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University of Veterinary Medicine Hannover
Centre for Systems Neuroscience Hannover
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University Louis Pasteur Strasbourg

Torpor and timing:
Impact of endogenously controlled hypothermia on the circadian system of two hamster species.

Thesis

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„Van Burd dött ik ober doch ne gohn, ne?“

„Och du Dösbattel, kannst du ok van Burd gohn?

Büst doch up See, is doch all Woter üm di rüm.“

Gorch Fock „Seefahrt ist not!“

Contents

Abbreviations

AA-NAT	Arylalkylamine-N-acetyltransferase
ARC	Arcuate nucleus
AVP	Arginine-vasopressin
CLOCK	Circadian locomotor output cycles kaput
CRY	Cryptochrome
CNS	Central nervous system
HP	Hibernation protein
DD	Constant darkness
DLG	Dorsal lateral geniculate
DMH	Dorso-medial hypothalamus
H1-4R	Histamine 1-4 receptor
LD	Long day
L:D	Light:dark
LP	Long photoperiod
MR	Metabolic rate
NA	Noradrenaline
OX1R	Orexin receptor 1
OX2R	Orexin receptor 2
PER	Period
REV-ERBα	Orphan nuclear receptor
RORα	Retinoid-orphan receptor
SCN	Suprachiasmatic nuclei
T_a	Ambient temperature
T_b	Body temperature
T	Endogenous period
TM	Tuberomammilary nucleus
VLPO	ventrolateral preoptic area
ZT	Zeitgeber-time

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Introduction

Introduction

Our earth rotates around itself once a day and around the sun once a year, which is why most organisms face periodic changes of their environment. The alteration of day and night is the most important Zeitgeber in our lives and build the framework for all doings. Activity and resting, food and water intake are only the most obvious behaviours that are timed. However, also most physiological processes like body temperature, hormone release, moods and emotions underlie rhythmicity triggered by the light dark cycle (FOSTER and KREITZMAN 2004). The ratio of day and night length changes throughout the year and with it the conditions of living. Humans mostly elude those seasonal variations by electric light, central heating and always available food stocks, but animals have evolved different strategies to live with and adapt to varying conditions of light, temperature, food supply or predator abundance. Seasonal influences are extremely marked at high latitudes, where changes in external conditions are most drastic. Some species, especially birds, migrate over long distances to exploit local resources as best as possible (GWINNER 1996). Mammals often anticipate profound environmental changes throughout the year facilitating survival in winter when energy supply is low, but metabolic costs are high. Generally reproduction and growth are shut down in less inviting seasons. Reproduction is strictly bound to times of the year when resources cover the demanding energy costs of pregnancy and lactation and warrant survival of the offspring. Some species increase their body weight during summer in order to live on fat reserves in winter, until food availability improves again in spring. Others, especially small mammals that can only store a low amount of fat relative to their body mass, even decrease their body weight towards winter – lower body mass costs less energy. Many mammals like sheep, deer, rabbits or bears change their fur to a better insulating coat to decrease heat loss. For endotherms, thermoregulation is the highest expenditure in cold seasons. The rate of heat production is proportionally enhanced to the rate of heat loss to maintain body temperature (T_b) at the desired level allowing maximal physical performance. Energy costs for endotherms are eight times higher than for ectotherms of comparable body size and ambient temperature (T_a) (ELSE and HULBERT 1981) and cannot always be afforded.

Torpor

To lower metabolic costs, many species evolved endogenously controlled hypothermia which is probably the most powerful tool for saving energy in harsh seasons. Those heterotherms appear to use mainly two different forms of torpor to adapt to their environment and their physiological needs as best as possible: hibernation or prolonged torpor in "true" hibernators and daily torpor in the daily heterotherms (Fig. 1). Deep hibernators like e.g. European hamsters (*Cricetus cricetus*), European ground squirrels (*Spermophilus citellus*) or Syrian hamsters (*Mesocricetus auratus*) show a controlled decrease in T_b to values approaching ambient temperature (T_a) during the hibernation season which can last 6-7 months. Periods of deep torpor are interrupted by regular arousals and brief 1-2 day periods of euthermia every 5 to 20 days (KÖRTNER and GEISER 2000). Mostly, the preferred T_b during deep hibernation ranges between 1 and 6 °C, but some species like the Arctic ground squirrel can even tolerate supercooling at -3 °C (BARNES 1989). Hibernating mammals reduce their metabolic costs by around 90% (HELDMAIER et al. 2004) which allows survival entirely on body fat that has been stored prior to winter, making this economizing strategy most effective. Thus, energy intake and expenditure in hibernators are not balanced on a daily, but rather a yearly basis.

Smaller animals like the Djungarian hamster (*Phodopus sungorus*), various bat species, hummingbirds and also lemurs predominantly undergo bouts of daily torpor (GEISER 2004, DAUSMANN 2004). During this relatively shallow form of torpor they decrease T_b depending on T_a to barely lower than 15 °C. Generally, daily torpor is restricted to the circadian resting phase of the animal, hence not exceeding a duration of some hours (GEISER 2004, HELDMAIER et al. 2004). During a bout of daily torpor the extent of hypothermia and hypometabolism, i.e. energy saving, is usually less pronounced than in deep hibernation. Nevertheless, a 60-70% reduction in energy requirements is reached, also because feeding-related activities are strongly reduced in torpid animals (RUF et al. 1991, 1993, SCHMID et al. 2000). The advantage of daily torpor compared to deep hibernation is that territorial and social activities can be maintained during the torpor season. Generally, daily torpor is less strictly seasonal in most species and therefore can also be used as a response to acute energy shortage.

It has been questioned whether different forms of hypothermia underlie different mechanisms, but so far physiological properties have turned out to be very similar (HELDMAIER et al. 2004).

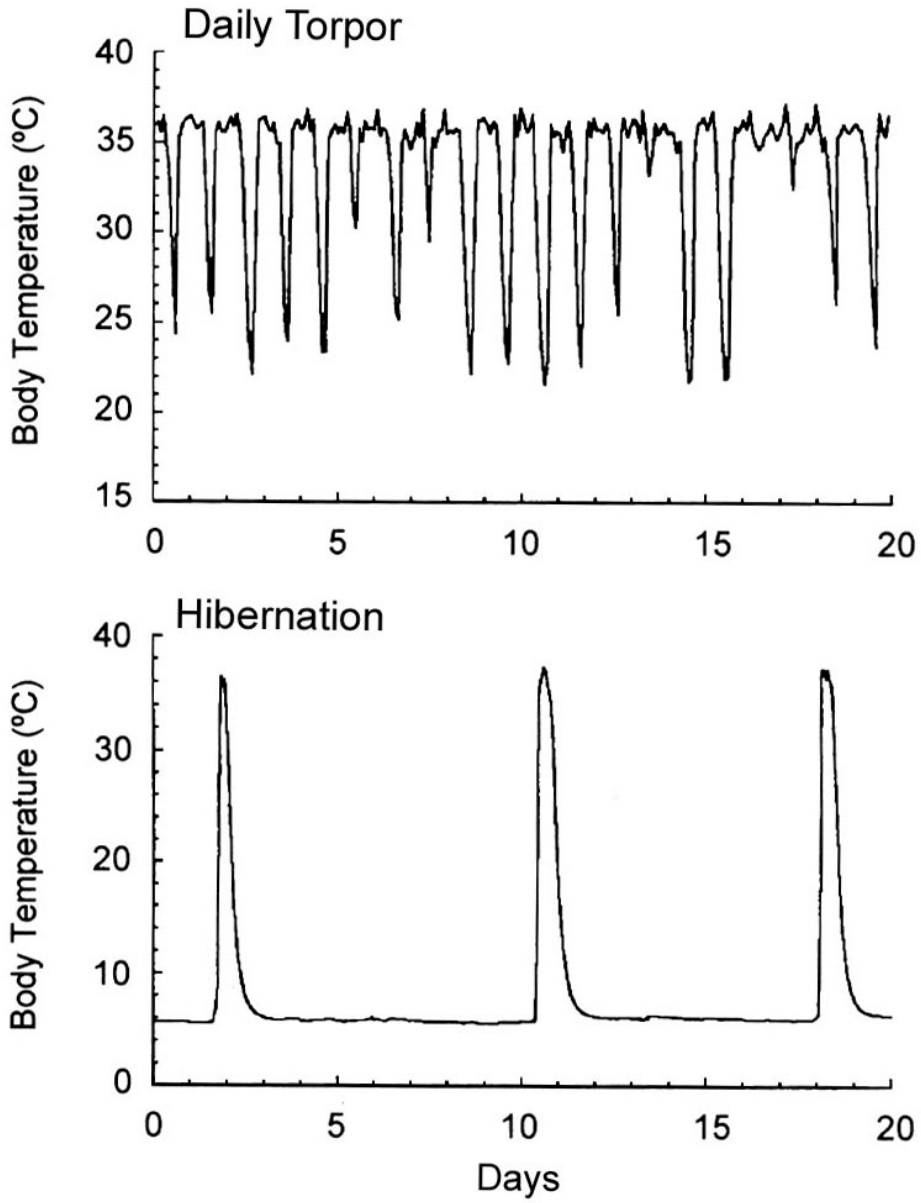

Figure 1: Examples of daily torpor (upper panel) and hibernation (lower panel) over 20 days in midwinter. The minimum T_b achieved during a daily torpor bout of this Djungarian hamster, which was kept at 15 °C T_a, does not drop below 20 °C. Note that daily torpor is not expressed every day. During deep hibernation preferred T_b of golden-mantled ground squirrels generally lies at around 6 °C at 5 °C T_a. Regular arousals occur every 7-8 days in this example (modified from RUBY et al. 2003).

First, metabolic rate is reduced, followed by a drop in T_b which can be precisely regulated and maintained at low degrees, before rapidly increasing again to the normometabolic and normothermic state (GEISER and HELDMAIER 1995, LOVEGROVE et al. 1999,

ORTMANN and HELDMAIER 2000). At present, the different classifications have been considered to simply represent gradual differences in timing, duration and amplitude of an analogue physiological state (HELDMAIER et al. 2004).

Cooling down poses several biochemical problems. Humans cannot even tolerate a decrease in T_b of more than 10 °C (VAN BREUKELEN and MARTIN 2002 a,b) before cardiac arrest and already at a T_b of 34 °C a state of profound exhaustion is reached in which consciousness is severely reduced. Hibernators are capable of attaining, withstanding and reversing low T_bs, so obviously something in their physiology must be different. It has been discussed whether those abilities to reduce rates of biochemical processes are based on specific adaptations and activation of important metabolic pathways, or whether they are undirected simple temperature effects the animal passively needs to tolerate (VAN BREUKELEN and MARTIN 2002b). Regular arousals between torpor bouts would then serve to recover from the "freeze". Certain is that the animal takes advantage of a low T_b in torpor for a profound reduction in biochemical reactions that serve to maintain balance in extremely hypoxic and nutrient deficient periods. Translational processes slow down dramatically during torpor, protein synthesis is affected at the levels of initiation and elongation (KNIGHT et al. 2000, VAN BREUKELEN and MARTIN 2001). Also initiation as well as elongation of transcription is temperature sensitive as expected from Q_{10} effects and ceases during deep hibernation in golden-mantled ground squirrels (BOCHAROVA et al. 1992, VAN BREUKELEN and MARTIN 2002a). Thus, generally central nervous system (CNS) activity is reduced. Nevertheless, it is crucial that functional integrity is maintained. The hibernating brain needs to accurately control the complex metabolic processes and to remain sensitive to external and internal stimuli. Continued CNS function could be demonstrated in Arctic ground squirrels, where metabolic rate was increased when T_a fell below 0 °C indicating that the CNS actively triggers heat production to maintain a constant T_b (BUCK and BARNES 2000).

It seems that specific adaptations keep the cellular and molecular machinery functional and resistant to damage and ensure correct and fast reactivation during interbout arousal. Although transcription is inhibited during torpor (BOCHAROVA et al. 1992), there is no evidence for mRNA loss during hypothermia (FRERICHS et al. 1998, O´HARA et al. 1999). Therefore, it follows that there must be an extension of mRNA half life. Indeed, Poly (A) tail lengths of liver transcripts are not reduced and there is an association of mRNA with a Poly (A) binding protein in torpor. This might prevent transcripts from being degraded and

facilitate translation after rewarming (KNIGHT et al. 2000). Special nuclear bodies (RNPs) have been described in torpid, but not euthermic hibernators (MALATESTA et al. 1994, 1999, 2001, TAMBURINI et al. 1996). They may store transcripts and various splicing factors that then could be rapidly processed for expression during arousal.

Finally, there exists evidence that special brain systems abide full activity during torpor and do not follow expected Q_{10} effects. The subject of this thesis is to describe systems that are supposed to remain functional in the physiological state of endogenously controlled hypothermia better.

The circadian system

One of the systems believed to be active during torpor is the circadian system. On the one hand, this system translates seasonal information to the organism, enabling the animal to anticipate environmental changes well in advance. On the other hand, it times daily events, thus controlling and interlocking various internal physiological processes on a circadian basis, which might be of benefit for animals with rare external time information during the torpor season. The circadian clock is situated in the suprachiasmatic nuclei (SCN) of the hypothalamus and oscillates endogenously in a ~24 hours rhythm (MOORE and EICHLER 1972, STEPHAN and ZUCKER 1972). This endogenous rhythm (τ) that is expressed in constant conditions without any external Zeitgebers (light-dark or temperature cycles) differs between individuals, but is under normal circumstances precisely entrained to the light-dark cycle.

According to current knowledge, the molecular basis of SCN´s oscillation are feedback loops of so-called clock genes which are expressed rhythmically (Fig. 2). Circadian rhythm generation is based on the transcriptional regulation of two sets of genes, *Pers* (*Per1, Per2, Per3*) and *Crys* (*Cry1, Cry2*), by an autoregulatory feedback loop. Transcription of these genes is driven by CLOCK:BMAL1 protein heterodimers acting on E-box sequences in their promoters. *Per* and *Cry* mRNA are built up and followed by an increase of cytoplasmatic concentrations of PER and CRY proteins.

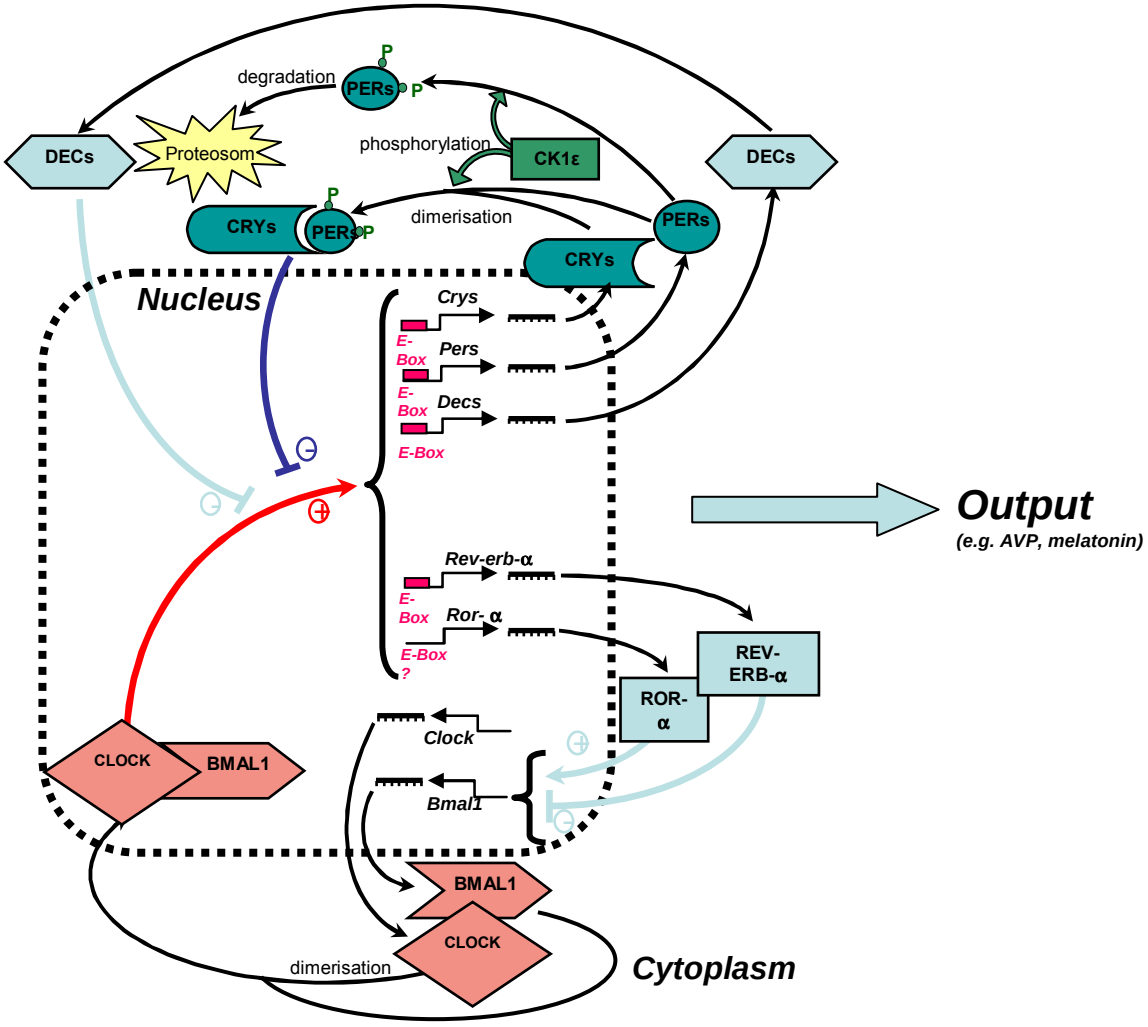

Figure 2: Molecular feedback loops of the circadian clock (SCN). CLOCK:BMAL1 heterodimers stimulate the transcription of *Pers* and *Crys*, *Decs, Reverbs* and *Rors* via e-Box elements. Once translated into proteins in the cytosol, PER and CRY form heterodimers that enter the nucleus, inhibit CLOCK/BMAL and thereby their own stimulation. DECs support this feedback loop. In addition REV-ERBs and RORs also inhibit and activate *Bmal1* transcription to fine tune and stabilize the oscillations of the core loop. Casein kinase 1ε (Ck1ε) modulates the oscillation by facilitating PER degradation. One autoregulatory cycle takes ~24 hours and is under normal circumstances entrained to the environment by external factors of which the most important is the light-dark cycle (provided by Paul PÈVET).

When their concentration in the cytoplasm is high enough, these proteins again form heterodimers which then are likely to enter the nucleus. Here the PER:CRY dimers bind to CLOCK:BMAL1 and thereby prevent its stimulatory action on their own transcription. Other factors like Casein Kinase 1ε, REV-ERB α and ROR α are thought to fine tune the oscillation by either facilitating PER degradation or acting on *Bmal1* expression. Entrainment to the light/dark cycle is arranged through receptors within the retina and their action on *Per1* and *Per2* transcription which is rapidly induced by light (FOSTER and KREITZMANN 2004).

Finally, the molecular circadian machinery forwards the "time" to the organism via different outputs of which the rhythmic melatonin secretion from the pineal gland is the best characterised. Melatonin release is exclusively under SCN control which restricts production of melatonin to night-time by controlling the activity of its rhythm-generating enzyme, arylalkylamine-N-acetyltransferase (*Aa-nat*). Hence, melatonin secretion precisely reflects the day-night ratio. Its most important function is to provide the organism with photoperiodic information and it has been demonstrated to be crucial for seasonal adaptations (STEINLECHNER et al. 1987, PÉVET 1988, BARTNESS 1989, STEINLECHNER 1992, STEINLECHNER and NIKLOWITZ 2002). Another clock output is considered to be arginine-vasopressin (AVP). This neuropeptide is expressed rhythmically in the SCN, peaking during the light phase of the circadian cycle (VAN ESSEVELDT et al. 2000). Its precise role in rhythm generation is not entirely clarified, but it appears that the amplitude in AVP rhythms accounts for the amplitude of behavioural rhythms (VAN ESSEVELDT et al. 2000).

One of the particularities of circadian clocks is that they are strongly temperature compensated. Free running circadian rhythms that are the endogenous rhythms of individual organisms without external Zeitgebers, show Q_{10} values that lie close to 1 in all investigated species (SWEENEY and HASTINGS 1960, PITTENDRIGH 1954, MENAKER 1954, JOHNSON et al. 2004, BELL-PEDERSEN et al. 2005). This makes sense especially for ectotherms. A temperature-sensitive process would be useless as a clock because it would run faster on a warm day than on a cold day. Also endotherms may display large T_b changes and for those hetreotherms as well time-keeping is crucial.

Photoperiodically induced daily torpor is an event that is precisely timed relative to the resting phase of the animal. This timing makes it possible that torpor does not interfere with foraging so that a positive energetic balance can be maintained. Hence, it seems likely that the

circadian system is not disturbed by T_{bs} of ~15 °C as reached during this hypothermia in the Djungarian hamster and is still capable of keeping time.

Also in deep hibernation a functional circadian clock appears useful for synchronising endogenous physiological functions and for timing the regularly occurring interbout arousals. This would even mean that the circadian clock tolerates temperatures below 10 °C.

Studies that addressed the question of a functional circadian clock during daily torpor or hibernation, though, have been controversial (FRENCH 1977, FLORANT et al. 1984, VANECEK et al. 1984, 1985, STANTON et al. 1986, KILDUFF et al. 1989, GRAHN et al. 1994, WOLLNIK and SCHMIDT 1995, KÖRTNER and GEISER 2000, RUBY et al. 2002, LARKIN et al. 2002, HUT et al. 2002, RUBY 2003, HELLER and RUBY 2004).

In chapter 2, 3 and 6 we investigated for the first time the effect of the two different forms of endogenously controlled hypothermia, daily torpor in Djungarian hamsters (Chapter 2, 3) and deep hibernation in European hamsters (Chapter 6), on the very core of the clock, the molecular feedback loops generating rhythms in the SCN. We set up experiments to investigate rhythmic clock and clock related gene expression in normo- and hypothermic animals and tried to reveal whether alterations in rhythm amplitude might be directly temperature dependent. Moreover, to better understand how daily torpor affects the phase of the circadian clock in Djungarian hamsters, we set up trans pineal microdialysis to directly measure melatonin release as a phase marker *in vivo* (Chapter 4, 5).

The histaminergic system

Another system that has already been shown to be upregulated and which plays an important role in deep hibernation is the histaminergic system. The neurotransmitter histamine plays a prominent role in various neurophysiological processes such as in the regulation of wakefulness and thermoregulation in nonhibernators (SCHWARTZ et al. 1991). Already in early studies, it was suggested that histamine acts as a “waking substance” (MONNIER et al. 1970) and hitherto many studies indicate that histamine is required for arousal. Lesioning or inactivation of the posterior hypothalamus has been shown to cause hypersomnia (LIN et al. 1989). Mice lacking histamine show a deficit in waking, attention and interest in a new environment (PARMENTIER et al. 2002). Histaminergic neurons fire at a rate dependent on the behavioural state. Their activity is high during waking and attention and low or absent

during sleep, inhibited by GABAergic neurons from the ventrolateral preoptic area (VLPO) (STEVENS et al. 2001).

In mammals, histamine synthesising neurons are exclusively found in the tuberomammillary nucleus (TM) and project to almost all parts of the brain (INAGAKI et al. 1988, PANULA et al. 1989) where 3 of the 4 cloned histamine receptors (H1-H3) mediate its action. The H1 and H2 receptors mostly excite neurons or potentiate excitatory inputs. By contrast, H3 receptor activation causes autoinhibition of TM neurons and inhibition of neurotransmitter release. H4 receptors are predominantly detected in the periphery (NGUYEN et al. 2001). In addition to histamine, the TM neurons co-express several other neurotransmitters and modulators of which γ-aminobutyric acid (GABA) is probably the most prominent (ERICSON et al. 1991). This is likely to be of functional relevance because TM neurons constitute an extensive far-reaching system that could mediate general inhibition throughout the CNS. General inhibition of CNS activity has also been reported during hibernation (HELLER 1979) and these findings have drawn interest to the histaminergic system in hibernation research. In golden-mantled ground squirrels (*Citellus lateralis*) the histaminergic projections have been demonstrated to be more extensive than in non-hibernators, such as the rat (SALLMEN et al. 1999) which could be interpreted as indication of an enhanced importance of histamine in the CNS of hibernators. Indeed, the rate of histamine turnover is high during hibernation; moreover, histamine infused into the hippocampus prolongs the hibernation bout implying that histamine might be involved in the maintenance of the hibernating state and inhibits the arousal (SALLMEN et al. 1999, 2003b). It could be demonstrated that the histaminergic system blocks the arousal from hibernation via inhibitory H3 receptors which are up-regulated in cortex and basal ganglia in hibernating ground squirrels (SALLMEN et al. 1999, 2003a).

In Chapter 7 we investigated the expression of the H3 receptor during daily torpor in Djungarian hamsters. If torpor indeed underlies mechanisms comparable to deep hibernation we may expect a resembling, but less pronounced up-regulation in Djungarian hamsters compared to deep hibernating golden-mantled ground squirrels.

Orexins

Moreover, we explored in chapter 7 whether reactivation of the other systems could be mediated by orexins. Orexins are a group of neuropeptides in the lateral hypothalamus that have been more and more linked to the arousal state of the animal and probably directly activate the histaminergic system (RODGERS et al. 2001, ISHII et al. 2005). Orexin positive neurons are found in the dorsal, perifornical, posterior and lateral hypothalamic areas and project throughout the hypothalamus including paraventricular, arcuate, lateral, perifornical and ventromedial areas as well as to several extrahypothalamic sites like the septal nuclei, the bed nucleus of the stria terminalis, the paraventricular and reunions nuclei of the thalamus, the zona incerta, the subthalamic nucleus, the central grey, the substantia nigra, the dorsal raphe nuclei, the parabranchial nucleus, the medullary reticular formation, the area postrema and the nucleus of the solitary tract (PEYRON et al. 1998). Two G-protein-coupled receptors (OX1R, OX2R) have been identified and are distributed widely throughout the brain (TRIVEDI et al. 1998). Binding studies indicate that OX1R is selective to orexin A, whereas OX2R binds with similar activity to both orexins (SAKURAI et al. 1998). OX2R appears to be more strongly associated with arousal, as it is the lack of this receptor that causes narcolepsy in dogs. The arousal effect depends on activation of the histaminergic neurons in the TM where solely OX2 receptors are expressed (ERIKSSON et al. 2001, HUANG et al. 2001, ERIKSSON et al. 2004). Moreover, orexin A injection into several brain areas has been shown to provoke enhanced thermogenesis and energy expenditure due to activation of sympathetic nervous system (GEERLING et al. 2003, KOTZ 2006). Therefore, we hypothesised that orexins might be a good candidate for provoking arousal from hypothermia and tested orexin A expression in the lateral hypothalamus in the course of a torpor bout in chapter 7.

All in all, this thesis aims at providing insight into brain systems that are likely to be important for timing, maintaining and reversing endogenously controlled hypothermia.

2

Daily torpor alters multiple gene expression in suprachiasmatic nuclei and pineal gland of the Djungarian hamster (*Phodopus sungorus*).

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Abstract

Circadian rhythms are still expressed in animals that display daily torpor, implying a temperature compensation of the pacemaker. Nevertheless, it remains unclear how the clock works in hypothermic states and whether torpor itself as a temperature pulse affects the circadian system. In order to reveal changes in the clockwork during torpor, we compared clock gene and neuropeptide expression by *in situ* hybridisation in the SCN and pineal gland of normothermic and torpid Djungarian hamsters (*Phodopus sungorus*). Animals from LD 8:16 were sacrificed at 8 time points throughout 24 hours. To investigate the effect of a previous torpor episode on the clock, we killed a group of normothermic hamsters one day after torpor. In normothermic animals *Per1* peaked at ZT4, whereas *Bmal1* reached maximal expression between ZT16 and ZT19. *Avp* mRNA in the SCN showed highest levels at ZT7. On the day of torpor only, *Avp* mRNA contents did not differ from the control group, showing increased mRNA levels during the torpor bout. Moreover, the *Bmal1* rhythm was advanced. On the day after the hypothermia *Bmal1* and *Avp* rhythms showed a severely depressed amplitude. Those distinct amplitude changes in *Bmal1* and *Avp* on the day after a torpor episode expression might give a hint that torpor affects the circadian system, probably by altered translational processes that might lead to a modified protein feedback on gene expression.

In the pineal gland, an important clock output, *Aa-nat* expression peaked between ZT16 and ZT22 in normothermic animals. *Aa-nat* levels were significantly advanced on the day of hypothermia, an effect which was still visible one day afterwards.

In this study we showed that daily torpor affects the phase and amplitude of rhythmic clock gene and clock controlled gene expression in the SCN. Furthermore, the rhythmic gene expression in a peripheral oscillator, the pineal gland, is also affected.

Introduction

One of the particularities of circadian clocks is the strong temperature compensation that has been found in all investigated species from flagellates to mammals. Circadian rhythms show a

Q_{10} close to 1 which has been shown for both homeotherms and heterotherms (PITTENDRIGH 1954, SWEENEY and HASTINGS 1960). However, temperature compensation does not mean that temperature has no impact on circadian organisation at all. It has been shown that changes in temperature may even significantly affect the endogenous period length (τ), both, *in vivo* and *in vitro* (THOMAS et al. 1993, RUBY et al. 1999). This suggests at least a sensitivity of the pacemaker to temperature. It appears, however that changes in body temperature (T_b) rather than ambient temperature (T_a) are necessary to bias circadian organisation in homeothermic animals. Thus, animal models like hibernators or species that display daily torpor can provide insight into the clock´s temperature compensation. Such an animal is the Djungarian hamster (*Phodopus sungorus*). This small rodent from Siberia shows diverse traits that vary seasonally, such as body weight, thermoregulation, reproduction, pelage, pituitary and gonadal hormone concentrations. The photoperiod is the cue for a proper timing of annual rhythms and it is well established that the pineal gland by way of its hormone melatonin conveys the photoperiodic message (PÉVET 1988, BARTNESS and GOLDMAN 1989). The pineal gland transforms the nervous signal coming from the suprachiasmatic nuclei (SCN) of the hypothalamus, the master circadian clock. The molecular basis of the clock are believed to be the clock genes and their expression has been shown to be photoperiod-dependent in Syrian hamsters, rats, mice and also Djungarian hamsters (STEINLECHNER et al. 2002a, CARR et al. 2003, TOURNIER et al. 2003, SUMOVA et al. 2003, JOHNSTON et al. 2005).

Another well-known seasonal adaptation of this species is the daily torpor which is expressed in short photoperiod. During this form of hypothermia body temperature is reduced from 37 °C to 15-25 °C for several hours, whereby around 20 % of daily energy consumption is saved (KIRSCH et al. 1991, RUF and HELDMAIER 1992). In the laboratory torpor occurs spontaneously after ~3 months in short photoperiod and the frequency of torpor bouts can be influenced by food availability and ambient temperature. On average, torpor bouts occur 2-4 times a week.

Daily hypothermia shows a very precise circadian organisation. Entry into torpor generally precedes the time of lights-on in nocturnal species and torpor bouts readily reentrain to phase shifts of the photocycle and they free-run in DD (KIRSCH et al. 1991, RUBY and ZUCKER 1992, RUF and HELDMAIER 1992). After ablation of the SCN, torpor bouts occur at random during the day (RUBY and ZUCKER 1992). These studies demonstrate that it is the circadian system that times daily torpor, and that the clock does not seem to be severely disturbed by low

temperatures during hypothermia. Nevertheless, a study by THOMAS et al. (1993) revealed that torpor shortens the τ of Djungarian hamsters, indicating at least a modulatory effect of temperature on the clock.

In this study we wanted to have a closer look on how the circadian clock works at low temperature at the molecular level and if a torpor bout itself, as a temperature pulse, has even an after-effect on the circadian system.

Material and Methods

Hamsters and experimental set-up

Male and female Djungarian hamsters (*Phodopus sungorus*) from our own breeding colony were raised under long photoperiod (LP; LD 16:8) until 3-6 months of age. Food and water were available *ad libitum* at any time of the experiment. Three months before the experiments started, the LD cycle was stepwise changed to short photoperiod (SP) by reducing the light phase for two hours every two weeks until the final photoperiod of. LD 8:16 was reached. T_a amounted to 18±2 °C. The light intensity during the light phase was between 150 and 300 lux at cage level. A dim red light provided less than 10 lux light intensity during the dark phase.

After three months, torpor bouts occurred spontaneously in most animals. To study the circadian clockwork during torpor, 3 groups of animals were sacrificed at eight time points throughout 24 hours starting with ZT1 (ZT0 = lights on). We first compared animals of a normothermic control group with animals that were killed on the day of torpor. For the normothermic control group (ZT1, 4, 7 n=8; ZT10, 13, 16, 19, 22, n=3) we chose animals that had never been observed torpid, but clearly showed a SP phenotype with decreased body weight and changed pelage. Animals that were killed during torpor had been implanted with radiofrequency transmitters at least one week before sacrifice to continuously measure body temperature (T_b) and to ensure that hamsters were killed during entry into daily torpor (ZT1, n=8), deep torpor (ZT4, n=8) and arousal (ZT7, n=8; all other time points n=4). To investigate the after-effect of the torpor bout on the circadian clock, a third group of animals was sacrificed in a normothermic state on the day following a torpor bout at the same time points (n=4) in LD conditions. Brain tissue was rapidly removed and frozen on dry ice. Samples were stored at -80 °C until analysis.

In situ hybridizations

Serial coronal sections (16 µm) were made through the brain regions containing SCN and pineal gland and were mounted onto Superfrost glass slides and stored at -80 °C.

We used riboprobes of *rPer1* and *mBmal1b* plasmids as described by MENDOZA et al. (2005) that were made available by Dr. H. Okamura, University of Kobe, Japan. *AVP* oligoprobes were kindly provided by Jens Mikkelsen, NeuroSearch, Ballerup, Denmark (MAYWOOD et al. 1995). Fixation and hybridisation were performed as previously described (TOURNIER et al., 2003). For each gene one hybridisation was made including all samples. Hybridized sections were then exposed to autoradiographic films (BioMax, Kodak, Amersham, France) for 5 days. Quantitative analysis of the autoradiograms was performed using Image J software. For each time point, three sections per SCN were analysed. Relative mRNA abundance values were calculated by defining the highest value of each experiment as 100%.

Satistical analysis

All data were analysed using SigmaStat1.0 software (Jandel Corp, San Rafael, CA, USA). To see whether a significant rhythm occurred within one group a one way *ANOVA* was carried out. Comparison between the 3 animal groups was made using a Kruskal Wallis test followed by a *Dunn´s* test *post hoc*. Statistical significance was defined as $P < 0.05$.

Results

In normothermic animals all genes analysed showed temporal changes in mRNA expression in the SCN. In these conditions *Per1* peaked during the day ($P<0.05$), whereas *Bmal1* reached high levels between ZT10 and ZT19 ($P<0.001$). *Avp* mRNA rhythm in the SCN showed maximal expression at ZT7 ($P<0.001$), *Aa-nat* expression in the pineal gland between ZT16 and ZT22 in non-torpid animals ($P<0.001$, Fig. 1).

On the day of torpor *Per1* ($P<0.001$) expression was significantly increased at ZT7 ($P<0.05$) and at ZT22 ($P<0.05$). *Bmal1* ($P<0.001$) showed significantly higher levels at ZT4 ($P<0.05$) during hypothermia, so the onset of expression was advanced. *Avp* rhythm ($P<0.001$) was not different compared to normothermic animals. *Aa-nat* expression ($P<0.001$) showed a

significantly advanced onset at ZT10 (P<0.05) on the day of torpor and reached its maximum at ZT13 (P<0.05, Fig. 1).

One day after torpor *Per1* rhythm (P<0.001) was comparable to the control group again. *Bmal1* showed low mRNA levels with no rhythm on the day following torpor and expression differed significantly from group n at ZT19 (p<0.05) and ZT22 (p<0.05), whereas *Avp* expression (P<0.05) was reduced at ZT4, ZT7 and ZT10 (P<0.05) compared to normothermic animals. *Aa-nat* rhythm (P<0.05) was still advanced and showed significant difference to the control group at ZT13 (P<0.05, Fig. 1).

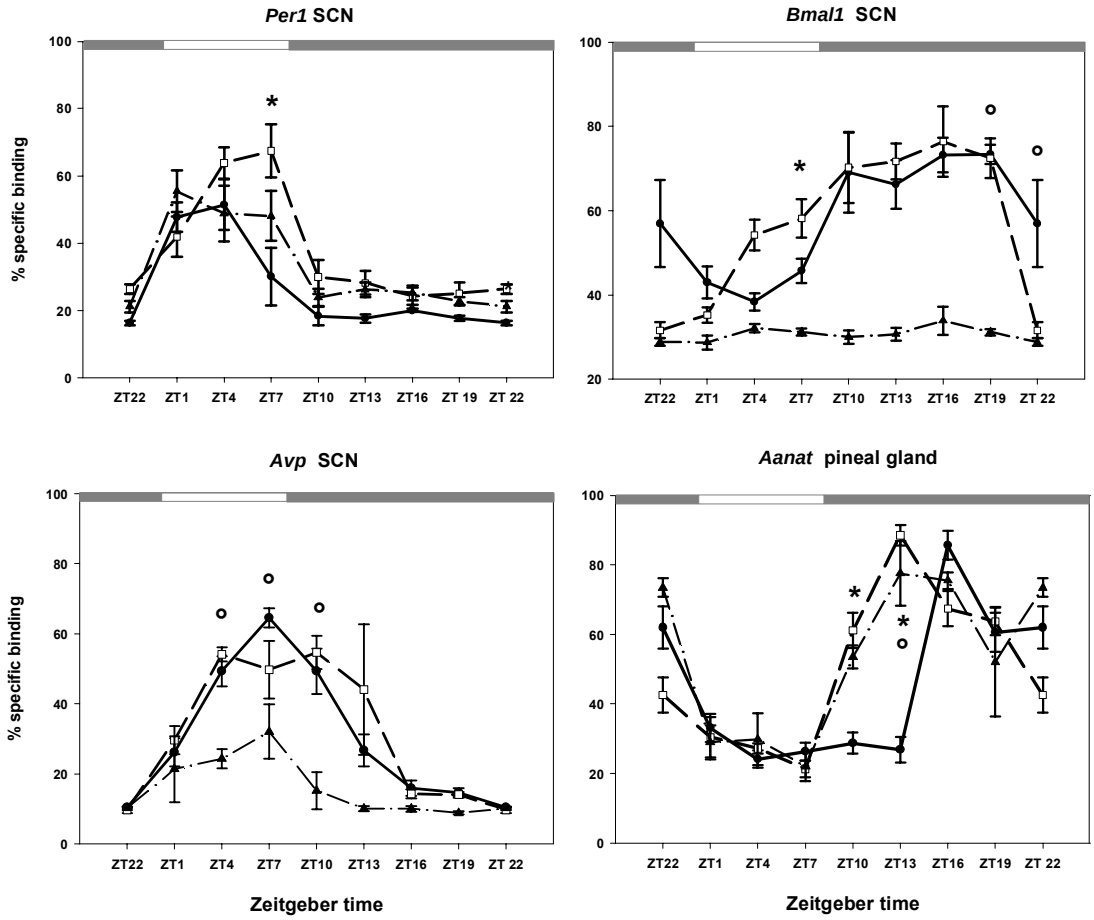

Figure 1: Expression profiles of clock genes and neuropeptides in SCN and pineal gland of Djungarian hamsters. Animals were sacrificed either in a normothermic state (ZT1, 4, 7 n=8; ZT 10, 13, 16, 19, 22 n=3; filled circles, solid line) on a day of torpor (ZT1, 4, 7 n=8; ZT 10, 13, 16, 19, 22 n=4; open squares, dotted line) or one day after a torpor bout in a normothermic state (n=4, filled triangles, dash-dot line). Data are presented as mean ± SEM. Solid and open bars represent the dark and light period. Significant differences are indicated by * for differences between normothermic vs day of torpor and ° for differences between normothermic and day after torpor.

Discusssion

In normothermic animals, expression of the investigated genes in the SCN showed clear rhythmic changes throughout 24 hours. Profiles are comparable to a previous study by Johnston et al. (2005), who showed similar expression patterns for *Per1*, *Bmal1* and *Avp* in the SCN in SP. On the day of torpor, expression of the investigated genes did show significant changes. mRNA levels of *Per1* as well as *Bmal1* were significantly higher during the hypothermia. This could either mean that low temperatures stimulate those genes´ expression or that a delay in protein synthesis results in an accumulation of mRNA. Moreover, the onset of *Bmal1* expression was significantly advanced on the day of torpor. These results are congruent with the prediction of ASCHOFF (1979) that τ becomes shorter in nocturnal species as temperature decreases and the findings of THOMAS et al. 1993) that torpor shortens Djungarian hamsters` circadian rhythms in constant conditions. This makes sense in order to maintain entrainment close to 24 hours on days where no light is seen, as it occurs in nature after a torpor bout which is longer than the light phase. Hereby T_b seems to be the important stimulus rather than T_a (LEE et al. 1990, THOMAS et al. 1993). Of course T_b is to a certain extent dependent on T_a, given that the torpid animal can not drop its temperature below T_a. In our study, animals did not drop their temperature below 22 °C although it is known from other studies that T_b can fall to 15 °C during a torpor bout at lower ambient temperatures (HELDMAIER and STEINLECHNER 1981). It would be worth investigating whether the feedback of hypothermia on the clock might be directly temperature dependent and increases with decreasing T_b. This seems likely as τ changes show a much stronger magnitude in other species like squirrels or pocket mice that show longer periods of hypothermia with a T_b decreased to 3-5 °C above T_a (FRENCH 1977, LEE et al. 1990).

One day after torpor we found severe changes in the amplitude of *Avp* and *Bmal1* expression profiles. *Bmal1* showed no rhythm at all and also *Avp* rhythm was significantly depressed. This is striking given that during hypothermia those rhythms were clearly expressed. A possible explanation for these results might be a modified protein feedback after the hypothermia. Although the transcriptional rhythms` amplitude of the investigated clock genes does not seem to be attenuated by temperature, this does not give information about translational, transport, or other post transcriptional processes. Indeed, protein synthesis seems to be more dramatically affected during hypothermia than gene transcription

(FRERICHS et al. 1998, KNIGHT et al. 2000) and this could lead to a lack of gene induction on the day after torpor. These results, however, need to be confirmed and extended by measuring protein synthesis. The *Per1* rhythm on the day after torpor did not differ from that of the control group. This might be due to the fact that *Per1* is directly induced by light and that our experiment was done in an LD cycle.

The best known clock output, the *Aa-nat* expression within the pineal, showed a late onset in normothermic control animals compared to other literature where *Aa-nat* activity was already elevated 2 hours after dark onset (HOFFMANN et al. 1981). We might have observed a different profile due to strain differences because it is known that melatonin profiles can differ considerably between different strains of the same species and it is likely that those differences may occur as well in *Aa-nat* as in the rate limiting enzyme in melatonin production (BARASSIN et al. 1999). Moreover, it is not clear if the animals from other studies had been torpid during the experiments, and we demonstrate here that torpor seems to change the *Aa-nat* expression profile.

On the day of torpor we observed a strong *Aa-nat* induction in the pineal gland at the very beginning of the night which is likely to be caused by the enhanced sympathetic activation associated with intense thermogenesis during arousal of the animal. Similar results have been obtained by LARKIN et al (2003) demonstrating an increased melatonin production during arousal. This could also potentially contribute to a phase advance (and thereby entrainment) of the circadian clock after a day of torpor. *Aa-nat* mRNA levels in the pineal gland were still advanced on the day after hypothermia which might indicate that there is nevertheless a longer lasting effect of the temperature pulse.

In this study we showed for the first time a direct impact of daily torpor on the molecular clockwork. Further studies have to confirm these results and to reveal whether protein synthesis is altered in the SCN during hypothermia and whether the impact of hypothermia is directly temperature dependent.

3

Daily torpor affects the molecular machinery of the circadian clock in Djungarian hamsters (*Phodopus sungorus*).

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In preparation

Abstract

Daily torpor in the Djungarian hamster (*Phodopus sungorus*) is a precisely timed event gated by the circadian clock situated in the suprachiasmatic nuclei (SCN) of the hypothalamus. Timing controlled hypothermia during which body temperature (T_b) decreases to ~15 °C implies temperature compensation of the circadian system. Nevertheless, it remains controversial as to how the molecular clockwork functions at those low T_bs and whether the torpor bout affects the circadian system. In this study we investigated rhythmic clock and clock related gene as well as protein expression in the SCN and pineal gland of torpid and normothermic Djungarian hamsters over a 48 hours cycle. We demonstrate clearly rhythmic gene expression of *Per1*, *Bmal1* and *Avp* in the SCN as well as *Aa-nat* in the pineal gland on a day of torpor. Alterations in phase and amplitude of these rhythms, however, may be due to decreased protein synthesis during hypothermia. This decreased protein feedback resulting from the hypothermia, might also be responsible for changes in gene expression observed one day after a torpor bout. Although we could not show direct temperature dependency of altered gene expression, we conclude that temperature has at least a modulatory effect on the circadian system.

Introduction

Temperature compensation is one of the fundamental properties of circadian clocks which prescinds their abilities from most other biological processes that are usually very sensitive to temperature in a predictable way. Biochemical reaction rates generally double or triple with every 10 °C increase in temperature, but the Q_{10} of circadian rhythms, at least in the range of temperature tested, lies close to 1 in all species investigated from flagellates to mammals (PITTENDRIGH 1954, SWEENEY and HASTINGS 1960). For ectotherms the need for a temperature compensated clock seems obvious, as their rhythms would otherwise depend on changes of ambient temperature (T_a) and consequently body temperature (T_b), so that synchrony with the most important Zeitgeber, the light-dark (LD) cycle, could not be warranted. But, also endothermic species undergo T_b changes of more than 10 °C. Such

seasonally occurring periods of hypothermia, like hibernation or daily torpor provide good models to study the clock´s temperature compensation in mammals in a precise and controlled range of temperature.

Daily torpor occurs in the Djungarian hamster (*Phodopus sungorus*) as one of various adaptations (body weight reduction, winter coat, gonadal regression) to reduce metabolic costs during harsh Siberian winters (FIGALA 1973). Those distinct changes in morphology and physiology are induced by decreasing photoperiod. Photoperiod is perceived and integrated by the circadian clock located in the hypothalamic suprachiasmatic nuclei (SCN). In LD conditions the endogenous pacemaker oscillates rhythmically over 24 hours, precisely reflecting a day length. This oscillation is based on positive (e.g. BMAL1) and negative (e.g. PER1) feedback loops of so-called clock genes that are expressed rhythmically and generate output signals like vasopressin (AVP) release from the SCN or melatonin secretion from the pineal gland. Their proportional expression has been shown to be photoperiod dependent in various species including the Djungarian hamster (STEINLECHNER et al. 2002a, TOURNIER et al. 2003, SUMOVA et al. 2003, JOHNSTON et al. 2005) and it is well established that seasonal information is conveyed to the organism via melatonin release from the pineal gland (STEINLECHNER et al. 1987, PÉVET 1988, BARTNESS and GOLDMAN 1989, STEINLECHNER 1992, STEINLECHNER and NIKLOWITZ 1992).

In short photoperiod, Djungarian hamsters spontaneously undergo bouts of daily torpor. During this hypothermia T_b is reduced from 37 to 15-25 °C for several hours a day, whereas ~20% of daily energy expenditure is saved (RUF and HELDMAIER 1992, KIRSCH et al. 1992). Torpor occurs spontaneously on average 2-4 times a week, but frequency, amplitude and duration of the bouts can be influenced by food availability and T_a. Lower T_as result in lower T_bs during torpor which never drop far below 15 °C, though (RUF et al. 1993).

The temporal organisation of daily torpor is accurately timed by the circadian system. Entry into hypothermia generally precedes the light phase, torpor bouts quickly reentrain to shifts of the photocycle, free run in constant darkness and occur at random times of the day after SCN lesions (KIRSCH et al. 1991, RUBY and ZUCKER 1992, RUF and HELDMAIER 1992). These behavioural findings suggest that the circadian clock compensates for the temperature effects. However, THOMAS et al. (1993) have shown that daily torpor shortens the free-running period (τ) of Djungarian hamsters probably in a directly temperature-dependent manner, indicating at least a modulatory effect of temperature on the circadian system.

Nonetheless, it is still not clear whether and how temperature affects the core clock on the molecular level or if modulations occur further downstream so that only certain behavioural outputs are altered.

In this study we wanted to clarify if the molecular machinery of the circadian clock is affected by the low T_bs of daily torpor and if there might be a sustained after-effect of the torpor bout (= low temperature pulse) on the circadian system. Therefore, we investigated clock gene (*Bmal1*, *Per1*) and clock related gene (*Avp*) as well as protein expression in the SCN in normothermic and hypothermic Djungarian hamsters. Furthermore, we studied the expression of *Aa-nat,* the rate limiting enzyme for melatonin in the pineal gland, another very well defined clock output. Moreover, by setting up different experiments in different T_as resulting at different T_bs, we addressed the question whether changes in clock gene expression might be directly temperature dependent.

Materials and Methods

Animals

Male and female Djungarian hamsters (*Phodopus sungorus*) from the breeding colony of the Institute for Zoology of the University of Veterinary Medicine Hannover were raised under long photoperiod (LP) of light-dark (LD) 16:8 (8:00 lights on; 16:00 lights off) and T_a of 22±2 °C until 3 to 6 months of age. Light intensity during the light phase was between 150 and 300 lux at cage level. Constant dim red light gave ~1lux light intensity during the dark phase.

Three months before the experiments, the light period was decreased stepwise for 2 hours every two weeks until a short photoperiod (SP) of LD 8:16 was reached. Then T_a was reduced to 18±2 °C. Bouts of daily torpor occurred spontaneously in most animals 12 weeks after the beginning of decreasing photoperiod. Each sampling was carried out within 2 weeks.

Experiment 1: mRNA expression at 18 ± 2 °C T_a

To assess acute changes in the circadian clockwork during daily torpor and after-effects of the hypothermia, mRNA expression of clock and clock-related genes were investigated in 3

groups of animals. Hamsters were sacrificed by CO_2 every 3 hours throughout a 24 hour cycle starting at ZT1 (ZT0 = lights on = 8.00). A control group consisted of normothermic animals (n) that clearly showed a winter adapted phenotype (pelage colour, decreased body weight), but had previously never been observed as torpid (n = 4-8 per time point). The second group (t) was sacrificed on a day of torpor (n = 4-8 per time point). Animals that were killed during the torpor bout had been implanted with radiofrequency transmitters to continuously measure T_b at least one week before sacrifice, to ensure that brains were removed precisely during entry into hypothermia (ZT1), deep torpor (ZT4) and arousal (ZT7). To investigate possible after-effects of a torpor bout, a third group (t+1) normothermic hamsters was culled on the day following a torpor bout (n = 4 per time point) (Fig.1). After sacrifice brain tissue was rapidly removed and frozen on dry ice. Samples were stored at -80 °C until analysis.

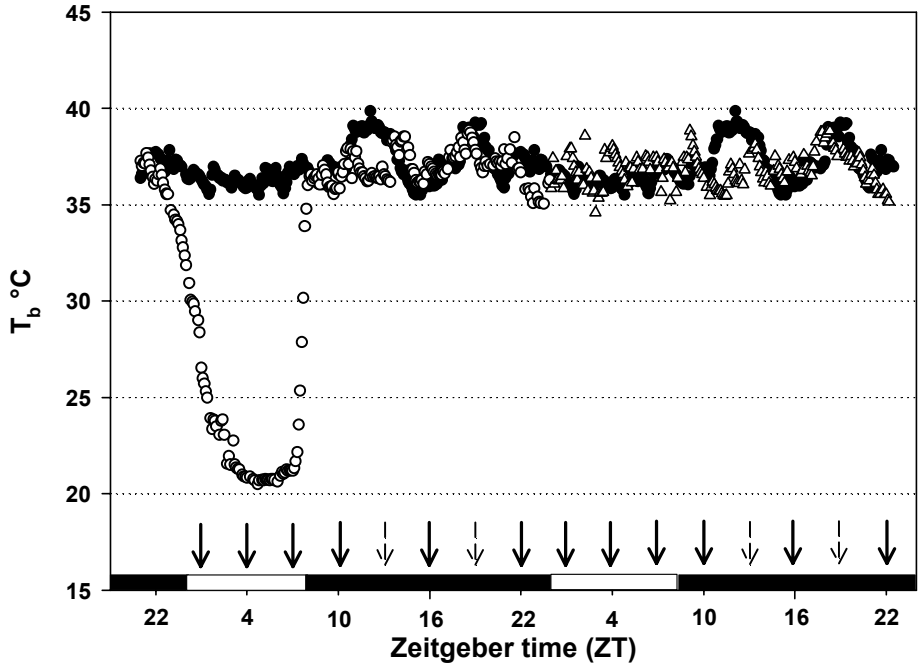

Figure 1: Experimental set-up to investigate changes in gene and protein expression during and after daily torpor in Djungarian hamsters. Three groups of animals were sacrificed throughout a 24 hour LD (8:16) cycle. Values of the normothermic group (group n, black circles) representing the results of one 24 hours cycle were double plotted for better visualisation. This group was compared to a group of animals sacrificed on a day of torpor (group t, open circles). A third group of hamsters was killed one day after a torpor bout (group t+1, open triangles). Arrows represent the sampling points in all experiments, dashed arrows the additional sampling points of experiment 1.

Experiment 2: Protein expression at 18 ± 2 °C T_a

In a second experiment we determined changes in protein expression during and after daily torpor. The experiment was carried out as described for experiment 1. Animals were perfused with 4% paraformaldehyde in 0.1m phosphatebuffer (PBS) (pH 7,4) before brains were removed. To reduce the animal number we decreased the number of control animals to n = 3 for each time point and n = 4 in the two other groups and sampled at ZT1, ZT4, ZT7, ZT10, ZT16 and ZT22, hence leaving out the sampling points ZT13 and ZT19.

Experiment 3: mRNA expression at 4°+2 °C T_a

As T_b during torpor also depends on T_a another experiment was set up at T_a of 4±2 °C to investigate whether changes in clock gene expression during torpor might be directly temperature dependent. One week before sampling T_a was decreased from 18±2 °C to 4±2 °C. Samples were taken at ZT1, ZT4, ZT7, ZT10, ZT16 and ZT22 with n = 3 in the control group and n = 4 in the others and brain tissue was rapidly removed and frozen on dry ice. Samples were stored at -80 °C until analysis.

*In situ hybridization*s

Serial coronal kryostat sections (16µ) of SCN and pineal gland were mounted on Superfrost Plus® glass slides and stored at -80 °C until further treatment.

Antisense and sense RNA probes were generated with an *in vitro* transcription kit (Maxiscript; Ambion, Austin, TX). We used riboprobes of *rPer1* (from plasmids provided by Dr. H. Okamura, University of Kobe, Kobe, Japan), *rVasopressin* (*Avp*) (DARDENTE et al., 2002), and *mBmal1b* (MENDOZA et al. 2005). Fixation and hybridization was carried out as previously described (TOURNIER et al. 2003). For the *Aanat* in situ hybridization we used a mixture of antisense oligonucleotide synthetic probes labelled with ^{35}S ATP, proceeding as reported by SINITSKAYA et al. 2006. Slides were exposed to autoradiographic films (Biomax, Kodak) together with ^{14}C standards for 5 days. Densiometric analysis of the autoradiograms was performed using Image J software. For each animal 3 SCN sections were analysed bilaterally and averaged. Relative mRNA abundance values were calculated by defining the highest value of an individual animal as 100%.

Immunohistochemistry

Brains were post-fixed for 24 hours in 4% paraformaldehyde in 0.1m phosphate buffer (pH 7,4) after removal and kryoprotected in 30% glucose for 3 days. The tissue including the hypothalamus was serially sectioned 25µ on a kryostat and 2 series were mounted on gelatine covered slides for PER1 and AVP staining respectively. After rinsing in 0.2 m PBS for 10 min, slides were cooked for 15 min in 0.01m citrate buffer to permeabilise the tissue. Endogenous peroxidase was blocked for 30 min in 1% H_2O_2 before the sections were rinsed 2x10 min in 0.2m PBS + 0.1% Triton. After blocking non specific binding sites for 1 h in 2% albumin bovine + 2% goat normal serum in 0.2m PBS + 0,1% Triton, sections were incubated with the primary antibody for 24 hours (PER1 1:8000, provided by David Weaver, AVP 1:8000 Fitzgerald), then washed again and incubated 1h with 5% anti rabbit IgG (Vector labs) in 0.2m PBS + 0.1% Triton. The signal was amplified with a 2% avidin/biotin complex (Vectastain Elite ABC Kit, Vector labs) and immunolabelling was developed using 1%nickel-3,3´-diaminobenzidine (Ni-DAB, Sigma).

BMAL1 immunohistochemistry was performed on free-floating sections following the same protocol, leaving out the first step (citrate buffer) and incubating with the first antibody (1:500, Santa Cruz) for 48 hours. Cells were counted in 4 SCN sections per animal using image J software.

Statistical analysis

All data were analysed using Graph Pad Prism 4.0 software. Rhythms in any group were detected using one-way analysis of variance (*ANOVA*). Comparisons between the 3 animal groups were made by a Kruskal Wallis test followed by a Dunn´s test *post hoc*. Statistical significance was defined as $p<0.05$.

Results

T_bs

To asses T_b at the nadir of torpor (ZT4) in the different experimental conditions (T_a 18±2 °C, T_a 4±2 °C), T_b was measured immediately after decapitation in the particular animals. In experiment 1, T_b dropped to 22.6 ± 0.65 °C, in experiment 2 to 18.5 ± 1.04 °C.

Per1

Per1 gene expression at 18 ± 2 °C T_a

In all groups, *Per1* expression showed a clear circadian rhythm ($p<0.05$) with a peak at ZT4 in the control group (n) as well as group t+1. During torpor (t) the peak was shifted to ZT7 and increased ($p<0.05$) compared to group n (Fig. 2a).

PER1 protein expression at 18 ± 2 °C T_a

The PER1 protein was rhythmic in all groups ($p<0.05$) and reached highest values at ZT10 in groups n and t+1. Group t differed from group n at ZT4 ($p<0.05$) and showed a decreased number of immunopositive cells (Fig. 2b).

Per1 gene expression at 4 ± 2 °C T_a

Per1 expression showed a similar pattern as in the 18±2 °C T_a experiment with rhythms in all groups ($p<0.05$) peaking at ZT4 in group n and t+1 and increased expression at ZT7 in group t ($p<0.05$) compared to group n (Fig. 2c).

Bmal1

Bmal1 gene expression at 18 ± 2 °C T_a

Bmal1 was rhythmically expressed in group n and group t ($p<0.05$). Highest amounts of mRNA were detected between ZT10 and ZT16. In group t expression was significantly increased at ZT4 ($p<0.05$). Group t+1 did not show a detectable rhythm and expression differed significantly from group n at ZT19 ($p<0.05$) and ZT22 ($p<0.05$) from group t at ZT13 ($p<0.05$) (Fig. 3a).

BMAL1 protein expression at 18 ± 2 °C T_a

BMAL protein levels were rhythmic in group n ($p<0.05$) with highest values between ZT22 and ZT4 ($p<0.05$). No rhythm could be revealed in group t or t+1. In group t+1 the cell number differed significantly at ZT4 from that of group n (Fig. 3b).

Bmal1 gene expression at 4 ± 2 °C T_a

Bmal1 showed a rhythmic expression in group n and group t ($p<0.05$) and peaked between ZT10 and ZT16. No rhythm was detected in group t+1, values differing from the control group n at ZT10 ($p<0.05$) (Fig. 3c).

Avp

Avp gene expression at 18 ± 2 °C T_a

Avp rhythm was significant in all 3 groups ($p<0.05$). In group n and t the clock related gene was expressed highest between ZT4 and ZT10. Group t+1 showed decreased values at ZT4 ($p<0.05$), ZT7 ($p<0.05$) and ZT10 ($p<0.05$) compared to group n (Fig. 4a).

AVP protein expression at 18 ± 2 °C T_a

AVP protein was rhythmic in group n and peaked at ZT16. No significant rhythm was detected in group t or group t+1. In group t we found decreased cell numbers at ZT10 ($p<0.05$) relative to group n. Group t+1 showed a decreased number of immunopositive cells at ZT10 ($p<0.05$) (Fig. 4b).

Avp gene expression at 4 ± 2 °C T_a

Avp expression showed comparable rhythms ($p<0.05$) to those in the 18 °C T_a experiment with rhythms peaking ZT4 and ZT10 in group n and t and decreased expression at ZT4 ($p<0.05$), ZT7 ($p<0.05$) and ZT10 ($p<0.05$) compared to group n (Fig. 4c).

Aa-nat

Aa-nat gene expression at 18 ± 2 °C T_a

Aa-nat was rhythmic in the pineal gland in all 3 groups ($p<0.05$). In group n the expression peaked between ZT16 and ZT22, in group t and t+1 between ZT13 and ZT22. Increased mRNA amounts were found in group t at ZT10 ($p<0.05$) and ZT13 ($p<0.05$), in group t+1 at ZT13 ($p<0.05$) compared to group n (Fig. 5a).

Aa-nat gene expression at 4 ± 2 °C T_a

Aa-nat rhythms were comparable to those in the 18 °C T_a experiment ($p<0.05$) with gene expression peaking at ZT16 in all groups. In group t expression was increased at ZT10 compared to group n ($p<0.05$) (Fig. 5b).

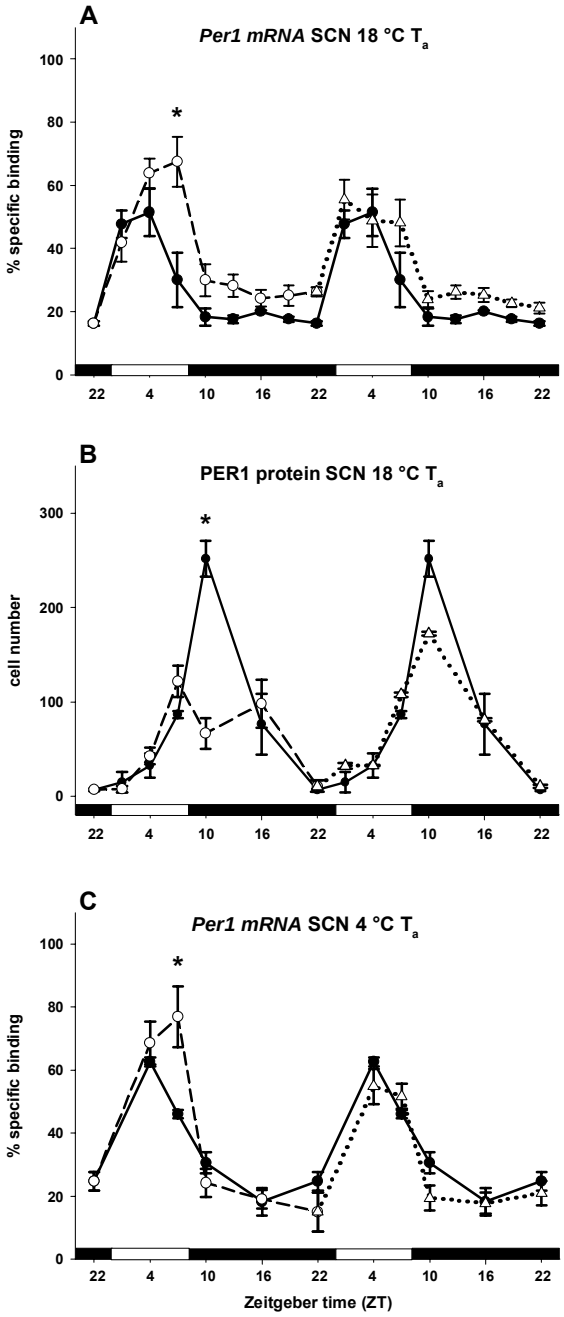

Figure 2a-c*:* mRNA and protein expression of *Per1*/PER1 in the SCN of Djungarian hamsters at different T_as. In each experiment, 3 animal groups were sacrificed throughout a 24 hour cycle. Values of the normothermic control group (n, black circles, solid line) represent the results of one 24 hour cycle and were double plotted. The second group (t, open circles, dashed line) was killed on a day of daily torpor. Finally samples of a third group (t+1, open triangles, dotted line) were taken one day after a torpor bout. Data are presented as mean ± SEM. Solid and open bars represent dark and light periods, respectively. Significant differences are indicated by * for differences between the control group n and group t or t+1, by ° for differences between group t and group t+1.

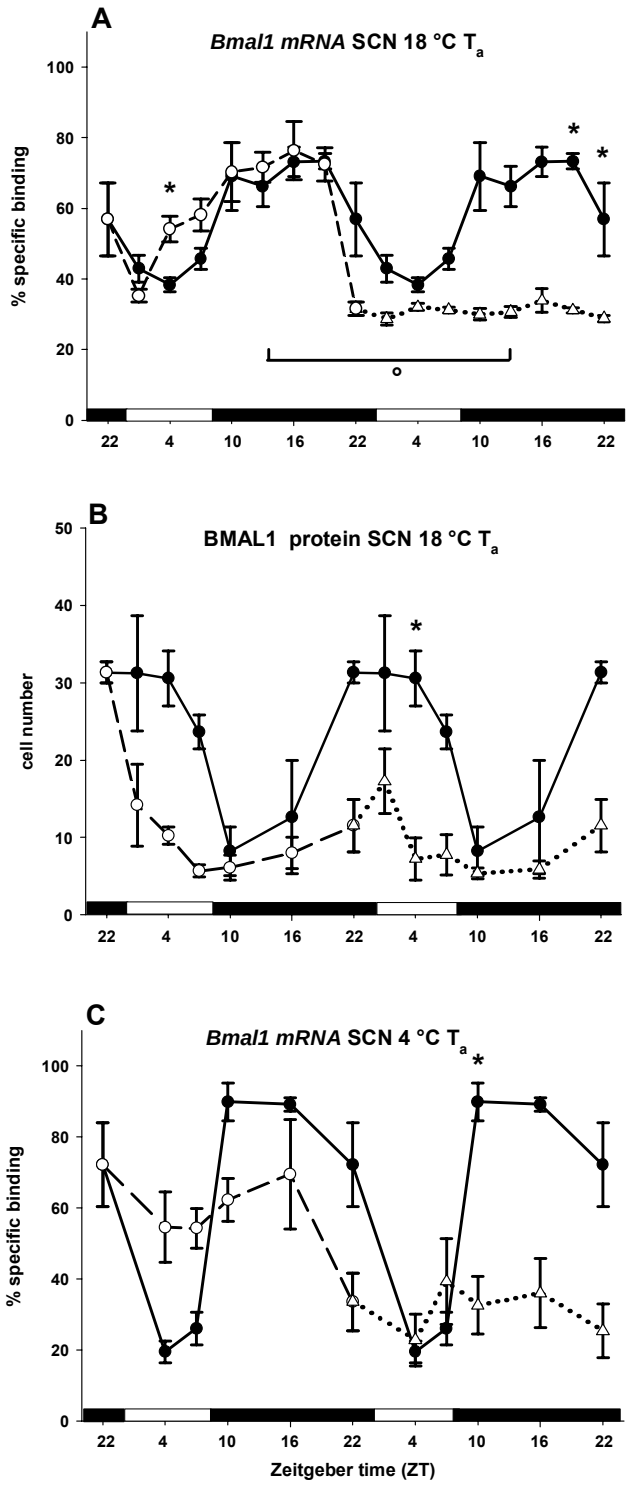

Figure 3a-c: mRNA and protein expression of *Bmal1*/BMAL1 in the SCN of Djungarian hamsters at different T_as. For further details see Fig. 2.

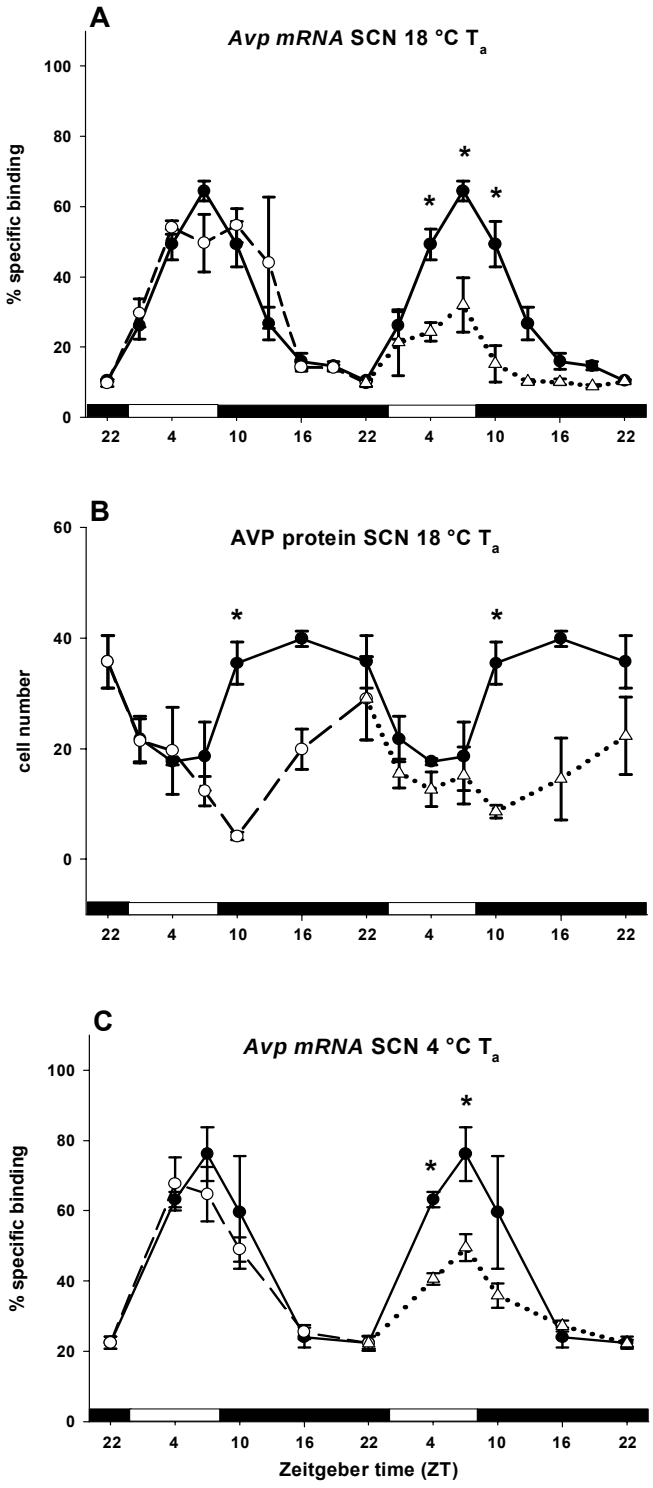

Figure 4a-c: mRNA and protein expression of *Avp*/AVP in the SCN of Djungarian hamsters at different T_as. For further details see Fig. 2.

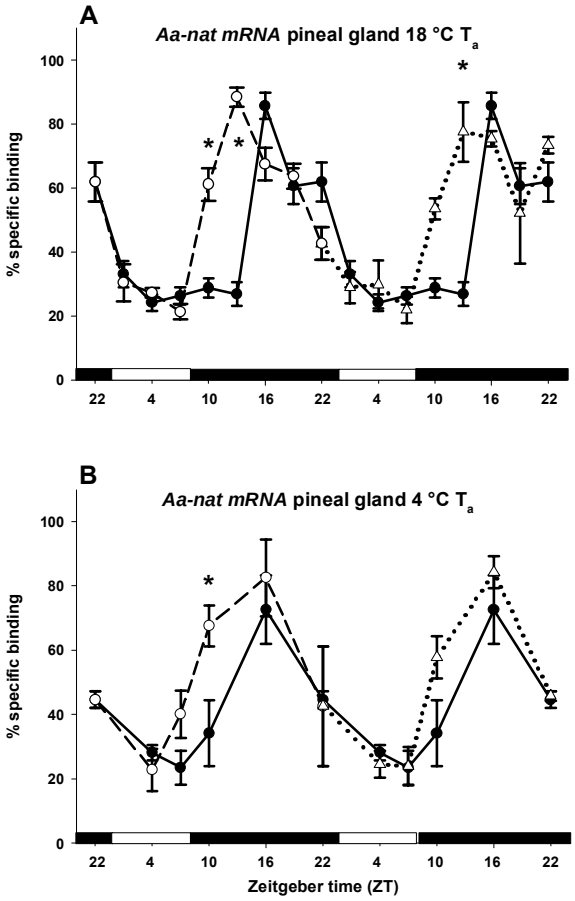

Figure 5a-b: *Aa-nat* mRNA in the pineal gland of Djungarian hamsters at different T_as. For further details see Fig. 2.

Discussion

In this study we show rhythmic clock and clock related gene expression during daily torpor in SCN and pineal gland of Djungarian hamsters in different T_as (and consequently T_bs), demonstrating that the clock´s molecular machinery still oscillates during torpor. Alterations in phase and amplitude of these rhythms however, point to temperature sensitivity. Decrease in protein expression during hypothermia, hence a decreased feedback might be responsible for changes in gene expression one day after a torpor bout.

All investigated genes showed a rhythmic mRNA expression in normothermic animals (group n) at both T_as. In this normothermic state T_a does of course not influence T_b which generally lies between 36 °C and 39 °C, and hence identical rhythms were found in both groups. These rhythms resemble those described by JOHNSTON et al. (2005) who showed similar expression patterns of *Per1, Bmal1* and *Avp* in the SCN of Djungarian hamsters in short photoperiod (SP), reflecting the typical short day feedbackloops with *Per1* as example for the negative and *Bmal1* as the marker for the positive limb of the circadian molecular machinery. Also protein expression of PER1, BMAL1 and AVP showed clear circadian rhythms at T_a of 18± 2°C peaking with a delay to mRNA expression of 6-9 hours for PER1 and AVP and a delay of 9-12 hours for BMAL1. This is in accordance with previously shown results (REPPERT and WEAVER 2001) and seems to be the normal delay for clock protein synthesis.

On the day of torpor (group t) all genes were rhythmically expressed at both T_as, suggesting temperature compensation of the clock at the transcriptional level in the investigated T_b range between ~18 and 39 °C. With regard to behavioural data these results are not surprising, as the circadian system is of utmost importance for the precise timing of daily torpor which is maintained over the whole winter season and can easily be adapted to shifts in the photocycle (KIRSCH et al. 1991, RUBY and ZUCKER 1992, RUF and HELDMAIER 1992). However, studies investigating transcriptional processes in hibernating ground squirrels, demonstrate a directly temperature-dependent transcript elongation which is arrested in deep hibernation (T_b ~3 °C) to reduce transcriptional expenses (BOCHAROVA et al. 1992, VAN BREUKELEN and MARTIN 2002a). Only few exceptions in systems that are of vital importance for controlling the hypothermia, as for example the histaminergic system, are made (SALMEN et al. 1999, 2003a) and here transcription appears to be even upregulated during hypothermia. Admittedly, daily torpor is only a relatively shallow form of hypothermia. Therefore, it is likely that the attenuating temperature effect on transcription is less pronounced at those still relatively high T_bs. In addition, it could be shown that despite reduced transcription total mRNA amounts in both brain and liver were surprisingly constant during deep hibernation, implicating an extension of mRNA half life (FRERICHS et al. 1998, O´HARA et al. 1999). This could e.g. happen through specific poly(A) binding proteins protecting poly(A)tail length (KNIGHT et al. 2000) or heterogenous ectopic RNP-derived structures (HERDS) that have

been described in torpid but not euthermic hibernators (MALATESTA et al. 1999, TAMBURINI et al. 1996) and might serve to store transcripts.

We did not observe differences in gene expression between the groups at 18±2 °C and 4±2 °C T_a. T_b during torpor depends to a certain extent on T_a , given that torpid animals cannot drop their T_b below T_a. Despite a 14 °C difference in T_a, T_bs only differed for 4±2,5 °C at the nadir of torpor between both groups. Probably this difference is not great enough to reveal changes in gene expression by in situ hybridisation and requires a more sensitive quantitative method like qPCR to determine differences in amount of mRNA. It is also possible that temperature compensation is not gradual but limited to a certain temperature range. In vitro studies have shown that electrical SCN activity is arrested below 16.6 °C (MILLER et al. 1994). In hibernating European hamsters (*Cricetus cricetus*) with T_bs ~8 °C during the torpor bout we did not observe any rhythmic clock gene expression (REVEL et al. submitted) so it may be that oscillation does not disappear gradually, but once a certain low temperature (probably 16.6 °C) is reached, rhythmic clock gene expression is no longer possible.

mRNA amounts of *Per1* and *Bmal1* were increased during the torpor bout. This might either result from a directed upregulation of gene expression or a passive accumulation of mRNA due to extension of mRNA half life in hypothermia (KNIGHT et al. 2000) and attenuated protein synthesis. Decline in protein synthesis during hypothermia has been demonstrated before and is likely to result from active suppression through regulatory phosphorylation of translational initiation factors rather than from simple Q_{10} effects (FRERICHS et al. 1998, VAN BREUKELEN and MARTIN 2001). Indeed we found a decreased number of immunopositive cells in all investigated genes during hypothermia supporting this hypothesis. Yet, *Avp* mRNA was not increased during torpor compared to the control group n (that had never shown torpor before the experiment), although protein expression was reduced as a consequence of torpor. This might result from a decreased BMAL1 feedback after hypothermia. The precise role of AVP in the SCN is still unclear. Lower amplitude in AVP rhythms causes lower amplitude in SCN electrical activity and may therefore account for less pronounced behavioural rhythms (VAN ESSEVELDT et al. 2000). Indeed behavioural rhythms are reduced or even completely absent during torpor season in the Djungarian hamster (unpublished observation), activity levels are generally low and amplify energy saving (RUF and HELDMAIER 1992). Lower AVP amplitude, as also seen on the day after torpor (t+1), could at least support this behaviour. We discussed previously (HERWIG et al.,

2006b) that the onset of *Bmal1* expression seems to be advanced on the day of torpor and that this advance may probably explain the shortened τ accompanying hypothermia in DD that has been described by Thomas et al. 1993. With the additional immunohistochemistry results that reveal a depressed amplitude in BMAL1 protein during torpor, it seems more likely that the increase in *Bmal1* mRNA amount is rather a passive accumulation of mRNA. Moreover, we did our experiments in LD conditions and therefore cannot predict the endogenous period.

On the day after torpor, we found a depression of *Bmal1*/BMAL1 as well as *Avp*/AVP rhythms in all experiments. It is striking that no *Bmal1*/BMAL1 rhythm was detected one day after torpor. However, PER1 protein rhythms were significantly dampened during hypothermia, probably resulting in a diminished feedback on *Bmal1* transcription. Diminished rhythms in one of the feedback loops of the circadian molecular machinery though, would support diminished output, namely *Avp*/AVP rhythms and consequently decreased behavioural rhythms. Moreover, also hypocaloric fed mice displayed decreased *Bmal1* amplitude (FEILLET et al. 2006). As torpor serves as an energy saving process, animals displaying torpor decrease their food intake which might have the same effect as food restriction. It is striking though, that we did not observe changes in *Per1* expression despite the lack of *Bmal1*/BMAL1 feedback, as this feedback seems necessary for restarting the next circadian cycle (REPPERT and WEAVER 2001). However, *Per1* expression is directly induced by light and as our experiments were carried out in an LD cycle the light input might be the more crucial factor here.

Besides the molecular machinery within the SCN, we also investigated *Aa-nat* expression in the pineal gland, a well studied output of the circadian clock. *Aa-nat* as the rate limiting enzyme for melatonin, has been shown to be directly under SCN control and is a good marker for the actual output of the circadian clock. Its expression was rhythmic in all groups. On the day of torpor (group t) we observed a strong *Aa-nat* induction at the very beginning of the night, most likely caused by the strong sympathetic activity during the arousal from hypothermia. Indeed already LARKIN et al. (2003) found an increased melatonin production accompanying the arousal from torpor. The advanced increase in *Aa-nat* expression was still visible on the day after the torpor bout. It has been discussed whether such “melatonin pulses” might contribute to a phase advance of the circadian system and consequently shorten τ in constant conditions (THOMAS et al. 2003). Our results would support such a hypothesis, but as the animals were always kept in LD conditions we do not know the endogenous phase.

However, we set up long term microdialysis experiments to continuously measure melatonin in the pineal gland (HERWIG et al. 2006a) to further investigate phase changes caused by daily torpor *in vivo* and in LD as well as constant conditions.

Overall, our results demonstrate transcriptional temperature compensation during daily hypothermia. No difference in gene expression could be observed in varying T_as and consequently T_bs. However, a decreased protein feedback results in altered clock and clock related gene expression after hypothermia.

4

Trans-pineal microdialysis in the Djungarian hamster (*Phodopus sungorus*): a tool to study seasonal changes of circadian clock activities.

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Abstract

The Djungarian hamster is a highly seasonal small mammal. The rhythmic secretion of melatonin by the pineal gland is controlled by the circadian clock, conveying the photoperiodic message to the organism. Trans-pineal microdialysis permits the *in vivo* study of this well-defined and precise clock output by measuring melatonin release directly in the pineal gland. The aim of this study was to adapt this method to the Djungarian hamster in order to monitor clock properties during photoperiodic changes. Male adult Djungarian hamsters were kept in a long photoperiod (LD 16:8) and melatonin release was measured hourly during the dark period for several weeks. Melatonin showed a regular secretion between ZT 17 and ZT 23.5 whereas the amplitude became stable only after the 3rd day of perfusion. To test how quickly changes in melatonin profile can be measured, 15 min light pulses were given at different time points throughout the scotophase. Light-pulses immediately interrupted the melatonin secretion at any time point during the scotophase and the temporal resolution for measurement could be reduced to 30 minutes. In accordance with studies in the rat, long term effects of light on the clock could only be observed when a light pulse was administered in the 2nd half of the night. For the first time we established a method to precisely measure a direct and reliable clock-output in a highly seasonal species which allows us now to study the circadian and seasonal properties of the clock in detail.

Introduction

The central biological clock, located in the suprachiasmatic nuclei (SCN) of the hypothalamus, is known to control the daily rhythm of many behavioural and physiological functions via endocrine and/or neuronal pathways (PERREAU-LENZ et al. 2004). In mammals, diurnal or nocturnal, the hormone melatonin, mainly synthesised in the pineal gland, is released during the night in a circadian manner and in proportion to the night length. Numerous studies on mammals have shown that the daily rhythm of melatonin synthesis is exclusively under SCN control, which is connected to the pineal gland through a multisynaptic pathway (MOORE 1996). Depending on inhibitory and stimulatory signals

from the clock, the release of noradrenaline (NA) into the pineal gland by the sympathetic fibres stimulates the melatonin synthesis pathway by activation of adrenergic receptors (PERREAU-LENZ et al. 2004). Melatonin is thus a stable and reliable output of the biological clock and detailed analysis of this output allows us to study clock properties (REITER 1993, PÉVET 2003).

Several approaches have been used to follow melatonin rhythm generation continuously over a long period of time, as for example repeated blood sampling with a jugular catheter. However, except for some large mammals such as sheep the amount and the volume of blood samples that can be taken from the same animal limit the data points per 24h cycle and the duration of the experiment (RAVAULT and CHESNEAU 1996). The indirect evaluation by analysis of the urinary melatonin metabolite 6-sulphatoxy-melatonin is also not very precise as data may be dependent on liver activity and show a phase-lag of two to four hours (RAYNAUD et al. 1993, STIEGLITZ et al. 1995, KENNAWAY et al. 2002).

It is the development of the trans-pineal microdialysis by AZEKAWA et al. (1990) and DRIJFHOUT et al. (1993), which allows the precise long term measurement of the melatonin rhythm with a high temporal resolution in its production site, in intact and freely moving animals. This technique permits longitudinal studies, in which each animal is its own control and has been useful especially in the rat to identify the nervous pathways and the neurotransmitters involved in distributing the circadian message (PERREAU-LENZ et al. 2004). Melatonin, however, through the change in duration of its nocturnal peak is known to be involved in the distribution of seasonal information (STEINLECHNER et al. 1987, BARTNESS et al. 1993, PÉVET 2003). In the last few years, it has been observed that a number of clock genes show day-length dependent changes in expression in the SCN supporting the idea that the clock directly generates the seasonal messages (VUILLEZ 1996, NUESSLEIN-HILDESHEIM et al. 2000, TOURNIER et al. 2003).

Trans-pineal microdialysis appears to be a good technical tool for studying clock properties and pathways involved in photoperiodic responses. Since rats only marginally respond to such seasonal changes, the development of trans-pineal microdialysis in a photoperiodic species is needed. We have set up such trans-pineal microdialysis approaches for the Djungarian hamster (*Phodopus sungorus*), a well known model in seasonal studies in which the role of photoperiod and melatonin is well defined (HOFFMANN 1979, HELDMAIER and

STEINLECHNER 1981, BARTNESS and GOLDMAN 1989, STEINLECHNER and NIKLOWITZ 1992).

Materials and methods

Animals

For the experiments male three to five-month-old Djungarian hamsters (*Phodopus sungorus*) from our own breeding colony were used. The animals weighed between 30 and 40 g and were housed in a temperature-controlled room (21 ± 2 °C) under a reversed 16:8 h light-dark (L:D) cycle (lights off from 10.00 a.m. to 6.00 p.m.) for at least three weeks before surgery. Light onset corresponds to Zeitgeber time ZT 0, thus in this photoperiodic condition night starts at ZT16. Water and food were available *ad libitum*.

All experiments were performed in accordance with 'Principles of Laboratory Animal Care´ (NIH pub.no., 86-23, revised 1985) and in accordance with French laws.

Surgery and dialysis

The animals were operated at least one week before the microdialysis experiment. They were anesthetised with intraperitoneal injections of 0.2 ml Zolétil and 0.1 ml Rompun (1:8). After the operation they were allowed to recover for one week in individual cages before starting the first perfusion.

Surgery was performed as previously described (BARASSIN et al. 1999) but with a modified dialysis probe. The active dialysis area measured 1 mm and the probe was implanted 0.8 mm ventral to the skull surface and 0.4 mm posterior to Lambda. Perfusion was performed at a flow rate of 2 µl/min

Experimental procedure

In all experiments, pineal melatonin release was measured during five subsequent days. Animals were always housed in long photoperiod (LD 16:8). Samples were collected hourly from ZT 14 (8.00 a.m., two hours before dark onset) to ZT 3 (9.00 p.m., three hours after light onset), except for the first two hours after the photic stimulus (exp 2) when samples were

collected every 30 min. The dialysates were stored at –20 °C until being assayed for melatonin by radioimmunoassay (RIA). In order to determine the location of the probe, cryostat sections of the brain/pineal (25µm) were stained with cresyl violet at the end of the experiments.

Experiment 1: To validate the transpineal microdialysis approach in the Djungarian hamster and to characterise the daily profile of the melatonin excretion under these conditions, in a first experiment daily melatonin rhythms were measured hourly for five subsequent days without any manipulations (n = 11).

Experiment 2: To assess how reliable changes in melatonin release can be measured, the influence of single 15 min light pulses on the melatonin secretion at different time points throughout the dark phase was tested in a second experiment. After two control days without any manipulation and hourly sampling, a 15 min light pulse (200 lux) was given on the third day at ZT 18 (*n = 4*), ZT 19 (*n = 3*), ZT 20 (*n = 3*), ZT 21 (*n = 3*), and ZT 22 (*n = 4*). In the first two hours after the light pulses, samples were collected every 30 min. The following two days the animals were kept in undisturbed LD 16:8 cycles.

Melatonin assay

Melatonin in the dialysates was measured by radioimmunoassay in 25 µl duplicate samples using specific rabbit antiserum (R19540, INRA, Nouzilly, France) and labelled (^{125} I) -2-iodomelatonin. The sensitivity limit of the assay was 0.5 pg/tube. For details see BARASSIN et al. (2003).

Data analysis and statistics

All melatonin data are expressed in absolute values (nM). For each animal the pattern of melatonin secretion was characterised and fitted by a non-linear regression analysis with Sigma Plot software (Jandel Scientific). All pineal Mel profiles were fitted with the following equation: $y = Y0 + (Ymax / ((1 + \exp(A*(IT50-x)))*(1 + \exp(x – DT50))))$ where y was the *n*th data point, x the time point of the *n*th point, Y0 the basal level measured during daytime, Ymax the maximum of the nocturnal peak, IT50 was defined as the time point of 50 % increase in melatonin levels and DT50 as the time point of 50 % decrease. The multiplicative factors in the exponentials were chosen arbitrarily to fit the observed slopes and in this work

fixed to 1.62 and 3.58 respectively. IT50 and DT50 were used to characterise the timings of the onset and offset of melatonin peak for each day of the experiments. The duration and the amplitude of the melatonin peak were determined as the difference between DT50, IT50 and Ymax, respectively.

First, an *ANOVA* for repeated measurements was carried out for each group of animals on *IT50*, *DT50* and *Ymax* with animals and control days (Ctrl 1 and Ctrl 2) as factors; a *Scheffé's* test was used as post hoc test. The differences were considered significant for a $P<0.05$(*).

Results

Under our experimental photoperiodic conditions (LD 16:8), extracellular pineal melatonin concentrations were low during the light period and peaked during darkness between ZT 20 and ZT 0. Figure 1 shows averaged nocturnal melatonin profiles of eleven animals measured for five successive days without any manipulation. The melatonin release showed a regular onset at ZT 18.49 ± 0.08 h and offset at ZT 0.13 ± 0.05 h corresponding to the time of lights-on in all animals. The amplitude showed a significant increase between day one and day four (*Scheffé's*: P = 0,054) as well as day one and day five (*Scheffé's*: P = 0.03). Moreover the amplitude varied between individuals correlating to the position of the probe within the pineal gland.

Figure 2 presents representative individual melatonin profiles of animals given light pulses at different time points throughout the dark phase. Ctrl 1 shows the normal melatonin pattern in the night before the photic stimulus. LP corresponds to the day where the 15 min light pulse was given, whereas Ctrl 2 presents the melatonin profiles two days after the light pulse. The 15 min photic stimulus given during darkness caused an immediate decrease in melatonin concentrations down to basal levels within one hour in every case.

After a light pulse at ZT 18 and ZT 19, melatonin concentrations increased again two hours after the light pulse and reached normal levels by the end of the night. Two days after the manipulation (Ctrl 2) no significant effect could be seen at the onset (*Anova*: ZT 18 P = 0.08; ZT 19 P = 0.24), offset (*Anova*: ZT 18 P = 0.59; ZT 19 P = 0.41) or amplitude (*Anova*: ZT 18 P = 0.68; ZT 19 P = 0.19).

The light pulse at ZT 20 caused an instant reduction in the hormone in the pineal gland which did not recover to normal (Ctrl 1) levels during the same night. Moreover the profile showed a significantly earlier offset (*Scheffé's*: 0.0015) of 1.45 ± 0.13 h. On Ctrl 2 only slight alterations could be seen at the onset (*ANOVA*: $P = 0.90$) but the offset took place 1.03 ± 0.08 hours earlier (*Scheffé's*: $P = 0.0056$) and the amplitude was significantly reduced (*Scheffé's*: $P = 0.016$).

At ZT 21 and ZT 22 the photic stimulus had a similarly acute effect. The melatonin concentrations did not or only hardly rose again after the light induced decrease. While the manipulation had no long term effect on the onset at ZT 21 (*ANOVA*: $P = 0.43$) the melatonin release set off significantly 0.95 ± 0.08 hours earlier (*Scheffé's*: $P = 0.025$) on Ctrl 2. At ZT 22 also the onset advanced significantly for 0.80 ± 0.13 hours (*Scheffé's*: $P = 0.009$), the offset shifting for 0.74 ± 0.28 hours (*Scheffé's*: $P = 0.001$).

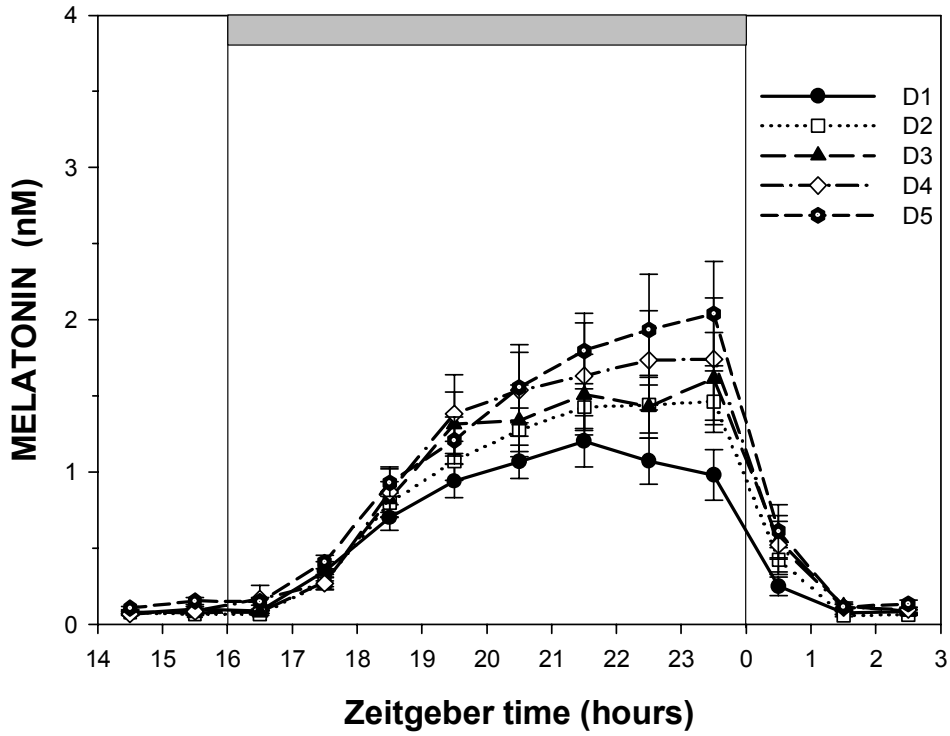

Figure 1: Averaged nocturnal profiles of pineal melatonin concentrations measured by *in vivo* microdialysis in *Phodopus sungorus* during 5 successive days (D1-D5) without any manipulations. Data are plotted as the mean ± SEM (n = 11). The grey bar represents the dark period (from ZT16 to ZT0) of the LD 16:8 cycle.

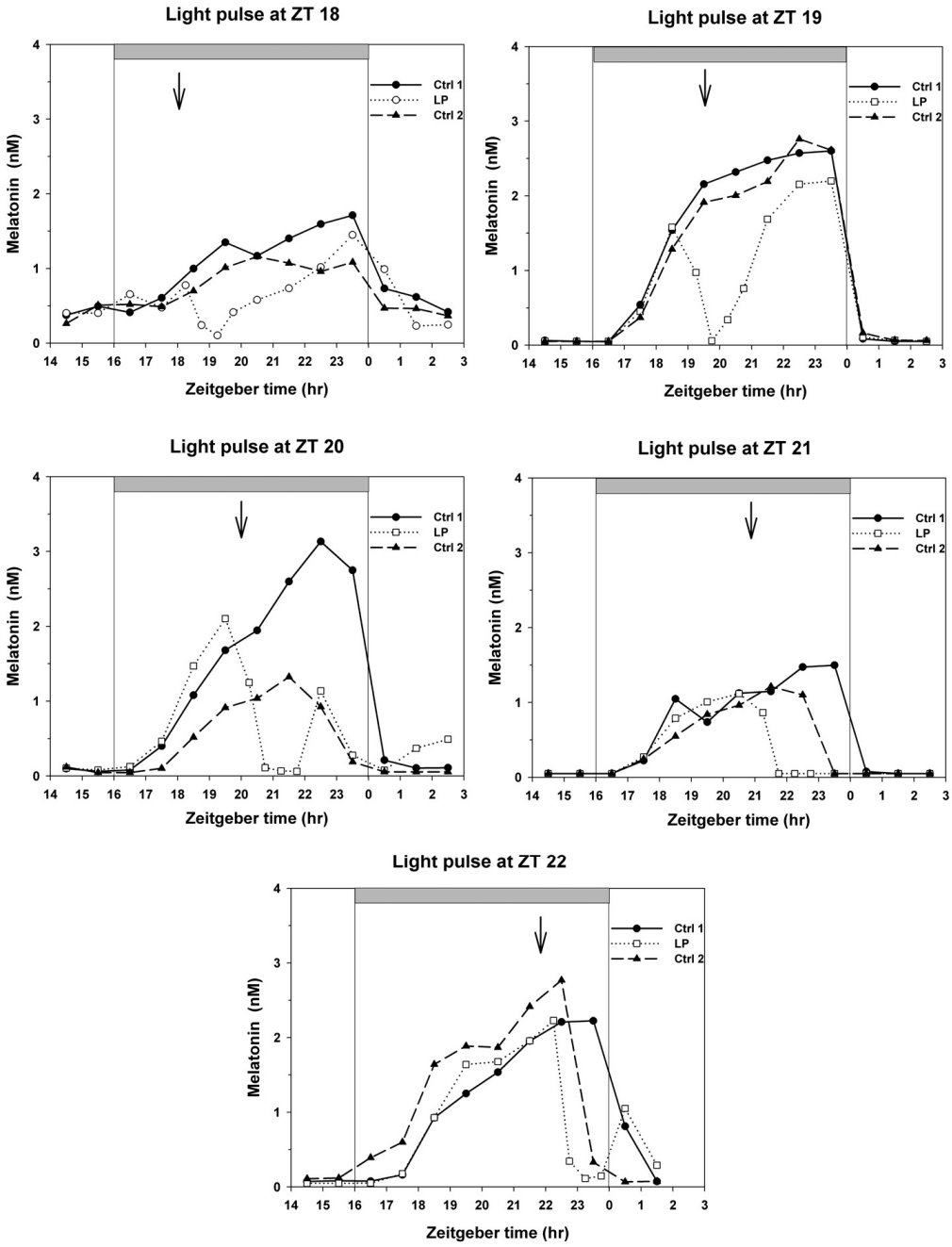

Figure 2: Examples of individual melatonin profiles in the pineal gland during the first control day (Ctrl 1), during the experimental day (LP) where a 15 min light pulse was given at different Zeitgeber times and during the second day after the photic stimulus (Ctrl 2). The grey bar represents the dark period of the LD 16:8 cycle the black arrow points to the 15 min light pulse.

The individual phase shifts of melatonin on- and offsets are presented in Figure 3a/b besides the means for each group. The individual values for the amplitude are illustrated in Figure 3c.

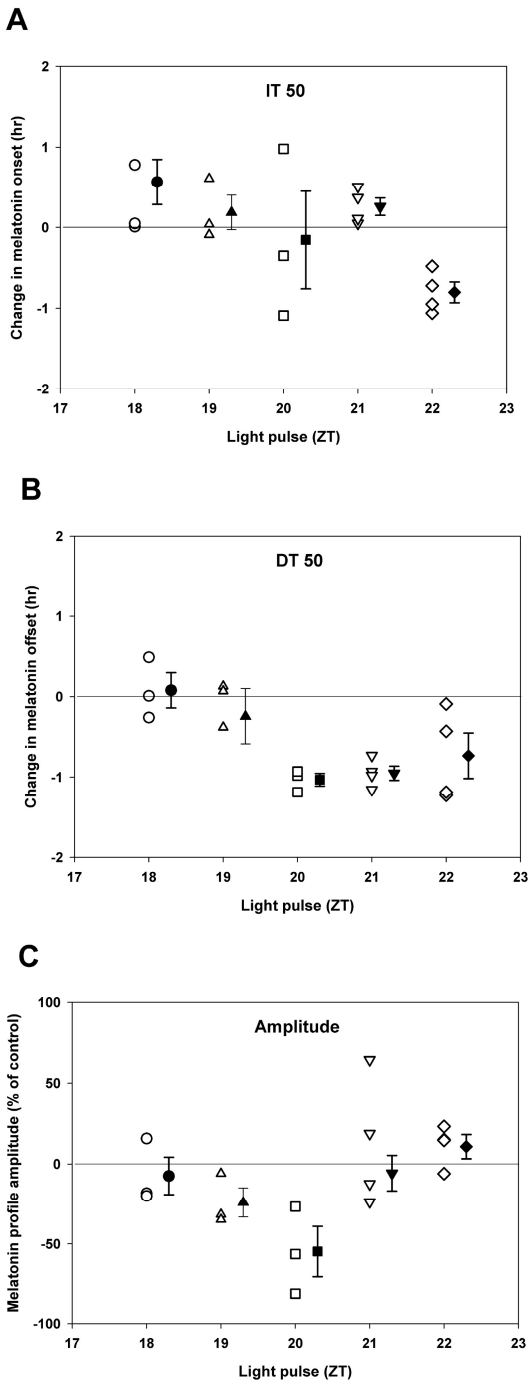

Figure 3: Effect of a 15 min light pulse given at different Zeitgeber times on the onset IT50 (A) and the offset DT 50 (B) of the nocturnal melatonin profiles revealed by *in vivo* microdialysis. Light pulses were given between ZT 18 and ZT 22 under LD 16:8 conditions and pineal dialysis samples were collected until 2 days afterwards (Ctrl 2). The variations in on- and offsets are presented as changes observed on the Ctrl 2 *vs*. a control day. Variations in the amplitude of the melatonin peak are presented in Figure 3 C. Open symbols: individual values. Closed symbols: means ± SEM.

Discussion

The melatonin rhythm in the pineal gland is an excellent marker of the circadian pacemaker because it accurately reflects and amplifies SCN electrical and metabolic activity in both diurnal and nocturnal mammals. Trans-pineal microdialysis, developed by AZEKAWA et al. (1990) and DRIJFHOUT et al. (1993) allows precise and continuous measurement of this marker directly in the pineal gland with a high temporal resolution and in the same animal for a period of up to five weeks (unpublished data). The adaptation of the trans-pineal microdialysis technique to a photoperiodic species as demonstrated in this study is important as melatonin is not only an SCN generated circadian signal but also conveys seasonal photoperiodic changes.

Undisturbed melatonin release showed profiles with regular on- and offsets during 5 consecutive days. In all animals tested and kept under LD 16:8, onsets occurred regularly two to three hours after the beginning of the dark period. Our results tally with pineal melatonin profiles or NAT activities described in this species (ILLNEROVA et al. 1984, HOFFMANN et al. 1985, STEINLECHNER et al. 1987). The offset differs from what has been described in the rat. Instead of declining about one hour before light onset, therefore being necessarily driven by an endogenous SCN-induced cessation of NA release (DRIJFHOUT et al. 1996) the melatonin peak in the Djungarian hamster did not decrease before ZT 0. This suggests that not an endogenous stimulus but the light itself terminates the melatonin release in LD 16:8. Interestingly STEINLECHNER et al. (1987) described a similar phenomenon for AA-NAT activities in long photoperiod, while in short photoperiod the AA-NAT activity already decreased before dawn. This observation of a light inhibitory signal in melatonin/AA-NAT production under LP but not under SP, also described in the Syrian hamster (PÉVET 1988) and *Arvicanthi ansorgei* (GARIDOU-BOOF et al. 2005) seems to be characteristic of photoperiodic species. Due to the short half life of circulating melatonin (20 min) (STANKOV et al. 1993), secretion dropped to day time levels within less than an hour.

The increase in amplitude within the first 3 days of melatonin measurement observed in this study has not been reported in other intra-pineal microdialysis studies in rats or birds, where the measured melatonin peak appeared to be stable from the first day of dialysis (BARASSIN et al. 1999). Very probably, this instability in amplitude during the first days of measurement

in the Djungarian hamster results from the technique itself. Implantation of the probe causes a more severe tissue trauma in the very small pineal gland of the Djungarian hamster which needs a longer time to recover. After the beginning of perfusion it takes some days until the dialysis system is equilibrated. The fact that after few days we obtained a reproducible nocturnal melatonin release similar to that described in the literature, demonstrates that this trauma is only temporary. Nevertheless, to use intra-pineal microdialysis in good conditions, the physiological experiments should not be started before 3 days of perfusion without sampling in this species.

As expected, all 15 min light pulses caused an immediate decrease in melatonin release down to basal levels within one hour. These results tally with all the other data in literature, describing immediate suppression of AA-NAT activity and melatonin synthesis by even short white light pulses (KLEIN and WELLER 1972, ILLNEROVA and VANECEK 1972). Depending on the time they were given, the photic stimuli showed different impacts on the melatonin rhythm. When the light pulse was given before midnight (ZT 20), only a transient decrease was seen, which recovered thereafter to normal levels and set off at the usual time. Light pulses after ZT 20 resulted in a generally sustained decrease in melatonin for the rest of the night. These results are in accordance with those of VANECEK and ILLNEROVA (1982), ILLNEROVA et al. (1986), showing an initial drop in AA-NAT activity and a subsequent rise when the light pulse was applied before midnight, and a sustained decrease when given after midnight in rats. Similar findings have been reported by Kanematsu et al. (1994) for the melatonin release. These different effects of light on melatonin release may result from the circadian change in sensitivity in the light pathway (PANGERL et al. 1990, HONMA et al. 1992). Moreover all components involved in melatonin regulation such as AA-NAT and HIOMT show complex and changing kinetics within a circadian phase (RIBELAYGA et al. 1999). Contrary to findings of Kanematsu et al. (1994) we did not find any long term alteration of the melatonin rhythm when the light pulse was given before midnight [ZT 20]. Very likely we had no such effect because our experiment was done in continued LD cycles, while Kanematsu et al. chose constant conditions for their experiments. Our results tally with studies, showing only a weak c-Fos induction in the SCN after a light stimulus in early on at night compared to a stronger induction late at night in hamsters (SUTIN and KILDUFF 1992). The protein c-Fos serves as marker for efferent SCN signals and is localised in VIP expressing cells. This efferent signal might modify the NA release in

the pineal gland and consequentially the melatonin rhythm (KALSBEEK et al. 1999). When the photic stimulus was applied in the middle of the night, at ZT 20, the melatonin rhythm was strongly reduced after two days. The duration was markedly shorter with an advanced offset, and the amplitude was significantly decreased. SUMOVA and ILLNEROVA (1998) showed a decreased amplitude of c-Fos immunoreactivity as well as an advance in the morning decline after a light stimulus around midnight in rats, suggesting a comparable effect of the light on the clock itself. In accordance with our study, LERCHL (1995) reported significant effects on melatonin amplitude and phase one night after a light pulse at midnight in the same species. However our results show that those alterations are still visible 2 days after the photic stimulus, tallying with results from STEINLECHNER et al. (2002b) who showed that already short light pulses can have a long-lasting or even destructive effect on circadian rhythms. It is known as well that during resetting of the mammalian circadian clock, not only the clock's phase is shifted, but amplitude of overt rhythms driven by the clock may be temporarily reduced or even abolished (ILLNEROVA 1992). Our results are not astonishing, knowing that hamsters in short photoperiod exposed to light pulses during every second or fourth night is virtually indistinguishable from a complete transfer to long-day photoperiod (HOFFMANN et al. 1981). It would make sense that nocturnal seasonally breeding animals can react very sensitively to even very little photoperiodic information to time the re-onset of reproduction correctly. A light pulse in the second half of the night resulted in an offset advance (ZT 21) or a complete phase advance in onset and offset (ZT 22) up to one hour. KANEMATSU et al. (1994) observed similar phase advances in rats, although they used a different protocol and released the animals in constant darkness after the light pulse. This means that even despite a present entrainment stimulus, namely the LD cycle, light at night has an enormous impact on the clock. In this study on- and offsets are not equally affected. In most cases the melatonin offset is more altered than the onset. This "uncoupling" of onset and offset supports the model of a complex two-component pacemaker system controlling the pineal melatonin production. The model by PITTENDRIGH and DAAN (1976) describes a clock with a morning (M) oscillator controlling the onset of the resting period and an evening (E) oscillator controlling the onset of locomotor activity. This theory was extended to pineal AA-NAT activity which appeared to be differentially affected by light pulses given at different circadian times in constant darkness (ILLNEROVA and VANECEK 1982). In this study we validated an important method in the Djungarian hamster,

a highly seasonal animal. We are now able to measure very precisely a direct and reliable clock output which is not only involved in circadian physiology but also responsible for seasonal changes, thus allowing us to study in detail the seasonal properties of the clock.

Acknowledgments

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5

In vivo melatonin measurement during torpor in the Djungarian hamster (*Phodopus sungorus*).

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Preliminary data

Introduction

As described in chapter 4, the rhythmic melatonin secretion from the pineal gland is a precise and very well-defined output of the circadian clock that reflects both seasonal and daily changes in light-dark (LD) ratio. It is exclusively regulated by the suprachisamatic nuclei (SCN) and therefore a very reliable phase marker for the clock´s activity. In chapter 2 and 3 we described alterations of the clock´s oscillation during and after daily torpor in the Djungarian hamster (*Phodopus sungorus*). Daily torpor mainly affected the amplitude of oscillation: decreased clock protein expression during the torpor bout probably led to decreased clock gene expression after the hypothermia. Yet, after arousal from hypothermia, which usually takes place at the end of the light phase, we observed a phase advanced increase in *Aa-nat* mRNA (the rate limiting enzyme for melatonin) relative to the normothermic control group. As melatonin feeds back on the clock and is capable of inducing phase shifts (UNDERWOOD 1982, REDMAN et al. 1983, LEWI et al. 1992, REDMAN 1997, AGEZ et al. 2007), it is possible that an advanced melatonin induction after arousal from daily torpor might also have an effect on the phase or, in constant conditions, the endogenous period (τ) of the circadian clock. Temperature-provoked phase and τ changes of circadian rhythms have been described before. Already ASCHOFF (1979) predicted that τ becomes shorter in nocturnal species as temperature decreases. Low temperature pulses of 25 °C caused a 2 hours shortening in the SCN´s τ of golden mantled ground squirrels (*Spermophilus citellus*) *in vitro* and consequently a nearly 5 hour phase advance after only 48 hours at low temperatures (RUBY and HELLER 1996). Also THOMAS et al. (1993) observed a shortening of τ in Djungarian hamsters that displayed daily torpor. To assess changes in phase and τ, it is necessary to continuously measure a clock output in the same individual because τ varies between animals. Measuring melatonin by trans-pineal microdialysis allows a long-term investigation of the most precise clock output known today and allows us to study in detail the effect of daily torpor on the phase and τ of the circadian clock.

Materials and methods

Animals

For the experiments we used male three to five-month-old Djungarian hamsters (*Phodopus sungorus*) from the breeding colony of the Laboratory for Neurobiology of Rhythms at the Louis Pasteur University, Strasbourg. The animals were housed in a temperature-controlled room (21 ± 2 °C) under a 16:8 h light-dark (LD) cycle (lights on from 4.00 a.m. to 8.00 p.m.). To facilitate working we inversed the photocycle to an LD 16:8 with lights on from 6.00 p.m. to 10.00 a.m. Three months before the experiments the light period was decreased step-wise for 2 hours every two weeks until a short photoperiod (SP) of LD 8:16 was reached (lights on from 10.00 p.m. to 6.00 a.m.). Then T_a was reduced to 18 ± 2 °C. Bouts of daily torpor occurred spontaneously in most animals 12 weeks after the start of decreasing the photoperiod. Light onset corresponds to Zeitgeber time zero (ZT0). Water and food were available *ad libitum*.

Body temperature (T_b)

After torpor bouts had been observed, the animals were implanted with thermo-sensitive radio-transmitters (Model VM-FH-LT, Mini-Mitter Co., Sunriver, OR, USA) in the abdominal cavity under isoflurane anesthesia. Radiofrequency signals from the implanted transmitters were averaged every 5 min by receivers placed under each cage and collected by an automated computer software (Dataquest, St. Paul, MN). After Mini-Mitter implantation the animals were allowed to recover for at least four weeks before microdialysis surgery.

Microdialysis surgery and dialysis

Microdialysis surgery and dialysis were performed as described in chapter 4. After implantation of the dialysis probe the animals were allowed to recover for at least two weeks.

Experimental procedure

Pineal melatonin release was measured during 8-16 days depending on the animals. To primarily complete the results in chapter 3, the animals were first kept under an LD 8:16 cycle. Samples were collected hourly throughout 24 hours by automatic fraction collectors

(Microsampler Univentor, Phymep, France). The dialysates were stored at –20 °C until being assayed for melatonin by radioimmunoassay (RIA, see chapter 4).
To gain insight into how torpor affects τ the animals were released in constant darkness for 3-4 days at the end of the experiments.

Data analysis and statistics

Due to the small number (n=3) of successfully monitored animals that displayed torpor after surgery we desisted from statistical analysis and provide here only a description of the observed results.

Results

As expected under our experimental photoperiodic conditions (LD 8:16), extracellular pineal melatonin concentrations were low during the light period and peaked during darkness. A double plot of a normothermic control day (n) is plotted together with a day of torpor (t) and the day following a torpor bout (t+1). To better illustrate the changes in melatonin after the arousal from a torpor bout, an extra figure shows the melatonin release on one normothermic day and on a day of torpor together with the T_b data.
Animal Hs 2 displayed two torpor bouts during the microdialysis experiment (Figs. 1 and 2). Relative to the normothermic control day the onset of melatonin release was delayed for ~20 min on the day of torpor in the first torpor episode (torpor 1, Fig. $1_{A,B}$), and for approximately one hour in the second (torpor 2, Fig. $2_{A,B}$). The animal aroused between ZT8 and 9 in both cases. The same pattern, a delay of melatonin secretion onset of approximately one hour, occurred in an additional animal which aroused from torpor between ZT7 and 8 (Hs 14, data not shown). No changes in the melatonin profile could be observed one day after a torpor bout (Fig. 1_A, 2_A).
Also animal Hs 15 showed two torpor bouts (Figs. 3 and 4). On the first day of torpor the melatonin increased ~30 min earlier on a day of torpor compared to the normothermic control day (Fig. $3_{A,B}$). During the second torpor episode the onset was approximately one hour advanced (Fig. $4_{A,B}$). The animal aroused between ZT 4 and 6. Again we did not observe changes in the melatonin profile on the day after a torpor bout (Fig. 3_A, Fig 4_A).
In DD none of the monitored animals displayed torpor.

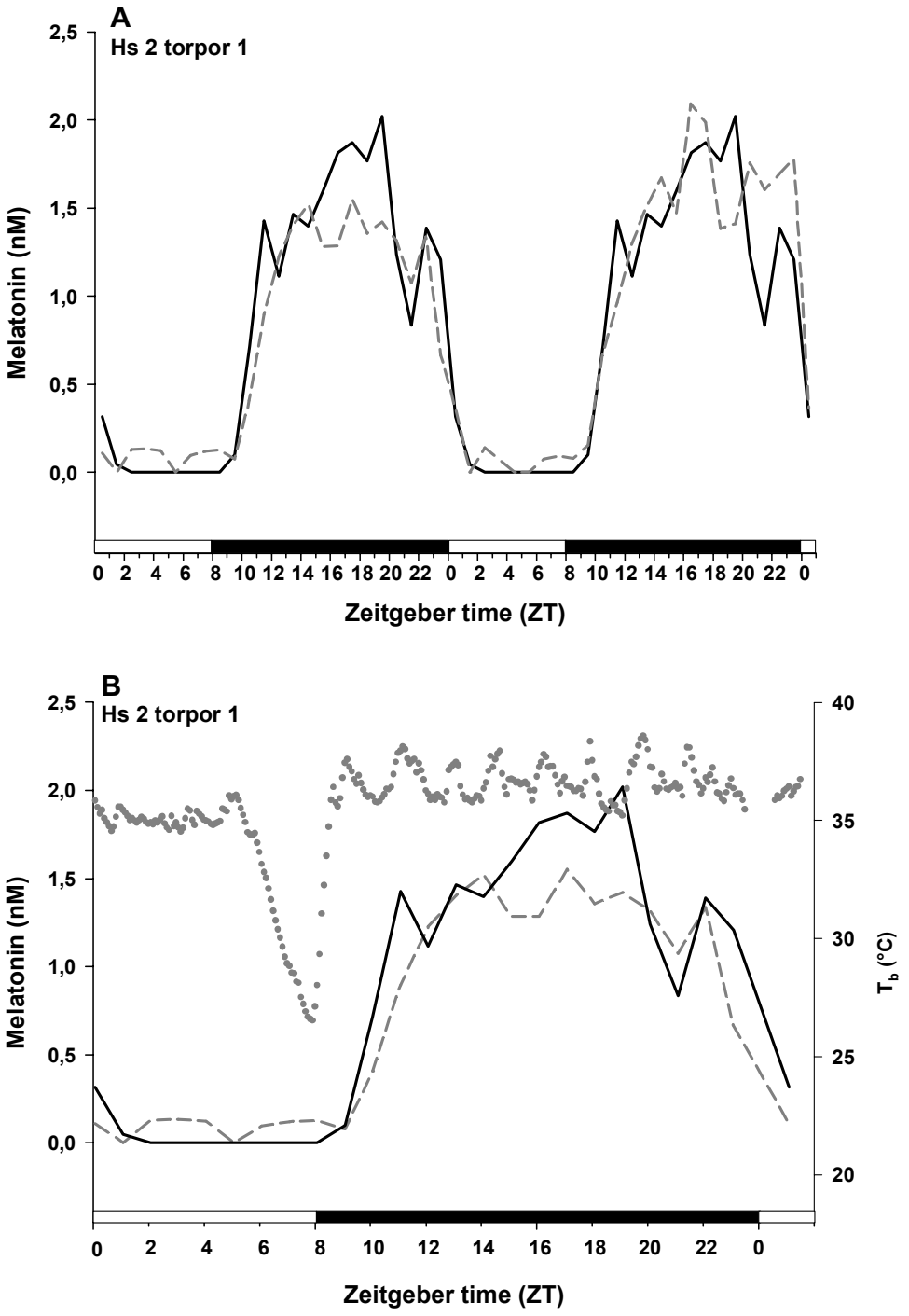

Figure 1: Profiles of pineal melatonin concentrations of animal Hs 2 (torpor 1) measured by *in vivo* microdialysis in *Phodopus sungorus*. **A.** Values of the normothermic control day (black solid line) were double plotted for better visualisation. This day was compared to a day of torpor (grey dashed line) and a day following the torpor bout. **B.** Pineal melatonin profile on the day of topor (grey dashed line) *versus* the normothermic control day (black solid line). The dotted line represents the body temperature (T_b) on the day of torpor. The white and black bar on the x-axis represents the LD cycle.

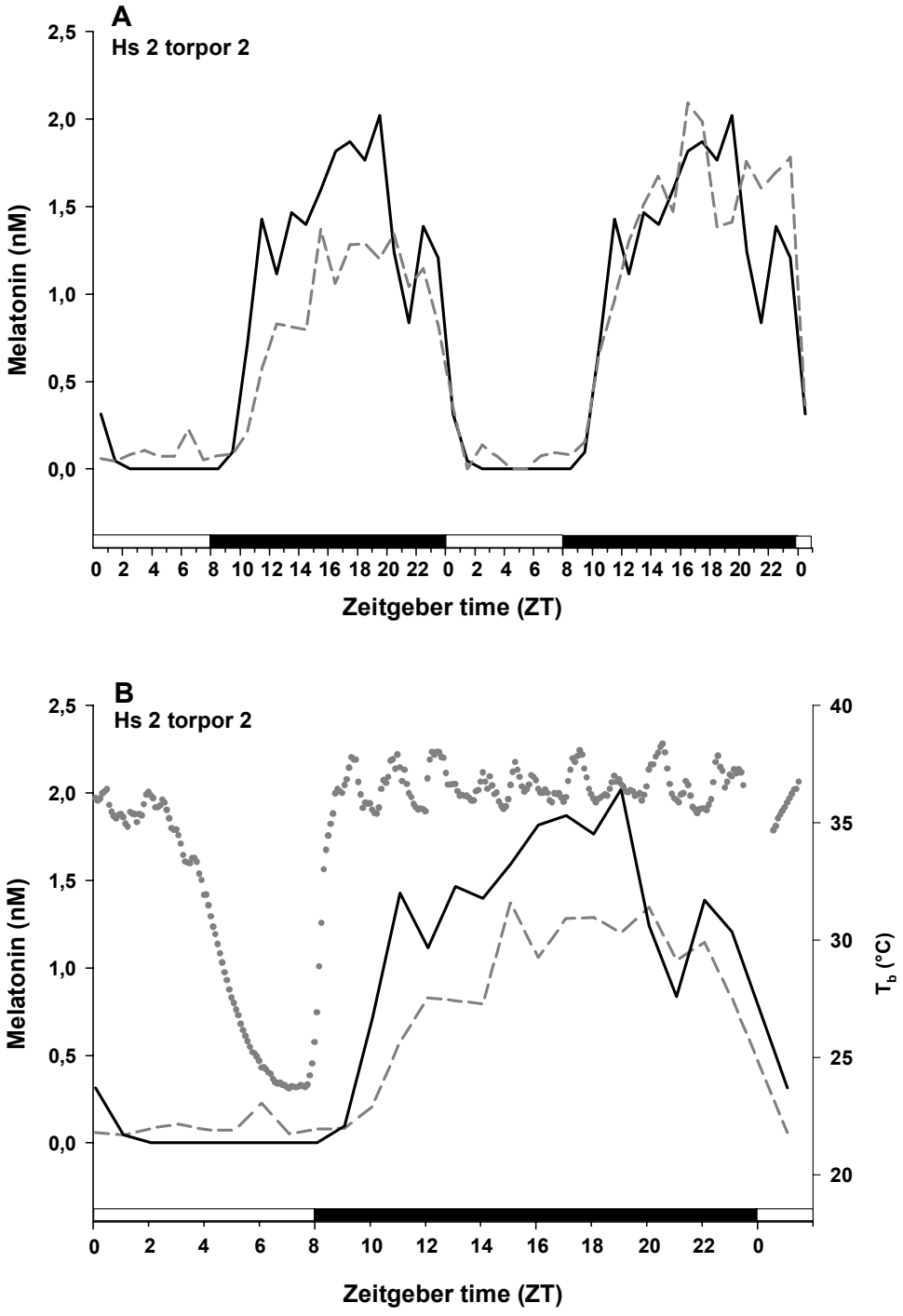

Figure 2: Profiles of pineal melatonin concentrations of animal Hs 2 (Torpor 2) measured by *in vivo* microdialysis in *Phodopus sungorus*. For details see figure 1.

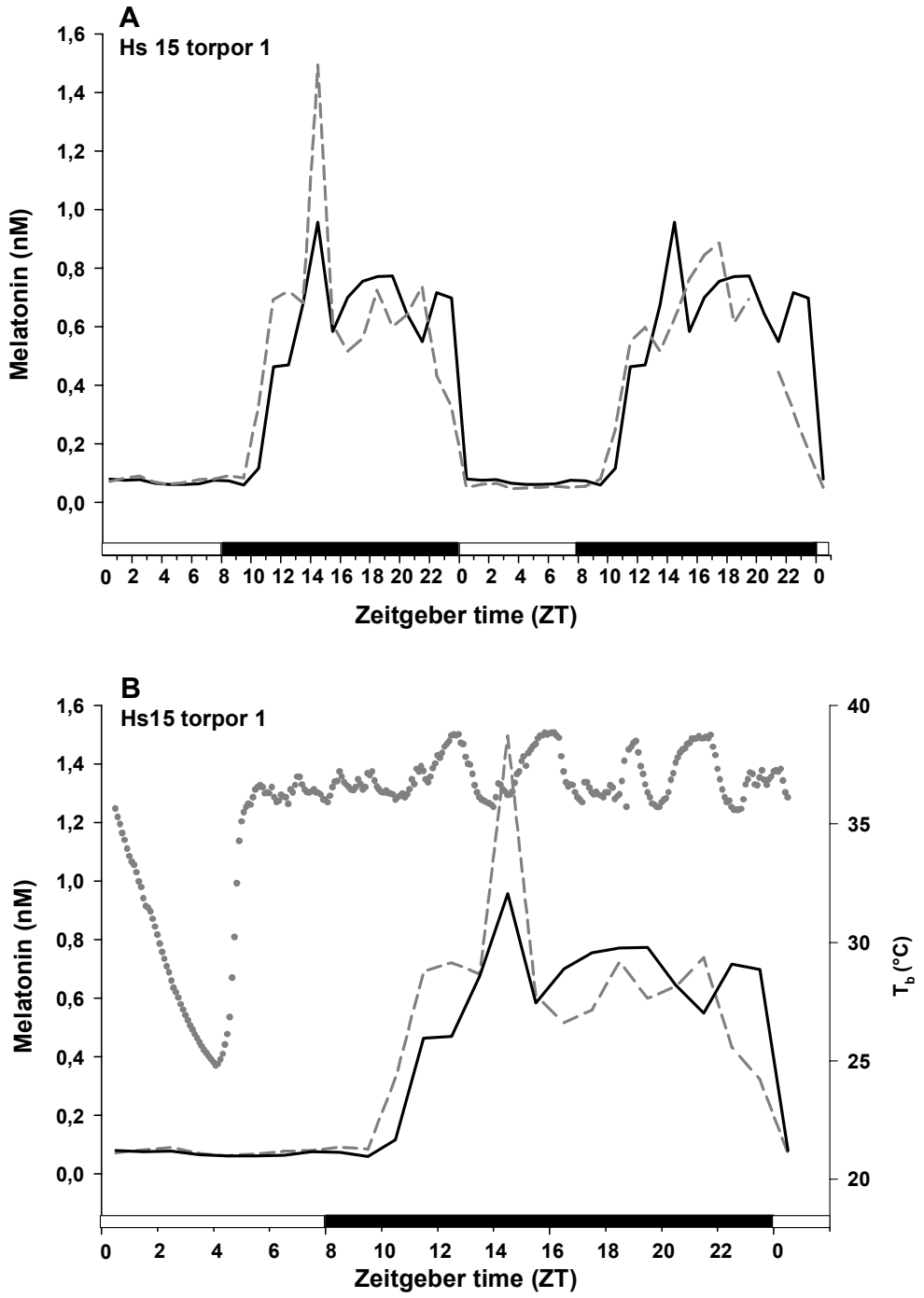

Figure 3: Profiles of pineal melatonin concentrations of animal Hs 15 (Torpor 1) measured by *in vivo* microdialysis in *Phodopus sungorus*. For details see figure 1.

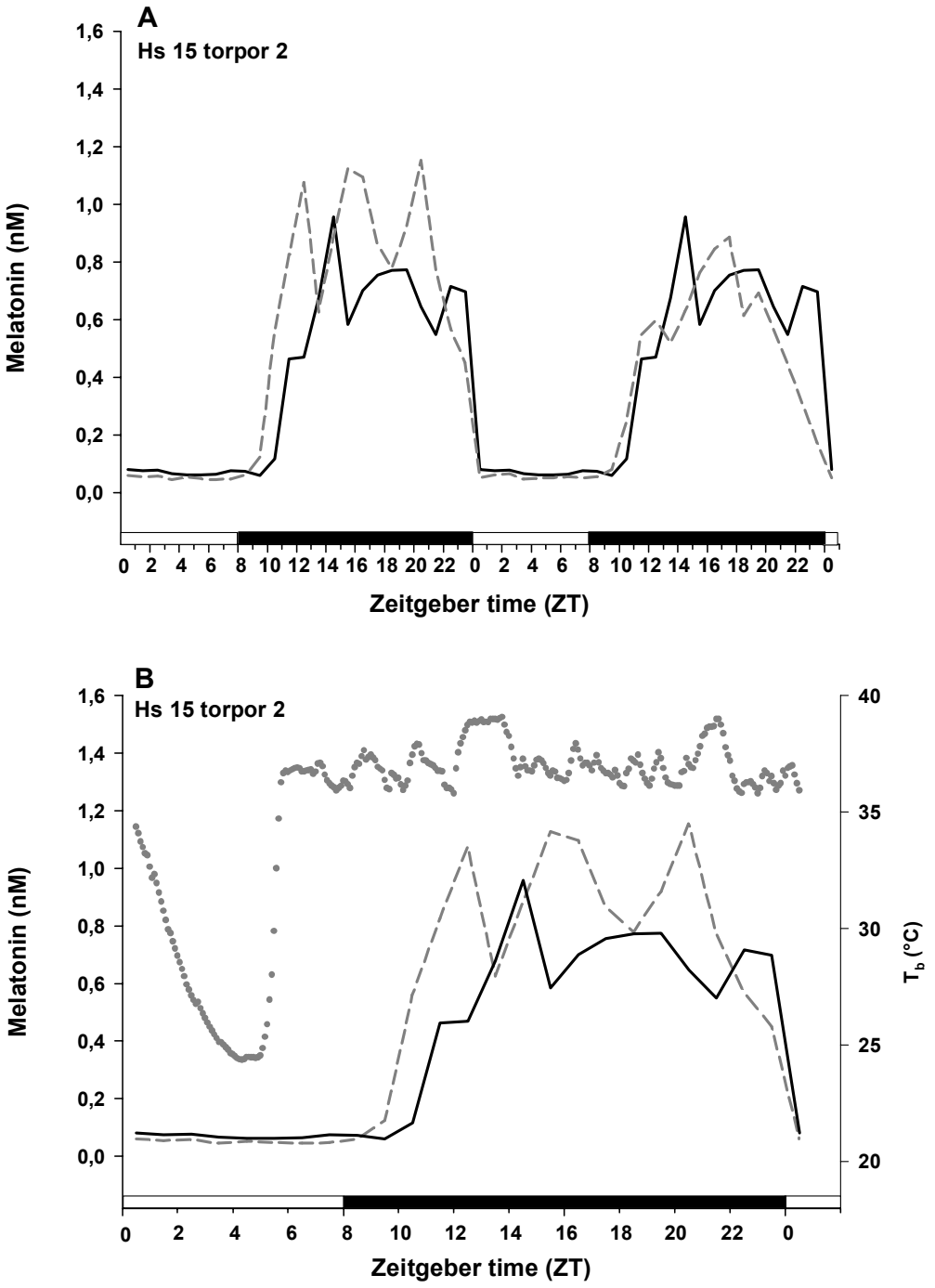

Figure 4: Profiles of pineal melatonin concentrations of animal Hs 15 (Torpor 2) measured by *in vivo* microdialysis in *Phodopus sungorus*. For details see figure 1.

Discussion

During the course of the experiment, 5 torpor episodes displayed by three animals could be observed. In all cases the torpor bout affected the onset of melatonin secretion in the night directly following arousal. It has previously been hypothesised that "melatonin pulses" after a torpor bout contribute to entrain and reset the circadian clock in the torpor season where animals retreat to an enclosed nest or burrow to enter torpor and light exposure is rare (RUBY 2003).

From our results in chapter 2 and 3 where we showed an advanced onset of *Aa-nat* as well as from the results of THOMAS et al. (1993), who showed a shortened τ in torpid Djungarian hamsters, we expected that the melatonin secretion would be advanced after arousal from torpor. This hypothesis is appealing because ecologically it would make sense. In DD, short day adapted Djungarian hamsters usually free run with a τ longer than 24 hours. Thus, in times with rare or no light exposure a regular phase advance after a torpor bout would ensure that the animal´s endogenous rhythm remains closer to the external LD cycle. Moreover, the sensitivity of the clock for melatonin is highest at the end of the day and very early night (REDMAN et al. 1983, LEWI et al. 1992, REDMAN 1997). In this experiment we saw both phase advance as well as phase delay of melatonin secretion after arousal from torpor.

Hs 2 showed two torpor episodes in which at both times the melatonin secretion was delayed. Regarding the T_b data it is noticeable that the torpor bouts of this animal occur relatively late on in the light phase and also the arousals are atypically late, i.e. already in the dark phase of the LD cycle. A comparable pattern with a late arousal between ZT7 and ZT8 and delayed melatonin secretion onset was observed in another animal (Hs 14, data not shown) where we only have data during one torpor bout. At first sight this appears to point against a role for melatonin resetting the clock, as an advance would be necessary to maintain entrainment. However, the clock´s sensitivity for melatonin decreases soon after dark onset (REDMAN et al. 1983, REDMAN 1997), so a delayed onset of melatonin secretion would probably have no effect at all.

In contrast, Hs 15 showed an advanced melatonin release as expected. The T_b data indicate that also the arousal from torpor occurred much earlier, namely in the early afternoon. This might point to a direct relation between time of arousal and time of melatonin release onset.

Such a relation has already been assumed by LARKIN et al. (2003) who demonstrated that arousal from torpor is accompanied by melatonin production due to the strong sympathetic activation during rewarming. Our observation would support this hypothesis. In Hs 15 the melatonin secretion starts indeed only after ~5 hours after arousal (whereas in Hs 2 it is elevated already 2 hours after rewarming), but this may well result from the presence of light which strongly inhibits melatonin release (chapter 4). As soon as the lights go off, melatonin concentrations rapidly increase.

Contrary to our results in chapter 2 and 3, where we observed a still advanced *Aa-nat* expression on the day following a torpor bout, we did not see an aftereffect on the melatonin release. It must be taken into account, though that in the experiments in chapter 2 and 3 we chose animals for the control group that have never been observed torpidly before. In the experiment in this chapter all investigated animals had displayed torpor before the experiment to increase the possibility that they still would do so after surgery. It is likely that displaying torpor generally alters the circadian organisation compared to animals that do not at all show hypothermia.

However, with only three investigated animals we cannot draw firm conclusions and need to study the issue of torpor affecting the circadian phase further.

Unfortunately none of the monitored animals showed torpor in DD. DD conditions started only at the end of the experiments and either the microdialysis probes broke or the animals did just not enter torpor.

Nevertheless, these results are a huge success, as the experimental setup is extremely difficult. Already the trans-pineal microdialysis approach in such a small animal which weighs only 20-30 g in short day conditions is tricky and the rate of successfully implanted animals lies by only 50%. In our setup it was not possible to monitor T_b and melatonin release more than 10 animals per experiment and the hourly melatonin sampling (which is done by collectors that have to be emptied every 10 hours) is very demanding. Moreover, this experiment was critical because we could not predict that the animals would display torpor at all. Torpor is a very sensitive process as animals are very vulnerable in this condition. Therefore, if the animals are disturbed or feel “unsafe” they do not enter hypothermia; so, we were very pleased to see that even despite the implanted Mini-Mitters and the microdialysis probe the animals showed hypothermia.

This study is proof of the fact that the microdialysis approach is valuable in order to investigate the effect of daily torpor on the melatonin release. Further, it can now confirm and broaden the observations of these preliminary data.

6

The circadian clock stops ticking during hibernation.

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Submitted

Abstract

Hibernation represents one of the most fascinating, yet enigmatic physiological phenomena (CAREY et al. 2003). During the harsh season this strategy allows substantial energy saving by reducing body temperature and metabolism (CAREY et al. 2003, GEISER 2004). Accordingly, biological processes are considerably slowed down and reduced to a minimum. However, the persistence of a temperature-compensated, functional biological clock in hibernating mammals has long been debated (KÖRTNER et al. 2000, RUBY 2003, HELLER and RUBY 2004). Here we show that the master circadian pacemaker no longer displays 24-h molecular oscillations under hypothermia, suggesting an arrest of the clock. We found that in the suprachiasmatic nucleus (SCN) of the European hamster, the expression of the clock genes *Per1*, *Per2* and *Bmal1* remains constant over 24-h in hypothermic animals. Similarly, the expression of the clock-controlled gene *arginine-vasopressin* is constantly suppressed. Finally, the melatonin rhythm generating enzyme, arylalkylamine-n-acetyl transferase, rhythmic expression in the pineal gland being controlled by the SCN, no longer displays day-night changes of expression, but constantly elevated mRNA levels over 24-h. Our results strongly suggest that during hibernation the circadian clock is stalled at the molecular level, and may no longer deliver rhythmic output signals.

Text

In mammals, the hibernation season consists of recurring bouts of hypothermia (or torpor) alternated with short periods of euthermia, during which body temperature (T_b) returns to ~37 °C (KILDUFF et al. 1993, KÖRTNER and GEISER 2000, CAREY et al. 2003, RUBY 2003, GEISER 2004). During deep torpor, energetically expensive cellular processes like transcription and translation are severely depressed, and physiological functions like heart rate, respiration, immune and renal functions, and neural activity run at greatly reduced rates (CAREY et al. 2003, GEISER 2004). The circadian system temporarily coordinates internal biological processes to ensure health and survival (KALSBEEK and BUIJS 2002, HASTINGS and HERZOG 2004, BELL-PEDERSEN et al. 2005). Circadian rhythms are

temperature compensated and run at a relatively constant pace under various temperatures (BELL-PEDERSEN et al. 2005). However, it has long been debated whether the circadian system remains functional during deep hibernation (KÖRTNER and GEISER 2000, RUBY 2003, HELLER and RUBY 2004). Although several studies have already addressed this question, uncertainty prevails since clock outputs rather than core clockwork mechanisms were examined (FRENCH 1977, FLORANT et al. 1984, VANECEK et al. 1984, 1985, STANTON et al. 1986, KILDUFF et al. 1989, GRAHN et al. 1994, CANGUILHEM et al. 1994, WOLLNIK and SCHMIDT 1995, HUT et al. 2002, RUBY et al. 2002, LARKIN et al. 2002). In mammals, the master circadian clock is located in the suprachiasmatic nuclei of the hypothalamus (SCN; KALSBEEK and BUIJS 2002, HASTINGS and HERZOG 2004, BELL-PEDERSEN et al. 2005). Circadian oscillations within SCN neurons result from recurrent expression of so-called *clock genes* that interact in complex, interlocked transcription / translation feedback loops (HASTINGS and HERZOG 2004, BELL-PEDERSEN et al. 2005). Assessing such molecular oscillations in the SCN of hibernating animals is a valuable approach for reconsidering the issue of a functional circadian clockwork during deep torpor.

For this we used the European hamster (*Cricetus cricetus)*, a well-defined hibernator (HERMES et al. 1989, CANGUILHEM et al. 1994, WOLLNIK and SCHMIDT 1995, MAGARINOS et al. 2006). Hamsters raised outdoor were transferred in September to a climatic room kept at 6±2 °C under short photoperiodic regimen. Individual T_bs were recorded via a telemetric system. Under these conditions, comparable to outdoor conditions in December, the bouts of hypothermia characteristic of the hibernation cycle were regularly expressed (Fig. 1a; CANGUILHEM et al. 1994, MAGARINOS et al. 2006). During deep torpor, basal metabolic rate is typically reduced to 2-4% of euthermic rates, and T_b drops close to ambient temperature (T_a; CAREY et al. 2003, GEISER 2004). In this experiment, the T_b of hypothermic hamsters was 8-10 °C (Fig. 1b). The European hamster enters hibernation preferentially around mid-night (CANGUILHEM et al. 1994, WOLLNIK and SCHMIDT 1995). We took advantage of this characteristic to design our experiment, as illustrated in Figure 1c. Radioactive *in-situ* hybridisation was used to assess gene expression in the SCN of *euthermic* versus *hypothermic* hamsters, killed during *daytime* (D), *nighttime* (N) or during the *subjective day* (SD).

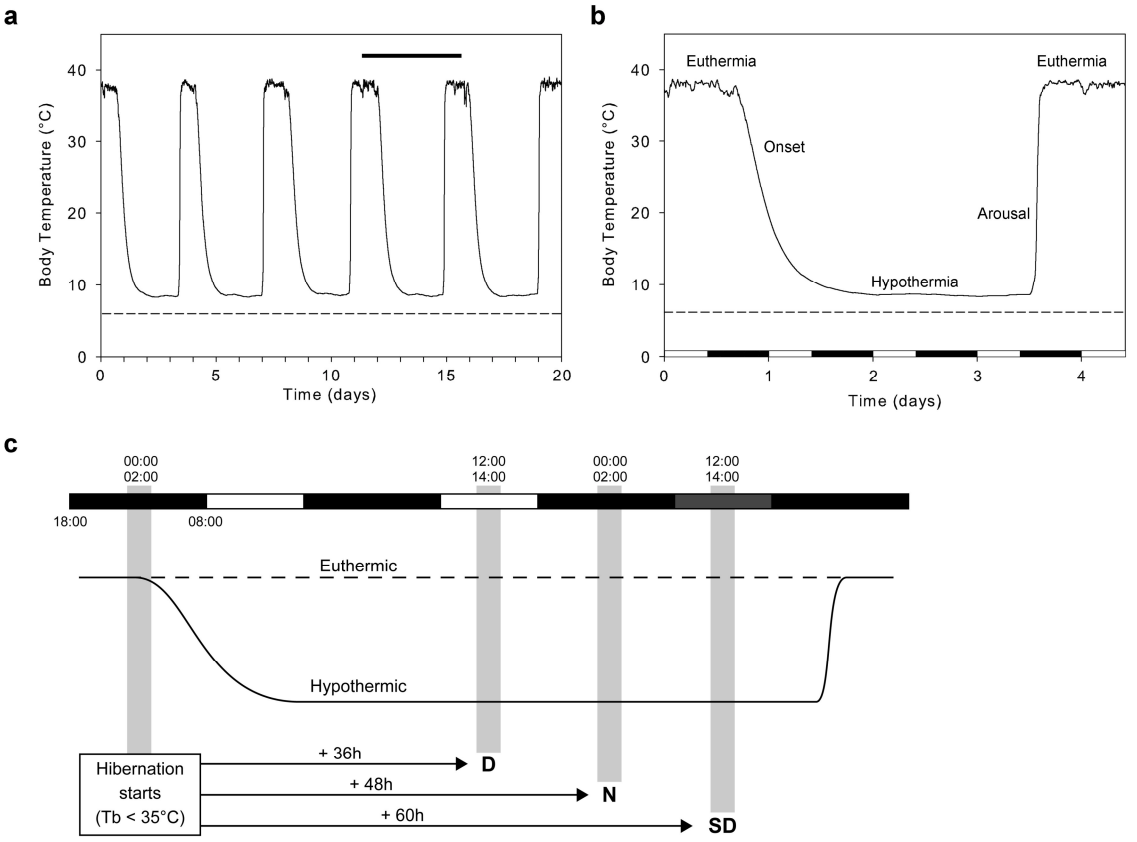

Figure 1: European hamster's hibernation pattern and experimental paradigm. **a**, **b**, Typical body temperature (T_b) recording (animal #B17) illustrating the major phases of the hibernation cycle of the European hamster. **a**, Three weeks of recording (25th Nov. to 14th Dec., 2005) showing 5 hibernation cycles. The horizontal black bar represents the period detailed in **b**. The ambient temperature of the climatic room was set at 6±2 °C (dotted line). **b**, Focus on a single hibernation cycle (6th Dec. to 10th Dec., 2005). Entrance into hibernation (T_b < 35 °C) preferentially occurred during night-time. **c**, Experimental paradigm used for the experiment. For the euthermic group, the hamsters had a T_b close to 37 °C, and were aroused from hibernation for more than 48-h. These animals were sacrificed at mid-day (13:00; D, day; n = 6), mid-night (01:00; N, night; n = 6) or mid-subjective day (13:00; darkness from the previous day; SD, subjective day; n = 5). For the hypothermic group, only the animals entering hibernation (T_b < 35 °C) between 00:00 and 02:00 (mid-night) were considered. Their sacrifice occurred 36-h (D, day; n = 4), 48-h (N, night; n = 5) or 60-h (SD, subjective day; no lights on for the last day; n = 7) later. Light and dark bars: day and night; Dark-grey bar: subjective day.

In the molecular core of the clock, the transcription factors CLOCK and BMAL1 drive the expression of the Period (*Per*) and Cryptochrome (*Cry*) genes, and in turn PER and CRY proteins heterodimerize and repress CLOCK/BMAL1-driven transcription (HASTINGS and HERZOG 2004, BELL-PEDERSEN et al. 2005). To compare the state of these molecular loops during hypo- versus euthermia, we examined the expression of 3 major clock genes: *Per1*, *Per2* and *Bmal1* (Fig. 2a,b,c). As expected in euthermic animals significant day-night changes of expression were observed, with high expression of *Per1* and *Per2* during the day and on the subjective day, and high expression of *Bmal1* during night-time. In contrast to euthermic animals, no day-night change of expression occurred in the SCN of hypothermic hamsters (Fig. 2a,b,c). In this condition, *Per1* mRNA levels were persistently elevated (Fig. 2a), whereas those of *Per2* were constantly depressed (Fig. 2b). For *Bmal1*, the nocturnal peak of expression was lost (Fig. 2c), and mRNA levels were intermediate between daytime and night-time values in euthermic hamsters. All in all, these results show that the robust day-night oscillations of *Per1*, *Per2* and *Bmal1* expression disappear during hibernation, strongly suggesting that the circadian clockwork ceases to function. A potential slow-down of the circadian oscillations and extended period of rhythm seem unlikely, because gene expression was not systematically restricted to intermediate levels. Importantly, we observed that the circadian molecular oscillations were clearly re-expressed during the interbout intervals. This "recovery" and re-synchronisation of the clock was presumably facilitated by the presence of the light-dark cycle.

If the molecular circadian clock is stopped during hypothermia, then the 24-h rhythm of its output signals should be lost Thus, we examined the expression of *arginine-vasopressin* (*Avp*), a *clock-controlled gene* whose rhythmic expression in the SCN directly depends upon the molecular circadian machinery (JIN et al. 1999, KALSBEEK and BUIJS 2002, HASTINGS and HERZOG 2004). As expected, *Avp* expression displayed a clear day-night change of expression in euthermic hamsters (Fig. 2d), with high mRNA levels during the day and on the subjective day. This was in marked contrast to hypothermic animals, in which *Avp* expression was persistently depressed (Fig. 2d). This result confirms and extends our previous conclusion, suggesting that the SCN molecular clockwork and at least one of its molecular output cease during hypothermia.

Interestingly, the SCN maintain a relatively high metabolic activity during hibernation, as assessed by [^{14}C] 2-deoxyglucose uptake and *c-Fos* expression {KILDUFF et al. 1982, 1989

BITTING et al. 1994). In the present study, examination of *c-Fos* mRNA levels in the SCN also revealed a dramatic increase of expression in hibernating hamsters relative to euthermic animals (Fig. 2e), without significant day-night differences. This suggests that the loss of circadian organisation in the SCN of hibernating hamsters does not simply result from reduced metabolic activity or transcription, and favours the hypothesis that the SCN may serve a non-circadian role during hibernation (RUBY 2003).

Finally, we questioned whether hibernation equally affects the rhythmic activity of SCN targets. Synthesis of melatonin by the pineal gland represents one of the best characterised outputs of the SCN (KALSBEEK and BUIJS 2002, PERREAU-LENZ 2003, GARIDOU et al. 2003, BELL-PEDERSEN et al. 2005). The SCN restricts production of melatonin to night-time by controlling the activity of its rhythm-generating enzyme, the arylalkylamine-N-acetyltransferase (AA-NAT). In the European hamster, the daily variations of AA-NAT activity result from changes in transcription of the *Aa-nat* gene (GARIDOU et al. 2003). The expression of this gene was therefore used as an index of the pineal gland activation by the SCN. As expected in euthermic animals, *Aa-nat* expression was increased more than 17 fold during night-time (Fig. 3a, GARIDOU et al. 2003). This was faithfully translated into changes of plasma melatonin, as measured by radioimmunoassay (Fig. 3b). In contrast, *Aa-nat* expression was persistently high during hibernation (Fig. 3a), comparable to that in euthermic animals during night-time, suggesting that melatonin synthesis is no longer rhythmic. This was confirmed at the melatonin level, although plasma melatonin concentrations were continuously low during hypothermia, as already described in other species. Interestingly, plasma melatonin level has been reported to rise rapidly after arousal from hibernation, whatever the time of arousal (FLORANT et al. 1984, VANECEK et al. 1984, 1985, STANTON et al. 1986). The fact that *Aa-nat* mRNA levels remain persistently elevated during hibernation may well explain these observations, and suggests a possible decoupling between transcriptional activity, and protein synthesis / enzymatic function.

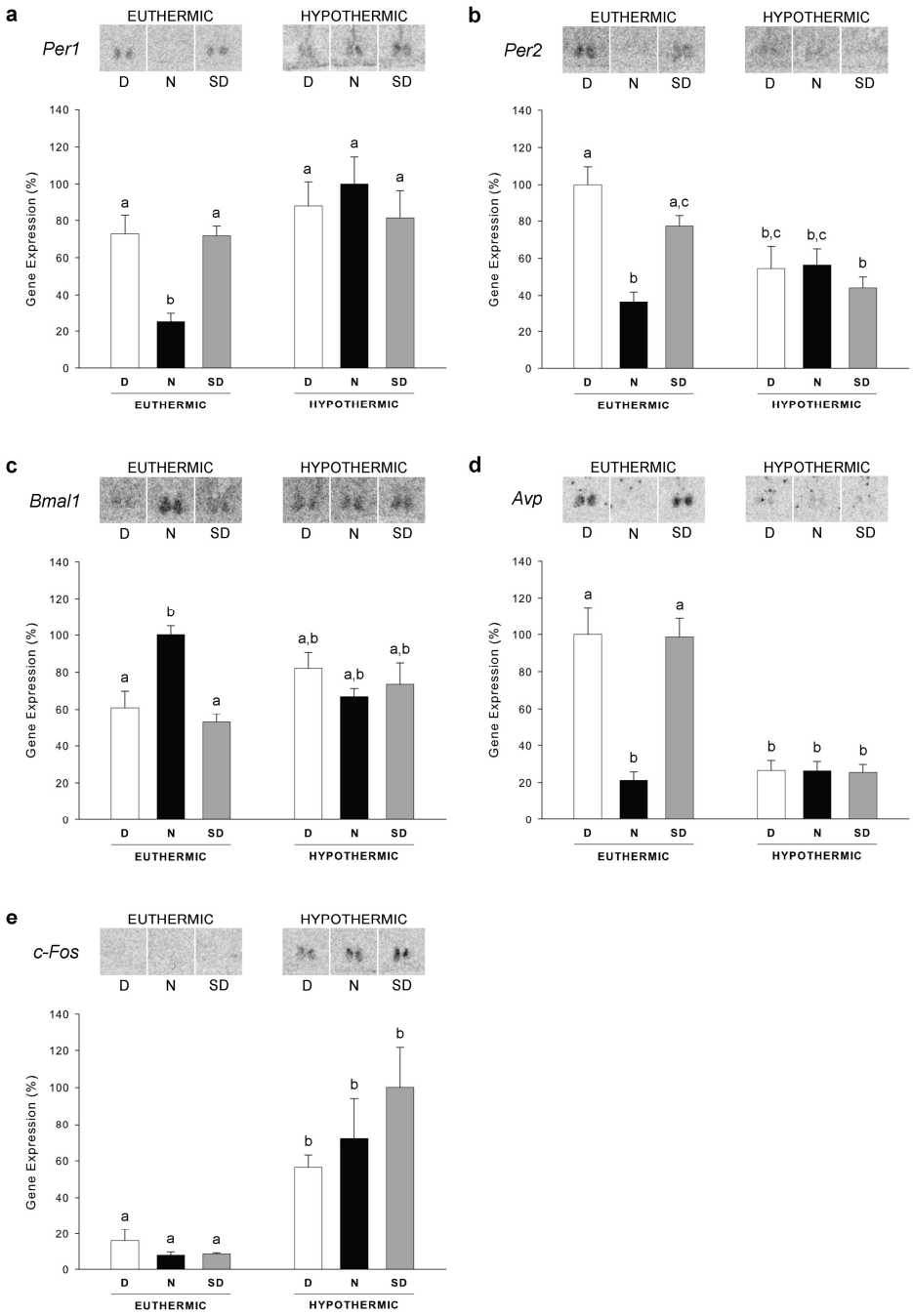

Figure 2: The circadian expression of clock genes in the suprachiasmatic nucleus is eradicated under deep hibernation. **a-e**, In-situ hybridisation was used to examine gene expression in euthermic and hypothermic hamsters during daytime (D), night-time (N) or during the subjective day (SD). For all genes, the upper panel displays representative autoradiograms for each condition; the lower panel shows the quantified mRNA levels. Expression of the clock genes *Per1* (**a**), *Per2* (**b**) and *Bmal1* (**c**) exhibits circadian fluctuations in euthermic hamsters (*Per1*, *Per2*: P < 0.001, N versus D and SD; *Bmal1*: P = 0.008, N versus D and SD), whereas their mRNA level is constant in hypothermic animals

(*Per1*, *Per2*, *Bmal1*: P > 0.05). During hibernation, *Per1* expression is elevated (**a**; no significant difference with daytime levels in euthermic animals; P > 0.05), *Per2* expression remains low (**b**; no statistical difference with night-time levels in euthermic animals; P > 0.05), and *Bmal1* expression is intermediate between daytime and night-time levels in euthermic hamsters (**c**; P > 0.05 versus D and SD; P > 0.05 versus N). Similarly, the expression of the clock controlled gene *Avp* (**d**) is rhythmic in euthermic (P < 0.001, N versus D and SD), but not in hibernating hamsters (P > 0.05), in which its expression remains low (for all hypothermic conditions, P > 0.05 versus euthermic N, and P < 0.001 versus euthermic D and SD). The expression of the immediate early gene *c-Fos* (**e**) is dramatically increased in hypothermic relative to euthermic hamsters (P < 0.001), without significant day-night fluctuations (P > 0.05). Data are shown as percentage of the maximum and represent the means ± s.e.m. (n = 4-7). Error bars denote s.e.m.. Different characters (a, b, c) indicate significant differences (P < 0.05).

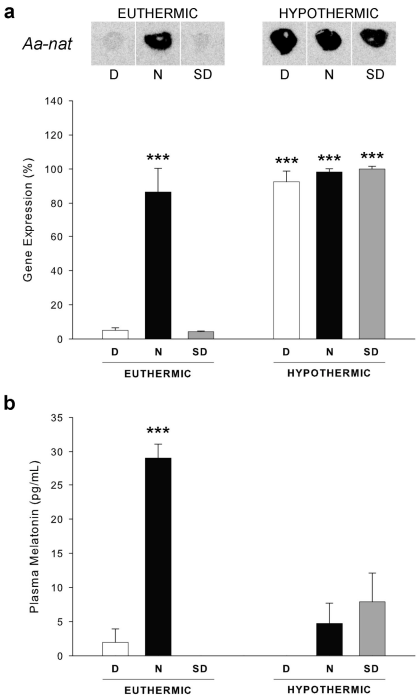

Figure 3: *Aa-nat* is constitutively expressed in the pineal gland of hibernating hamsters. **a**, Representative autoradiograms and quantification of *Aa-nat* mRNA demonstrate marked day-night changes of expression in euthermic animals (P < 0.001, N versus D and SD), while in hypothermic hamsters it is persistently expressed (P > 0.05) and displays high mRNA levels (for all hypothermic conditions, P > 0.05 versus euthermic N, and P < 0.001 versus euthermic D and SD). Data are shown as percentage of the maximum and represent the means ± s.e.m. (n = 4-7). Error bars denote s.e.m.. ***, p < 0.001. **b**, A rhythmic concentrations of plasma melatonin is observed in euthermic animals (P < 0.001, N versus D and SD), but not in hibernating hamsters which exhibit constantly low plasma melatonin levels (for all hypothermic conditions, P > 0.05 versus euthermic D and SD). Data are means ± s.e.m. (n = 4-7). Error bars denotes s.e.m. . ***, p < 0.001.

Overall, our results show that the circadian clock of the SCN stops generating 24-h rhythms during hibernation. This is the first physiological condition in which an arrest of the master circadian clock over several days is described. The issue has been debated for many years with various evidence either denying or supporting the existence of a functional pacemaker during deep torpor (FRENCH 1977, FLORANT et al. 1984, VANECEK et al. 1984, 1985, STANTON et al. 1986, KILDUFF et al. 1989, GRAHN et al. 1994, WOLLNIK and SCHMIDT 1995, KÖRTNER and GEISER 2000, RUBY et al. 2002, LARKIN et al. 2002, HUT et al. 2002, RUBY 2003, HELLER and RUBY 2004). These contradictions may stem from species difference as well as from the variety of markers used to assess the functional state of the clock. Based on the recording of small circadian fluctuations in T_b, some reports have suggested the maintenance of circadian rhythms in hibernating ground squirrels (GRAHN et al. 1994, RUBY et al. 2002, LARKIN et al. 2002). However, consistent with other reports we did not detect such variations in the European hamster (CANGUILHEM et al. 1994, WOLLNIK and SCHMIDT 1995, HUT et al. 2002,). In this study we assessed gene transcription. Thus, we cannot rule out the possibility that the circadian clock might continue to function based on separate mechanisms like post-transcriptional regulations. Furthermore, the technique we used may not be suitable for detecting either small subsets of cells that retain temperature-compensated time measurement, or desynchronization amongst individual SCN oscillators (GRAHN et al. 1994, HELLER and RUBY 2004). However, we did not systematically observe intermediate mRNA levels, and the rhythmicity of both *Aa-nat* expression and plasma melatonin levels were lost during hibernation. The possibility that an extra-SCN oscillator could take over the circadian clock function is also unlikely, since the SCN is one of the most active brain structure during hibernation (KILDUFF et al. 1982, 1989). Furthermore, we and others previously demonstrated that arousals are randomly distributed over 24-h, supporting the absence of a circadian timer during hibernation (CANGUILHEM et al. 1994, WOLLNIK and SCHMIDT 1995). Electrophysiological studies indicate that the circadian clock of hibernators still functions at temperatures lower than 37 °C, but is likely to stop at temperatures below 16.6 °C (MILLER et al. 1994, RUBY 2003, HELLER and RUBY 2004). The absence of action potentials, together with the vanishment of rhythmic oscillation in clock gene expression, make temperature compensation unlikely at low T_b (5-10 °C). This property of the clock may be limited to a range of temperatures, as rhythmic clock gene expression still occurs during daily torpor, a shallower form of

hypothermia during which T_b decreases to 15-20 °C for only several hours (HERWIG et al. 2006b).

In conclusion, our data demonstrate that in the European hamster the molecular clock of the SCN is arrested during deep hibernation and no longer delivers circadian signals.

METHODS

Animals

All experiments were performed in accordance with the rules of the French Department of Agriculture (license no 67-38) and the European Committee Council Directive of November 24, 1986 (86/609/EEC). The adult male European hamsters used in this study were bred in-house. The colony was established from animals caught in the fields near Strasbourg, France between 1994 and 1996 Hamsters were raised under semi-natural conditions (natural photoperiod) with *ad libitum* access to water and food. In September, animals born in May of the previous year (16-17 months old) were transferred to a climatic room kept at 6±2 °C, under a 10-h light (150 lux; lights off at 18:00) / 14-h dark (2 lux dim red light) cycle. The core body temperature (T_b) was continually registered via thermo-sensitive radio-transmitters (Model VM-FH-LT, Mini-Mitter Co., Sunriver, OR, USA) implanted in the abdominal cavity under halothane anesthesia, before placing the hamsters in the climatic room. Radiofrequency signals from the implanted transmitters were averaged every 5 min by receivers placed under each cage and collected by an automated computer software (Dataquest, St. Paul, MN). Straw was given as nest-building material. All the animals considered in this study had completed at least 3 bouts of hypothermia. Sacrifice occurred as depicted in Figure 1b. Animals were anesthetised with N_2O and killed by decapitation. Brains were rapidly removed, snap-frozen at -30 °C, and stored at -80 °C until *in-situ* hybridization. Trunk blood was collected, centrifuged at 1,500 x g for 15 min and plasma was stored at -20 °C until melatonin assay.

In-situ hybridisation

Radioactive *in-situ* hybridisation was performed as previously described (GARIDOU et al. 2003), using riboprobes for rat *Period1* (bases 638-1618 from GenBank accession number

NM_001034125), *Period2* (bases 1170-1930 from NM_031678; donated by Dr. H. Okamura, Department of Anatomy and Brain Science, Kobe University School of Medicine, Japan), *Bmal1* (bases 75-1809 from AB012600) and *arginine vasopressin* (bases 68-539 from M25646), or for Syrian hamster *c-fos* (bases 625-1025 from AF061881) and *Aa-nat* (bases 1-1045 from AF092100). After they were fixed, acetylated, and dehydrated, 20-µm thick coronal brains sections were hybridised overnight at 54 °C with ^{35}S-UTP labelled riboprobes The sections were then treated with Rnase (10 µg/ml), washed (SSC 0.1x, 60 °C), dehydrated and exposed to a BioMax MR Film (Kodak) for 3-5 days along with ^{14}C-radioactive standards to allow standardisation of densitometric measurements across films. X-ray films were scanned on an Epson 4990 transmittance scanner, and background subtracted, calibrated optical density (OD) measurements of mRNA levels were performed using ImageJ (NIH).

Melatonin radioimmunoassay

Plasma melatonin was analysed by a radioimmunoassay previously validated in European hamsters and described in the reference (GARIDOU et al. 2003).

Data and Statistical Analyses

Experiments were performed twice with similar results, and the data reported here are those from the second experiment. Results are shown as a percentage of the maximum and represent the mean ± SEM. Data were analysed by two-way analysis of variance (ANOVA), followed by HSD Tukey's analysis. Statistical significance was set at $P < 0.05$.

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Authors` contributions

FGR and AH contributed equally to this work, and performed all molecular and statistical analysis. PP conceived the original experiment and coordinated the study. MS performed the physiological studies and monitored the animals on a daily basis for several years. MLG, HD, JSM, MMP and VS performed the preliminary experiments. All authors discussed the results and commented on the manuscript.

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Histamine H3 receptor and Orexin A expression during daily torpor in the Djungarian hamster (*Phodopus sungorus*).

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Submitted

Abstract

Controlled hypometabolism and hypothermia lead to a reduction in CNS activity. Nevertheless, brain systems of importance for controlling and maintaining the hypothermic state have been shown to increase activity. During hibernation the histaminergic system seems to actively inhibit other neurotransmitter pathways through the inhibitory auto – as well as hetero-histamine H3 receptor. In deep hypothermia H3 receptors have been shown to be up-regulated in several brain areas. In this study we demonstrate an up-regulation of H3 receptors in several brain nuclei including arcuate nucleus, dorsomedial hypothalamus, suprachiasmatic nucleus dorsal lateral geniculate nucleus and tuberomammilary nucleus during daily torpor, a shallow form of hypothermia in the Djungarian hamster (*Phodopus sungorus*). This suggests that inhibition through histaminergic pathways may play a more general role in maintaining low body temperature and torpor state in mammals. Moreover, we showed that mRNA for orexins, a group of neuropeptides that have been shown to increase wakefulness, remain unchanged during the arousal from daily torpor. Therefore, it is likely that this "arousal" pathway is not involved in the arousal from hypothermia.

Introduction

Controlled hypothermia is a powerful strategy for reducing the high metabolic costs of a constant body temperature (T_b) during periods of prolonged food shortage. Different forms of hypothermia have evolved. In contrast to long enduring hibernation bouts lasting several days, some small mammals undergo bouts of daily torpor where the circadian timing system is used to precisely time hypo-metabolism and hypothermia (HELDMAIER et al. 2004). Decreasing day-length provides an anticipatory mechanism of acclimatisation to winter for the Djungarian hamster, also known as Siberian hamster (*Phodopus sungorus*), evoking physiological adaptations to winter conditions, including daily torpor. During a torpor bout the extent of hypothermia and hypo-metabolism is less pronounced than in deep hibernation and consequently less energy is saved (HELDMAIER and RUF 1992, GEISER and HELDMAIER 1995). However, the physiological properties are very similar, and simply

represent gradual differences in timing, duration and amplitude of an analogous physiological state.

Invariably is associated with a depression of CNS activity but it is crucial that functional integrity is maintained. The hibernating brain needs to accurately control the complex metabolic processes and to remain sensitive to external and internal stimuli (HELLER 1979). It has been shown that the histaminergic system, involved in the regulation of sleep-wake cycle, T_b and energy metabolism (SCHWARTZ et al. 1991), is important in initiating and maintaining the hibernating state in ground squirrels (*Citellus lateralis*) (SALLMEN et al. 1999, 2003a,b). Contrary to many studies that have shown a general depression of neuronal transcription and translation activity in this state (FRERICHS et al. 1998, VAN BREUKELEN et al. 2002a), perhaps serving to maintain homeostatic balance in extremely hypoxic and nutrient deficient periods, histamine turnover in the hypothermic brain is known to increase. The increased histamine activity seems to be linked to the up-regulation and elevated binding of the inhibitory histamine H3 receptors in several brain areas including cortex, caudate nucleus and putamen as well as globus pallidus and substantia nigra (SALLMEN et al. 2003a). The inhibitory $G_{i/o}$ protein coupled receptor has auto- as well as heteroreceptor functions and it is likely that histamine thereby influences and regulates other neurotransmitter systems. In the Siberian hamster, H3 receptors are expressed in various nuclei of the hypothalamus, the principal brain region controlling autonomic functions and for which a role for seasonal energy regulation has been assumed (BARRETT et al. 2005). In this study we investigated whether H3 receptors are also involved in controlling daily torpor. If torpor indeed underlies mechanisms comparable to deep hibernation, the histaminergic system might also actively inhibit CNS activity and H3 receptors should be up-regulated in the brain of torpid Djungarian hamsters.

Molecular mechanisms underlying and preparing the arousal from daily torpor, accompanied by strong activation of the sympathetic nervous system, are still unknown. Orexins are a group of peptides that have been linked to the arousal state of animals (RODGERS et al. 2001, ISHII et al. 2005). Orexin neurons are mainly located in the lateral hypothalamus and project to other hypothalamic regions such as the paraventricular, arcuate, lateral, perifornical and ventromedial nuclei, areas that have all been associated with feeding and wakefulness (KHOROOSHI et al. 2005, KOTZ 2006). The absence of a functional orexin OX2R receptor causes narcolepsy in dogs and the absence of the peptide orexin in humans also results in

narcolepsy whilst orexin administration enhances arousal (KOTZ 2006). As orexins have also been related to non-shivering thermogenesis in brown adipose tissue (YASUDA et al. 2005, KOTZ 2006), i.e. the principal mechanism for regaining normal T_b after hypothermia, we also investigated whether orexin may be important for the arousal from daily torpor.

Material and Methods

Animals

Male Djungarian hamsters (*Phodopus sungorus*) were raised in the breeding colony of the University of Veterinary Medicine, Hanover, Germany. Animals were born between April and July and raised under natural photoperiod. Body weight and pelage score were monitored to ensure hamsters responded appropriately to changing photoperiod (FIGALA et al. 1973). Animals were implanted with radiofrequency transmitters to monitor body temperature and torpor state. They were acclimatised to short photoperiod of 8:16h light:dark for 2 weeks prior to transfer to climatic chambers with an ambient temperature (T_a) of 18 °C on the same photoperiod. Brains were removed during entry into torpor (n=6; T_b:30,94±1,61 °C) at ZT1 with ZT0 corresponding to "lights-on", deep torpor (n=7; T_b:23,87±1,34 °C) at ZT4, arousal (n=6; T_b:33,63±1,23 °C) at ZT8 and in a normothermic state following the torpor bout (n=6; ZT12). Control brains of normothermic animals were sampled at the same time points (n=6 each). Brain tissue was rapidly removed and frozen on dry ice. Cryostat sections of 14 µm thickness were thaw mounted on SuperfrostPlus® sildes and stored at -80 °C until further use.

In situ hybridisation

A probe for the histamine H3 receptor has been described previously (10). ^{33}P-UTP (3000Ci/mmol, ICN) labelled sense and antisense riboprobes were generated with SP6 polymerase (Sph1 digestion) and T7 polymerase (Spe1 digestion), respectively. The probes were purified with Chromaspin 100 columns (BD Bioscience) Sections were fixed for 15 min in 4% paraformaldehyde, rinsed twice in 1× PBS 5 min each and then acetylated for 10 min in 0.1 µM triethanolamine, 0.375% acetic anhydride. After rinsing in 1× PBS for 5 min, sections were dehydrated in graded ethanol series (70%, 80%, 90%, 95%, 100%, and Chloroform 5 min each) and finally dried at room temperature (RT). The cover-slipped slides were

hybridised with the riboprobe in a humid chamber in a solution containing 50% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 2× SSC, 500µg/ml salmon sperm DNA, 0.3% β-mercaptoethanol at 54 °C over night. Post-hybridisation washes with 1x10 min 2XSSC-50% formamide (RT), 2x30 min 2XSSC-50% formamide (60 °C, 1x30 min RNAse A (20 µg/ml in TEN buffer) 37 °C, 2x15 min 2XSSC-50% formamide 60 °C, 1x30 min 0.5XSSC 60 °C were performed before dehydration in graded ethanol series (70%, 90%, 95% (with 250 mM ammonium acetate) and 100%, 1 min each) and dried at RT.

For Orexin A in situ hybridisation an oligoprobe with the sequence 5'-GCT CTG CGC CTG CGC GGC GGC CCA TGG TCA GGA TGC CAG CTG - 3' was designed. Linearised plasmids were labelled with ^{35}S-ATP (NEN, NEG034H) using 10x tailing buffer and TdT (Promega, M1875) and purified on NAPTM5 columns (SephadexTM G25 DNA Grade, Amersham). Fixation was performed as described above. Sections were hybridised with the oligoprobe in a humid chamber at 37 °C over night. Post hybridisation slides were briefly rinsed in 1xSSC before being washed in 1xSSC for 3x30 min at 55 °C and 1xSSC for 60 min at RT. Finally, sections were dehydrated and dried as described above.

Slides were exposed to BioMax MR films (Kodak) for one week. Sections were scanned with Image Pro-Plus software, ver. 6.0 (Media Cybernetics) quantitative analysis was performed using Image J software.

Statistical analysis

To assess whether hybridisation signals showed a time-dependence in either normothermic or torpid animals and single time points differed between the two groups a two-way analysis of variance (ANOVA) with a *post hoc* Bonferroni test was performed. The data were analysed using GraphPad Prism 4.00 for Windows, GraphPad Software, San Diego California USA.

Results

Photoperiodic response in the Djungarian hamster

All animals used in the experiment had shown a clear photoperiodic response. Body weight decreased by 23.1% from 34.02g ± 4.79g in August to 25.89g ± 3.10g in December. Mean

pelage score changed during this time from 1 to 4 (according to 16), that is from summer agouti colour to nearly white winter coat.

Body temperature

Radio-telemetry successfully revealed the daily body temperature and torpor response in our Djungarian hamsters. Hamsters entered torpor approximately 1h before lights on and terminated torpor approximately 8h later at lights off and showed a 17 °C drop in body temperature compared to normothermic hamsters (Fig. 1a,b)

Histamine H3 receptor (H3R) mRNA expression

H3R expression is shown in Figure 2. Expression was significantly up-regulated during torpor in the arcuate nucleus (ARC) ($p<0.001$; Fig. 2a), dorso medial hypothalamus (DMH) ($p<0.01$; Fig. 2b), the suprachiasmatic nucleus (SCN) ($p<0.050$; Fig. 2c), dorsal lateral geniculate nucleus (DLG) ($p<0.010$; Fig. 2d) and tuberomammilary nucleus (TM) ($p<0.001$; Fig. 2g). Within the suprachiasmatic nucleus, H3R expression showed a diurnal change, with lowest expression at ZT8 and peak expression between ZT1 and ZT4 ($p=0,005$; Fig. 2c) in normothermic animals. This rhythm was eradicated in torpid animals where expression was upregulated at ZT8 ($p<0.050$; Fig. 2c). Also in the tuberomammilary nucleus H3R expression appeared to be time dependent ($p=0.048$) in the normothermic animals with high night-time expression compared to low daytime levels. No significant changes were observed in the cortex (Fig. 2e), caudate/putamen (Fig. 2f), ventromedial hypothalamic nucleus (VMH, data not shown), and ventral posterior medial nucleus of the thalamus (VPM, data not shown). No time-dependent expression was seen in other structures in torpid or normothermic animals.

Orexin A mRNA expression

Orexin A mRNA expression was confined to the lateral hypothalamus (LHA; Fig. 3). Expression was rhythmical in normothermic animals (*ANOVA* $p=0.04$) with highest expression at ZT4 and lowest at ZT12. There was no rhythmical change in expression observed in torpid hamsters. In torpid animals, orexin A mRNA was significantly down-regulated relative to normothermia animals at ZT4 (*t-test* $p=0.03$), and also during arousal at ZT8 (*t-test* $p=0.04$).

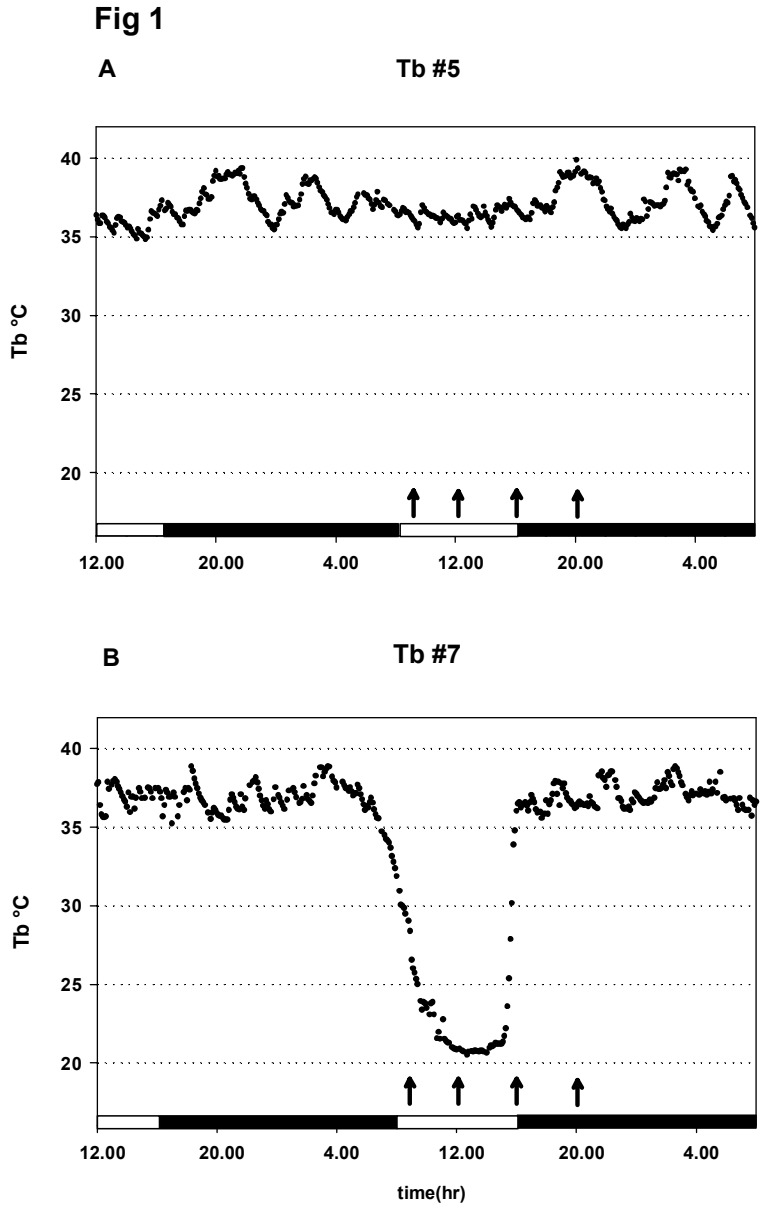

Figure 1: Representative body temperature rhythms (T_b) in short photoperiod adapted Djungarian hamsters (LD8:16). Panel A illustrates T_b of a normothermic animal, panel B of an individual that displayed daily torpor at an ambient temperature (T_a) of 18±2 °C. T_b was recorded at 6 min intervals with i.p. implanted radiofrequency transmitters. Black arrows indicate the sampling points at ZT1, ZT4, ZT8 and ZT12, with ZT0 corresponding to lights on.

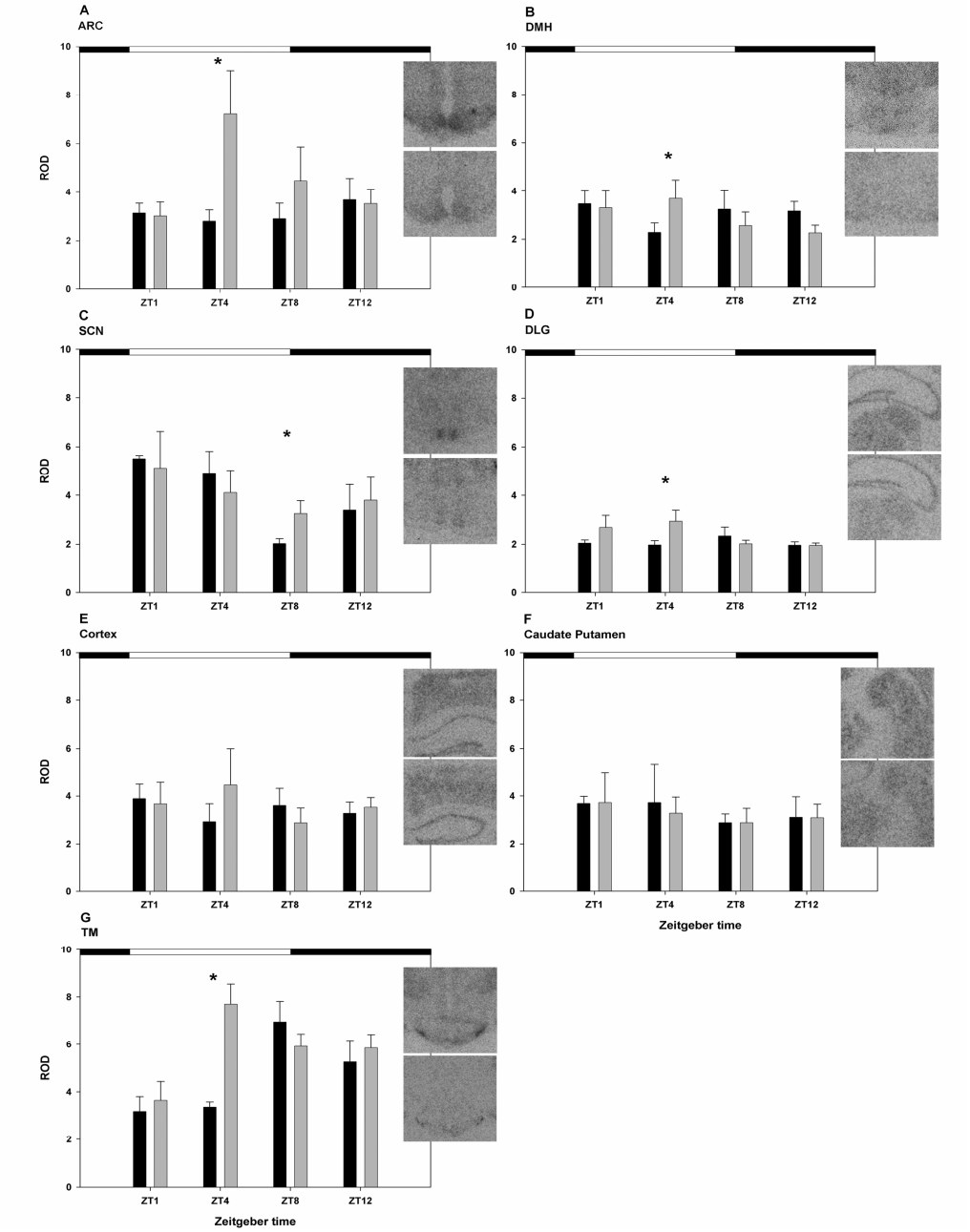

Figure 2: H3 receptor expression in various brain regions of normothermic (black bars, n=6) and hypothermic (grey bars, n=6) Djungarian hamsters. Animals were kept in short photoperiod (LD8:16). Samples were taken at ZT1, ZT4, ZT8 and ZT12 (ZT0=lights on) along the torpor cycle and hybridised with a ^{33}P labelled H3 receptor riboporobe. Significantly increased H3 receptor mRNA levels were found in the ARC (panel A, $p<0.001$), DMH (panel B, $p<0.050$), SCN (panel C, $p<0.010$), DLG (panel D, $p<0.010$) and TM (panel G, $p<0.001$) during torpor, but not in cortex (panel E) or caudate putamen (panel F). Statistical significances are indicated through * for $p<0.05$. The upper and lower images represent sections from torpid and normothermic animals respectively at ZT4 in the ARC, DMH, DLG, TM, Cortex, Caudate Putamen, at ZT8 in the SCN.

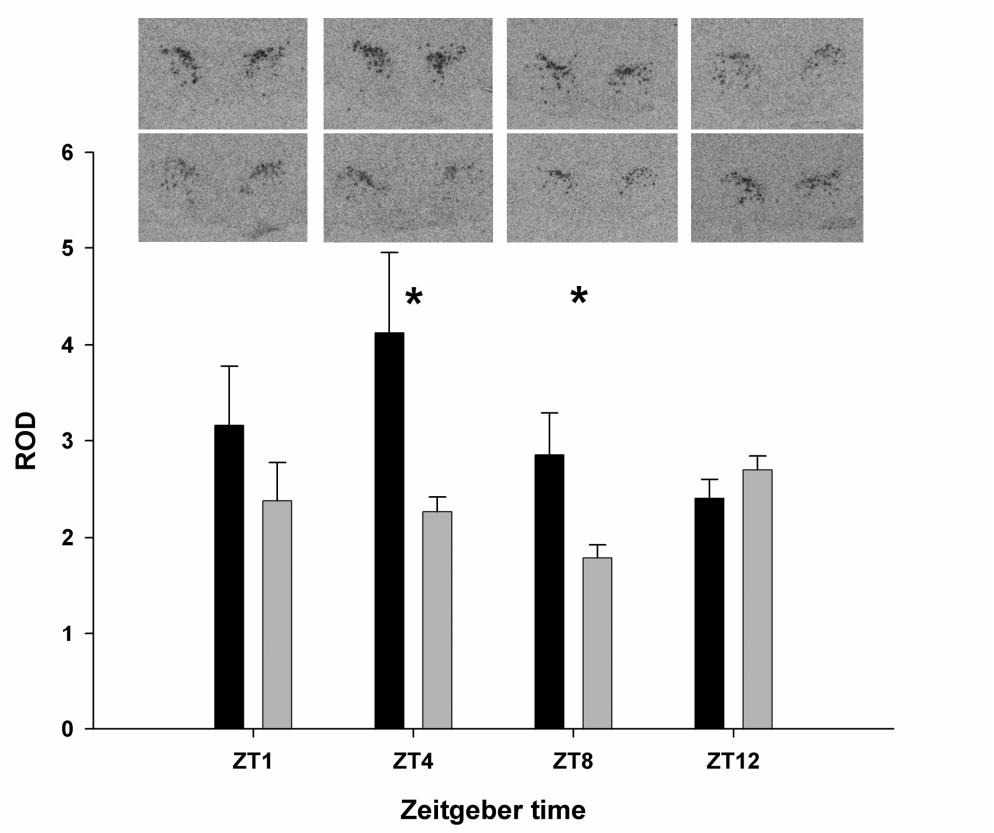

Figure 3: Orexin A expression in the LHA of normothermic (black bars, n=6) and hypothermic (grey bars, n=6) Djungarian hamsters. Animals were kept in short photoperiod (LD8:16). Samples were taken at ZT1, ZT4, ZT8 and ZT12 (ZT0=lights on) along the torpor cycle and hybridised with a ^{35}S labelled orexin A oligoprobe. Orexin A mRNA was significantly reduced during torpor (ZT4, p=0.03) as well as arousal (ZT8, p=0.04). The upper and lower panels show representative sections from normothermic and torpid animals, respectively.

Discussion

H3 receptors

We show significant increases of histamine H3 receptor mRNA in the arcuate nucleus, dorsomedial hypothalamus, suprachiasmatic nucleus, dorsal lateral geniculate nucleus and tuberomammilary nucleus during daily torpor. Increased histamine H3 receptor mRNA expression during hypothermia has already been described in the brain of hibernating golden-

mantled ground squirrels (SALLMEN 2003a). Our data in a torpid model suggest that the histaminergic system may play an important role in the hibernation process – a state where transcription as well as translation are generally depressed (CHEN et al. 2001, VAN BREUKELEN and MARTIN 2001, 2002a,b). During torpor, the animal takes advantage of low T_b to induce a profound reduction of metabolic rate, but specific adaptations exist to keep the cellular and molecular machinery functional in order to ensure a rapid reactivation during inter-bout arousal (KNIGHT et al. 2000, VAN BREUKELEN and MARTIN 2002a,b). SALLMEN et al. 2003 suggested that the specific up-regulation of inhibitory H3 receptors could mediate the active down-regulation of various neurotransmitter systems at cold temperatures and may therefore be important in the control of hibernation state. Our data, showing significant changes in expression over the course of a shallow torpor bout, suggest that deep hibernation and daily torpor may share common histamine-dependent features over the course of a low-temperature bout.

In our study H3 receptors were up-regulated in various hypothalamic nuclei implying involvement in metabolic processes and autonomic regulation. As previously described (BARRETT et al. 2005, ROSS et al. 2005) H3 receptor mRNA is expressed in the arcuate nucleus, a structure which is subject to photoperiod-dependent seasonal variation in expression in this species. Since histamine-synthesizing neurons are confined to the tuberomamillary nucleus (SCHWARTZ et al. 1991) and are not present in the arcuate nucleus, H3 receptors might inhibit hypothalamic neurons of the arcuate nucleus which are involved in acute responses to energy demand (EBLING et al. 1998). These neurons contain neuropeptide Y, agouti-related protein, and the inhibitory neurotransmitter GABA, both of which are key regulators of feeding behaviour, and local regulation by H3 receptor up-regulation may serve to suppress this behaviour during torpor (RUF et al. 1991). In our study we did not observe changes in H3 receptor expression in the dmpARC, a subdivision of the arcuate nucleus which has been shown to be an area of the brain that is highly responsive to photoperiod (BARRETT et al. 2005). Here we found low H3 receptor expression as would be expected for hamsters housed in short photoperiod (BARRETT et al. 2005). The lack of H3 receptor induction during torpor suggests that the dmpARC is not directly involved in acute physiological responses to energy demand over the course of a torpor bout.

In the tuberomammilary nucleus H3 recptors were significantly up-regulated during torpor. Tuberomammilary neurons innervate the whole brain and are the sole source neuronal

histamine. The activity of tuberomammilary neurons is strongly associated with behavioural state. They fire tonically during waking, less so during slow wave sleep and not at all during rapid eye movement sleep. Further, histamine has been shown to influence the ability to sustain wakefulness likely through activation of the H1 receptor (VANNI-MERCIER et al. 1984, MONTI et al. 1994). During torpor, this system might be inhibited through H3 receptors and hence serve to maintain a low state of arousal.

At the lowest point of the body temperature cycle, up-regulation of H3 receptors were also detected in the DMH, an important thermoregulatory site within the hypothalamus. Disinhibition of DMH neurons by microinjections of the $GABA_A$ receptor antagonist bicuculline methiodide has been shown to markedly elevate core T_b in freely moving rats at room temperature, while similar microinjections evoked rapid and dramatic increases in temperature within brown adipose tissue (BAT) (DIMICCO and ZARETSKY 2002, ZARETSKAIA et al. 2002). As non-shivering thermogenesis via BAT is the main re-warming mechanism employed to establish normothermy, inhibition of DMH neuronal activity may be mediated via the H3 receptor during hypothermia in order to reduce thermogenesis. The decreased expression of H3 receptor at the end of the torpor phase would consequently permit activation of DMH neurons, an increase in BAT activity, renal and cardiac pathways, which have all been shown to be activated following electrical stimulation of the dorsomedial nucleus in rats, as well as dorsomedial-dependent altered cutaneous vasoconstriction, all adaptive processes during torpor when thermogenesis is suppressed (DIMICCO and ZARETSKY 2002, CAO et al. 2004).

H3 receptor expression was up-regulated in two structures involved in circadian timing. In the SCN, expression was up-regulated at the end of the torpor bout, and elevated expression was also detected during hypothermia in the thalamic dorsal lateral geniculate nucleus. The dorsal lateral geniculate nucleus is a subdivision of the lateral geniculate nucleus which presents the main thalamic relay of retinal information to the striatal cortex and is part of the non-image-forming visual network originating in the melanopsin-expressing retinal ganglion cells (BERSON et al. 2002, HATTAR et al. 2002, 2006). Entry into daily torpor is a precisely timed event for which an entrained circadian system is a key feature in this species, always precedes the onset of the photo-phase, and is endogenously timed by the circadian clock (RUBY and ZUCKER 1992, RUBY 2003). In contrast, arousal times exhibit considerable individual variation and can occur during both the light or dark phase. Compared to torpor

entry, precise endogenous timing of arousal may have less selective advantage, as the animal needs to be able react to external stimuli and if needed arouse early. Since daily torpor is timed by the circadian clock, possible H3 receptor mediated inhibition during torpor through H3 receptors may appear paradoxical. However, inhibition of neurotransmitters in the pacemaker during the variably-timed arousal period might prevent re-setting of endogenous rhythms evoked by sympathetic activation.

In contrast to the studies of SALLMEN et al. 2003 on hibernating ground squirrels, we did not detect increased H3 receptor mRNA expression in the caudate putamen or cortex. It is possible that H3 receptor up-regulation in the basal ganglia may serve to inhibit movement during deep hypothermia, but in contrast, mobility is only partially reduced during a daily torpor bout. A similar reason may explain why we found no changes in cortical H3 receptors in torpid animals. In hibernating ground squirrels mRNA expression was significantly increased and the authors suggest that this increase plays an important role in controlling the release of newly activated neurotransmitters during arousal (SALLMEN et al. 2003a).

Orexin A

Orexin fibers projecting through the whole hypothalamus to hypothalamic structures such as paraventricular nucleus, arcuate nucleus, lateral, perifornical and ventromedial nucleus of the hypothalamus, have been associated with enhanced feeding behaviour and arousal (IDA et al. 1999, KOTZ et al. 2002, KOTZ 2006). The arousal effect of orexin A has been shown to involve the histaminergic neurons in the tuberomammilary nucleus (HUANG et al. 2001, YAMANAKA et al. 2002). The stimulation of pronounced spontaneous physical activity after orexin A injection to several brain areas has been shown to be accompanied by enhanced thermogenesis and energy expenditure due to activation of sympathetic nerve activity (IDA et al. 1999, KOTZ et al. 2002, GEERLING et al. 2003). We may therefore predict that orexin A could play a role in arousal from torpor and be up-regulated during the re-warming.

In situ hybridisation revealed orexin expression in the LHA which was elevated within the initial period of the light phase of normothermic hamsters but did not change across a circadian cycle in torpid hamsters. Activation of histaminergic neurons by orexin may constitute part of the mechanism of arousal in a sleep-wake cycle. It was therefore surprising that we did not observe any tendency for increased expression in both torpid and normothermic hamsters at the time of arousal for nocturnal activity (YAMANAKA et al.

2002, ISHIZUKA et al. 2002). However, it is possible that local peptide release may be enhanced during arousal despite reduced gene expression. It is also plausible that orexin-stimulated arousal to wakefulness in normothermic experimental animals and arousal from low body temperature represent different physiological processes which employ different neurochemical substrates. Although at present this temporal pattern of orexin expression seems counter-intuitive in torpid hamsters, the absence of an increase at ZT4 may have a causal link to state of torpor as enhanced release of histamine from the tuberamammillary neurons via the OXR2 receptor may be part of a mechanism to prevent entry into the torpid state (YAMANAKA et al. 2002).

In conclusion, we show that the inhibitory H3 receptor exhibits significant up-regulation in several hypothalamic structures during the process of torpor in Siberian hamsters. These observations, together with the earlier studies showing a role for this receptor in deep hibernation in ground squirrels (SALLMEN et al. 2003a), suggest that inhibition through histaminergic pathways may play a vital role in maintaining the low body temperature and torpor in mammals more generally. In contrast, we did not show expected changes in orexin expression during torpor, suggesting that the orexin-mediated "arousal" pathway may not operate during natural bouts of hypothermia.

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Discussion

Discussion

The circadian system

Hibernating animals have been successful models for studying fundamental properties of many physiological systems. The extremely low metabolic rate (MR) and body temperature (T_b) reached during hypothermia pose great challenges for all kinds of biological processes. Furthermore, the manner in which hibernators deal with these problems are of great interest for various biological fields. Over the past 40 years interest in the role of the circadian system in control and expression of torpor has been aroused. Convergent lines of evidence have supported a role for the biological clock in timing daily torpor and controlling several aspects of deep hibernation (KÖRTNER and GEISER 2000, RUBY 2003). However, the two distinct patterns of hypothermia differ in their interaction with the circadian system. When photoperiodically induced, daily torpor is integrated into the usual daily activity and rest cycle of the animal which pursues foraging, territorial and social activities throughout winter (KIRSCH et al. 1991, RUF et al. 1991, DEBOER and TOBLER 1994, KÖRTNER and GEISER 2000, RUBY 2003). Torpor should not interfere with foraging, so as to maintain a positive energetic balance. Thus, the need for a fully functional circadian clock may be obvious.

In Chapters 2 and 3 we provide for the first time a detailed analysis of the molecular basis of the circadian system in the suprachiasmatic nuclei (SCN) and pineal gland of torpid and normothermic Djungarian hamsters (*Phodopus sungorus*), indeed indicating an oscillating pacemaker during torpor. These results could be expected from previous studies that clearly demonstrated maintenance of circadian T_b and activity rhythms despite daily hypothermia, though disappearing after SCN lesions (GEISER 1985, RUF and HELDMAIER 1987, KIRSCH et al. 1991, RUBY and ZUCKER 1992, RUBY et al. 2002, THOMAS et al. 1993, RUBY 2003). Interestingly enough, however, remains the question as to how the circadian system of daily heterotherms maintains entrainment, despite rare light availability and a daily low temperature pulse. For nocturnal animals that retreat into their burrows to enter torpor before dawn, light exposure is limited to the end of the day. Additionally, although the circadian system has been shown to be temperature compensated (PITTENDRIGH 1954,

MENAKER 1959, SWEENEY and HASTINGS 1960, RUBY et al. 1999), temperature pulses and transitions are nevertheless capable of phase-shifting the clock and affecting its endogenous period length (τ) (PALVIDIS and ZIMMERMANN 1968, RUBY and HELLER 1996, RUBY et al. 1999).

Thus, the question arises as to whether daily torpor feeds back on the organisation of circadian rhythms. Behavioural observations have supported the idea of a feedback of daily torpor on the circadian system (THOMAS et al. 1993), but what kind of changes that feedback might induce in the clock or its outputs remained unclear.

In chapter 2 and 3 we could demonstrate that the daily low temperature pulse indeed affected the molecular circadian clockwork. Although transcription appeared to be temperature compensated, the amount of immunopositive cells was decreased during the torpor bout in all investigated genes, supporting former findings of decreased translational processes in the cold (FRERICHS et al. 1998, VAN BREUKELEN and MARTIN 2001). Hence, it seems that no specific biochemical adaptations compensate for protein synthesis during hypothermia. It is likely that the decreased protein feedback after hypothermia changes the amplitude of the circadian oscillation as observed in our study for *Bmal1*/BMAL1 and *Avp*/AVP expression on the day after a torpor bout. This raises the question as to how the circadian system would then be able to keep the time throughout the whole torpor season, because according to our results oscillation would simply fade away. It must be taken into account though that daily torpor is not an "everyday" event. In Djungarian hamsters it occurs on average 2-4 times a week (RUF et al. 1991). On the other days the animals remain normothermic, hence normal transcription/translation cycles in the SCN can take place to reset the clock and assure consistent entrainment to the photocycle.

In chapter 3 we could not show a direct temperature dependent alteration of the molecular clockwork. We decreased T_a from 18 °C to 4 °C and consequently the animals´ T_b from ~22 °C to ~18 °C during hypothermia and could not reveal differences in gene or protein expression; so, either this decrease of T_b of only 4 °C was not great enough to provoke essential changes that could be revealed by *in situ* hybridization in the investigated molecular mechanisms, or the clock does not react to temperature in a gradual way and is somehow "buffered" until a certain lower threshold.

It is likely that daily torpor may not only affect the amplitude, but also the phase of the pacemaker in order to maintain entrainment. The τ of Djungarian hamster is longer than 24

hours and on a day without light perception (e.g. a day of torpor on which the animal does not leave its burrow) the system would need a phase advance. A shortened τ in torpid Djungarian hamsters supported this hypothesis (THOMAS et al. 1993). It was assumed that a phase advance might happen through non-photic cues, such as an advance in melatonin secretion which resets the clock via melatonin receptors in the SCN (RUBY 2003).

In chapter 4 and 5 we made the effort to test this assumption and set up *in vivo* microdialysis experiments to be able to measure melatonin continuously. Well established in rats, it was challenging to adapt this method to the very small (~30 g) Djungarian hamster and we validated this method as best as possible (Chapter 4). Moreover, we successfully set up an experiment with torpid Djungarian hamsters which is very difficult, because daily torpor is a very sensitive event and any kind of disturbance prevents it. Due to the low number of animals investigated that did not show an equal advance of melatonin secretion after a torpor bout, we cannot draw any firm conclusions from our preliminary data. Yet, it seems that the onset of melatonin release is linked to the time of arousal from torpor. Thus, it is possible that melatonin contributes to entrainment, but this stills remains to be clarified by further studies.

All in all, in chapter 2-5 we could clearly demonstrate an effect of daily torpor on the circadian oscillation of the core clock, supporting the idea that the pacemaker is not entirely insensitive to temperature.

This has been assumed before and it was discussed for a long time as to whether a functional circadian clock in deep hibernation, during which the animal drops its T_b even below 10 °C, could be realistic and would be necessary (MENAKER 1959, POHL 1961, FLORANT et al. 1984, VANECEK et al. 1984, 1985, STANTON et al. 1986, JANSKY et al. 1989, GRAHN et al. 1994, CANGUILHEM et al. 1994, WOLLNIK and SCHMIDT 1995, KÖRTNER and GEISER 2000, HUT et al. 2002, RUBY et al. 2002, LARKIN et al. 2002, RUBY 2003). In deep hypothermia that lasts for several days or even weeks, clearly a day is no longer divided into phases of activity and rest and the need for a timing system is not apparent. Moreover, many animals remain underground throughout the entire hibernating season. However, such animals might benefit from a clock that maintains synchrony of internal rhythms and times regular events like arousals (KÖRTNER and GEISER 2000, RUBY 2003). Regular arousals appear to be counterproductive, because the animals waste a lot of energy through endogenous heat production. In spite of this, all hibernators regularly reheat throughout the hibernation season and several hypotheses have been put forward. One is that accumulation of

certain metabolites during torpor requires normothermic periods for re-establishing homeostatic balance (GALSTER and MORRISON 1970, FRENCH 1985), but this could never be clearly demonstrated. Another theory is based on the observation that hibernators predominantly sleep during normothermic periods. It has been proposed that an accumulation of a sleep deficit induces arousals (DAAN et al. 1991, TRACHSEL et al. 1991). Indeed, it could be shown by EEG studies that the function of sleep may be impaired by hypothermia as slow wave activity (SWA) patterns after arousals resemble those of sleep deprived animals (DEBOER and TOBLER 1994, 2003). Another explanation for periodic arousals deals with the lengthening of the normal circadian rhythm due to low T_b during hibernation. This theory is favoured as hypothermic bouts become longer with decreasing T_a, but this relationship only holds true until the T_b threshold for thermoregulation during torpor is reached (KÖRTNER and GEISER 2000). Below this point the animal defends its T_b by actively increasing its metabolic rate and hypothermic bouts shorten (GEISER and BROOME 1993). Also the persistence of daily T_b fluctuations during deep hibernation in some species would not support a slowing down of the endogenous period, but imply a fully functional circadian clock (GRAHN et al. 1994). The persistence of circadian rhythms and especially of daily T_b fluctuations in deep torpor has been discussed controversially as they can interfere with fluctuation in T_a and hence, lead to false positive conclusions regarding rhythm persistence (WOLLNIK and SCHMIDT 1995, RUBY et al. 2002). In addition, T_b rhythms are often absent when T_b falls below 10 °C and are restored when T_a and consequently T_b is increased to 12 °C pointing to a probable direct effect of temperature on rhythm generation (RUBY et al. 2002). Hence, the question of a functional circadian clock during hibernation has never really been resolved. This divergence might result from the fact that so far mainly peripheral rhythms (T_b, activity) have been measured that do not always reflect the endogenous clock´s time, but can be masked by external factors like light or T_a.

Chapter 6 provides for the first time insight into the molecular SCN mechanisms and lack of any rhythmic gene expression strongly supports the hypothesis of a non-functional circadian system during deep torpor. It is the first physiological state in which an arrest of the master circadian clock over several days is described. However, we did not find overall depressed mRNA levels suggesting that the clock somehow remains in an “alert state” to facilitate fast rhythm generation after arousal. This assumption is underlined by the fact that after 48 hours of normothermia, gene oscillation had fully recovered. Moreover, the question arises as to

whether the SCN might serve a non-circadian role in hibernation related to energy balance (RUBY et al. 1998, RUBY 2003). This was assumed from findings that SCN-lesioned golden-manteled ground squirrels (*Spermophilus lateralis*) were not able to defend their high winter body mass after cold exposure despite rich food supply (RUBY et al.1998). This excessive weight loss in the cold was accredited to increased energy costs associated with arousals that occurred more frequently, but also ground squirrels with partial lesions that maintained robust T_b rhythms lost substantial amounts of weight. So far a non-circadian role of the SCN is not entirely clear, but some authors have suggested an influence on regulation of blood glucose and glucose tolerance that is unrelated to its role as circadian pacemaker (NAGAI et al. 1994, LA FLEUR et al. 2001).

If the SCN does not continue to oscillate during hibernation the question obtrudes as to how the animal then remains synchronised with the environment, can arouse regularly and how it "knows" when to finally rewarm in spring and thereby end the hibernation season. Interbout arousals might not be an actively timed process, but simply occur after a certain accumulation of negative factors like e.g., metabolites or sleep deficits as discussed above. Alternatively arousal could be the result of a depletion of metabolites as has been suggested for melatonin (STANTON et al. 1986). A solution for maintaining entrainment to yearly cycles appears to be a circannual clock that might work independently from the circadian system. Such a system was proposed after the observation that many long-lived hibernators display a stable circannual cycle of reproduction, activity, food consumption, body mass and torpor under constant T_a and photoperiod in the laboratory (PENGELLEY and KELLY 1966, KENAGY 1980, MROSOVSKY 1980, WARD and ARMITAGE 1981, PÉVET et al. 1989). Furthermore, this cycle can persist after ablation of the SCN, whereas the circadian rhythm is abolished (RUBY et al. 1996, 1998). The long-term timing and structure of the circannual cycle is altered after SCN ablation, though. Therefore, it appears that the circadian clock has only a modulating function on circannual rhythms (ZUCKER et al. 1983). Feedback of the circadian on the circannual clock might be provided during euthermic periods throughout the hibernation season. We show in chapter 6 that the circadian oscillation fully recovers within 48 hours after rewarming. What the common circannual pacemaker or signal exactly might be remains unknown. Yet, a recent study from KONDO et al. (2006) described a circannually controlled hibernation-specific protein (HP) in chipmunks (*Tamias sibiricus*) which is synthesised in the liver and transported to the brain prior to and during the hibernation season.

Such HP regulation proceeds independently of photoperiod and T_b changes, but animals only display torpor when HP levels in the brain are increased. Blocking HP activity decreased duration of hibernation in a dose-dependent manner, leading to the suggestion that this hormonal signal is essential for developing tolerance to hibernation in the brain. The identification of a signalling pathway and target sites of HP in future studies may be useful in order to develop a molecular approach to mechanisms governing circannual rhythms.

The histaminergic system

Since the discovery of sedative actions of classical antihistamines, it has become clear that histamine has important functions in the central nervous system (CNS). Today the role of histamine as a neurotransmitter is clearly established and has been shown to be involved in many vital brain functions such as body temperature regulation, feeding and sleeping (SCHWARTZ et al. 1991). Moreover, it could be demonstrated to be implicated in the hibernation process by actively inhibiting other neurotransmitter systems through the inhibitory histamine 3 (H3) receptor (SALLMEN et al. 2003a).

In chapter 7 we could demonstrate an up-regulation of H3 receptors during daily torpor in various brain nuclei including arcuate nucleus, dorso medial hypothalamus, suprachiasmatic nucleus and dorsal lateral geniculate, important relay stations for energy balance, thermoregulation, timing and vision. This suggests that inhibition through histaminergic pathways is crucial for maintaining low body temperature and low arousal state in mammals.

Besides histamine regulating the activity of other brain areas and thereby the arousal state of the animal, it also plays a role in thermoregulation. When for example rats are forced to produce heat at low T_as, concentration of hypothalamic histamine decreases and conversely increases at high T_as, when animals needed to “cool” (FUJIMOTO et al. 1990). Increased histamine turnover during a torpor bout (SALLMEN et al. 1999) and inhibition of the dorso-medial hypothalamus (DMH), a very important thermoregulatory site, through H3 receptors as we demonstrated here, would nicely fit with the idea of an impact of histamine on thermoregulation.

Interestingly, a recent study by CANONACO et al. (2005) stated a neuroprotective role for the neurotransmitter system against induced neurodegenerative processes in Syrian hamsters (*Mesocricetus auratus*). In this hibernator the neurotoxicant 3-nitropionic acid (3NP) which is

used for mimicking the typical pathological features of Alzheimer´s disease and schizophrenia, did not lead to abnormal motor behaviours such as hindlimb dystonia and clasping observed in rats. These behavioural differences were strongly related to fewer neuronal alterations such as crenated cell membranes, swollen mitochondria and darkened nuclei and could be related to the expression activity of H1, H3 receptor subtypes in the amygdala. Increased H1 expression, together with low H3 expression are thought to be key protective components (PATKAI et al. 2001, OTSUKA et al. 2003, CANONACO et al. 2005). High levels of H3 receptors during torpor and hibernation might then appear contradictory at first sight, as the phenotype with up-regulated H3 receptors in the study by CANONACO et al. (2005) was symptomatic. In that sense hypothermia could be interpreted as stress and up-regulation of H3 receptors could prevent neurodegeneration. However, it must be taken into account that H3 receptors in Djungarian hamsters are also regulated by photoperiod with low levels occurring during winter so that the basal level is much lower than in summer and up-regulation only results in relatively high mRNA levels that are probably not higher than in the summer state (BARRETT et al. 2005). Moreover, inhibition of movement, which is described as the pathological phenotype after 3NP treatment and up-regulation of H3 receptors, is even desired during hypothermia.

Orexins

Having demonstrated that the histaminergic system might be involved in maintaining the hypothermic state and preventing arousal of Djungarian hamsters, we asked the question as to what might cause the disinhibition of this mechanism and hence, be the signal for arousal from daily torpor. Orexin A and B (also known as hypocretins 1 and 2) are a group of neuropeptides that are strongly associated with feeding and wakefulness of animals (RODGERS et al. 2001, KOTZ 2006). They increase wakefulness and have moreover been shown to play a role in non-shivering thermogenesis in brown adipose tissue, the main mechanism for regaining normal T_b after hypothermia. Moreover, they directly activate the histaminergic system (HUANG et al. 2001, ISHIZUKA et al. 2002). Therefore, we hypothesised that orexin might be involved in the arousal from daily torpor.

Yet, we did not observe the expected up-regulation of orexin during the rewarming after daily torpor, but a down-regulation at the nadir of hypothermia and still slightly decreased orexin expression during arousal (Chapter 7). However, torpor is not sleep and increased

wakefulness, as described after orexin injections, is likely to be a different form of arousal than the arousal from hypothermia. Down-regulation of orexin during torpor makes sense, though, as it may depress enhanced release of histamine from the tuberomammilary nucleus (TM) via orexin 2 receptors (OX2R) and therefore support a low arousal state (YAMANAKA et al. 2002).

It should be taken into account that the histaminergic system, as well as orexins, are not only implicated in arousal. Both of them have also been demonstrated to be involved in metabolic processes and feeding behaviour (HAAS and PANULA 2003, KOTZ 2006, MASAKI and YOSHIMATSU 2006). Central injections of histamine or H1 receptor agonists decrease food intake in rats and mice (MASAKI et al. 2004), whilst orexin injections increase feeding (KOTZ 2006). Also daily torpor seems to be strongly linked to the metabolic state of the animal and can be induced by fasting despite a long photoperiod of Djungarian hamsters (RUF et al. 1993). Yet, if induced by fasting, daily torpor is not circadian gated, i.e. not timed in relation to the activity/rest rhythm. It seems that there is no direct link between onset of torpor and immediate fuel availability, like blood glucose concentrations as torpor cannot be induced by insulin injections and blood glucose concentrations only decrease after and not before torpor onset during spontaneous torpor bouts (DARK et al. 1999). Food restriction results in a substantial weight loss before torpor is triggered, suggesting that torpor induction is not only a product of metabolic fuel unavailability, but rather dependent on direct feedback from body fat stores or perhaps an interaction of these factors (RUBY and ZUCKER 1992). Indeed, reduced leptin concentrations, going along with body weight reduction of winter adapted Djungarian hamsters, are permissive for displaying daily torpor (FREEMAN et al. 2004). It has been postulated that the effects of leptin as a satiety factor are mediated by the histaminergic system which then triggers food intake, lipolysis and energy expenditure (MASAKI and YOSHIMATSU 2006). However, this interaction remains controversial (LECKLIN et al. 2000) and further work is needed to clarify the relation between leptin and histamine.

Conclusions

Overall we could demonstrate a functional, but altered circadian system during daily torpor, a shallow form of regulated hypothermia, but no rhythmical output during deep hibernation.

Our data suggest that in mammals the circadian system compensates for temperature effects to a relatively large extent and is able to distribute adequate rhythmic output. However, once a certain low temperature is reached, time keeping is no longer possible. What temperature this might be and what mechanisms underlie the temperature compensation will be the focus of further studies.

Up-regulation of H3 receptors in various brain structures during daily torpor as well as deep hibernation in golden-mantled ground squirrels (SALLMEN et al. 2003a) imply that inhibition through histaminergic pathways may play a central role in maintaining low body temperature and torpor in mammals. Orexins however, do not seem to be involved in the reactivation of brain systems during arousal from hypothermia. Failing to see an increase in orexin during arousal, we suggest that orexin-stimulated arousal to wakefulness in normothermic experimental animals and arousal from low body temperature might represent different physiological processes which employ different neurochemical substrates. Therefore, the question as to what finally provokes the rewarming from hypothermia remains to be revealed.

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Summary

Annika Herwig

Torpor and timing: Impact of endogenously controlled hypothermia on the circadian system of two hamster species.

Seasonal influences become increasingly important at high latitudes, where variations in external conditions are most pronounced. Mammals living in such drastic conditions have developed different forms of torpor in order to reduce metabolic costs in harsh periods when energy is rare. Deep hibernators like the European hamster (*Cricetus cricetus*) regularly decrease their body temperature (T_b) for several days to temperatures approaching ambient temperature (T_a) and thereby save a maximum of energy. In contrast, some small mammals like the Djungarian hamster (*Phodopus sungorus*) undergo shallower bouts of daily torpor. Induced by short photoperiod they spontaneously use their circadian resting time for only a few hours of precisely timed hypothermia during which their body temperature decreases to a minimum of 15 °C. Invariably low T_bs is accompanied by a depression of CNS activity. Nevertheless, it is crucial that functional integrity is maintained, which is why systems of vital importance during hypothermia are supposed to remain active at such low T_bs.

One of the important keys during torpor is believed to be the circadian system. It measures photoperiod and thereby determines the onset of the torpor season. Moreover, it synchronises internal processes on a circadian basis. In this thesis we could demonstrate for the first time that the clock´s molecular machinery is still active during daily torpor in Djungarian hamsters. Alterations in phase and amplitude of clock gene expression rhythms, however point to temperature sensitivity. Decrease in protein expression during hypothermia, hence a decreased feedback might be responsible for transcriptional alterations during and after a torpor bout.

To investigate phase changes seen in gene expression more precisely, we set up a long term microdialysis experiment to continuously measure melatonin, a well defined clock output, *in vivo* directly in the pineal gland. This method could be adapted to the very small Djungarian hamster for the first time and provides a good tool for studying a circadian signal like melatonin in a seasonally heterothermic animal.

During deep hibernation in European hamsters that decreased their T_b for several days to ~8 °C in our experimental conditions, we could not observe any rhythmic clock gene expression and thereby show that the clock stops oscillating at those low temperatures. We conclude from our data that the circadian clock seems to be temperature compensated in a wide temperature range but once a certain low temperature is reached oscillation is no longer possible.

The histaminergic system has been shown to play an important role in hibernation as well. In hibernating ground squirrels (*Spermophilus lateralis*) it probably serves to purposefully suppress other neurotransmitter systems via the inhibitory autoregulatory histamine 3 (H3) receptor. In our study we also show up-regulation of this receptor during daily torpor in Djungarian hamsters in various nuclei including arcuate nucleus, dorso medial hypothalamus, suprachiasmatic nucleus and dorsal lateral geniculate, hence important relay stations for energy balance, thermoregulation, timing and vision. Those findings point to a role for the histaminergic system in daily torpor comparable to that in deep hibernation, namely actively inhibiting other neurotransmitter systems. We investigated whether reactivation of the other systems could be caused by orexins. Orexins are a group of neuropeptides that has been shown to increase wakefulness and seems moreover to be related to non-shivering thermogenesis in brown adipose tissue, the main mechanism for regaining normal T_b after hypothermia. Contrary to expectations we found orexin down-regulated during torpor as well as during arousal from torpor. This contradicts the role of orexin during arousal from hypothermia and suggests distinct mechanisms for arousal related to wakefulness and arousal from hypothermia.

Zusammenfassung

Annika Herwig

Torpor und Timing: Der Einfluss endogen kontrollierter Hypothermie auf das circadiane System zweier Hamsterspezies.

Einflüsse saisonaler Umweltveränderungen (z.B Photoperiode und Umgebungstemperatur) verstärken sich mit zunehmender geographischer Breite, und Säugetiere, die unter solch extrem variablen Bedingungen leben, haben unterschiedliche Formen von Torpor entwickelt, um in energetisch ungünstigen Zeiten metabolische Kosten einzusparen. Echte Winterschläfer wie der Europäische Hamster (*Cricetus cricetus*) reduzieren in der Winterschlafsaison ihre Körpertemperatur regelmäßig für mehrere Tage auf Werte nahe der Umgebungstemperatur und sparen dabei ein erhebliches Maß an Energie. Kleinere Säugetiere wie der Dsungarische Zwerghamster (*Phodopus sungorus*) zeigen dagegen eine abgeschwächte Form der Hypothermie: täglichen Torpor. Induziert durch eine kurze Photoperiode senken die Tiere ihre Körpertemperatur während der Ruhephase spontan für einige Stunden auf bis zu 15 °C ab. Ein Absenken der Körpertemperatur führt auch zu sinkender Aktivität des zentralen Nervensystems, dessen Funktionieren jedoch überlebenswichtig ist. Daher wird angenommen, dass wichtige Gehirnsysteme auch während einer Hypothermie aktiv bleiben.

Da Torpor ein im Jahres- und Tagesverlauf zeitlich präzise integrierter Vorgang ist, gilt die innere Uhr als Schlüsselsystem für heterotherme Tiere. Zum einen misst und integriert sie die Tageslänge und bestimmt so den Beginn der Torporsaison im Jahr, zum anderen synchronisiert sie interne physiologische Prozesse im Tagesverlauf. In dieser Arbeit konnte zum ersten Mal gezeigt werden, dass die innere Uhr während des täglichen Torpors in Dsungarischen Zwerghamstern „weitertickt“, also die rhythmische Expression der so genannten Uhrengene weiterhin stattfindet. Änderungen in Phase und Amplitude der rhythmischen Uhrengen- expression deuten jedoch auf eine Temperatursensibilität der Uhr hin. Reduzierte Protein- expression während der Hypothermie und somit ein reduziertes Protein-Feedback könnte die Ursache für eine veränderte Genexpression nach einem Torporschub sein.

Um Phasenverschiebungen der Uhr, die in der Genexpression sichtbar wurden, genauer zu untersuchen, wurde die trans-pineale Mikrodialyse etabliert. Diese Methode ermöglicht eine

kontinuierliche *In-vivo*-Messung von Melatonin im Pinealorgan. Die rhythmische, nächtliche Sekretion von Melatonin aus dem Pinealorgan ist ein sehr gut definierter „Zeiger" der inneren Uhr, der präzise ihre Aktivität widerspiegelt und darüber hinaus als das wichtigste Signal gilt, um saisonale Informationen an den Organismus zu übermitteln. In dieser Arbeit wurde erstmalig die Mikrodialyse bei den sehr kleinen Dsungarischen Zwerghamstern erfolgreich eingesetzt.

Im Gegensatz zum täglichen Torpor konnten wir in tiefem Torpor bei Europäischen Hamstern keine rhythmische Expression von Uhrengenen feststellen. Demnach steht die innere Uhr bei dieser geringen Körpertemperatur von ~8 °C still und generiert keinerlei Oszillation. Diesen Daten zufolge scheint die innere Uhr zwar in einem weiten Temperaturbereich temperatur-kompensiert zu sein. Sobald die Körpertemperatur jedoch auf einen bestimmten Wert abgesunken ist, findet keine endogene Zeitmessung mehr statt.

Es konnte bereits gezeigt werden, dass auch das histaminerge System eine wichtige Rolle beim Torpor spielt. In winterschlafenden Erdhörnchen (*Spermophilus lateralis*) scheint es über den inhibitorischen Histamin-3-Rezepor aktiv andere Neurotransmittersysteme zu unterdrücken. Beim Dsungarischen Zwerghamster zeigte sich, dass dieser Rezeptor auch während des täglichen Torpors in vielen Kerngebieten des Gehirns verstärkt exprimiert wird. Dazu gehören vor allem der Nucleus arcuatus, der Nucleus dorsomedialis hypothalami, der Nucleus suprachiasmaticus und der Nucleus corporis geniculati dorsalis lateralis, also wichtige Schaltstationen für Energiehaushalt, Thermoregulation, zeitliche Integration und das visuelle System. Diese Ergebnisse deuten hin auf eine vergleichbare Rolle des histaminergen Systems im täglichen und tiefen Torpor, nämlich die aktive Inhibierung bestimmter Neuro-transmittersysteme. Ob diese Systeme möglicherweise durch Orexine wieder aktiviert werden, war eine weitere Fragestellung dieser Arbeit. Orexine sind eine Gruppe von Neurotransmittern, die nachweislich den Wachzustand verstärken und darüber hinaus eine Rolle in der zitterfreien Wärmeproduktion des braunen Fettgewebes spielen, dem wichtigsten Mechanismus, um die Körpertemperatur nach einem Torporschub wieder zu erhöhen. Entgegen unserer Annahme konnten wir im lateralen Hypothalamus keine verstärkte Orexinexpression während des Aufwachvorgangs aus dem täglichen Torpor nachweisen. Dies spricht gegen eine Rolle der Orexine im Aufwachprozess aus der Hypothermie und lässt vermuten, dass die gesteigerte Aufmerksamkeit im Wachzustand anderen Mechanismen unterliegt als das Aufwachen aus dem Torpor.

Résumé

Annika Herwig

Torpeur et mesure du temps : Incidence de l'hypothermie contrôlée sur le système circadien de deux espèces de Hamsters.

L'influence des saisons est extrêmement marquée au niveau des hautes latitudes du globe, avec des variations des conditions environnementales très prononcées. Les Mammifères vivant dans de telles conditions drastiques, ont développés différentes formes d'hibernation (ou torpeur) pour réduire les coûts métaboliques pendant ces périodes rudes lorsque les ressources énergétiques se font rares.

Les Mammifères hibernants comme le Hamster d´Europe (*Cricetus cricetus*), dont la température corporelle (T_b) diminue pendant plusieurs jours pour atteindre des niveaux voisins de la température ambiante (T_a), peuvent ainsi préserver au maximum leurs réserves d'énergie. Au contraire, quelques petits Mammifères comme le Hamster de Djungarie (*Phodopus sungorus*) montrent des hypothermies journalières de moindre amplitude. Ils utilisent les quelques heures de leur temps de repos circadien pour effectuer avec précision des hypothermies contrôlées, et diminuer leur Tb jusqu'à un seuil minimal de 15 °C. De telles T_b basses qui se prolongent, peuvent avoir des conséquences sur l'activité du système nerveux central (SNC). Il est donc crucial que l'intégralité fonctionnelle du SNC soit maintenue, ainsi, la limitation de la durée d'hypothermie permet un « réveil » régulier à des T_as basses. Pendant la torpeur, un des systèmes régulateurs importants serait le système circadien, car il réagit à la photopériode et synchronise les phénomènes internes sur une base de temps circadienne. Dans ce travail de thèse, nous avons pu montrer, pour la première fois, que la machinerie moléculaire de « l'horloge » biologique endogène reste active pendant la torpeur chez le Hamster de Djungarie mais une chute de la T_b diminue l'amplitude de l'expression des gènes de « l'horloge » biologique circadienne. La diminution de l'expression des protéines pendant l'hypothermie, induirait une diminution du rétrocontrôle de la transcription pendant et après une période de torpeur.

Pour mieux comprendre les changements d'expression de ces gènes, nous avons déterminé par microdialyse trans-pineal les sécrétions de mélatonine (hormone reflétant l'activité de la

principale sortie de « l'horloge »), pendant une longue durée. Cette méthode, qui a été adaptée pour la première fois au Hamster de Djungarie (animal de petite taille), s'avère être un bon outil pour étudier les signaux saisonniers comme la mélatonine chez les animaux hibernants.
Pendant l'hibernation du Hamster d´Europe, la diminution de la T_b atteint ~8 °C pendant quelques jours dans nos conditions expérimentales. A basse température, nous n'avons pas pu observer de rythme de l'expression des gènes de « l'horloge » biologique ce qui indiquerait dans ces conditions un arrêt des oscillations. A partir de nos résultats, nous avons conclu que « l'horloge » circadienne semble compenser les effets d'une diminution de la Tb dans un large gradient mais au delà d'un certain seuil de température les oscillations sont arrêtées.
Le système histaminergique joue aussi un rôle important pendant l'hibernation. Chez l'Ecureuil terrestre à manteau doré (*Spermophilus lateralis*), ce système est impliqué *via* une inhibition autorégulatrice du récepteur de l'histamine 3 (H3). Dans notre étude chez le Hamster de Djungarie, nous avons montré une expression de ce récepteur pendant la torpeur. Les différents noyaux où ce récepteur est exprimé (le noyau arqué, l'hypothalamus dorso-médian, les noyaux suprachiasmatiques et le noyau géniculé dorso-latéral) sont des zones relais pour les régulations de la balance énergétique, de la thermorégulation, de « l'horloge » endogène et de la vision. Ces découvertes établissent l'importance du système histaminergique pendant la torpeur journalière, rôle qui est comparable à celui observé durant l'hibernation profonde, et qui se caractérise par une inhibition d'autres systèmes neurotransmetteurs. Nous nous sommes aussi intéressés à la réactivation de ce système par les orexines (neuropeptides impliqués dans l'éveil) car elles sembleraient aussi jouer un rôle dans la thermogénèse sans frisson du tissu adipeux brun (mécanisme qui permet le retour à une T_b normale [ou euthermie] après la phase d'hypothermie). Contrairement à notre attente, nous avons mis en évidence une inactivation de l'effet des orexines, aussi bien pendant les phases de torpeur que pendant les phases de réveil. Cela va à l'encontre du rôle des orexines pendant la phase de réchauffement à la fin de la torpeur. De ce fait, les orexines pourraient contrôler l'expression de mécanismes différents lors des phases d'éveil et lors du réchauffement.
Cette thèse nous a permis d'apporter de nouveaux éléments concernant les mécanismes de régulation de deux systèmes importants (le système circadien et le système histaminergique) qui contrôlent l'hypothermie au cours de l'hibernation chez les Mammifères.

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Publications

Posters

Herwig A, Tritschler L, Bothorel B, Pévet P, Steinlechner S and Saboureau M.
Photosensitivity in the Djungarian hamster (*Phodopus sungorus*) revealed by trans-pineal microdialysis.
NEUREX Annual Meeting, Freiburg, Germany, April 23/04/2004

Herwig A, Tritschler L, Bothorel B, Pévet P, Steinlechner S and Saboureau M.
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Best poster prize, *36ème Congrès de la Société Francophone de Chronobiologie*, Rennes, France, 17-19/05/2004

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Torpor alters multiple gene expression in Djungarian hamsters SCN and pineal gland. *Center for systems neuroscience (ZSN)*, Hanover, Germany, 18/11/2005

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Submitted

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