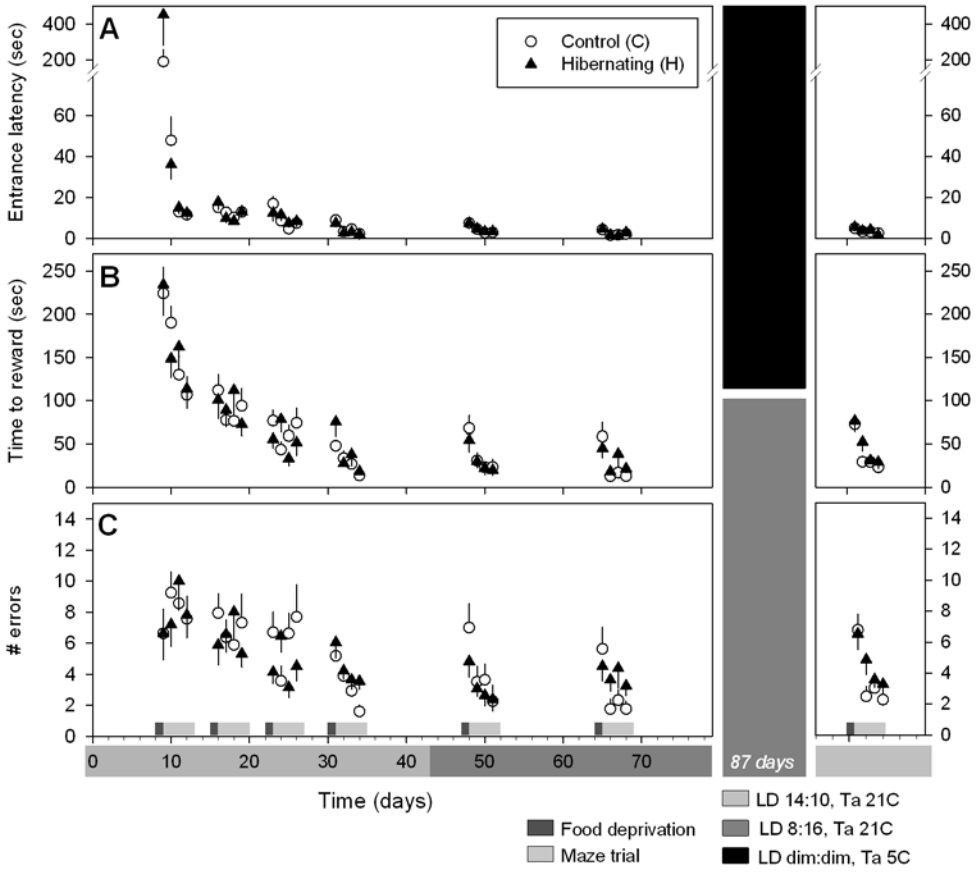

Figure 3 *Performance in the spatial memory task before and after hibernation. A) Maze entrance latency, B) the time needed to find the food reward and C) the number of errors made. Trials were averaged per animal per day. The group average was plotted for every day (Control group open circles (n=8), hibernation group (black triangles (n=8)). The error bars indicate the standard error*

After the 87 days during which the H and C group received different treatment the performance in the spatial task was retested. Both the animals in the C group and the H group performed at the same level as on days 31-34 prior to hibernation. On day 171-174 the animals in the C group showed an

entrance latency of on average 3.6 seconds (SEM 0.9). The time needed to find the reward was on average 34.4 seconds (SEM 4.0), and on average 3.4 errors (SEM 0.4) were made.

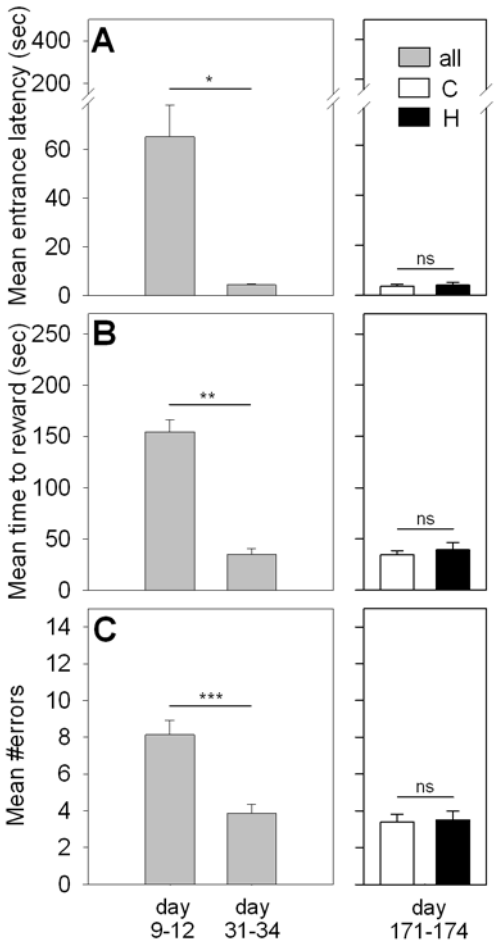

Figure 4 *average performance values for day 9-12 (A), day 31-34 (B) before hibernation and day 171-174 (C) after hibernation. Hamsters make significantly less errors F) p < 0.001, need less time to enter the maze D) p <0.001 and find the food faster E) p<0.001, indicating that they learned the location of the food. On days 171-174 the performances for the C group and the H group were at the same level (latency, p = 0.794; time to reward, p = 0.458, # errors, p = 0.363)*

The hamsters in the H group needed on average 4.1 seconds (SEM 1.2) to enter the maze. The time needed to find the reward was on average 39.4 seconds (SEM 7.1) and the hamsters in the H group made on average 3.5 errors (SEM 0.5) before they found the food. The animals in the C group and the H group performed at the same level after hibernation. Differences between C and H were not significant for the maze entrance latency (p = 0.794, t = -0.266, n = 16), time needed to find the reward (p = 0.458, t = -0.764, n = 16) and the number of errors made (p = 0.363, t = -0.941, n = 16).

Discussion

Syrian hamsters were highly capable of learning the location of a food reward in a complex maze. In 24 trials distributed over three weeks the time needed to find the food and the number of errors made were significantly decreased. In the five weeks of short photoperiod following acquisition the performance remained at the same level. When the hamsters were tested again after circa 3 months, during which one group hibernated in continuous dim red light at 5 °C and the other remained in a short photoperiod at 21 °C, both groups performed at the same level as before. A previous study by (Oklejewicz et al. 2001b) showed long term memory retention in a passive avoidance test in Syrian hamsters, but at a substantially lower performance level then is known from rats and mice. The result of our study demonstrates that, in the right functional context, Syrian hamsters have a fully functional long term memory system. Our study also clearly shows that spatial memory is not lost during hibernation at 5 °C in Syrian hamsters. The finding that spatial memory in hamsters is retained over hibernation is consistent with recent studies in marmots and bats. Alpine marmots retain memory in two operant conditioning tasks over six months of hibernation (Clemens et al. 2009). Greater mouse-eared bats do not loose spatial memory after approximately two months of hibernation (Ruczynski and Siemers 2010). Clemens et al (2009) also performed an open field test in the Alpine marmots showing that locomotor activity is high in naïve, and low in habituated animals. The spontaneous activity after hibernation is equally low as in the habituated animals, suggesting that the marmots remember the context of the open field arena. We find that hamsters have a high (~65 sec) maze entrance latency if they are confronted with the maze in the first trials. This latency rapidly decreased to ~10 sec during the following trials. After hibernation both the control hamsters and those that hibernated immediately showed the same low maze entrance latency time. This suggests that the hamsters also remember the context of the maze. Our findings deviate from a study in European ground squirrels by Millesi et al. (2001). These authors studied several types of memory in European ground squirrels before and after the winter. One group of ground squirrels was housed at high temperatures preventing the occurrence of hibernation with torpor. The other group hibernated at 5 °C. All ground squirrels retained social memory over this period, but the hibernating animals forgot the location of a food reward in a maze and performed worse in an operant conditioning task after hibernation (Millesi et al. 2001).

The hippocampus is an important brain region for learning and memory, in particular for the acquisition of spatial memory (Gilbert and Brushfield 2009). It also plays a role in retention of stored spatial memory traces (Lisman 1999), potentially involving different functions for the CA1 and CA3 sub regions (Leutgeb et al. 2004). Synaptic plasticity is thought to be important for learning and memory (Martin et al. 2000), in particular for memory acquisition and early consolidation. Neurons of the hippocampus are thought to be involved in pattern recognition and completion during the retrieval of spatial memory. The differences in spatial memory between Syrian hamsters and ground squirrels may be related to differences in neuronal plasticity in the hippocampus during hibernation. Ground squirrels show dramatic reductions in the extent of dendritic branching and in the number of boutons of the CA1/CA3 pyramidal neurons (Popov and Bocharova 1992; Popov et al. 1992; Popov 2003) during torpor. Synaptic efficacy in the mossy fibre system is also decreased during torpor (Strijkstra et al. 2003; Arendt et al. 2003; von der Ohe et al. 2007). The cycle in synaptic plasticity over the torpor arousal cycle may have repercussions for hippocampus dependent memory performance after hibernation. In Syrian hamsters we could neither detect a decrease in synaptophysin in the mossy fibre system (Boerema et al. 2008b), nor a decrease in dendrites as indicated by a MAP-2 staining

(Boerema, unpublished data). This would be consistent with the fact that Syrian hamsters do not loose spatial memory over the hibernation period. It also fits the life-history of both species. Ground squirrels do not eat during hibernation and find food immediately in the vicinity of the burrow after hibernation. Spatial memory loss over the hibernation cycle probably entails no cost for these animals, whereas hamsters need their memory intact and unharmed in order to find their food stores during hibernation.

Syrian and Siberian hamsters, mice and ground squirrels all show Alzheimer-like hyperphosphorylation of the tau protein in deep hibernation (Arendt et al. 2003; Härtig et al. 2007) as well as in daily torpor (Boerema et al. 2008a). Tau hyperphosphorylation normally occurs in the process of ageing in hamsters (Härtig et al. 2005) and other species (Hartig et al. 2000). It is also observed in several neurodegenerative disorders (Delacourte and Buée 2000). The tangles resulting from hyperphosphorylation of the tau protein are one of the hallmarks for the diagnosis of Alzheimer's disease in humans, which is associated with cognitive decline and memory loss (Goedert et al. 1989b; Braak and Braak 1991b, 1994). At first thought, a direct relation between memory loss and tau hyperphosphorylation seems likely. This idea is supported by the findings of memory loss during daily torpor in Siberian hamsters (Palchykova et al. 2006) and deep torpor in European ground squirrels (Millesi et al. 2001), which coincide with tau hyperphosphorylation (Arendt et al. 2003; Boerema et al. 2008a). The data presented in this study do not directly support this view. Syrian hamsters do not lose acquired and consolidated spatial memory traces over the hibernation period, but they do have massive tau hyperphosphorylation during torpor, especially in the cortex and the hippocampus (Härtig et al. 2007; Stieler et al. 2011). Tau hyperphosphorylation occurs in the Dentate Gyrus (DG), the CA1 and the CA3 regions of the hippocampus, areas important for the retention of spatial memory (Lisman 1999), in European ground squirrels (Arendt et al. 2003), and in Syrian hamsters during torpor. Other evidence against a direct negative role for torpor-induced hyperphosphorylation in memory loss comes from a study showing that mice perform better in the Morris water maze after a single bout of food deprivation induced torpor immediately after the training (Nowakowski et al. 2009). Both memory studies involving shallow daily torpor in Djungarian hamsters (Palchykova et al. 2006) and mice (Nowakowski et al. 2009) interfere with the memory acquisition/consolidation process whereas our study in Syrian hamsters and the study by Millesi et al (2001) in ground squirrels study long term effects on acquired and already consolidated memory traces. Tau protein hyperphosphorylation in the hippocampus might have different effects on consolidation vs. retention of memory traces. The sleep deprivation effects caused by daily torpor (Deboer and Tobler 1994) may also interfere with memory consolidation and thereby explain the negative effect on memory caused by torpor in these studies.

This still leaves the difference in memory performance over the hibernation cycle in ground squirrels and hamsters unexplained. There are some first indications of differential hyperphosphorylation in the CA1 and CA3 region between in hamsters and ground squirrels (Boerema & van der Zee, unpublished data). The cellular distribution of PKCγ, an important signal transduction system for learning and memory processes (Van Der Zee and Douma 1997), is changed towards the nucleus in the CA1 and CA3 areas and remains functionally located in the dendrites in the DG during torpor in European ground squirrels (Van der Zee et al. 2004). In Syrian hamsters PKCγ is translocated around the nucleus in the CA3 and the DG, but remains functionally located in the dendrites in the CA1 region (van der Zee, unpublished data). Together with the decrease in synaptophysin during torpor (Strijkstra et al. 2003), suggesting depletion of vesicles by use in neurotransmission, this would suggest an active hippocampal input (DG) system. In Syrian hamsters the hippocampal input system would be offline,

which is in line with the absence of a decrease in synaptophysin in the mossy fibre system of the DG (Boerema et al. 2008b).

The hippocampus is thought to be an important brain structure for the control of torpor bout duration, by integrating temperature information from the hypothalamus arriving in the DG via the septum and actively suppressing the Brain stem Reticular Formation (BSRF) (Heller and Colliver 1974; Heller 1979a). The hippocampus remains electrically active at low temperatures in hamsters (Chatfield and Lyman 1954; Arant et al. 2011). In ground squirrels hippocampal histamine infusions prolong torpor bout duration with a few hours (Sallmen et al. 2003b). Histamine increases spike amplitudes of pyramidal cells in hippocampal slices of Syrian and Turkish hamsters, also under cold conditions (Nikmanesh et al. 1996; Arant et al. 2011). Torpor bout duration is much longer in ground squirrels (up to 3 weeks, (Hut et al. 2002a)) than in hamsters (4-6 days, (Oklejewicz et al. 2001a; Boerema et al. 2008b)). In fact, torpor bout duration in Syrian hamsters is much shorter than would be expected based on the relation between body mass and torpor bout duration of hibernating animals (French 1985). Hamsters are also easily arousable throughout the torpor bout ((Lyman and Chatfield 1950). This is contrary to ground squirrels, which show resistance to arousal, particularly in the first half of the torpor phases (Twente and Twente 1968). These observations are consistent with the view of a relatively inactive hippocampus in hamsters, leading to low suppression of the BSRF, causing relatively short torpor bouts. The fact that histamine infusion causes an extension of only a few hours of torpor also suggests that ground squirrels already maximally use their hippocampus to prolong torpor bout duration. The main difference between Syrian hamsters and ground squirrels therefore is the duration of the torpor phase and its associated tau hyperphosphorylation in the hippocampus. It may well be that short exposure to tau hyperphosphorylation, as in Syrian hamsters, does not immediately affect the hippocampus-dependent spatial memory traces. Prolonged exposure of the hippocampus to tau hyperphosphorylation, combined with an active use of this structure during torpor for other purposes than memory retention might affect the spatial memory traces, causing memory loss in ground squirrels. This view adequately fits the life-history of both species. Ground squirrels sacrifice less relevant spatial memory for the energy savings associated with long torpor bouts. They can do this, because they possess considerable fat stores as energy source. Hamsters do not have these fat reserves and need their spatial memory on line, in order to be able to access their energy reserves in the form of food stores. They do this at the cost of shorter torpor bouts.

In summary, our results show that spatial memory is not affected by hibernation in Syrian hamsters. This suggests that the occurrence of tau hyperphosphorylation in the brain during torpor in itself is not immediately detrimental for memory traces. This does not rule out the possibility that prolonged exposure of neurons to tau hyperphosphorylation causes memory loss, as in ground squirrels.

Acknowledgment

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Part II

Natural and induced daily torpor

Chapter 6

Reversible PHF-like phosphorylation of the microtubule associated protein tau during daily torpor in Djungarian hamsters.

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Abstract

Torpor in hibernators is a natural model system for the study of reversible PHF-like phosphorylation of the tau protein. Torpor in hibernators is characterized by several days of torpor. We investigated if the relatively short daily torpor leads to reversible tau protein hyperphosphorylation. Djungarian hamsters were sacrificed after 7h of torpor and after 6h of euthermia following daily torpor. Hyperphosphorylation of the tau protein was investigated with the AT8 antibody. Brain slices showed high AT8 immunoreactivity in daily torpid hamster brains, as compared to euthermic hamster brains after daily torpor. Western blotting confirmed this result. We conclude that also daily torpor is associated with reversible PHF-like phosphorylation of the tau protein.

Introduction

Several mammalian species use the natural hypometabolic state of torpor to reduce metabolic requirements. During hypometabolism, hypothermia occurs as a consequence of the reduced heat production. In the hypometabolic hypothermic state of torpor, electrical brain activity is reduced. Electroencephalographic research suggests that torpor affects neuronal connectivity (Strijkstra & Daan 1998), and induces a state in the brain similar to phenomena observed in brains affected with Alzheimer's disease brains. Hyperphosphorylation of the tau protein, a hallmark for Alzheimer's disease, has been shown for torpid deep hibernators such as European ground squirrels (Arendt et al. 2003) and Syrian hamsters (Härtig et al. 2007).

Hyperphosphorylation of the tau protein may be associated with the extremely low temperature reached in deep torpor. Currently, no data is available for hyperphosphorylation of the tau protein in species that use less extreme torpor. The Djungarian hamster, a well-studied daily torpor species, uses daily torpor bouts of 4-12 hours, reaching body temperatures of >15°C (Heldmaier and Ruf 1992). We investigated tau protein hyperphosphorylation in torpid Djungarian hamsters.

Materials & Methods

Twelve Djungarian hamsters (*Phodopus sungorus*) where derived from the breeding colony maintained at the Zoological Institute, University of Veterinary Medicine, Hannover, Germany. Hamsters where exposed to natural photoperiod and temperature in outside enclosures. The occurrence of daily torpor was confirmed by regular inspection of animals in nest boxes. During this time of year, daily torpor typically starts around 07:00 hours (civil twilight starts at about 07:30 hours) and ends at about 16:00 hours (civil twilight ends at about 17:30 hours). Six animals were sacrificed during torpor at on average 14:02 hours (+/- 19 min; torpor group: T). Average body temperature of these animals was 19.57 °C (+/- 1.37 °C) confirming their torpid state. Another six animals where sacrificed in euthermic conditions following a confirmed daily torpor bout at on average 22:08 hour (+/- 13 min; euthermic group: EU).

Four animals of both T and EU groups were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS. Brains were dissected out and post-fixated for 12 hours in 4% paraformaldehyde in 0.1 M PBS at 6 °C. Brains were subsequently stored in PBS with 1% sodium-azide at 6 °C. Before slicing, brains were cryoprotected by submergence in 30% sucrose and 1% sodium-azide in 0.1 M TBS (pH 7.4) at 6 °C for 48 hours. Following cryoprotection, brains were cut coronally in 20 µm sections on a cryostat. Two animals of both T and EU groups were decapitated and the brain instantaneous dissected out and frozen on dry ice. The brains were subsequently stored at -80 °C for later protein analysis by western blotting.

Syrian hamster hibernation material was used for reference. Material was obtained from hibernating Syrian hamsters sacrificed after four days of torpor. Sampling methods have been described previously (Härtig et al. 2007). Immunocytochemical staining was performed as described below. Western

blotting material was extracted as described previously (Härtig et al. 2007). Western blotting was performed as described below.

Immunocytochemistry

For immunocytochemistry, sections were mounted on polysine coated glass slides and air dried for 24 hours. Slides where treated with 0.3% H2O2 in Tris-Buffered Saline (TBS) for 45 min to eliminate endogenous peroxidase activity, after which the slides were thoroughly rinsed with TBS. Non-specific binding was blocked with 3% Bovine Albumin Serum (BSA) in TBS, containing 0.5% Triton X-100 (T) (Sigma, Germany) for 3 hours. Slides were then incubated overnight at room temperature with the monoclonal antibody AT8 (mouse; 1:1000; Perbio, Etten-Leur, The Netherlands) in BSA-TBS-T. Afterwards, the sections were thoroughly rinsed in TBS and treated with byotinylated Goat anti-mouse IgG and preformed streptavidin/byotinyl-peroxidase complexes, followed by a nickel-enhanced diaminobenzidine reaction.

Western blotting

For protein analysis by western blotting, cortex samples were dissected from the frozen brains and the tissue was homogenized by sonification. Per 1 mg tissue, 15µl ice-cold buffer (TRIS-HCl 20mM, NaCl 150mM, MgCl2 2 mM, NaF 5mM, Na3VO4 1 mM, DTT 1 mM, NP40 1%) was used. To preserve proteins and their phosphorylated state, standard amounts of a complete protease inhibitor cocktail (Roche Diagnostics, Woerden, The Netherlands) and complete phosphatase inhibitor cocktail (Roche Diagnostics, Woerden, The Netherlands) were added fresh to the buffer. Homogenates were centrifuged for 30 min at 20000 x g at 4 °C. The clear supernatant was removed and its protein concentration determined by Bradford assay. Protein concentration was standardized to 1.5 µg/µl. Proteins were separated in a 10% SDS-polyacrylamide gel, with 35 µg of protein loaded per lane. Protein was blotted to PVDF membrane and treated with blocking buffer (PBS, 2% BSA, Tween20 0.1%) for 1 hour at room temperature. Immunoreaction was performed by applying the monoclonal antibodies AT8 (mouse; 1:800; Perbio, Etten-Leur, The Netherlands) and Actin (mouse, 1:500000, MP-Bio., Illkirch, France), diluted in blocking buffer at 5°C overnight. The membranes were thoroughly washed in washing buffer (PBS, Tween20 0.1%) and probed with the secondary antibody Goat anti-mouse HRP conjugate (Santa Cruz, sc-2005, 1:5000) in blocking buffer. Blots were developed with a Pierce ECL-System (Pierce, Rockford, IL).

Results and Discussion

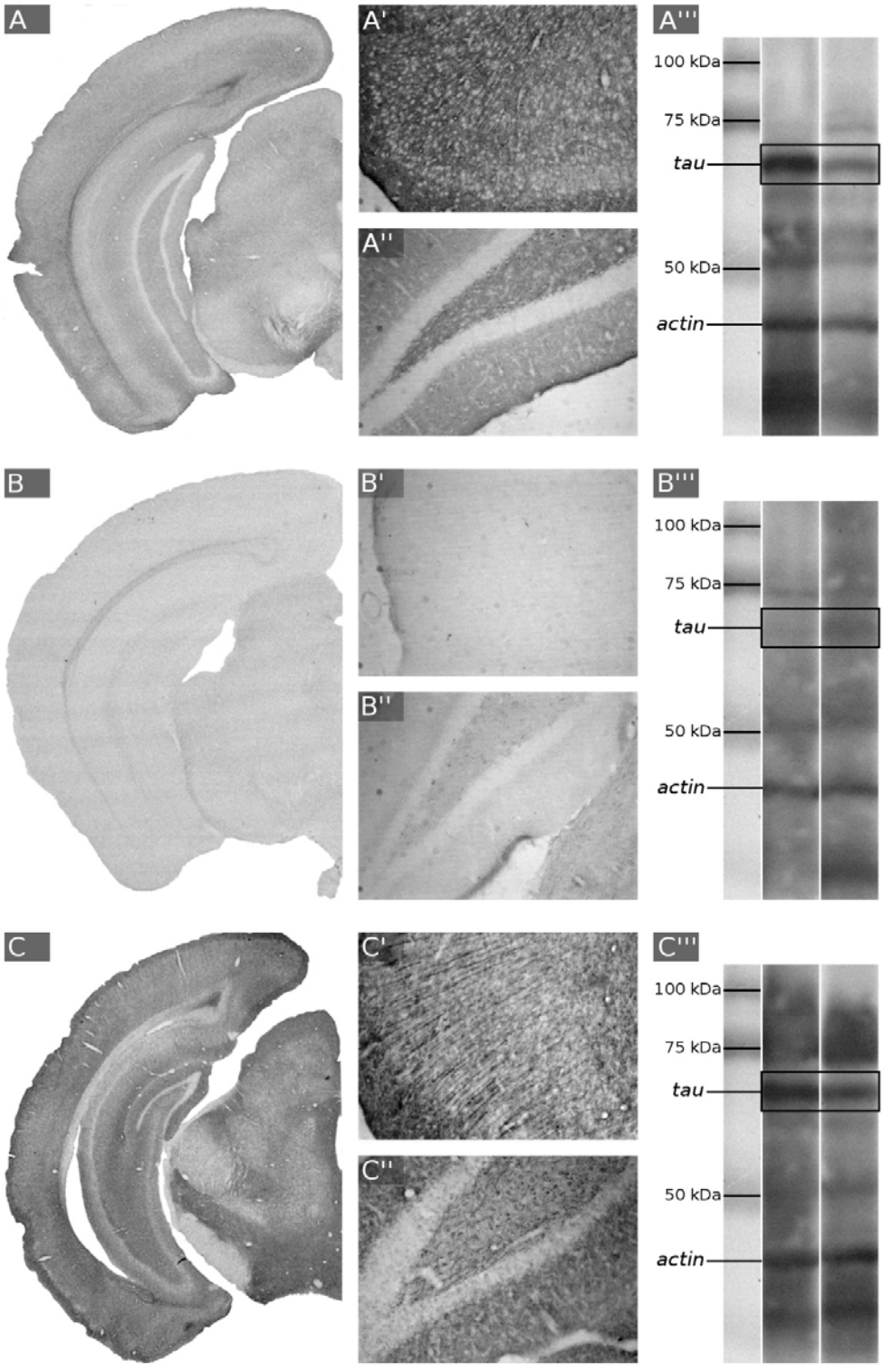
Figure 1 shows representative results of the immuncytochemical staining and western blotting of brain material of daily torpid Djungarian hamsters (A-A'''), euthermic Djungarian hamsters after daily torpor (B-B''') and deep torpid Syrian hamsters (C-C''') with the AT8 antibody. Panel A shows a coronal section of one hemisphere of a torpid Djungarian hamster; detail images of cortex and the dentate gyrus of the hippocampus are shown in panel A' and A''. It is clear that AT8 staining is generally present in all panels.

In panel B a coronal section of a euthermic Djungarian hamster after daily torpor is shown, with detail images of cortex (B') and dentate gyrus of the hippocampus (B''). AT8 immunoreactivity is virtually absent in all panels. Panel C shows a coronal section of a torpid Syrian hamster brain with detail images of the cortex (C') and the dentate gyrus of the hippocampus (C''). AT8 immunorectivity is generally present. In particular, the pyramidal neurons of the cortex appear heavily stained.

Panel A''' shows western blotting results for cortex material of two torpid Djungarian hamsters. A clear band is present at about 70 kDa indicating the presence of hyperphosphorylated protein tau. Furthermore, this result demonstrates that the AT8 antibody correctly recognizes the hyperphosphorylated protein tau in the Djungarian hamster. Panel B''' shows the western blotting results for two euthermic Djungarian hamsters after daily torpor. No band can be observed at the molecular weight associated with the hyperphosphorylated protein tau, indicating the absence of hyperphosphorylated protein tau in euthermic Djungarian hamsters after torpor.

Panel C''' shows the western blotting results for cortex material of two torpid Syrian hamsters, serving as a positive control. The western blots show a clear band at ~70kDA, indicating the presence of hyperphosphorylated protein tau in the cortex of the torpid Syrian hamster brain. Negative controls where performed by omitting the primary (AT8) antibody. This omission resulted in the absence of the band at ~70kDA, indicating that the band indeed represent hyperphosphorylated tau as recognized by the AT8 antibody.

Figure 1 *AT8 immunoreactivity in Djungarian hamsters during daily torpor (A-A'''), in euthermic Djungarian hamsters after daily torpor (B-B''') and in Syrian hamsters after 4 days of deep torpor (C-C'''). Panels A, B & C show a coronal section of one brain-hemisphere. PHF-like phosphorylation of tau can be observed in torpid (A) but not in euthermic Djungarian hamster (B). Syrian hamster deep torpor (C), serves as a positive control. In Panels A', B' & C', a detail view of the cortical area is shown. In both torpid Djungarian (A') and Syrian hamster (C'), high AT8 immunoreactivity can be observed throughout the cortex. In particular the axons of cortical neurons in the torpid Syrian Hamster appear heavily stained. In the euthermic Djungarian hamster (B'), AT8 immunoreactivity is virtually absent. A similar pattern appears from the detail views of the dentate gyrus of the hippocampus (panels A'', B'' & C''). Panels A''', B''', C''' show the results of the AT-immunoreactivity in western blots of cortical protein extracts of two torpid Djungarian hamsters (A'''), two euthermic Djungarian hamsters (B''') and two torpid Syrian hamsters (C'''). In torpid tissues (A''' & C''') a clear band is present at a molecular weight of ~70 kDa, indicating the presence of hyperphosphorylated protein tau. In both euthermic Djungarian hamsters this band is absent. Omission of the AT8 antibody resulted in the absence of a band at ~70kDa. The western blots confirm specific immunoreactivity of the used AT8 antibody in Djungarian hamsters.*


A
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The present study demonstrates reversible paired-helical-filament (PHF)-like phosphorylation of the microtubule-associated-protein tau as indicated by the AT8 antibody during daily torpor in Djungarian hamsters. Formation of PHF-like phosphorylated tau was detected the cortex and hippocampus after about seven hours of daily occurring torpor. This suggests that hyperphosphorylation of tau occurs even after a limited time in torpor in Djungarian hamsters. In deep hibernators, hyperphosphorylation can be shown after 24 hours to 4-7 days. This may be specific for daily torpor, although critical evidence for presence or absence of hyperphosphorylation of tau after several hours of torpor is currently lacking in deep hibernators. In any case, daily torpor also appears to be a valid natural model system for research on PHF-like tau phosphorylation dynamics in the brain.

Torpor is used by various species of many different mammalian families (Heldmaier et al. 2004). Deep torpor during hibernation and daily torpor are effective strategies of endotherms to reduce energy expenditure. This enables animals to refrain from exposed activity during unfavourable times of year. Small mammals appear are more likely to use short torpor bouts on a daily basis.

Benefits of torpor are related to enhanced survival for which animals have adaptations enabling them to cope with the extreme states of hypometabolism and hypothermia. These adaptations may be useful for finding naturally evolved solutions to medical problems (Bradbury 2001). Furthermore, mild hypothermia is neuroprotective (Zhou et al. 2001). Costs of torpor, however, have received much less attention. The hyperphosphorylation that occurs in torpor may indicate such a torpor related cost, either in the classical sense suggesting that hyperphosphorylation indicates a pathological pathway to the occurrence of paired helical filaments (which coincidentally is conveniently restored by hibernators, and also in daily torpor), or in the more adaptive sense that hyperphosphorylation is a natural way of coping with problems occurring during inactivity in the torpid brain.

We previously showed that reversible PHF-like phosphorylation of tau occurs in torpid European ground squirrels, which are obligate deep hibernators (Arendt et al. 2003), and in Syrian hamsters, which are facultative deep hibernators (Härtig et al. 2007). We extend this now to daily torpor in Djungarian hamsters. The fact that torpor affects memory performance both in deep hibernators (Millesi et al. 2001) and in Djungarian hamsters (Palchykova et al. 2006) supports the idea that torpor affects the brain also in a functional way, thus representing a cost of torpor.

In conclusion, daily torpor, as well as torpor in deep hibernation, represents a brain state associated with (and perhaps requiring) hyperphosphorylation of the tau protein, a state which is readily reverted in subsequent euthermia. This makes daily torpor, as deep torpor in hibernators, a model system suitable to analyse the mechanisms of PHF-like phosphorylation of tau and its cellular consequences in the framework of Alzheimer's disease.

Chapter 7

Working for food shifts nocturnal mouse activity into the day.

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Abstract

Nocturnal rodents show diurnal food anticipatory activity when food access is restricted to a few hours in daytime. Timed food access also results in reduced food intake, but the role of food intake in circadian organization per se has not been described. By simulating natural food shortage in mice that work for food we show that reduced food intake alone shifts the activity phase from the night into the day and eventually causes nocturnal torpor (natural hypothermia). Release into continuous darkness with ad libitum food, elicits immediate reversal of activity to the previous nocturnal phase, indicating that the classical circadian pacemaker maintained its phase to the light-dark cycle. This flexibility in behavioral timing would allow mice to exploit the diurnal temporal niche while minimizing energy expenditure under poor feeding conditions in nature. This study reveals an intimate link between metabolism and mammalian circadian organization.

Chapter 7

Introduction

Mice and rats are nocturnal, but their activity shifts to the day when food access is restricted to a few hours in daytime. In such restricted schedules the food received is limited both in time and in quantity (typically 60–70% of normal daily food intake). This procedure induces food anticipatory activity (FAA) a few hours before the food is delivered (Mistlberger 2009). FAA is thought to be the behavioural output of a separate circadian oscillator called the food entrainable oscillator (FEO) which is entrained by periodic food availability and capable of driving peripheral (Stokkan et al. 2001; Pezuk et al. 2010) and behavioural rhythms (Stephan 2001). Under these conditions the FEO drives the activity rhythm independently of the suprachiasmatic nucleus (SCN), which controls nocturnal activity under standard ad libitum conditions (Kawamura and Inouye 1979; Davidson 2009a). The anatomical substrate of the FEO remains elusive in spite of intense investigation and debate (Krieger et al. 1977; Mistlberger and Rechtschaffen 1984; Challet et al. 1996a, 1996b, 1997, 1998; Stephan 2001; Mieda et al. 2006; Gooley et al. 2006; Landry et al. 2006, 2007; Landry and Mistlberger 2007; Feillet et al. 2008; Mistlberger et al. 2009; Pendergast et al. 2009; Moriya et al. 2009; Storch and Weitz 2009; Davidson 2009a). In nature, food availability is hardly periodic to most rodents, and diurnal activity has often been observed in nocturnal animals (Hoogenboom et al. 1984; Levy et al. 2007; Gattermann et al. 2008). A crucial difference between lab and field is that animals in the field have to work (i.e. spend time and energy) to obtain food. To study the effect of reduced food intake on circadian organization, we simulated natural food scarcity by having mice work for their food at increasing levels of workload under a 12 h light - 12 h dark (LD) cycle, without restricting food access to a specific time of day.

Methods

All animal experiments were approved by the University of Groningen ethical committee (DEC 5011, 5114, 5454) and carried out following (inter-) national animal welfare regulations. Adult male mice (CBA/CaJ; Jackson Laboratory, Bar Harbor, Maine, USA) were kept under standard laboratory conditions (see Text S1 – Detailed Methods). Sample sizes were as follows: Fig. 1&2: control n = 12, experimental n= 12; Fig. 3: n= 12. Running wheel activity was recorded continuously and stored in 2-min bins. Onsets and offsets of activity (Fig. 1E) were determined over 8 days just before the switch to DD or right after the switch to DD following the procedure previously described (Spoelstra et al. 2004). In the work for food protocol, a small 45 mg food pellet (F0165; Bio-Serv, Frenchtown NJ, USA) was delivered in the mouse cage after a set number of wheel revolutions using computer controlled pellet dispensers (Med Associates Inc., St. Albans VT, USA). During the 'work for food' protocol, mice started on a high reward schedule (100 revolutions/pellet). This generated essentially an ad libitum food condition, because excess pellets were observed in each individual kept under these conditions. During the reward reduction phases of the experiment, workload was increased daily by an extra 10–20 revolutions per pellet up to 200 revolutions per pellet (medium reward schedule) or 300–400 revolutions per pellet (low reward schedule). Throughout the experiment body mass was closely monitored at least every 3rd day at different times of day and high workload levels were individually titrated to keep the mice above 18 grams and 75% of their initial body mass. Body temperature was measured by customised temperature loggers (Thermochron iButton®, DS1922L, Maxim Integrated Products Inc., Sunnyvale CA, USA) implanted in the abdominal cavity of the mice and set to a sampling rate of once every 20 minutes (see Text S1 – Detailed Methods).

Results

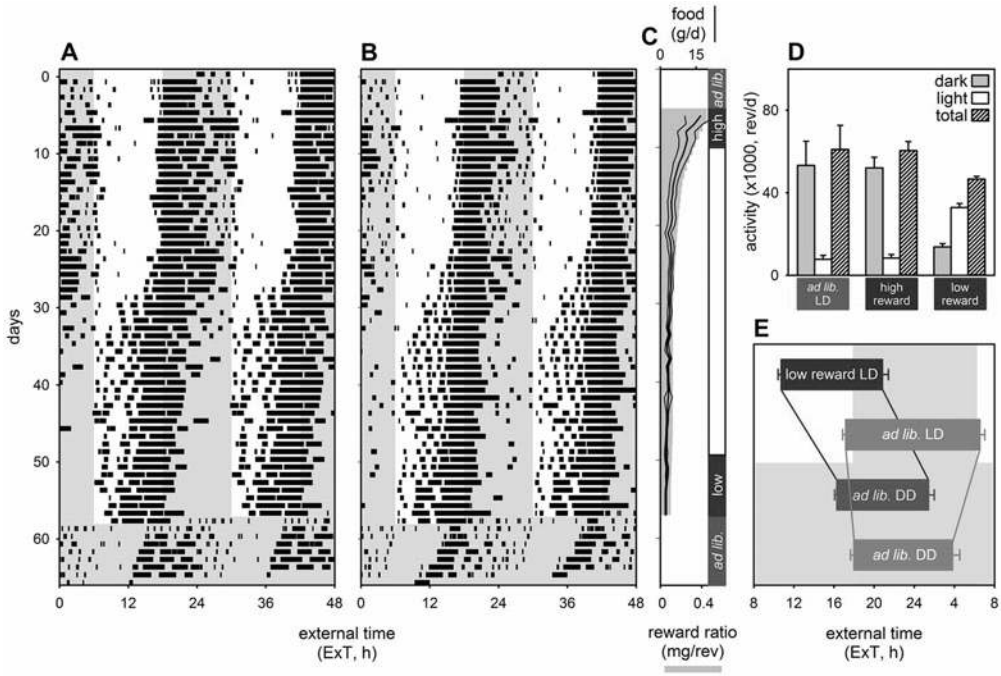

Figure 1 *Low food reward ratio induces diurnal activity, with minor effects on daily activity levels and circadian pacemaker phase.(A, B) Two example actograms show the gradual shift from nocturnal to diurnal wheel running. (C) Gradual reward ratio reduction (grey bars) reduces total food obtained (line, in g/d; thin lines indicate s.e.m.). (D) Total activity per 24 h was slightly reduced but shifted largely to the day when the reward ratio was reduced (red compared to blue and cyan). The immediate shift to the previous dark phase after release in DD with ad libitum food indicates normal entrainment of the circadian pacemaker to the previous LD cycle (A, B; days 59–66). (E) Average activity onsets and offsets for working mice during the last 8 days under LD (red bar) and first 8 days in DD (green bar), and for the ad libitum control mice (grey bars) during the same time intervals.*

Reducing food reward per unit workload (i.e. wheel running in mammals or perch hopping in birds) reduced daily food intake (Fig. 1C) as described in other species (Wiersma and Verhulst 2005). As a result mice spontaneously shifted their activity to the light phase of the day (Fig. 1A–B). This procedure increased activity in daytime while total activity per 24 h remained stable or was slightly reduced (Fig. 1D). Detailed comparison of activity onsets and offsets during the last 8 days in LD and the first 8 days in DD shows that low reward ratios advance activity onsets by 5.5 h (paired T-test, p,0.0001) and offsets by 4.6 h (paired T-test, p =0.0003) relative to the estimated phase of the main circadian pacemaker during entrainment (as extrapolation from activity patterns after release in DD with ad libitum food; Fig.1E red vs. green bars).

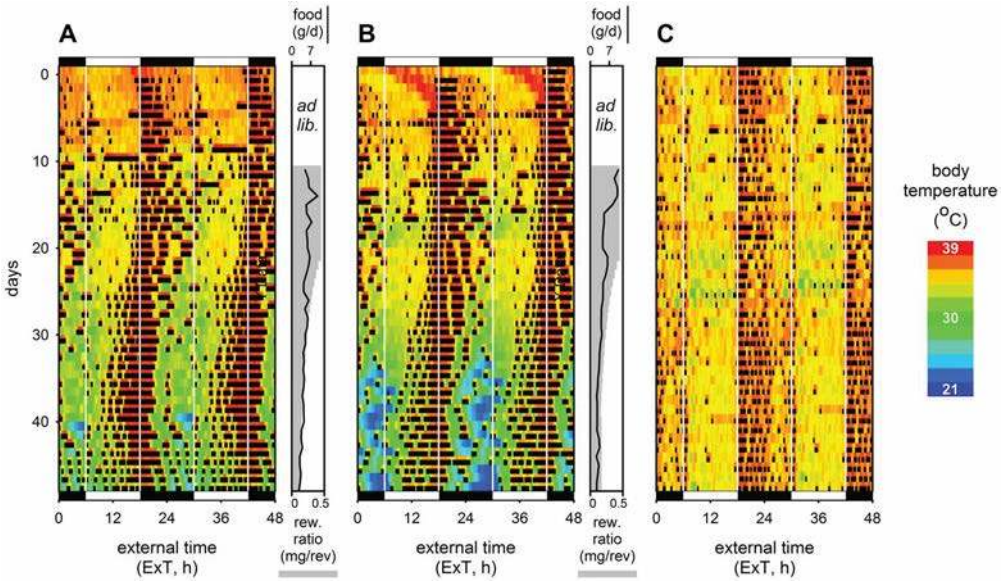

Figure 2 *Reward reduction causes diurnal activity and eventually reduces body temperatures. (A, B) Two example actograms and body temperature patterns of mice under a reduced reward ratio protocol (grey bars) and (C) a control mouse kept under ad libitum conditions. Low daily food rewards (side panels, black lines) eventually elicited torpor response (blue colors) after activity patterns shifted to the light (see also Fig. S3). No evidence of torpor was found in any of the control mice. Black and white bars indicate the light-dark cycle to which the mice were exposed throughout the experiment.*

Control mice on ad libitum food access throughout the experiment did not show advanced activity onsets in LD but rather delayed offsets (paired T-test: onsets, 0.8 h advanced, p = 0.08; offsets, 2.7 h delayed p = 0.01; Fig.1E grey bars). Low reward ratio advanced activity onsets in LD by 6.4 h (p,0.0001) and offsets by 9.7 h (p,0.0001) when compared to control mice (Fig.1E, red vs. top grey bar). After release in DD and ad libitum food conditions, the estimated phase of the circadian pacemaker during entrainment was 1.7 h (p = 0.0004) advanced for the onset of activity and 2.4 h advanced for the offset of activity (green vs. grey bar) when compared to the ad libitum control mice. Taken together, these data indicate that reduced food intake shifts activity from the dark phase into the light phase. Upon release into DD with food available ad libitum, activity patterns instantaneously reverted to the previous dark phase (Fig. 1A, B). This may suggest that the circadian pacemaker had remained entrained to the LD cycle, albeit with a slightly advanced phase relative to that of mice kept on ad libitum food supply throughout the experiment (Fig. 1E).

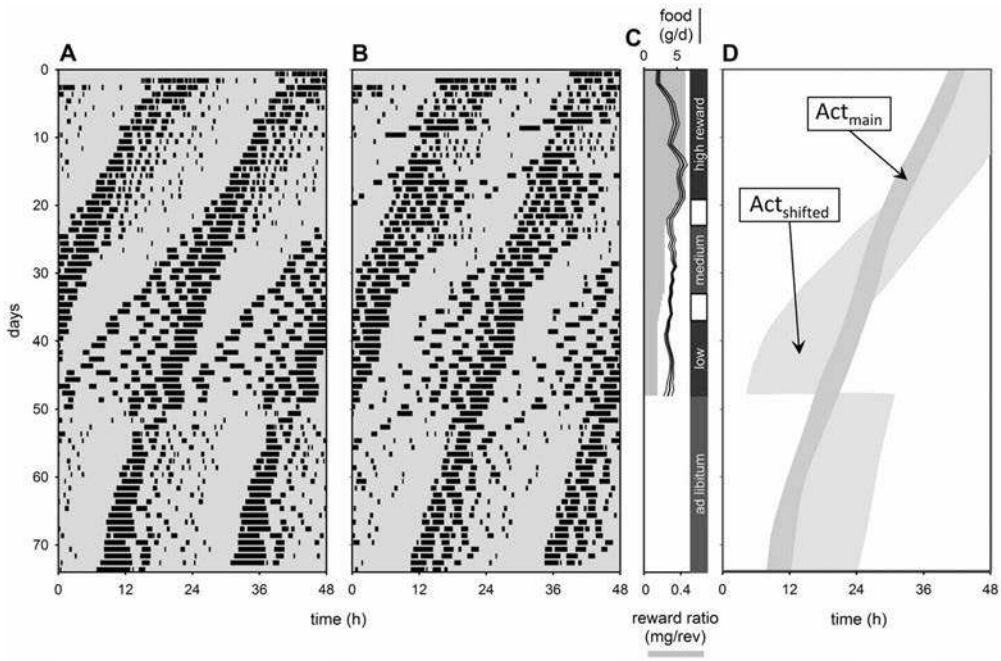

Figure 3 *Reduced reward ratio modifies free-running circadian activity patterns in continuous dim light (DD). (A, B) Two examples of free-running activity rhythms in mice that work for food are shown in double plotted actogram format. (C) Gradual reward ratio reduction (grey bars) and food obtained (line, in g/d; thin lines indicate s.e.m.) shifted circadian activity patterns. (D) Graphical interpretation of observed activity patterns where a shifted activity component (Act$_{shifted}$, light grey area) is discerned from a non-shifted main activity component (Act$_{main}$, dark grey area). Four different work levels were applied: high reward ratio (C, blue bar), medium reward ratio (C, purple bar), low reward ratio (C, red bar), and ad libitum (C, green bar).*

Daily rhythms in core body temperature (T_b) were associated with activity both in mice that work for their food and in ad libitum fed mice (Fig. 2). High Tb episodes gradually shifted to the light phase when reward ratio was reduced in mice working for food (Fig. 2A–B), but remained in the dark phase in control mice (Fig. 2C). While resting T_b rarely approached 30 °C in control mice, mice under low reward ratio gradually reduced resting T_b, eventually resulting in daily torpor well below 30 °C (Fig. 2, Fig. S1) (Schubert et al. 2010). In some mice activity shifted to the light phase well before torpor occurred (Fig. S2), indicating that the behavioral shift is not caused by the occurrence of torpor (Fig. 2B). Unlike control mice that show lowest T_b always during the light phase, lowest T_b in mice with a low reward ratio occurred in the last part of the night (Fig. 2A–B). The timing of torpor would thus coincide with the coldest phase of the daily cycle under natural conditions. This may have functional significance since at that time of day burrow retreat and torpor will have larger energetic benefits. To test whether the shift in activity patterns from nocturnal to diurnal depends on the presence of a light dark cycle, we applied the work for food protocol in mice kept under continuous dim red light (DD, Fig. 3A–D). Here we identified a main activity component (Act$_{main}$), which seems unaffected by the work for food protocol, and a less intense activity component (Act$_{shifted}$) that followed the main activity component under high reward ratio and ad libitum conditions. Act$_{shifted}$ component shifted and preceded the main

activity component under medium and low reward ratio conditions (Fig. 3D). When mice under low reward ratio conditions were fed ad libitum, the preceding $Act_{shifted}$ component immediately shifted back to its normal phase position and followed Act_{main}. This observation is paralleled by the immediate return from diurnal to nocturnal activity patterns when working mice in an LD cycle were placed on ad libitum food access (Fig. 1. A–B).

Discussion

Our data indicate that the output of the SCN driving activity cycles is modified downstream. Since the activity pattern gradually shifts towards diurnality when energy intake is reduced (Fig. 1A–B; 2A–B), it is plausible that this activity pattern is controlled by a slave oscillator that changes its phase position with respect to the SCN. Activity-rest oscillators outside the SCN have been demonstrated both in temporally restricted fed animals (FEO (Stephan 2001)) and in rodents given access to low concentrations of methamphetamine in the drinking water (methamphetamine sensitive circadian oscillator, MASCO (Honma et al. 1986; Mohawk et al. 2009). The FEO, the MASCO, and the activity regulating oscillator presented here are all expressed under modified metabolic conditions. They may very well turn out to rely on the same physiological mechanism and anatomical structure. Reduced reward ratios in mice that run their wheel to obtain food resemble natural conditions, where travelling distance between food patches increases when food becomes scarce (Schubert et al. 2010). Our data may reveal the strategy that mice adopt to cope with reduced energy intake under natural conditions. Two alternative strategies to maintain energy balance under such conditions can be hypothesized (Fig. 4): A) foraging activity time per day remains constant and mice reduce energy expenditure during rest to match reduced energy intake; B) resting metabolic rate remains constant and mice increase foraging time per day to balance daily intake and expenditure. Both strategies were quantified and graphically represented (Fig. 4) using measures of energy expenditure from previous work (see Text S1 – Model Calculations). Although one might expect a combination of these strategies, the normal activity levels (Fig. 1) and the reduction in body temperature (Fig. 2) indicates that CBA/CaJ mice that work for food follow strategy A to maintain their energy balance when energy intake is reduced.

These results provide novel insight to the current debate on the FEO, in which mice show diurnal (food anticipatory) activity when feeding is restricted during a few hours in the light phase. This restricted diurnal meal timing is thought to uncouple activity rhythms from the circadian pacemaker (SCN) signal (Stephan 1983, 2001). The timed food restriction protocol used to study entrainment of the FEO carries the confounding risk that two variables are manipulated simultaneously: meal timing and reduced food intake (70–75% for 3 h food access in mice; (Froy et al. 2008)). Mendoza et al. (2007) showed that diurnal activity occurs also when 6 short meals are presented at 4 h intervals, a procedure which still involves imposed meal timing. Mice in the present study chose themselves when to work for their food, indicating that reduced food intake alone, and not meal timing, was crucial to the occurrence of diurnal activity. Diurnal activity patterns in house mice were recently observed to persist for months under semi-natural conditions with competition for food access (Daan et al. 2011).

This diurnal behaviour would allow mice to forage during the warmer part of the day and to time rest and hypothermia (torpor) at the end of the night. This temporal niche switching would minimize daily energy expenditure, possibly at the cost of increased predation risk during the day.

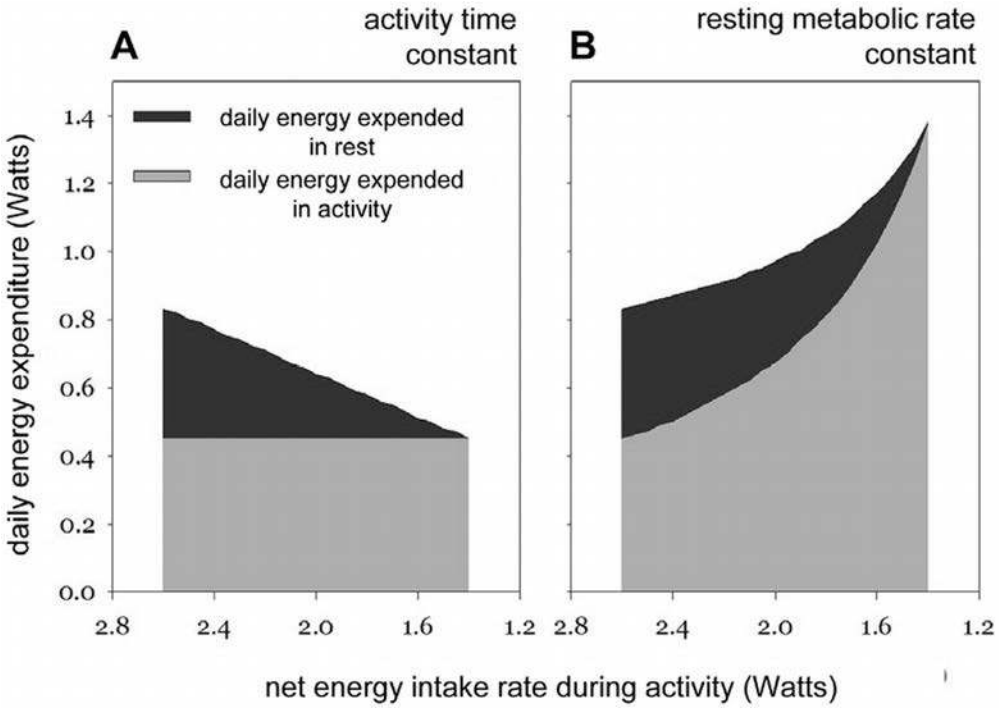

Figure 4 *Energy budget calculations for two strategies to cope with reduced energy intake during foraging activity. Using known values for energy expenditure during rest and activity (see Text S1 - Model Calculations) we illustrate how mice may cope with reduced energy intake either by (A) reducing resting metabolic rate while duration of daily foraging activity remains constant (strategy A), or by (B) expanding daily foraging while metabolic rates during rest and activity remain constant (strategy B). With decreasing daily energy intake during activity, strategy A predicts a gradual reduction in body temperature during the rest phase, and reduced daily energy expenditure (DEE), while strategy B leads to a gradual increase in activity duration and DEE. Both strategies reach a limit when net energy intake during foraging drops below ~1.4 Watts.*

The 'work for food' paradigm presented here reveals adaptive flexibility in circadian organization and a strong link between metabolism and the circadian system in an intact mammal. It avoids possible confounding effects of food timing and rationing, that are inherent to the classical food entrainment protocol. Spontaneous diurnal activity elicited in mice that work for food may open new approaches to describe an alternative circadian activity-rest oscillator, and will lead to a better understanding of temporal organization of behaviour under natural conditions.

Acknowledgments

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Supporting Information

Detailed Methods

Adult male mice (CBA/CaJ; Jackson Laboratory, Bar Harbor, Maine, USA) were kept in translucent cages (12.5 x 12.5 x 20 cm). They were provided with running wheels (14 cm diameter) and water *ad libitum*. Cages were placed in a climate controlled room (21°C, Rel.Hum. 60%) under either continuous incandescent dim red light (< 1 lux) or a 12-h:12-h light-dark cycle using white fluorescent tube lights (Philips TLD 58W/840) providing 76 lux (SD=20 lux) at the level of the cage.

Activity onsets were defined as those time points where activity in a 1 h running mean became higher than the average activity level in a running mean with the time base of the circadian cycle as determined by periodogram analysis over the same data. Offsets were determined when 1 h running mean activity levels became lower than the activity level in a running mean with the time base of the circadian cycle as determined by periodogram analysis over the same data [1].

Implanted temperature loggers (Thermochron iButton ®, DS1922L, Maxim Integrated Products Inc., Sunnyvale CA, USA) were customized by taking the loggers from their metal jacket, sealing them in plastic foil, and coating them with Elvax (Minimitter, Bend OR, USA) followed by 2 h 2% glutaraldehyde sterilisation, three rinses with sterile saline, overnight incubation in 1 million IU/30ml Na-penicillin, followed by 2 rinses with sterile saline. Weight of the encapsulated temperature loggers was ~2 g. Before abdominal logger implantation, animals were operated under isoflurane (Abbott Laboratories Ltd., Kent, UK) inhalation anesthesia (2%) followed by 1mg/kg finadine® analgetics post-operation treatment. After operation animals were closely observed during a week recovery period.

Model calculations of two alternative energetic strategies in mice to cope with reduced net energy intake (see Fig.4)

Model solutions are examples using parameter values from other mouse strains (Spoelstra et al. 2004; Vaanholt et al. 2007a, 2007b), not necessarily matching the body mass and activity patterns of the strain used in our experiments. They aim to demonstrate the basic qualitative principle, not to yield specific quantitative predictions for the present experimental data.

For simplicity we assume that a mouse is either foraging or resting, such that the fraction of time spent resting (R) equals 1-A, if A is the time spent actively foraging. In order to stay energetically in balance, the daily metabolizable (net) energy intake M_d (Watt) has to match the daily energy expenditure (DEE), here indicated as E_d (Watt).

M_d equals A times the net intake rate during foraging m_a (Watt). E_d equals the sum of the rates of energy expenditure during foraging (e_a) and during rest (e_r), each weighted by the fraction of time spent in these two states. Thus maintaining energy balance simply means:

$$A*m_a = A*e_a + (1-A)*e_r \qquad \textit{(equation 1)}$$

Food abundance to a mouse is reflected in its net rate of metabolizable energy intake (m_a) during activity (foraging). When m_a decreases due to poorer conditions, energy balance can be maintained by changing A or e_r. We assume that e_a cannot be decreased without simultaneously jeopardizing m_a further, and we assume e_a is a constant.

In our example simulations of the two strategies, we use the following realistic values. Under *ad libitum* feeding conditions, at 20°C, a mouse spends 72.3 kJ/day (DEE), or 0.837 Watt [2]. Its RMR is 0.571 Watt [2]. The time active is 7.7 h/day or A= 0.321 [3]. The contribution of rest to DEE is then 0.571*(1-0.321)*24*60*60= 33.5kJ/day, or 0.388 Watt. Hence, the contribution of activity to DEE is 0.837-0.388= 0.449 Watt. This yields a figure for the metabolic rate during activity of 0.449/0.321 = 1.399 Watt. The metabolizable energy intake during activity is thus estimated at 0.837/0.321 = 2.607 Watt.

In summary, the parameter values for the model calculations were as follows:

Active fraction of the day in ad lib:	A = 0.321
Energy expenditure during activity:	e_a = 1.399 Watt
Contribution of activity to DEE:	$E_a = A*e_a$ = 0.449 Watt
Rest fraction of the day:	R = 1-A = 0.679
Energy expenditure during rest:	e_r = 0.571 Watt
Contribution of rest to DEE:	$E_r = (1-A)*e_r$ =0.388 Watt
Total daily energy expenditure DEE:	$E_d = E_a + E_r$ = 0.837 Watt
Metabolizable energy intake during activity:	m_a = 2.607 Watt

While the metabolizable energy intake rate m_a is deduced from its *ad lib* value we can now solve equation 1 for two different strategies:

Strategy A: Activity time A is kept constant at 0.321:

The unknown variable e_r can be solved from the equation 2:

$$e_r = A * (m_a - e_a) / (1-A) \qquad \textit{(equation 2)}$$

Thus, e_r drops linearly with decreasing m_a (Fig.4A). There are only positive solutions for $m_a > e_a$ = 1.399 Watt.

Strategy B: Resting metabolic rate e_r is kept constant at 0.571

The unknown variable A can now be solved from the equation 3:

$$A = e_r/(m_a - e_a + e_r) \qquad \textit{(equation 3)}$$

Thus, A increases hyperbolically with decreasing m_a (Fig.4B). The limit to A obviously is 1.0. At this limit $m_a = e_a$. This is the same as for strategy A.

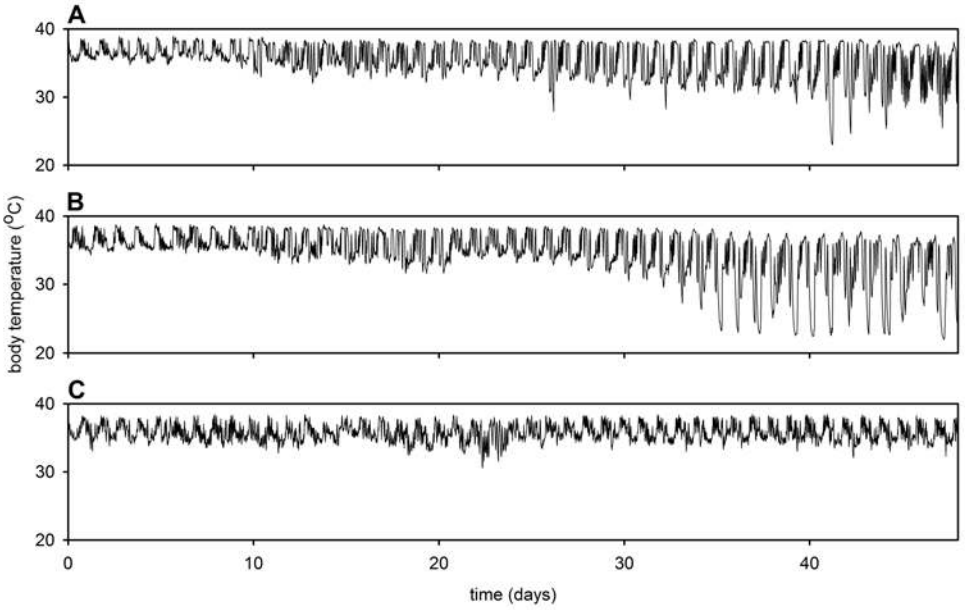

Figure S1 *Reduced food reward affects body temperature patterns. Body temperature patterns of two representative animals with gradually reduced food reward (A, B) and an* ad libitum *control animal (C). Body temperature occurring during the rest phase is reduced, eventually leading to torpor. Data are from the same animals as presented in Fig.1 (panel identifiers correspond).*

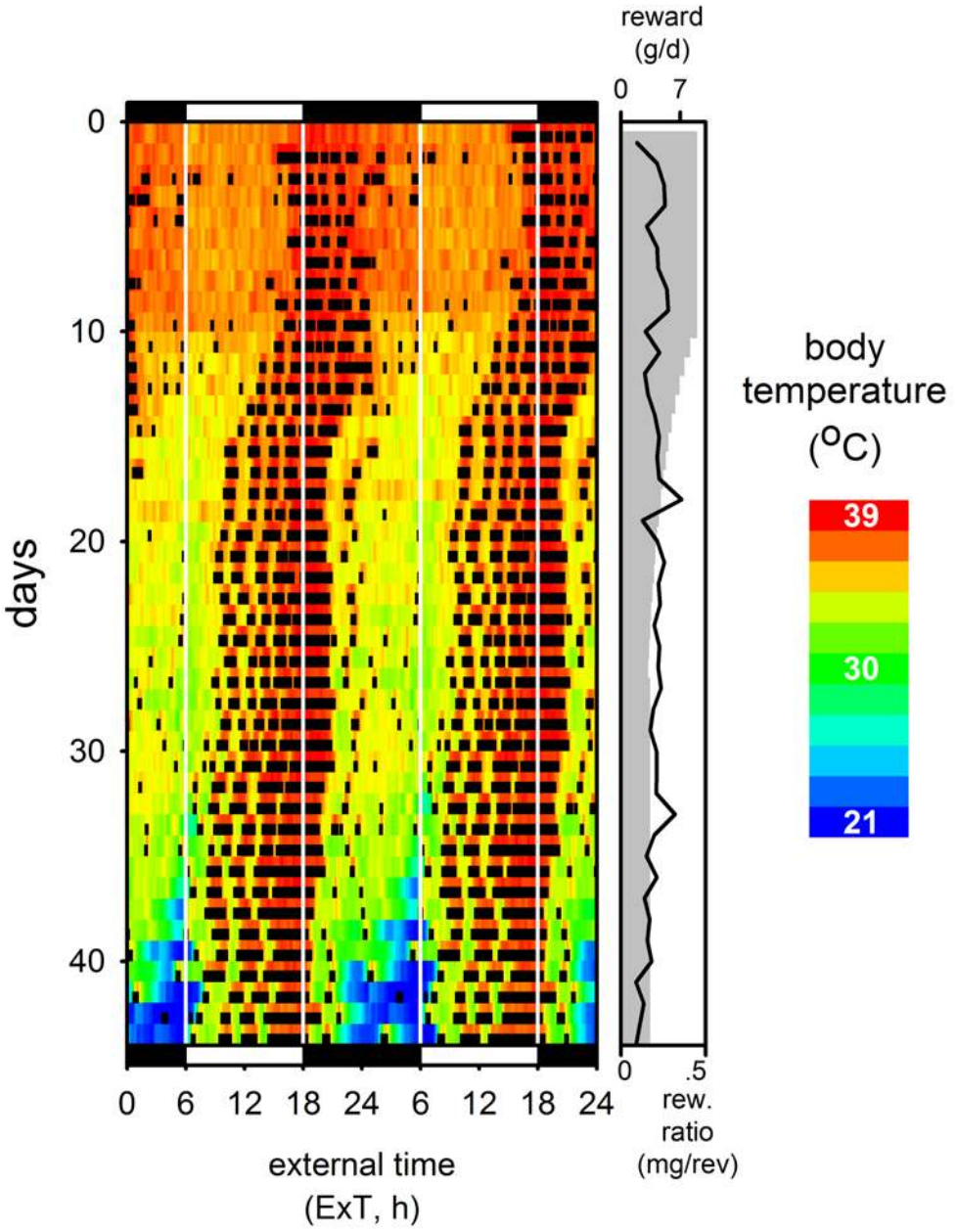

Figure S2 *Reduced food reward shifts activity rhythms before torpor occurs. This example shows that some animals in the experiment presented in Fig. 2 and Fig. S1 clearly showed a shifted activity rhythm well before torpor occurs. The opposite (torpor occurs before the activity rhythm is shifted) was never observed. These data indicate that torpor is not necessary to shift the activity rhythms into the day. It is rather the shifted activity rhythms that allow torpor to occur in the dark phase.*

Chapter 8

Workload induced food limitation induces daily torpor, diurnal activity and brain tau hyperphosphorylation in CD-1 mice.

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Abstract

Mice are nocturnal and euthermic under standard laboratory housing conditions. When natural food shortage is simulated by letting mice work for their food, they start to show diurnal activity and a sharp reduction in body temperature (torpor) during the rest phase at night. Working for food does not involve an imposed temporal feeding schedule or food deprivation, but it has remained unclear whether the effects are caused by food shortage, or by the working effort itself, or both. We designed an experiment in which for each working mouse a yoked control mouse that did not have to work for food was included. Yoked control mice received identical amounts of food at the same time as their matched working mice. Both the working and the yoked mice reduced food intake, lost body mass, increased diurnal wheel running activity, and went into torpor. The effects were slightly less pronounced in the yoked control mice. We conclude that food shortage itself is able to induce torpor in mice. The shift towards diurnal activity of the yoked control mice may be attributable to either reduced food intake or the timing of food availability imposed by the working mice. The strength of the effects is most likely influenced by the total energy intake available to the mice. Furthermore, torpor in mice, like natural daily torpor in Djungarian hamsters, was also associated with increased tau protein hyperphosphorylation in the brain. This indicates that use of torpor may be associated with a cost: the occurrence of tau hyperphosphorylation in the brain.

Introduction

In contrast to the standard laboratory situation, animals in nature have to invest time and energy to obtain food. They face time constraints when obtaining food, and/or a low energy return on their investment. Small endotherms such as rodents have evolved strategies to cope with energy shortage, such as intermittent hypothermia (torpor). Several experimental paradigms are in use to mimic food shortage: caloric restriction, restricted feeding (RF) schedules and letting animals work for their food (Perrigo and Bronson 1983; Perrigo 1987; Vaanholt et al. 2007b; Schubert et al. 2008; Hut et al. 2011).

Caloric restriction in studies on ageing and life expectancy is usually done by providing food during a limited time interval each day (Froy and Miskin 2007). This is essentially the same experimental paradigm as the restricted feeding schedule used to study the 'food-entrainable oscillator' (FEO) in chronobiology studies (reviewed in (Stephan 2002; Davidson 2009b)), although the questions addressed are totally different.

The experimental treatment always involves two aspects of feeding: restriction of the total food intake, and altered circadian timing of food intake. In the work for food (WFF) paradigm the experimenter does not restrict food intake to a particular time of day: this is left to the animal to decide (Hut et al. 2011). It introduces, however, another dualistic aspect. In WFF simultaneously the activity (wheel running) and the resulting food intake are affected. We are keen on differentiating between these aspects in their effects on hypothermia and circadian organisation of activity.

During restricted feeding (RF) schedules, access to food is only allowed during certain times of the day. When animals have access to the food, it is available *ad libitum*. Whether RF results in caloric restriction depends on the duration of the food availability. If the access to the food is limited to the day in rats and mice they become active a few hours before the food is available. This behaviour is known as food anticipatory behaviour (FAA) (Mistlberger 2009). Scheduled daytime feeding also affects body temperature. Mice shift the maximum of their daily body temperature into the day and the amplitude of the body temperature cycle is larger, the minima are more pronounced, but RF for 4 hours per day does not induce torpor (Moriya et al. 2009).

In the working for food paradigm foraging effort is manipulated by letting animals travel for their food, as they would have to in nature when scarcity of food increases the distance between food patches. Usually the travel distance per unit of food is manipulated, for instance by increasing the flight distance in birds (Wiersma et al. 2005). In mice walking distance can be manipulated by rewarding a pre-set number of wheel revolutions with a food reward (Perrigo and Bronson 1983; Vaanholt et al. 2007b; Schubert et al. 2008; Hut et al. 2011). The advantage of this method is that it does not impose an external timing schedule upon animals, while simultaneously limiting the availability of energy. The work for food paradigm is a laboratory tool to effectively titrate the energy balance in mice without confounding effects of manipulated feeding time signalling.

Increased foraging effort can trigger several responses. Mice become active during a larger part of the day, especially when increased foraging costs are combined with a metabolic challenging condition such as lactation (Perrigo 1987). CBA/CaJ mice shift their activity into the day and become diurnal when working for food (Hut et al. 2011).

Mice also start employing torpor to save energy. The frequency and duration of torpor depend on the height of the foraging costs (Schubert et al. 2010). Since the WFF paradigm involves changes in the intensity and timing of activity as well as in food intake, we designed an experiment to disentangle activity and food in their effects on hypothermia. We manipulated foraging effort in a group of mice, and matched each mouse to another individual - the yoked control - that did not have to run for food, but received exactly the same amount of food at the same time as the working mouse. We measured effects on the circadian profiles of body temperature and activity in the working mice, the yoked controls and in a group of *ad libitum* fed control mice.

Besides the behavioural responses, we investigated brain physiology during torpor. The occurrence of hypothermia, or torpor, in small endotherms in response to prolonged food deprivation is well known. This has been described e.g. in blackbirds (Biebach 1977), willow tits (Reinertsen and Haftorn 1984), pigeons (Heller 1988) and mice (Hudson and Scott 1979; Webb et al. 1982; Koizumi et al. 1992). This body temperature response to caloric restriction has also been measured in many strains of laboratory mice (Rikke et al. 2003; Dikic 2008).

During hibernation in ground squirrels and Syrian hamsters, deep torpor causes hyperphosphorylation of tau protein in the CNS (Arendt et al. 2003; Härtig et al. 2007). This hyperphosphorylation is reversed upon the return to euthermia. Tau hyperphosphorylation in humans is associated with diseases involving tau pathology, such as Alzheimer's disease (Goedert et al. 1989b). Natural hibernation is a valuable model system to study tau hyperphosphorylation dynamics under physiological conditions (Arendt et al. 2003). Reversible tau hyperphosphorylation has also been observed in less extreme, daily torpor in Djungarian hamsters (Boerema et al. 2008a).

It is unknown whether tau hyperphosphorylation also occurs during naturally invoked torpor in mice. Being able to study torpor associated hyperphosphorylation dynamics in mice would be beneficial, since mice are common laboratory animals, contrary to most hibernators. In addition to the behavioural responses including torpor, we also investigated tau hyperphosphorylation during torpor to indicate possible physiological repercussions of this energy saving behavior. We sacrificed hard working mice and their yoked controls during and after work-induced torpor. We screened the brains of torpid and euthermic mice to assess whether tau hyperphosphorylation during torpor occurs in mice.

Materials and methods

Animals and housing

Male Hsd:ICR (CD-1) mice (age: 3 months; n=36) were raised in our local breeding colony (Zoological laboratory, Haren, the Netherlands). The colony founders were obtained from Harlan (France) in 2005 and were bred as described previously (Schubert et al. 2008). Prior to the experiment, the mice were kept on a 12:12 h light/dark cycle at an ambient temperature of 21 (+/- 1) ˚C. Initially, mice were housed individually in Macrolon type 2 cages (33x15x13 cm), on sawdust bedding, with nesting material (Enviro-dri®, Shepherd specialty papers, USA) and without a running wheel. Water and food (RMHB 2181, Arie Blok, Woerden, the Netherlands) were available *ad libitum*.

Body temperature and activity were measured using telemetry. Mice (n=24) were surgically implanted with transmitters in the abdominal cavity, under inhalation anaesthesia (2.5% Isoflurane, 97.5% O_2), prior to the experiments. 12 Mice received TA-F20 transmitters (Data Sciences International, St. Paul, USA) and 12 mice received Series 3000 XM-FH transmitters (Mini Mitter, Bend, OR, USA). Mice were allowed to recover for 8 days in their home cages. Following recovery for the implanted mice, all 36 mice were transferred to cages (Macrolon type 2 long) equipped with a running wheel. Animals were switched over to the experimental diet of 45 mg precision food (NOYES Precision Pellets™, PJAI-0045, Formula A/I, Research Diets Inc, New Brunswick, USA). Food was provided *ad libitum* in the cage until the onset of training on the workload. Then food was provided by food dispensers (Med Associates Pellet Dispenser ENV-203, Sandown Scientific, Hampton, UK), linked to a steering computer (Series 3 programmable logic controller PLC, General Electric). During the remainder of the experiment a food pellet was dispensed each time a mouse had completed a number of wheel revolutions (X). The reward ratio was gradually reduced (thereby increasing the workload) by adjusting X upwards in small steps. During the initial learning phase, the mice received additional pellets in their cage up to a total maximum of 6 g food/ day.

Data acquisition

Telemetry body temperature signals were acquired in 2 minute intervals and the transmitter activity counts were summed in 2 minute bins, using Dataquest 4 software (Data Sciences Int.). Wheel running activity was acquired by our home built event recording system (ERS). Wheel revolutions were summed in 2 minute bins and stored. The number of pellets mice received was both recorded and measured daily (2 hours preceding lights on) by weighing the pellets in the food dispensers, the food added to the dispensers and the additional food given in the cage (all masses: 0.1 g precision). Body mass of the animals was also measured every day in the 2 hours preceding lights off, between External time (ExT) 16:00 and 18:00.

Experimental design

At the start of the experiment, mice were assigned to the three experimental groups. The mice assigned to the Workload (W) group, had to work for their food by running in their wheel. The mice assigned to the Yoked control (Y) group received one food pellet each time a matched individual in the W group received one. The mice in the third group, the Control (C) group, were fed pellets *ad libitum* throughout the experiment. Initially all groups contained 12 animals. One mouse in the W group died during the experiment. Only complete sets of C, W and Y mice were included in the analysis, resulting in final group sizes of n=11 (C, W & Y group). The average body mass at the start of the experiment was 38.6 g (SD 3.4) for the W group, 38.8 g (SD 3.8) for the Y group, and 38.2 g (SD 2.1) for the C group. Mice with a transmitter implanted were allocated to the W or the Y group. These mice were matched in pairs on body mass and transmitter type, a third animal from the C group was matched to each W+Y couple on body mass.

Workload schedule

The experiment was divided in three phases. During phase 1 (baseline; days -7 till -1), all mice had access to a running wheel, and were fed precision pellets *ad libitum*. During the last two days of the baseline phase, rewarding of wheel revolutions also started at 100 rev/pellet (reward ratio = 0.45 mg/rev). In phase 2 (training; days 0 till day 15), mice in group W started at 100 rev/pellet, (reward

ratio = 0.45 mg/rev) and were provided with 6 g of additional food in their cages. In the following days the workload was increased every day with ~15 rev/pellet, resulting in a decrease of the reward ratio with 0.058 mg/day. During the first 4 days of the increasing workload the amount of extra food provided in the cage was reduced, until the W mice were solely dependent on the food earned by working. The workload then was gradually increased to 250 rev/pellet. During phase 3 (working, day 16 till sacrifice), the animals were working for their food. The workload was maintained and further increased per individual if necessary, until torpor occurred in the W animal of each pair.

Sacrifice

To analyse the influence of torpor and rewarming from torpor on the brain, mice were sacrificed at different times, for each trio as soon as the W mouse consistently showed torpor. The mice were euthanized three at a time, one individual each from the W, Y and C group, by an intraperitoneal injection of an overdose of 6% sodium pentobarbital. Half of the animals were sacrificed while the W animals were in torpor. Rectal (T_b) and mouth (T_m) temperatures confirmed the torpid state of the W mice (T_b = 25.3 °C (SD 2.9), T_m = 24.5 °C (SD 2.3)). The other mice were sampled when the W mice were euthermic, following a torpor bout. Rectal and mouth temperatures confirmed the euthermic state of the W mice (T_b = 36.7 °C (SD 2.0), T_m = 37.4 °C (SD 2.1)). Four of the Y mice also appeared to be in torpor (T_b = 24.5 °C (SD 4.2), T_m = 24.2 °C (SD 3.7)). All other Y and all C mice were euthermic when sampled (T_b = 36.0 °C (SD 2.0), T_m = 36.0 °

protease inhibitors and Roche phosstop® phosphatase inhibitors (Roche, Woerden, the Netherlands)]. Lysis buffer was added in a 1:9 tissue to buffer ratio. Lysates were centrifuged at 20841 g for 30 min at 4°C. Clear supernatant was removed and protein concentrations were determined by Bradford assay. Protein concentrations were standardized to 2.5 mg/ml by adding ice-cold lysis buffer and 5x Laemmli-buffer. Proteins were separated on 4-20% gradient Nu Page Novex Bis-Tris mini gels (Invitrogen, Breda, the Netherlands) using 57µg of total protein per well and were subsequently transferred to an 0.2µm PVDF-membrane, using the I-blot dry blotting system (Invitrogen, Breda, the Netherlands).

Immunodetection of western blots

The membrane was subsequently probed for tau hyperphosphorylation using the AT8 antibody (Perbio, MN1020, 1:1000) and total tau protein irrespective of the phosphorylation state using anit tau antibody (Sigma, clone DC 25, T8201, 1:5000). The lower half of the membrane was cut off and probed for Hypoxanthine-guanine Phosphoribosyl Transferase (HPRT) with the HPRT (FL-218) antibody (Santa Cruz, sc-209751:100000). The Membrane was blocked for 1 h with blocking buffer (0.05% Tween-20, 2.0% BSA in TBS (pH 8.0), prior to antibody incubation. 5mM Na3VO4, 50 mM NaF and Phosstop were added to all buffers in order to maximize the inhibition of phosphatases also during washing, blocking and antibody incubation steps (Sharma and Carew 2002). The membrane was stripped between the application of AT8 and Tau antibodies, by 2 times 15 minutes incubation in stripping buffer (24mM glycine + 1.25 % SDS + 0.1% Tween-20 in 50mM Tris, (pH 2.0) heated to 60 °C prior to use, followed by thorough rinsing in washing buffer. Detection of bound primary antibodies was performed with goat-anti-mouse (SC-2005) IgG-HRP (Santa Cruz), diluted 1:10000 in blocking buffer. Detection of bound secondary antibody was done by enhanced chemiluminescense; using Super Signal West Dura (Pierce, Rockford, IL, USA) diluted 1:1 in TBS (pH 8.0) in a Biorad Chemidoc XRS+ imaging system.

Optical density of western blots

The expression of the tau-protein and the hyperphosphorylation of the tau-protein were analysed in cortex material of 6 Workload (W) mice and 4 yoked control (Y) mice sampled in torpor and 5 W and 4 Y mice sampled in euthermia following torpor. Several lanes had be omitted from the analysis because of artifacts, preventing proper quantification of the signal, resulting in 6 euthermic mice (EU) and 8 torpid mice (T) in the final OD analysis. Optical density of the bands on the membranes was analyzed with the image software provided with the chemidoc system: Biorad Imagelab version 3.0 beta. The HPRT signal was first normalized by dividing the signal in each band through the average of all 14 bands included in the analysis. Subsequently the Tau and AT8 OD values were corrected for the amount of loaded protein by dividing them through the normalized HPRT signal. The OD analysis was performed in 2 ranges of molecular weights: high molecular weight tau species from 65-68 kDa and lower molecular weight tau species from 55-61 kDa. The AT8 signal was corrected for the amount of Tau protein present by dividing the signal by the total normalized tau expression within the molecular weight range analysed, in order to be able to distinguish between differences in hyperphosphorylation and changes in total amount of tau protein. All OD-values were expressed as a percentage of the average signal of the euthermic (EU) mice. One-way ANOVA's were used to test for significant differences between euthermic and torpid mice within each molecular weight range.

The experiments were approved by the animal experimental ethics committee of the University of Groningen under license number DEC-5114A.

Results

Food intake, body mass, wheel running activity

Figure 1 shows the data on reward ratio (group W; panel A), food intake (panel B) and body mass (panel c) for the experiment. Note that the data are truncated at day 25, from where onwards the numbers of mice declined due to sacrifice for brain tissue collection. The reward ratio was decreased from 0.44 mg/rev (SD 0.02) in the beginning of the training phase to 0.14 mg/rev (SD 0.02) on day 25 (figure 1b). The food obtained by the W animals and as a consequence also by the Y animals, decreased rapidly from 8.02 g/day (SD 2.41) on day -1 to 4.02 g/day (SD 1.39) on day 4. During the rest of the experiment (day 5 – 26), the amount of food obtained by the mice was stable around on average 3.81 g/day over these days for the W mice (SD between days 0.58), even though the reward ratio was constantly decreased (figure 1c). Increasing the workload (in group W) resulted in a decrease in body mass in the W and the Y group (figure 1a). Body mass decreased from on average 38.49 g (SD 3.63; n=11) on day -1 to 30.00 g (SD 1.02; n=11) on day 25. The yoked control mice also lost weight but not as much as the W animals. Body mass decreased from on average 38.39 g (SD 3.79; n=11) on day -1 to 32.21 g (SD; n=11) on day 25 for the Y group. The *ad libitum* fed control (C) mice did not lose body mass. Body mass was on average 38.66 g (SD 1.57; n=11) on day -1 and 38.85 g (SD 1.65, n=11) on day 25 for the C group.

Figure 2 shows the wheel running activity for the three groups during the experiment. The total amount of wheel running was slightly higher for the C group during the baseline (figure 2). This is most likely caused by the fact that the W and Y animals were implanted with transmitters. Animals in the C group ran on average 14598 rev/day (SD 5963) during phase 1 (baseline). They slightly increased activity to 18183 rev/day (SD 10103) during the training, but did not really increase further from day 15 -25 (18436 rev/day (SD 10428)). The W and the Y animals more than doubled running wheel activity during the training. The W animals ran on average 10468 rev/day (SD 4975) during the baseline, and 14732 rev/day (SD 2831) during the phase 2 (training). The yoked controls ran on average 9322 rev/day (SD 5107) and 11728 rev/day (SD 4603) during the training phase. The W animals increased further in phase 3 (high workload), to 23631 rev/day (SD 2292). The Y animals did not increase their activity any further and remained at 10840 rev/day (SD 5603) in phase 3.

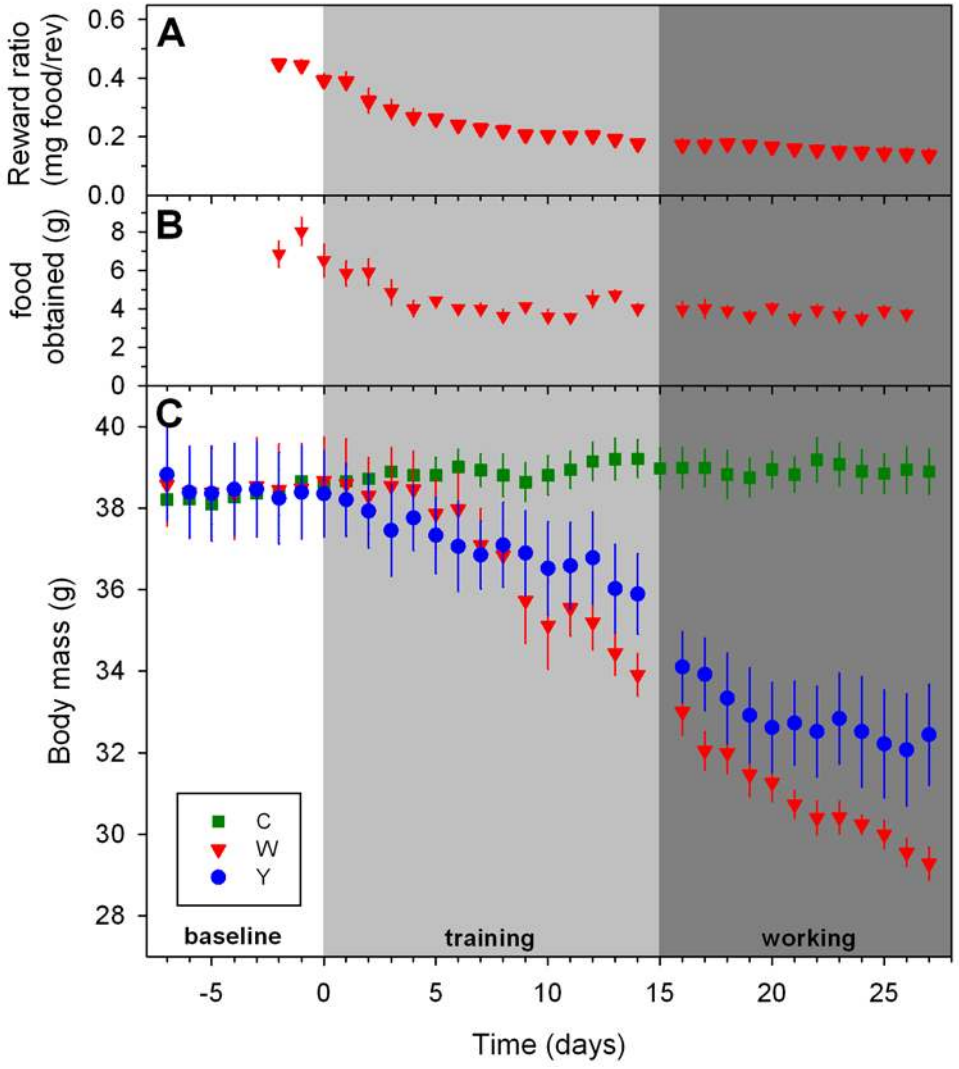

Figure 1 *Reward ratio, food obtained and body mass. Average body mass (± s.e.m.) over time (panel C) of the ad lib fed mice (C; n=11; green squares), mice working for food (W; n=11; red triangles) and Yoked control mice (Y; n=11; blue circles). Panel A shows the average workload with standard error for the W mice, expressed as the average reward ratio (mg food per wheel revolution). The workload is steadily increased for all mice during the training. During phase 1 the workload was further increased for individual mice until torpor occurred in the W mice. Panel B shows the average amount of food obtained by the W mice (and as a consequence also the Y mice). Data are plotted until day 27 after which sample sizes declined.*

Figures 2 A-C show the distribution of activity over the day. The peak in activity occurs following lights-off (ExT 18-20) for all animals. The period with the lowest activity is around lights-on (ExT 5-7). Peak and trough stayed at approximately the same time for all groups in the training and working phase (fig 2 A-C). In both the W and the Y group the activity pattern changed during the working schedule. Both groups started to run during the light phase, and decreased the number of revolutions during the dark phase. This effect is much larger in the W animals. The C animals hardly ran during the light phase. The small amount of activity around ExT 17 is most likely attributable to the weighing of the food and the mice performed at this time of day.

The distribution of wheel running over the light and dark phase is summarized in the Diurnality index D. Figure 3 shows the average D for the 3 groups over time. A D-index of 1 means that there is only activity during the light phase, a D-index of -1 means that there is only activity during the dark phase. During baseline all three groups were nocturnal with a D-index of approximately -0.8. As soon as the W mice have to work for their food they start to become more diurnal. During the training phase the D-index started to increase, to approximately -0.1. During the working phase the Y animals also became more diurnal, but at a later point in time. The W animals increased their D-index even more and became more diurnal than nocturnal (D-index > 0). The ad lib fed animals remained predominantly nocturnal in their wheel running behaviour during the whole experiment (D-index ~-0.8 - 0.9).

The centre of gravity (COG) of the running wheel activity per day is plotted for all mice in figure 4, from the start of the baseline until the moment of sacrifice for each mouse. Also the group averages on day 0 and on the moment of sacrifice are plotted. The mice in the C group did not shift their activity. The average COG at day 0 was at ExT 22:09 (SE 19 min). The average COG did not change over the experiment for the C group ($t = 0.0650$, $p = 0.949$, $n = 11$) and was at ExT 22:07 (SE 31 min) see figure 2 C). The animals in the W group phase advanced and shifted their centre of gravity to an earlier phase ($t = 11.098$, $p < 0.001$, $n = 11$). Most mice started to phase advance already during the training phase. W mice shifted their activity forward by 7.2 hours from ExT 21.14 (SE 19 min) on day 0 to ExT 14:04 (SE 28 min). Most, but not all Y animals did phase advance their activity as well. Yoked controls always started phase advancing later than their matched W mouse. None of the Y mice started shifting in the training phase. The Y animals phase advanced on average 4.1 hours from ExT 21:20 (SD 21 min) on day 0 to ExT 17:13 (SD 47 min) on the day of sacrifice ($t = 6.269$, $p < 0.001$, $n = 11$).

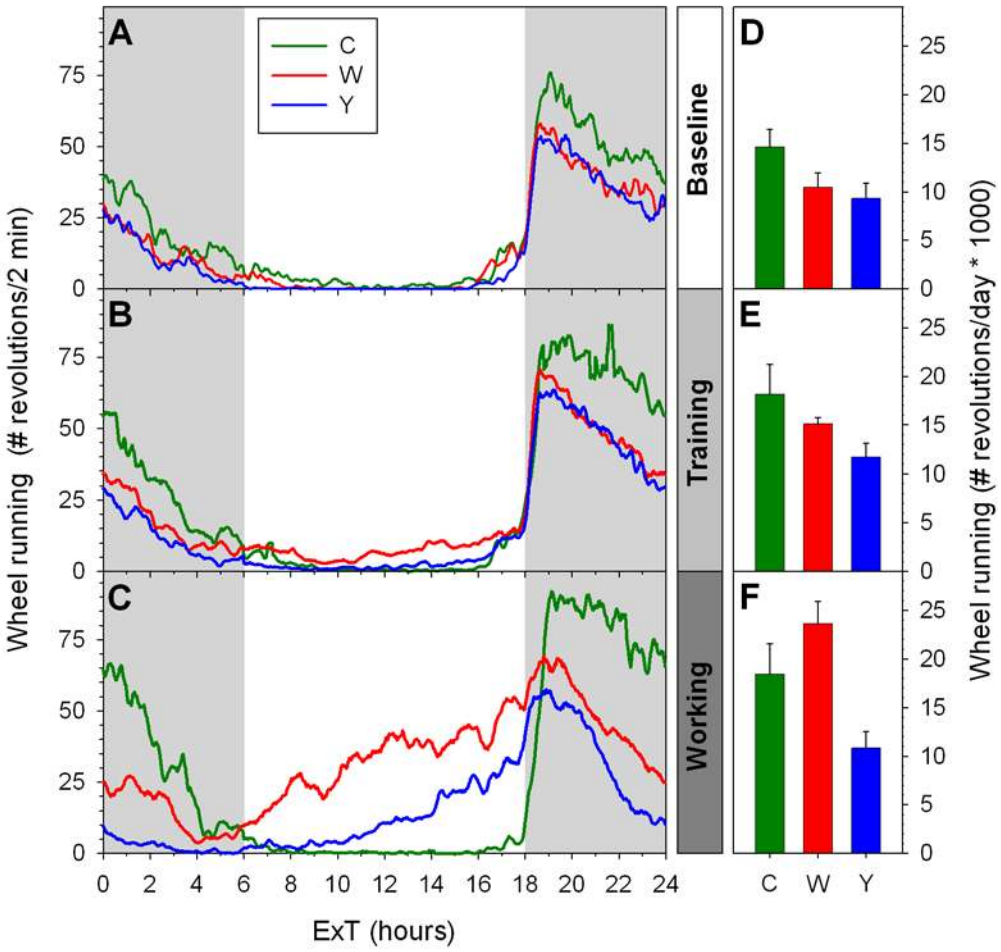

Figure 2 *Daily distribution of wheel running activity. The distribution of the average wheel running activity for each experimental group over de day during the baseline (1), the training phase (2) and the working phase (3) of the experiment. Time is indicated as External Time (ExT; (Daan et al. 2002)). The curves are based on running means with a window of 16 min for each individual, prior to averaging. The total activity in revolutions/day + standard error of the mean for the experimental phases is shown in panels D-F. C mice have green bars, W mice have red bars and Y mice have blue bars. W mice run significantly more than baseline during the training (t= 2.594; p = 0.012; n=11) and the working phase (t = 7.371; p < 0.001; n = 11). Both the W and the C group run more during the working phase than the yoked controls. (C: t = 2.632; p = 0.011; n = 11; W: t = 4.431; p < 0.001; n = 11), The W mice do not run significantly more than the C mice during phase 3 (working) (analysed by 2-way RM ANOVA with post hoc t- tests).*

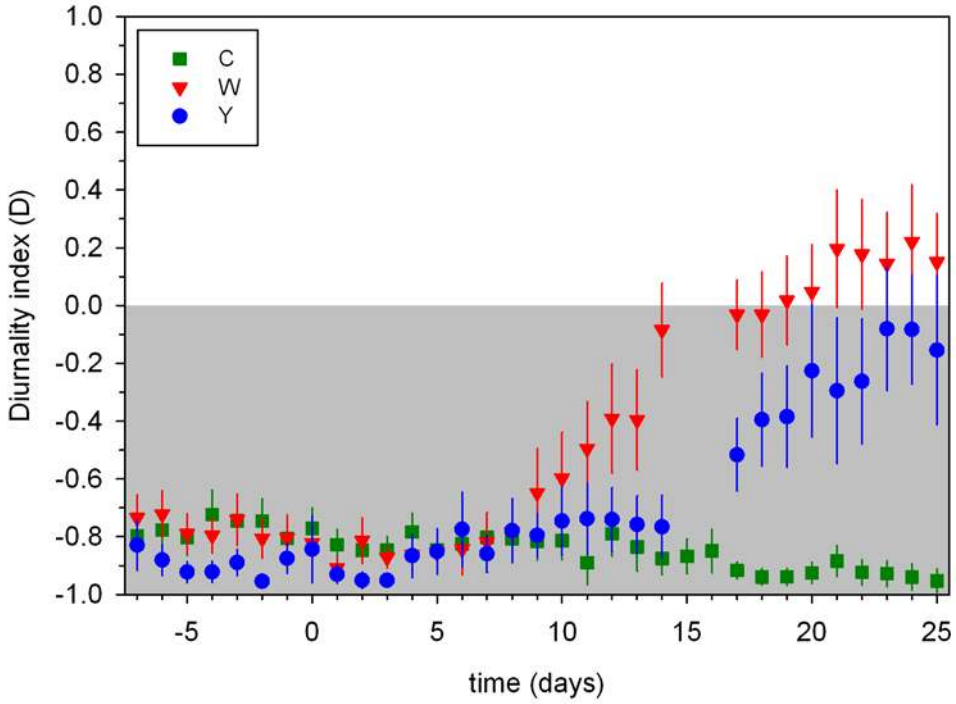

Figure 3 *Diurnality index. Average Diurnality index (D-index) calculated for the wheel running activity of the different experimental groups over time with standard errors of the mean. Days 15 and 16 are not plotted for the W and Y mice because of PLC failure.*

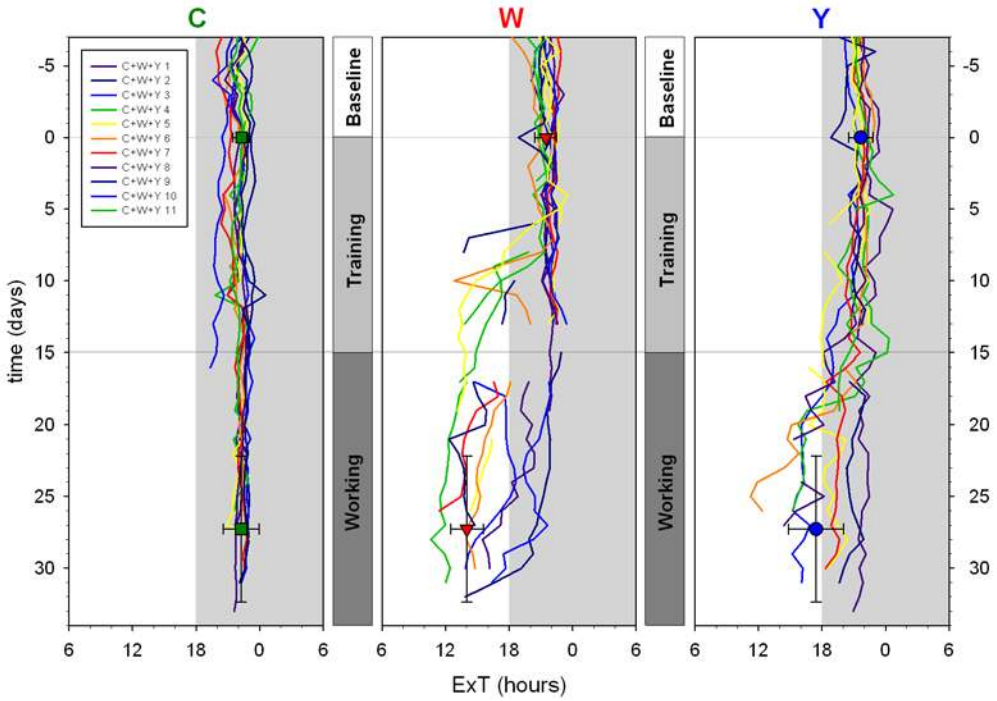

Figure 4 *Daily Center of gravity of wheel running activity. The daily center of gravities are plotted for individual mice of the three experimental groups (separate panels). The individuals are colour coded; each matched pair of W,Y and C mice have the same colour. Group averaged centerpoints of gravity at the start of the training (day 0) and at the time of sacrifice are also plotted. The vertical error bars represent the s.d. in the sacrifice time, horizontal bars the s.d. of the centerpoint of gravity.*

Body temperature

The working for food regime has a considerable impact on the regulation of body temperature in mice from both the W and the Y groups. Figure 5 shows examples of body temperature thermograms combined with actograms of the wheel running activity of a matched W-and Y pair. The temperature is colour coded, higher body temperatures are orange and lower body temperatures are blue. Peaks in body temperature follow the running wheel activity; if mice are running they have a high body temperature. When the W mice start working for their food (from day 0 onward) the amplitude of the body temperature rhythm becomes larger, the minimal body temperatures decrease.

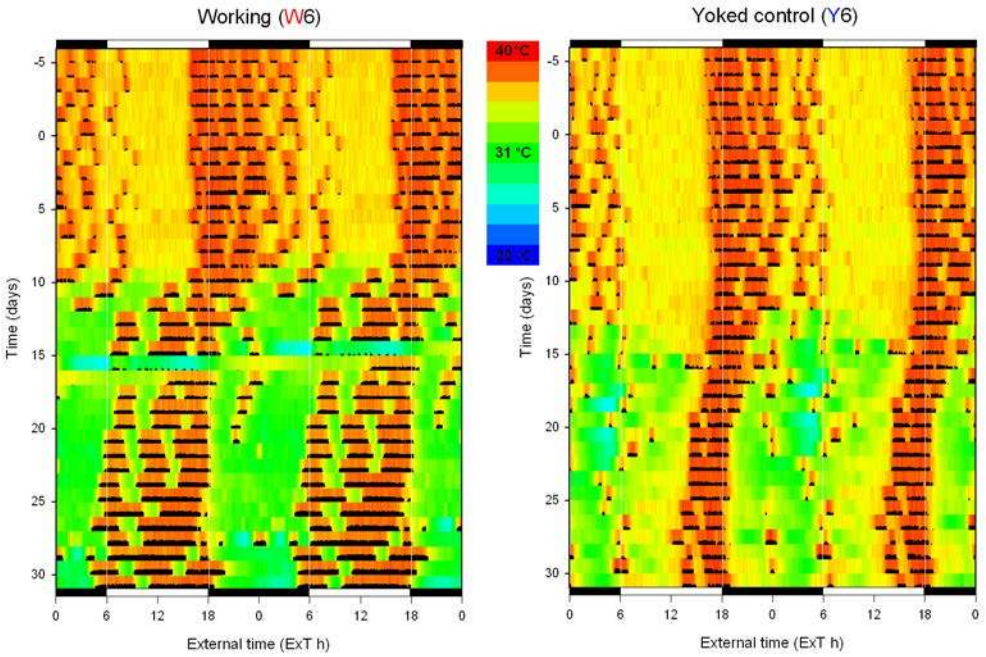

Figure 5 *Example of wheel running activity and body temperature during the experiment of a working mouse and its yoked control.Wheel running activity is double plotted for a working mouse (W6) in the left panel and for its yoked control (Y12) in the right panel. The height of the bars indicates the amount of activity. In the background body temperature for both individuals is plotted in colour. Note the huge effect of the PLC failure around days 15 and 16 on body temperature and activity of the W mouse.*

The decrease in minimal body temperature coincides with a phase advance of the activity rhythm (see Wl6 figure 5). At some point W mice started to show torpor bouts: episodes containing minimal body temperatures below 30 °C. Similar changes occurred in the yoked control mice, but the magnitude of the effects is smaller in this group of mice. Y mice started to phase advance later and the advance was smaller. The decreases in minimal body temperature were also less extreme in this group.

The effects on body temperature are summarized in in figure 6, which shows the average circadian body temperature profiles of the W (red) and Y (blue) mice during the baseline (panel A), training (panel B) and working (panel C) phases of the experiment. The differences between the W and Y mice

are small. During the baseline both the W and Y mice showed a circadian profile of an average body temperature of approximately 37 °C +/- 1. The peak of the body temperature was in the beginning of the dark phase (around Ext 19), coinciding with the peak in running wheel activity (see figure 2). The minimal body temperatures were reached in the end of the dark phase. During the training phase the W mice started to work for their food, earning approximately 3.8 g. of food per day (see fig 1). Both the W and the Y mice showed a body temperature profile that is overall on average 1 °C lower than during baseline, apart from the peak value around ExT 18:10. The W mice also started to show an additional drop in body temperature just before lights on. The amplitude of the body temperature rhythm increased. During the working phase the average body temperature curves of both groups were quite different from baseline. Body temperature started to rise earlier and the maximum of the body temperature was lower, especially in the W mice. Both the W and the Y mice showed a dramatic reduction in body temperature between ExT 02:00 and ExT 08:00 with a minimum at the end of the dark phase, between ExT 04:00 and ExT 06:00. The reduction of body temperature is caused by the occurrence of torpor (see figure 5 & table 1) in both the W and the Y mice. The reduction in body temperature was a little less pronounced in the Y mice.

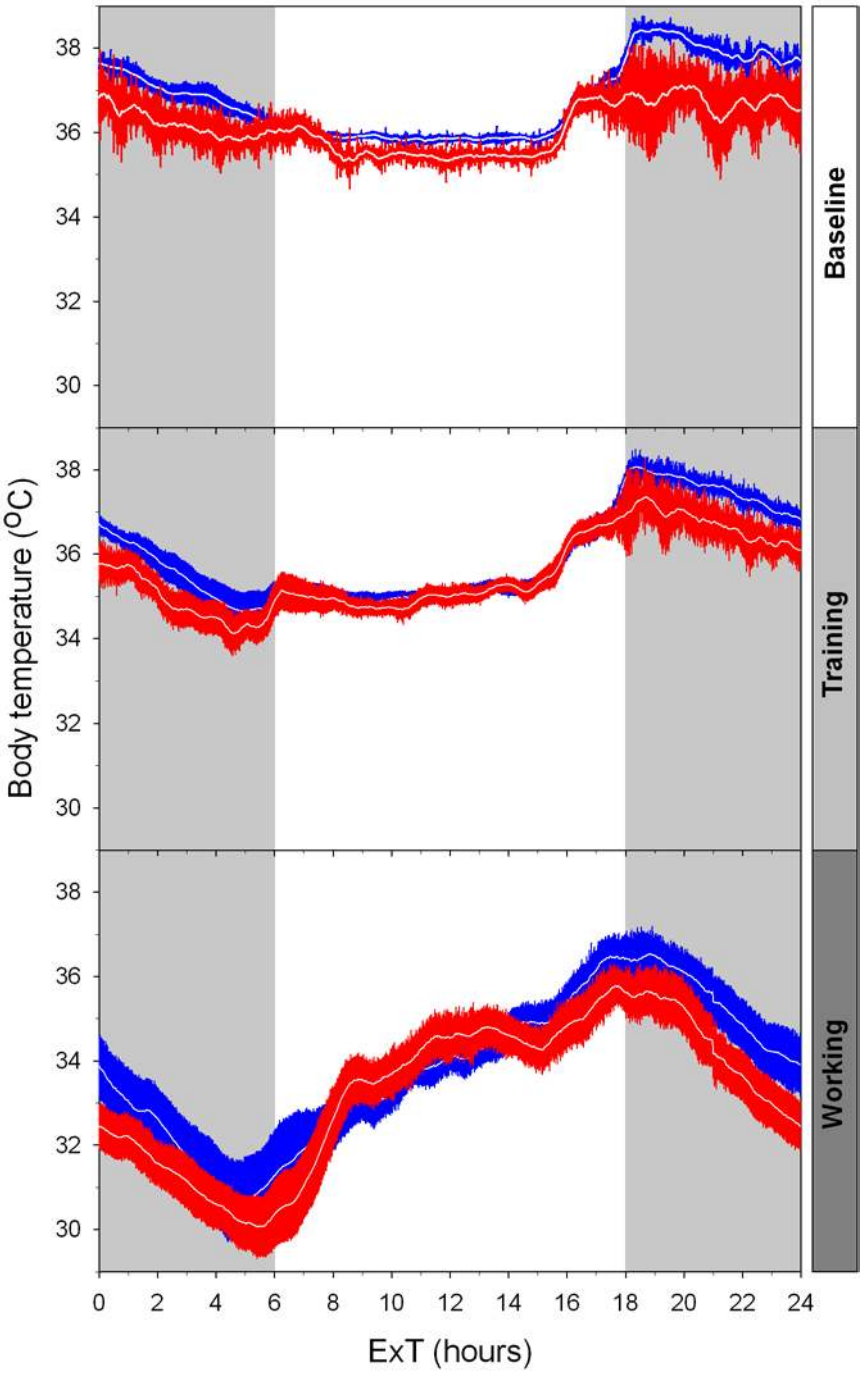

Figure 6 *Mean body temperatures (± s.e.m.) during the 3 phases for the W mice (red; n=11) and the Y mice (blue; n=11).*

Torpor

During the working schedule both the W and Y mice reduced their body temperature (see figure 5 & 6). Body temperature depressions with a minimum below 30 °C were arbitrarily defined as time in torpor. The number of days with torpor and the time spent in torpor were scored for all individuals. In the C mice no torpor was observed upon visual inspection of the mice. Also no torpor was observed during the baseline phase in the W and the Y mice. The results of the torpor scoring for the W and the Y mice during the training and the working phase of the experiment are presented in table 1. Torpor occurred in 9 W mice during training and in all W mice during the working phase. 9 Y mice showed torpor during the training phase and 9 Y mice showed torpor during the working phase. The W mice spent more time in torpor than the Y mice. Of the total time during training and working, the W mice spent on average 11.7% of the time in torpor and the Y mice spent significantly less at 8.3% of the time in torpor ($t = 2.371$, $p = 0.039$).

Tau hyperphosphorylation

The results of the analysis of cortex material of mice are shown in figure 7. The tau protein could be detected at two molecular weight ranges (panel A) in both euthermic (EU) and torpid (T) mice. There are no differences in the amount of tau protein present between the groups: neither at 55-61 kDa (One-Way-ANOVA, $F = 2.708$, $p = 0.126$; panel E) nor at 65-68 kDa (ANOVA on ranks, $H = 0.417$, $p = 0.573$, panel C). The tau proteins of lower molecular weight show a strong mobility shift towards a higher molecular weight in the torpid mice. This is possibly due to additional phosphorylation of the tau protein in torpor. The 65-68 kDa tau always shows AT8 hyperphosphorylation (panel B). Phosphorylation at this weight is not different between euthermic and cold mice (One-Way-ANOVA, $F = 0.0021$, $p > 0.1$; panel D). Mice in torpor do have increased hyperphosphorylation in the brain, but only on the tau proteins with a molecular weight between 55-61 kDa. The AT8 hyperphosphorylation is approximately 2 times higher in the torpid mice (Kruskal-Wallis One Way ANOVA, $H = 4.267$, $p = 0.043$; panel F).

Table 1 *Incidence of torpor in WFF induced food shortage.*

mouse id		# of days		# days with torpor				torpor (hours)				% time torpid	
		training	working	training		working		training		working			
W	Y			W	Y	W	Y	W	Y	W	Y	W	Y
1	11	15	11	0	0	11	0	0.0	0.0	81.4	0.0	10.0	0.0
2	14	15	17	1	0	17	14	4.9	0.0	85.8	103.2	12.2	13.9
6	12	15	7	1	1	7	6	8.8	4.4	53.1	29.1	8.1	4.4
7	5	15	10	1	1	10	12	5.3	6.0	66.0	102.9	10.6	16.2
9	4	15	8	2	4	8	3	25.7	23.6	66.8	9.5	12.0	4.3
13	23	15	9	1	1	9	7	8.8	7.2	91.3	62.6	15.4	10.8
15	21	15	9	1	1	9	8	8.6	3.0	69.8	37.4	10.9	5.6
16	17	15	11	3	2	11	11	41.2	11.1	100.7	100.5	21.1	16.6
24	3	15	14	1	1	14	2	5.7	6.9	80.1	15.0	10.8	2.8
37	38	15	10	3	6	10	8	13.7	27.5	69.9	46.5	13.9	12.3
40	39	15	3	0	3	3	0	0.0	22.1	16.2	0.0	3.4	4.6
avg		15.0	9.9	1.3	1.8	9.9	6.5	11.2	10.2	71.0	46.1	11.7	8.3
stdev		0.0	3.6	1.0	1.8	3.6	4.8	12.2	9.7	22.5	40.8	4.4	5.8
se		0	1.1	0.3	0.6	1.1	1.4	3.7	2.9	6.8	12.3	1.3	1.7
t												*2.371*	
p												*0.039*	

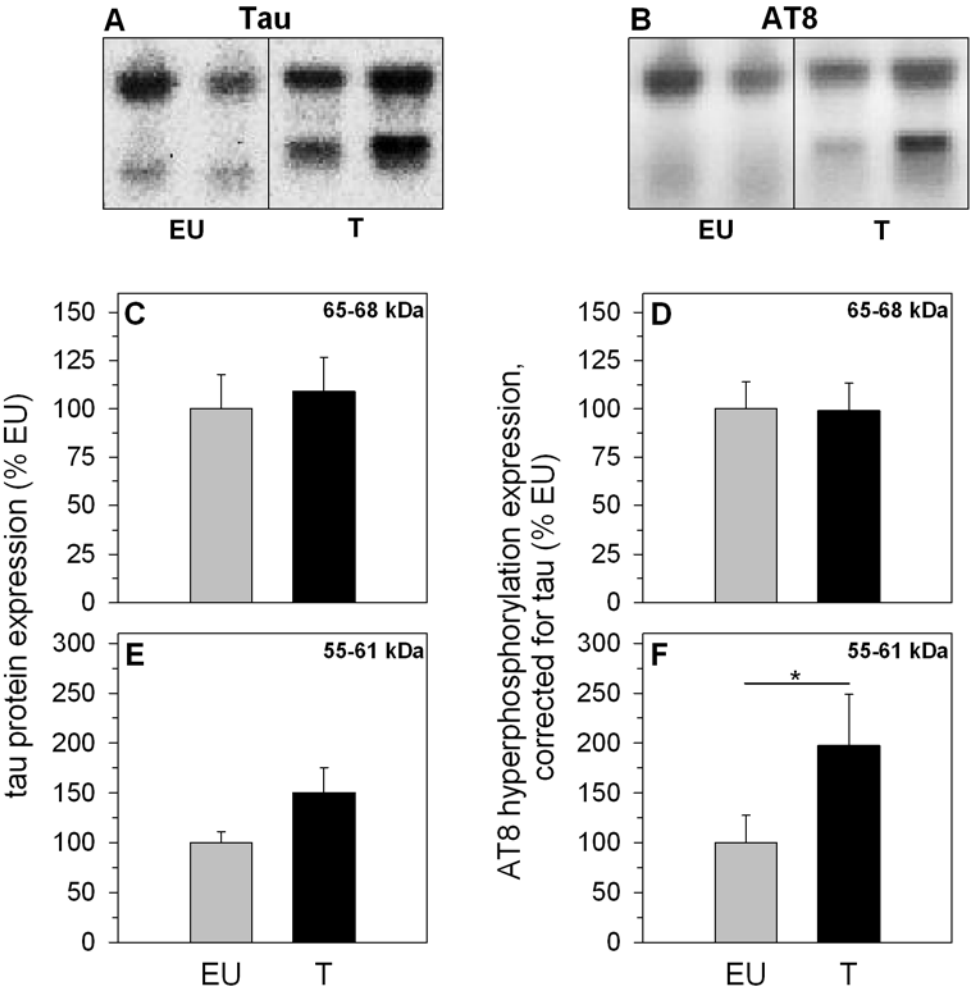

Figure 7 *Representative examples western blot examples of euthermic (EU) and torpid mice (T). Immunodetection was done with Tau (panel A), and AT8 (panel B). Panels C-F show the results of the OD analysis of the amount of Tau expression (C,E) and the amount of serine 201 / Threonine 205 (AT8) hyperphosphorylation on the tau protein (D,F). The analysis was separately done for two molecular weight ranges: 65-68 kDA (C, D) and 55-61 kDa (E, F). OD values were averaged (+ s.e.m.) and plotted for all mice sampled in euthermia (n=6, red bars) and mice sampled in torpor (n=8, blue bars). OD values were expressed as a percentage of the average euthermic OD. The * denotes a significant difference in AT8 hyperphosphorylation between the euthermic and torpid mice.*

Discussion

Circadian rhythms

Our data (figure 3) confirm that male CD-1 mice that have to work for their food gradually become diurnal (Hut et al. 2011). When confronted with an increased foraging effort (WFF): the W mice shift most of their activity in to the day, resulting in a D-index > 0, even though the peak in activity remains at approximately the same position just after lights off, at ExT 19:00. This is consistent with results obtained using a similar WFF protocol in male CBA/CaJ mice (Hut et al. 2011). WWF also induces torpor as was previously observed in male CD-1 mice (Schubert et al. 2010) and CBA/CaJ mice (Hut et al. 2011).

Hut et al. (2011) showed that diurnal activity in mice working for their food depends on the voluntary food intake by wheel running and not on meal timing as in studies manipulating meal timing and food availability simultaneously (Mendoza et al. 2007). Schubert et al. (2010) showed that the frequency and intensity of torpor depend on the workload (reward ratio) in mice. Both studies did not discriminate between effects of the workload itself and of the reduced food intake it entailed.

In our study the yoked control mice showed similar effects on body temperature and activity as the working mice. The Y mice also phase advanced their activity and became more diurnal resulting in a D-index of close to 0 (figure 3). Torpor was also observed in the Y mice. The circadian temperature profiles of the W and the Y mice are also similar under all conditions (figure 6). The Y mice do not have to work for their food and run much less in their wheels than the W mice (average for Y: 10840 rev/day; for W: 23631 rev/day in phase 3), but had exactly the same food (on average 3.81 g/day (SD 0.58)). Thus the results clearly show that food shortage, not the workload induces the torpor in mice. We have to be aware that the W mice had no external timing signal inducing the shift of activity into the day, while the Y mice received food in a daily pattern generated by the W mice. Hence, while the incidence of torpor in the Y mice is a consequence of reduced food supply, the increased diurnality of the Y mice can be attributed either to this feeding schedule, or to food restriction, or to both.

Incidence of torpor

Although the experimental effects on body temperature and activity are similar between the W and Y mice, the effects were slightly smaller in the Y mice. Y mice phase advanced later and to a lesser extent (CoG Y: -3.1 hrs. vs. CoG W:-7.2 hrs.; figure 4). Y mice showed less diurnal activity (figure 3). The amplitude of the circadian body temperature oscillation was slightly smaller (figure 6). The incidence of torpor was lower in the Y mice (Y: 8.3% vs. W: 11.7% of the time) (table 1), which is consistent with the observation that male CD-1 mice show more torpor with increasing workload (Schubert et al. 2010). The Y mice did not lose as much weight as the W mice and the average body mass was almost stable during days 20-25. Because the Y mice were less active than the W mice they presumably also spent less energy. This suggests that not only food intake matters, but the total energy budget is of importance for the strength of the effects. The Y-mice are confronted with food shortage but are partly able to compensate by spending less energy in activity. Therefore the Y mice phase advanced their activity later than and not as much as the W mice. As soon as the Y mice started to reduce body temperature and show torpor the Y mice seem to reach a state of energy balance, since the average body mass stabilized.

Tau hyperphosphorylation

Food shortage induced torpor in mice was associated with hyperphosphorylation of forms of the tau protein at lower molecular weights (55-61 kDa). This is different from the hyperphosphorylation observed in ground squirrels (Arendt et al. 2003), Syrian hamsters (Härtig et al. 2007) and Djungarian hamsters (Boerema et al. 2008a) during torpor, which was most prominently found at a higher (~70 kDa) molecular weight. Other studies in non-transgenic mice have found tau hyperphosphorylation at the lower molecular weight range, after starvation (Yanagisawa et al. 1999) or after Cold water induced stress (CWS) (Okawa et al. 2003). Anaesthesia associated hypothermia also causes tau hyperphosphorylation in mice (Planel et al. 2007). Hypothermia during torpor causes tau hyperphosphorylation in the brains of hibernators and mice, although there are some species differences in which forms of the tau protein are most affected.

In conclusion, the WFF paradigm proves to be a valuable tool to study the circadian behaviour in relation to metabolism in mice. Food shortage itself causes the induction of torpor in mice. This is aggravated when the animals have to spend energy in working for their food, a situation which simultaneously elicits the shifts of activity from night to day. Torpor in mice is further associated with tau protein hyperphosphorylation, which makes mice a suitable species to study its regulation in a normal physiological context.

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Chapter 9

Reversible tau hyperphosphorylation in 5'-AMP-induced hypothermia in mice.

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Abstract

Neurofibrillar tangles, resulting from hyperphosphorylation of the tau protein, are a hallmark of Alzheimer's disease. Animals in deep hibernation and in daily torpor, both forms of naturally occurring hypothermia, show tau hyperphosphorylation. This process is fully reversible during rewarming to euthermy. A state of torpor-like hypothermia can be pharmacologically induced in mice by suppressing metabolism with 5'-AMP. In this study we focus on tau hyperphosphorylation in mouse brains after inducing torpor-like hypothermia in mice at ambient temperatures of 21 °C and 30 °C. 11 Mice were sacrificed during torpor-like hypothermia and 11 after spontaneous rewarming to euthermy at an ambient temperature of 21 °C. 6 Additional animals were sacrificed during torpor induced at an ambient temperature of 30°C. The brains were analyzed by immunocytochemistry (21 °C animals) and immunodetection of westernblots (21 °C and 30 °C animals). Tau hyperphosphorylation was indicated using AT8 (ICC and westernblot) and AT100 (westernblot) antibodies. AT8 and AT100 immunoreactivity was present in brains of torpid mice at a T_a of 21 °C. Hyperphosphorylation was reversed in brains of euthermic mice sacrificed after torpor at T_a 21 °C. In the cortex of torpid mice at T_a 30 °C, hyperphosphorylation could not be detected with the AT8 antibody, but was detected with the AT100 antibody. The areas most affected in the ICC brains included the stratum lucidum of the hippocampus and layers I-VI of the cortex. The results show that, as in natural torpor, also 5'-AMP induced torpor-like hypothermia leads to reversible tau hyperphosphorylation. The accessibility of this mouse model makes it a valuable tool to study the mechanisms of Alzheimer associated PHF-like tau phosphorylation *in vivo*.

Introduction

Alzheimer's disease (AD) is the primary cause of senile dementia. AD is characterized by two brain abnormalities: formation of plaques consisting of amyloid-beta peptide (Braak and Braak 1991b) and intracellular neurofibrillar tangles consisting of Paired-Helical Filaments (PHF's) of hyperphosphorylated tau protein (Goedert et al. 1991).

Tau is mainly present in neurons and plays a role in microtubule stabilization and proliferation (Weingarten et al. 1975; Drubin and Kirschner 1986). The binding capacity of tau to microtubules is affected by its phosphorylation state, influencing the ability for microtubule assembly (Jameson et al. 1980). It is hypothesized that tau is involved in the regulation of axonal transport (Ebneth et al. 1998; Dixit et al. 2008).

Hyperphosphorylation of tau can cause destabilization of microtubules and may lead to self-aggregation of the protein and formation of paired helical filaments in AD (Goedert et al. 1989b; Haase et al. 2004; Iqbal et al. 2009). The processes that lead to tau phosphorylation and its link to neurodegeneration are not well understood (Geschwind 2003). There is a need for good physiological animal models to study the mechanisms of tau hyperphosphorylation and its natural reversal *in vivo*. Such a model has the potential of studying all elements of naturally occurring tau hyperphosphorylation. The phenomenon of natural mammalian hypothermia offers such a potential study system (Arendt et al. 2003).

Mammalian hibernation is one of the natural adaptations to seasonal food shortage. Hibernating animals reduce their metabolic rate by actively suppressing metabolism, resulting in a state of torpor (Heldmaier and Ruf 1992; Heldmaier et al. 2004). Hibernators, such as European ground squirrels (*Spermophilus citellus*) and Syrian hamsters (*Mesocricetus auratus*), express hyperphosphorylated tau during torpor, which is reversed when the animals return to euthermy (Arendt et al. 2003; Härtig et al. 2007; Stieler et al. 2008; Su et al. 2008; Stieler et al. 2011).

Several small mammals, such as Djungarian hamsters (*Phodopus sungorus*), use daily torpor to balance their energy budget (Song et al. 1995; Heldmaier et al. 2004). Mice (*Mus musculus*) are also able to do this when they have to work for their food (Schubert et al. 2010; Hut et al. 2011), or as a reaction to fasting (Hudson and Scott 1979; Webb et al. 1982; Dikic 2008). Metabolic suppression in daily torpor is limited compared to deep torpor and body temperatures are not allowed to drop below approximately 12°C. Reversible tau hyperphosphorylation has nevertheless also been observed during daily torpor in Djungarian hamsters (Boerema et al. 2008a). Tau hyperphosphorylation was also present during workload induced torpor in mice (Boerema et al, chapter 8, this thesis).

A state of torpor can be induced in mice by pharmacological means (see (Bouma et al. 2011a) for a recent overview). Administration of H_2S (Blackstone et al. 2005), or adenosine 5'-monophosphate (5'-AMP) (Zhang et al. 2006), lead to metabolic suppression followed by body temperature reduction in mice, mimicking the torpid state of mice and other species. It has been argued that 5'-AMP is a natural substance involved in the induction of torpor, because the 5'-AMP level is elevated in mice entering torpor upon fasting (Zhang et al. 2006; Lee 2008). Whether AMP is indeed involved in the regulation of natural torpor is still under debate (Swoap et al. 2007; Swoap 2008). Experimental 5'-AMP administration in mice is nonetheless useful to assess metabolic and temperature effects on the brain.

In this study we examined if 5'-AMP-induced hypothermia leads to tau hyperphosphorylation and whether this process is reversible. We have further explored effects of temperature on tau hyperphosphorylation by using two different ambient temperatures, allowing for cold (21 °C) and near euthermic situations (30 °C).

Methods

Animals

Male C57bl/6J mice (age: 4 weeks; n=28) were purchased from Harlan (Horst, the Netherlands). Mice were 20 weeks old and weighed 25.7 g (SD 1.6) at the start of the experiment. Prior to the experiment they were kept on a 12:12 light/dark cycle, at an ambient temperature of 21 (+/- 1) ˚C. Mice were housed individually on sawdust bedding with nesting material (Enviro-dri®, Shepherd specialty papers, USA). Water and food (RMHB 2181, Arie Blok, Woerden, the Netherlands) were available *ad libitum* throughout. The experiments were approved by the animal experimental ethics committee of the University of Groningen under license number DEC-5114B.

Telemetry

Body temperature and activity were measured using telemetry. TA-F20 transmitters (Data Sciences International, St. Paul, USA) were surgically implanted in the abdominal cavity prior to the experiments. Mice were allowed to recover for two weeks. Following recovery, one week was used to collect baseline recordings of body mass, activity patterns and body temperatures. Temperature signals were acquired in 1 minute intervals and activity was summed in 1 minute bins, using Dataquest 4 software (Data Sciences International, St. Paul, USA).

Torpor induction

Torpor is the behavioural state of homeotherms characterized by inactivity and low reactivity, as the consequence of metabolic suppression (Heldmaier and Ruf 1992; Strijkstra 2009). We induced torpor-like hypothermia in mice by intraperitoneal (i.p.) injection of 7.5 µmol/g adenosine 5'-monophosphate disodium salt (5'-AMP), obtained from Sigma Aldrich (Sigma, St Louis, USA) and dissolved in sterile saline to a concentration of 1.2M. Just before the injections, stock solution was further diluted with sterile saline to a final concentration of 0.75 M. This procedure resulted in torpor bouts of approximately 4 h at T_a = 21°C and 1.5 h at T_a = 30°C (Strijkstra, personal communication). Injections were given approximately 1 h after lights-on in the inactive phase of the animals.

Sacrifice

Animals were sacrificed during or after 5'-AMP-induced torpor. 11 Mice were sacrificed during torpor-like hypothermia at T_a = 21°C on average 240 min (SD 6.9) after 5'-AMP injection (T21; group 1). Average core body temperature (T_b) at that time was 22.7°C (SD 0.6). A further 11 animals were sacrificed during euthermia following torpor-like hypothermia, on average 576 min. (SD 13.2) after

injection at T_a = 21°C (EU; group 2). These mice had been hypothermic for on average 237 min (SD 24.9), and had been rewarming for 112 min. (SD 47.8). By the time of sacrifice, they had reached euthermy (as defined as higher than 2 standard deviations below the average of the 24h baseline T_b) (34.3 ± 0.5 °C) for 227 min (SD 59). Body temperature at the time of sacrifice was 36.0°C (SD 0.5). In six mice torpor-like hypothermia was induced at an ambient temperature of 30 °C (T30, group 3). These mice were sacrificed during torpor, 75.5 min (SD 23.97) after the 5'-AMP injection, because at this temperature torpor-like hypothermia is shorter and lasts about 1.5 h Body temperature of these mice at the time of sacrifice was on average 31.7°C (SD 1.3).

Western blotting

The 18 frozen brains were cut coronally on a freezing microtome into thick (~1.5 mm) slices. From these slices, on average 22 mg cortex material was dissected out on ice. Tissue was homogenized in ice-cold lysis buffer, containing [20 mM Tris-HCl, 2 mM MgCl2, 150 mM NaCl, 5 mM NaF, 1 mM Na3VO4, 1% NP-40, with Roche complete mini protease inhibitors and Roche phosstop® phosphatase inhibitors (Roche, Woerden, the Netherlands)]. Lysis buffer was added in a 1:9 tissue to buffer ratio. Lysates were centrifuged at 20841 *g* for 30 min at 4°C. Clear supernatant was removed and protein concentrations were determined by Bradford assay. Protein concentrations were standardized to 2.5 mg/ml by adding ice-cold lysis buffer and 5x Laemmli-buffer.

Proteins were separated on 4-20% gradient Nu Page Novex Bis-Tris mini gels (Invitrogen, Breda, the Netherlands) using 57µg of total protein per well and subsequently transferred to an 0.2µm PVDF-membrane, using the I-blot dry blotting system (Invitrogen, Breda, the Netherlands). Membranes were blocked for 1 h with blocking buffer (0.05% Tween-20, 2.0% BSA in TBS (pH 8.0). Additionally 5mM Na_3VO_4, 50 mM NaF and Phosstop were added to all buffers in order to maximize the inhibition of phosphatases also during washing, blocking and antibody incubation steps (Sharma and Carew 2002).

Immunodetection of western blots

The same membrane was subsequently probed with several antibodies (see table 1). The membrane was stripped between the applications of different antibodies. Immunodetection was performed by incubating the membrane overnight at 5 °C, with the primary antibody, diluted in blocking buffer (see table 1 for dilutions). Detection of bound primary antibodies was performed with either goat-anti-mouse (SC-2005) IgG-HRP (Santa Cruz), diluted 1:10000 in blocking buffer (Tau, AT8, AT100, Tau-1), or donkey-anti-rabbit (Amersham) for Beta-tubulin-III, diluted 1:15000 in blocking buffer. After washing the membrane in washing buffer, detection of bound secondary antibody was done by Enhanced chemiluminescense, using Super Signal West Dura (Pierce, Rockford, IL, USA) diluted 1:1 in TBS (pH 8.0) in a Biorad Chemidoc XRS+ imaging system. As a positive control brain material of a torpid Syrian hamster was included in each gel. Prior to each probe the membrane was stripped of bound antibodies by 2 times 15 minutes incubation in stripping buffer (24mM glycine + 1.25 % SDS + 0.1% Tween-20 in 50mM Tris, (pH 2.0) heated to 60 °C prior to use, followed by thorough rinsing in washing buffer.

Optical density analysis of western blots

The tau-protein and hyperphosphorylation of the tau-protein were examined by western blotting of cortex material of 5 mice from each group, 1 mouse from each group was omitted from the analysis because of artifacts that prevented proper quantification of the signal. Optical density of the bands on the membranes was analyzed with the image software provided with the Chemidoc system (Biorad Imagelab, version 3.0 beta). The β-tubulin-3 signal was first normalized by dividing the signal in each band through the average of all 15 bands included in the analysis. Subsequently the Tau, AT8, AT100 and Tau-1 OD values were corrected for the amount of loaded protein by dividing them through the normalized β-tubulin-III values. Because of the mobility shift observed in the tau protein expression between group 1+3 and group 2, the OD analysis was performed in two ranges of molecular weights: high molecular weight tau species from ~65 kDa - ~68 kDa and lower molecular weight tau species from ~55 kDa - ~61 kDa. The AT8, AT100 and Tau-1 OD values were further corrected for the amount of Tau protein present by dividing the signal by the total normalized tau expression within the molecular weight range analyzed. This allowed us to distinguish differences in hyperphosphorylation

from changes in total amount of tau protein. For total Tau, the expression at 65 kDa was separately analyzed.

All OD-values were subsequently expressed as a percentage of the average signal of the euthermic control animals (Group 1, EU) and square-root transformed to correct for non-normal distribution of the data. Two-way ANOVA's with experimental group and molecular weight range as factors (Tau, AT8, AT100 and Tau-1 OD analysis) and a one-way ANOVA (mobility shift of 58 kDA tau analysis) with Student-Newman-Keuls post-hoc testing were used to test for significant differences.

Table 1 *Antibodies and dilutions used for the detection of the protein tau independent of phosphorylation state and the hyperphosphorylation of tau (wb = applied in immunodetection of western blots; icc = applied in immunocytochemistry).*

antibody	Supplier	Target	Dilution	Secondary antibody	Supplier	dilution
AT8 *MN1020*	PerBio	Tau protein phosphorylated at Serine202 & Threonine 205	1:1000 (icc)	goat-anti-mouse IgG Biotin SP, *#115-065-166*	Jackson	1:500
			1:1000 (wb)	goat-anti-mouse IgG-HRP, *SC-2005*	Santa Cruz	1:10000
AT100 *MN1060*	PerBio	Tau protein phosphorylated at Serine214 & Threonine 212	1:1000 (wb)	goat-anti-mouse IgG-HRP, *SC-2005*	Santa Cruz	1:10000
Tau-1 *MAB3420*	Chemicon	Tau protein not phosphorylated at part of the AT8 epitope	1:1000 (wb)	goat-anti-mouse IgG-HRP, *SC-2005*	Santa Cruz	1:10000
Tau *clone DC 25, T8201*	Sigma-Aldrich	Total protein tau, irrespective of phosphorylation state	1:5000 (wb)	goat-anti-mouse IgG-HRP, *SC-2005*	Santa Cruz	1:10000
B-Tubulin-3 *T8578*	Sigma-Aldrich,	β-Tubulin	1:1500 (wb)	donkey-anti-rabbit *NA934*	Amersham	1:15000

Results

Body temperature profiles

5'-AMP injections (7.5 µmol/g) led to hypothermia in all mice. A representative example of the difference between the average baseline body temperature and the body temperature during the induced torpor-like hypothermia bout is shown in figure 1. Torpor-like hypothermia was induced in the inactive phase of the mice, 1 hour after lights on (at ExT 7).

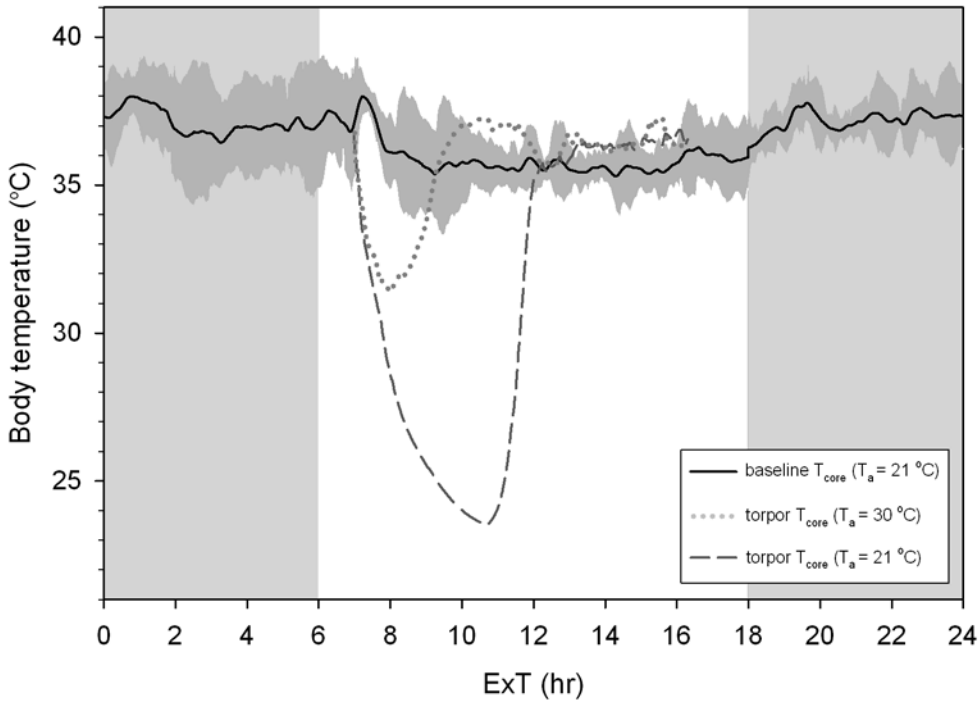

Figure 1 *Representative example of daily baseline body temperature of a mouse (5 days average, smoothed with a 10 min running mean; black line). The dark grey shaded area indicates 2 times the standard deviation of the average body temperature, which was used to define the return to euthermia after induced torpor-like hypothermia. Body temperature of the same individual during the 5'-AMP-induced torpor-like hypothermia at Ta = 21 °C is plotted in blue. Body temperature of a representative individual during 5'-AMP-induced torpor-like hypothermia at Ta = 30 °C is plotted in red. The light dark cycle is indicated in light grey. Time is indicated as External Time (ExT=0 is midnight; (Daan et al. 2002)). 5'-AMP was always given 1 hour following lights on.*

Figure 2A shows the averaged body temperature curves after 5'AMP injection at T_a = 21 °C. The data are synchronized at the time point of injection. Mice housed at an ambient temperature of 21 °C (groups 1 and 2) reached their lowest body temperature of on average 23.0 °C (SD 0.6) on average 238

min (SD 18) after the injection. The average rate of cooling, determined over the time interval between the 5'AMP injection and the lowest body temperature was 0.06 °C/min (SD 0.005).

Figure 2B shows the rewarming curve for the mice in group 2, synchronized to the onset of rewarming. After spontaneous rewarming, mice reached euthermic temperatures on average 349 min (SD 67) after injection. The rate of rewarming was determined over the time interval between the lowest body temperature and the body temperature two standard deviations lower than baseline body temperature. The rewarming rate in the animals of group 2 was on average 0.1 °C /min (SD 0.03).

The mice housed at an ambient temperature of 30 °C (group 3) reached their lowest body temperature of on average 31.7 °C (SD 1.3) 75.5 min. (SD 23.97) after the injection. These animals cooled at approximately the same rate (0.065 °C /min) as the mice housed at T_a 21 °C. The total time spent in hypothermia of the mice in group 3 was significantly lower than in the mice housed at 21 °C (groups 1 + 2), (75.5 min vs. 238 min, $p<0.01$).

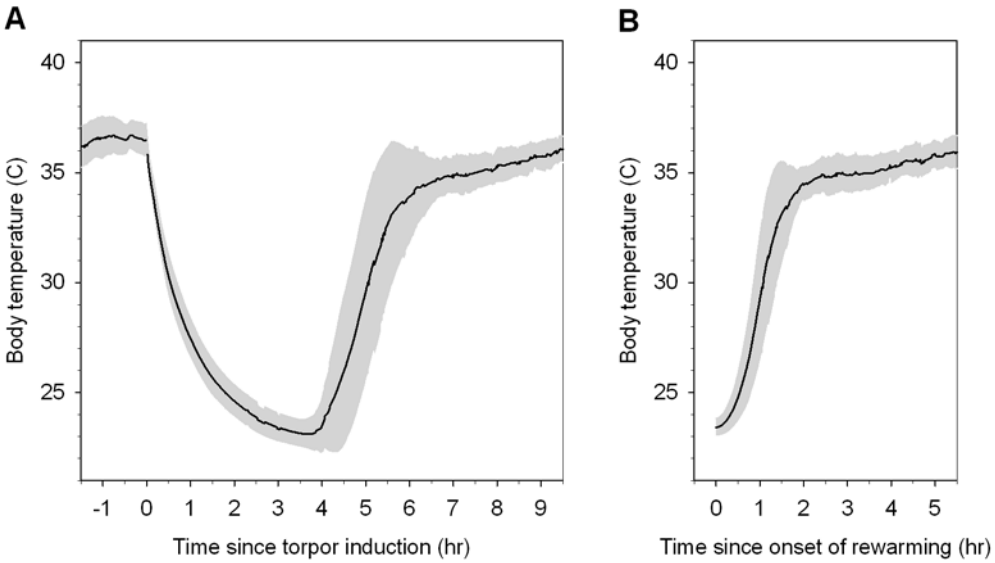

Figure 2 *Panel A: average body temperature with the standard deviation around the mean (shaded area) of all mice in response to 5'-AMP injections at an ambient temperature of 21 °C. Injections were done at t=0. During the cooling all animals (n=22) are included in the average. The rewarming part of the curve shows only the animals not sampled in torpor-like hypothermia (n=11). The average cooling rate of the animals was 0.06 °C/min. (SD 0.005). Panel B: average body temperature of the 11 animals allowed to return to euthermia. The onset of rewarming (t=0) was defined as the first time point after the minimum in body temperature during torpor-like hypothermia. The average rewarming rate was 0.1 °C/min (SD 0.03).*

Immunocytochemistry: AT8

Figure 3A shows immunocytochemical (ICC) staining of phosphorylated tau by the AT8 antibody in coronal sections including hippocampal and cortical regions. During 5'-AMP-induced torpor at an ambient temperature of 21 °C, AT8 staining is detected at several locations in the brain. The tau protein in the cortex, hypothalamus and hippocampus appears to be hyperphosphorylated during torpor, while the thalamus is devoid of staining in the mice of group 1 (Figure 3A).

The positive AT8 immunoreactivity is almost completely reversed after spontaneous rewarming to euthermia, following the 5'-AMP-induced torpor-like hypothermia in the mice of group 2 (Figure 3D). Panels B and E show detailed views of the mossy fiber system in the stratum lucidum of the hippocampus at 100x magnification during torpor-like hypothermia (B) and during euthermia following torpor-like hypothermia (E). Panels E and F show detailed views of Layers I-III in the motor cortex at 200x magnification during torpor-like hypothermia (C) and subsequent euthermia (F). A clear pattern of hyperphosphorylated tau during induced torpor-like hypothermia emerges for the hippocampal and cortical areas, and many other areas in the brain. Within the hippocampus, especially the immunoreactivity in the mossy fiber system in the stratum lucidum appears to be high. The staining in this area has also completely disappeared after rewarming from torpor-like hypothermia.

Figure 3I shows the optical density of the AT8 staining in layer I-III of the cortex in 5'-AMP-induced torpor-like hypothermia and after rewarming to euthermia. A significant difference in optical density between cortical optical density between the mice in group 1 (EU) and group 2 (T21) (Mann-Whitney-U test: $U_A = 25$; $z = -2.51$; $p = 0.006$) was found. The difference in optical density between mice sampled in 5'-AMP-induced torpor-like hypothermia and mice sampled after rewarming to euthermia in the stratum lucidum appears even larger (Mann-Whitney-U test: $U_A = 90$; $z = -3.63$; $p < 0.001$). The included negative controls (omission of 1^{st} and 2^{nd} antibody) did not show specific staining.

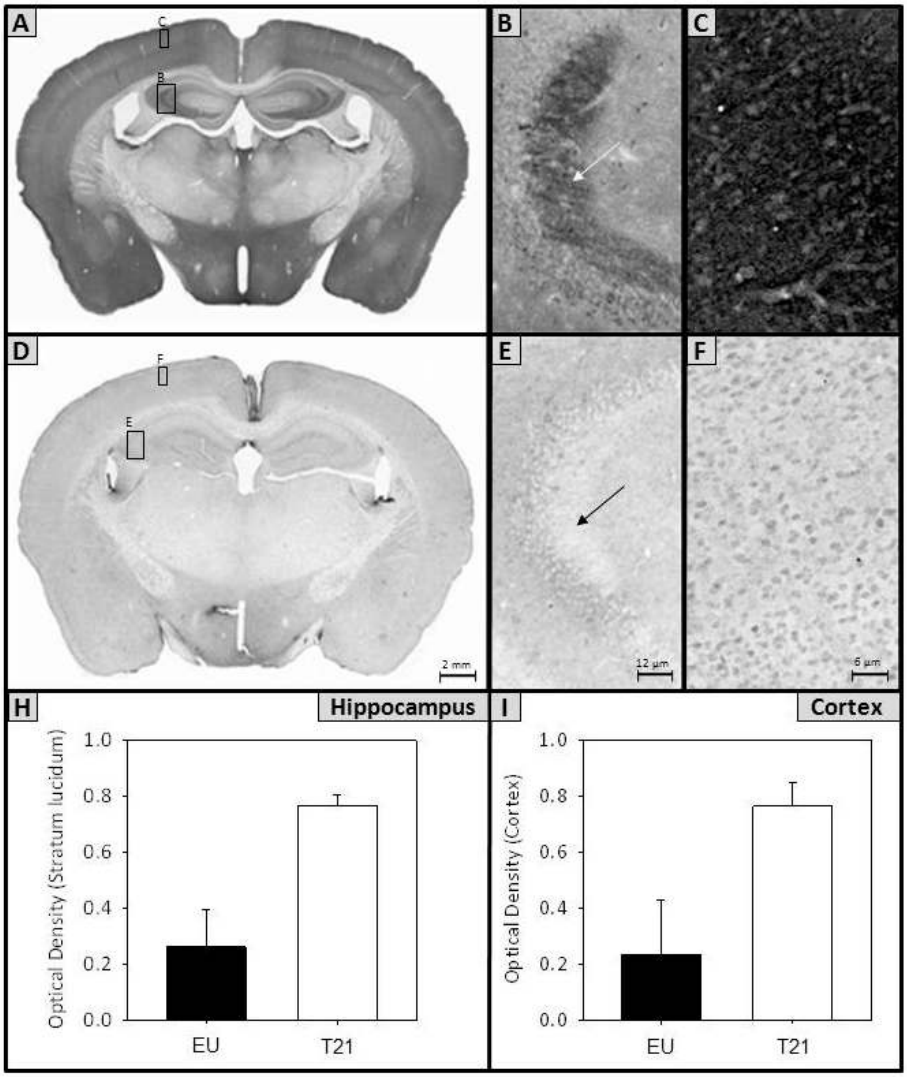

Figure 3 *Immunocytochemical detection of phosphorylated tau with the monoclonal antibody AT8 in 5'AMP-induced torpor-like hypothermia (panels A,B,C) compared to arousal after 5'-AMP induced torpor-like hypothermia at 21 °C (panels D,E,F). The cortex (C) and the hippocampus (B) show heavy AT8 staining. Staining in the hippocampus (E) and the cortex (panel F) almost disappears after rewarming from torpor-like hypothermia. Note the difference in the stratum lucidum of the CA3 area of the hippocampus (arrows in panels B & E). Panels G, H: average optical density + s.e.m. in regions of interest in the stratum lucidum of hippocampus and in the cortex. Completely black is set to 1 and the average background staining in the thalamus is set to 0. Significant differences in optical density were found between torpor-like hypothermia and euthermic animals in both stratum lucidum and cortex (Mann Whitney U: stratum lucidum UA = 90, z = -3.63, p <0.001, cortex UA = 25, z = -2.51, p = 0.006, group 1 (EU): n=5; group 2 (T21): n = 5).*

Western blotting

Representative examples of the immunodetection for total Tau, AT8, AT100 and Tau-1, with their β-tubulin-III expression for all 3 groups of mice are shown in figure 4. The tau protein is always detected around 55-61 kDA and around 68 kDa simultaneously in all three experimental groups. A third band representing the tau protein at approximately 65 kDa, is only present in the mice of group 2 (T21) (figure 4, top left panel). In the mice of group 2 (T21) the tau protein expressed between 55-61 kDA also shows significant ($F_{2,12}$ = 6.750; p = 0.01; One-Way Anova) mobility shift towards a higher molecular weight, which is most likely due to the additional phosphorylation load in this condition.

The optical density analysis of the bands representing the total amount of tau protein present is shown in figure 5. There are no detectable differences in the total amount of tau protein detected at 68 kDA (figure 5A) and between 55-61 kDa (figure 5C). The 3rd band detected at ~65kDa in the mice in group 2 (T21) is only dominantly present in the mice of group 2 (T21) and is expressed at approximately 400% of the level observed in the mice in group 1 (EU), (figure 5B). The tau protein expression in the experimental groups depends significantly on the molecular weight (2-way ANOVA, interaction, F = 3.913, p = 0.010). The band at 65 kDa is significantly more expressed (figure 5B) in the mice of group 2 (T21) (group 1 vs. group 2: q = 5.567, p = 0.001; group 1 vs. group 3: q = 3.449, p = 0.020).

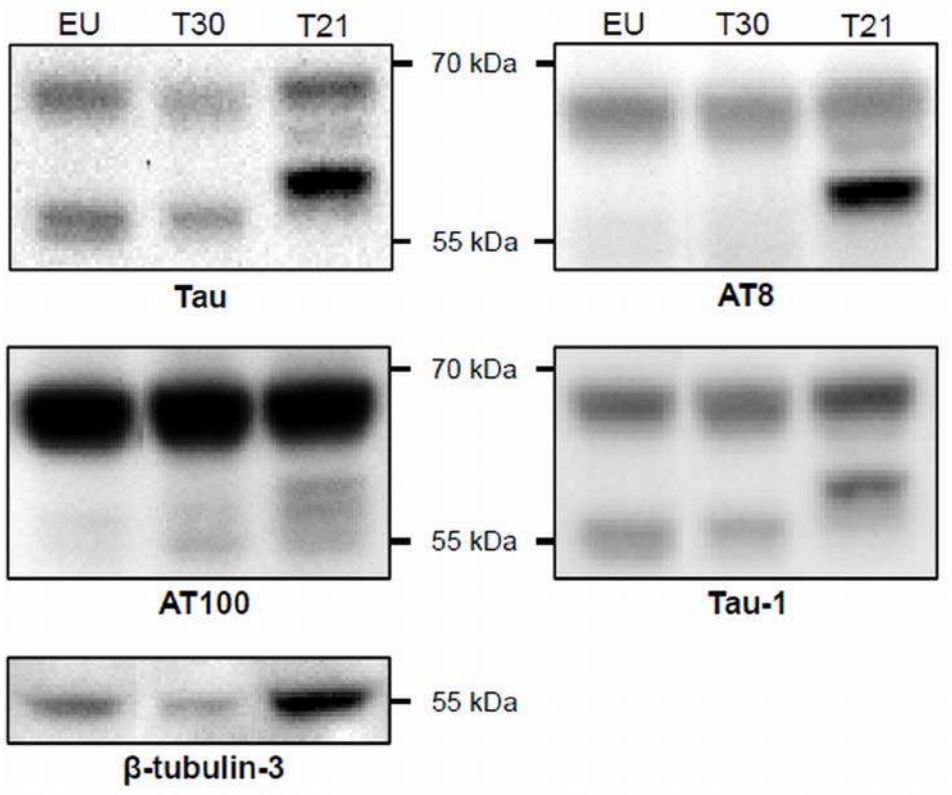

Figure 4 *Representative examples of immunodetection with Tau, AT8, AT100, Tau-1 and β-tubulin-3 antibodies of western blots of mice in group 1 (EU), group 2 (T21) and group 3 (T30).*

The hyperphosphorylation of the tau protein was assessed by AT8 and AT100 and Tau-1 immunodetection. Representative examples of the blotting results are shown in figure 4 (AT8 top right panel, AT100 bottom middle panel, Tau-1 bottom right panel). AT8, AT100 and Tau-1 immunoreactivity are present in all three groups of mice in the higher molecular weight tau forms between 65-68 kDa. There are no significant differences in the amount of tau protein phosphorylation, measured by AT8, AT100 and Tau-1, between the experimental groups in this molecular weight range. The phosphorylation of the lower molecular weight forms of tau between 55-61 kDa shows a different pattern. There is a clear band present for AT8 in the mice from group 2 (T21). Hardly any AT8 expression is detected in the mice in group 1 (EU) and group 3 (T30) in this molecular weight range (figure, 4 top left panel). When the OD values are analyzed, the mice in group 2 (T21) show a significantly higher expression of AT8 hyperphosphorylation in this molecular weight range (group 1 vs. group 2: $q = 6.136$ $p < 0.001$; group 2 vs. group 3: $q = 5.380$, $p < 0.001$) (figure 6B).

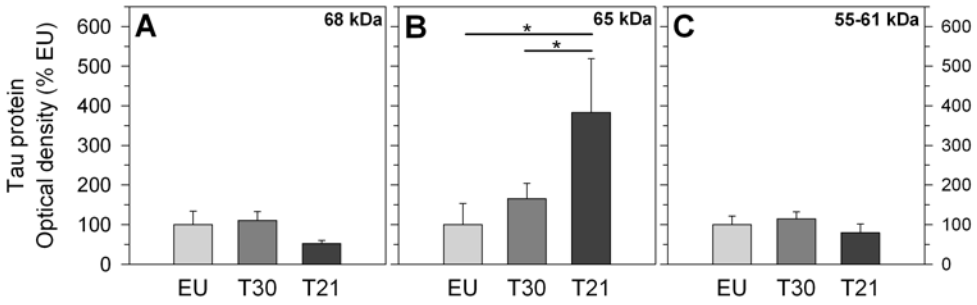

Figure 5 *Optical density (OD) analysis of the tau protein present in the three groups (1 EU; 2 T21; 3 T30) of mice at: 68 kDa (panel A), 65 kDa (panel B) and in the range of 55-61 kDa (panel C). OD's were corrected prior to averaging for the amount of protein loaded by the β-tubulin-3 signal. The OD is expressed as a percentage of the average OD of the mice in group 1 (EU). Asterisks denote significant differences ($p < 0.05$) between groups.*

The Tau-1 antibody also detects tau in phosphorylated form (Binder et al. 1985), but is partly complementary to the AT8 antibody. Tau-1 recognizes part of the AT8 epitope when it is not phosphorylated (Biernat et al. 1992). Tau-1 is clearly expressed between 55-61 kDa in the mice of group 1 (EU) and group 3 (T30) (figure 4 bottom right panel), when AT8 expression is absent. Tau-1 is also expressed in the mice in group 2 (T21), simultaneously with the AT8 expression. When the OD values of all mice are analysed, the Tau-1 expression in the mice of group 2 (T21), is at approximately 50% of the expression observed in groups 1+3 (figure 6F). This is significantly lower (group 1 vs. group 2: $q = 4.304$ $p = 0.006$; group 2 vs. group 3: $q = 6.151$, $p < 0.001$) then the Tau-1 expression of the mice in groups 1 and 3.

No AT100 hyperphosphorylation (Serine 214 & Threonine 212) is observed in the euthermic mice (group 1, EU) at the lower molecular weight tau forms (figure 4, middle left panel). AT100 Hyperphosphorylation is present in the torpor-like hypothermia mice of groups 2 and 3 (figure 4, middle left panel). The AT100 expression is separated in several bands at approximately 55, 58 and 61 kDa. The AT100 signal between 55-61 kDa is approximately 2 times higher in the torpid mice of groups 2 and 3 (figure 6D), which is significant (group 1 vs. group 2, $q = 4.643$, $p = 0.009$; group 1 vs. group 3: $q = 4.015$, $p = 0.009$).

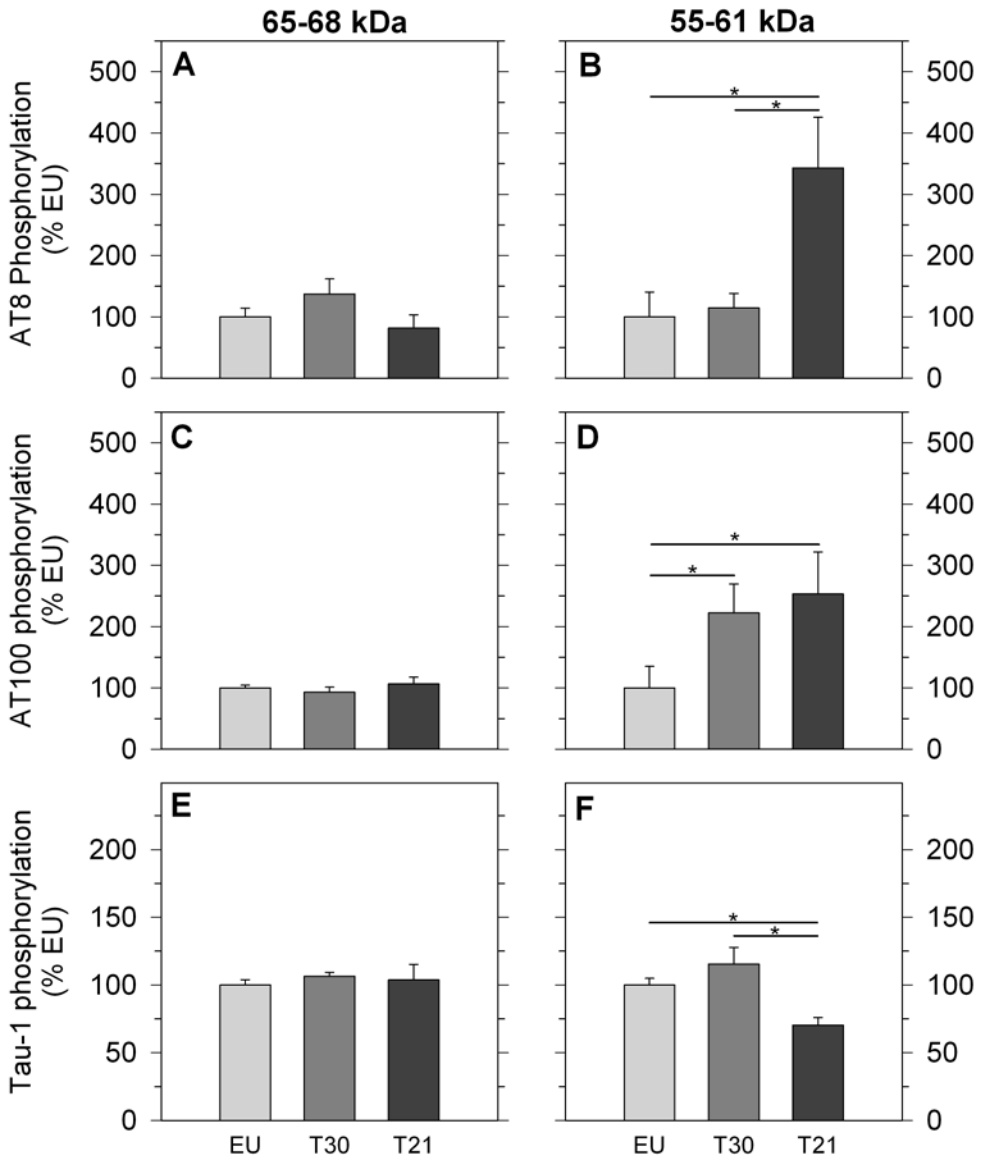

Figure 6 *Optical density analysis of the hyperphosphorylation of the tau protein in two molecular weight ranges: 65-68 kDa (panels A, C and E) and 55-61 kDa (panels B,D and E). The hyperphosphorylation was measured using 3 phospho-dependent antibodies: AT8 (panels A, B), AT100 (panels C, D) and Tau-1 (panels E,F). The average hyperphosphorylation with s.e.m. is plotted, conventions as in figure 5. Prior to averaging, optical densities were corrected for the amount of protein loaded using the β-tubulin-3 signal. Subsequently signals were corrected for the amount of tau protein present within each molecular weight range.*

Discussion

We studied the effect of 5'-AMP-induced torpor-like hypothermia on reversible paired-helical-filament (PHF)-like phosphorylation of the microtubule-associated-protein tau in mice housed at two different temperatures, T_a=21°C and T_a=29°C. All mice showed hypothermia after intra-peritoneal administration of 5'-AMP. The responses observed were similar between animals. Cooling rates at T_a = 21 °

We explicitly screened for the hyperphosphorylated state of the tau-protein, by applying the phosphorylation dependent AT8, AT100 and Tau-1 antibodies. These antibodies are commonly used for post-mortem diagnosis of Alzheimer's disease (Biernat et al. 1992; Goedert et al. 1995). We always detected hyperphosphorylation of the AT8, AT100 and Tau-1 epitopes in the heavier molecular weight forms between 65-68 kDa of the tau protein. The phosphorylation observed in this molecular weight range is not different between the three experimental groups. This is different from the findings in hamsters, ground squirrels, and black bears, where the most prominent bands of tau affected by hyperphosphorylation were detected at relatively high molecular weights, around 68-72 kDa (Arendt et al. 2003; Härtig et al. 2007; Boerema et al. 2008a; Stieler et al. 2011). Most likely this is related to species differences in the isoforms of tau present in the brain (Janke et al. 1999). We also detected tau at lower molecular weights (55-61 kDa), which is at the same level as reported by Planel and colleagues, who also studied tau protein hyperphosphorylation in C57bl/6J mice (Planel et al. 2007). The AT8 immunoreactivity at the higher molecular weight range observed in the western blots is also not found in the ICC staining: here the brain is almost devoid of staining after the return to euthermia (figure 3). The absence of a mobility shift in the expression of the tau protein around these molecular weights also suggests that this form of the tau protein is not heavily differentially phosphorylated between conditions. At this moment it is unknown what causes the difference in detection between ICC and the western blot for the heavy forms of the tau protein, but apparently the heavier molecular weight epitopes cannot be recognized by the AT8 antibody upon aldehyde fixation.

In the molecular weight range between 55 and 61 kDa we also detected reversible tau hyperphosphorylation at Serine 201 & Threonine 205 (AT8) and Serine214 & Threonine 212 (AT100) in the cortex of mice after torpor-like hypothermia induction at T_a = 21 °C. This is consistent with previous observations of tau hyperphosphorylation during deep torpor in European ground squirrels and hamsters, showing both AT8 and AT100 phosphorylation during torpor (Arendt et al. 2003; Härtig et al. 2007) and Arctic ground squirrels showing phosphorylation at Serine 214 and Threonine 205 during torpor (Su et al. 2008). No significant increase in AT8 hyperphosphorylation was present in the mice of group 3. This is consistent with the strong negative correlation between body temperature and the amount of AT8 immunoreactivity observed in anesthetized mice by (Planel et al. 2007). The increase in AT8 hyperphosphorylation is mainly attributable to decreased phosphatase activity at lower temperatures (Planel et al. 2004, 2007). We also show that the Tau-1 signal at 55-61 kDa is complementary to the AT8 expression in group 1 and 3. In the mice of group 2 the Tau-1 expression is still present, although significantly lower than in groups 1 and 3 (figures 4, 6F). The fact that there is still Tau-1 expression indicates that probably not all AT8 epitopes that can be phosphorylated are also phosphorylated in the mice in group 2.

We observed AT100 hyperphosphorylation in the mice in group 3 (T30) (figure 6D). This would suggest that AT100 phosphorylation precedes AT8 phosphorylation, which is in contradiction with recent studies suggesting that the AT8 epitope is phosphorylated before the AT100 epitope in the pathological process of filamentous tau formation (Zheng-Fischhöfer et al. 1998; Yoshida and Goedert 2006; Luna-Muñoz et al. 2007). Phosphorylation of tau at the AT8 epitope is also thought to be negatively affected by AT100 phosphorylation (Yoshida and Goedert 2006). The phosphate turnover rate is severely affected by temperature in Arctic ground squirrels. At higher temperatures the tau protein in general is phosphorylated faster (Stieler et al. 2011). In our study the cooling rate was lower in the mice in group 3. This means that these mice remained longer at high temperatures than the mice in group 2, even though the total time in torpor-like hypothermia was shorter in the mice in group 3. The higher temperature might facilitate the rapid phosphorylation of the AT100 epitope, via

the AT8 epitope. Because brain temperature does not drop below 30 °C in group 3, it could be that the AT8 epitope is subsequently dephosphorylated again in these mice. Alternatively the differential regulation of phosphorylation between the mice in group 2 and 3 might be indicative of the difference between physiological and pathological phosphorylation of the tau protein. The kinase thought to be associated with pathological phosphorylation of the tau protein (tau hyperphosphorylation) is GSK3-β, whereas physiological modulation of tau phosphorylation is, amongst others, associated with CDK5 (Plattner et al. 2006). During deep torpor and its associated low body temperatures in hamsters and ground squirrels GSK3-β is inhibited and CDK5 is activated (Stieler et al. 2008, 2011).

Previous observations of tau hyperphosphorylation in (non-tau-transgenic) mice include higher phosphorylation of tau after starvation (Yanagisawa et al. 1999), following cold water induced stress (Okawa et al. 2003), after altered glucose metabolism (Planel et al. 2004) and after repeated exposure to ether (Ikeda et al. 2007). Apart from the ether stress, all of these manipulations probably reduced body temperature of the mice, thereby inducing tau phosphorylation, as a consequence of lowered body temperature, as is previously reported in anaesthetized mice (Planel et al. 2007; Run et al. 2009). Further manipulations in extending the 5'AMP induced torpor-like hypothermia at different ambient temperatures and sampling brain material at different time points along the temperature curve might yield valuable insights in the interplay of metabolism, temperature or the time in torpor-like hypothermia, affecting the (hyper)phosphorylation state of the tau protein.

Taken together the results show that that reversible hyperphosphorylation of tau occurs even after a brief time in torpor-like hypothermia in mice at 21 °C and that AT100 hyperphosphorylation can be detected already in the cortex of mice in torpor-like hypothermia at 30 °C . It has been previously shown that reversible PHF-like phosphorylation of tau occurs in torpid European ground squirrels (body temperatures around 7 °C) (Arendt et al. 2003), which are obligate deep hibernators, in torpid Syrian hamsters (body temperatures around 7 °C) (Härtig et al. 2007), which are facultative deep hibernators and in Djungarian hamsters and mice with daily torpor and body temperatures around 21 °C (Boerema et al. 2008a). We extend this finding now to artificially induced torpor-like hypothermia in C57bl/6J mice with body temperatures similar to torpid Djungarian hamsters. Because of the differences between AT8 hyperphosphorylation and AT100 hyperphosphorylation, 5'AMP induced torpor-like hypothermia in mice also seems to be a useful system for the study of the early events in the phosphorylation chain leading to AD.

Chapter 10

Epilogue

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Chapter 10

During hibernation and torpor mammals are subject to the most extreme fluctuations in metabolism and body temperature observed in homeotherms. In ground squirrels metabolic rate can vary between 1-5 % of basal metabolic rate (BMR) during deep torpor, and 6-8 times BMR during rewarming from torpor. The body temperature ranges between sub-zero and euthermic (36-38 °C) values. The low temperatures during torpor have an impact on the state of the brain. Markers of synaptic plasticity are influenced in torpor, and the neuroskeletal protein tau becomes hyperphosphorylated in a way similar to that found in humans affected with Alzheimer's disease (AD). In hibernating ground squirrels these effects are reversed during periodic euthermic phases of rewarming from torpor, but on the behavioural level hibernation remains associated with spatial memory loss.

The main results

The first part of this project aimed to evaluate if these brain related characteristics of torpor and hibernation are specific for ground squirrels, or associated with deep hibernation in general. We measured brain physiology and spatial memory performance in another species capable of deep hibernation, the Syrian hamster. We found that neuronal connectivity in the hippocampus (chapter 3) and spatial memory performance (chapter 5) appear unaffected by hibernation in the Syrian hamster. It is possible that these effects do not occur in hamsters since they are hoarders and need their memory to find their food stores during the periodic euthermic phases in hibernation. On the other hand, hyperphosphorylation of the microtubule associated protein tau was increased during torpor in Syrian hamsters and de-phosphorylated upon rewarming (chapter 3, 4), similar to previously measured European ground squirrels (Arendt et al. 2003).

In chapter 3 we compared characteristic aspects of tau hyperphosphorylation in Alzheimer's Disease (AD) with tau hyperphosphorylation in hibernation. As in AD, there is a cholinergic specificity of tau hyperphosphorylation in the basal forebrain in hibernation. Chapter 4 elucidates the dynamics of tau hyperphosphorylation during the transitions from torpor to euthermia and vice versa. We found that most of the torpor associated tau hyperphosphorylation already occurs at relatively high brain temperatures (26 - 30 °C) during entry in to torpor. The phosphorylation load in the cortex and the hippocampus subsequently increases marginally with progression of time in torpor. Most of the de-phosphorylation of the tau protein during arousal from torpor takes place at similar (26 - 30 °C) brain temperatures as the phosphorylation in torpor entry.

In part II of the thesis we further studied tau hyperphosphorylation in two species known for the prevalence of daily torpor in response to short winter days, i.e. Djungarian hamsters, and to food shortage, House mice. We found reversible tau hyperphosphorylation during daily torpor in Djungarian hamsters (chapter 6). We then studied torpor in mice. We induced daily torpor in mice by letting them work for their food (WFF) in a (chapters 7 & 8). WFF has a remarkable effect on the circadian organization of behaviour as mice shift their activity from the night to the day. By combining the WFF paradigm with yoked controls that receive the same food without working for it, chapter 8 shows that the nocturnal torpor is a response to food limitation. Torpor in mice is also associated with tau hyperphosphorylation. Chapter 9 takes this a final methodological step further. A torpor-like hypothermic state in mice is induced pharmacologically. This torpor-like state is also associated with hyperphosphorylation of the tau protein and the hyperphosphorylation is reversed upon return to euthermia.

Interspecies differences in neuronal plasticity and behaviour during hibernation and torpor.

In chapter 2 we studied synaptic plasticity in Syrian hamsters by measuring synaptophysin levels in the stratum lucidum of the hippocampal CA3 area in a similar way as in a previous study in European ground squirrels (Strijkstra et al. 2003). To our surprise, we did not find differences in synaptophysin staining intensity between torpor, periodic euthermy and euthermy in summer conditions in Syrian hamsters. Other markers for changes in neuronal plasticity (MAP2, PSA-NCAM, Doublecortin) also did not change in expression pattern over the torpor-arousal cycle in Syrian hamsters. These results are not included in chapter 2 and are shown in Box 3. Previously, MAP2 (Hut et al. 2001; Arendt et al. 2003) and PSA-NCAM (Arendt et al. 2003) were found decreased in torpor and be restored in subsequent euthermy in hibernating European ground squirrels. Other groups also reported a torpor-arousal cycle for several neuronal plasticity markers in golden mantled ground squirrels (*Spermophilus lateralis*) (von der Ohe et al. 2006, 2007) and in European hamsters (*Cricetus cricetus*) (Magariños et al. 2006). Popov and colleagues (2011) recently reported on adult neurogenesis in hibernating Siberian ground squirrels (*Spermophilus undulatus*). A staining for doublecortin (DCX), a marker for newly divided and maturing neurons, revealed that the number of maturing neuronal cells in the Sub Granular Zone (SGZ) of the hippocampal dentate gyrus is not reduced during torpor. DCX positive cells with branched apical dendrites were only observed in summer euthermic animals (Popov et al. 2011). These authors also observed retraction of dendritic branches of DCX positive neurons in the SGZ in torpor, similar to their previous finding of reduced branching of dendrites in the CA3 area during torpor (Popov et al. 1992). In the Syrian hamster we also do not find differences over the hibernation cycle in the number of maturing neurons in the SGZ , consistent with Popov's Siberian ground squirrels (Box 3; figure 4). In contrast to (Popov et al. 2011) we did not observe differences in the amount of dendritic branching in DCX labelled neurons during hibernation in Syrian hamsters (Box 3; figure 4). This finding is consistent with the absence of MAP2 dynamics in the hippocampal CA3 area over the hibernation cycle in Syrian hamsters (Box 3; figure 3).

Our results thus show that Syrian hamsters differ from other deep hibernators such as ground squirrels, with respect to their synaptic plasticity dynamics in the hippocampus over the hibernation cycle. At the behavioural level an interspecific difference also becomes apparent. European ground squirrels lose spatial memory and operant conditioning memory over hibernation, only if torpor is allowed during hibernation at low ambient temperature (Millesi et al. 2001). Syrian hamsters, however, In contrast to this, retain spatial memory over the hibernation season, even if deep torpor during hibernation at ambient temperatures of 5 °C is abundantly present.

Tau protein hyperphosphorylation dynamics in torpor

The other major brain characteristic observed in hibernating ground squirrels was hyperphosphorylation of the microtubule associated tau protein during torpor. Contrary to the lack of hippocampal synaptic plasticity dynamics, we established reversible tau hyperphosphorylation during hibernation in Syrian hamsters. We demonstrated the occurrence of reversible tau hyperphosphorylation during hibernation in another species than the European ground squirrel (Härtig et al. 2007 (chapter 3). Recent publications confirm the occurrence of reversible hyperphosphorylation during hibernation in Syrian hamsters (Stieler et al. 2011), Arctic ground squirrels (*Spermophilus parryii*) (Stieler et al. 2008; Su et al. 2008; Stieler et al. 2011) and black bears (*Ursus americanus*) (Stieler et al. 2011). We observed that in Syrian hamsters the tau hyperphosphorylation in the basal forebrain was specific for cholinergic neurons (Härtig et al. 2007

(chapter 3). Almost all cholinergic neurons contained hyperphosphorylated tau protein in torpor, whereas hardly any GABA-ergic neurons with hyperphosphorylated tau protein were found. This cholinergic tau hyperphosphorylation specificity, leading to loss of basal forebrain neurons, is considered to be an early event in the pathological chain of events of AD (Davies and Maloney 1976; Arendt et al. 1983). Tau hyperphosphorylation was not only present in deep torpor, but also occurred in shallow daily torpor in Djungarian hamsters and induced torpor in laboratory house mice (Boerema et al. 2008a, chapters 8 & 9).

Chapter 4 zoomes in on the dynamics associated with the transitions from cold to warm, and from warm to cold, during hibernation. We obtained body temperatures and (estimates of) brain temperature during cooling to torpor and rewarming from torpor. We sampled animals at a range of brain temperatures during cooling, rewarming and torpor. We found indications of a stepwise change in phosphorylation state in the brain. The fastest changes in tau hyperphosphorylation load in the cortex and the hippocampus occurred at brain temperatures between 26 - 30 °C during both cooling to torpor and rewarming from torpor. This finding deviates from the linear relation between temperature and tau hyperphosphorylation during anaesthesia induced hypothermia, as found in mice (Planel et al. 2007; Run et al. 2009). On average the total tau hyperphosphorylation increased only marginally between the initial onset of torpor below 26-30 °C towards the end of the torpor bout. Similar small increases during torpor in the tau phosphorylation load in several brain areas have been reported for Syrian hamsters and Arctic ground squirrels (Stieler et al. 2011). This suggests that onset and offset of tau hyperphosphorylation is not only a passive temperature driven effect, but may be a functional relevant regulatory process in torpor brain physiology.

The results presented in this thesis, together with recent publications of other groups, thus show that tau hyperphosphorylation is a general feature, occurring in both deep and shallow torpor across species. The hyperphosphorylation of the tau protein starts already at brain temperatures just below the euthermic range, early during the entry into torpor.

On the function of periodic euthermy in hibernation

This question, already addressed by (Dubois 1896) and (Daan 1973b), remains still unanswered in this thesis. Previous hypotheses have focused on the need for periodic euthermy to recover from some kind of damage accumulating in torpor. Especially hypotheses involving restoration of brain function (Daan et al. 1991; Trachsel et al. 1991; Strijkstra and Daan 1998; Strijkstra 1999) are attractive. The brain is of utmost importance for survival of hibernators, since they need to be able to initiate arousal at least once. The brain is a delicate structure, with a high metabolic turnover under normal physiological conditions, quickly suffering from metabolic distress. It might be susceptible to damage during hibernation. When approaching sub-zero temperature in torpor, metabolism and heat protection appear up regulated to protect the brain from freezing, whereas peripheral tissues show some tolerance to sub-zero temperatures in ground squirrels (Eisentraut 1956; Barnes 1989; Strijkstra et al. 1999; Buck and Barnes 2000). In hibernators, (parts of) the brain keep functioning at low temperatures (Kilduff et al. 1982; Krilowicz et al. 1988, 1989). This activity may allow them to sustain cardiac and pulmonary functions at low temperature levels, where non-hibernating homeothermic animals would have died from cardiac or pulmonary arrest (Kayser and Malan 1963). It is likely that the benefits of maintaining some functional neuronal activity in the brain at low temperatures are associated with costs.

Torpor is associated with hyperphosphorylation of the tau protein, as confirmed in both deep and shallow torpor in this thesis. Tau protein hyperphosphorylation is known for its association with pathological and neurodegenerative phenomena, such as AD in humans. One of the characteristics of AD is a reduced glucose uptake in the brain (Jagust et al. 1991; Hoyer 1993). Starvation, a metabolically difficult situation, is associated with tau hyperphosphorylation in mice (Yanagisawa et al. 1999) and altered glucose metabolism leading to hypothermia is also associated with tau hyperphosphorylation (Planel et al. 2004). Tau hyperphosphorylation in hibernation might signal a 'poor energetic state' of the brain. It is therefore tempting to speculate on the possibility that periodic euthermy during hibernation is necessary to reverse the accumulation of tau protein hyperphosphorylation occurring in torpor.

Some of the results obtained in this project and those from the study by (Stieler et al. 2011) argue against this idea. In Syrian hamsters most of the tau hyperphosphorylation measured in late torpor has already accumulated during torpor entry. Prolonged torpor does not lead to a substantial further increase in tau hyperphosphorylation in Syrian hamsters (Stieler et al. 2011, chapter 4) and Arctic ground squirrels (Stieler et al. 2011). It is therefore unlikely that the degree of hyperphosphorylation represents an accumulating cost of torpidity provoking a need for de-phosphorylation in periodic euthermy. That requires a periodic euthermic phase. On the other hand, as we shall see below (Perspective), it is possible that the initial tau hyperphosphorylation has a neuroprotective effect, while prolonged hyperphosphorylation eventually is damaging and eventually requires return to euthermy.

A role for tau hyperphosphorylation in torpor physiology?

Instead of leading to arousals tau hyperphosphorylation may have a function in the entrance and/or maintenance of the state of torpor. The tau protein is known to be involved in axonal vesicle transport (Ebneth et al. 1998; Trinczek et al. 1999; Dixit et al. 2008). Both phosphorylation and hyperphosphorylation of the tau protein affect the binding capacity of tau to the microtubules. Most likely, they thereby also affect the efficiency of vesicle transport. If the transport of neurotransmitter vesicles is impaired or blocked, the ability of neurons to relay action potentials by neurotransmission is hampered. This is consistent with the observation that the brain temperatures (26-30 °C) at which the largest changes in phosphorylation take place are similar to the temperatures (28 – 30 °C) where wakefulness resembling, spontaneous electrical activity patterns, of the neurons in the cortex and the hippocampus of hibernating hamsters change (Chatfield et al. 1951; Chatfield and Lyman 1954).

Because of the reduction in transcription and translation during toprpor (van Breukelen and Martin 2001, 2002), post-translational modifications become increasingly important as a method of regulation. Dynamics in protein phosphorylation continue in torpor, as shown in this thesis. Other forms of regulation by post-translational modifications to proteins, such as protein SUMOylation (Lee et al. 2007), protein Ubiquitylation and protein Acetylation (Storey 2010) are also present in torpor. Gong and colleagues (2006) have recently offered a novel hypothesis on the relation between glucose metabolism, tau hyperphosphorylation and neurofibrillary degeneration. This hypothesis may be relevant for tau hyperphosphorylation in torpor physiology. These authors hypothesise that the decreased glucose metabolism leads to decreased tau O-GlcNAcylation, which increases the phosphorylation of tau and eventually leads to neurofibrillary degeneration in AD. Protein O-GlcNAcylation is a form of protein Glycosylation. It is an abundant form of post-translational modification in eukaryotic organisms, similar to protein phosphorylation (Hart 1997). Protein O-GlcNAcylation is an important regulatory process, since modifications in the gene coding for the O-

GlcNAc transferase enzyme are incompatible with life (Shafi et al. 2000). In some cases O-GlcNAcylation and phosphorylation can even act on the same proteins and/or sites, influencing each other (Comer and Hart 2000). The tau protein, amongst other cytoskeletal proteins, is known to be subject to O-GlcNAcylation. Increased O-GlcNAcylation negatively affects the amount of tau hyperphosphorylation and vice versa (Liu et al. 2004). The production of the enzyme necessary for protein O-GlcNAcylation is directly dependant on the glucose available (Gong et al. 2004, 2006). Thereby the decrease in glucose metabolism in AD can cause tau protein hyperphosphorylation. In mammalian hibernation, the process of protein O-GlcNAcylation may serve a functional physiological role during torpor.

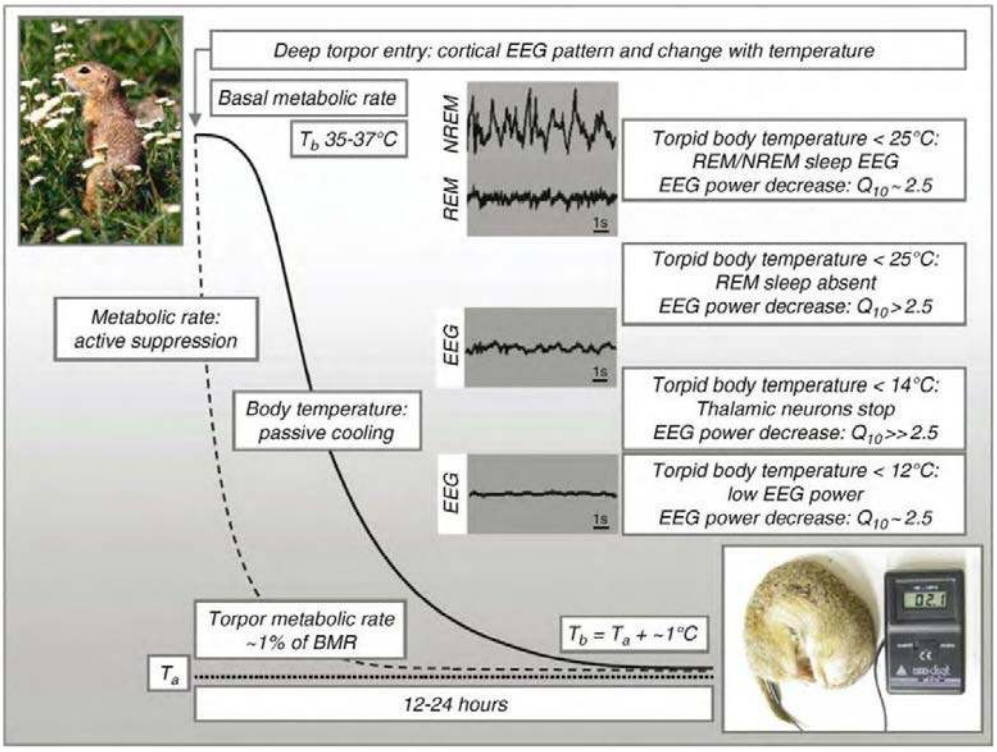

Figure 1 *Schematic representation of deep torpor entry from a euthermic body temperature (T_b) to a deep torpor body temperature just over ambient temperature (T_a) in the European ground squirrel. Active metabolic suppression (dotted line) from basal metabolic rates at euthermia is followed by a passive body temperature decrease (solid line). The 10 s EEG traces initially resemble Non-REM and REM sleep; REM sleep is lost below 25°C. At lower temperatures the EEG shows progressive changes in power with different rates, indicating regulated activity changes (Q10 > 2-3) and passive changes (Q10 ~2-3), ultimately resembling a flat line below ~10°C.*

The entrance in to torpor is a well-orchestrated cell biological process (Carey et al. 2003; Storey 2003; MacDonald and Storey 2005). In the brain, however, torpor entry may cause a discrepancy between metabolic possibilities of the suppressed cellular energy production and the still present electrical activity of neurons. Metabolic rate decreases rapidly at torpor entry (Heldmaier and Ruf 1992; Geiser

2004), but body temperature goes down much more slowly, as a consequence of passive cooling. The effects on the cortical EEG signal, and thereby on the underlying neuronal activity, are associated with body temperature, and not to metabolic rate. Frequency of the cortical EEG slows down with a constant Q_{10} of ~2.5 (Deboer and Tobler 1995; Deboer 1998; Strijkstra et al. 2000), indicating passive temperature dependence. In contrast, the power of the EEG signal shows stepwise changes with significant deviations from a Q_{10} of ~2.5 (Strijkstra et al. 1999). Around 25 °C, the decrease in EEG power is enhanced, and REM sleep cannot be detected from the EEG below this temperature (Strijkstra et al. 2000; Strijkstra 2006). Around 14 °C, the decrease in power is further enhanced, most likely due to the disappearance of electrical activity from the thalamic neurons (Krilowicz et al. 1988). Below 14 °C, the little power that is left in the EEG signal, decreases again with temperature with a Q_{10} of ~2.5. These effects are summarized in figure 1, showing the sequence of events during torpor entry in the European ground squirrel (figure adapted from (Strijkstra 2009 p.1834).

The discrepancy between glucose availability, as a consequence of decreased metabolic rate, and the glucose demands of electrically active neurons, may result in low brain glucose levels during torpor entry. This would affect the balance between phosphorylation and O-GlcNAcylation of the tau protein, tilting the balance in favour of phosphorylation. The high temperatures at which the largest changes in tau hyperphosphorylation occur during torpor entry (chapter 4, this thesis) fit this view. The apparent cholinergic specificity of tau hyperphosphorylation in hibernation (Härtig et al. 2007) may also be consistent with this idea since Rapid Eye Movement sleep (REM-sleep) is associated with firing of cholinergic neurons in the Reticular Activating System (Jones 2004). This area consists of several areas in the basal forebrain, midbrain and brain stem (Moruzzi and Magoun 1949; Magoun 1952). REM-sleep is one of the first electrical characteristics to disappear from the EEG, between 30-25 °C during entry into torpor (Deboer et al. 1994; Deboer 1998; Strijkstra 2006).

In this scenario, tau hyperphosphorylation plays an active role in early torpor entry by blocking the neurotransmission ability of cholinergic neurons. This would effectively further decrease the metabolic demands of the brain, allowing it to enter the state of torpor.

Hibernation and torpor as a model system for Alzheimer's disease?

There are many similarities between the tau hyperphosphorylation observed during torpor and Alzheimer's Disease (AD), but there are also differences. In AD, brain regions with a high degree of synaptic plasticity are affected by tau pathology first (Braak and Braak 1991a, 1994). During torpor, plastic regions such as the enthorinal cortex and the hippocampus, are indeed affected (Arendt et al. 2003; Härtig et al. 2007; Boerema et al. 2008a; Stieler et al. 2011). In Syrian hamsters the brainstem (amongst other brain regions), a less plastic region of the brain, is also broadly and equally fast affected by tau hyperphosphorylation (see figure 2). This aspect of tau hyperphosphorylation in hibernation does not fit the normal progression of hyperphosphorylation and pathological staging of AD (Braak and Braak 1991a).

At the behavioural level, the observed hyperphosphorylation is not always associated with cognitive decline, such as a decrease in memory performance. European ground squirrels and Djungarian hamsters share the combination of tau hyperphosphorylation (Arendt et al. 2003; Boerema et al. 2008a (chapter 6)) and decreased spatial memory performance (Millesi et al. 2001; Palchykova et al. 2006) after torpor. This is not the case for Syrian hamsters which retain spatial memory performance over hibernation (chapter 5).

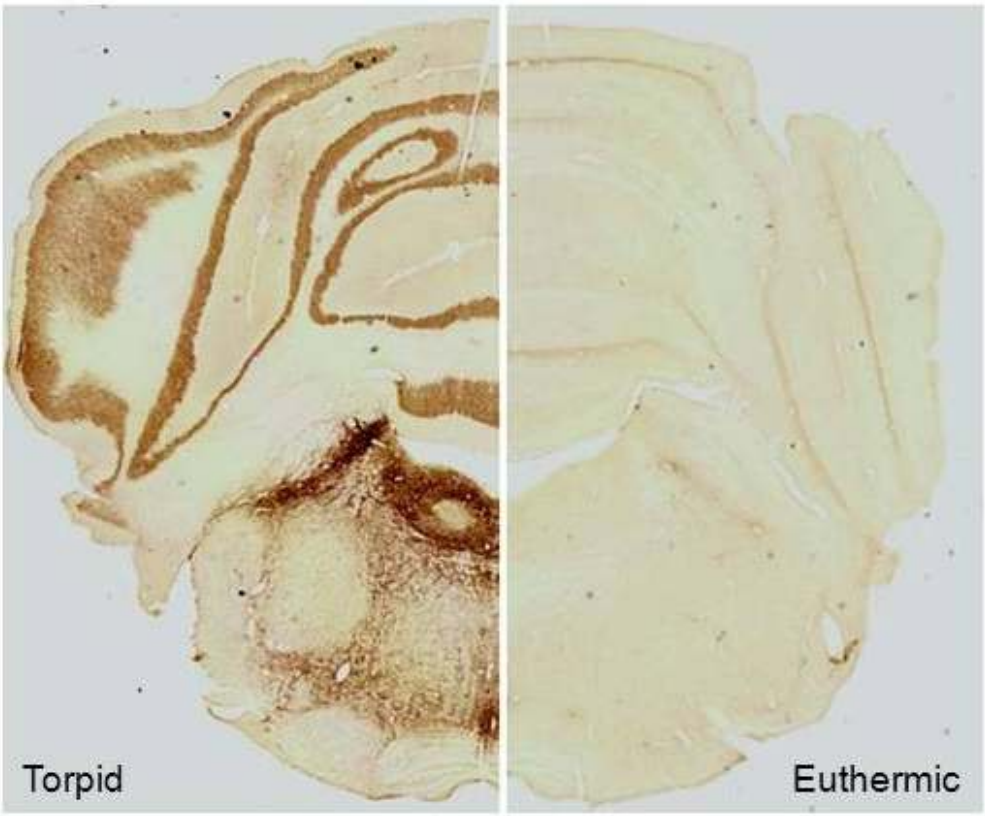

Figure 2 *AT8 immunoreactivity in the brainstem and the cerebellum of two Syrian hamsters. The left panel shows an animal sampled late in torpor (>96 hours after onset of torpor). The right panel shows an animal sampled late in arousal (>8.5 hours after induction of arousal). The brain stem shows a highly area specific tau hyperphosphorylation staining pattern. The hyperphosphorylation is reversed during periodic euthermy (data not shown). The general staining procedure is according to the methods described for the AT8 staining in chapter 3, but the staining was performed on glass mounted slides with a primary antibody dilution of 1:750 (Staining performed by Else Eising).*

In Greater mouse-eared bats (*Myotis myotis*) (Ruczynski and Siemers 2010) and Alpine marmots in (*Marmota marmota*) (Clemens et al. 2009) there were also no apparent effects of hibernation on memory. Although not measured so far, it is likely that these species also have hyperphosphorylation of the tau protein in the brain during torpor. AD is associated with a disturbed sleep-wake behaviour and loss of circadian organization of behaviour (Vitiello and Prinz 1989; Mirmiran et al. 1992). Syrian hamsters and European ground squirrels lose their circadian organisation of behaviour during hibernation (Oklejewicz et al. 2001a; Hut et al. 2002b), whereas Djungarian hamsters and mice retain their circadian rhythmicity of behaviour an even time their torpor in the inactive phase of the day (Kirsch et al. 1991; Hut et al. 2011). Therefore, the occurrence of tau protein hyperphosphorylation per

se may not necessarily lead to detrimental effects in the brain with direct deleterious functional consequences.

One other dissimilarity of AD and tau hyperphosphorylation is that hibernators do not suffer from AD. AD is a human multi-factorial disease, also involving hyperphosphorylation of the tau protein, and eventually causing the death of the patient. In mammalian hibernators, survival rates over hibernation are generally good. In fact, hibernation has been reported to prolong life-span in Turkish hamsters and European ground squirrels (Lyman et al. 1981; Zivadinovic and Andjus 1996). This seems at variance with the neurodegenerative consequences of the tau pathology observed in AD. Although hibernators are not affected by AD, and are maybe not even a perfect replica of all the Alzheimer pathology, they are nevertheless the only *in vivo* model available for the study of physiological regulation of tau protein (hyper)phosphorylation and de-phosphorylation at the moment. Considering the huge physiological changes a hibernator repeatedly goes through in a very short time, torpor may be an important model system for several medical problems related to cellular metabolism in general.

Perspectives

Prime targets for future research on the functional need for periodic euthermy in maintenance of brain processes would be the integrity of thermoregulatory centres in the hypothalamus and the brainstem, primarily because of their crucial role in rewarming and regulation of vital functions during torpor. Even in relation to AD pathology, it is hypothesised that tau protein hyperphosphorylation is initially a neuroprotective response, which derails with progression of time, and finally may become neurodegenerative (Lee et al. 2005). Obviously, hibernators prevent the latter and arouse from torpor preventing this neuronal damaging state, although different functional consequences, for example with regard to memory performance after hibernation in ground squirrels, may be accepted. Experiments in which tau hyperphosphorylation is prevented during torpor might shed light on the neuroprotective role of tau hyperphosphorylation and the potential role in the physiological regulation of torpor entry. Perhaps the possibility to artificially induce torpor by injection of 5'AMP (Zhang et al. 2006) or exposure to H_2S gas (Blackstone et al. 2005) may provide the tools to perform experiments in which torpor bout duration is substantially elongated. Such experiments manipulating time in torpor may shed light on the potential function of periodic euthermy in hibernation.

Another promising research direction would be the relation between the function of the immune system during hibernation and potential neurodegenerative effects of torpor. Neurodegeneration in AD is associated with neuroinflammation (Hauss-Wegrzyniak et al. 1998; McGeer et al. 2000). In mammals, the peripheral immune system is heavily down regulated during deep torpor and daily torpor (Prendergast et al. 2002; Bouma et al. 2010a, 2010b, 2011b). Not much is known about the function of the brain immune system in torpor, but microdialysis probes inserted in the brains of torpid ground squirrels caused hardly any microglial activation and cell damage (Zhou et al. 2001), suggesting also a diminished function of the brain immune system in torpor. The role of the Central Nervous System Immune system in neurodegeneratieve diseases is dualistic. Both neuroprotective and neurodegenerative effects of cytokines have been reported (see (Rojo et al. 2008) for a review). For instance the expression of the cytokines Interleukin-6 and Interleukin-1 are linked to an up regulation of the cdk5/p35 complex, which hyperphosphorylates the tau protein. It could be that the suppression of the immune system allows hibernators to endure prolonged tau protein hyperphosphorylation in torpor by preventing additional, neuroinflammation mediated, hyperphosphorylation of the tau protein.

Concluding remark

This thesis expands our knowledge on brain physiology during torpor by monitoring dynamics of a potential neurodegenerative process indicator, i.e. tau hyperphosphorylation, in hibernation and torpor. We demonstrated that hyperphosphorylation of the tau protein occurs across species and is associated with both deep and shallow (daily) torpor. Although torpid animals do not have Alzheimer's disease, torpor associated (physiological) hyperphosphorylation shares some of its characteristic with (pathological) tau hyperphosphorylation in AD. We demonstrated the occurrence of reversible tau hyperphosphorylation in artificially induced torpor-like hypothermia in C57bl/6J mice. This uncovers the full potential of using mice, as the standard laboratory animal model system with all its molecular knowledge and tools, in studying physiological regulation of tau phosphorylation and its similarities with tau hyperphosphorylation in Alzheimer's disease.

Box 3: Markers of structural synaptic plasticity in Syrian hamster hibernation.

Plasticity markers

In ground squirrels several indicators of synaptic plasticity have been reported to cycle between torpor, arousal and summer euthermia (Popov and Bocharova 1992; Popov et al. 1992; Hut et al. 2001; Strijkstra et al. 2003; Arendt et al. 2003; von der Ohe et al. 2006, 2007) over the hibernation cycle. In Syrian hamsters, we duplicated measurements of several of these markers. Synaptophysin and MAP2 were measured in the stratum lucidum (SL) of the hippocampal CA3 area. Synaptophysin is a trans-membrane protein located in synaptic vesicles (Wiedenmann and Franke 1985). It was measured as an indicator of synaptic efficacy (see (Boerema et al. 2008b (chapter 2)) for a more detailed report). Microtubule associated protein 2 (MAP2) is a protein associated with the cytoskeleton of dendrites (Friedrich and Aszódi 1991). As such, it indicates the presence of dendrites in the SL. PSA-NCAM was measured in the hylus of the dentate gyrus of the hippocampus, an area known for the presence of adult neurogenesis. PSA-NCAM, Polysialic Acid Neuronal Cell Adhesion Molecule that is expressed on the surface of neurons and glial cells is implicated in cell-cell adhesion and adult neurogenesis (Seki and Arai 1993). As such, it is involved in synaptic plasticity. Adult neurogenesis is a process in which new neurons are formed, mature and become functional (van Praag et al. 2002). It is thought to be important for learning and memory and as such associated with synaptic plasticity. We measured dDoublecortin (DCX) as a measure of the number of maturing neurons (Francis et al. 1999; Gleeson et al. 1999) resulting from adult neurogenesis in the dentate gyrus of the hippocampus.

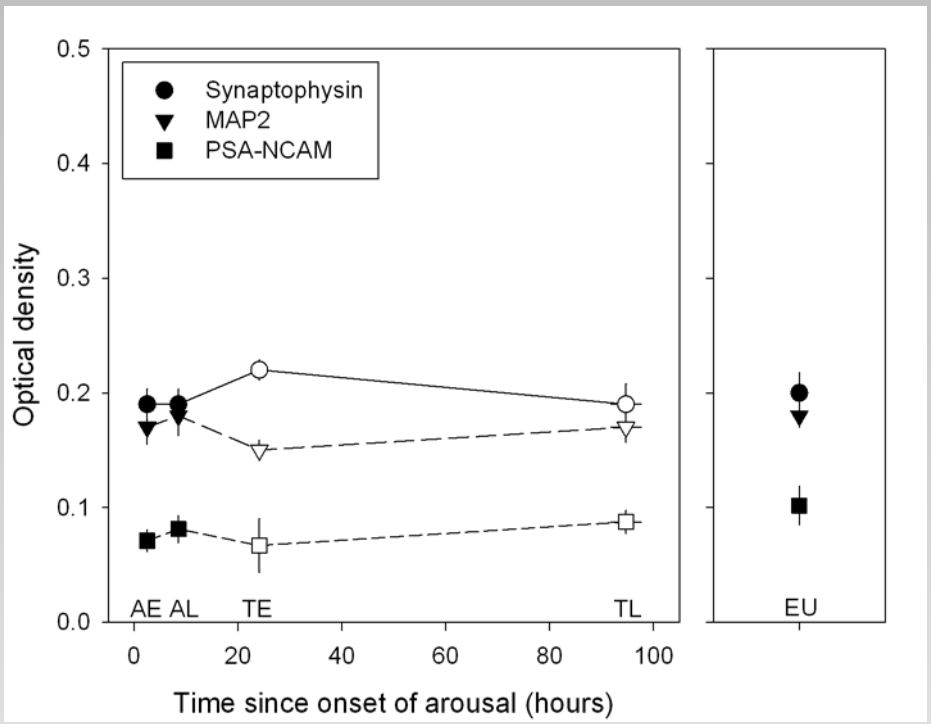

Figure 1 *Markers for synaptic plasticity (Synaptophysin, MAP2 and PSA-NCAM) in the hippocampus of Syrian hamsters in different phases of the torpor-arousal cycle during hibernation and in summer control conditions. The average optical density (OD) of the staining (+/- SEM) is shown for hamsters sacrificed ~2.5 hours after arousal (AE), ~8.5 hours after arousal (AL), 24 hours in torpor (TE), and ~96 hours in torpor (TL) and continuous euthermic hamsters in long day summer conditions (EU) all groups n=5. Open circles indicate hypothermic animals, closed circles indicate euthermic animals. OD's for Synaptophysin and MAP2 were measured in the stratum lucidum of the hippocampal CA3 area. PSA-NCAM OD was measured in the hylus of the hippocampal dentate gyrus area. No significant differences were detected between groups (Kruskal-Wallis one-Way Anova for Synaptophysin: H = 3.602, p = 0.463; MAP2: H = 6.002, p=0.199; PSA-NCAM H = 3.537, p = 0.427; n=25 for all tests).*

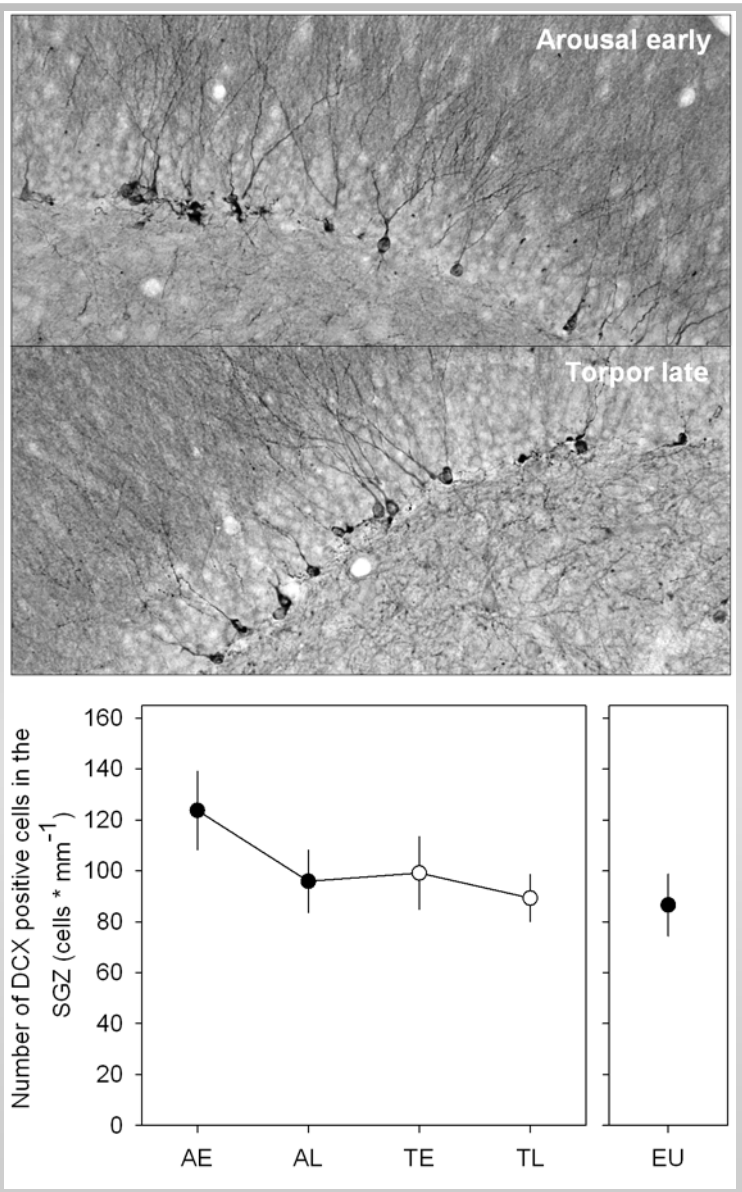

Figure 2 *Doublecortin (DCX) immunoreactivity in the Sub Granular Zone (SGZ) of the dentate gyrus. Panel A & B show representative examples of DCX positive cells in a euthermic hamster (2.5 hours after the onset of arousal, panel A) and a torpid hamster (>96 hours after the onset of torpor, panel B). Panel C shows the average number of DCX positive cells (+/- SEM) present in the SGZ in hibernating and euthermic hamsters (all groups n=5). Open circles indicate hypothermic animals, closed circles indicate euthermic animals. The amount of cells appears to be a little higher in the AE animals, but this did not survive statistical evaluation, overall there were no significant differences between experimental groups (One-way Anova, F = 1.310, p = 0.297, n = 5).*

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Nederlandse samenvatting

Winterslaap en torpor

Bij het woord winterslaap denken mensen meestal aan dieren die, om de barre omstandigheden tijdens de winter te ontwijken, maandenlang koud zijn en overwinteren in hun holen zonder te eten of te drinken. Kortom een voor de dieren een rustige periode waarin niet al te veel interessants gebeurt wat de moeite van het bestuderen waard zou kunnen zijn. Dit beeld klopt op hoofdlijnen, maar is niet helemaal juist. Om te beginnen, gebeuren er juist allerlei interessante dingen in de winterslaapperiode. Dit proefschrift gaat dieper in op een aantal van die zaken.

De meeste warmbloedige dieren die in winterslaap gaan, winterslaap gedrag komt voor van muizen tot beren en zelfs bij lemuren, zoeken inderdaad een beschutte plaats op als ze in winterslaap gaan. Vervolgens verlagen ze hun stofwisseling om energie te besparen. De stofwisseling is de snelheid waarmee voedsel en zuurstof in energie en bouwstoffen voor het lichaam worden omgezet. Als gevolg daarvan, koelen dieren af, soms tot temperaturen vlak boven de omgevingstemperatuur. Deze periodes met een verlaagde stofwisseling worden torpor genoemd. Hoewel torpor een onderdeel van de winterslaap is, betekent dit niet dat dieren tijdens winterslaap continu in torpor zijn. Eigenlijk geldt dit alleen voor beren en onder bepaalde condities voor vetstaartmaki's. Beren zijn namelijk zo groot en goed geïsoleerd dat ze zelfs met een zeer lage stofwisseling niet tot onder de 30 graden afkoelen tijdens torpor. Vetstaartmaki's gaan in de tropen bij een relatief hoge omgevingstemperatuur in winterslaap, waardoor ze niet zover afkoelen.

Grofweg zijn er drie manieren waarop dieren torpor gebruiken om energie te besparen. De eerste is winterslaap met ononderbroken torpor, zoals bij beren. Dieren die continu in torpor gaan koelen niet af onder de ca. 30 ˚C (beren), of gebruiken de variatie in de omgevingstemperatuur om regelmatig tot boven de ca. 30 ˚C op te warmen (vetstaartmaki's). De tweede manier is diepe winterslaap. Dat is winterslaap met lange periodes van diepe torpor (meerdere dagen – weken), afgewisseld met korte periodes (0.5 – 2 dagen) waarin de stofwisseling weer opgereguleerd wordt tot normale waarden en de dieren volledig opwarmen. Deze manier wordt bijvoorbeeld gebruikt door kleine tot middelgrote knaagdieren, zoals hamsters, marmotten en grondeekhoorns, maar ook door vleermuizen. Tijdens diepe torpor koelen deze dieren af tot temperaturen vlak boven de omgevingstemperatuur. Dat kan zelfs lichaamstemperaturen rond het vriespunt opleveren. De derde manier is het gebruik van dagelijkse torpor. Dagelijkse torpor wordt vooral gebruikt door kleine dieren, zoals bijvoorbeeld muizen. Deze dieren gaan niet echt in winterslaap, maar integreren een torporperiode van een paar uur in hun dagelijkse activiteitspatroon. Dieren die korte dagelijkse torpor gebruiken koelen niet af onder de ca 14 °C tijdens torpor. Figuur 1 in hoofdstuk 1 laat voorbeelden van het verloop van de lichaamstemperatuur tijdens dagelijkse en diepe torpor in goudhamsters te zien.

Koude hersenen

Vooral de afwisseling tussen torpor en periodes met een normale stofwisseling in diepe winterslaap, intrigeert biologen al meer dan honderd jaar. Het tussendoor opwarmen kost erg veel energie, tot 80% van de totale hoeveelheid energie die tijdens het hele winterslaapseizoen uitgegeven wordt. Dit beïnvloedt de conditie, overlevingskansen en het voortplantingssucces van bijvoorbeeld Europese grondeekhoorns in de periode na de winterslaap negatief. In theorie zou het voor deze dieren dus voordeliger zijn om net als een beer gewoon een paar maanden koud te blijven en niet tussendoor op te warmen.

De reden waarom winterslapers toch tussendoor opwarmen is nog steeds niet bekend, maar er zijn wel een aantal hypotheses over geformuleerd. In Groningen is veel onderzoek gedaan naar hersengerelateerde hypotheses met betrekking tot deze vraag. Hersenen gebruiken onder normale warme omstandigheden veel energie en zijn erg gevoelig voor verstoringen in de energietoevoer. Dat maakt hersenen in theorie erg gevoelig voor de enorme reductie in stofwisseling die optreedt tijdens de winterslaap. Juist de hersenen zijn erg belangrijk voor een dier, ook in winterslaap. Elke winterslaper moet om te overleven minimaal één keer opwarmen (namelijk aan het einde van de winterslaap) en heeft daarvoor intacte en goed functionerende hersenen nodig om het opwarmproces op te starten en te aan te sturen. Een van de hersengerelateerde hypotheses is dat winterslapers moeten opwarmen om te slapen. In Groningen zijn een aantal voorspellingen die voortvloeien uit die hypothese getoetst. Inderdaad blijkt torpor als slaapdeprivatie te werken, en verlengt verdere slaapdeprivatie de duur van de warme episodes, zoals voorspeld. Maar het slaap-EEG gedraagt zich anders dan verwacht. Dit leidde tot de iets algemenere hypothese dat de warme periodes in winterslaap er voor zorgen dat geen blijvende schade ontstaat aan de zenuwcellen in de hersenen.

In Europese grondeekhoorns zijn inderdaad diverse aanwijzingen gevonden dat de hersenen inderdaad aan grote cyclische veranderingen onderhevig zijn tijdens torpor en de warme periodes in winterslaap. Het aantal contacten tussen zenuwcellen gaat omlaag tijdens torpor en in bepaalde hersengebieden vermindert ook het vermogen tot chemische communicatie tussen zenuwcellen, de basis van het functioneren van de hersenen als geheel. Daarnaast treedt er een proces op waarbij er zich tijdens torpor grote hoeveelheden fosfaatgroepen op specifieke plekken aan een eiwit dat onderdeel uitmaakt van het zogenaamde neuronaal skelet van de zenuwcellen binden. Dit proces wordt *tau-eiwit hyperfosforylatie* genoemd. Het neuronaal skelet van een zenuwcel zorgt voor de stevigheid en de structuur van zenuwcellen. Daarnaast is het neuronaal skelet betrokken bij allerlei transportprocessen binnen de cel. Uiteindelijk kunnen de ophoping van fosfaatgroepen er toe leiden dat het tau eiwit gaat opkrullen, waardoor het neuronaal skelet beschadigt raakt en de zenuwcel uiteindelijk zelfs kan afsterven. Wat er overblijft van zo'n afgestorven zenuwcel wordt dan een tangle genoemd. Als tangles en de voorloper daarvan, de ophopingen van fosfaatgroepen op het tau-eiwit, waargenomen worden in de hersenen van mensen, dan betekent dat meestal dat ze aan de ziekte van Alzheimer, of een andere hersenziekte leden voor ze stierven.

Verrassend genoeg bleken de hiervoor beschreven veranderingen in de zenuwcellen volledig omkeerbaar in winterslapende Europese grondeekhoorns. Zodra de dieren opgewarmd zijn uit torpor zijn alle, potentieel negatieve, effecten op de zenuwcellen verdwenen. Deze verandering ondersteunt het idee dat opwarmen uit torpor noodzakelijk is om onderhoud aan de hersenen te plegen. Ook zou het onderzoeken van de dynamiek van de Alzheimer achtige veranderingen in de hersenen van winterslapers ons inzicht in een aspect van de ziekte van Alheimer kunnen bieden.

Onderzoeksvragen in dit proefschrift

Bij aanvang van dit PhD project in 2005 waren de hiervoor beschreven effecten van torpor op de hersenen alleen gemeten in Europese grondeekhoorns. Het eerste doel was daarom vast te stellen of de in grondeekhoorns gevonden herseneffecten ook te meten waren in een andere diep winterslapende soort: de Syrische hamster (Goudhamster). De resultaten daarvan worden beschreven in de ***hoofdstukken 2 en 3*** van dit proefschrift. Daarnaast wilden we een stap verder gaan en meten wat er gebeurt in de hersenen tijdens het proces van opwarmen en afkoelen. ***Hoofdstuk 4*** beschrijft een experiment waarin we onderzoeken doen naar het verloop van de hoeveelheid fosfaatgroep ophopingen in de hersenen van hamsters met een verschillende temperatuur tijdens het opwarmen uit torpor en tijdens het afkoelen aan het begin van de torpor fase. In ***hoofdstuk 5*** wordt tenslotte onderzocht of diepe torpor in hamsters, net als in grondeekhoorns, ook schade aan het ruimtelijk geheugen veroorzaakt. Samen vormen deze vier hoofdstuken het ***eerste deel***, ***"Winterslaap"***, van het proefschrift.

In het ***tweede deel*** van het proefschrift, ***"Natuurlijke en geïnduceerde dagelijkse torpor"*** wordt de vertaalslag van diepe naar minder diepe dagelijkse torpor gemaakt. In ***hoofdstuk 6*** worden de effecten van dagelijkse torpor op de hersenen van Siberische hamsters (dwerghamsters) gemeten. Daarnaast maken we de stap naar het onderzoeken van muizen, een veelgebruikte diersoort in medisch onderzoek. ***Hoofdstuk 7*** beschrijft een experiment waarin muizen moeten werken voor hun voer, met torpor en een dramatisch verschoven dag-/nachtritme tot gevolg. In ***hoofdstuk 8*** wordt verder ingegaan op het mechanisme van de torporinductie en de effecten hiervan op de hersenen. ***Hoofdstuk 9*** beschrijft een experiment waarin een torpor-achtige staat in muizen volledig kunstmatig wordt opgewekt door een stofje (5'-adenosine-monofosfaat) in muizen te injecteren bij twee verschillende temperaturen. De effecten op de hersenen worden gemeten. Tenslotte worden de resultaten besproken en geïntegreerd in ***hoofdstuk 10***.

De resultaten

In ***hoofdstuk 2*** is het eiwit synaptophysine gemeten in de hersenen van goudhamsters tijdens de winterslaap. Dit eiwit is in sommige hersengebieden een goede indicator van het vermogen van zenuwcellen om chemisch met elkaar te communiceren. Dit kom omdat synaptophysine voorkomt in de blaasjes die neurotransmitter vervoeren in de cel. Als er weinig synaptophysine gemeten wordt zijn er dus ook weining blaasjes en kunnen zenuwcellen waarschijnlijk minder goed met elkaar communiceren. In grondeekhoorns is er een duidelijke cyclus gemeten in de hoeveelheid van dit eitwit in een specifiek subgebied van de hippocampus (een hersengebied dat onder andere betrokken is bij het ruimtelijk geheugen). Tijdens torpor was de hoeveelheid synaptophysine laag, maar tijdens de warme periodes in de winterslaap was de hoeveelheid synaptophysine weer terug op het niveau van dieren tijdens de zomer. We verwachtten een zelfde cyclus te vinden in goudhamsters, maar tot onze verbazing vonden we dat de hoeveelheid synaptophysine niet varieert tijdens de winterslaap in goudhamsters in ditzelfde hersengebied. Wellicht heeft dat te maken met het feit dat hamsters hun geheugen wel nodig hebben tijdens winterslaap of, omdat de hippocampus van hamsters op een andere manier betrokken is bij de regulatie van de duur van de torporperiodes.

In ***hoofdstuk 3*** onderzoeken we op dezelfde manier als in hoofdstuk 2 opgehoopte fosfaatgroepen op het tau-eiwit (tau-eiwit hyperfosforylatie). We vinden in goudhamsters, net als in grondeekhoorns, een duidelijk cyclisch patroon tijdens de winterslaap. Tijdens torpor is er veel tau-eiwit hyperfosforylatie. Zodra de hamsters opgewarmt zijn is de tau-eiwit hyperfosforylatie verdwenen. Daarnaast valt het op

dat de tau-eiwit hyperfosforylatie specifiek lijkt te zijn voor een bepaald type (de cholinerge) zenuwcellen. Dit type zenuwcellen is ook het type dat als eerste getroffen wordt in mensen met de ziekte van Alzheimer. Hamsters in torpor lijken dus een aantal kenmerken van mensen met de ziekte van Alzheimer te hebben en zouden wellicht een goed onderzoeksmodel voor tau-eiwit hyperfosforylatie tijdens deze ziekte kunnen zijn.

In ***hoofdstuk 4*** zoomen we in op wat er gebeurt tijdens de periodes in de winterslaap met de grootste veranderingen in lichaamstemperatuur. Tijdens het opwarmen uit torpor gaan hamsters in een periode van 2.5 uur van 5 °C naar ca. 37 °C en tijdens het afkoelen overbruggen ze dit verschil in tegenovergestelde richting in ongeveer 12 uur. Het eerste dat opvalt is dat hamsters niet overal in hun lichaam even snel opwarmen. We hebben hersen-, schedel- en lichaamstemperatuur gemeten tijdens het opwarmen uit torpor. We zien dat hamsters eerst hun hersenen opwarmen en daarna pas de rest van hun lichaam. Op sommige momenten tijdens het opwarmen kan er een temperatuurverschil van wel 18 °C tussen de hersenen en het onderlichaam zijn. We hebben vervolgens bij verschillende hersentemperaturen de hoeveelheid tau-eiwit hyperfosforylatie in diverse hersengebieden gemeten. We zien dat de hoeveelheid fosfaatgroepen op het tau-eiwit niet geleidelijk toeneemt als de temperatuur afneemt en vice versa. In plaats daarvan vinden we dat er een grenstemperatuur van ongeveer 28 °C is. Onder die temperatuur is er tau-eiwit hyperfosforylatie, die nauwelijks nog verder toeneemt als dieren langer in torpor zijn. Boven die temperaturr verdwijnen de fosfaatgroep ophopingen.

Hoofdstuk 5 beschrijft een experiment waarin het ruimtelijk geheugen van hamsters getest wordt voor en na de winterslaap. Hamsters hebben de plaats van een voedselbeloning in een ingewikkelde doolhof geleerd, voorafgaand aan de winterslaap. Vervolgens is een groep dieren drie maanden in winterslaap gegaan bij 5 °C terwijl een andere groep dieren bij 20 °C gehuisvest is, zodat ze niet in winterslaap konden gaan. Aan het einde van die drie maanden is het geheugen opnieuw getest. Beide groepen dieren wisten na die periode nog steeds waar het voedsel in de doolhof lag. Dat betekent dat het ruimtelijke geheugen in goudhamsters niet wordt aangetast tijdens de winterslaap. Dit wijkt af de situatie in grondeekhoorns, die ruimtelijke geheugen wel kwijt zijn na de winterslaap. Het past wel in de verschillende leefsituaties van de beide soorten. Grondeekhoorns eten en drinken niets tijdens de hele winterslaap. Hamsters eten wel in de warme periodes in de winterslaap en moeten waarschijnlijk de route naar hun voedselvoorraad onthouden. Zij hebben er dus belang bij om hun geheugen intact te houden. Dit past ook in het beeld van hoofdstuk 2, waar we zien dat de hoeveelheid synaptophysine in de hippocampus, die erg belangrijk is voor het ruimtelijk geheugen, niet omlaag gaat tijdens torpor in Goudhamsters.

In ***hoofdstuk 6*** laten we zien dat er ook tau-eiwit hyperfosforylatie optreedt tijdens de minder diepe en veel kortere dagelijkse torpor in Siberische hamsters. Ondanks dat deze dieren niet verder afkoelen, dan tot ongeveer 20 °C in ongever 6 à 7 uur, zien we toch de ophopingen van fosfaatgroepen op het tau-eiwit in ht neuronaal skelet. Zeven uren nadat de hamsters weer opgewarmd zijn, is de situatie weer volledig omgekeerd en zijn de ophopingen van fosfaatgroepen weer verdwenen.

In ***hoofdstuk 7*** induceren we op een natuurlijke manier torpor in huismuizen door ze hard te laten werken voor hun voer in een loopwiel. Daarbij vinden we verassend genoeg ook sterke effecten op het dagelijkse ritme van de muizen. Normaal gesproken zijn muizen nachtactief als ze in het lab gehuisvest worden. Zodra ze echter moeten werken voor hun voer, verschuiven ze opeens een groot gedeelte van hun activiteit naar de dag, waarbij de torporperiodes in de nacht terecht komen. We denken dat de

muizen dit doen, om de torpor op het koudste moment van de dag te kunnen doen. Op deze manier kunnen ze maximaal profiteren van de energiebesparing die de torpor hun oplevert.

Hoofdstuk 8 beschrijft opnieuw een experiment waarin muizen moeten werken voor hun voer. We proberen inzicht in het mechanisme van de torporinductie te krijgen door een groep muizen te laten werken voor voer en op hetzelfde moment voor elke werkende muis automatisch een andere muis exact dezelfde hoeveelheid voer te geven, zonder zonder dat die muis ervoor hoeft te werken. We zien dat er topor samen met ritmiekverschuivingen optreden in beide groepen muizen. De hoeveelheid torpor en de verschuiving van het dagelijkse activiteitspatroon zijn wel groter in de muizen die moeten werken voor hun voer. Dit toont aan dat niet zozeer het werken, maar meer het gebrek aan voedsel (en dus energie) muizen noodzaakt om in torpor te gaan. Daarnaast hebben we ook weer gekeken naar de effecten van de geïnduceerde torpor op de hersenen van de muizen. Het blijkt dat ook deze vorm van dagelijkse torpor gepaard gaat met tau-eiwit hyperfosforylatie tijdens de koude periode.

In ***hoofdstuk 9*** induceren we op een niet natuurlijke manier een torpor-achtige staat in muizen door ze te injecteren met een 5′-adenosine-monofosfaat. Deze stof zorgt ervoor dat de muizen afkoelen tot vlak boven de omgevingstemperatuur. We hebben muizen geïnjecteerd bij twee verschillende omgevingstemperaturen (21 °C en 30 °C). We zien dat de duur van de torpor-achtige staat afhankelijk is van de omgevingstemperatuur. Bij de hogere temperatuur wordt de periode waarin de muizen een lagere lichaamstemperatuur vertonen korter. Vervolgens hebben we op verschillende manieren in de hersenen gekeken naar de hoeveelheid tau-eiwit hyperfosforylatie. We zien bij de muizen bij een omgevings temperatuur van 21 °C dezelfde cyclus in hyperfosforylatie tijdens torpor en opwarming als bij goudhamsters, Siberische hamsters en muizen die moeten werken voor voer. In de muizen die bij 30 °C gehuisvest waren, was er geen duidelijke toename in de hoeveelheid hyperfosorylatie tijdens torpor. Die bevinding komt overeen met de temperatuurgrens voor tau-eiwit hyperfosforylatie van 28 °C in goudhamsters die we in hoofdstuk 4 gevonden hebben.

In ***hoofdstuk 10*** worden de gevonden resultaten besproken en ingepast in de bestaande literatuur over winterslaap en tau-eiwit hyperfosforylatie. Daarnaast wordt er een iedee geopperd voor een mogelijke rol van tau-eiwit hyperfosforylatie als regelmechanisme in het torpor proces.

Conclusies

Dit proefschrift draagt bij aan onze kennis van de hersenen tijdens extreme omstandigheden, zoals torpor. We laten zien dat er veel gaande is in de hersenen van winterslapers tijdens torpor. We tonen aan dat fosfaat ophopingen op het tau eiwit tijdens torpor voorkomen bij diverse soorten en dat deze ophopingen plotseling verschijnen en verdwijnen bij een hersentemperatuur van 28-30 °C in goudhamsters. De tau-eiwit hyperfosforylatie tijdens torpor heeft sterke overeenkomsten, maar ook verschillen met de hyperfosforylatie tijdens de ziekte van Alzheimer bij mensen. Hoewel winterslapers duidelijk niet aan de ziekte van Alzheimer lijden, kunnen vooral de torporinductie modellen in muizen wel degelijk veel waarde hebben voor het bestuderen van de regulatie mechanismen die betrokken zijn bij de tau-eiwit hyperfosforylatie, één van de factoren die ook een rol speelt bij de ziekte van Alzheimer in mensen.

Fryske gearfetting

Wintersliep en torpor

By it wurd wintersliep wurdt meastal tocht oan dieren dy't moannen oanien kâld binne en de winter trochbringe yn har hoalen sûnder te iten of te drinken, om sa de grime omstannichheden fan 'e winter te ûntwiken. Koartsein, in foar de dieren rêstich skoft, dêr't suver neat yn bart dat de muoite fan it bestudearjen wurdich wêze kinne soe. Yn grutte halen is dit byld ynoarder, mar it is dochs ek wer net hielendal korrekt. Om te begjinnen, der dogge har yn it wintersliepskoft krekt alderhanne nijsgjirrige dingen foar. Yn dit proefskrift wurdt op in pear dêrfan neier yngien.

De measte waarmbloedige dieren dy't in wintersliep hâlde — wintersliep komt foar by ûnderskate dieren, fan mûzen oant bearen, en sels by lemueren — sykje in smout plakje op, as se yn wintersliep geane. Om enerzjy te besparjen, sette se it dêrnei mei harren stofwiksel op in sêft sin. De stofwiksel is it proses fan it omsetten fan iten en soerstof yn enerzjy en boustoffen foar it lichem. It gefolch fan in fermindere stofwiksel is, dat de dieren ôfkuolje, somtiden ta temperatueren dy't mar krekt boppe de omjouwingstemperatuer út komme. Sa'n skoft fan in ferlege stofwiksel wurdt torpor neamd. Al is torpor in ûnderdiel fan de wintersliep, dieren binne yn 'e wintersliep net de hiele tiid yn torpor. Eins jildt dat allinnich foar bearen, en ûnder beskate kondysjes foar vetsturt lemueren. Bearen binne nammentlik sa grut en sa goed isolearre, dat se sels mei in tige lege stofwiksel net oant ûnder de 30°C ôfkuolje yn torpor. Vetsturt lemueren libje yn de tropen, dêr't in relatyf hege omjouwingstemperatuer is, dy't makket dat se yn 'e wintersliep net sa bot ôfkuolje.

Rûchwei binne der trije wizen hoe't dieren torpor brûke om enerzjy te besparjen. De earste is wintersliep mei net ûnderbrutsen torpor, lykas by bearen. Dieren dy't de hiele tiid yn torpor binne, kuolje net ôf ûnder likernôch 30°C (bearen), of se brûke de fariaasje yn de omjouwingstemperatuer om geregeldwei op te waarmjen oant boppe likernôch 30°C (vetsturt lemueren). De twadde wize is djippe wintersliep. Dat is wintersliep mei lange skoften fan djippe torpor (fan in dei- oant in wykmannich), ôfwiksele mei koarte skoften (in heale dei oant twa dagen) fan it opregulearjen fan de stofwiksel ta normale wearden; dy soargje derfoar dat de dieren wer hielendal opwaarmje. Dizze metoade wurdt bygelyks brûkt troch lytse oant middelgrutte kjifdieren [knaagdieren] — lykas hamsters, marmotten en grûnykhoarntsjes —, mar ek troch flearmûzen. Yn djippe torpor kuolje de dieren ôf oant krekt boppe de omjouwingstemperatuer; dat kin sels lichemstemperatueren fan om it friespunt opsmite. De tredde wize is it brûken fan deistige torpor. Dy wurdt foaral brûkt troch lytse dieren, lykas mûzen. Sokke dieren geane net echt yn wintersliep, mar yntegrearje torpor-skoften fan in pear oeren yn it deistige aktiviteitepatroan. Dieren dy't koarte deistige torpor brûke, wurde yn torpor net kâlder as likernôch 14°C. Figuer 1 yn haadstik 1 lit foarbylden sjen fan it ferrin fan de lichemstemperatuer yn goudhamsters by deistige en djippe torpor.

Kâlde harsens

Foaral de ôfwikseling tusken torpor en skoften mei in normale stofwiksel yn djippe wintersliep hat al mear as hûndert jier de niget fan biologen. It tuskentroch opwaarmjen kostet in hiele protte enerzjy (oant 80% fan de totale enerzjy dy't yn it hiele wintersliepseizoen brûkt wurdt). Yn it skoft nei de wintersliep hat dit in negatyf effekt op de kondysje, de oerlibbingskânsen en it fuortplantingssúkses fan bygelyks Europeeske grûnykhoarntsjes. Yn teory soe it dus foardiel opleverje moatte om, krekt as in bear, gewoan in pear moanne kâld te bliuwen en net tuskentroch op te waarmjen.

De reden wêrom't wintersliepers tuskentroch dochs opwaarmje, is noch altiten net bekend, mar der binne wol guon hypotezen oer formulearre. Yn Grins binne mei it each op dizze fraach gâns hypotezen ûndersocht dy't in relaasje lizze mei de harsens. De harsens brûke ûnder normale waarme omstannichheden in protte enerzjy, en binne tige gefoelich foar fersteuringen yn 'e tafier fan enerzjy. Dat makket harsens yn teory o sa gefoelich foar de grutte reduksje yn stofwiksel dy't him foardocht yn de wintersliep. De harsens binne tige fan belang foar in dier, krekt yn de wintersliep. Elke winterslieper moat, sil er oerlibje, op syn minst ien kear opwaarmje (nammentlik oan 'e ein fan de wintersliep) en dêrfoar moatte de harsens yntakt wêze en goed funksjonearje, want dy moatte it opwaarmingsproses yn gong sette en oanstjoere. Der wurdt wol ûndersteld, dat wintersliepers opwaarmje om sliepe te kinnen. De resultaten en waarnimmingen dy't út ûndersyk nei oanlieding fan dy hypoteze nei foaren kamen, laten by einsluten ta de hypoteze dat de waarme perioaden yn de wintersliep wolris de funksje hawwe kinne om der foar te soargjen dat de senuwsellen yn de harsens gjin skea oprinne.

Undersyk nei Europeeske grûnykhoarntsjes hat ûnderskate oanwizingen opsmiten dat it wier sa is, dat de harsens yn torpor en yn de waarme perioaden yn de wintersliep oan grutte syklyske feroaringen bleatsteld binne. Yn torpor giet it tal kontakten tusken senuwsellen omleech en yn beskate gebieten fan de harsens ferminderet ek it fermogen ta gemyske kommunikaasje tusken senuwsellen, de basis fan it funksjonearjen fan de harsens as gehiel. Dêrnjonken docht him yn torpor it proses foar, dat grutte hoemannichten fosfaatgroepen har op spesifike plakken bine oan in aaiwyt dat ûnderdiel is fan it saneamde neuronale skelet fan de senuwsellen (tau-aaiwyt hyperfosforylaasje). Dit neuronale skelet jout stevichheid en struktuer oan de senuwsellen. Tagelyk is it behelle by alderlei transportprosessen binnen de sel. By einsluten kin it gearheapjen fan fosfaatgroepen derta liede, dat it tau-aaiwyt op begjint te kroljen; dêrtroch rekket it neuronale skelet skansearre en kin it sels sa fier komme, dat de senuwsel ôfstjert. Wat der fan sa'n ôfstoarne senuwsel oerbliuwt, wurdt dan in tangle neamd. As tangles en de foarrinners dêrfan, de gearheappingen fan fosfaatgroepen op it tau-aaiwyt, yn de harsens fan minsken foarkomme, dan betsjut dat almeast dat se de sykte fan Alzheimer of in oare harsensykte hienen, foardat se stoaren.

It die elk nij, doe't bliken die, dat de hjirfoar beskreaune feroaringen yn de senuwsellen by Europeeske grûnykhoarntsjes yn de wintersliep folslein omkearber wiene. Sagau't de dieren opwaarme binne út torpor, binne alle, potinsjeel negative, effekten op 'e senuwsellen weiwurden. Dy feroaring jout stipe oan it idee dat it opwaarmjen út torpor needsaaklik is foar ûnderhâld oan de harsens. It ûndersykjen fan de dynamyk fan de Alzheimer-eftige feroaringen yn de harsens fan wintersliepers soe ús ek ynsjoch yn in aspekt fan de sykte fan Alzheimer biede kinne.

Undersyksfragen yn dit proefskrift

By it begjin fan dit PhD-projekt yn 2005 wiene de hjirfoar beskreaune effekten fan torpor op de harsens eins allinne noch mar metten yn Europeeske grûnykhoarntsjes. It earste doel wie dêrom om nei te gean, oft sokke effekten ek te mjitten wiene yn in oare soarte mei in djippe wintersliep: de Syryske hamster (Goudhamster). De resultaten dêrfan wurde beskreaun yn ***haadstik 2 en 3*** fan dit proefskrift. Dêrnjonken woene wy in stap fierder gean en neigean wat him yn de harsens ôfspilet by it proses fan opwaarmjen en ôfkuoljen. ***Haadstik 4*** beskriuwt in eksperiment dêr't wy yn sjogge nei it ferrin fan de hoemannichte fosfaatgroep-gearheappingen yn de harsens fan hamsters mei in ferskillende temperatuer by it opwaarmjen út torpor en by it ôfkuoljen oan it begjin fan de torpor-faze. Yn ***haadstik 5***, as lêste, wurdt neigien oft djippe torpor yn hamsters, krekt as yn grûnykhoarntsjes, it romtlik ûnthâld ek skea docht. Mei-inoar foarmje dizze fjouwer haadstikken it ***1ste diel, "Wintersliep"***, fan it proefskrift.

Yn it ***2de diel*** fan it proefskrift, ***"Natuerlike en yndusearre deistige torpor"***, wurdt de fertaalslach fan djippe nei minder djippe deistige torpor bestudearre. Yn ***haadstik 6*** wurde de effekten fan deistige torpor op de harsens fan Sibearyske hamsters metten. Dêrnjonken wurdt de fertaalslach nei mûzen makke, in diersoarte dy't in protte yn laboratoaria brûkt wurdt. ***Haadstik 7*** beskriuwt in eksperimint, dat mûzen wurkje moatte foar har foer, mei as gefolch torpor en in dramatyske ferskowing yn it dei/nacht-ritme. Yn ***haadstik 8*** wurdt fierder yngien op it meganisme fan de torpor-ynduksje en de effekten dêrfan op de harsens. ***Haadstik 9*** beskriuwt in eksperimint, dat mûzen op in folslein keunstmjittige wize yn in torpor-eftige steat brocht wurde, troch har by twa ferskillende temperatueren mei in stofke (5'-adenosine-monofosfaat) te ynjektearjen. De effekten op de harsens wurde metten. As lêste wurde yn ***haadstik 10*** de resultaten besprutsen en yntegrearre.

De resultaten

Yn ***haadstik 2*** is it aaiwyt synaptofysine yn de harsens fan goudhamsters yn de wintersliep metten. Yn guon gebieten fan de harsens is dit aaiwyt in goede yndikator fan it fermogen fan senuwsellen om gemysk mei-inoar te kommunisearjen, omdat it foarkomt yn de blaaskes dy't neurotransmitters yn de sel ferfiere. Yn grûnykhoarntsjes komt der in dúdlike syklus oan it ljocht yn hoefolle fan dit aaiwyt oft der foarkomt yn in spesifyk subgebiet fan de hippokampus (it harsengebiet dat ûnder oaren behelle is by it romtlik ûnthâld). Yn torpor wie it kwantum synaptofysine leech, mar yn de waarme perioaden yn de wintersliep wie dat wer werom op it nivo fan dieren yn de simmer. Wy ferwachten inselde syklus te finen yn goudhamsters, mar ta ús fernuvering fûnen wy dat it kwantum synaptofysine yn ditselde gebiet fan de harsens by goudhamsters yn de wintersliep net fariearret. Lichtwol hat dat dermei te krijen, dat hamsters har ûnthâld nedich binne yn de wintersliep, of omdat de hippokampus fan hamsters op in oare wize behelle is by it regulearjen fan de doer fan de torpor-perioaden.

Yn ***haadstik 3*** undersykje wy op deselde wize as yn haadstik 2 de hoemannichte gearheappe fosfaatgroepen op it tau-aaiwyt (tau-aaiwyt hyperfosforylaasje). Wy fine yn goudhamsters likegoed as yn grûnykhoarntsjes in dúdlik syklysk patroan yn de wintersliep. Yn torpor is der in protte tau-aaiwyt hyperfosforylaasje, mar sagau't de hamsters opwaarmje, is dat oer. Dêrnjonken falt it op, dat de tau-aaiwyt hyperfosforylaasje spesifyk liket te wêzen foar in beskaat type senuwsellen (de golinerge). Dit is ek it type senuwsellen dat fûn wurdt yn minsken mei de sykte fan Alzheimer. It liket der dus op, dat hamsters yn torpor guon skaaimerken ien en mien hawwe mei minsken mei de sykte fan Alzheimer; dêrom soene se lichtwol in goed ûndersyksmodel foar tau-aaiwyt hyperfosforylaasje by dizze sykte wêze kinne.

Yn ***haadstik 4*** besjogge wy neier wat him foardocht yn de perioaden yn de wintersliep mei de grutste feroaringen yn lichemstemperatuer. By it opwaarmjen út torpor geane hamsters yn in tiid fan 2,5 oere fan 5°C nei likernôch 37°C, en by it ôfkuoljen dogge se der likernôch 12 oeren oer om ditselde trajekt, mar dan oarsom, ôf te lizzen. Alderearst falt it op, dat hamsters net op alle plakken yn it lichem like fluch opwaarmje út torpor. Wy hawwe de temperatuer metten fan de harsens, de holleplasse en it lichem as gehiel. Wy sjogge, dat earst de harsens opwaarmje en earst dêrnei it fierdere fan it lichem. By it opwaarmjen kin der op in stuit in temperatuerferskil fan wol 18°C tusken de harsens en it ûnderlichem wêze. Dêrnei is by ferskillende temperatueren fan de harsens, yn ûnderskate gebieten, de tau-aaiwyt hyperfosforylaasje metten. Dêr komt út, dat de hoemannichte fosfaatgroepen op it tau-aaiwyt net stadichwei oanwint as de temperatuer ôfnimt en vice versa, mar dat der yn stee dêrfan in grinstemperatuer fan likernôch 28°C is. Dêrûnder nimt de tau-aaiwyt hyperfosforylaasje amper noch ta as dieren langer yn torpor binne, dêrboppe wurde de gearheappingen fan fosfaatgroepen wei.

Haadstik 5 beskriuwt in eksperimint dat it romtlik ûnthâld foar en nei de wintersliep test. Foar de wintersliep hawwe hamsters leard wêr't, yn in kompleks doalhôf, it plak is dêr't se, as beleanning, har iten fine kinne. Dêrnei is in groep dieren trije moanne yn wintersliep gien, wylst in oare groep dieren tahâlde op in plak dêr't it 20°C wie, sadat se net yn wintersliep gean koene. Oan de ein fan dy trije moanne is it ûnthâld op 'en nij test. Beide groepen wisten noch wêr't it iten yn it doalhôf lei, dus yn hamsters wurdt it romtlik ûnthâld troch de wintersliep net oantaast. Dat is oars by grûnykhoarntsjes, dy't it plak fan it iten troch de wintersliep al ferjitte. Soks slút oan by de ferskillende libbenssitewaasjes fan de beide soarten. Hamsters hawwe belang by har romtlik ûnthâld. Yn de waarme perioaden yn de wintersliep ite se en dêrfoar moatte se nei alle gedachten it paad nei de itensfoarried ûnthâlde. Dat past ek yn it byld fan haadstik 2, dêr't wy sjogge dat it kwantum synaptofysine yn de hippokampus, dat tige wichtich is foar it romtlik ûnthâld, yn torpor ek net omleech giet.

Yn ***haadstik 6*** wurdt sjen litten, dat der ek tau-aaiwyt hyperfosforylaasje is yn de minder djippe en folle koartere deistige torpor yn Sibearyske hamsters. Yn sa'n 6 à 7 oeren kuolje dizze dieren ôf, mar net fierder as oant likernôch 20°C. Likegoed sjogge wy gearheappingen fan fosfaatgroepen op it neuronale skelet. Sân oeren neidat de hamsters wer opwaarme binne, is de sitewaasje wer folslein oarsom en binne de gearheappe fosfaatgroepen ferdwûn.

Haadstik 7 beskriuwt in oar eksperimint. Wy hawwe op in natuerlike wize torpor yn húsmûzen yndusearre, troch se yn in rintsjil hurd wurkje te litten foar harren foer. It die ús tige nij, dat wy dêrby ek sterke effekten op it deistich ritme fan de mûzen fûnen. As mûzen yn it lab sitte, binne se gewoanwei nachts aktyf. Sagau't se lykwols wurkje moatte foar harren foer, skoot ynienen in grut part fan har aktiviteit nei de dei op, en komme de torpor-bouts — de fazen mei lege lichemstemperatuer — yn de nacht telâne. Wy tinke, dat de mûzen de torpor mei opsetsin nei it kâldste momint fan de dei ferskowe. Sa kinne se maksimaal profitearje fan de besparring yn enerzjy dy't de torpor harren opleveret.

Yn ***haadstik 8*** wurde de útkomsten beskreaun fan in eksperimint dat liket op dat fan haadstik 5. Mei dit eksperimint besykje wy om grip te krijen op it meganisme fan de torpor-ynduksje. De iene groep mûzen litte wy wurkje foar harren foer, tagelyk krijt de oare groep likefolle foer sûnder dat se derfoar wurkje hoege. Wy sjogge dat har yn beide groepen ferskowingen yn it torpor-ritme en aktiviteitepatroan foardogge. It tal kearen dat de mûzen yn torpor geane en de ferskowing fan it deistige aktiviteitepatroan binne grutter by de mûzen dy't foar harren foer wurkje moatte. Dat lit sjen, dat net salyk it wurkjen, mar mear it tekoart oan iten (en dus enerzjy) mûzen derta twingt om yn

torpor te gean. Dêrnjonken hawwe wy ek wer sjoen nei de effekten fan de yndusearre torpor op de harsens fan de mûzen. It docht bliken dat dizze foarm fan deistige torpor ek lykop giet mei tau-aaiwyt hyperfosforylaasje yn de kâlde perioade.

It yn ***haadstik 9*** beskreaune eksperimint slút oan by dat fan haadstik 7. Wy hawwe op in net-natuerlike wize mûzen yn in torpor-eftige steat brocht, troch se te ynjektearjen mei in 5'-adenosine-monofosfaat. Dy stof soarget derfoar, dat de mûzen ôfkuolje oant krekt boppe de omjouwingstemperatuer. Wy hawwe mûzen ynjektearre by twa ferskillende omjouwingstemperatueren, fan 21°C en 30°C. It docht bliken dat de doer fan de torpor-eftige steat ôfhinget fan de omjouwingstemperatuer. By de hegere temperatuer wurdt de perioade koarter dat de mûzen in legere lichemstemperatuer hawwe. Dêrnei hawwe wy op ûnderskate manieren sjoen nei de graad fan tau-aaiwyt hyperfosforylaasje. By in omjouwingstemperatuer fan 21°C hawwe de mûzen deselde syklus yn hyperfosforylaasje yn torpor en opwaarming as goudhamsters, Sybearyske hamsters en mûzen dy't foar harren foer wurkje moatte. De mûzen dy't yn in omjouwingstemperatuer fan 30°C tahâlden, lieten gjin dúdlike tanimming fan de hyperfosforylaasje yn torpor sjen. Dat strykt mei de temperatuergrins dy't yn haadstik 4 fûn waard.

Yn ***haadstik 10*** wurde de resultaten dy't wy fûn hawwe besprutsen en ynpast yn de besteande literatuer oer wintersliep en tau-aaiwyt hyperfosforylaasje. Der wurdt spekulearre oer de mooglike funksje fan dizze hyperfosforylaasje as regelmeganisme yn it torpor-proses.

Konklúzjes

Dit proefskrift draacht by ta ús kennis fan de harsens ûnder ekstreme omstannichheden, lykas torpor. Wy litte sjen dat der yn de harsens fan wintersliepers by torpor gâns te rêden is. Sa komme der by ûnderskate soarten by torpor fosfaatgearheappingen op it tau-aaiwyt foar; dy gearheappingen ferskine en ferdwine samar ynienen by in harsentemperatuer fan 28-30°C. Dizze tau-aaiwyt hyperfosforylaasje hat grutte oerienkomsten mei de hyperfosforylaasje by de sykte fan Alzheimer by minsken, al binne der ek ferskillen. Kleardernôch hawwe wintersliepers de sykte fan Alzheimer net. Mar likegoed kinne foaral de modellen fan torpor-ynduksje by mûzen sûnder mear fan grut belang wêze foar it bestudearjen fan de regulaasjemeganismen dy't anneks binne mei tau-aaiwyt hyperfosforylaasje, ien fan de faktoaren dy't ek in rol spilet by de sykte fan Alzheimer.

Dankwoord

De mensen die mij een beetje kennen, kunnen vast wel ongeveer inschatten wanneer dit dankwoord voor het grootste deel geschreven is… Inderdaad, 's nachts om een uur of 3, ongeveer 2 dagen nadat het proefschrift eigenlijk bij de drukker had moeten zijn. Ik denk dat ik tot, wanhoop van velen, het "Just in time", of soms het "Just out of time" management tot een ware kunst verheven heb. Hoewel die flexibiliteit een uitermate handige en wellicht onmisbare eigenschapis als je met onvoorspelbare en gemakkelijk te verstoren winterslapende dieren (lees hamsters) te maken hebt, vergt die manier van werken soms toch ook een hoop flexibiliteit van de mensen in je directe omgeving.

Als eerste gaan daarom mijn dank en liefde uit naar Judith en Sietse. Jullie zijn er altijd voor me als dat nodig is, en dat is vaak. Tijdens het project stonden jullie me altijd terzijde met aanmoedigingen, raad, een helpende hand (gezellig met zijn drieën 90 hamsterkooien verschonen op zaterdagochtend bijvoorbeeld) en kritische vragen over de inhoud van dit boekje. Tige dank! Zonder jullie was dit proefschrift er waarschijnlijk niet gekomen en met jullie is het veel meer waard!

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Het leuke aan het schrijven van een dankwoord is dat alle herinneringen aan de afgelopen jaren weer boven komen. Ik heb het voorrecht gehad om gedurende 11 jaar achter mijn interesses aan te mogen jagen in de uiterst stimulerende omgeving van eerst de vakgroep diergedrag en later de vakgroepen chronobiologie/gedragsbiologie en moleculaire neurobiologie. Het begon allemaal tijdens mijn studie biologie met een klein keuze onderwerpje van het vak Oriëntatie op Biologie en Samenleving. Opeengepropt met een stuk of wat mensen in een van de woonkamers van de tijdvrije ruimte maakte ik voor het eerst kennis met Domien en Arjen. Zij wakkerden met succes, mijn nieuwsgierigheid naar de functie van melatonine en daarmee naar het doen van onderzoek aan. Deze goede ervaring kreeg een vervolg tijdens de beide chronobiologie cursussen waarbij we ons vrijwillig door Arjen lieten slaapdepriveren op de afdeling psychiatrie van het, toen nog, Acedemisch Ziekenhuis. Daarna volgde de ethologie cursus waarbij Bernd ons achter kuikens aan liet jagen. In deze periode heb ik van jullie geleerd dat onderzoek doen echt leuk is, Bedankt! Daarna volgden nog de diverse doctoraal onderwerpen (een stuk of 3, geloof ik), en natuurlijk de onvermijdelijke infectie met het winterslaap virus door Arjen...

Uiteindelijk heeft dit er toe geleid dat ik mijn promotie onderzoek ook aan winterslaap ben gaan doen. De aanloop was lang. Dankzij Frank Nürnberger was er in 2003 wat geld beschikbaar om een 1e experiment met winterslapende hamsters op te zetten. Op basis daarvan hebben Serge en Arjen zich tot 2 keer toe ingespannen om een subsidie van ALW te verwerven voor een PhD project, helaas zonder succes. Uiteindelijk werd er op de valreep op creatieve wijze door Paul, Serge, Domien, Eddy en Arjen uit alle hoekjes geld geschraapt en mijn PhD project van in 1e instantie 2.5 jaar was een feit. Dankzij Serge is die 2.5 jaar uiteindelijk aangevuld tot bijna de volle 4 jaar. Tijdens mijn project kon ik bouwen op de ervaring van vijf supervisors. Ondanks die riante hoeveelheid begeleiders had ik toch erg veel vrijheid om het project zelf vorm te geven en daar ben ik erg blij mee.

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List of publications

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Submitted manuscripts

F. Talaei, M.N. Hylkema, H.R. Bouma, A.M. Strijkstra, **A.S. Boerema**, M. Schmidt, R.H. Henning
Mechanisms of reversibility of remodeling of lung tissue during hibernation in the Syrian hamster: involvement of endogenous H_2S formation. *Subitted to the Journal of Experimental Biology*

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“Iedereen in winterslaap”; Article by Maartje Kouwen. *EOS, February 27, 2012*

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