Investigation of hypothalamic regulatory mechanisms during spontaneous daily torpor and fasting-induced torpor in the Djungarian hamster (*Phodopus sungorus*)

Dissertation

with the aim of achieving the doctoral degree

Doctor rerum naturalium (Dr. rer. nat.)

at the Faculty of Mathematics, Informatics and Natural Sciences

Department of Biology

University of Hamburg

submitted by

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2017 in Hamburg

Day of oral defense: 02.02.2018

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“An expert is a person who has made all the mistakes that can be made in a very narrow field.”

(Niels Bohr)

Abstract

The physiology of Djungarian hamsters (*Phodopus sungorus*) is well adapted to cope with harsh seasonal changes of environmental conditions. Triggered by decreasing day length, the Djungarian hamster starts to develop its winter phenotype (reduced bodyweight, molt to winter coat, gonadal regression) in advance of the upcoming winter season. ThesZe winter adaptions serve to reduce energy expenditure in energetically unfavourable times. During winter, the hamsters express spontaneous daily torpor (SDT), which can reduce energy expenditure by up to 65%. SDT is a strictly seasonal as well as circadian controlled state of metabolic depression with severely reduced body temperature. Besides SDT, Djungarian hamsters are able to use fasting-induced torpor (FIT) in times of food scarcity. This type of torpor can occur at any season and time of day and differs in physiological properties such as torpor depth, torpor duration as well as fuel utilization when compared to SDT. The general physiological properties of torpor in this species have been studied intensively over the past decades. Nevertheless, the regulatory mechanisms underlying torpor are insufficiently known to date.

The hypothalamus has become object of major interest in the field of torpor research since this brain area is known for its role in regulating food intake, energy homeostasis as well as body temperature and hosts the master circadian clock.

In my doctoral thesis I set out to investigate hypothalamic gene expression profiles in Djungarian hamsters during different torpor states (torpor entrance, mid torpor, arousal, post torpor) at particular times (ZT1, ZT4, ZT7, ZT16) to uncover torpor state dependent as well as circadian regulation patterns. Besides investigating hamsters undergoing SDT, also short- and long day adapted hamsters undergoing FIT were included.

In the first part of my thesis I conducted a transcriptomic study by using Illumina sequencing. Here, I set out to investigate expression profiles of all hypothalamic transcripts during torpor entrance. Subsequently, identified genes of interest were verified by RT-qPCR considering all four torpor states. The aim of the second study was to analyse the hypothalamic mRNA expression of selected genes by RT-qPCR in hamsters showing SDT and FIT. The investigated genes were related to orexigenic (*Agrp*, *Npy*), circadian clock (*Bmal1*, *Per1*) and thyroid hormone (*Dio2*, *Mct8*) system. This study enabled the direct comparison of gene expression profiles between SDT and FIT to uncover possible differences in molecular control mechanisms. The third study was designed to investigate a possible involvement of insulin, leptin and glucose in torpor expression of both, SDT as well as FIT, by using ELISA to determine serum concentrations. Furthermore, hypothalamic insulin and leptin sensitivity was assessed by relative gene expression analyses of selected gens (*OB-Rb*, *Socs3*, *Ptpn1*).

The transcriptomic study showed that within the hypothalamus 181 genes were up- and 103 genes were down regulated during torpor entrance. Within the group of the 20 most strongly up regulated genes a remarkable number of genes coding for structure proteins was found, including five different collagens, *Dnah2* and *Myo15a*. Also the procoagulation factor *Vwf* was amongst the most strongly up regulated group. We were able to verify up regulated mRNA expression of *Dnah2*, *Myo15a* and *Vwf* by RT-qPCR. During all other investigated time points these genes showed down regulated expression values. This suggests protective molecular adaptions in the hypothalamus, which are specifically induced during torpor initiation, comprising variations in neuronal plasticity, transport of biomolecules and coagulation.

The comparison of expression patterns between SDT and FIT showed up regulated mRNA expression of the orexigenic genes *Agrp* and *Npy* solely during FIT. During SDT the expression of these two genes remained unaltered. This provides evidence for a balanced energy state of hamsters using SDT, but a negative energy balance of hamsters using FIT. The relative expression analysis of the clock genes *Bmal1* and *Per1* demonstrated a disturbed circadian rhythmicity during FIT. In contrast, the circadian rhythmicity during SDT remained comparable to those of active hamsters. During both, SDT as well as FIT, *Dio2* expression was down regulated, indicating reduced hypothalamic T3 availability during torpor. In summary, this study demonstrates that SDT and FIT may partly be controlled by different regulatory mechanisms.

No significant differences were found in circulating insulin, leptin and glucose concentrations over the course of a torpor bout. However, glucose and insulin were slightly down regulated during torpor entrance of SDT and FIT-LD, indicating a possible role of glucose in torpor initiation mechanisms. Insulin and leptin concentrations were slightly decreased during mid torpor of SDT and FIT-SD. This might suggest an involvement of these two hormones in torpor maintenance. Investigation of hypothalamic controlled leptin sensitivity showed down regulated *OB-Rb* expression during the post torpid state of SDT, whereas in FIT *Socs3* mRNA expression was up regulated during the post torpid state. These data imply reduced leptin sensitivity after a day with torpor in winter adapted Djungarian hamsters.

Taken together, the results of my thesis provide new insights in molecular mechanisms of torpor regulation in Djungarian hamsters.

Zusammenfassung

Die Physiologie des Dsungarischen Zwerghamsters (*Phodopus sungorus*) ist bestens darauf ausgerichtet, mit saisonal bedingten, energetisch ungünstigen Herausforderungen zurechtzukommen. Durch Veränderungen in der Tageslänge, werden bereits vor Wintereinbruch Anpassungen an die bevorstehende Jahreszeit eingeleitet (Körpergewichtsreduktion, Fellwechsel, reproduktive Inaktivität), um Energie einzusparen. Während der Wintersaison selbst zeigen Dsungarische Zwerghamster spontanen täglichen Torpor (SDT), durch den der generelle Energieverbrauch um bis zu 65% gesenkt werden kann. Spontaner täglicher Torpor ist ein saisonal und zirkadian kontrollierter hypometaboler Zustand bei dem die Körpertemperatur drastisch herabgesetzt wird. Zusätzlich zu SDT kann diese Spezies in Zeiten von Ressourcenknappheit auch fasten induzierten Torpor (FIT) zeigen. Diese Form des Torpors unterscheidet sich in mehreren Parametern von saisonalem Torpor. So kann FIT zu jeder Jahres- und Tageszeit auftreten und weist Abweichungen in Torpordauer, Torportiefe sowie des genutzten Energiesubstrates auf. Die generellen physiologischen Eigenschaften von Torpor sind bereits vielfach und sehr gut untersucht. Dennoch sind die regulatorischen Mechanismen, die diesem Phänomen zugrunde liegen noch immer weitestgehend unbekannt. Aufgrund seiner regulatorischen Funktionen, die die Kontrolle von Nahrungsaufnahme, der Energiebalance, der Körpertemperatur sowie der zirkadianen Uhr umfassen, wird angenommen dass der Hypothalamus eine Schlüsselrolle bei der Initiierung und Aufrechterhaltung von Torpor spielt.

Im Rahmen meiner Doktorarbeit habe ich Genexpressionsprofile im Hypothalamus während unterschiedlicher Torporstadien (Torporeintritt, tiefe Torporphase, Aufwachphase, post torpide Phase) zu definierten Zeitpunkten (ZT1, ZT4, ZT7, ZT16) im Dsungarischen Zwerghamster untersucht, um torporstadien abhängige sowie zirkadiane Regulationsmuster identifizieren zu können. Zusätzlich zu kurztagadaptierten Hamstern während SDT wurden auch gefastete Langtag- und Kurztagtiere während FIT untersucht. In der Transkriptomstudie, die den ersten Teil meiner Doktorarbeit ausmacht, wurde mit Hilfe der Illumina Sequenzierung das Expressionsprofil aller Transkripte des Hypothalamus während dem Torporeintritt untersucht. Nachfolgend wurden differenziell regulierte Gene unter Berücksichtigung aller Torporstadien mittels RT-qPCR verifiziert. In einer zweiten Studie wurden Genexpressionsanalysen ausgewählter Gene während SDT und FIT mittels RT-qPCR analysiert, die im Zusammenhang mit dem orexigenen System (*Agrp*, *Npy*), der zirkadianen Uhr (*Bmal1*, *Per1*) und dem Schilddrüsenhormonsystem (*Dio2*, *Mct8*) stehen. Dies ermöglichte den direkten Vergleich der Expressionsprofile zwischen SDT und FIT. In der dritten Studie galt es, eine mögliche Beteiligung von Insulin, Leptin und Glucose an der Regulation von SDT und FIT zu prüfen. Hierzu wurden die Serumkonzentrationen dieser drei Komponenten mittel ELISA bestimmt. Zudem

wurde die Insulin- und Leptinsensitivität im Hypothalamus durch Bestimmung der relativen mRNA Expression ausgewählter Gene (*OB-Rb*, *Socs3*, *Ptpn1*) untersucht.

Bei der Transkriptomstudie zeigte sich, dass während dem Torporeintritt bei SDT insgesamt 181 Gene hoch- und 103 Gene herunterreguliert wurden, wobei innerhalb der 20 am stärksten hochregulierten Gene eine außergewöhnlich hohe Anzahl an Strukturgenen vertreten war. Diese Strukturgene umfassten fünf unterschiedliche Kollagene sowie *Dnah2* und *Myo15a*. Des Weiteren zählte auch der Koagulationsfaktor *Vwf* zu den am stärksten hochregulierten Genen. Die hochregulierte Expression von *Dnah2*, *Myo15a* und *Vwf* konnte mittels RT-qPCR verifiziert werden, zeigten aber während aller weiteren untersuchten Torporstadien eine Herunterregulation der mRNA Expression. Diese Ergebnisse geben Hinweise für protektive, molekulare Anpassungen im Hypothalamus, die spezifisch für die Torporeintrittsphase sind. Diese Anpassungen umfassen Veränderungen in der neuronalen Plastizität, dem Transport von Biomolekülen und der Koagulation.

Der Vergleich von Expressionsprofilen ausgewählter Gene während SDT und FIT zeigte, dass die orexigenen Gene *Agrp* und *Npy* einzig während FIT eine erhöhte Expression aufwiesen, wohingegen diese während SDT unbeeinflusst in ihrer Expression blieben. Dies deutet auf eine ausgeglichene Energiebalance der SDT-Gruppe und einen negativen Energiestatus der FIT-Gruppe. Die relative Expressionsanalyse der Uhrengene *Bmal1* und *Per1* deuten auf eine Phasenverschiebung der zirkadianen Rhythmik während FIT. Bei der SDT-Gruppe ist die Rhythmik der zirkadianen Uhr dagegen weiterhin vergleichbar mit der Rhythmik aktiver Hamster. Sowohl während SDT als auch während FIT war die mRNA Expression von *Dio2* herunterreguliert, was auf eine verringerte T3-Verfügbarkeit im Hypothalamus hindeutet. Insgesamt bestätigt diese Studie nur teilweise die Hypothese, dass es sich bei SDT und FIT um zwei Torporformen handelt, die durch unterschiedliche Regulationsmechanismen gesteuert werden.

Die Bestimmung der Serumkonzentration von Insulin, Leptin und Glukose zeigte keine statistisch signifikanten Veränderungen in torpiden Hamstern. Nichtsdestotrotz schienen Glucose und Insulin während dem Torporeintritt bei SDT und FIT-LD tendenziell reduziert zu sein. Dies könnte auf eine Beteiligung von Glucose während der Initiierung beider Torporformen deuten. Die Insulin- und Leptinkonzentration war während der tiefen Torporphase in SDT und FIT-SD leicht erniedrigt, was auf eine mögliche Funktion dieser beiden Enzyme bei der Aufrechterhaltung des torpiden Status hindeuten könnte. Die Untersuchung der Leptinsensitivität zeigte ein herrunterreguliertes *OB-Rb*-Expressionslevel während SDT und ein erhöhtes *Socs3*-Expressionslevel während FIT-SD in post torpiden Hamstern. Beides weist auf eine herabgesetzte Leptinsensitivität nach einem Tag mit Torpor in winteradaptierten Hamstern.

Zusammenfassend liefert diese Arbeit neue Erkenntnisse über molekulare Mechanismen bei der Torporregulation von Dsungarischen Zwerghamstern.

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Chapter 1

General Introduction

1.1. Seasonally changing environments

Our living on earth is affected by periodic daily as well as annual changes of the environment. These changes are caused by the fact that the earth rotates around itself once a day and around the sun once a year, while the earth axis is tilted towards the ecliptic of the sun at approximately 23.44 degrees. At polar and temperate latitudes this tilted rotation is responsible for seasonal variations in diurnal cycles and temperature over the course of the year. Thus, the annual cycle is divided into the four seasons spring, summer, autumn and winter depending on how the poles are pointed towards the sun. The variations of day length and ambient temperature (T_a) are more pronounced at higher latitudes (Trenberth, 1983).

Physiology and behavior of all living animals are influenced by the rhythm of day and night and photoperiodic alterations over the course of a year. Generally, environmental conditions are less favorable in winter and animals need to anticipate and provide against the cold season well in advance. Alteration of day length is the only reliable Zeitgeber predicting seasonal changes (Turek & Campbell, 1979; Bartness *et al.*, 2002). In mammals, photoperiodic information is integrated by a neuronal pathway which translates the exogenous information into an endogenous signal by detecting the photoperiodic information via the eye in melanopsin-containing retinal ganglion cells and transmitting this information to the suprachiasmatic nuclei (SCN) of the hypothalamus which constitutes the circadian clock (Schwartz *et al.*, 2001; Foster & Hankins, 2007). The SCN controls the nocturnal secretion of melatonin from the pineal gland. The duration of melatonin secretion decreases when days are getting longer during spring. In turn, the nocturnal duration of melatonin secretion increases when days are getting shorter during autumn. Thus, photoperiodic modification of melatonin production in mammals clearly indicates the upcoming season and represents the driving factor for seasonal adaptions by providing a neurochemical index of night length (Pevet, 1988; Bartness & Goldman, 1989; Ebling, 2015).

1.2. Seasonal adaptions in mammals

As endotherms, mammals are able to constantly maintain a high body temperature (T_b) of about 37 °C (homeotherm) independent of external factors. The evolution of homeothermy enabled the occupation of thermal niches, increased brain size, increased aerobic capacity during exercise resulting in enhanced exercise performance and increased metabolic efficiency (Hayes & Garland, 1995). But there are also some drawbacks of an endothermic living. Especially mammals living at high and temperate latitudes are challenged by environmental seasonal changes. To compensate low T_as during the cold winter months energy intake requirements are increased to cover increasing

thermoregulatory costs, whereas quality and quantity of food availability is at their lowest. To cope with these environmental challenges, mammals have evolved numerous adaptive strategies in behavior, morphology and physiology to reduce their energy demand and facilitate survival in winter (Schmidt-Nielsen, 1979). Among these are for example increased insulation by development of a dense winter fur to decrease heat loss, increased thermogenic capacity of brown adipose tissue, nest building and huddling, reproductive inactivity to avoid the high energetic costs of pregnancy and lactation and the accumulation of energy reserves by hoarding food or by increasing body weight to accumulate internal fat reserves (Bronson, 1985; Heldmaier, 1989; Gilbert *et al.*, 2010). To further enhance energy saving capacities in unfavorable times, many mammalian species are able to undergo torpor (Fig 1). Seasonal torpor is a precisely controlled state characterized by a marked reduction of metabolic rate, T_b, heart rate, ventilation and locomotor activity which enables mammals to reduce energy expenditure and hence energy intake requirements while remaining largely energetically balanced (Ruf & Geiser, 2015). Torpor can largely be classified into two forms: hibernation (deep torpor) and daily torpor (shallow torpor) which gradually differ in timing, duration, and amplitude of physiological inhibition. Mammalian hibernators show torpor bouts with an average bout duration of 198 hours with a minimum metabolic rate of 0.037 ml O_2 g^{-1} h^{-1} whereas mammalian daily heterotherms display torpor bouts with an average duration of only 8.2 hours with a minimum metabolic rate of 0.430 ml O_2 g^{-1} h^{-1} (Ruf & Geiser, 2015).This thesis is focused on daily torpor, a strategy commonly used in small mammals with a body mass of less than 100g. Depending on their small internal energy stores they are not able to accumulate adequate internal fat depots which are required during prolonged torpor episodes. Thus small mammals need to stay awake during their natural activity phase to maintain their daily foraging activities (Geiser & Ruf, 1995; Geiser, 2008; Ruf & Geiser, 2015).

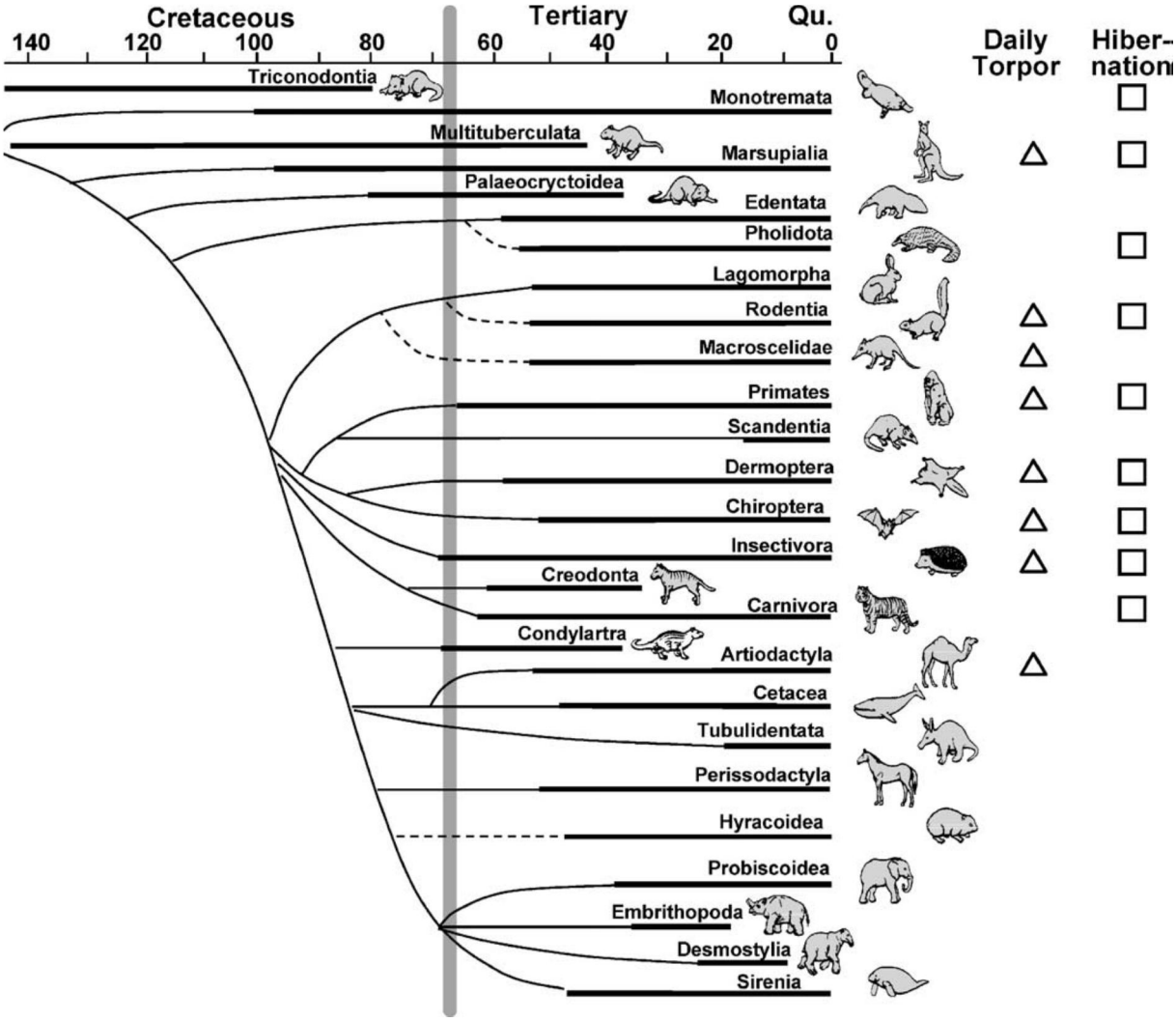

Fig 1. Distribution of the occurrence of torpor among mammalian species (Heldmaier *et al.*, 2004). Triangles (△) indicate mammalian orders using daily torpor and squares (☐) indicate mammalian orders using hibernation when torpor has been reported in at least one species.

1.2.1. The Djungarian hamster – a model in the field of seasonal adaptions

The Djungarian hamster (*Phodopus sungorus*, also known as Siberian hamster) started to become a model in the field of seasonal adaptions when the scientist Dr. J. Figala brought the first hamster breeding pairs to Germany in the early 1960s. Djungarian hamsters are native to the steppes of southwestern Siberia and northeastern Kazakhstan. This habitat is characterized by pronounced annual changes in environmental conditions with long days (~16 hours daylight) during the summer months and warm ambient temperatures (T_a) up to 30 °C, whereas days in winter are getting short (~8 hours daylight) and T_a drops to cold values down to -45 °C (Flint, 1966). Thus, vegetation and thereby quality and quantity of food availability is low during the winter season when energy requirements are high due to low T_a. To overcome this energetic bottleneck, the Djungarian hamster changes morphology, physiology and behavior in anticipation of winter facilitating efficient energy

savings (Figala *et al.*, 1973; Scherbarth & Steinlechner, 2010). These adaptions are triggered by changing day length and consequent alteration of pineal melatonin production. *P. sungorus* starts to develop its winter phenotype in advance at a critical photoperiod of 13.5 hours light and 10.5 hours dark in September (Hoffmann, 1982). The animals start to gradually reduce their body weight by voluntarily entering a hypophagic catabolic state. Thereby they lose up to 40 % of total body weight, mainly by a reduction of fat mass but also by reducing lean mass. The decreased food consumption is not caused by food scarcity, but also occurs when food is available *ad libitum* (Steinlechner *et al.*, 1983). Initially, this behavior appears to be paradoxical since it results in an unfavorable surface-to-volume ratio, facilitating increased heat loss. However, Djungarian hamsters are small and therefore not able to store a sufficient amount of fat reserves to cover their high energy turnover. Instead they decrease their general energy demands by reducing body weight resulting in an overall reduced need of food consumption to maintain energy homeostasis (Scherbarth & Steinlechner, 2010).

The energetic disadvantage of an increased surface-to-volume ratio is compensated by the development of a well-insulated white winter fur that replaces the dark greyish-brown summer fur (Figala *et al.*, 1973; Heldmaier, 1981; Kauffman *et al.*, 2001). Responsible for changes in fur color and density are seasonal variations in plasma concentrations of prolactin with high levels in summer and low levels during winter (Duncan & Goldman, 1984a; Duncan & Goldman, 1984b). The higher insulation does not result from elevated hair density but rather appears to be caused by increased fur regrowth rate (Paul *et al.*, 2007).

In addition to body weight reduction and fur change, Djungarian hamsters become reproductively inactive to avoid the energetic costs of mating and rearing offspring. Gonads are entirely regressed during winter, a process which is initiated in early autumn and accompanied by decimated serum concentrations of sex hormones. The return to a reproductive active state occurs spontaneously after ~30 weeks and is already completed in early spring to allow early breeding (Hoffmann, 1979; Schlatt *et al.*, 1995).

The most drastic strategy to save energy in this species is the expression of spontaneous daily torpor (SDT), a voluntary hypometabolic state.

The development of the winter phenotype occurs gradually until the climax of each adaption is reached after 15 to 20 weeks (Scherbarth & Steinlechner, 2010).

1.2.1.1. Characteristics of spontaneous daily torpor (SDT)

SDT is under seasonal control and restricted to the winter months. Djungarian hamsters start to express SDT after about 12 weeks under short day conditions (SD) once all other adaptions, including the accompanying endocrine systems (reduced leptin, prolactin and testosterone levels), are

established (Cubuk *et al.*, 2016). SDT has been shown to be under circadian control. The occurrence of torpor is restricted to the hamsters' natural resting phase. Djungarian hamsters usually enter the torpid state at dawn by actively depressing metabolic rate to 25 % below resting metabolic rate (Heldmaier & Ruf, 1992). SDT is entered from an energetically balanced state of glucose metabolism, indicated by a respiratory quotient (RQ) of 0.88 ± 0.02, which progressively declines towards lipid based metabolism with an RQ of 0.68 ± 0.01 at the end of a torpor bout (Diedrich *et al.*, 2015). It has been shown that in a winter adapted hamster metabolic rate is on average 2.63 $mlO_2\ g^{-1}\ h^{-1}$ during the active period and reaches a minimum of 2.1 $mlO_2\ g^{-1}\ h^{-1}$ during the resting period whereas the metabolic rate during a torpor bout falls to 0.709 $mlO_2\ g^{-1}\ h^{-1}$ (Heldmaier *et al.*, 2004). The decrease of metabolic rate is followed by a gradual reduction of T_b to a minimum value of ~15 °C. Djungarian hamsters remain torpid during their diurnal resting period showing a plateau phase with almost constant low metabolic rate and decreased T_b and arouse prior to their nocturnal activity period. The euthermic state is recovered within 30 minutes by both, shivering and non-shivering thermogenesis (Kirsch *et al.*, 1991; Heldmaier & Ruf, 1992; Heldmaier *et al.*, 2004). Torpor bouts in this species have an average duration of six hours (Fig 2A). The frequency of torpor expression is highly variable between individuals (with comparable body constitution and equal ambient conditions) and even within the same animal, which allows an individual adjustment of energy expenditure in response to energetic challenges. At best, SDT enables energy savings of up to 65 % when torpor is used frequently (Heldmaier & Steinlechner, 1981; Kirsch *et al.*, 1991). The restriction of torpor expression to the resting phase allows the animal to stay active throughout the winter season. Thus, these animals are able to maintain social, territorial and foraging activities (Ruf *et al.*, 1991).

In Djungarian hamsters, SDT can be easily induced in laboratory conditions by adjusting the photoperiod to an artificial short light-dark-cycle (8:16-h light:dark cycle). The occurrence of torpor is neither dependent on very low T_as nor on food scarcity. Instead SDT occurs spontaneously at moderate T_as and when food is provided *ad libitum* without acute energy deficiency (Heldmaier & Steinlechner, 1981; Vitale *et al.*, 1985). Nevertheless, low T_as have been shown to exhibit a modulatory effect on torpor depth and duration (Ruf *et al.*, 1993). Regarding the easy handling of these animals and the possibility to reliably induce winter acclimatization, Djungarian hamsters have become an attractive model for the investigation of spontaneously occurring torpor.

1.2.1.2. Characteristics of fasting-induced torpor (FIT)

Times of food scarcity are not restricted to the winter months but might occur throughout the whole year, e.g. caused by periods of drought or flooding. Djungarian hamsters are able to survive prolonged times of food shortage by using fasting-induced torpor (FIT). This type of torpor can occur at any time of the year and is not restricted to the diurnal resting period of the animal. Thus, FIT is neither under seasonal nor under circadian control (Steinlechner *et al.*, 1986; Ruby & Zucker, 1992). FIT does not require a period of complex physiological preparations. Instead, the only prerequisite for the occurrence of FIT seems to be a severe depletion of internal energy reserves. Only when body weight is already decreased by approximately 30% (below 30 g) Djungarian hamsters start to display FIT bouts. FIT seems to be induced as direct response to an acute energetic challenge when the animals` survival is vulnerable (Ruby *et al.*, 1993). Here, the torpid state is entered from a lipid based metabolism, indicated by a RQ of 0.79 ± 0.001, and remains on lipid based metabolism throughout the entire torpor bout (Diedrich *et al.*, 2015). Like in SDT, a depression of the metabolic rate initiates the entrance into FIT followed by a decrease of T_b. With an average duration of 1.5 hours FIT bouts are shorter than SDT bouts, mainly caused by the missing plateau phase during mid torpor, and also T_b values are not as deep as during SDT (Fig 2B). Thus, the energy saving accrued from a FIT bout is less effective compared to a SDT bout (Diedrich & Steinlechner, 2012; Diedrich *et al.*, 2015). However, FIT can be used more than once a day resulting in multi bout events. In this case, the hamster enters a second torpor bout immediately after the arousal from the first torpor bout (Steinlechner *et al.*, 1986; Ruby & Zucker, 1992; Diedrich *et al.*, 2015).

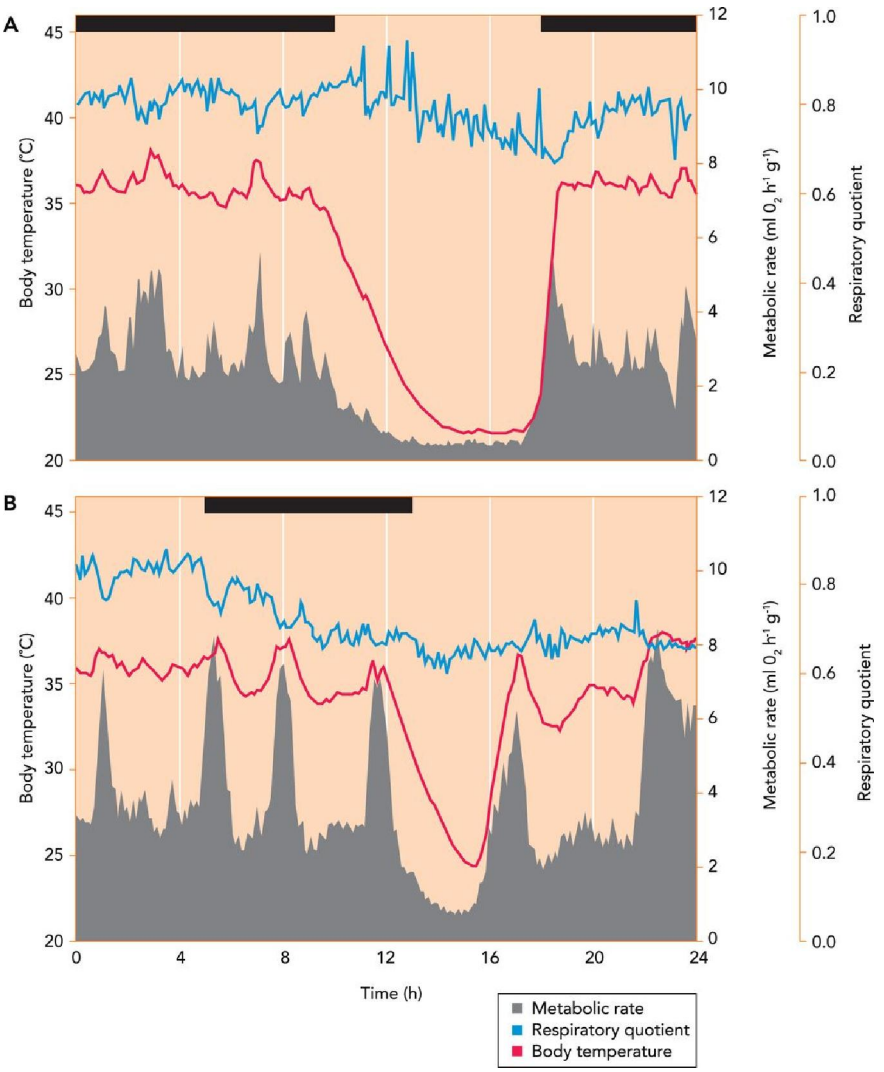

Fig 2. Torpor bout characteristics in *P. sungorus* (Cubuk *et al.*, 2016). Continuous 24 hour record of metabolic rate (grey field), respiratory quotient (blue line) and body temperature (red line) of a torpid Djungarian hamster. The upper panel shows an exemplary spontaneous daily torpor bout (A) and the lower panel an exemplary fasting-induced torpor bout (B).

The main traits of SDT and FIT coincide, but they clearly show some distinct physiological characteristics in prerequisites before first torpor bout occurs, differences in torpor depth, duration, frequency, fuel utilization and circadian control.

1.3. The hypothalamus – regulatory role in the control of torpor

It is commonly suggested that during torpor a general reduction of cellular metabolism (i.e. depression of metabolic pathways, transcription, translation, protein degradation) takes place. However, functional brain activity is maintained during this hypometabolic state (Braulke & Heldmaier, 2010; Jastroch *et al.*, 2016). The hypothalamus has become subject of major interest as a possible key structure of torpor regulation. This brain area includes several nuclei that have been shown to be involved in the control of food intake, energy balance and T_b as well as circadian rhythms (Schwartz *et al.*, 2000; Abizaid *et al.*, 2006). Inhibition of the sympathetic noradrenergic pathway has been shown to result in the complete disappearance of torpor (Braulke & Heldmaier,

2010). This implies that an intact noradrenergic signaling of the sympathetic nervous system, which is controlled by the hypothalamus, is essential for torpor expression. Moreover, in the torpid state the hypothalamus shows an increased neuronal activity in different structures, such as the SCN, paraventricular nucleus (PVN), median preoptic area (MPOA), arcuate nucleus (ARC) and supraoptic nucleus (SON) (Park & Dark, 2007). The general regulatory features of the hypothalamus, together with the elevated neuronal activity of various hypothalamic areas make this brain area a promising target for the investigation of torpor regulatory mechanisms.

1.3.1. The circadian clock

The master circadian clock is located in the SCN of the hypothalamus and controls a variety of physiological as well as behavioral processes in all living organisms by timing daily events and also translating seasonal information. Among others, these processes comprise the daily sleep and wake cycle as well as fluctuations in T_b, feeding behavior and hormone secretion (Moore & Eichler, 1972; Stephan & Zucker, 1972). As mentioned before, also the expression of SDT is under control of the circadian clock.

The circadian organization of daily rhythms in mammals is controlled by autonomous transcription-translation feedback loops which take approximately 24 hours to complete. These feedback loops are regulated by a set of highly conserved rhythmically expressed clock genes (Fig 3). The genes *Clock* and *Bmal1* are considered as elements of the positive limb, whereas *Pers* (*Per1*, *Per2*, *Per3*) and *Crys* (*Cry1*, *Cry2*) are considered as elements of the negative limb of the primary feedback loop. Here, the transcription factor CLOCK interacts with BMAL1 to build a CLOCK:BMAL1 complex. The CLOCK:BMAL1 complex initiates transcription of *Per* and *Cry* genes via e-Box elements on their promoter region, leading to an accumulation of these proteins in the cytoplasm. When a certain threshold of PER and CRY protein concentrations is exceeded, they form heterodimers and translocate back to the nucleus. Here, the PER:CRY complex inhibits the CLOCK:BMAL1 complex and thereby their own transcription. Thus, the cytoplasmic protein concentrations of PER and CRY decrease again thereby releasing the inhibitory effect on the CLOCK:BMAL1 complex. Now a new transcription cycle can be activated and a new transcription-translation feedback loop is initiated (Reppert & Weaver, 2002; Takahashi *et al.*, 2008). Since this endogenous rhythm does not exactly take 24 hours, it needs to get entrained by an environmental *Zeitgeber* which is provided by the light-dark cycle synchronizing the clock´s rhythm with the 24 hours rhythm of a day (Gillette, 1991). Secondary feedback loops support the primary feedback loop by the transcription of other clock genes (*Decs*, *Reverbs*, *Rors*), affecting the primary feedback loop at different sites of action (Preitner *et al.*, 2002; Sato *et al.*, 2004).

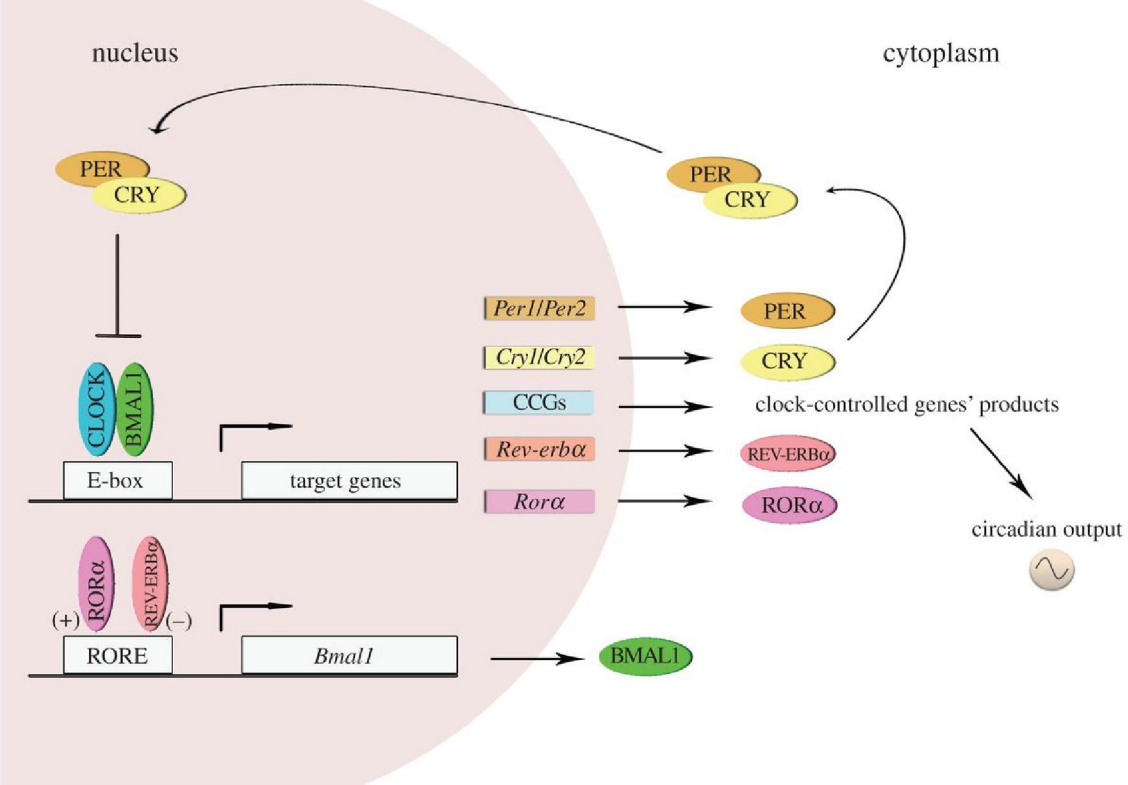

Fig 3. Molecular mechanisms of the mammalian circadian clock system (Golombek *et al.*, 2014). The core circadian feedback loop is activated by CLOCK:BMAL1 heterodimers that bind to e-BOX elements of *Pers* and *Crys* to initiate their transcription. Consequently, protein levels of PER and CRY increase in the cytoplasm and translocate as PER:CRY heterodimers back to the nucleus where they inhibit the action of the CLOCK:BMAL1 complex. This inhibits their own transcription until the cytoplasmic concentration of PER and CRY falls below a certain threshold and a new feedback loop can start. This core oscillation is underpinned by a secondary loop comprising *Reverbs* and *Rors* which affect the expression of *Bmal1*.

Lesion studies have demonstrated that the absence of a functional SCN results in a disordered SDT onset but does not prevent SDT. In this case SDT is no longer restricted to the animals´ resting phase but can occur at any time of day. Thus, a functional circadian clock system is crucial for the proper timing of SDT expression during their diurnal resting phase. In contrast, FIT can still be provoked in SCN ablated hamsters by food deprivation which could be explained by the generally missing circadian rhythmicity of FIT onset (Ruby & Zucker, 1992).

1.3.2. Adipose signal mediated regulation of energy balance

The maintenance of a balanced energy homeostasis is controlled by a highly complex process comprising hormonal and neuropeptide signaling pathways. The ARC of the hypothalamus is known as the main regulatory center of energy homeostasis. The control of energy balance relies on peripheral adiposity signals, mainly from gastrointestinal tract and adipose tissue, that are transduced into a neuronal response within the ARC. Leptin, a hormone primarily produced by

adipocytes, and insulin, which originates from the pancreas, are such adipose signals (Schwartz *et al.*, 2000; Varela & Horvath, 2012). Leptin and insulin secretion are positively correlated with the predominant amount of blood glucose and concentration of plasma leptin and insulin increases in proportion to the amount of stored fat content. They enter the brain from the circulation via the blood stream in proportion to their plasma concentration transducing the body's energetic state to the brain (Bagdade *et al.*, 1967; Baura *et al.*, 1993; Considine *et al.*, 1996; Schwartz *et al.*, 1996). High levels of insulin and leptin indicate an energy surplus and result in elevated transcription of the catabolic neuropeptide proopiomelanocortin (POMC) which leads to decreased food intake and simultaneously increased energy expenditure. In contrast, low circulating concentrations of leptin and insulin, conditioned by low body fat mass, activate the transcription of the anabolic neuropeptides agouti-related protein (AGRP) and neuropeptide Y (NPY) to stimulate food intake and decrease energy expenditure (Schwartz *et al.*, 2000).

Leptin receptors (OB-Rb) and insulin receptors (IR) are highly concentrated in the ARC. Insulin and leptin sensitivity is increased by binding to their respective receptor. The receptor binding leads to an activation of a complex signal transduction pathway, which is not fully understood to date (Cheung *et al.*, 1997; Cowley *et al.*, 2001; Lin *et al.*, 2010). But it is known that leptin binding to the extracellular domain of OB-Rb activates the Janus kinase (JAK). JAK in turn phosphorylates OB-Rb, leading to the activation of signal transducer and activator of transcription 3 (STAT3). STAT3 can bind to the promoter region of *Pomc*, *Agrp* and *Npy* and provokes elevated *Pomc* expression while inhibiting the expression of *Agrp* and *Npy* (Vaisse *et al.*, 1996; Mesaros *et al.*, 2008; Ernst *et al.*, 2009). Simultaneously, the binding of insulin and leptin to their respective receptors activates the phosphatidylinositol-3-kinase (PI3K) which results in phosphorylation and inactivation of Forkhead box protein O1 (FoxO1), a repressor of *Pomc* expression. The phosphorylation of FoxO1 triggers its export out of the nucleus. This in turn facilitates STAT3 binding to the *Pomc*, *Agrp* and *Npy* promoters (Fig 4) (Hill *et al.*, 2008; Plum *et al.*, 2012; Varela & Horvath, 2012).

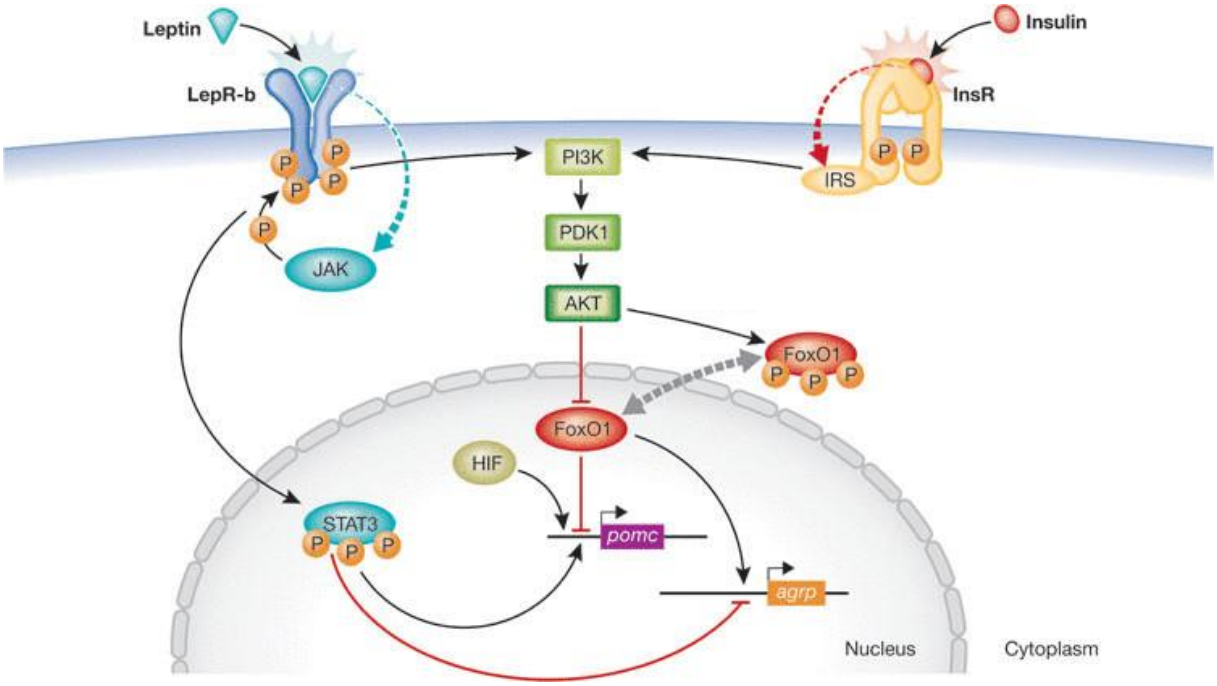

Fig 4. Leptin and insulin action in the ARC (Varela & Horvath, 2012). The receptor binding of insulin and leptin leads to the inactivation of the *Pomc* expression repressor FoxO1 by the activation of PI3K. Additionally, STAT3 is activated by binding of leptin to the leptin receptor resulting in initiation of *Pomc* expression, while expression of *Agrp* is inhibited resulting in reduced food intake and enhanced energy expenditure.

Djungarian hamsters are leptin resistant during summer, when leptin sensitivity is inhibited by elevated suppressor of cytokine signaling 3 (SOCS3, leptin signaling inhibitor) levels. Also insulin sensitivity seems to be decreased by elevated levels of protein tyrosine phosphatase non-receptor type 1 (PTPN1, insulin signaling inhibitor). Hamsters become leptin and insulin sensitive in autumn when they start to develop their winter phenotype (Klingenspor *et al.*, 2000; Rousseau *et al.*, 2002; Tups *et al.*, 2004; Tups *et al.*, 2006). There is strong evidence that this adipose signaling pathway or at least particular components of this pathway are somehow involved in torpor regulation. Ablation of the ARC by monosodium glutamate injections prevents SDT expression. This indicates that ARC mechanisms are involved in the regulation of SDT and that a functional ARC is crucial for torpor expression (Pelz *et al.*, 2008). Furthermore, intracerebroventricular NPY injections are sufficient to induce a torpor-like state in Djungarian hamsters. However, this torpor pattern seems to resemble FIT bouts rather than SDT bouts (Paul *et al.*, 2005). Another study has shown that the NPY induced torpor-like state is mediated by the NPYY1 receptor (Pelz & Dark, 2007).

Circulating leptin concentrations are decreased in SD adapted hamsters (2.06 ng/ml) as compared to animals held in LD (19.03 ng/ml). Exogenous leptin treatment by implanted osmotic minipumps has been shown to block SDT in most, but not all investigated hamsters (Freeman *et al.*, 2004). Thus, low circulating leptin concentrations are a necessary prerequisite for the onset of SDT in *P. sungorus* but are not sufficient to induce torpor.

1.3.3. The thyroid hormone system

Thyroid hormones are involved in the regulation of energy balance as well as T_b and are known to be able to modulate metabolic activity by affecting adaptive thermogenesis. They are regulating energy balance in peripheral tissues but also by central mechanisms within the brain (Bianco *et al.*, 2005; Herwig *et al.*, 2008; Hollenberg, 2008; Murphy & Ebling, 2011).

The prohormone L-thyroxine (T4, inactive metabolite) is the primary secretory product of the thyroid gland. Also Triiodorthyronine (T3, bioactive metabolite) is synthesized in the thyroid gland, but to a much lesser content. These synthesized thyroid hormones are secreted into the blood stream. The majority of circulating thyroid hormones are bound to transport proteins and thereby remain biological inactive until they arrive at their target tissue (Bianco *et al.*, 2014). Here they are actively transported into the tissue via specific transporters and can intracellularly be converted into different metabolites by deiodinase enzymes. The type-II-deiodinase (DIO2) catalyzes the activation of T4 by removing an iodine residue from the phenolic ring which converts T4 into the active metabolite T3. Besides, DIO2 can also convert the T3 metabolite reverse triiodothyronine (rT3) into the inactive diiodothyronine (T2). The type-III-deiodinase (DIO3) inactivates thyroid hormones by deiodination of the tyrosyl ring of T4. Thereby, DIO3 catalyzes the conversion of T4 to rT3 and of T3 to T2 (Kohrle, 1999; Gereben *et al.*, 2008; Herwig *et al.*, 2008).

Thyroid hormones need to be actively transported to enter their target tissues. Thus they rely on transporter proteins like monocarboxylate transporters, organic anion transporters or L-type amino acid transporters (Visser, 2000; Hennemann *et al.*, 2001; Friesema *et al.*, 2003; Wirth *et al.*, 2014). To date, the monocarboxylate transporter 8 (MCT8) is the only thyroid hormone transporter found in the brain of Djungarian hamsters (Herwig *et al.*, 2009). MCT8 plays an important role for thyroid hormone transport into the hypothalamus via the blood brain barrier (Friesema *et al.*, 2003).

Hypothalamic thyroid hormone availability shows seasonal alterations controlled by photoperiod dependent regulation of deiodinase gene expression in tanycytes (Fig 5) (Watanabe *et al.*, 2004; Barrett *et al.*, 2007; Nakao *et al.*, 2008; Herwig *et al.*, 2009; Herwig *et al.*, 2013). Tanycytes are a specific type of glial cells lining the third ventricle that are thought to be key players in regulating seasonal adaptations of mammals. The nocturnal melatonin secretion modulates TSHβ production by the pars tuberalis which maintains the DIO2 expression in tanycytes. Under SD conditions the longer duration of melatonin production decreases TSHβ production which in turn up regulates expression of *Dio3*. Thus, T3 availability is reduced in SD adapted animals by the enhanced production of inactive thyroid hormone metabolites resulting in the catabolic state of winter acclimatized Djungarian hamsters (Murphy & Ebling, 2011).

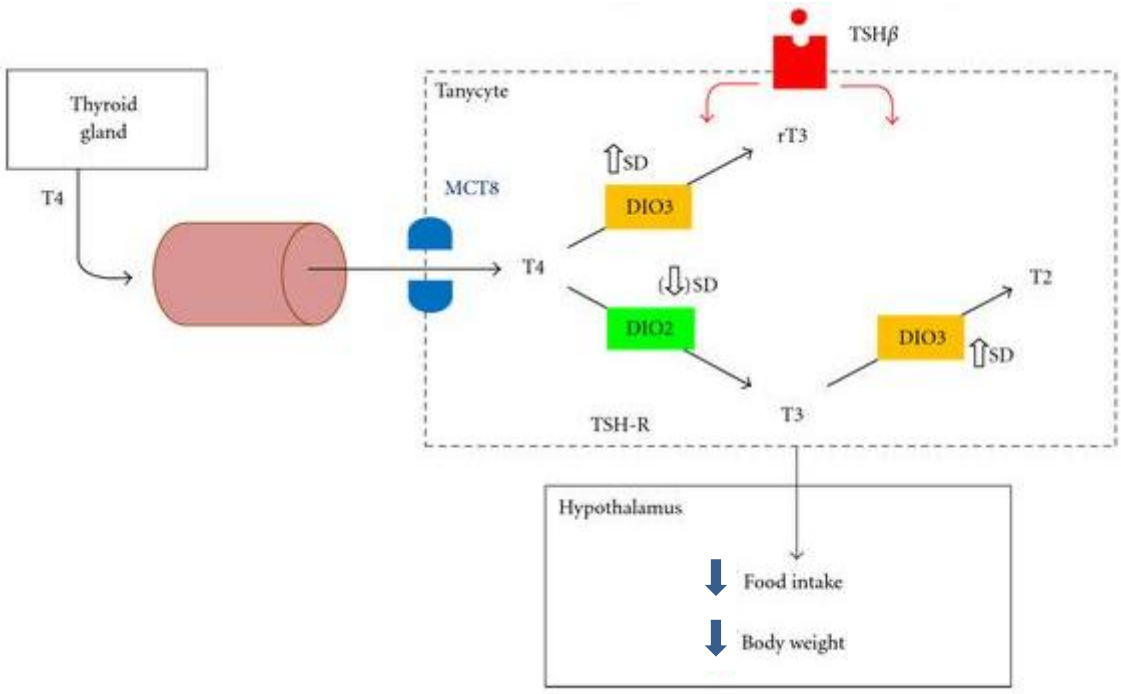

Fig 5. Modulation of deiodinase enzyme gene expression under short photoperiod (Murphy & Ebling, 2011). The decreased TSHβ production under short day conditions induces up regulated *Dio3* expression while *Dio2* expression seems to be down regulated. Thus, thyroid hormones are predominantly converted into inactive metabolites which results in decreased food intake and weight loss.

In addition, it has been shown that peripheral as well as central hypothalamic T3 availability has a direct effect on torpor behavior. A reduction of serum thyroid hormone levels by the pharmacological agent methimazole or perchlorate (these drugs inhibit the synthesis of thyroid hormones) results in increased torpor expression frequency, depth and duration. In contrast, enhanced peripheral T3 availability as well as increased hypothalamic T3 levels by local T3 releasing implants or microdialyis are able to block torpor in winter adapted animals (Murphy *et al.*, 2012; Bank *et al.*, 2015; Bank *et al.*, 2017a). These findings suggest an important role of T3 availability as a crucial prerequisite for the occurrence of torpor and a modulatory effect on torpor depth and duration.

1.4. Aim of the thesis

Over the past decades many studies extensively investigated the phenomenon of torpor to identify its regulatory mechanism. To date, the pathways underlying the metabolic depression during torpor are only partially known and understood. The precise neurological and endocrine mechanisms and pathways of torpor initiation and regulation are still a great mystery. Revealing this mystery would give new insights in the field of energy metabolism and the general regulatory mechanisms of energy balance. This knowledge could benefit research in obesity and furthermore facilitate the development of strategies for possible clinical applications like transplantation medicine and major cardiac and brain surgery.

Experiments of chapter two were conducted to give an overview of whole transcriptomic expression pattern in the hypothalamus during torpor entrance by Illumina sequencing to uncover new candidate genes which might be involved in torpor initiation mechanisms. The next generation sequencing technique enabled a complete screening for potential candidate genes showing a distinct regulation during the initiation of torpor as compared to non-torpid animals at corresponding Zeitgeber time (ZT) held under the same SD conditions. In a next step, genes of interest were investigated by relative mRNA expression analysis at four different torpor states (torpor entrance, mid torpor, arousal, post torpor) by using real-time qPCR (RT-qPCR) to verify the results received by Illumina sequencing analysis and to provide information about the circadian regulation of investigated genes.

In chapter three I set out to investigate hypothalamic expression profiles of genes related to orexigenic, circadian clock and thyroid hormone pathways. These systems have already been proposed to be involved in the regulation of torpor. Expression analyses were conducted at different torpor states and ZTs to uncover whether these systems play a role in initiation, maintenance or recovery from the torpid state and to consider potential circadian regulations of investigated genes. Furthermore, relative differences of mRNA expression were not just investigated in *ad libitum* fed SDT expressing hamsters but also in food restricted hamsters showing FIT. The variations of physiological characteristics between SDT and FIT raise the question whether these hypometabolic states reflect two differentially controlled forms of torpor rather than the same phenomenon. This study allowed direct comparison of hypothalamic expression profiles between SDT and FIT to uncover possible differences in regulatory mechanisms.

The last study in chapter four was designed to investigate alterations of insulin, leptin and glucose levels as well as alterations in hypothalamic adipose signaling pathways over the course of a torpor bout in SDT and FIT expressing Djungarian hamsters. Circulating insulin, leptin and glucose

concentrations were determined in serum samples. To uncover torpor dependent alterations of hypothalamic insulin and leptin signaling, I investigated the gene expression pattern of a leptin receptor, a leptin signaling inhibitor and an insulin signaling inhibitor over the course of a torpor bout. The adipose signals insulin and leptin and also glucose itself represent promising targets for torpor research since they have the ability for manipulating energy expenditure and T_b.

The purpose of this thesis was to investigate how the brain, in particular the hypothalamus, regulates the complex physiological processes of metabolism and T_b during the torpid state in Djungarian hamsters and to identify mechanisms contributing to the initiation and maintenance of torpor.

Chapter 2

Transcriptome analysis of hypothalamic gene expression during daily torpor in Djungarian hamsters (*Phodopus sungorus*)

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Published in in the journal of Frontiers in Neuroscience (2017)

(11:122. doi: 10.3389/fnins.2017.00122)

The original manuscript is included in this thesis, but layout, figure numbers, table numbers and references have been reformatted. The author of this thesis was involved in the design of all experiments supported by Annika Herwig. All *in vivo* experiments were carried out by me. Analyses of transcriptomic data was carried out by me with the support of Andrej Fabrizius and have been interpreted by myself. Gene expression analysis by RT-qPCR was performed by Julia Kemmling and me and data were analysed as well as interpreted by myself. Manuscript was written by myself and revised by Annika Herwig.

2.1. Abstract

Animals living at high or temperate latitudes are challenged by extensive changes in environmental conditions over seasons. Djungarian hamsters (*Phodopus sungorus*) are able to cope with extremely cold ambient temperatures and food scarcity in winter by expressing spontaneous daily torpor. Daily torpor is a circadian controlled voluntary reduction of metabolism that can reduce energy expenditure by up to 65% when used frequently. In the past decades it has become more and more apparent, that the hypothalamus is likely to play a key role in regulating induction and maintenance of daily torpor, but the molecular signals, which lead to the initiation of daily torpor, are still unknown.

Here we present the first transcriptomic study of hypothalamic gene expression patterns in Djungarian hamsters during torpor entrance. Based on Illumina sequencing we were able to identify a total number of 284 differentially expressed genes, whereby 181 genes were up- and 103 genes downregulated during torpor entrance. The 20 most up regulated group contained eight genes coding for structure proteins, including five collagen genes, *Dnha2* and *Myo15a*, as well as the procoagulation factor *Vwf*. In a proximate approach we investigated these genes by quantitative real-time PCR (RT-qPCR) analysis over the circadian cycle in torpid and normothermic animals at times of torpor entrance, mid torpor, arousal and post torpor. These RT-qPCR data confirmed up regulation of *Dnah2, Myo15a* and *Vwf* during torpor entrance, but a decreased mRNA level for all other investigated time points. This suggests that gene expression of structure genes as well as the procoagulation factor are specifically initiated during the early state of torpor and provides evidence for protective molecular adaptions in the hypothalamus of Djungarian hamsters including changes in structure, transport of biomolecules and coagulation.

Keywords: metabolic depression, seasonal adaptations, circadian, hypothalamus, Illumina, RNA-Seq

2.2. Introduction

Metabolic depression (torpor) is a commonly used strategy of mammals to survive winter. A reduction in energy expenditure as well as energy requirements is necessary to compensate for harsh environmental conditions during winter season when ambient temperature (T_a) drops and food availability is reduced (Jastroch *et al.*, 2016).

The Djungarian hamster (also known as Siberian hamster, *P. sungorus*) has evolved a number of physiological and morphological adaptations (e.g. voluntary reduction of body weight, molt to dense white winter coat, gonadal regression, torpor) to seasonally reduce energy requirements (Figala, 1973; Scherbarth & Steinlechner, 2010). In Djungarian hamsters, winter adaptations are driven by

photoperiod and can easily be induced by changes of the artificial light-dark cycle at moderate T_a in the laboratory (Steinlechner & Heldmaier, 1982; Vitale *et al.*, 1985). The most effective adaptive trait is the expression of daily torpor that spontaneously occurs after 10 - 12 weeks in short days once all other physiological adaptations are completed and the corresponding hormonal systems are in winter state (reduced levels of prolactin, testosterone and leptin) (Cubuk *et al.*, 2016). Daily torpor is initiated by an active depression of metabolic rate (25% below the level of resting metabolic rate), accompanied by reduced heart rate and ventilation as well as a decrease in body temperature (T_b) to > 15 °C and reduced physical locomotor activity (Heldmaier & Ruf, 1992; Heldmaier *et al.*, 2004). Torpor bouts are usually timed into the light phase of the light-dark cycle and have been shown to be under circadian control. The average duration of a torpor episode is six hours and it is terminated by a spontaneous arousal prior to the hamsters´naturally active phase (Kirsch *et al.*, 1991). The incidence of daily torpor is highly variable between individuals as well as in the same animal (1-7 torpor bouts per week) and can save up to 65% of total energy requirements, when torpor is used on a daily basis (Heldmaier, 1981; Kirsch *et al.*, 1991; Ruf *et al.*, 1991).

Spontaneous daily torpor is dependent on signaling of various hormonal systems changing with seasons and morphology, nutritional state as well as circadian timing mechanisms, hence, the hypothalamus is the brain area most likely involved in its regulation. Manipulations of prolactin levels lead to reduced torpor incidence and when testosterone, leptin or T3 are supplemented peripherally torpor is almost completely blocked (Ouarour *et al.*, 1991; Ruby *et al.*, 1993; Freeman *et al.*, 2004; Bank *et al.*, 2015). It has already been shown, that lesion of various hypothalamic nuclei (suprachiasmatic nuclei, arcuate nucleus, paraventricular nucleus) alters torpor behavior. Moreover, the pharmacological activation of NPY mechanisms in arcuate nucleus induces torpor like hypothermia and hypothalamic application of T3 is able to block the expression of torpor (Ruby & Zucker, 1992; Ruby, 1995; Paul *et al.*, 2005; Pelz & Dark, 2007; Dark & Pelz, 2008; Pelz *et al.*, 2008). However, although torpor physiology has been extensively studied in this species, the regulatory systems in the brain ultimately initiating entrance into torpor on some days but not on others are entirely unknown.

Here we carried out a next generation sequencing (NGS) study to impartially screen for potential candidate genes playing a role in molecular hypothalamic torpor induction mechanisms. NGS allows the investigation of all transcripts of a genome by counting the number of mRNA sequencing reads of a specific tissue. To date, only few transcriptomic studies are available investigating gene expression patterns in the 13-lined ground squirrel (*Ictidomys tridecemlieatus*) during hibernation in various tissues, like cerebral cortex, hypothalamus, heart, skeletal muscle, brown adipose tissue and white adipose tissue (Hampton *et al.*, 2011; Hampton *et al.*, 2013; Schwartz *et al.*, 2013; Grabek *et al.*, 2015; Schwartz *et al.*, 2015). Except for one study investigating brown adipose tissue during entrance

into torpor (Grabek *et al.*, 2015), these studies were focused on time points before animals enter hibernation season, while being in deep hibernation or during the interbout arousals. The Djungarian hamster is an excellent animal model to investigate gene expression patterns during torpor entrance, because torpor is precisely timed into the circadian cycle and allows precise sampling with timed controls that are winter adapted but do not show torpor on that particular day. Moreover, substantial knowledge exists about hypothalamic mechanisms regulating seasonal adaptations in body weight and reproduction in this species (Ebling & Barrett, 2008).

Here we present a summary of differentially expressed genes during torpor entrance in the hypothalamus of *P. sungorus*. Moreover, we provide information about circadian regulation of mRNA expression patterns for selected candidate genes by relative gene expression analysis in torpid and normothermic hamsters.

2.3. Materials and Methods

2.3.1. Animals

All experiments and procedures were conducted in accordance with the German Animal Welfare Law and approved by the local animal welfare authorities (No. 114_14, Hamburg, Germany). All animals originated from our own breeding colony at the Institute of Zoology University of Hamburg. Djungarian hamsters (*P. sungorus*) were bred and raised under artificial long photoperiod (16:8-h light:dark cycle) at 21 °C ± 1 °C T_a. The animals were individually housed in plastic cages (Macrolon Type III). Before and during the experiments, hamsters were fed a hamster breeding diet (Altromin 7014, Germany) *ad libitum* and had free access to drinking water. For the experiments, three to four months old Djungarian hamsters were transferred to short day conditions (8:16-h light:dark cycle) at constant T_a of 18 °C ± 1 °C. After 12 weeks in short days they were implanted i.p. with DSI-transmitters (Model TA-F10, St. Paul, MN, USA) under 1.5 – 2% isoflurane anesthesia and carprofen (5 mg/kg) analgesia as previously described to monitor T_b on line (Bank *et al.*, 2015). T_b was recorded every three minutes.

2.3.2. Experiment 1: Transcriptomic analysis of hypothalamic gene expression at torpor entrance

2.3.2.1. Sampling

Between week 13 and week 16, three animals were euthanized by carbon dioxide during entrance into torpor (T_b 30.4 °C ± 0.6 °C) at Zeitgebertime 1 (ZT1; ZT0 = lights on) (Fig 6, group 1). Additionally,

three hamsters were culled in a normothermic state (T_b 36.1 °C ± 0.7 °C) at the same ZT as control group (Fig 6, group 5). The brain was dissected from each hamster, frozen on dry ice and stored at -80 °C.

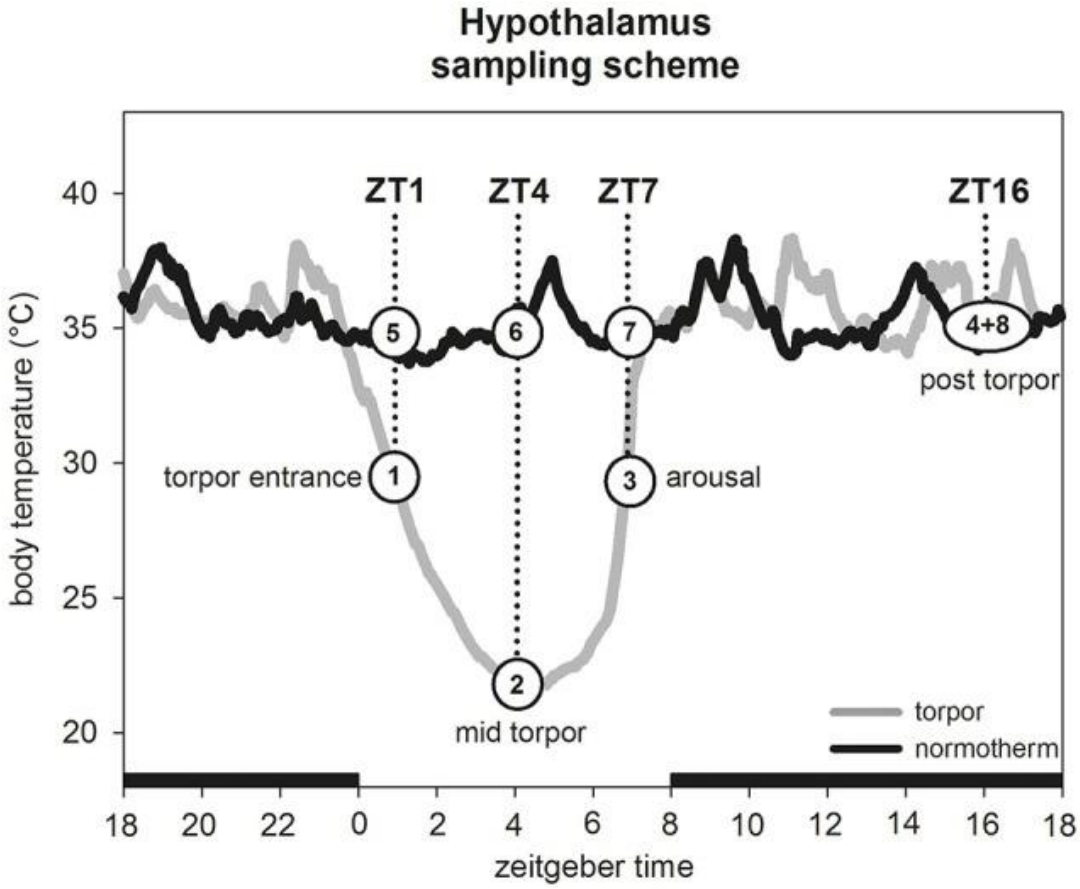

Fig 6. Hypothalamus sampling scheme. For experiment 1 one hamster group (n=3) was sampled during torpor entrance (1) and a corresponding normothermic group (n=3) at same ZT (5). For experiment 2 four hamster groups (n=5 for each group) were sampled in the torpid state during torpor entrance at ZT1 (1), mid torpor at ZT4 (2), arousal at ZT7 (3) and post torpor at ZT16 (4). For each time point a corresponding normothermic group (n=5 for each group) was sampled (5 – 8).

2.3.2.2. Isolation of total RNA

Hypothalamic blocks were cut from frozen tissues between Bregma -0.20 mm and -2.70 mm, laterally at the hypothalamic sulci and dorsally 3 – 4 mm from the ventral surface. Tissue samples were homogenized in 500 µl TriFast using a micropestle. Total RNA was obtained using peqGOLD Trifast™ (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. Total RNA was purified with the Crystal RNA MiniKit (Biolabproducts, Bebensee, Germany) including an on-column digestion with RNase-free DNase (Qiagen, Hilden, Germany). RNA integrity was proven by gel electrophoresis, total RNA was quantified spectrometrically and RNA purity was assessed by the 260/280 nm ratio on a NanoDrop 1000 spectrophotometer.

2.3.2.3. Illumina sequencing

In total, 2 µg total RNA per sample were used for transcriptome analysis. Library preparation and Illumina sequencing were performed by *GENterprise* Genomics (Mainz, Germany). For library preparation the TruSeq RNA Library Preparation Kit (Illumina, San Diego, CA) was used. All RNA samples had a RIN ≥ 6.9. The samples were sequenced by Illumina NextSeq 500 with a calculated output of 50 million paired-end reads (2 x 150 bp) per sample. The raw Illumina data are available at the NCBI SRA database under the accession numbers SAMN062002211 to SAMN06200226 (Bioproject PRJNA360070). Since currently no annotated *Phodopus sungorus* genome is available, the reads were mapped against the genome of the Chinese hamster (*Cricetulus griseus*), that showed best compliance, using the CLC-Genomics Workbench 7.5.1 (Qiagen, Hilden, Germany). Only reads with intact pairs mapping with an 85% read identity and 85% read length were used for RPKM (reads per kilobase per million mapped reads) calculation (Mortazavi *et al.*, 2008). Supplementary table 1 shows the total number of reads and the number of reads mapped in pairs for each sample. Statistically significant expression changes between normothermic and torpid hamsters were calculated by an empirical analysis of digital gene expression (DGE) statistics, performing an "Exact Test" (Robinson & Smyth, 2008). This tool is implemented in CLC-Genomics Workbench 7.5.1. To correct for multiple testing, a false discovery rate (FDR) correction of p-values was applied (see supplementary table 2).

Transcripts with an RPKM-value ≥ 0.1 in one of the samples were chosen for further analysis. Transcripts with a fold change ≥ 1.2 and an FDR-corrected p-value ≤ 0.05 were considered as differentially expressed. The identified differentially expressed transcripts were functionally classified using the PANTHER Classification System (Protein ANalysis THrough Evolutionary Relationships; www.pantherdb.org) version 10.0 (Mi *et al.*, 2013). Differentially expressed transcripts were additionally analysed using the PANTHER Overrepresentation Test (release 20160715) applying the PANTHER GO-slim terms as annotated, followed by Bonferroni correction for multiple testing. The PANTHER Overrepresentation Test was conducted for all 284 differentially expressed genes as well as the 181 up regulated genes and the 103 down regulated genes respectively. *Mus musculus* was selected as reference organism for the GO annotation and for the statistical calculation of overrepresented GO-terms. Overrepresented terms with a Bonferroni corrected p-value ≤0.05 were considered as significant.

2.3.3. Experiment 2: Relative quantification of hypothalamic gene expression in different torpor stages

2.3.3.1. Sampling

To validate our Illumina results, we selected eight genes for further investigations by real-time PCR (RT-qPCR) analysis. A group of seven genes immediately attracted attention for their potential role in structural changes (five collagens, myosin and dynein). Additionally, the von Willebrand factor (*Vwf*) was chosen for its role in blood clotting. To provide more detailed information about gene expression changes over a circadian cycle in torpid and normothermic state, 40 hamsters were used for gene expression analysis by RT-qPCR. A total of 20 animals were culled by carbon dioxide on a day with torpor expression at ZT1 (entrance into torpor: T_b 30.8 °C ± 0.5 °C, n=5), ZT4 (mid torpor: T_b 22.5 °C ± 1.5 °C, n=5), ZT7 (arousal: T_b 30.4 °C ± 0.4 °C, n=5) and ZT16 (active phase after torpor bout: T_b 35.7 °C ± 0.6 °C, n=5) (Fig 6, group 1 – 4). Five normothermic animals were sampled for each time point as respective control group (ZT1: Tb 35.7 °C ± 0.5 °C; ZT4: T_b 35.7 ± 0.4 °C; ZT7: T_b 35.6 ± 0.4 °C; ZT16: T_b 36.2 ± 1.3 °C) (Fig 6, group 5 – 8). Brains were dissected, frozen on dry ice and stored at -80 °C for RT-qPCR analysis.

2.3.3.2. Isolation of RNA and cDNA synthesis

Hypothalami were dissected from frozen brains and isolation of total RNA was performed as described for experiment 1. For RT-qPCR templates and generation of standard plasmids, cDNA was synthesized from every total RNA sample using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) and oligo-(dT)18 oligonucleotide primers following manufacturer's instructions. Reverse transcription was conducted using 1 µg total RNA per sample.

2.3.3.3. Cloning and sequencing

For standard plasmids, coding sequence fragments (100 – 200 bp long) of collagen alpha-1(XXIV) chain-like (*LOC103164493*), collagen, type XX, alpha 1 (*Col20a1*), collagen, type XVII, alpha 1 (*Col17a1*), collagen, type XVIII, alpha 1 (*Col18a1*), collagen, type V, alpha 3 (*Col5a3*), dynein, axonemal, heavy chain 2 (*Dnah2*), myosin XVA (*Myo15a*), von Willebrand factor (*Vwf*) and hypoxanthine phosphoribosyltransferase (*Hprt*) were amplified by gene specific primers (Table 1). All primers were designed on the P. sungorus specific sequences generated by Illumina sequencing using the Onlinetool OligoAnalyzer 3.1. The primers were designed with a melting temperature at 60 °C ± 1.1 °C. After cloning of the amplicons using the pGEM®-T Easy Vector System (Promega, Madison,

USA) following the manufacturer's instructions, the cDNA fragments were Sanger-sequenced by GATC Biotech (Konstanz, Germany).

Table 1. *P. sungorus* specific primer sequences.

gene		5´3´sequence	melting temperature	amplicon length
LOC103164493	forward reverse	CATGCAGCAGTAACGCCAACC GTGGCAATTGTGCTTCACCAACTC	59.4 °C 59.2 °C	136 bp
Col20a1	forward reverse	GCTCCTACCTCCACGTCTGTCTC CTGCCATAGGTGTCACCTGCAC	60.5 °C 60.2 °C	174 bp
Col17a1	forward reverse	CATAACCTCCTCCTGGGCTGATG GCTCTTCCTACAGTGCTCCCATG	59.2 °C 59.4 °C	126 bp
Col18a1	forward reverse	CAGGACCAAAGGGTGACAAAGGAG GGCCAGGTACACTTGAGCTGAAG	59.7 °C 59.8 °C	189 bp
Col5a3	forward reverse	GAACAAGGAGACCTCAAGGCTGAG CTGCAAGACAGTGGCATTTCGTTC	59.2 °C 58.9 °C	166 bp
Dnah2	forward reverse	CTTCGTGCTCAATGATATGGGCCG CTGCGATGGCTCTTGTCAATGCTG	60.6 °C 60.1 °C	102 bp
Myo15a	forward reverse	CATGGCACCCAGGAGATGATCTTG CACGCTTGGCATTGTAGGCATTG	59.7 °C 59.4°C	136 bp
Vwf	forward reverse	CCACAAGGTCATTTCTCCAGCCAC GGTCCGACAGAGGTGAGCATAAG	60.1 °C 59.1 °C	109 bp
Hprt	forward reverse	AGTCCCAGCGTCGTGATTAGTGATG CGAGCAAGTCTTTCAGTCCTGTCCA	60.4 °C 60.5 °C	140 bp

2.3.3.4. Real-time qPCR and analysis of expression data

RT-qPCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) on an ABI Prism 7300 Real Time PCR System (Applied Biosystems). Due to the large number of samples, PCRs were performed on two 96-well plates for each target gene. For comparability, the normothermic ZT16 group was applied to all plates as inter-plate calibrator. *Hprt* was selected as reference gene, based on the stability of expression values across all samples. All

samples were run in triplicates (for 5 biological replicates per group), using 1 µl cDNA as template in a reaction volume of 20 µl, and a series of six 10-fold dilutions of specific standard plasmids were used to generate the standard curve to calculate RT-qPCR efficiencies. Additionally, a no-template control was included on each plate in duplicates for each target gene. Quantification was performed with the following cycling parameters for 40 cycles: 50 °C 2 min; 95 °C 10 min; 95 °C 15 s; 60 °C 15 s; 72 °C 30 s. Amplification specificity was controlled by dissociation curve analysis referring to the RT-qPCR run.

First evaluation of RT-qPCR results was carried out using the 7300 System Software v1.4.0 (ABI Prism, Applied Biosystems) and subsequently exported to Microsoft Excel 2010 to identify differences in expression levels using the ΔΔCT method. All statistical testings and figures were done with SigmaPlot 12.5 (Systat Software Inc). All results were statistically analyzed by two-way ANOVA with time of day (Zeitgebertime) and metabolic state (torpid/normothermic) as factors, followed by Tukey´s test for pairwise comparison of relative expression levels between torpid and normothermic hamsters and within the torpid and normothermic groups.

2.4. Results

2.4.1. Transcriptomic expression analysis in the hypothalamus of *P. sungorus* during torpor entrance

We identified a total number of 27.830 transcripts with 284 transcripts being differentially expressed in hamsters during torpor entrance as compared to ZT matched normothermic hamsters (Table 2). A total of 181 transcripts were significantly upregulated whereas 103 transcripts were significantly downregulated. All transcripts identified had an RPKM-value ≥ 0.1 and a FDR p-value ≤ 0.05.

Table 2. Overview of Illumina sequencing data and transcriptomic expression analysis.

		Up regulated	Down regulated
Identified genes	27 830		
Differentially expressed genes	284	⬆ 181	⬇ 103

2.4.2. Functional classification of differentially expressed genes

Differentially expressed transcripts during torpor entrance were classified according to gene ontology categories. It has to be noted, that some genes were included in more than one category.

Of the 181 up regulated genes 154 could be classified and assigned to 389 biological processes (Fig 7A). The majority of up regulated genes are involved in metabolic (18%) and cellular processes (18%), followed by localization (45 genes, 12%), biological regulation (9%), developmental processes (9%), cellular component organization or biogenesis (7%), multicellular organismal processes (7%), biological adhesion (6%), immune system processes (5%), response to stimulus (5%), apoptotic processes (2%) and reproduction (2%).

A total number of 82 out of the 103 down regulated genes could be classified and assigned to 117 biological process hits (Fig 7B). The majority of down regulated genes was assigned to metabolic processes (33%), followed by cellular processes (20%), biological regulation (10%), developmental processes (9%), multicellular organismal processes (5%), localization (4%), immune system processes (4%), cellular component organization or biogenesis (3%), reproduction (3%), response to stimulus (3%), apoptotic processes (3%) and biological adhesion (3%).

The PANTHER overrepresentation test showed significant enrichments of the GO-slim terms only for the up regulated group of genes, comprising “transmembrane transporter activity” (9.64-fold, p=0.034) in the domain molecular function and “extracellular matrix” (6.02-fold, p=0.000397) in the domain cellular component.

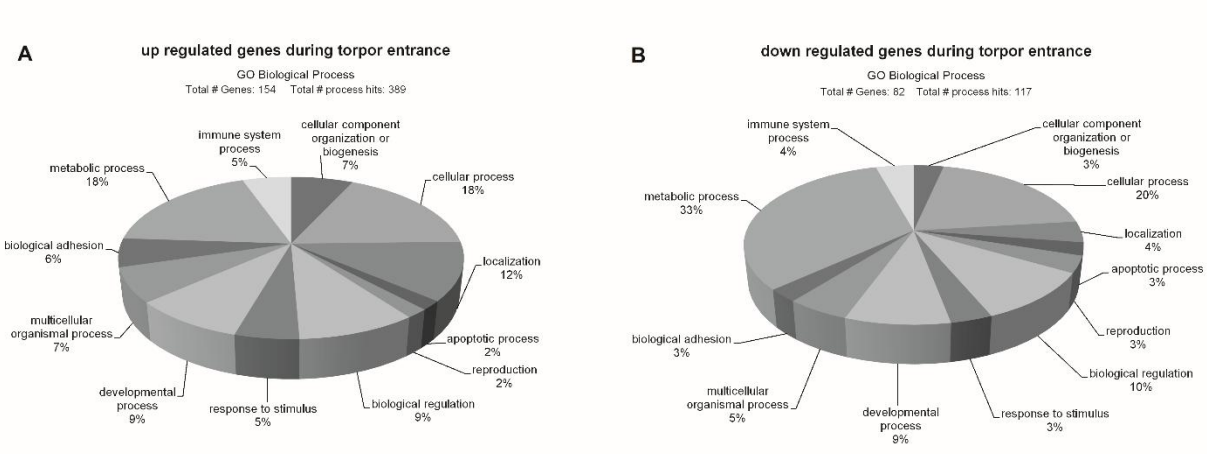

Fig 7. Ontology of genes up- (A) and down regulated (B) during torpor entrance in the hypothalamus. Sequences were classified according to http://pantherdb.org and assigned into biological process hits. Some genes were assigned to more than one category.

2.4.3. Analysis of most affected genes during torpor entrance

To determine the most affected genes during torpor entrance, we ranked the identified genes into the 20 most up- and 20 most down regulated genes, based on their fold changes.

Most up regulated genes (Table 3) showed fold changes in a range of 1.55 to 2.66. Within this group we found 8 genes coding for structure proteins (*LOC103164493*, *Col20a1*, *Myo15a*, *Col17a1*, *Micalcl*,

Dnah2, *Col18a1*, *Col5a3*), 4 involved in transporter function (*Abca6*, *Atp2a1*, *Kcnh3*, *Atp1a4*), 2 with signaling function (*OR2K2*, *LOC100773864*) and one gene each involved in stress defense (*Klk8*), coagulation (*Vwf*) and cell death (*Steap3*). Three genes have so far unknown function (*LOC103160902_1*, *LOC100766933*, *Catip*).

Table 3. Most up regulated genes in the hypothalamus during torpor entrance.

Function	Gene	Gene symbol	Fold change
Structure	Collagen alpha-1(XXIV) chain-like	*LOC103164493*	2.03
	Collagen, type XX, alpha 1	*Col20a1*	1.97
	Myosin XVA	*Myo15a*	1.93
	Collagen, type XVII, alpha 1	*Col17a1*	1.77
	MICAL C-terminal like	*Micalcl*	1.77
	Dynein, axonemal, heavy chain 2	*Dnah2*	1.67
	Collagen, type XVIII, alpha 1	*Col18a1*	1.62
	Collagen, type V, alpha 3	*Col5a3*	1.55
Transporter	ATP-binding cassette, sub-family A (ABC1), member 6	*Abca6*	2.07
	ATPase, Ca^{++} transporting, cardiac muscle, fast twitch 1	*Atp2a1*	1.9
	Potassium voltage-gated channel, subfamily H, member 3	*Kcnh3*	1.6
	ATPase, Na^{+}/K^{+} transporting, alpha 4 polypeptide	*Atp1a4*	1.58
Signalling	Olfactory receptor, family 2, subfamily K, member 2	*OR2K2*	2.66
	Cyclin-dependent kinase 11B-like	*LOC100773864*	1.67
Stress defense	Kallikrein-related peptidase 8	*Klk8*	1.87
Coagulation	von Willebrand factor	*Vwf*	1.59
Cell death	STEAP family member 3, metalloreductase	*Steap3*	1.69
Unknown	EF-hand calcium-binding domain-containing protein 8	*LOC103160902_1*	1.94
	Protein ARMCX6-like	*LOC100766933*	1.73
	Ciliogenesis associated TTC17 interacting protein	*Catip*	1.63

Fold changes of the most down regulated genes (Table 4) ranged between -1.5 and -4.0. This group contained 7 genes coding for transcription factors (*Stk31*, *LOC100768314*, *LOC102638674*, *LOC100756005*, *LOC102632383*, *LOC102642077*, *Smim11*), 4 with enzymatic activity (*Top2a*, *Clk1*, *Coq3*, *LOC100772408_2*), 2 with transporter functions (*Slc47a1*, *Nipsnap3b*), one gene each involved

in cellular structure (*Cornifin-A*), signaling (*Psmc3ip*), rRNA maturation (*Rrp15*) and ORF (*Swt1*) and 3 genes with so far unknown function (*LOC100754037*, *LOC103159055*, *LOC100753290*).

Table 4. Most down regulated genes in the hypothalamus during torpor entrance.

Function	Gene	Gene symbol	Fold change
Trancription factor	Serine/threonine kinase 31	*Stk31*	-2.69
	Zinc finger protein 93-like	*LOC100768314*	-1.69
	Zinc finger protein 26-like	*LOC102638674*	-1.62
	Zinc finger protein 420-like	*LOC100756005*	-1.61
	Zinc finger protein 431-like	*LOC102632383*	-1.56
	Zinc finger protein 431-like	*LOC102642077*	-1.55
	Small integral membrane protein 11	*Smim11*	-1.47
Enzyme	Topoisomerase (DNA) II alpha 170kDa	*Top2a*	-2.08
	CDC-like kinase 1	*Clk1*	-1.93
	Coenzyme Q3 methyltransferase	*Coq3*	-1.51
	2-hydroxyacylsphingosine 1-beta-galactosyltransferase	*LOC100772408_2*	
Transporter	Solute carrier family 47 (multidrug and toxin extrusion), member 1	*Slc47a1*	-1.87
	Nipsnap homolog 3B	*Nipsnap3b*	-1.48
Structure	SMALL PROLINE-RICH PROTEIN 1A	*Cornifin-A*	-4.04
Signaling	PSMC3 interacting protein	*Psmc3ip*	-1.71
rRNA maturation	Ribosomal RNA processing 15 homolog	*Rrp15*	-1.61
ORF	SWT1 RNA endoribonuclease homolog	*Swt1*	-1.56
Unknown	Chromosome unknown open reading frame, human C5orf46	*LOC100754037*	-2.99
	Uncharacterized LOC103159055	*LOC103159055*	-2.87
	Chromosome unknown open reading frame, human C5orf63	*LOC100753290*	-1.51

2.4.4. Verification of most up regulated gene expression by RT-qPCR

To verify the Illumina results, we calculated hypothalamic relative mRNA expression of *Col17a1, Dnah2, Myo15a* and *Vwf* during torpor entrance by RT-qPCR (Fig 8). Up regulation could be confirmed for *Dnah2* (RT-qPCR: 1.6-fold, p=0.016; Illumina: 1.7-fold, p<0,001), *Myo15a* (RT-qPCR: 2.5-fold, p=0,005; Illumina: 1.9-fold, p=0.035) and *Vwf* (RT-qPCR: 1.6-fold, p<0,001; Illumina: 1.6-fold. p=0.046). Up regulation of *Col17a1* did not reach significance in the RT-qPCR analysis (RT-qPCR: 1.2-

fold, p=0.462; Illumina: 1.8-fold, p=0.027). Also the other collagens identified by Illumina did not reach significance by RT-qPCR (*Col5a3*: 1.3-fold, p=0.550; *Col18a1*: 1.2-fold, p=0.450; *Col20a1*: 2.0-fold, p=0.361; *LOC103164493*: 1.4-fold, p=0.563) (data not shown).

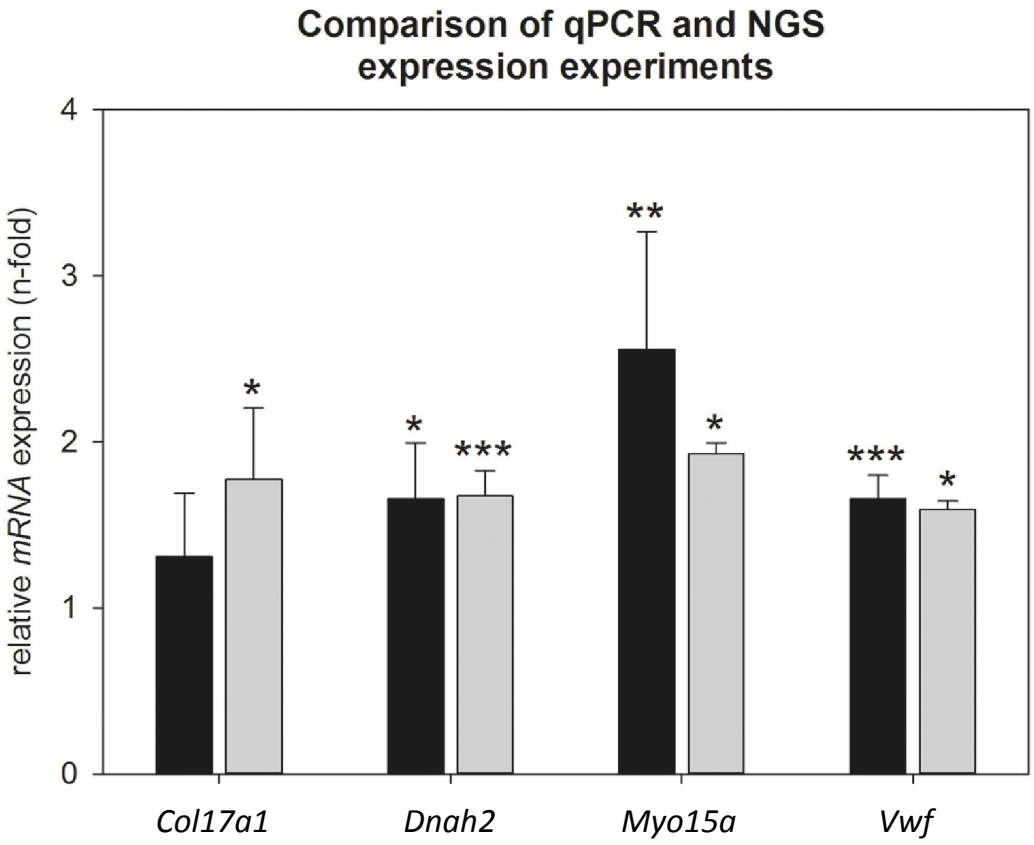

Fig 8. Comparison of NGS- and RT-qPCR data during torpor entrance. Expression changes were calculated by comparison of torpid animals at ZT1 to normothermic animals at ZT1 in both experiments. Grey bars represent torpid animals at ZT1(±SEM) analyzed by NGS and black bars represent torpid animals at ZT1(±SEM) analyzed by RT-qPCR. Significant differences to their relative control groups are marked with *=p<0.05, **=p<0.01, ***=p<0.001.

2.4.5. Relative gene expression patterns over the circadian cycle in torpid and normothermic hamsters

To determine, whether differential candidate gene expression is restricted to torpor entrance and to assess circadian regulation, we investigated relative mRNA expression at ZT1, ZT4, ZT7 and ZT16 in animals undergoing torpor and animals remaining normothermic. Differences within each investigated time point are shown relative to normothermic control group at same ZT respectively (Fig 9A, 9C, 9E, 9G). Circadian variations for normothermic animals are shown relative to the normothermic ZT1 group. Circadian variations for torpid animals are presented relative to torpor ZT1 group (Fig 9B, 9D, 9F, 9H).

There was no effect of time of day on *Col17a1* mRNA levels for normothermic animals, but there was an effect of time of day for torpid animals (two-way ANOVA: $p<0.001$). *Col17a1* mRNA expression was reduced in the post torpor group (ZT16) as compared to torpor entrance (ZT1, Tukey´s test: $p=0.004$) and to arousal (ZT7, Tukey´s test: $p=0.002$) (Fig 9B).
There were no significant changes in mRNA expression during torpor entrance (ZT1), mid torpor (ZT4) or arousal (ZT7) relative to normothermic control groups at the same ZTs. Post torpor (ZT16), mRNA expression was 0.57-fold down regulated (Tukey´s test: $p=0.014$) (Fig 9A).

There was an effect of time of day on *Dnah2* mRNA levels both, in normothermic and torpid animals (two-way ANOVA: $p<0.001$). Normothermic animals showed lowest mRNA expression at ZT1 (Tukey´s test: ZT1 vs ZT4 $p=0.006$, ZT1 vs ZT7 $p<0.001$, ZT1 vs ZT16 $p=0.004$), that increased at ZT4, peaked at ZT7 (Tukey´s test : ZT7 vs ZT4 $p=0.011$) and decreased again at ZT16 (Tukey´s test: ZT16 vs ZT7 $p=0.015$) (Fig 9D). Over the investigated torpor stages (ZT1, 4, 7) no significant changes were found, but post torpor (ZT16) mRNA expression was 0.38-fold down regulated as compared to torpor entrance (ZT1, Tukey´s test $p<0.001$), mid torpor (ZT4, Tukey´s test $p=0.003$) and arousal (ZT7, Tukey´s test $p<0.001$) (Fig 9D).
Relative to their normothermic control groups, *Dnah2* expression was 1.64-fold up regulated during torpor entrance (ZT1, Tukey´s test $p=0.016$), 0.56-fold down regulated during mid torpor (ZT4, Tukey´s test $p=0.014$), 0.40-fold down regulated during arousal (ZT7, Tukey´s test $p<0.001$) and 0.26-fold down regulated post torpor (ZT16, Tukey´s test $p<0.001$) (Fig 9C).

There was an effect of time of day on *Myo15a* mRNA levels both, in normothermic and torpid animals (two-way ANOVA: $p<0.001$). Normothermic animals showed low mRNA expression at ZT1 that increased at ZT4 (Tukey´s test $p=0.019$) and ZT7 (Tukey´s test $p<0.001$), before decreasing again at ZT16 (Tukey´s test: ZT16 vs ZT7 $p=0.030$). Torpid animals showed highest mRNA expression during torpor entrance (ZT1) differing significantly from mid torpor (ZT4, Tukey´s test $p=0.010$) and post torpor (ZT16, Tukey´s test $p<0.001$). mRNA expression at mid torpor (ZT4) was also down regulated as compared to arousal (ZT7, Tukey´s test $p=0.047$) and mRNA expression during arousal (ZT7) was up regulated relative to post torpor (ZT16, Tukey´s test $p<0.001$) (Fig 9F).
Myo15a expression was 2.51-fold up regulated during torpor entrance (ZT1, Tukey´s test $p=0.005$), 0.32-fold down regulated at mid torpor (ZT4, Tukey´s test $p=0.002$), 0.40-fold down regulated during arousal (ZT7, Tukey´s test $p=0.006$) and 0.29-fold down regulated in post torpor group (ZT16, Tukey´s test $p<0.001$) (Fig 9E) as compared to the normothermic control groups.

There was an effect of time of day on *Vwf* mRNA levels both, in normothermic and torpid animals (two-way ANOVA: $p<0.001$). Normothermic animals showed low mRNA expression at ZT1 that increased at ZT4 (Tukey´s test $p=0.038$) and further at ZT7 (Tukey´s test: ZT1 vs ZT7 $p<0.001$, ZT4 vs

ZT7 p=0.017), before decreasing at ZT16 (Tukey´s test: ZT7 vs ZT16 p<0.001). The mRNA expression in torpid animals was significantly up regulated during torpor entrance (ZT1) as compared to mid torpor (ZT4, Tukey´s test p=0.002) and post torpor (ZT16, Tukey´s test p<0.001) and during arousal (ZT7) compared to post torpor (ZT16, Tukey´s test p=0.002) (Fig 9H).

Vwf expression was 1.61-fold up regulated during torpor entrance (ZT1, Tukey´s test p<0.001), 0.58-fold down regulated during mid torpor (ZT4, Tukey´s test p=0.004), 0.51-fold down regulated during arousal (ZT7, Tukey´s test p<0.001) and 0.60-fold down regulated in post torpor group (ZT16, Tukey´s test p=0.003) (Fig 9G) as compared to the normothermic control groups.

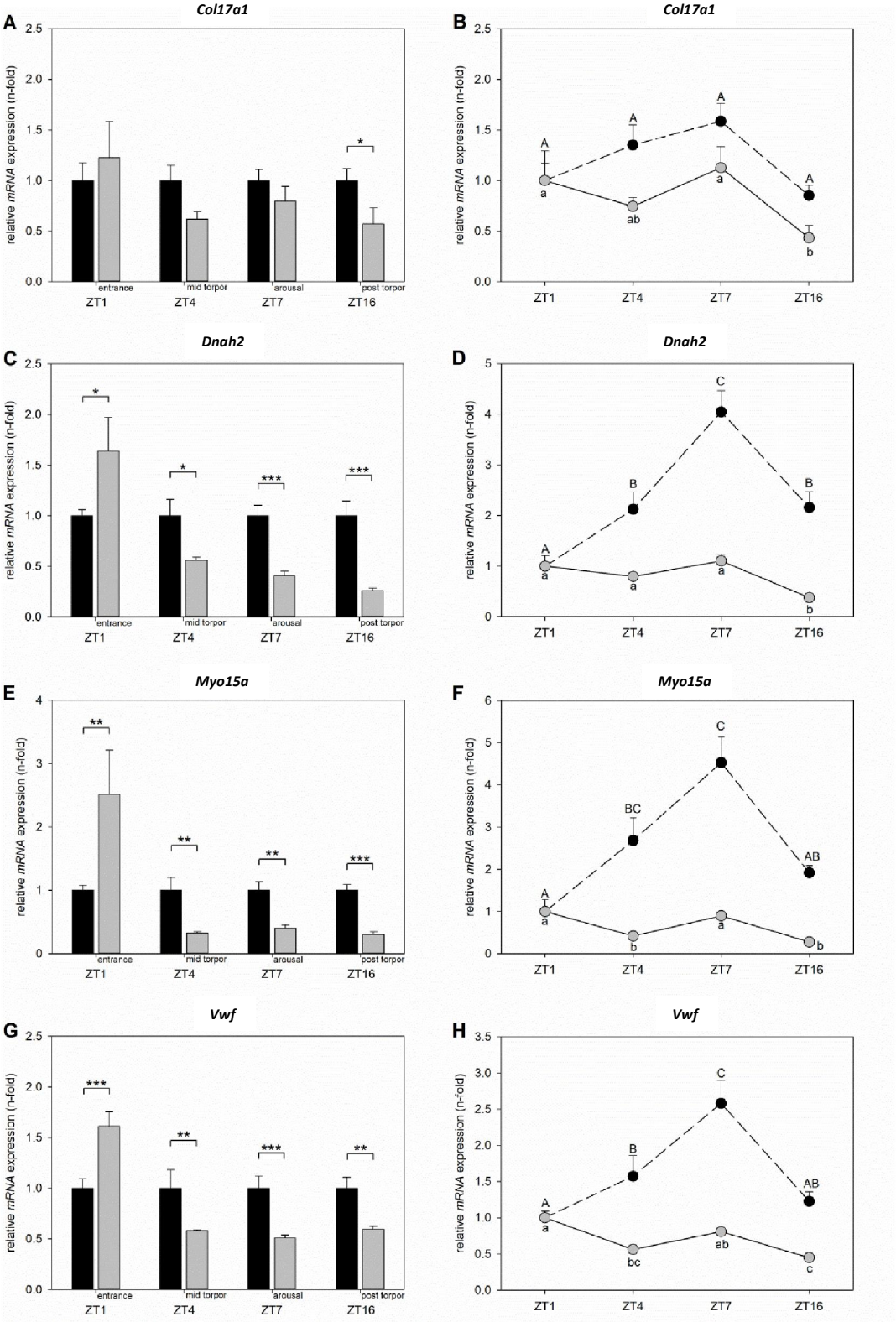

Fig 9. Circadian regulation of *Col17a1*, *Dnah2*, *Myo15a* and *Vwf* in torpid and normothermic Djungarian hamsters. Bar graphs on the left side show differences in *mRNA* expression of *Col17a1* (A), *Dnah2* (C), *Myo15a* (E) and *Vwf* (G) in torpid animals (grey bars, ±SEM) relative to normothermic control group at same ZT (black bars, ±SEM). Significant differences are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs on the right side show relative differences in *mRNA* expression of *Col17a1* (B), *Dnah2* (D), *Myo15a* (F) and *Vwf* (H) over the course of a day within normothermic animals relative to normothermic ZT1 group marked with upper case

(black circles, ±SEM) and within torpid animals relative to torpid ZT1 group marked with lower case (grey circles, ±SEM). Data points with different characters are significantly different ($p<0.05$).

2.5. Discussion

Our data show 284 differentially expressed genes out of 27830 identified genes in the hypothalamus of *P. sungorus* during entrance into the torpid state, implying that just a small set of genes is affected by the metabolic depression initiating torpor entrance. These results are in accordance with previous studies showing that transcript levels of most genes are unaffected during torpor (Storey & Storey, 2004). In accordance with the fact that daily torpor is a state of extreme metabolic adjustment, the majority of differentially regulated genes was found in cellular and metabolic processes for both, up and down regulated genes.

The majority of the top 20 down regulated genes were transcription factors, which could be responsible for a delay or suppression of mRNA transcription during the torpid state. It has been shown before, that transcriptional initiation as well as elongation rates are reduced during hibernation in golden-mantled ground squirrels (van Breukelen & Martin, 2002). Also in *P. sungorus* metabolic depression is associated with reduced transcriptional initiation (Berriel Diaz *et al.*, 2004). This may contribute to the generally suppressed protein synthesis during torpor that has been demonstrated in various tissues from different species (Gulevsky *et al.*, 1992; Frerichs *et al.*, 1998; Hittel & Storey, 2002).

Within the top 20 up regulated group our data show a remarkable number of genes coding for structure proteins. Except for the up regulation in collagen genes we were able to verify these results by RT-qPCR for *Dnah2*, *Myo15a* and the procoagulation factor *Vwf*.

Collagens are extracellular matrix structural components, which are involved in neuronal development of the brain. Collagens play a role in axonal guidance, synaptogenesis and establishment of brain architecture (Chernousov *et al.*, 2006; Fox, 2008; Hubert *et al.*, 2009). A study of Schwartz *et al*. identified an up regulation of several collagen genes in the cerebral cortex, but not in the hypothalamus, of thirteen-lined ground squirrels during deep hibernation and interbout arousals, indicating synaptic plasticity during hibernation (Schwartz *et al.*, 2013). Although we obtained a significant up regulation in mRNA expression of five collagen genes during torpor entrance by NGS and a significant enrichment of extracellular matrix components in up regulated gens, we were not able to verify these results by RT-qPCR. There was only a trend of increased *Col17a1* during torpor entrance as well as slightly lower mRNA levels during all other torpor stages and no diurnal changes could be detected in normothermic animals. Investigation of all other

collagens identified in the 20 up regulated group showed a similar picture with a slight up regulation at torpor entrance and trend to lower mRNA levels during the other torpor stages that did not reach significance (data not shown). There was a high variability in the mRNA expression levels of RT-qPCR samples, especially at torpor entrance, which might have caused the non-significant result. Different groups of animals were used for NGS and RT-qPCR study and data might reflect inter-individual differences. A larger sample size might help to resolve expression patterns in collagen genes more precisely. Hence, whether collagens are involved in synaptic remodeling and plasticity during torpid states remains to be revealed.

Elevated expression of *Dnah2*- and *Myo15a* mRNA during torpor entrance could be identified by both, NGS and RT-qPCR approach. Myosin and dynein are structural components of cytoskeleton and represent two out of three superfamilies of molecular motor proteins in neurons. They are able to transport biomolecules, such as vesicles, protein complexes and mRNAs in axons, dendrites and pre- and postsynaptic regions. Intracellular transport is necessary for neuronal morphogenesis, function and survival (Hirokawa *et al.*, 1998; Vale, 2003; Hirokawa *et al.*, 2010). During deep hibernation, elevated mRNA levels of three different myosin types and one dynein have been detected in the cerebral cortex of *S. tridecemlineatus, indicating dynamic structural changes* (Schwartz *et al.*, 2013).
In our study, hamsters showed elevated *Dnah2* and *Myo15a* expression only during torpor entrance (ZT1), whereas mRNA expression was reduced at all other investigated torpor states (mid torpor, arousal, post torpor) compared to normothermic animals. The higher expression of *Dnah2* and *Myo15a* during torpor entrance could be important to ensure maintenance of synaptic transmission and neuron survival during torpor by an elevated transport of biomolecules. It might also be possible that higher mRNA amounts are produced at the beginning and stored during the torpid state to provide transcripts for a fast utilization of these molecular motors during arousal. However, we think this possibility is unlikely because mRNA levels are already declining during mid torpor (ZT4).
In normothermic animals *Dnah2* as well as *Myo15a* show a diurnal regulation in its mRNA expression with a peak at ZT7 in normothermic animals. This might suggest a higher demand of these motor proteins during the hamster´s naturally active phase.
Taken together changes in structural protein shows evidence for plasticity in the hypothalamus of torpid hamsters and thereby confirm studies in deep hibernation that have proposed plastic changes in the brain before.

In addition to structure gene expression changes, we chose to investigate *Vwf* in more detail, because of its function in blood clotting. In torpid animals the reduced heart rate, ventilation and T_b results in a decreased blood flow that increases relatively fast to its euthermic flow rate during arousal. In contrast to all other mammalian species, torpor expressing mammals are able to survive

these periods of low blood flow and consequent reperfusion without apparent formations of deep vein thrombi, stroke or pulmonary embolism (Lyman & O'Brien, 1961; Frerichs *et al.*, 1994).
vWF is a major factor involved in platelet adhesion and thrombus formation (Denis & Wagner, 2007). Higher vWF levels increase the risk for thrombosis and embolism whereas deficiency in vWF activity leads to the human bleeding disorder von Willebrand´s disease (Sadler, 1998; Sadler, 2005). Moreover, Zhao *et al*. identified vWF as an important protein regulating the occurrence of cerebral ischemia and showed that a lack of vWF is able to reduce infarct volume (Zhao *et al.*, 2009). Based on this knowledge, a reduced level of Vwf would be expected during the torpid state to prevent blood clotting during periods of low blood flow. Indeed, in plasma samples of hibernating thirteen-lined ground squirrels vWF collagen binding is 10-fold decreased and in lung tissues *Vwf* mRNA expression is 3-fold down regulated during torpor (Cooper *et al.*, 2016). Unexpectedly, our NGS and RT-qPCR data show an elevated level of *Vwf* mRNA during torpor entrance in the hypothalamus. The elevated level of *Vwf* mRNA might either not directly translate into protein variation or alternatively translate into protein without damaging effects, namely inactive vWF. vWF is a large multimeric glycoprotein which can be cleaved in smaller multimers by ADAMTS13, a zinc-containing metalloprotease enzyme. These smaller multimers of vWF have a strongly decreased activity resulting in a reduced platelet adhesion and aggregation (Chauhan *et al.*, 2006; Zhao *et al.*, 2009). In this case, no damage of brain structures would be expected even when higher *Vwf* levels are present. Moreover, apart from the up regulation during torpor entrance, *Vwf* expression was lower in torpid animals at all other investigated states, supporting the hypothesis of low *Vwf* levels facilitating blood flow during the torpid state. Diurnal changes of *Vwf* could be detected in either group. Normothermic animals displayed highest *Vwf* level at ZT7, suggesting a higher demand of *Vwf* at the beginning of the active time. In torpid animals *Vwf* level is lowest at mid torpor (ZT4) and post torpor (ZT16). Taken together, our data provide evidence for readjustment of blood clotting during different torpor stages as well as times of day.

In general, the diurnal mRNA expression of all investigated genes of this study is less pronounced in torpid animals, which is likely to be caused by the suppression of transcription and translation during torpor. The transcriptional depression during torpor has been shown to result from both, down regulated transcriptional initiation and suppressed elongation (van Breukelen & Martin, 2002; Berriel Diaz *et al.*, 2004). Low T_b during torpor affects biochemical process, leading to a decline in gene expression caused by the temperature sensitivity of transcriptional elongation (van Breukelen & Martin, 2002; Berriel Diaz *et al.*, 2004).

The NGS technology allows a whole transcriptome survey of gene expression changes and our analysis provide an overview of gene expression changes during torpor initiation in *P. sungorus* for the first time.

Although we could not determine signaling pathways regulating torpor initiation with this approach, we identified molecular adaptions in the hypothalamus of *P. sungorus* initiated during the early state of torpor. Our data provide evidence for synaptic remodeling and plasticity, an elevated transport of biomolecules and readjustment of coagulation. Comparable gene expression changes have already been found in deep hibernators. This would support the hypothesis that daily torpor and hibernation are similar physiological states only differing in amplitude and duration. Interestingly, the molecular changes already occur within the short time span of daily torpor. These adaptations may, just like in deep hibernation, help the brain cells to better survive or reduce cell damages during the extreme physiological conditions in the torpid state. In the future, precise anatomical investigation of identified genes is necessary to eventually gain insights into their functions.

Chapter 3

Hypothalamic control systems show differential gene expression during spontaneous daily torpor and fasting-induced torpor in the Djungarian hamster (*Phodopus sungorus*).

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Published in in the journal of Plos One (2017)

(12(10): e0186299. doi: 10.1371/journal.pone.0186299)

The original manuscript is included in this thesis, but layout, figure numbers, table numbers and references have been reformatted. The author of this thesis was involved in the design of all experiments supported by Annika Herwig. All *in vivo* experiments were carried out by me. Gene expression analysis by RT-qPCR was performed by Hanna Markowsky and me and data were analysed as well as interpreted by myself. Manuscript was written by myself and revised by Annika Herwig.

3.1. Abstract

Djungarian hamsters are able to use spontaneous daily torpor (SDT) during the winter season as well as fasting-induced torpor (FIT) at any time of the year to cope with energetically challenging environmental conditions. Torpor is a state of severely reduced metabolism with a pronounced decrease in body temperature, which enables animals to decrease their individual energy requirements. Despite sharing common characteristics, such as reduced body mass before first torpor expression and depressed metabolism and body temperature during the torpid state, FIT and SDT differ in several physiological properties including torpor bout duration, minimal body temperature, fuel utilization and circadian organization. It remains unclear, whether SDT and FIT reflect the same phenomenon or two different physiological states. The hypothalamus has been suggested to play a key role in regulating energy balance and torpor. To uncover differences in molecular control mechanisms of torpor expression, we set out to investigate hypothalamic gene expression profiles of genes related to orexigenic (*Agrp*/*Npy*), circadian clock (*Bmal1*/*Per1*) and thyroid hormone (*Dio2*/*Mct8*) systems of animals undergoing SDT and FIT during different torpor stages. Orexigenic genes were mainly regulated during FIT and remained largely unaffected by SDT. Expression patterns of clock genes showed disturbed circadian clock rhythmicity in animals undergoing FIT, but not in animals undergoing SDT. During both, SDT and FIT, decreased *Dio2* expression was detected, indicating reduced hypothalamic T3 availability in both types of torpor. Taken together, our results provide evidence that SDT and FIT also differ in certain central control mechanisms and support the observation that animals undergoing SDT are in energetical balance, whereas animals undergoing FIT display a negative energy balance. This should be carefully taken into account when interpreting data in torpor research, especially from animal models of fasting-induced hypometabolism such as mice.

3.2. Introduction

The use of torpor in times of energetically challenging environmental conditions is a common strategy, which has been identified in most orders of mammals. Reduction of metabolic rate and body temperature (T_b), the two main characteristics of torpor, enable the animal to reduce energy expenditure and lower energy requirements with almost no evidence of tissue or organ damage after rewarming from the torpid state (Arendt *et al.*, 2003; Carey *et al.*, 2003; Heldmaier *et al.*, 2004; Fleck & Carey, 2005; Melvin & Andrews, 2009; Ruf & Geiser, 2015; Jastroch *et al.*, 2016).

The Djungarian hamster (*Phodopus sungorus*, also known as Siberian hamster) uses spontaneous daily torpor (SDT) to save energy during the harsh winters of Central Asian steppes. SDT in

Djungarian hamsters is the final trait of various adaptions (severe body weight loss, molt to white winter fur, gonadal regression) to the winter season (Figala *et al.*, 1973; Scherbarth & Steinlechner, 2010). The onset of winter acclimatization is triggered by decreasing day length during autumn, driven by modification of melatonin production in the pineal gland, and can easily be induced by adjusting the light-dark cycle in the laboratory (Steinlechner & Heldmaier, 1982; Illnerova *et al.*, 1984; Vitale *et al.*, 1985). SDT is controlled by the circadian clock and usually limited to the resting phase, allowing the hamsters to maintain foraging activities during the night throughout winter. The torpid state is initiated by an active metabolic depression (25 % below the level of resting metabolic rate), followed by a drop in T_b to a minimum value of approximately 15 °C, reduced ventilation, heart rate and locomotor activity with an average duration of six hours per torpor bout (Kirsch *et al.*, 1991; Heldmaier & Ruf, 1992; Heldmaier *et al.*, 2004). When SDT is used frequently, Djungarian hamsters are able to save up to 65 % of total energy requirements during the winter season (Ruf *et al.*, 1991).

Food scarcity, hence energy depletion, is not restricted to the winter months, but can occur at any time of the year and is able to induce fasting-induced torpor (FIT) in many small mammals, including mice (Webb *et al.*, 1982; Dark *et al.*, 1994; Brown & Staples, 2010; Diedrich & Steinlechner, 2012; Diedrich *et al.*, 2015). This form of torpor can occur not only during any season but also at any time of day. Although SDT and FIT in Djungarian hamsters both involve metabolic depression and decreased T_b, they show some distinct physiological characteristics including differences in preparatory time period before the first torpor episode occurs, circadian control, fuel utilization as well as differences in torpor depth and duration (Diedrich & Steinlechner, 2012; Diedrich *et al.*, 2015). The physiological variations between these two forms of torpor suggest that they might be regulated by distinct central control mechanisms.

Several hypothalamic systems have been linked to the regulation of energy balance and torpor. In the arcuate nucleus (ARC), food intake and energy expenditure are closely linked to the expression of orexigenic (*Npy*/*Agrp*) and anorexigenic (*Pomc*/*Cart*) genes. When orexigenic neuropeptides are activated, food consumption is enhanced and energy expenditure reduced (Stephens *et al.*, 1995; Hahn *et al.*, 1998; Schwartz *et al.*, 2000). Intracerebroventricular injections of NPY induce torpor like hypothermia in *P. sungorus*, likely mediated by NPYY1 receptors. This hypothermic state seems to resemble torpor patterns of FIT bouts rather than SDT bouts (Paul *et al.*, 2005; Pelz & Dark, 2007). Moreover, ARC lesions by monosodium glutamate injections prevent SDT expression (Pelz *et al.*, 2008).

The suprachiasmatic nuclei (SCN) host the circadian clock, which controls the circadian organization of daily rhythms in biochemistry, physiology and behavior by autonomous transcription-translation feedback loops. These feedback loops consist of positive (BMAL1/CLOCK) and negative (PER/CRY) elements, activating and inactivating each other's transcription in a roughly 24 hour rhythm that is

synchronized to the light-dark cycle of the day (Takahashi *et al.*, 2008). SDT is clearly timed by the circadian clock and expression of clock genes remains largely rhythmic (Kirsch *et al.*, 1991; Herwig *et al.*, 2006; Herwig *et al.*, 2007). The ablation of the SCN leads to a disordered SDT onset, which is no longer restricted to the daytime, but does not prevent the expression of torpor (Ruby & Zucker, 1992). In FIT, a circadian rhythmicity of torpor timing is absent, thus FIT can occur at any time of a day.

Thyroid hormones are known for their role in regulating energy balance by peripheral but also central mechanisms (Bianco *et al.*, 2005; Herwig *et al.*, 2008). The hypothalamic thyroid hormone system has been shown to be a crucial driver of seasonal adaptions, including long-term shifts in energy balance (Barrett *et al.*, 2007; Hanon *et al.*, 2008; Herwig *et al.*, 2009). Thyroid hormones are transported into the hypothalamus by transporters (e.g. *Mct8*) where they are activated or inactivated by the type-II-deiodinase (*Dio2*) and type-III-deiodinase (*Dio3*), respectively (Kohrle, 1999; Friesema *et al.*, 2003). These two enzymes are co-expressed in tanycytes of the third ventricle and expression is regulated in a season dependent manner (Barrett *et al.*, 2007; Herwig *et al.*, 2009; Herwig *et al.*, 2013; Bank *et al.*, 2017b). Moreover, T3 availability affects torpor behavior. Chemical inhibition of T3 production promotes torpor expression, whereas excess T3 in the periphery as well as locally in the hypothalamus, is able to prevent torpor in winter adapted animals (Murphy *et al.*, 2012; Bank *et al.*, 2015; Bank *et al.*, 2017a).

Here we set out to investigate differences in regulatory mechanisms of SDT and FIT induced torpor. For this purpose we compared hypothalamic expression profile of *Agrp* and *Npy* (orexigenic system), *Bmal1* and *Per1* (circadian clock system) and *Dio2* and *Mct8* (thyroid hormone system) over different torpor stages of fasted and *ad libitum* fed hamsters. Alterations in the orexigenic as well as circadian system demonstrate that SDT and FIT are at least partly regulated by distinct mechanisms.

3.3. Material and Methods

3.3.1. Animals

The experiments were performed in accordance with the German Animal Welfare Law and approved by the local animal welfare authorities (No. 4_16 and No. 114_14, Hamburg, Germany).

All Djungarian hamsters (*P. sungorus*) were descended from our own breeding colony at the Zoological Institute of the University of Hamburg, Germany. Hamsters were bred and raised under artificial long day conditions (LD) with a light-dark-cycle of 16 hours light and 8 hours dark and an ambient temperature of 21 ± 1 °C. The hamsters were housed individually in plastic cages (Macrolon

Type III) with free access to drinking water before and during experiments. They were fed a hamster breeding diet (Altromin 7014, Germany) *ad libitum* before experiments started.

3.3.2. Experimental setup and sampling

At the age of three to five months a total number of 70 hamsters was transferred to short day conditions (SD) with a light-dark-cycle of 8 hours light and 16 hours dark and an ambient temperature of 18 ± 1 °C to develop their winter phenotype (catabolic state, white winter fur, occurrence of SDT, gonadal regression). Animals not clearly showing a winter phenotype after 10 weeks of SD exposure were excluded from the experiments. A group of 20 hamsters remained under LD, representing the summer phenotype (anabolic state, brownish-grey summer fur, reproductively active). Animals were separated into three SD-groups (SDT, FIT-SD, non-torpid SD animals (NT-SD)) and one LD-group (FIT-LD). Each group consisted of 20 male and female hamsters.
After 12 weeks under SD all animals were implanted i.p. with DSI-transmitters (Model TA-F10, St. Paul, MN, USA) under isoflurane anesthesia (2.0 – 2.5 %, Forene, Abott, Wiesbaden, Germany) and analgesia by s.c. injection of carprofen (5 mg/kg, Paracarp, IDT Biologika, Germany). The LD-group was implanted with DSI-transmitters in the same way. Surgeries were carried out as previously described (Bank *et al.*, 2015). Core T_b of each hamster was measured in three minute intervals until the end of the experiment to precisely determine the T_b while sampling during different torpor stages as well as to calculate torpor duration and depth.

The three SD groups were sampled according to torpor state and Zeitgeber time (ZT, ZT0=lights on), whereas the LD group was sampled according to torpor state only (Fig 10).

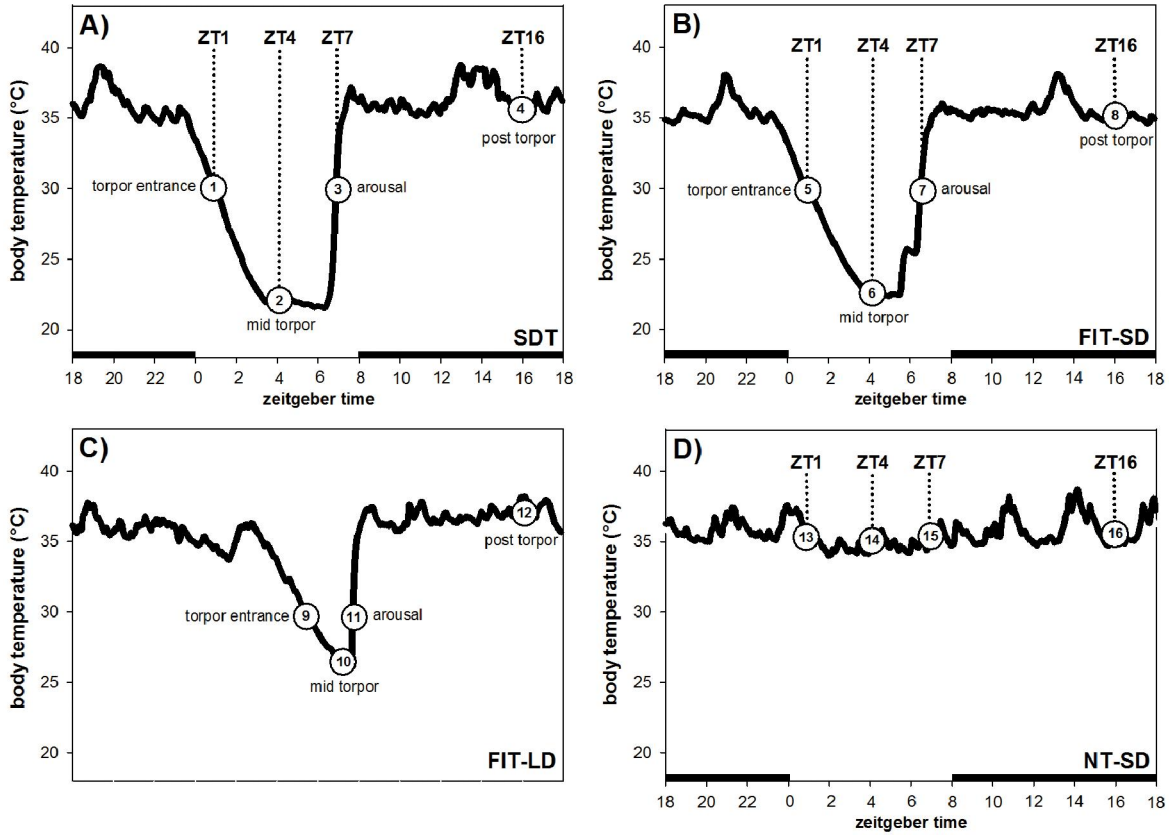

Fig 10. Sampling points for hamsters undergoing SDT (A), FIT-SD (B) and FIT-LD (C) and of hamsters remaining NT-SD (D). Black bars in the x-axis represent the dark phase of the light-dark cycle.

The spontaneous torpor group kept under SD (SDT) was fed *ad libitum* throughout the experiment. These animals were culled at different stages of a spontaneous daily torpor bout: during torpor entrance at ZT1 (n=5, Tb: 30.8 °C ± 0.5 °C), mid torpor at ZT4 (n=5, Tb: 22.5 °C ± 1.5 °C), arousal at ZT7 (n=5, Tb: 30.4 °C ± 0.4 °C) and in a post torpid state at ZT16 (n=5, Tb: 35.7 °C ± 0.6 °C) (Fig 10A, group 1 – 4; Table 5).

For the SD fasting-induced torpor group (FIT-SD), daily food consumption of each hamster was individually recorded during week 11 in SD for seven consecutive days to calculate average food intake. Throughout the experiment the hamsters were provided 60 % of their daily food intake at ZT6 for five days, followed by three days of *ad libitum* feeding. The animals were weighed twice a week to monitor body weight loss. In case of a critical body weight loss over 25 % of their LD body mass the hamsters were fed additional 10%. To ensure sampling during FIT bouts, we collected brain samples only after at least two days of food restriction, when the hamsters had already shown torpor bouts. Once the hamsters displayed FIT, they were culled during torpor entrance at ZT1 (n=5, Tb: 30.1 °C ± 0.4 °C), mid torpor at ZT4 (n=5, Tb: 21.3 °C ± 0.9 °C), arousal at ZT7 (n=5, Tb: 30.3 °C ± 0.5 °C) and in a post torpid state at ZT16 (n=5, Tb: 35.0 °C ± 2.7 °C) (Fig 10B, group 5 – 8; Table 5).

For the LD fasting-induced torpor group (FIT-LD), average daily food consumption for each hamster was determined as described for FIT-SD. The food was provided daily at ZT14. During the first experimental week hamsters were fed 30 % of their daily food intake followed by four weeks with 60 % feeding to induce FIT (Diedrich *et al.*, 2015). The hamsters were weighed twice a week and received additional 10 % food, if they exceeded a body weight loss of over 25 %. Hamsters not showing torpor within these four weeks were fed *ad libitum* again for three days, followed by four days with 30 % food and one week with 60 % food. Since FIT is not under circadian control, these animals were not sampled at a particular ZT, but during torpor entrance at ZT5 – ZT9 (n=5, Tb: 29.7 °C ± 0.4 °C), mid torpor at ZT5 – ZT10 (n=5, Tb: 25.4 °C ± 1.4 °C), arousal at ZT7 – ZT11 (n=5, Tb: 30.7 °C ± 0.5 °C) and in a post torpid state at ZT20 (n=5, Tb: 35.1 °C ± 1.9 °C) (Fig 10C, group 9 – 12; Table 5).

The SD non-torpid group (NT-SD) was fed *ad libitum* throughout the experiment. These animals were winter adapted and already spontaneously expressed torpor. They were culled on a day without torpor in a non-torpid state at ZT1 (n=5, Tb: 35.7 °C ± 0.5 °C), ZT4 (n=5, Tb: 35.7 °C ± 0.4 °C), ZT7 (n=5, Tb: 35.6 °C ± 0.4 °C) and ZT16 (n=5, Tb: 36.2 °C ± 1.3 °C) as respective control (Fig 10D, group 13 – 16; Table 5).

Table 5. Sampling T_b data of animals undergoing SDT, FIT-SD, FIT-LD and remaining NT-SD. SDT and FIT-SD were sampled according to torpor state and ZT, FIT-LD was sampled according to torpor state and NT-SD was sampled according to ZT.

	torpor entrance **ZT1**	**mid torpor** **ZT4**	**arousal** **ZT7**	**post torpor** **ZT16**
SDT	30.8 °C ± 0.5 °C	22.5 °C ± 1.5 °C	30.4 °C ± 0.4 °C	35.7 °C ± 0.6 °C
FIT-SD	30.1 °C ± 0.4 °C	21.3 °C ± 0.9 °C	30.3 °C ± 0.5 °C	35.0 °C ± 2.7 °C
FIT-LD	29.7 °C ± 0.4 °C	25.4 °C ± 1.4 °C	30.7 °C ± 0.5 °C	35.1 °C ± 1.9 °C
NT-SD	35.7 °C ± 0.5 °C	35.7 °C ± 0.4 °C	35.6 °C ± 0.4 °C	36.2 °C ± 1.3 °C

All hamsters used in this experiment were sacrificed by CO_2 inhalation. Brains were dissected, immediately frozen on dry ice and stored at -80 °C until further use.

3.3.4. Isolation of total RNA and cDNA synthesis

Hypothalamic blocks were cut from frozen brain samples as described in Cubuk *et al*. (Cubuk *et al.*, 2017a). Hypothalamic samples were homogenized in 1 ml TriFast by using a micropestle. Total RNA was isolated using peqGOLD Trifast™ (Peqlab, Erlangen, Germany) and purified by using the Crystal RNA MiniKit (Biolabproducts, Bebensee, Germany) including an on-column digestion with RNase-free DNase (Quiagen, Hilden, Germany) according to the manufacturer`s instructions. Total RNA was quantified spectrometrically, RNA purity was assessed by the 260/280 nm ratio on a NanoDrop 1000 spectrophotometer and RNA integrity was proven by formaldehyde agarose gel electrophoresis. 1 µg of total RNA of each sample was used for cDNA synthesis, carried out with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) using oligo-(dT)-primer (0.5 µg/µl) according to manufacturer`s instructions. Total cDNA samples were stored at -20 °C until usage as template for Real Time qPCR (RT-qPCR) or standard plasmid generation.

3.3.5. Cloning and sequencing

Standard plasmids were generated from 90 – 200 bp long coding sequence fragments of agouti related neuropeptide (*Agrp*), brain and muscle Arnt-like protein-1 (*Bmal1*), iodothyronine deiodinase 2 (*Dio2*), monocarboxylate transporter 8 (*Mct8*), neuropeptide Y (*Npy*), period circadian clock 1 (*Per1*), hypoxanthine phosphoribosyltransferase (*Hprt*), actin beta (*Actb*), ribosomal protein lateral stalk subunit P0 (*Rplp0*) and 18S ribosomal RNA (*Rn18s*) by gene specific primers (Table 6). All primers were designed on *P. sungorus* specific sequences obtained from our previous Illumina study (Cubuk *et al.*, 2017a). 18 – 25 bp long primers were designed using the online tool OligoAnalyzer 3.1 with a melting temperature of 60 °C ± 1.1 °C. The amplicons were cloned into the pGEM®-T Easy Vector System (Promega, Madison, USA) according to manufacturer`s instructions and sequenced by the commercial sequencing platform GATC Biotech (Konstanz, Germany).

Table 6. *P. sungorus* specific primer sequences used for standard plasmid generation and RT-qPCR.

gene		5´3´sequence	melting temperature	amplicon length
Agrp	forward	GCC TTT GCC CAA CAT CCG TTG	59.8	99 bp
	reverse	GCT ACT GCC GCT TCT TCA ATG CC	60.9	
Bmal1	forward	GCT CAA GAG ACC CCA GGT TAT CC	59.1	145 bp
	reverse	GGC TCA TGA TGA CAG CCA TCG C	60.8	
Dio2	forward	TGA AGA AAC ACA GGA GCC AAG AGG A	60.0	111 bp
	reverse	CAT TAT TGT CCA TGC GGT CAG CCA	59.8	
Mct8	forward	GTC CTC TCA TTC CTG CTC CTG G	59.2	151 bp
	reverse	GTC CCA CCA GCT CAA ATG CAA TG	59.0	
Npy	forward	CCA GGC AGA GAT ACG GCA AGA GAT C	60.7	119 bp
	reverse	CCA TCA CCA CAT GGA AGG GTC C	60.0	
Per1	forward	CTC TTC TTC TGG CAA TGG CAA GGA C	60.0	120 bp
	reverse	GCA CTC AGG AGG CTA TAG GCA ATG	59.4	
Actb	forward	ACC TCA TGA AGA TCC TGA CCG AGC	60.3	120 bp
	reverse	CCA TCT CTT GCT CGA AGT CCA GGG	61.1	
Hprt	forward	AGT CCC AGC GTC GTG ATT AGT GAT G	60.4	140 bp
	reverse	CGA GCA AGT CTT TCA GTC CTG TCC A	60.5	
Rplp0	forward	GCA ACA GTC GGG TAA CCA ATC TGC	60.4	153 bp
	reverse	CTTCGGGCTCATCATCCAGCAG	60.1	
Rn18s	forward	GCT CCT CTC CTA CTT GGA TAA CTG TG	59.2	126 bp
	reverse	CGG GTT GGT TTT GAT CTG ATA AAT GCA	59.4	

3.3.6. Real-time qPCR (RT-qPCR) and analysis of expression data

RT-qPCR was performed to compare relative gene expression values of investigated genes between and within SDT, FIT-SD, FIT-LD and the non-torpid control group over the course of a day and during different torpor stages.

RT-qPCR experiments were carried out on an ABI Prism 7300 Real Time PCR System (Applied Biosystems, Darmstadt, Germany) using Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany). Due to the large number of samples, RT-qPCRs were performed on five 96-well plates (Biolabproducts, Bebensee, Germany) for each target gene with a non-torpid ZT16 sample

applied to all plates as inter-plate calibrator. *Hprt*, *Actb*, *Rplp0* and *Rn18s* were employed as putative non-regulated controls. Since none of these reference genes showed stability of expression values across all investigated states, we used *Hprt* as reference gene for NT-SD and SDT and *Rplp0* as reference gene for FIT-SD and FIT-LD. To calculate PCR efficiency, a series of six 10-fold dilutions of target gene specific standard plasmids was added to the plate from which standard curves were generated. Specificity of each amplification reaction was validated by dissociation curve analysis. All samples were run in triplicates, using 1 µl cDNA as template for each reaction. Furthermore, a no-template control was run on each plate in duplicates. RT-qPCR was conducted with a standard cycling protocol using 40 amplification cycles (50 °C 2 min; 95 °C 10 min; 95 °C 15 s; 60 °C 15 s; 72 °C 30 s).
First evaluation of RT-qPCR data was done with the 7500 Software v2.0.6 (ABI Prism, Applied Biosystems). Afterwards RT-qPCR data were exported to Microsoft Excel 2010 to estimate expression levels of investigated genes using the ΔΔCT method. Differences in relative mRNA expression were assessed during torpor entrance, mid torpor, arousal and post torpor and are shown relative to NT-SD at same ZT respectively. Relative mRNA expression levels over the circadian cycle of investigated genes are shown for SDT, FIT-SD and NT-SD relative to the corresponding ZT1 sample of each group. Since FIT-LD was not sampled at specific ZT time points, but sampling was only defined by torpor state, this group was excluded from circadian rhythmicity analysis.

3.3.7. Statistical analysis

All statistical analysis and figures were performed with SigmaPlot 12.5 (Systat Software Inc). Differences in torpor duration and depth between SDT, FIT-SD and FIT-LD were statistically tested by Mann Whitney Rank Sum test (U-test). The RT-qPCR data were statistically tested by two-way ANOVA with the factors torpor group (SDT, FIT-SD, FIT-LD, NT-SD) and torpor state/time of day (torpor entrance, mid torpor, arousal, post torpor) followed by Tukey test or by one-way ANOVA or Kruskal-Wallis test, if normality test failed in at least one of the investigated groups. P-values ≤ 0.05 were considered as significant.

3.4. Results

3.4.1. Torpor depth and duration in fasting-induced and spontaneous daily torpor

We calculated the mean torpor duration and minimal T_b of hamsters undergoing SDT, FIT-SD and FIT-LD. Torpor was defined as T_b < 32 °C for more than 30 minutes. Animals undergoing SDT had a mean torpor duration of 306.0 ± 14.46 minutes with a minimal T_b of 23.2 ± 0.27 °C. FIT-SD animals showed

a slightly lower mean torpor duration of 279.02 ±7.19 minutes and minimal T_b of 23.2 ± 0.16 °C. FIT-LD animals showed significantly shorter torpor bouts with a mean torpor duration of 178.6 ± 13.53 minutes (SDT vs. FIT-LD: U-test, P<0.001; FIT-SD vs. FIT-LD: U-test, P<0.001) as well as a significantly higher minimal T_b of 26.3 ± 0.33 °C (SDT vs. FIT-LD: U-test, P<0.001; FIT-SD vs. FIT-LD: U-test, P<0.001) compared to SDT and FIT-SD (Fig 11).

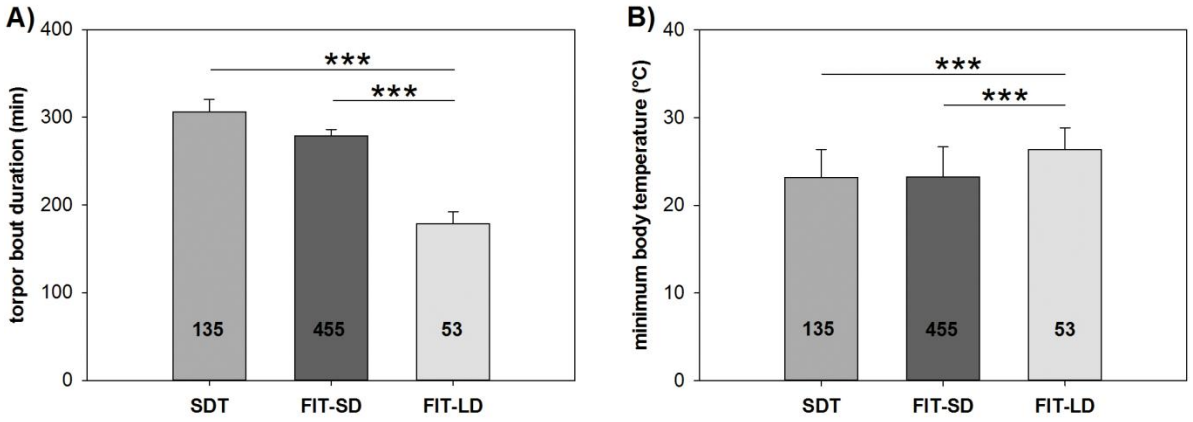

Fig 11. Average torpor bout duration (A) and depth (B) of hamsters undergoing SDT, FIT-SD and FIT-LD. Numbers within bars indicate the number of torpor episodes within each group used for statistical analysis. Significant differences between these three types of torpor are marked with *=p<0.05, **=p<0.01 and ***=p<0.001.

3.4.2. Hypothalamic gene expression of genes involved in orexigenic, circadian and thyroid hormone regulatory mechanisms

To determine whether spontaneous daily torpor and fasting-induced torpor might underlie different regulatory systems, we investigated hypothalamic mRNA expression levels of *Npy*, *Agrp*, *Per1*, *Bmal1*, *Dio2* and *Mct8* as representatives for orexigenic, circadian and thyroid hormone regulatory mechanisms.

3.4.2.1. Relative mRNA expression of *Agrp* and *Npy* over the course of a torpor bout

There was an effect on *Agrp* mRNA expression in torpor group during torpor entrance (one-way ANOVA, p=0.003) and mid torpor (one-way ANOVA, p=0.034). During torpor entrance, *Agrp* mRNA expression was 0.52-fold down regulated in FIT-LD relative to NT-SD at ZT1 (FIT-LD vs. NT-SD: Tukey test, p=0.002). During mid torpor, expression of *Agrp* in FIT-SD showed a 3.05-fold up regulation compared to NT-SD (FIT-SD vs. NT-SD: Tukey test, p=0.034). SDT and FIT-LD showed no significant differences compared to NT-SD during mid torpor. There were no significant changes in *Agrp* expression during arousal or post torpor among all investigated torpor groups (Fig 12).

There was no effect of time of day or torpor state in *Agrp* expression for NT-SD (Kruskal-Wallis test, p=0.574) and SDT (one-way ANOVA, p=0.178). In FIT-SD there was an effect (one-way ANOVA, p=0.034) showing elevated mRNA expression during mid torpor at ZT4 compared to torpor entrance at ZT1 (ZT4 vs. ZT1: Tukey test, p=0.022) (Fig 12).

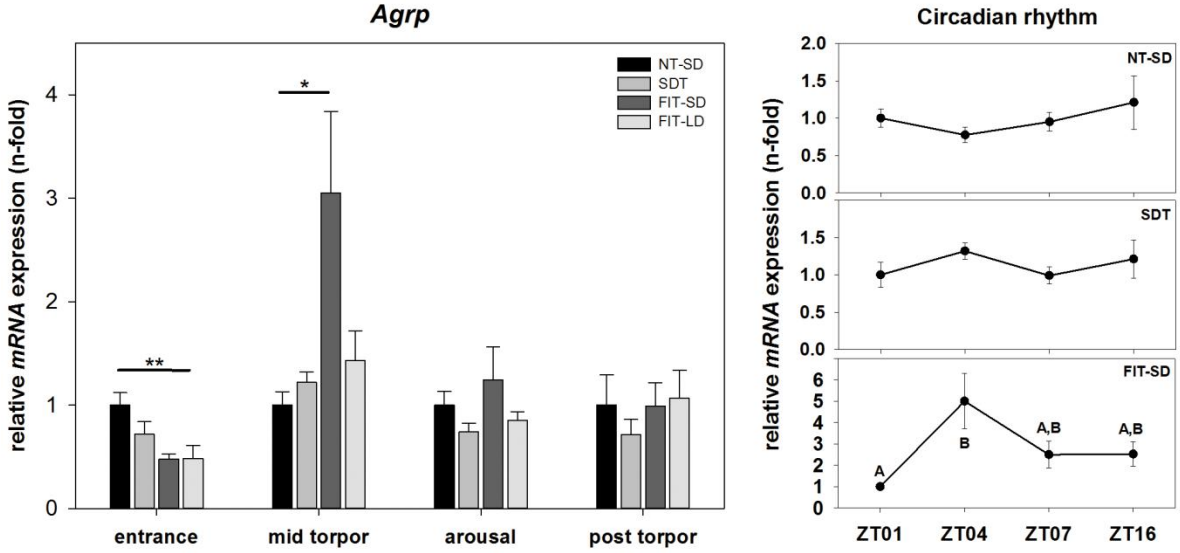

Fig 12. Relative mRNA expression of *Agrp*. Bar graphs (with n=5 for each bar) show fold changes of mRNA expression for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) relative to NT-SD (black bars, ±SEM) for torpor entrance (ZT1), mid torpor (ZT4), arousal (ZT7) and post torpor (ZT16) respectively. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show differences in mRNA expression (±SEM) at four different time points (with n=5 for each time point) over the course of a day relative to ZT1 for hamsters remaining active (NT-SD), undergoing SDT or FIT-SD. Significant differences are marked with different upper cases (p<0.05).

There was an effect of torpor group on *Npy* mRNA expression during the post torpid state (Kruskal-Wallis test, p=0.045). RT-qPCR analysis revealed no significant differences of *Npy* expression during torpor entrance, mid torpor or arousal between SDT, FIT-SD or FIT-LD. However, there was a trend towards up regulation in FIT-SD during mid torpor compared to NT-SD (FIT-SD vs. NT-SD: Tukey test, p=0.090). Only during the post torpid state a significant difference could be found between SDT and FIT-SD (SDT vs. FIT-SD: Tukey test, p<0.05) (Fig 13).

There was no effect of time of day or torpor state in *Npy* expression for NT-SD (one-way ANOVA, p=0.195), SDT (one-way ANOVA, p=0.275) or FIT-SD (one-way ANOVA, p=0.144) (Fig 13).

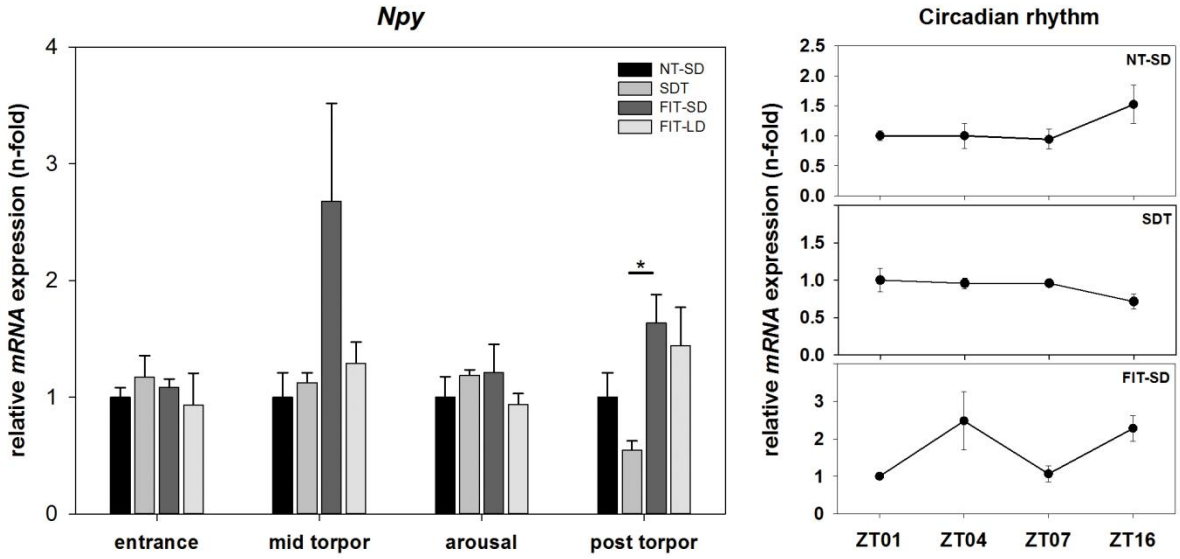

Fig 13. Relative mRNA expression of *Npy*. Bar graphs (with n=5 for each bar) show fold changes of mRNA expression for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) relative to NT-SD (black bars, ±SEM) for torpor entrance (ZT1), mid torpor (ZT4), arousal (ZT7) and post torpor (ZT16) respectively. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show differences in mRNA expression (±SEM) at four different time points (with n=5 for each time point) over the course of a day relative to ZT1 for hamsters remaining active (NT-SD), undergoing SDT or FIT-SD. Significant differences are marked with different upper cases (p<0.05).

3.4.2.2. Relative mRNA expression of *Bmal1* and *Per1* over the course of a torpor bout

There was an effect of torpor group on *Bmal1* mRNA expression during torpor entrance (one-way ANOVA, p<0.001), arousal (one-way ANOVA, p=0.002) and post torpor (one-way ANOVA, p<0.001), as well as of torpor state (NT-SD: one-way ANOVA, p=0.002; SDT: one-way ANOVA, p=0.003; FIT-SD: Kruskal-Wallis test, p=0.024). During torpor entrance, *Bmal1* mRNA expression was 0.25-fold down regulated in FIT-LD compared to NT-SD (FIT-LD vs NT-SD: Tukey test, p=0.003), but 1.41-fold up regulated in FIT-SD compared to NT-SD (FIT-SD vs NT-SD: Tukey test, p=0.031) with a higher expression level compared to SDT and FIT-LD (FIT-SD vs. SDT: Tukey test, p<0.001; FIT-SD vs. FIT-LD: Tukey test, p<0.001). During arousal *Bmal1* in FIT-LD was 0.48-fold down regulated relative to NT-SD (FIT-LD vs. NT-SD: Tukey test, p=0.012) and the expression level was significantly lower than in SDT (FIT-LD vs. SDT: Tukey test, p=0.045) and FIT-SD (FIT-LD vs. FIT-SD: Tukey test, p=0.001). In the post torpid state *Bmal1* expression was 0.41-fold down regulated in FIT-LD (FIT-LD vs. NT-SD: Tukey test, p<0.001). FIT-SD showed a significantly higher expression level than SDT (FIT-SD vs SDT: Tukey test, p=0.003) and FIT-LD (FIT-SD vs. FIT-LD: Tukey test, p<0.001) (Fig 14).

There was an effect of time of day or torpor state in *Bmal1* expression. In NT-SD *Bmal1* expression was low at ZT1 and ZT4, significantly increased 1.56-fold at ZT7 and remained 1.66-fold up regulated

at ZT16 (ZT1 vs. ZT16: Tukey test, p=0.052; ZT4 vs. ZT7: Tukey test, p=0.011; ZT4 vs. ZT16: Tukey test, p=0.002). In SDT *Bmal1* expression was low at ZT1 and ZT4, increased 1.62-fold at ZT7 before slightly decreasing again to 1.29-fold at ZT16 (ZT1 vs. ZT7: Tukey test, p=0.004; ZT1 vs. ZT16: Tukey test, p=0.020; ZT4 vs. ZT7: Tukey test, p=0.043). In FIT-SD *Bmal1* expression was low at ZT1, ZT4 (1.06-fold) and ZT7 (1.11-fold), but 1.50-fold up regulated at ZT16 (ZT4 vs. ZT16: Tukey test, p<0.05) (Fig 14).

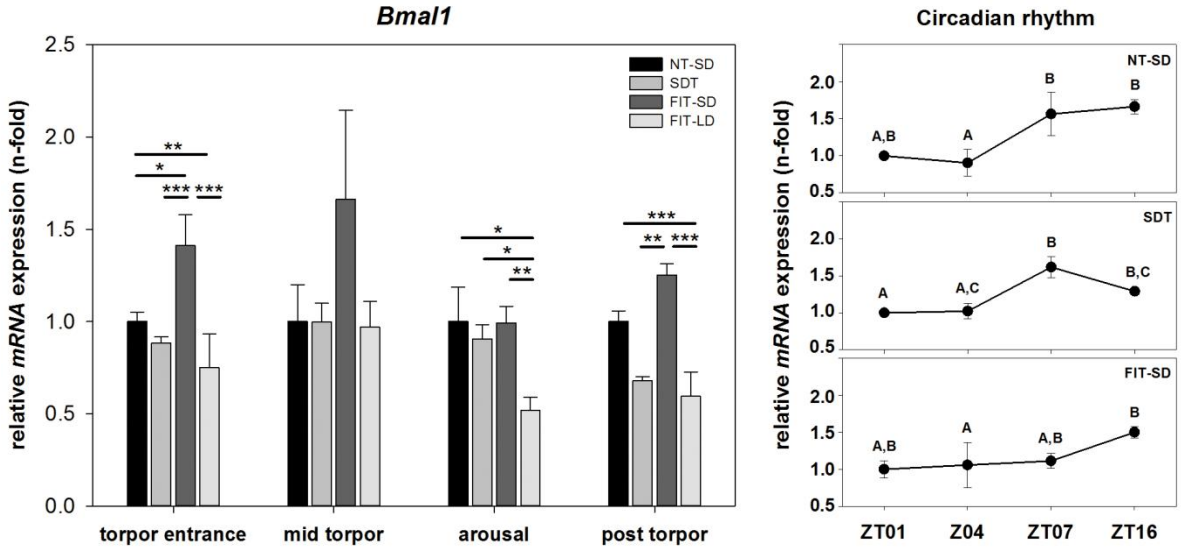

Fig 14. Relative mRNA expression of *Bmal1*. Bar graphs (with n=5 for each bar) show fold changes of mRNA expression for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) relative to NT-SD (black bars, ±SEM) for torpor entrance (ZT1), mid torpor (ZT4), arousal (ZT7) and post torpor (ZT16) respectively. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show differences in mRNA expression (±SEM) at four different time points (with n=5 for each time point) over the course of a day relative to ZT1 for hamsters remaining active (NT-SD), undergoing SDT or FIT-SD. Significant differences are marked with different upper cases (p<0.05).

There was an effect of torpor state on *Per1* mRNA expression (two-way ANOVA, p<0.001) as well as of torpor group (two-way Anova, p<0.001) showing a significant interaction between torpor state and torpor group (two-way ANOVA, p=0.002). During torpor entrance, *Per1* expression in FIT-SD showed a significantly higher value compared to SDT (FIT-SD vs. SDT: Tukey test, p=0.010) and FIT-LD (FIT-SD vs. FIT-LD: Tukey test, p=0.002). During mid torpor, *Per1* in FIT-SD was 2.35-fold up regulated relative to NT-SD (FIT-SD vs. NT-SD: Tukey test, p=0.001) and was significantly higher expressed than in SDT (FIT-SD vs. SDT: Tukey test, p<0.001) and FIT-LD (FIT-SD vs. FIT-LD: Tukey test, p<0.001). During arousal, *Per1* expression was 1.72-fold up regulated in SDT (SDT vs. NT-SD: Tukey test, p=0.012) and 1.50-fold up regulated in FIT-SD (FIT-SD vs. NT-SD: Tukey-test, p=0.039) compared to NT-SD, with a significantly lower expression value of FIT-LD compared to SDT (FIT-LD vs. SDT: Tukey test, p<0.001) and FIT-SD (FIT-LD- vs. FIT-SD: Tukey test, p<0.001). In the post torpid state, *Per1* was significantly lower expressed in FIT-LD than in FIT-SD (FIT-LD vs. FIT-SD: Tukey test, p=0.013) (Fig 15).

There was an effect of time of day or torpor state in *Per1* expression. In NT-SD *Per1* expression was slightly increased 1.32-fold at ZT4 and increased 2.67-fold at ZT7, before decreasing again to a fold change of 1.65 at ZT16 (ZT1 vs. ZT7: Tukey test, p<0.001; ZT4 vs. ZT7: Tukey test, p<0.001). In SDT *Per1* expression remained on a low expression level of 1.02-fold at ZT4, increased 5.09-fold at ZT7 and decreased again to a fold change of 1.94 at ZT16 (ZT1 vs. ZT7: Tukey test, p<0.001; ZT1 vs. ZT16: Tukey test, p<0.001; ZT4 vs. ZT7: Tukey test, p<0.001; ZT4 vs. ZT16: Tukey test, p=0.002; ZT7 vs. ZT16: Tukey test, p<0.001). In FIT-SD *Per1* expression was 2.70-fold up regulated at ZT4, increased by 3.47-fold at ZT7 before decreasing to 2.04-fold at ZT16 (ZT1 vs. ZT4: Tukey test, p=0.008; ZT1 vs. ZT7: Tukey test, p<0.001; ZT1 vs. ZT16: Tukey test, p=0.020; ZT7 vs. ZT4: Tukey test, p=0.013; ZT7 vs. ZT16: Tukey test, p=0.005) (Fig 15).

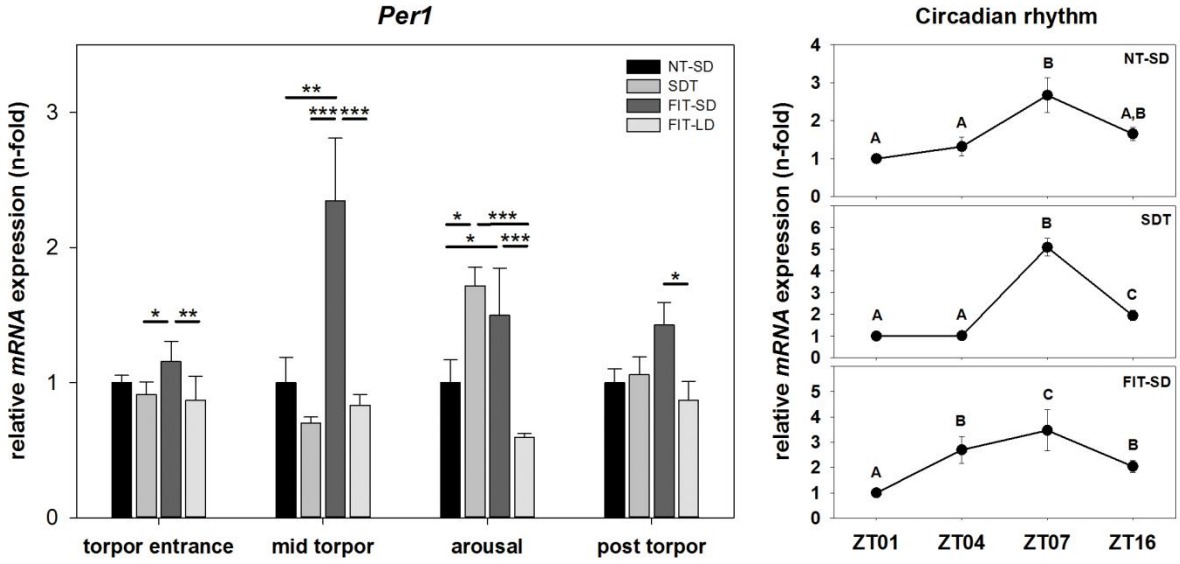

Fig 15. Relative mRNA expression of *Per1*. Bar graphs (with n=5 for each bar) show fold changes of mRNA expression for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) relative to NT-SD (black bars, ±SEM) for torpor entrance (ZT1), mid torpor (ZT4), arousal (ZT7) and post torpor (ZT16) respectively. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show differences in mRNA expression (±SEM) at four different time points (with n=5 for each time point) over the course of a day relative to ZT1 for hamsters remaining active (NT-SD), undergoing SDT or FIT-SD. Significant differences are marked with different upper cases (p<0.05).

3.4.2.3. Relative mRNA expression of *Dio2* and *Mct8* over the course of a torpor bout

There was an effect of torpor state on *Dio2* mRNA expression (two-way ANOVA, p=0.002) as well as of torpor group (two-way ANOVA, p<0.001) showing no significant interaction between torpor state and torpor group. During torpor entrance, *Dio2* mRNA expression in FIT-SD was 0.53-fold down regulated compared to NT-SD (FIT-SD vs. NT-SD: Tukey test, p<0.001) and was significantly lower

than in SDT (FIT-SD vs. SDT: Tukey test. p=0.015). FIT-LD showed a 0.77-fold down regulation compared to NT-SD (FIT-LD vs. NT-SD: Tukey test, p<0.001) and had a significantly lower expression level compared to SDT (FIT-LD vs. SDT: Tukey test, p<0.001) and FIT-SD (FIT-LD vs. FIT-SD: Tukey test, p<0.001). During mid torpor, FIT-SD was 0.49-fold down regulated compared to NT-SD (FIT-SD vs. NT-SD: Tukey test, p<0.001) and *Dio2* expression was significantly lower than in SDT (FIT-SD vs. SDT: Tukey test, p<0.001). FIT-LD was 0.76-fold down regulated compared to NT-SD (FIT-LD vs NT-SD: Tukey test, p<0.001) with reduced expression in FIT-LD compared to SDT (FIT-LD vs. SDT: Tukey test, p<0.001) and FIT-SD (FIT-LD vs. FIT-SD: Tukey test, p=0.002). During arousal, *Dio2* expression was significantly lower in FIT LD than in NT-SD (FIT-LD vs. NT-SD: Tukey test, p<0.001), SDT (FIT-LD vs. SDT: Tukey test, p<0.001) and FIT-SD (FIT-LD vs. FIT-SD: Tukey test, p<0.001) with a fold change of 0.31 relative to NT-SD. *Dio2* expression was significantly lower in FIT-SD than in SDT (FIT-SD vs. SDT: Tukey test, p=0.027). In the post torpid state, *Dio2* expression of FIT-SD was 0.36-fold down regulated relative to NT-SD (FIT-SD vs. NT-SD: Tukey test, p=0.038). Lowest expression level was shown by FIT-LD with a fold change of 0.35 (FIT-LD vs NT-SD: Tukey test, p<0.001; FIT-LD vs. SDT: Tukey test, p<0.001; FIT-LD vs. FIT-SD: Tukey test, p<0.001) (Fig 16).

There was no effect of time of day or torpor state in *Dio2* expression for NT-SD and SDT but there was an effect in *Dio2* expression for FIT-SD. During FIT-SD mRNA expression was elevated post torpor at ZT16 compared to mid torpor at ZT4 (ZT16 vs. ZT4: Tukey test, p=0.012) (Fig 16).

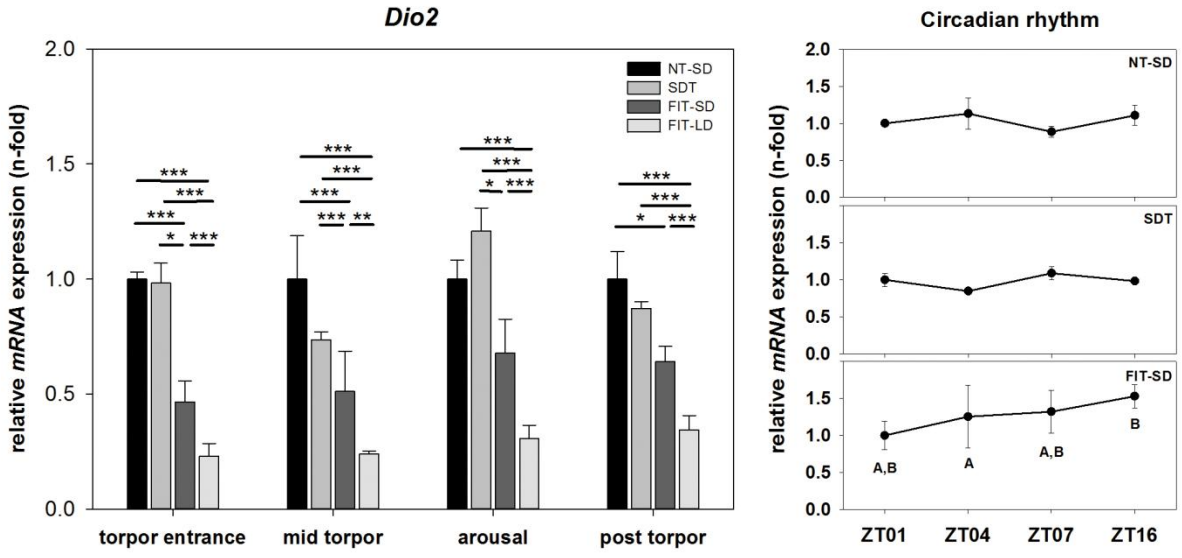

Fig 16. Relative mRNA expression of *Dio2*. Bar graphs (with n=5 for each bar) show fold changes of mRNA expression for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) relative to NT-SD (black bars, ±SEM) for torpor entrance (ZT1), mid torpor (ZT4), arousal (ZT7) and post torpor (ZT16) respectively. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show differences in mRNA expression (±SEM) at four different time points (with n=5 for each time point) over the course of a day relative to ZT1 for hamsters remaining active (NT-SD), undergoing SDT or FIT-SD. Significant differences are marked with different upper cases (p<0.05).

There was an effect of torpor state on *Mct8* mRNA expression (two-way ANOVA, $p<0.001$) as well as of torpor group (two-way ANOVA, $p<0.001$) showing a significant interaction between torpor state and torpor group (two-way ANOVA, $p<0.001$). During torpor entrance, *Mct8* mRNA expression of FIT-LD was 0.48-fold down regulated and differed significantly from NT-SD (FIT-LD vs NT-SD: Tukey test, $p<0.001$), SDT (FIT-LD vs. SDT: Tukey test, $p<0.001$) and FIT-SD (FIT-LD vs. FIT-SD: Tukey test, $p<0.001$). During mid torpor, *Mct8* expression was 0.34-fold down regulated in SDT (SDT vs. NT-SD: Tukey test, $p=0.044$). FIT-LD was 0.32-fold down regulated (FIT-LD vs. NT-SD: Tukey test, $p<0.001$) and was significantly lower expressed than in FIT-SD (FIT-LD vs. FIT-SD: Tukey test, $p=0.011$). During arousal, *Mct8* expression value was significantly lower in FIT-LD than in SDT (FIT-LD vs. SDT: Tukey test, $p=0.018$) and FIT-SD (FIT-LD vs. FIT-SD: Tukey test, $p=0.012$). In the post torpid state, *Mct8* was 0.40-fold down regulated in SDT and differed significantly from NT-SD (SDT vs. NT-SD: Tukey test, $p=0.006$) and FIT-SD (SDT vs. FIT-SD: Tukey test, $p<0.001$). *Mct8* expression in FIT-LD was 0.31-fold down regulated and differed significantly from NT-SD (FIT-LD vs. NT-SD: Tukey test, $p<0.001$) and FIT-SD (FIT-LD vs. FIT-SD: Tukey test, $p<0.001$) (Fig 17).

There was no effect of time of day or torpor state in *Mct8* mRNA expression for NT-SD, but there was an effect of torpor state in SDT and FIT-SD. In SDT *Mct8* mRNA expression during arousal at ZT7 was higher than in the post torpid state at ZT16 (ZT7 vs. ZT16, $p=0.002$). *Mct8* expression in FIT-SD was elevated in the post torpid state at ZT16 compared to torpor entrance at ZT1 and mid torpor at ZT4 (ZT1 vs. ZT16: Tukey test, $p=0.001$; ZT4 vs. ZT16: Tukey test, $p<0.001$) (Fig 17).

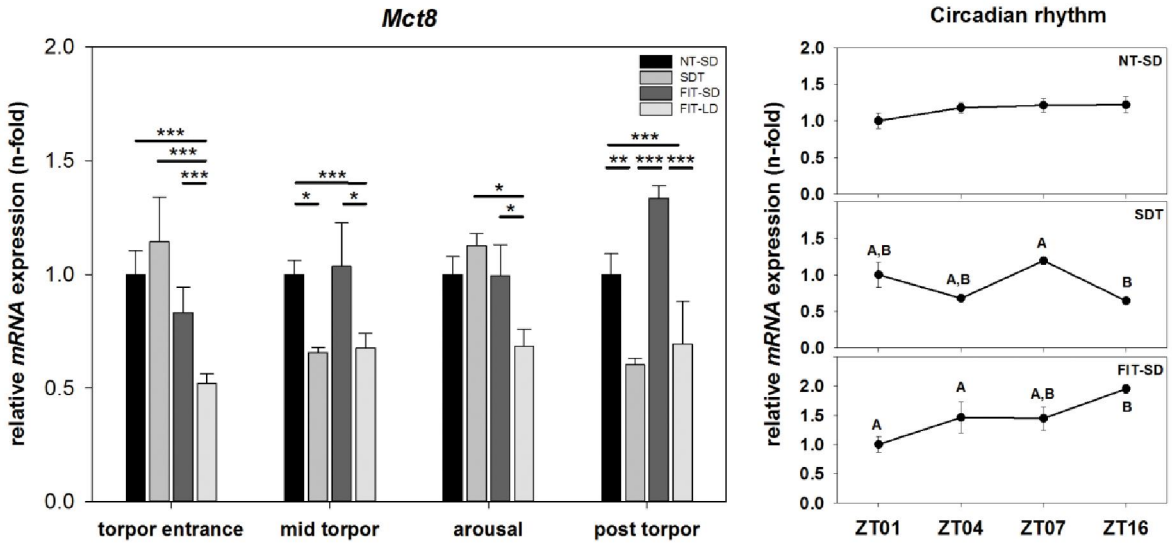

Fig 17. Relative mRNA expression of *Mct8*. Bar graphs (with n=5 for each bar) show fold changes of mRNA expression for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) relative to NT-SD (black bars, ±SEM) for torpor entrance (ZT1), mid torpor (ZT4), arousal (ZT7) and post torpor (ZT16) respectively. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show differences in mRNA expression (±SEM) at four different time points (with n=5 for each time point) over the course of a day relative to ZT1 for hamsters remaining active (NT-SD), undergoing SDT or FIT-SD. Significant differences are marked with different upper cases (p<0.05).

3.5. Discussion

In this study, we investigated differences between spontaneous daily torpor and fasting-induced torpor on the physiological and molecular levels in the Djungarian hamster. As previously shown, FIT-LD bouts differed from SDT in depth and duration. In FIT-LD bouts, the plateau phase of torpor maintenance, with almost constant low metabolic rate, is missing. This leads to shorter and shallower torpor episodes, consisting of torpor entrance directly followed by arousal. The mid torpor phase however, is mainly responsible for the energy savings accrued during torpor. Therefore single FIT bouts are less effective in saving energy than SDT bouts. The lower energy savings achieved from FIT bouts can be compensated by multiple FIT bouts per day (Geiser, 1991; Diedrich & Steinlechner, 2012; Diedrich *et al.*, 2015). In our study, no significant difference in depth or duration between FIT-SD and SDT in winter adapted animals was apparent. This is in agreement with earlier findings, showing differences in torpor depth and duration only between SDT and FIT-LD but not for SDT and FIT-SD. However, there was a higher mean torpor incidence of animals expressing FIT-SD compared to SDT (Diedrich *et al.*, 2015). Hence, winter adapted hamsters seem to increase the torpor frequency to increase energy saving, whereas summer adapted hamsters are able to use multiple torpor episodes per day to adjust energy requirements.

To dissect out differences in potential control mechanisms of FIT and SDT, we investigated differential gene expression in hypothalamic regulatory centers involved in the control of acute and seasonal energy balance over the course of a torpor bout.

Expression analysis of *Agrp* and *Npy* showed dissimilar mRNA expression patterns between SDT and FIT-SD. *Agrp* and *Npy* are up regulated when leptin and insulin actions are decreased in the hypothalamus to stimulate food intake and reduce energy expenditure (Stephens *et al.*, 1995; Shutter *et al.*, 1997; Schwartz *et al.*, 2000).

During SDT these two neuropeptides displayed no elevated mRNA expression levels in either of the investigated torpor states. This suggests that hamsters do not experience hunger before, during or after a spontaneously occurring torpor bout and indicates a balanced energy homeostasis that is still maintained while expressing SDT. Thus, no obvious energetic deficit appears to influence whether an animal enters torpor on that particular day or remains active. This is in accordance with physiological data, showing that spontaneous torpor is entered from a state of glucose metabolism (RQ ~1), hence metabolic balance (Diedrich *et al.*, 2015).

Animals undergoing FIT-SD showed significantly up regulated *Agrp* expression during mid torpor and also *Npy* expression was up regulated during this state, although not reaching significance. *Npy* expression was also up regulated during the post torpid state of fasted hamsters. The up regulated mRNA levels of *Agrp* and *Npy* during FIT-SD point towards an acute negative energy balance in FIT expressing animals. This is in accordance with the observation that hamsters using FIT already exhibit a low respiratory quotient (0.79 ± 0.01) when entering the torpid state indicating a lipid-based metabolism, hence negative energy balance (Diedrich *et al.*, 2015). Also the increased *Npy* expression at night possibly indicates a state of hunger, when fasted animals are active again. The differences in *Agrp* and *Npy* expression between SDT and FIT were smaller than expected. Since whole hypothalamus was used for gene expression analysis, it might be possible that differences appear less pronounced than in specific nucleus analysis, caused by the signal to noise ratio. However, the negative energy balance caused by reduced food supply in SD animals seems to predominantly be compensated by an elevated torpor frequency rather than a change in single torpor bout characteristics *per se*. Since we only sampled the FIT-SD group after at least two days of food restriction, to ensure FIT expression, the hamsters were already able to adjust their torpor frequency to maintain energy balance, which is possibly reflected in relatively balanced *Agrp* and *Npy* expression.

Interestingly, we were not able to detect altered expression patterns of *Agrp* or *Npy* within the FIT-LD group. This could result from the extremely long fasting period, which was required to induce torpor in the obese LD adapted animals. A comparative study investigating the effect of acute food deprivation and chronic food restriction on peptides regulating food consumption, has shown that

acute food deprivation leads to elevated *Agrp* expression in the hypothalamus of rats, whereas in chronically food restricted animals *Agrp* expression remained unaffected (Bi *et al.*, 2003). The same study found up regulation of *Npy* caused by both, acute food deprivation and chronic food restriction, however, with a significantly lower extent of up regulation in chronically food restricted rats (Bi *et al.*, 2003). In this approach, rats were fasted for 14 days to reach a chronically food restricted state, which is way shorter than the food restriction of over one month in our study. The extremely long period of food restriction could be the reason why we did not observe any alterations in *Npy* expression within the FIT-LD group. It is also possible that the long fasting period and accompanying body weight loss led to a decrease in energy requirements, so that 60 % food supply was sufficient to maintain energy homeostasis.

No circadian rhythm was found in either, *Npy* or *Agrp* expression. This is accordance with *in-situ* hybridization data of Ellis *et al*. who did not show a circadian rhythm for these genes in long or short photoperiod either (Ellis *et al.*, 2008). The difference observed for *Agrp* expression in FIT-SD between ZT1 and ZT4 reflects the torpid state rather than a circadian regulation.

Bmal1 and *Per1* are parts of the molecular circadian clockwork. *Bmal1* acts as positive regulator in the circadian feedback loop and interacts with CLOCK to activate transcription of *Per* and *Cry* genes, whereas *Per1* is considered as negative regulator, inhibiting its own transcription by building a repressor complex of the PER1 protein together with CRY, which translocate to the nucleus and inhibits the CLOCK:BMAL1 complex (Takahashi *et al.*, 2008).

Our results showed a circadian rhythm of *Bmal1* and *Per1* mRNA expression during SDT. As previously described, this indicates that the circadian clockwork is not disrupted during daily torpor and ensures the proper timing of SDT into the animal´s resting phase (Kirsch *et al.*, 1991; Ruby & Zucker, 1992; Ruf & Heldmaier, 1992; Herwig *et al.*, 2007). Some differences however, could be observed in clock gene expression between SDT and NT-SD, suggesting at least a modulatory effect of daily torpor. Compared to NT-SD, expression of *Per1* was significantly upregulated at ZT7, *Bmal1* expression already started to decrease at ZT16, whereas the expression level of *Bmal1* still remains high at ZT16 in the NT-SD group. The higher level of *Per1* combined with the earlier decrease of *Bmal1* during SDT could lead to a shortened free-running period in torpid animals that has previously been described for Djungarian hamsters undergoing torpor (Thomas *et al.*, 1993). A direct effect of SDT on the circadian clock has previously been demonstrated, indicating alterations in phase and amplitude of the circadian clock during SDT (Herwig *et al.*, 2007).

Expression of these two clock genes during FIT-SD was clearly different from SDT and NT-SD. *Per1* expression peak was advanced whereas *Bmal1* was constantly low expressed throughout the torpid state with a slight up regulation at ZT16. Hence, the circadian feedback loop appears to be shifted in fasted hamsters. Fasting in general leads to an increased SIRT1 deacetylase activity (Rodgers *et al.*,

2005; Cakir *et al.*, 2009). SIRT1 is able to deacetylate BMAL1, which in turn can inactivate the BMAL1:CLOCK complex resulting in a disruption of the normal circadian feedback loop (Hirayama *et al.*, 2007; Asher *et al.*, 2008; Nakahata *et al.*, 2008). Since it is known that the major exogenous Zeitgeber for FIT expression is the feeding schedule rather than the light-dark cycle (Ruby & Zucker, 1992; Paul *et al.*, 2004), it is likely that the circadian rhythm in FIT expressing animals was synchronized with the feeding schedule.

The FIT-LD group was not sampled at specific ZTs so that we were not able to investigate circadian rhythms in long term fasted hamsters.

Dio2 showed decreased expression throughout the torpor bout in animals undergoing FIT-SD and FIT-LD. *Dio2* was also lower during mid torpor in SDT, but did not reach significance, most likely caused by the high standard error of the NT-SD group. The decreased *Dio2* expression observed in our study suggests a reduced conversion of T4 into the active metabolite T3 and would thereby cause low T3 availability in the hypothalamus during torpor. This is in accordance with earlier studies, demonstrating that high T3 levels specifically in the hypothalamus are able to block torpor in Djungarian hamsters, whereas systemically low T3 concentrations increase torpor frequency, depth and duration (Murphy *et al.*, 2012; Bank *et al.*, 2015; Bank *et al.*, 2017a). Here, we confirm that a lowered *Dio2* expression and potential decrease in local T3 availability appears to be a permissive factor for both, SDT as well as FIT in summer and winter adapted animals. Although starvation of hamsters has previously been shown to increase *Dio2* expression of SD adapted animals, we did not observe an up regulation in food restricted hamsters. In the previous study, however, hamsters were starved for 48 hours and killed in a non-torpid state (Herwig *et al.*, 2009), whereas animals in the current study still received 60 % of their daily food consumption for more than two days. The differences in metabolic state and food supply may cause the diverging expression patterns.

Another factor which could influence the local thyroid hormone status of torpid Djungarian hamsters in hypothalamic neurons is the transporter protein *Mct8*. Our data showed decreased *Mct8* expression during mid torpor and in the post torpid state in SDT hamsters. Decreased expression of *Mct8* could support low T3 availability in the hypothalamus by reducing the thyroid hormone transport and possibly, together with the low *Dio2* mRNA level, facilitate the occurrence of torpor. This however, only seems to be true for SDT, since *Mct8* in FIT-SD animals showed no alteration relative to NT-SD during the torpor bout. Nevertheless, a number of different transporters, like organic anion transporters and L-type amino acid transporters, with the potential to transport thyroid hormones, have been identified and could affect the thyroid hormone status (Visser, 2000; Wirth *et al.*, 2014).

It has been shown that *Mct8* expression is higher in winter adapted animals compared to animals in summer state (Herwig *et al.*, 2009). This fits with our observation of the overall decreased *Mct8*

mRNA level within the FIT-LD group for all investigated torpor states, which is more likely caused by the animals' seasonal state than by torpor state.

No circadian rhythm was found in either, *Dio2* or *Mct8* mRNA expression within the NT-SD groups. The isolated differences observed for *Dio2* expression in FIT-SD and for *Mct8* in SDT and FIT-SD seem to reflect differences caused by the torpid state rather than a circadian regulation.

Taken together, our data do not clearly support the hypothesis that SDT and FIT represent distinct physiological states. Although the circadian system is differentially regulated in the different torpor forms, gene expression changes in the orexigenic system only partly reflect the physiological data. The thyroid hormone system rather appears to be regulated by torpor *per se*, irrespective of the torpor form used. Studies on single hypothalamic nuclei including examination on protein level might better disentangle the regulatory mechanisms of the two torpor forms. However, the form of torpor used should be carefully taken in consideration when investigating and interpreting the phenomenon of torpor and its underlying mechanisms.

Chapter 4

Torpor state dependent modulation of glucose, insulin and leptin in Djungarian hamsters (*P. sungorus*) during spontaneous daily torpor and fasting-induced torpor

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Unpublished

This chapter was written as a manuscript, but is unpublished. The author of this thesis was involved in the design of all experiments supported by Annika Herwig. All *in vivo* experiments were carried out by me. Gene expression as well as serum concentration analysis was performed by Hanna Markowsky and me and data were analysed as well as interpreted by myself. Manuscript was written by myself and revised by Annika Herwig.

4.1. Abstract

The physiology of Djungarian hamsters is well suited to cope with seasonal energetic challenges. The expression of spontaneous daily torpor is part of complex, well prepared adaptations to save energy during winter. Additionally, this species is able to cope with acute energetic disadvantages by using another type of torpor, the so called fasting-induced torpor. Both torpid states are characterized by a reduction of metabolic rate with decreased body temperature. The torpid state can be maintained for several hours per day and by using torpor, energy requirements can be reduced by up to 65% in times of food shortage, cold ambient temperatures and other environmental challenges. The adipose signals insulin and leptin are involved in the regulation of energy homeostasis by providing information about the energetic state to neuronal structures which control food intake and energy expenditure. They are positively correlated with blood glucose levels and involved in signaling pathways regulating thermogenesis and energy balance. Here we set out to investigate potential roles of glucose, insulin and leptin in SDT and FIT expressing hamsters. Serum concentrations were measured at four different torpor states to identify possible torpor state dependent alterations. Furthermore, we investigated hypothalamic insulin and leptin sensitivity by calculating relative mRNA expression levels of *OB-Rb*, *Socs3* and *Ptpn1*.

No clear statistically significant differences could be found in serum concentrations of glucose, leptin or insulin. However, serum glucose and insulin concentrations were slightly reduced during torpor entrance in SDT as well as FIT-LD suggesting a possible involvement in torpor initiation mechanisms. Additionally insulin and leptin concentrations were slightly decreased during mid torpor in SDT and FIT-SD expressing animals suggesting a role in torpor maintenance. Chosen candidate genes of hypothalamic insulin and leptin signaling pathways did not show torpor dependent alterations, but indicated reduced leptin sensitivity after a day with SDT as well as FIT in winter adapted hamsters.

4.2. Introduction

Many mammals, living in temperate and polar habitats, have evolved strategies to adjust their energy requirements in times of energetic challenging environmental conditions including the expression of seasonal torpor (Heldmaier *et al.*, 2004; Melvin & Andrews, 2009; Ruf & Geiser, 2015; Jastroch *et al.*, 2016).

The Djungarian hamster (*Phodopus sungorus*, also known as Siberian hamster) is well adapted to the harsh winters of Central Asian steppes. Already in autumn, when ambient temperatures are still moderate and food availability is abundant, Djungarian hamsters start to develop its winter phenotype. The onset of winter acclimatization is triggered by the decrease in day length, driving

modification of melatonin production in the pineal gland (Steinlechner & Heldmaier, 1982; Illnerova *et al.*, 1984). Winter acclimatization in this species comprises severe body weight loss (up to 40%), molt to a well-insulated white fur as well as gonadal regression. Spontaneous daily torpor (SDT) is the ultimate trait of winter acclimatization and starts to occur after ~12 weeks under short day conditions (SD), once all other adaptions (including accompanying reductions in prolactin, leptin and testosterone) are completed (Figala *et al.*, 1973; Scherbarth & Steinlechner, 2010). STD is initiated by an active depression of metabolic rate to 25% below the level of resting metabolic rate and a drop of T_b to minimum values of ~15 °C. During the torpid state, a plateau phase of almost constant low metabolic rate and decreased T_b is maintained until the animal spontaneously starts to arouse. SDT is a precisely circadian controlled phenomenon that is limited to the animals' diurnal resting phase and ends prior to the natural nocturnal active phase of *P. sungorus* with an average duration of six hours (Kirsch *et al.*, 1991; Heldmaier & Ruf, 1992; Heldmaier *et al.*, 2004). The incidence of torpor expression is highly variable between individuals and even within the same animal, allowing an individual adjustment of energy needs. By expressing SDT, hamsters are able to save up to 65% of total energy requirements when torpor is used on daily basis (Ruf *et al.*, 1991).

Besides SDT, Djungarian hamsters are able to use fasting-induced torpor (FIT) in response to prolonged times of food scarcity. This type of torpor is neither under seasonal nor under circadian control and can occur at any time of year or day (Steinlechner *et al.*, 1986; Ruby & Zucker, 1992). A body weight loss of approximate 30%, and thereby severe depletion of energy reserves, seems to be the only prerequisite for FIT (Ruby & Zucker, 1992; Ruby *et al.*, 1993). Like in SDT, FIT is initiated by the depression of metabolic rate, followed by decreased T_b, but the plateau phase during torpor maintenance is almost completely missing. Thus, FIT bouts are shorter and the drop in T_b is less pronounced than in SDT bouts, hence overall saving less energy (Diedrich & Steinlechner, 2012; Diedrich *et al.*, 2015). Determination of the respiratory quotient (RQ) during the torpor entrance phase showed that SDT is based on glucose metabolism (RQ=0.88 ± 0.02) whereas FIT is already based on lipid metabolism (RQ=0.79 ± 0.001) (Diedrich *et al.*, 2015). Taken together, SDT and FIT clearly share some main characteristics of torpor, but there are also remarkable differences between SDT and FIT. The general physiological characteristics of torpor have been well studied over the past decades but less is known about the underlying endocrine and neurological regulatory mechanisms leading to the initiation and maintenance of torpor.

The regulation of energy homeostasis relies on peripheral signals, mainly from the gastrointestinal tract and adipose tissue. The adipose signals leptin and insulin provide feedback to neuronal mechanisms to control food intake and energy expenditure. Leptin and insulin secretion is positively correlated with the predominant amount of blood glucose and concentrations of plasma leptin and insulin increase in proportion to the amount of stored fat (Bagdade *et al.*, 1967; Considine *et al.*,

1996; Mizuno *et al.*, 1996; Schwartz *et al.*, 1996; Mueller *et al.*, 1998). These adipose signals attain the brain via the blood stream where they increase leptin and insulin sensitivity by binding to the leptin receptor (OB-Rb) and the insulin receptor. These receptors are mainly expressed in the nucleus arcuatus (ARC) of the hypothalamus (Schwartz *et al.*, 2000; Niswender *et al.*, 2003). Just little is known about how insulin and leptin pass the blood-brain barrier, but movement from the blood stream to the hypothalamus appears to involve a saturable transport system (Baura *et al.*, 1993; Prigeon *et al.*, 1996).

It has been shown that low circulating leptin concentrations are a permissive factor for SDT in winter adapted animals and that exogenous leptin treatment is able to inhibit torpor in most, but not all winter adapted hamsters (Freeman *et al.*, 2004). Djungarian hamsters are leptin resistant during spring and summer and become leptin sensitive in autumn and winter, when they voluntarily start to reduce their body weight (Klingenspor *et al.*, 2000; Rousseau *et al.*, 2002). Here, the control of leptin sensitivity seems to be regulated by the expression of suppressor of cytokine signaling 3 (*Socs3*), a leptin signaling inhibitor, and by the expression of *OB-Rb* (Mercer *et al.*, 2000; Mercer *et al.*, 2001; Tups *et al.*, 2004). Also protein level of PTPN1, which is the main inhibitor of insulin signaling, is down regulated within the ARC in SD animals, suggesting elevated insulin sensitivity in winter hamsters (Tups *et al.*, 2006).

Alterations in insulin and leptin concentrations are able to influence energy expenditure by regulating thermogenesis (Rothwell & Stock, 1981; Haynes *et al.*, 1999; Roh *et al.*, 2016). Also changes of circulating glucose concentration itself can be sensed by the central nervous system, most likely by glucosensitive tanycytes which are located in the ependymal layer of the third ventricle, and glucose seems to be involved in the local regulation of energy balance (Frayling *et al.*, 2011; Roh *et al.*, 2016). It has been shown that glucoprivation is able to induce a torpor-like state in *P. sungorus*. Low glucose availability might cause the inhibition of thermogenesis and thereby lead to the torpor-like state (Dark *et al.*, 1994). Taken together, the available data suggest that glucose, insulin and leptin are involved in torpor regulation.

However, very little is known about glucose, insulin and leptin signaling in the brain of torpid animals. Here we set out to survey potential roles of these three in the regulation of torpor in Djungarian hamsters by investigating circulating glucose, insulin and leptin levels as well as hypothalamic mRNA expression profiles of *OB-Rb*, *Socs3* and *Ptpn1* at four different torpor states in SDT and FIT expressing animals.

4.3. Material and Methods

4.3.1. Animals and housing

Djungarian hamsters (*Phodopus sungorus*) were bred from our own colony at the Zoological Institute, University of Hamburg, and raised under artificial long photoperiod (LD, 16:8-h light:dark cycle) at constant ambient temperature of 21 °C ± 1 °C. Hamsters were weaned at an age of three weeks and individually housed in plastic cages (Macrolon Type III) with free access to drinking water throughout the experiment and fed a hamster breeding diet (Altromin 7014, Germany) *ad libitum* before experiment started. At an age of three to five months a cohort of 70 hamsters of both sexes was transferred to an artificial short photoperiod (SD, 8:16-h light:dark cycle) at constant ambient temperature of 18 °C ± 1 °C (representing the winter phenotype) and a cohort of 20 hamsters remained under LD conditions (representing the summer phenotype).

All experiments and procedures were conducted in accordance with the German Animal Welfare Law and approved by the local animal welfare authorities (No. 4_16 and No. 114_14, Hamburg, Germany).

4.3.2. Experimental setup and sampling

Three SD-groups were sampled during spontaneous daily torpor (SDT), fasting-induced torpor under SD (FIT-SD) and in a non-torpid state (NT-SD). Each group contained 20 hamsters of both sexes with five animals per investigated torpor state. Additionally, one LD-group, with 20 hamsters of both sexes and five animals per torpor state, was sampled during fasting-induced torpor under LD (FIT-LD) and one *ad libitum* fed non-torpid LD-group (NT-LD) at midday (ZT8, n=5). For the SD-groups we only selected hamsters, which clearly showed winter adaptions in body weight and fur color after ten weeks of SD exposure. All hamsters were implanted with DSI-transmitters (Model TA-F10, St. Paul, MN, USA) to record their body temperature in three minute intervals. DSI-transmitters were implanted i.p. under isoflurane anesthesia (2.0 – 2.5 %, Forene, Abott, Wiesbaden, Germany). Analgesia was maintained via s.c. injection of carprofen (5 mg/kg, Paracarp, IDT Biologika, Germany). Surgeries were performed as described in Bank *et al*. (Bank *et al.*, 2015).

The SD-groups were sampled according to torpor state and Zeitgeber time (ZT, ZT0=lights on) during torpor entrance at ZT1; mid torpor at ZT4, arousal at ZT7 and post torpor at ZT16. Since FIT is not under circadian control, the LD group was sampled according to torpor state only, but also during torpor entrance, mid torpor, arousal and post torpor.

The experimental groups were fed and/or fasted and sampled as previously described in Cubuk *et al*. (Cubuk *et al.*, 2017b).

All hamsters were sacrificed by CO_2 inhalation and final decapitation. Blood for serum analysis was sampled by cardiac puncture. Serum was extracted by centrifugation of blood samples for 15 min at 4 °C and 3000 rpm. Brains were dissected and immediately frozen on dry ice. Serum and brain samples were stored at -80 °C until further use.

4.3.3. Serum measurements of glucose, insulin and leptin

Serum glucose was measured using the Accu-Chek Aviva system (Roche, Mannheim, Germany) with a measure range of 10 – 600 mg/dl. 2 µl serum per test stripe was applied for each sample. Serum insulin was assessed by the Ultra Sensitive Mouse Insulin ELISA Kit (Chrystal Chem, Downers Grove, IL, USA) with a sensitivity of 100 pg/ml using 5 µl sample and an intra-assay and inter-assay precision of CV ≤ 10.0 %. Serum leptin was determined by using a Mouse Leptin ELISA Kit (Chrystal Chem, Downers Grove, IL, USA) with a sensitivity of 200 pg/ml using 5 µl sample and an intra-assay and inter-assay precision of CV ≤ 10.0 %. Elisa measurements were done according to manufacturer`s instructions. Differences in glucose, insulin and leptin concentrations were statistically tested by one-way ANOVA followed by Tukey test using the factor torpor group between SDT, FIT-SD, FIT-LD and NT-SD within the four investigated torpor states or the factor time of day at ZT1, ZT4, ZT7 and ZT16. P-values ≤ 0.05 were considered as significant. All statistics and figures were done with SigmaPlot 12.5 (Systat Software Inc.).

4.3.4. Total RNA isolation and quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from hypothalamus samples, which were cut from frozen brain tissues between Bregma -0.20 mm and -2.70 mm, dorsally 3 – 4 mm from the ventral surface and laterally at the hypothalamic sulci. Total RNA isolation was performed using peqGOLD Trifast™ (Peqlab, Erlangen, Germany) and isolated RNA was purified using the Crystal RNA MiniKit (Biolabproducts, Bebensee, Germany) according to the manufacturer's instructions. An on-column digestion with RNase-free DNase (Quiagen, Hilden, Germany) was included. RNA quantity and quality were verified by spectrophotometry and gel electrophoresis. 1 µg total RNA per sample was used as template for cDNA synthesis. cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) and oligo-(dT)18 oligonucleotide primers according to the manufacturer's instructions.

Gene specific primers for hypoxanthine phosphoribosyltransferase (*Hprt*), ribosomal protein lateral stalk subunit P0 (*Rplp0*), protein tyrosine phosphatase, non-receptor type 1 (*Ptpn1*) and suppressor of cytokine signaling 3 (*Socs3*) were designed on *P. sungorus* specific sequences obtained from our

previous Illumina study (Cubuk *et al.*, 2017a). Primers for leptin receptor isoform b (*OB-Rb*) were designed in conserved coding sequence regions based on sequence alignments from *Rattus norvegicus* (Accession no. U52966.1) and *Mus musculus* (Accession no. AF098792.1) (Table 7). Melting temperature and dimer formation of primers was controlled by OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer).

RT-qPCR was carried out on an ABI Prism 7300 Real Time PCR System (Applied Biosystems, Darmstadt, Germany) with Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) using a 40 amplification cycles protocol (50 °C 2 min; 95 °C 10 min; 95 °C 15 s; 60 °C 15 s; 72 °C 30 s). *Hprt* was used as reference gene for NT-SD and SDT and *Rplp0* as reference gene for FIT-SD and FIT-LD. A non-torpid sample was applied to each 96-well plate as inter-plate calibrator. PCR efficiency was calculated by standard curves, generated by a series of six 10-fold dilutions of target gene specific standard plasmids. For standard plasmid generation 100 – 200 bp long coding sequence fragments of *Hprt*, *Rplp0*, *Ptpn1*, *Socs3* and *OB-Rb* were cloned into the pGEM®-T Easy Vector System (Promega, Madison, USA) according to manufacturer`s instructions and sequenced by the commercial sequencing platform GATC Biotech (Konstanz, Germany). Validation of RT-qPCR amplification specificity was determined by dissociation curve analysis in each RT-qPCR run. Relative expression levels of investigated genes were estimated using the ΔΔCT method and relative differences in gene expression were calculated within each torpor stage relative to the respective NT-SD group, as well as over the course of a day relative to the corresponding ZT1 group. Since FIT-LD was not sampled according to specific ZTs, this group was excluded from circadian rhythmicity analysis. Additionally, relative differences between NT-SD at ZT4 and NT-LD at ZT8 were calculated. Statistical testing and figures were done with SigmaPlot 12.5 (Systat Software Inc.). Differences in gene expression values were statistically tested by one-way ANOVA with the factor torpor group (NT-SD, SDT, FIT-SD, FIT-LD) or with the factor time of day (ZT1, ZT4, ZT4, ZT16) followed by Tukey test for pairwise comparison. P-values ≤ 0.05 were considered as significant.

Table 7. *Phodopus sungorus* specific primer sequences used for RT-qPCR and standard plasmid generation.

gene		5´3´sequence	melting temperature	amplicon length
Ptpn1	forward	CAG TGC GAC AGT TGG AGT TGG A	59.6	155 bp
	reverse	GCT GAG TGA GCC TGA CTC TCG	59.5	
Socs3	forward	CTG AGC GTC AAG ACC CAG TCA G	60.0	130 bp
	reverse	GCA CCA GCT TGA GTA CAC AGT CG	59.6	
OB-Rb	forward	CAA GTG CAT CGC CAG TGA GC	59.3	162 bp
	reverse	CCC TCC AGT TCC AAA AGC TCA TCC	59.8	
Hprt	forward	AGT CCC AGC GTC GTG ATT AGT GAT G	60.4	140 bp
	reverse	CGA GCA AGT CTT TCA GTC CTG TCC A	60.5	
Rplp0	forward	GCA ACA GTC GGG TAA CCA ATC TGC	60.4	153 bp
	reverse	CTT CGG GCT CAT CAT CCA GCA G	60.1	

4.4. Results

4.4.1. Glucose, insulin and leptin concentrations in fasting-induced and spontaneous daily torpor

There was an effect of torpor group on glucose level during the post torpid state only (one-way ANOVA, p=0.035). During torpor entrance, mid torpor and arousal there were no significant differences in serum glucose level between NT-SD, SDT, FIT-SD and FIT-LD. However, during torpor entrance there was a trend towards lowered glucose concentration in SDT (186.2 mg/dl ± 12.9 mg/dl) compared to NT-SD (252.8 mg/dl ± 20.5 mg/dl) (NT-SD vs. SDT: Tukey test, p=0.099). During the post torpid state, glucose concentration was significantly lower in FIT-SD (183.8 mg/dl ± 25.8 mg/dl) compared to FIT-LD (298.2 mg/dl ± 19.4 mg/dl) (FIT-SD vs. FIT-LD: Tukey test, p=0.021). There were no circadian changes of serum glucose concentrations in NT-SD, SDT and FIT-SD (Fig 18)

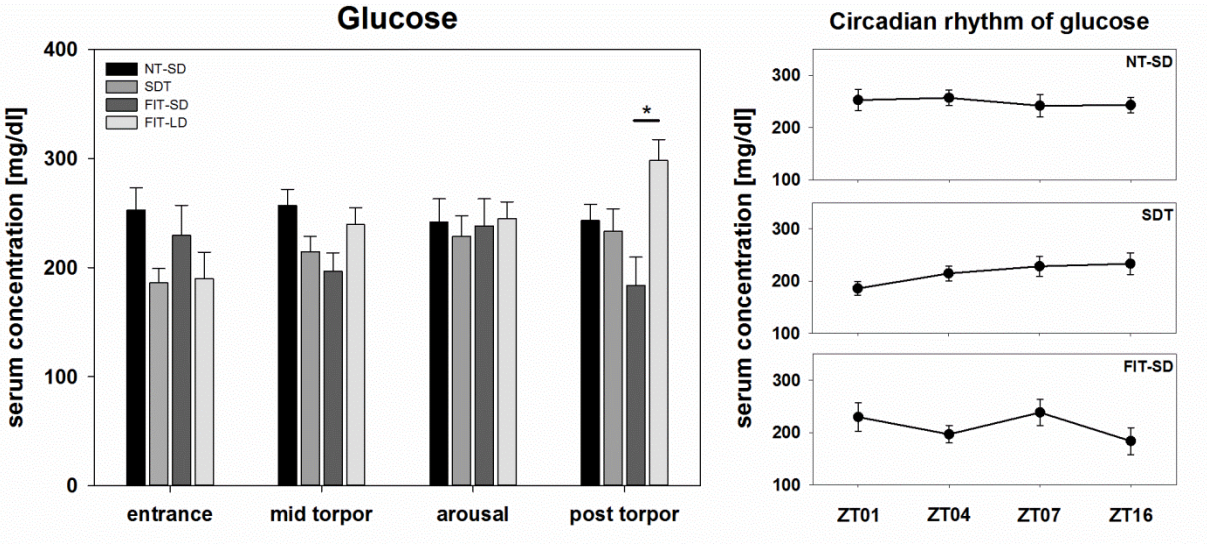

Fig 18. Serum glucose concentration. Bar graphs show glucose concentrations for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) during torpor entrance at ZT1, mid torpor at ZT4, arousal at ZT7 and post torpor at ZT16 and for hamsters remaining NT-SD (black bars, ±SEM) at ZT1, ZT4, ZT7 and ZT16 as respective control group. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show glucose concentrations (±SEM) over the course of a day at ZT1, ZT4, ZT7 and ZT16 for NT-SD, SDT and FIT-SD. Significant differences are marked with different upper cases (p<0.05).

There were no significant differences in serum leptin (Fig 19) or insulin (Fig 20) concentrations, neither between NT-SD, SDT, FIT-SD and FIT-LD within the investigated torpor states nor over the course of a day within NT-SD, SDT or FIT-SD.

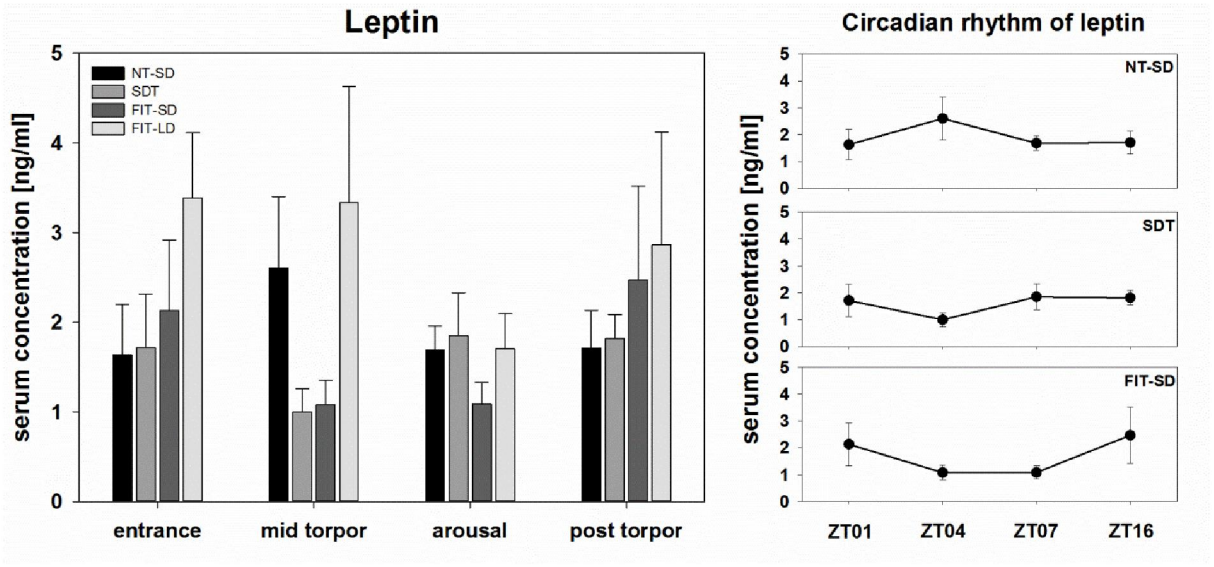

Fig 19. Serum leptin concentration. Bar graphs show leptin concentrations for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) during torpor entrance at ZT1, mid torpor at ZT4, arousal at ZT7 and post torpor at ZT16 and for hamsters remaining NT-SD (black bars, ±SEM) at ZT1, ZT4, ZT7 and ZT16 as respective control group. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show leptin concentrations (±SEM) over the course of a day at ZT1, ZT4, ZT7 and ZT16 for NT-SD, SDT and FIT-SD. Significant differences are marked with different upper cases (p<0.05).

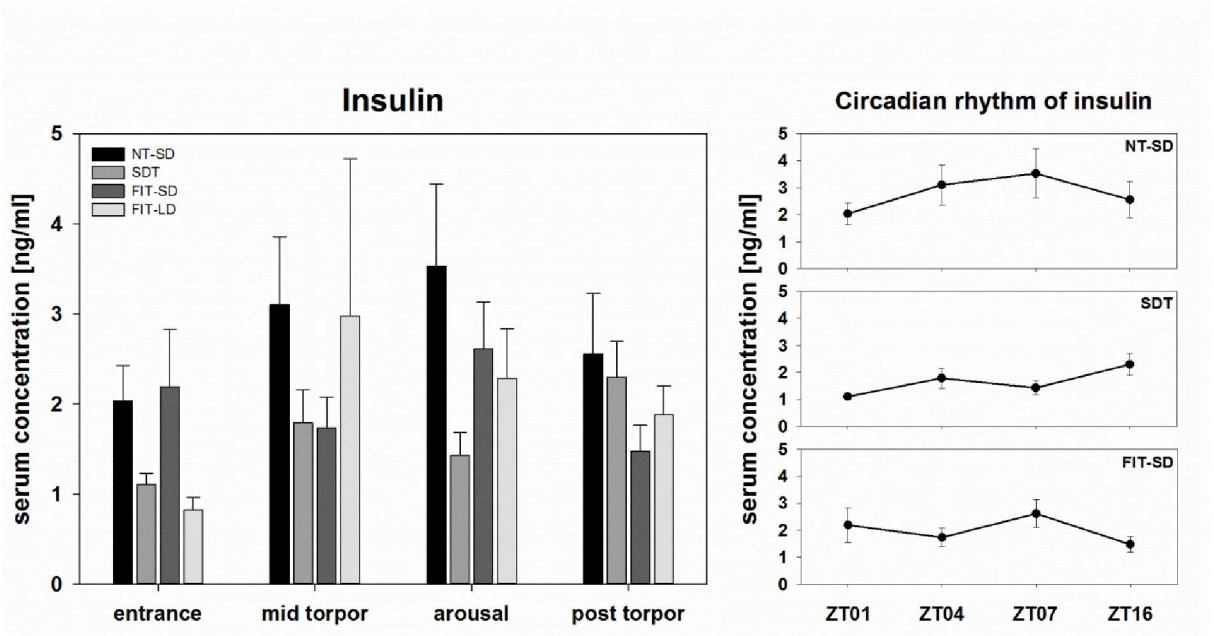

Fig 20. Serum insulin concentration. Bar graphs show insulin concentrations for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) during torpor entrance at ZT1, mid torpor at ZT4, arousal at ZT7 and post torpor at ZT16 and for hamsters remaining NT-SD (black bars, ±SEM) at ZT1, ZT4, ZT7 and ZT16 as respective control group. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show insulin concentration (±SEM) over the course of a day at ZT1, ZT4, ZT7 and ZT16 for NT-SD, SDT and FIT-SD. Significant differences are marked with different upper cases (p<0.05).

4.4.2. Hypothalamic gene expression of genes involved in insulin and leptin signaling

Seasonal dependent comparison of *OB-Rb*, *Socs3* and *Ptpn1* mRNA expression showed no significant difference in *OB-Rb* and *Ptpn1* mRNA expression between winter adapted non-torpid animals and summer adapted non-torpid animals (Fig 21 A+B). But there was a significant difference in *Socs3* mRNA expression between winter adapted non-torpid animals and summer adapted non-torpid animals with a 2.39-fold higher *Socs3* expression in summer adapted non-torpid animals (t-test, p=0.008) (Fig 21C).

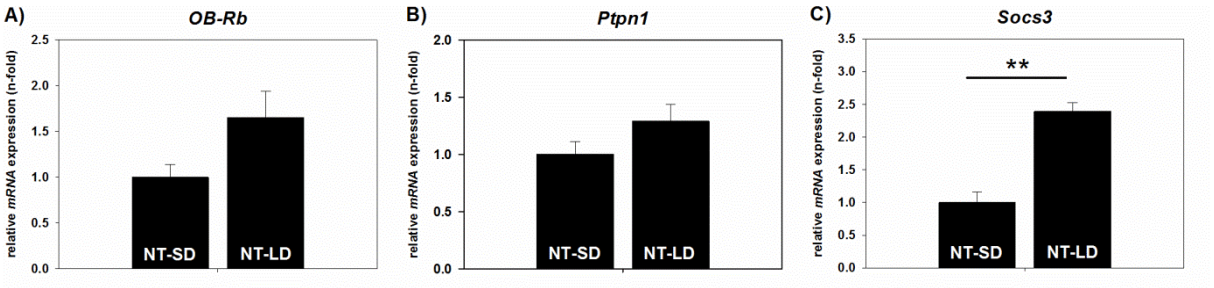

Fig 21. Relative mRNA expression of *OB-Rb* (A), *Ptpn1* (B) and *Socs3* (C) in non-torpid summer (NT-LD) and non-torpid winter (NT-SD) adapted hamsters. Fold changes of mRNA expression are shown for non-torpid hamsters under LD (ZT8) relative to non-torpid hamsters under SD (ZT4) at midday. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001.

There was an effect on *OB-Rb* mRNA expression on torpor group during torpor entrance (one-way ANOVA, p=0.001), mid torpor (one-way ANOVA, p=0.006) and post torpor (one-way ANOVA, p<0.001), but there was no effect during arousal (Fig 22). During torpor entrance, *OB-Rb* mRNA expression was 0.32-fold down regulated in FIT-LD relative to NT-SD at ZT1 (FIT-LD vs. NT-SD: Tukey test, p=0.003) and showed a lower expression value than SDT (FIT-LD vs. SDT: Tukey test, p=0.002). During mid torpor, *OB-Rb* mRNA expression in FIT-LD was 0.32-fold down regulated relative to NT-SD (FIT-LD vs. NT-SD: Tukey test, p=0.008), showed a significantly lower expression level than in SDT (FIT-LD vs. SDT: Tukey test, p=0.015) and a trend towards lowered mRNA expression compared to FIT-SD (FIT-LD vs. FIT-SD: Tukey test, p=0.061). In the post torpid state, *OB-Rb* was 0.52-fold down regulated in SDT and showed significantly lower expression than NT-SD (SDT vs. NT-SD: Tukey test, p=0.002), FIT-SD (SDT vs. FIT-SD: Tukey test, p<0.001) as well as FIT-LD (SDT vs. FIT-LD: Tukey test, p=0.001).

There was no effect of time of day on *OB-Rb* expression in NT-SD, but there was an effect in SDT (one-way ANOVA, p=0.001) and FIT-SD (one-way ANOVA, p=.0.13) (Fig 22). In SDT, *OB-Rb* mRNA expression was highest at ZT1 and decreased gradually over the course of a day until the nadir was reached at ZT16 with a fold change of 0.37 (ZT16 vs. ZT1: Tukey test, p=0.002; ZT16 vs. ZT4: Tukey

test, p=0.008). In FIT-SD, expression of *OB-Rb* was low at ZT1, increased 1.65-fold at ZT4, decreased again to 1.03-fold at ZT7 and showed highest expression value at ZT16 with a 1.91-fold up regulation in mRNA expression (ZT1 vs. ZT16: Tukey test, p=0.031; ZT7 vs. ZT16: Tukey test, p=0.016).

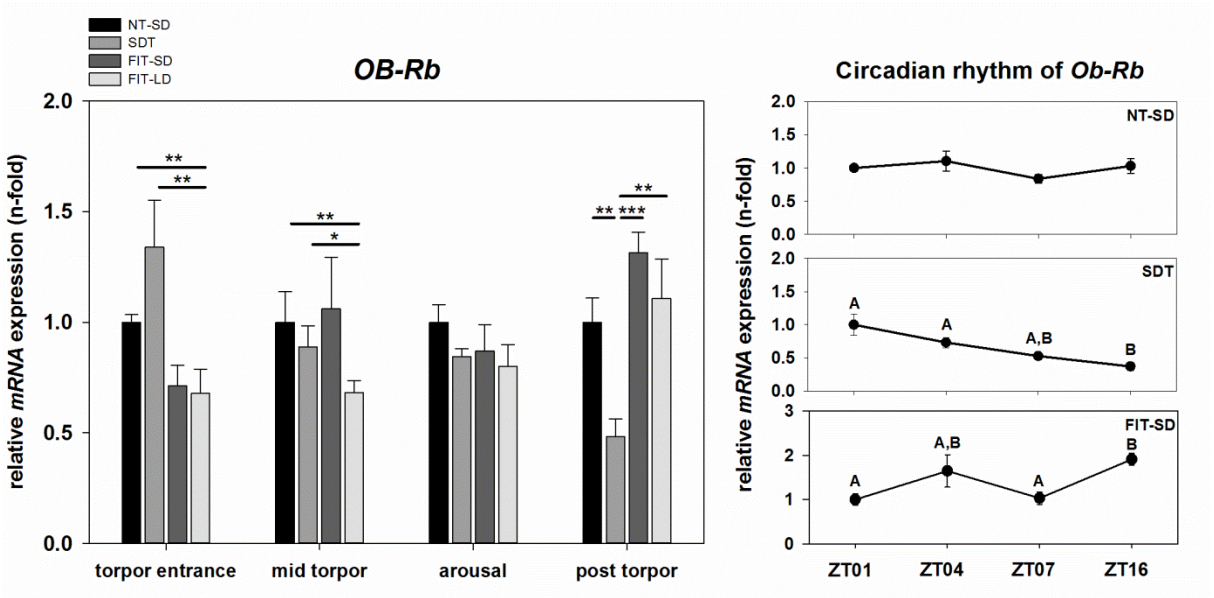

Fig 22. Relative mRNA expression of *OB-Rb*. Bar graphs show fold changes of mRNA expression for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) relative to NT-SD (black bars, ±SEM) for torpor entrance (ZT1), mid torpor (ZT4), arousal (ZT7) and post torpor (ZT16) respectively. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show differences in mRNA expression (±SEM) at four different time points over the course of a day relative to ZT1 for hamsters remaining NT-SD, undergoing SDT and FIT-SD. Significant differences are marked with different upper cases (p<0.05).

There was an effect on *Socs3* mRNA expression on torpor group during torpor entrance (one-way ANOVA, p=0.015), arousal (one-way ANOVA, p=0.002) and post torpor (one-way ANOVA, p<0.001), but not during mid torpor (Fig 23). During torpor entrance, *Socs3* expression showed a trend towards 2.06-fold up regulation in FIT-LD relative to NT-SD (FIT-LD vs. NT-SD: Tukey test, p=0.071) and higher expression than SDT (FIT-LD vs. SDT: Tukey test, p=0.010). During arousal, *Socs3* expression was 2.06-fold up regulated in FIT-LD relative to NT-SD (FIT-LD vs. NT-SD: Tukey test, p=0.001). In the post torpid state, *Socs3* was 2.67-fold up regulated in FIT-SD and 2.19-fold up regulated in FIT-LD relative to NT-SD (FIT-SD vs. NT-SD: Tukey test, p<0.001; FIT-LD vs. NT-SD: Tukey test, p<0.001; FIT-SD vs. FIT-LD: t-test, p=0.026), with higher expression in both, FIT-SD and FIT-LD, as compared to SDT (FIT-SD vs. SDT: Tukey test, p<0.001; FIT-LD vs. SDT: Tukey test, p<0.001) and highest expression in FIT-SD (FIT-SD vs. FIT-LD: Tukey test, p=0.044).

There was an effect of time of day on *Socs3* mRNA expression in SDT (one-way ANOVA, p<0.001) as well as FIT-SD (one-way ANOVA, p p<0.001) (Fig 23). In SDT, expression value of Socs3 was low at ZT1 and ZT4, increased 1.62-fold at ZT7 and decreased again at ZT16 to a fold change of 0.95 (ZT1 vs. ZT7:

Tukey test, p<0.001; ZT4 vs. ZT7: Tukey test, p=0.013; ZT16 vs. ZT7: Tukey test, p=0.005). In FIT-SD, *Socs3* mRNA expression was low at ZT1 and increased over the course of a day until highest expression value was reached at ZT16 (ZT1 vs. ZT16: Tukey test, p=0.001; ZT4 vs. ZT16, Tukey test, p=0.003).

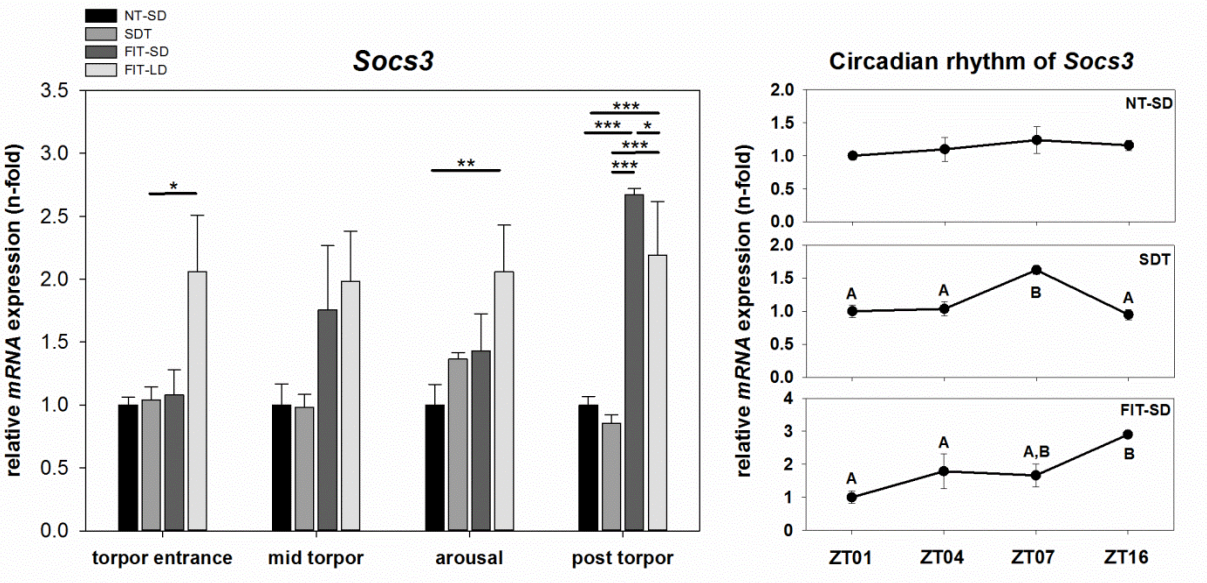

Fig 23. Relative mRNA expression of *Socs3*. Bar graphs show fold changes of mRNA expression for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) relative to NT-SD (black bars, ±SEM) for torpor entrance (ZT1), mid torpor (ZT4), arousal (ZT7) and post torpor (ZT16) respectively. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show differences in mRNA expression (±SEM) at four different time points over the course of a day relative to ZT1 for hamsters remaining NT-SD, undergoing SDT and FIT-SD. Significant differences are marked with different upper cases (p<0.05).

There was an effect on *Ptpn1* mRNA expression of torpor group during torpor entrance (one-way ANOVA, p<0.001) and mid torpor (Kruskal-Wallis test, p=0.003), but not during arousal or in the post torpid state (Fig 24). During torpor entrance, *Ptpn1* mRNA expression was 0.47-fold down regulated in FIT-SD and 0.23-fold down regulated in FIT-LD relative to NT-SD (FIT-SD vs. NT-SD: Tukey test, p=0.002; FIT-LD vs. NT-SD: Tukey test, p=0.004). Furthermore, *Ptpn1* was significantly lower expressed in FIT-SD and in FIT-LD as compared to SDT (FIT-SD vs. SDT: Tukey test, p=0.024; FIT-LD vs. SDT: Tukey test, p=0.048). During mid torpor, *Ptpn1* in SDT was significantly higher expressed than in FIT-SD and FIT-LD (SDT vs. FIT-SD: Tukey test, p<0.05; SDT vs. FIT-LD: Tukey test, p<0.05).

There was an effect of time of day on *Ptpn1* mRNA expression in NT-SD (one-way ANOVA, p=0.023), SDT (one-way ANOVA, p<0.001) and FIT-SD (one-way ANOVA, p=0.005) (Fig 24). In NT-SD, expression of *Ptpn1* was unaltered at ZT1, ZT4 and ZT7 but slightly decreased at ZT16 (ZT1 vs. ZT16: Tukey test, p=0.014). In SDT, *Ptpn1* mRNA expression was unaltered at ZT1 and ZT4, decreased at ZT7 and remained down regulated at ZT16 (ZT1 vs. ZT7: Tukey test, p=0.007; ZT4 vs. ZT7: Tukey test, p<0.001;

ZT4 vs. ZT16: Tukey test, p<0.001). In FIT-SD, *Ptpn1* mRNA expression was unaltered at ZT1 and ZT4, started to increase at ZT7 and was highest at ZT16 (ZT1 vs. ZT7: Tukey test, p=0.045; ZT1 vs. ZT16, p=0.004).

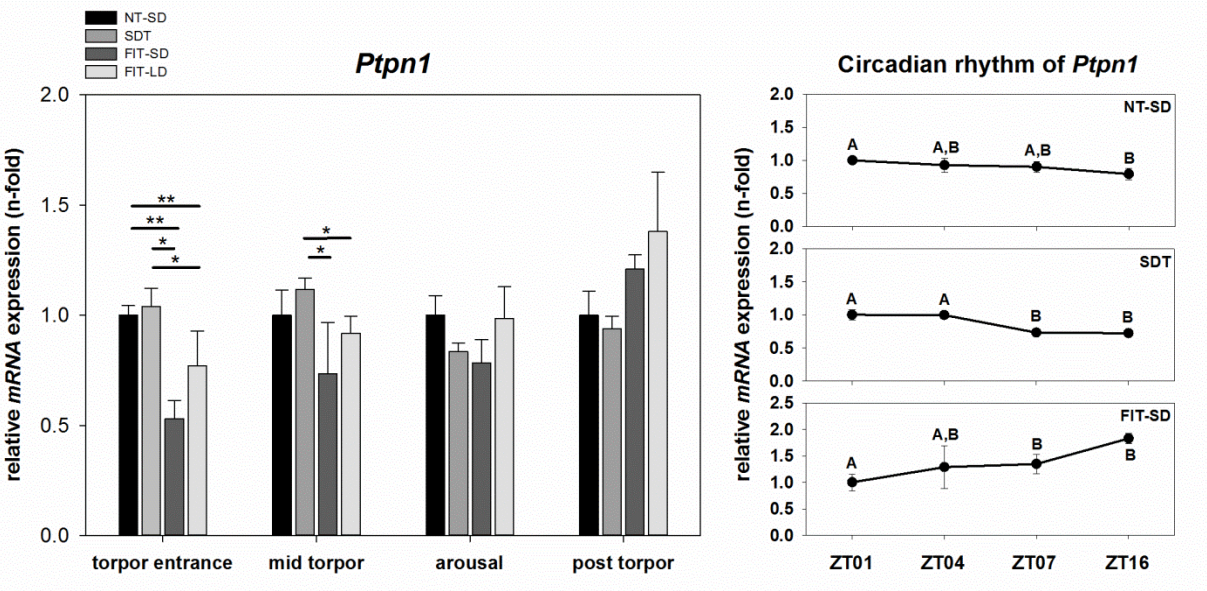

Fig 24. Relative mRNA expression of *Ptpn1*. Bar graphs show fold changes of mRNA expression for hamsters undergoing SDT (mid grey bars, ±SEM), FIT-SD (dark grey bars, ±SEM) and FIT-LD (light grey bars, ±SEM) relative to NT-SD (black bars, ±SEM) for torpor entrance (ZT1), mid torpor (ZT4), arousal (ZT7) and post torpor (ZT16) respectively. Significant differences within each torpor state are marked with *=p<0.05, **=p<0.01 and ***=p<0.001. Line graphs show differences in mRNA expression (±SEM) at four different time points over the course of a day relative to ZT1 for hamsters remaining NT-SD, undergoing SDT and FIT-SD. Significant differences are marked with different upper cases (p<0.05).

4.5. Discussion

In this study we assessed a possible role of glucose and the adipose signals insulin and leptin in the regulation of SDT and FIT in Djungarian hamsters by investigating peripheral circulating levels as well as central hypothalamic gene expression pattern of components of the insulin and leptin signaling pathways.

Serum analysis of glucose, insulin and leptin showed neither significant alterations in serum concentrations between the investigated torpor groups nor within these torpor groups over the course of a day. The only exception was a lowered glucose level during the post torpid state in FIT-SD compared to FIT-LD, suggesting that the SD group was more challenged by food restriction than the LD group. This is not surprising, since winter adapted hamsters show a season dependent voluntary reduction in body weight (Steinlechner *et al.*, 1983). The energetic state of these animals is affected by two factors, the overall reduced energy reserves due to the short photoperiod plus the forced

weight loss by lowered food availability. Thus, in FIT-SD the combination of reduced energy reserves and food restriction might result in a lower glucose level in comparison to the FIT-LD group which was challenged by food restriction only. Nevertheless, in our study the average body weight reduction of FIT-SD hamsters (17.5% ±2.4) did not differ significantly from those of FIT-LD hamsters (19.3% ±1.9).

The fact that we were not able to detect significant alterations in glucose, insulin and leptin levels is most likely caused by the high standard error of the means. However, glucose concentrations were slightly reduced during torpor entrance in SDT. This is in line with earlier findings, showing a lowered glucose level during torpor entrance when minimum metabolic rate is reached and during mid torpor in *P. sungorus*. It has been suggested that the lowered glucose level is caused by the glucose based metabolism which has been shown to be used during torpor entrance in *P. sungoruns* (Heldmaier *et al.*, 1999). This however, seems to be unlikely since glucose concentrations during torpor entrance in FIT-LD also appeared to be reduced. It has been shown that during torpor entrance in FIT-LD the metabolism is already based on lipid utilization (Diedrich *et al.*, 2015). Thus, the drop in blood glucose shown for both torpor forms cannot be explained by the kind of fuel utilization. Instead, it might be possible that lowered glucose availability during torpor entrance causes the inhibition of thermogenesis and thereby initiates the drop in T_b and reduces energy expenditure during entrance into the torpid state. In fact, it has been demonstrated that glucoprivation by the administration of a glucose oxidation disrupting glucose analogue is able to cause reduced T_bs resulting in a torpor-like state in Djungarian hamsters (Dark *et al.*, 1994). The trend of decreased glucose levels in SDT and FIT-LD during torpor entrance supports the hypothesis that glucose availability might play a role in torpor initiation. Nevertheless, a distinct verification of reduced glucose availability during torpor initiation is needed. In addition to the reduced serum glucose, insulin was slightly decreased during torpor entrance in SDT and FIT-LD. Furthermore, insulin as well as leptin concentrations were insignificantly decreased during mid torpor in SDT and FIT-SD. Leptin and insulin are able to influence energy expenditure by regulating thermogenesis. Central administration of leptin and high doses of insulin stimulate BAT activity which in turn causes elevated energy expenditure (Rothwell & Stock, 1981; Haynes *et al.*, 1999). Thus, decreased levels during mid torpor could contribute to the maintenance of the plateau phase of torpor with almost constant low T_b and metabolic rate by inhibiting BAT activity. This effect does not seem to play a role for FIT-LD where insulin and leptin concentrations remained comparable with those of NT-SD. This is not surprising since the plateau phase of torpor maintenance is almost completely missing during FIT in summer adapted animals (Diedrich *et al.*, 2015). FIT-LD episodes mainly consist of torpor entrance directly followed by arousal. Thus, low T_b is not maintained over a longer period. Paradoxically, insulin was still decreased during arousal in SDT, whereas leptin levels are already elevated in this state. It might be possible that the elevated leptin

concentration is sufficient to countermand the inhibiting effect on thermogenesis. It is assumed that leptin generally has a more important role in the hypothalamic control of energy balance than insulin. This has been shown by studies in mice, where leptin deficiency leads to obesity with severely elevated food consumption despite high insulin levels. In contrast, insulin deficiency alone is not sufficient to induce obesity (Schwartz *et al.*, 2000). This suggests a minor role of insulin in the regulation of long term energy homeostasis.
As already mentioned for glucose, also reliable verification of alterations in insulin and leptin is still needed, since we were not able to detect statistical significance of observed differences. *In vivo* real time measurements of glucose, insulin and leptin would provide a suitable method for more detailed analysis by conducting repeated measurements within the same animal.

In the second part of this study we had a closer look on components of central hypothalamic insulin and leptin signaling pathways by investigating relative expression patterns of *OB-Rb* (leptin receptor), *Socs3* (leptin signaling inhibitor) and *Ptpn1* (insulin signaling inhibitor).

OB-Rb mRNA expression did not significantly differ between non-torpid *ad libitum* fed SD and LD hamsters, but appeared to be slightly down regulated in the SD group. Mercer *et al*. demonstrated that the expression of *OB-Rb* is decreased in SD hamsters (Mercer *et al.*, 2001). This seems to be paradoxical since reduced *OB-Rb* levels would suggest decreased leptin sensitivity although SD adapted hamsters are leptin sensitive in contrast to leptin resistant LD hamsters (Klingenspor *et al.*, 2000; Rousseau *et al.*, 2002). The elevated leptin sensitivity might be caused by alterations in *Socs3* expression, which was lowered in SD animals compared to LD animals. The down regulated *Socs3* expression shown here is in accordance with earlier findings. Within the ARC *Socs3* is already reduced after few days in SD, thereby causing elevated leptin sensitivity. In contrast, SOCS3 protein levels are chronically maintained in LD adapted animals, resulting in the known leptin resistance of Djungarian hamsters (Tups *et al.*, 2004). The close association of leptin and insulin would anticipate a comparable expression pattern of *Socs3* and *Ptpn1*. Indeed, earlier investigations showed a similar expression pattern of *Ptpn1* mRNA within the hypothalamus, by using *in situ* hybridization with decreased expression values in SD, causing elevated insulin sensitivity and signaling (Tups *et al.*, 2006). In our study we were not able to verify a significantly reduced *Ptpn1* mRNA expression in non-torpid SD animals possibly caused by the fact that we investigated whole hypothalamus samples by RT-qPCR instead of distinct hypothalamic nuclei by *in situ* hybridization. This might decrease the ability to detect differences in mRNA expression due to the numerous different nuclei.

In SDT, gene expression analysis showed no torpor state dependent alteration in *OB-Rb* mRNA expression compared to NT-SD. Also the expression pattern of *Socs3* in SDT did not differ from those of NT-SD. This was true for all investigated torpor states. Only in the post torpid state *OB-Rb*

expression was down regulated. This indicates reduced leptin signaling at night when hamsters are active again, which might be a consequence of torpor expression. Also in FIT-SD, *OB-Rb* and *Socs3* showed no torpor state dependent regulation of mRNA expression over the course of a torpor bout, but an elevated *Socs3* mRNA level in the post torpid state compared to NT-SD. This suggests that animals undergoing FIT-SD are less leptin sensitive at night after a day with torpor similar to post torpid animals after SDT.

In NT-SD, no circadian rhythm was found in either, *OB-Rb* or *Socs3* expression. Ellis *et al*. already showed that these two genes show now diurnal variation in gene expression under SD conditions (Ellis *et al.*, 2008). Thus, the differences shown for *OB-Rb* as well as for *Socs3* during SDT and FIT-SD reflect alterations in the animals' metabolic state rather than a circadian regulation.

In FIT-LD, the generally higher *Socs3* expression values in association with decreased *OB-Rb* expression, at least during torpor entrance and mid torpor, indicate an overall elevated inhibition of leptin signaling in this group. The resulting reduced leptin sensitivity is rather caused by the seasonal state of the Djungarian hamster than by the type of torpor. One might speculate that this expression pattern of *Socs3* illustrates the known leptin resistance in summer adapted hamsters, although, it also has been shown that LD adapted Djungarian hamsters are able to become leptin sensitive by 48 hours food deprivation (Tups *et al.*, 2004). However, the animals used here were only food restricted to 60% of their daily food consumption. The comparatively mild food restriction might be not sufficient to induce leptin sensitivity, even though *Socs3* expression values in FIT-LD were slightly lower than those shown for *ad libitum* fed LD hamsters. Indeed, it has been shown that long-term food restriction has no effect on *Socs3* mRNA expression in Djungarian hamsters (Tups *et al.*, 2004).

Our data suggest that hypothalamic leptin signaling is not directly involved in torpor regulation since leptin sensitivity seems not to be crucial for the occurrence of FIT in summer adapted hamsters.

In FIT-SD as well as FIT-LD, down regulated *Ptpn1* mRNA expression might indicate a reduced insulin inhibitory action during torpor entrance and mid torpor, while no differences in *Ptpn1* expression could be observed in SDT. Also the circadian rhythmicity of *Ptpn1* expression for NT-SD and SDT indicated low expression values at ZT1 that gradually increased over the course of a day. As opposed to this, *Ptpn1* expression was elevated at ZT1 and gradually decreased over the course of a day during FIT-SD. This might indicate enhanced insulin sensitivity during fasting induced torpor but unaltered insulin sensitivity in SDT. Unfortunately, we were not able to investigate hypothalamic gene expression patterns of the insulin receptor. Therefore, we do not know how insulin sensitivity is affected by this. Thus, it remains questionable if insulin sensitivity is influenced by food restriction and/or winter acclimatization and whether alterations of insulin signaling are involved in torpor regulation or not.

Taken together, our data support the idea of torpor dependent adaptions in circulating glucose, leptin and insulin concentrations. At this, glucose might be involved in the regulation of torpor initiation, whereas insulin and leptin might contribute to torpor maintenance during the mid torpor state. Since there were no torpor state dependent alterations in *OB-Rb* or *Socs3* mRNA expression detectable for all three torpor groups, hypothalamic leptin signaling pathways are rather responsible for the regulation of long term energy homeostasis than being directly involved in acute torpor regulation. Further, more detailed studies are still needed to verify the obtained results and to nail down the role of glucose, insulin and leptin in naturally occurring forms of torpor.

Chapter 5

General Discussion

The expression of torpor to reduce individual energy requirements and minimize energy expenditure in energetically challenging times is a fascinating and puzzling phenomenon. The characteristics of torpor have been intensively studied over the last decades and to date substantial knowledge exists about the physiological properties of this hypometabolic state. Lots of attention has been payed to identify pathways underlying and regulating torpor. Even though some systems have been linked to the regulation of energy balance and torpor, the particular molecular mechanisms leading to the initiation and control of torpor expression are still unknown. In this thesis I set out to shed more light on endocrine and neurological mechanisms contributing to the initiation and maintenance of spontaneous daily torpor as well as fasting-induced torpor in Djungarian hamsters.
Differential gene expression analysis is a fundamental tool for identifying genes involved in a given biological process. For this purpose two different gene expression profiling approaches were conducted by using Illumina sequencing and RT-qPCR.

For the initial study I used Illumina sequencing (also known as RNA-seq) to get a global picture of alterations in hypothalamic gene expression during SDT. As part of next generation DNA sequencing, the Illumina sequencing technology facilitates the investigation of all transcripts of a genome. RNA-seq has become more and more popular over the last decade. This technique shows a high coverage and is suitable to examine the expression pattern of a large number of genes by producing high dimensional data. This unbiased kind of study is not limited by prior knowledge of sequences and enables the identification of known as well as novel transcripts. RNA-seq can be conducted without a reference genome by assembling the generated short reads *de novo* into a transcriptome. Afterwards, transcripts can be discovered by comparing them to existing annotations.
This method is limited by the requirement of high quantity and integrity of RNA (1 – 2 µg, RIN>8) to generate adequate sequencing data. Furthermore, the bioinformatics needed for this kind of study are ambitious and complex. Thus, the methodology must be carefully designed in order to establish a robust statistical analysis of expression data (Mortazavi *et al.*, 2008; Wang *et al.*, 2009; Zhao *et al.*, 2014).

In contrast to RNA-seq, the application of RT-qPCR experiments as well as data interpretation is relatively easy and less time intensive. This standard method is widely established in laboratories and the RNA amount needed for analysis can be very low. RT-qPCR is still the most commonly used method for investigating alterations in gene expression. Gene expression quantification by RT-qPCR is more specific because it investigates just a small number of particular candidate genes at a time. This method is useful to examine the expression of few genes with a known sequence (Jozefczuk & Adjaye, 2011; Gadkar & Filion, 2014).

5.1. Hypothalamic transcriptome – differential expression pattern during torpor entrance in SDT

The present thesis investigated the whole hypothalamic transcriptome of torpid Djungarian hamsters for the first time. With this method it was possible to identify genes showing a differential gene expression during the entrance into SDT in order to uncover as yet unconsidered genes involved in torpor regulatory mechanisms.

During torpor entrance, out of 27830 identified genes 284 genes were differentially expressed, with 181 genes being up regulated and 103 genes being down regulated. This indicates that transcript levels of most genes are unaffected during this state. Just a small set of genes within the hypothalamus seems to be affected by the initiation of SDT. Global mRNA levels do not differ between torpid and non-torpid animals. Thus, a general suppression of transcription is not responsible for the reduced metabolism during torpor. Hence, the initiation of torpor does not reflect a simple shut down of functional processes. This suggestion is in accordance with results of earlier studies received from cDNA array screening of genes in various tissues of hibernators which also indicated that transcript levels of most genes are unaffected during the hypometabolic state (Hittel & Storey, 2001; Storey & Storey, 2004). The classification of altered transcripts showed that the majority of differentially regulated genes belong to cellular and metabolic processes reflecting the state of severe metabolic adjustment during torpor.

Ranking of the 284 altered genes showed that a considerable number within the top 20 up regulated group represented genes coding for structure proteins, whereas the majority of the top 20 down regulated genes comprised transcription factors. Transcription factors are proteins involved in the initiation, elongation and termination of transcription (Latchman, 1997). Hence, adaptions in gene expression of transcription factors in turn influence the expression pattern of their target genes. It has been shown that the general transcriptional initiation is decreased during the expression of SDT in Djungarian hamsters. Additionally, transcriptional elongation is reduced due to its temperature sensitivity. These two factors together, lead to reduced transcriptional activity during SDT in *P. sungorus* (Berriel Diaz *et al.*, 2004). Also in golden-mantled ground squirrels transcriptional initiation has been shown to be reduced by 28 % during hibernation bouts (van Breukelen & Martin, 2002). The decreased expression of transcription factors detected in the hypothalamus during torpor entrance could contribute to the reduced transcriptional activity found in torpid animals.

An additional benefit of this transcriptomic study was that the transcripts established by Illumina sequencing enabled us to design *P. sungorus* specific primers for all following experiments in order to

investigate torpor dependent alterations in the expression of particular genes. It is challenging to complete these types of studies with non-model organisms, which often lack an abundance of readily available sequenced genomes. Moreover, an annotated *P. sungorus* genome is still not available to date.

The hypothalamus is a very complex structured brain area including several nuclei exhibiting various regulatory functions. By investigating whole hypothalamus samples, possible relevant changes in gene expression of small groups of neurons might be lost in the signal to noise ratio. Potential physiologically significant gene expression differences were not captured with this initial study. Thus, it would be worth to investigate distinct hypothalamic nuclei in a continuative high-throughput sequencing study, to provide a more detailed insight in transcriptomic regulation patterns during SDT to identify as yet unknown genes which might play an important role in neuroendocrine pathways regulating torpor. Isolation of specific hypothalamic nuclei can be conducted by laser-capture microdissection. In this case it would be necessary to pool the dissected nuclei of different hamsters to accumulate adequate RNA amounts, since the Illumina sequencing technique requires a relatively high amount of RNA. Furthermore, besides the torpor entrance state also other torpid states (e.g. mid torpor, arousal) should be taken into account by this approach to allow the investigation of whole torpor bouts. Additionally, it would be worth to conduct this approach not just for SDT expressing animals but also for animals undergoing FIT. A comparison of whole transcriptomic differential gene expression is a promising experimental design to answer the question whether SDT and FIT are two distinct types of torpor controlled by different molecular pathways or not.

5.2. Enhanced synaptic plasticity during SDT

As already mentioned, within the group of top 20 up regulated genes a conspicuous number of genes coding for structure proteins was found. This group comprises five different collagen genes as well as *Myo15a*, *Dnah2* and *Micalcl* with a significant enrichment of extracellular matrix components considering all 181 up regulated genes.

Elevated gene expression of several collagens has already been found in the cerebral cortex of thirteen lined ground squirrels during deep hibernation as well as during interbout arousals. The authors concluded that elevated collagen expression levels provide evidence for synaptic plasticity during hibernation (Schwartz *et al.*, 2013). Besides being extracellular matrix structural components, collagens are also bio-active adhesion molecules. Collagens are mainly known for their capability to form elongated fibers to add tensile strength to tissues. Moreover, in the vertebrate central nervous system, collagens and collagen-like molecules play a role in the neuronal development of the brain

by the formation of neural circuits. They are involved in axonal guidance, synaptogenesis and establishment of brain architecture (Chernousov *et al.*, 2006; Fox, 2008; Hubert *et al.*, 2009). Our sequencing results support the hypothesis of existent synaptic remodeling and plasticity during the torpid state. Unfortunately, we were not able to verify the obtained up regulated mRNA expression for the five identified collagen genes during torpor entrance by RT-qPCR analysis. Although the relative expression analysis indicated a trend of increased *Col17a1* mRNA expression during torpor entrance and slightly down regulated *Col17a1* expression during mid torpor, arousal and post torpor, these data did not reach significance, except for the decreased mRNA expression during the post torpid state. Also the investigation of all other identified collagens showed an mRNA expression pattern like *Col17a1* with a tendency of up regulated levels during torpor entrance and down regulated expression values during all other investigated torpor states that did not reach significance. Especially the expression data of the torpor entrance group showed high variability between different hamsters which probably is responsible for the non-significant results. It is quite difficult to sample animals during torpor entrance at the exact same state since metabolic rate and T_b gradually drop to low values within a relatively short time period. Thus, animals possibly have been sampled a little bit earlier or slightly later during torpor entrance which might have an effect and could cause the obtained variability in mRNA expression data. Furthermore, it has to be considered that the NGS and RT-qPCR experiments have been conducted with different groups of animals so that the data might also reflect inter-individual differences. Since the verification of enhanced mRNA expression of collagen genes was not possible it remains to be revealed, whether collagens actually trigger synaptic plasticity and remodeling during torpor initiation in SDT expressing Djungarian hamsters. A larger sample size might facilitate more precisely resolved expression data. Having a higher number of relative expression data by elevating the number of investigated animals would result in lowered variance and thereby cause higher kurtosis which, in turn, leads to more precision of the data.

Also relative expression analysis for *Myo15a* and *Dnah2* mRNA expression were conducted during the four torpor states torpor entrance, mid torpor, arousal and post torpor in SDT expressing hamsters by using RT-qPCR. Comparable to the transcriptomic data, the RT-qPCR expression data showed up regulated mRNA expression levels during torpor entrance, but down regulated mRNA expression at all other investigated torpor states compared to the non-torpid control group. This was true for both, *Myo15a* as well as *Dnah2*. Thus, we were able to verify an elevated expression level of these two structural components of the cytoskeleton during the initiation of torpor. Both genes showed a circadian regulation in its mRNA expression which peaked at ZT7 in non-torpid animals indicating a higher demand of motor proteins during the hamsters' activity phase.

The superfamilies myosin and dynein are molecular motors which enable the transport of various biomolecules within the brain (Hirokawa *et al.*, 1998; Vale, 2003). Dyneins are mechanoenzymes

which are able to transport biomolecules in the axon and dendrites along microtubules by hydrolyzing ATP. Myosin mainly fulfills its role as molecular motor in synaptic regions moving along actin filaments by ATP hydrolysis. These molecular motors facilitate the transport of vesicles and protein complexes. Also mRNAs coding for large protein complexes can be transported by this way, so that the protein synthesis occurs locally. This intracellular transport within axons, dendrites and synapses is essential for neuronal function and plays a key role in brain wiring, neuronal survival, neuronal plasticity and nervous system development (Hirokawa & Takemura, 2005; Hirokawa *et al.*, 2010). Therefore, the increased expression of *Myo15a* and *Dnah2* provides evidence that they could contribute to the maintenance of synaptic transmission and neuronal survival during the torpid state by an elevated transport of fundamental biomolecules.
It has been shown that during deep hibernation and inter bout arousals the expression of myosin (*Myo1d*, *Myo9b*, *Myh6*) and dynein (*Dync1h1*) is elevated in the cerebral cortex of thirteen-lined ground squirrels, also pointing towards neuronal plasticity and remodeling (Schwartz *et al.*, 2013). During SDT, an elevated transport seems to play a role especially during torpor initiation, since expression values already have been decreased during the mid torpor state and remained down regulated during arousal and in the post torpid state.

To date it is not well understood how Djungarian hamsters and other torpor using animals are able to counter cellular damage when oxygen supply is reduced during the hypometabolic state, which is followed by reperfusion during arousal. Here we showed increased levels of several structure genes during the initiation of torpor suggesting increased synaptic plasticity, neuronal remodeling and elevated transport of biomolecules. These mechanisms might serve to counter damaging effects occurring during torpor.

5.3. Readjustment of coagulation during SDT

During the torpid state, the drop in T_b, ventilation and heart rate leads to a reduced blood flow which rapidly increases again during arousal. Thus, torpor using mammals are challenged by fluctuations in blood pressure throughout the torpor season, with lowered oxygen supply while being torpid and reperfusion when arousing from the hypometabolic state. Torpor using mammals show a natural tolerance to ischemia and can handle this challenge with no evidence of neuronal damage or neuronal dysfunction and without thrombi formation or stroke after complete rewarming from the torpid state (Lyman & O'Brien, 1961; Frerichs *et al.*, 1994).

The *Vwf* gene is coding for a procoagulation factor, named von Willebrand factor. This adhesive multimeric glycoprotein is involved in blood clotting by the enforcement of platelet plug initiation

and aggregation. vWF concentration correlates positively with thrombosis risk and negatively with bleeding risk. Thus, a high vWF level leads to an increased risk of thrombosis formation whereas vWF deficiency can result in von Willebrand´s disease, a known human bleeding disorder (Sadler, 1998; Sadler, 2005; Peyvandi *et al.*, 2011).

Expression analysis of *Vwf* at different torpor states in the hypothalamus showed down regulated mRNA expression of this gene during mid torpor, arousal and also in the post torpid state. The reduced *Vwf* expression level provides evidence for suppressed blood clotting during the torpid state of Djungarian hamsters. It may protect from massive thrombosis, when the blood flow rate is decreased during torpor. This is in accordance with a study of Cooper *et al*. (2016). Also in hibernating thirteen-lined ground squirrels *Vwf* mRNA expression is down regulated in lung tissue samples. Furthermore, in plasma samples, vWF collagen binding activity is 10-fold decreased during deep hibernation indicating elevated prevention of blood clotting. After arousal in spring, vWF concentration increases gradually until it reaches the non-hibernating level after about 16 days (Cooper *et al.*, 2016).

Paradoxically, our data showed up regulated hypothalamic *Vwf* mRNA expression during torpor entrance. This was shown by both experiments, RNA-seq and RT-qPCR. One explanation could be that these mRNA transcripts are stored during the torpid state rather than being directly translated into protein in order to facilitate fast protein synthesis after arousal. Clotting activities are important after arousal to avoid bleeding when animals are active again. But a simple storage of *Vwf* mRNA seems to be unlikely, because elevated mRNA levels where solely detectable during torpor entrance and where already decreased during the mid torpor state. An immediate translation into vWF with severely decreased activity seems to be more likely. vWF can be regulated post-transcriptionally by a zinc-containing metalloprotease enzyme, named ADAMTS13. The cleavage of vWF into smaller multimers by ADAMTS13 modulates the proadhesive function of this procoagulation factor resulting in reduced platelet aggregation (Levy *et al.*, 2001; Ruggeri & Ruggeri, 2004; Chauhan *et al.*, 2006; Zhao *et al.*, 2009). In this case, no damaging effect of elevated vWF concentrations in the brain would be expected during the torpid state.

The investigation of circadian rhythmicity in *Vwf* expression showed a circadian regulation in non-torpid as well as in torpid hamsters. In non-torpid animals *Vwf* expression peaked at ZT7 indicating a higher demand of *Vwf* expression during dusk, which represents the beginning of the animal's naturally active phase. The diurnal mRNA expression during torpor was less pronounced compared to non-torpid animals, but still showed a circadian regulation with lowest expression levels at ZT4 during mid torpor and at ZT16 in the post torpid state. Taken together, these data indicate a circadian dependent readjustment of blood clotting in both, SDT expressing and active animals.

5.4. Physiological comparison of SDT and FIT

In our second RT-qPCR based approach, we aimed to analyze specific hypothalamic systems, which are likely to be involved in torpor regulation. Here, we analyzed two different forms of torpor – SDT and FIT.

Since hamsters of both sexes were used for this study we analyzed whether sex differences might influence torpor depth and duration. As expected, our data could not reveal sex differences within the investigated SD groups. Sex differences in winter adapted hamsters are not expected, because Djungarian hamsters are reproductively inactive during their winter state. This inactivity is caused by gonadal regression with dramatically reduced serum concentrations of sex hormones (Scherbarth & Steinlechner, 2010). Thus, sex differences in such torpor characteristics under SD are unlikely. The LD group showed no sex differences in torpor depth or duration either.

In accordance with earlier studies, our data showed a significant difference in torpor depth and duration between SDT and FIT-LD with shorter and shallower torpor bouts during FIT-LD (Geiser, 1991; Diedrich *et al.*, 2015). The decreased torpor depth and duration of FIT-LD bouts result most likely from the missing plateau phase of torpor maintenance during the mid torpor state. It is assumed that the lack of a period with constant low metabolic rate leads to less pronounced energy saving (Heldmaier & Steinlechner, 1981; Diedrich & Steinlechner, 2012). But in contrast to SDT, FIT bouts can occur more than just once a day so that the lower energy savings achieved from FIT-LD might be compensated by the expression of multiple FIT bouts per day (Steinlechner *et al.*, 1986; Ruby & Zucker, 1992; Diedrich *et al.*, 2015). Indeed, we detected multi bout events in some but not all fasted, summer adapted hamsters undergoing FIT, but never in SDT using winter adapted hamsters.

Between SDT and FIT-SD no significant differences in torpor depth or duration were observed in winter adapted Djungarian hamsters. Also Diedrich *et al*. (2015) showed that torpor depth and duration does not differ between SDT and FIT-SD. In this study it was also shown that in winter adapted hamsters an additional energetic challenge caused by food restriction rather increased the mean torpor incidence than causing multiple FIT bouts per day (Diedrich *et al.*, 2015). The adjustment of torpor frequency might be efficient to compensate the additional energetic challenge evoked by food restriction.

The choice of whether increasing torpor incidence (without alterations in torpor depth and duration compared to SDT) or displaying multiple torpor bouts (with shorter torpor duration and shallower drop in T_b compared to SDT) to enhance energy savings seems to arise from seasonal dependent modulation of fasting induced torpor due to the summer or winter phenotype of the animal.

5.5. Circadian clock system

To date, it is well known that SDT usually is restricted to the daily resting phase of Djungarian hamsters. This is important to allow hamsters a nonrestrictive continuation of foraging and territorial and social activities throughout the torpor season (Ruf *et al.*, 1989; Kirsch *et al.*, 1991; Ruby & Zucker, 1992; Herwig *et al.*, 2007). In contrast, the occurrence of FIT is not restricted to a particular lapse of time within the daily activity and rest cycle of Djungarian hamsters and thus might interfere with behavioral activities (Steinlechner *et al.*, 1986; Ruby & Zucker, 1992). The variations in torpor timing between SDT and FIT raise the question whether the autonomous transcription-translation feedback loop of the master circadian clock shows differences in its regulation, depending on whether SDT or FIT is used. We investigated hypothalamic relative mRNA expression of *Bmal1* and *Per1* over the course of a day in hamsters undergoing SDT and FIT-SD. We were not able to investigate circadian rhythms for the FIT-LD group, since this group was sampled independent of specific ZTs.
Bmal1 and *Per1* are circadian clock genes playing and important role in the primary feedback loop of the master circadian clock. In this connection, *Bmal1* is considered to function as a positive regulator whereas *Per1* represents a negative regulator of the circadian feedback loop (Reppert & Weaver, 2002; Takahashi *et al.*, 2008).

For the NT-SD group, our data showed a clear circadian rhythm of *Bmal1* as well as *Per1* mRNA expression over the four investigated time points. As expected, our results also showed a circadian rhythm of *Bmal1* and *Per1* mRNA expression during SDT. The rhythmicity of circadian expression pattern during SDT was comparable to those found in hamsters which remained active, indicating an oscillating circadian clock during SDT.
It has already been shown that a proper working circadian clock plays an important role during SDT in order to determine the timing of SDT initiation. Daily torpor, as part of the “normal” circadian organization, has been shown to be disordered when the SCN is ablated. SCN ablation does not prevent the occurrence of torpor but torpor bouts start to occur randomly at any time of the day (Ruby & Zucker, 1992). Hence, the use of SDT could interfere with foraging activities causing a decline in energy intake which in turn might result in energy depletion. This would be counterproductive and narrow the energetic benefit of torpor expression.
Although the circadian clock keeps ticking, our results indicated a possible modulatory effect of SDT on *Bmal1* and *Per1* mRNA expression. *Per1* expression was significantly elevated at ZT7 compared to NT-SD and *Bmal1* expression started to decrease earlier than in the NT-SD group. These differences might lead to alterations in the feedback loop of the circadian clock resulting in a shortened free-running period on a day with torpor. The usual free-running period of SD adapted Djungarian hamsters is longer than 24 hours. Thus, on a day with torpor Djungarian hamsters might see no day

light at all. This implies that a resynchronization of the circadian clock with the environmental light-dark cycle is not possible. In this case a shorter free-running period would ensure an endogenous rhythm more close to the environmental 24 hours light-dark cycle in order to maintain entrainment. In fact, it has already been shown that low T_bs during torpor can feedback on circadian pacemakers causing a shortened free-running period in torpid Djungarian hamsters (Thomas *et al.*, 1993). A direct effect of SDT on the circadian clock has also been demonstrated by circadian studies in which torpid hamsters where sampled throughout a 24 hour time period indicating alterations in phase and amplitude of the circadian clock during SDT as well (Herwig *et al.*, 2006; Herwig *et al.*, 2007).

Analysis of *Bmal1* and *Per1* in animals undergoing FIT-SD showed a completely different mRNA expression pattern compared to SDT. During FIT-SD, *Per1* mRNA expression was advanced with significantly elevated mRNA levels at ZT4 compared to SDT and NT-SD. *Bmal1* expression remained on a constant level throughout all investigated torpid states and showed a slight up regulation only during the post torpid state at ZT16. The results indicate a shift of the circadian feedback loop in hamsters undergoing FIT-SD.

It has been shown that a timed calorie-restricted feeding schedule can affect the master circadian clock, leading to a molecular phase advance of *Per1* and *Cry2* expression in mice. However, the timed hypocaloric feeding did not change the endogenous free-running period (Mendoza *et al.*, 2005). Also in rats a phase advance in circadian rhythms (e.g. rhythms of T_b or locomotor activity) has been shown when the animals were food restricted, depending on the time when food was provided (Challet *et al.*, 1997). Thus, a good argument can be made that in hamsters undergoing FIT-SD, the daily light-dark cycle as exogenous Zeitgeber was most likely overwritten by the time when food was provided, resulting in a synchronization of the circadian clock with the feeding schedule.

One explanation how food restriction might lead to a disrupted transcription-translation feedback loop could be explained by increased activity of silent mating type information regulation 2 – homolog 1 (SIRT1). SIRT1 is a NAD^+-dependent deacetylase, which has been shown to be increased by fasting in various tissues including the brain. During fasting, the amount of cellular [NAD^+] increases as a result of nutrient depletion which in turn leads to an elevated SIRT1 level. This has been shown for both, *Sirt1* mRNA as well as SIRT1 protein level (Rodgers *et al.*, 2005; Cakir *et al.*, 2009). SIRT1 is able to deacetylate BMAL1 preventing the complex formation of BMAL1 with CLOCK. Without the BMAL1:CLOCK complex formation the expression of *Pers* and *Crys* cannot be initiated. Therefore, the normal circadian feedback loop can be disrupted by enhanced SIRT1 activity (Hirayama *et al.*, 2007; Asher *et al.*, 2008; Nakahata *et al.*, 2008).

5.6. Orexigenic system

Severe body weight reduction has been shown to be a common permissive factor of SDT and FIT. Nevertheless, the body weight loss is based on different conditions. Before the first SDT bout occurs, hamsters enter a catabolic state resulting in a gradual decrease of body weight which is caused by a controlled and voluntary reduction of food intake until an individual body weight set point is reached (Steinlechner *et al.*, 1983; Knopper & Boily, 2000). In contrast, the rather uncontrolled body weight loss preceding FIT results from a forced reduction of food intake by food scarcity.
It has been shown that winter adapted Djungarian hamsters use SDT from a balanced energetic state, whereas FIT expressing hamsters exhibit a negatively imbalanced energetic state (Diedrich *et al.*, 2015).
Here we investigated constituents of the orexigenic system playing a role in regulating food intake and energy expenditure to maintain energy homeostasis. This was conducted to survey a possible involvement of these constituents in controlling torpor expression as well as to uncover possible regulatory differences within the orexigenic pathway between SDT and FIT. Thus, we measured serum glucose, insulin and leptin over the course of a torpor bout. Furthermore, we determined relative mRNA expression of *OB-Rb*, *Socs3*, *Ptpn1*, *Npy* and *Agrp* at different torpor states for hamsters undergoing SDT, FIT-SD and FIT-LD relative to hamsters remaining NT-SD.

Before discussing alterations in serum glucose, insulin and leptin concentrations over the course of a torpor bout within or between SDT, FIT-SD and FIT-LD, it has to be noted that we solely detected a significant difference of the glucose level between FIT-SD and FIT-LD. For all other results the shown differences did not reach statistical significance. Nevertheless, a tendency of torpor state dependent alterations of glucose, insulin and leptin concentration seemed to be apparent.
Our data showed that, due to the fasting induced body weight reduction, food restriction results in lowered insulin and leptin levels of summer adapted hamsters comparable to those of winter adapted hamsters. At the end of the experiments all three investigated groups showed a comparable decreased of body weights with an average body weight loss of 17.06% ± 19 for SDT, 17.53% ± 2.4 for FIT-SD and 19.27% ± 1.9 for FIT-LD. Comparably reduced leptin concentrations and body weight loss of food restricted and SD adapted *ad libitum* fed hamsters have been shown before (Tups *et al.*, 2004; Diedrich *et al.*, 2015).
The difference in serum glucose found between FIT-SD and FIT-LD in the post torpid state indicates that food restriction represents a greater challenge in SD adapted than in LD adapted hamsters. This effect is most likely due to the seasonal state of investigated hamsters. The energetic state of the LD group was challenged by reduced food supply only whereas the SD group already decreased body weight and lipid stores prior to the fasting experiment as a result of the general winter adaptions.

Thus, the food restricted SD group was additionally impaired by the reduced food supply which has been shown to tighten the already proceeding body weight depletion (Steinlechner *et al.*, 1983; Morgan *et al.*, 2003). Even though, our study showed that the average body weight loss did not significantly differ between these two groups a difference in fat depot depletion and of the energetic state might still be existent.

Glucose as well as insulin concentrations were slightly reduced during torpor entrance in animals undergoing SDT and FIT-LD. A decreased glucose level during torpor entrance has already been shown in *P. sungorus*. Heldmaier *et al.* (1999) demonstrated that in torpid Djungarian hamsters blood glucose already starts to decrease during early torpor entrance and that the reduced glucose level reaches statistical significance with minimum metabolic rate. The decrease of glucose was considered to result from the glucose based metabolism which is used during the initiation of torpor and changes to lipid based metabolism over the course of a torpor bout (Heldmaier *et al.*, 1999). This switch from glucose to lipid metabolism has also been shown by Diedrich *et al.* (2015). Here we showed that a drop in glucose during torpor entrance also seems to be true for FIT-LD animals. It is known that during FIT torpor is entered from a state of lipid metabolism (Diedrich *et al.*, 2015). Thus, the decreased glucose concentration cannot simply be explained by glucose utilization since FIT is entered from a state of lipid utilization.

One explanation for the drop in glucose could be that the commonly reduced glucose availability during torpor entrance might lead to the inhibition of thermogenesis and thereby induce the gradual decrease of T_b to reduce energy expenditure while entering torpor. Indeed, it has been shown that glucoprivation by the administration of 2-deoxy-D-glucose is able to induce a torpor-like state with decreased T_b in *P. sungorus*. 2-deoxy-D-glucose is a glucose analogue disrupting glucose oxidation and inhibiting glucose availability (Dark *et al.*, 1994). Conversely, SDT incidence decreases when hamsters are fed with a high-carbohydrate diet (Ruf *et al.*, 1991). The reduced insulin level shown during torpor entrance in SDT and FIT-SD is most likely a result of the lowered glucose concentration since insulin is secreted from pancreatic cells in order to regulate blood glucose levels. When serum glucose is elevated, insulin is released into the blood stream to lower blood glucose levels by facilitating the transport of glucose into cells (Rebrin *et al.*, 1995; Lewis *et al.*, 1997; Sindelar *et al.*, 1998). Thus, high insulin concentrations are not expected when blood glucose is low. Taken together, our data suggest reduced glucose availability during torpor entrance for both types of torpor, SDT as well as FIT-LD, which is in line with the hypothesis that glucoprivation might be a trigger for torpor initiation in *P. sungorus*.

During mid torpor, serum insulin and leptin concentrations were decreased in SDT as well as FIT-SD, but without reaching statistical significance. In contrast, serum concentration of these two adipose signals during mid torpor in FIT-LD remained comparable to those of NT-SD. Both, insulin as well as

leptin, are known to be involved in the regulation of thermogenesis. Increased activity of brown adipose tissue (BAT) can be provoked by the administration of leptin as well as insulin which in turn results in elevated energy expenditure (Rothwell & Stock, 1981; Haynes *et al.*, 1999). Therefore, low circulating adipose signals can facilitate lowered BAT activity and are able to reduce heat production. BAT is a specialized adipose tissue which is able to produce heat by non-shivering thermogenesis (Heldmaier & Buchberger, 1985). Reduced circulating concentrations of insulin and leptin might facilitate the inhibition of thermogenesis by reduced BAT activity and thereby contribute to the prolonged period of low metabolic rate and T_b during mid torpor. Following this hypothesis, it makes sense that reduced levels of these two signals were not found in animals undergoing FIT-LD. During FIT-LD, the characteristically prolonged mid torpor state, with maintained low T_b, is missing. When the nadir of metabolic rate and T_b is reached, the hamsters almost immediately start to arouse and return to their euthermic active state (Diedrich & Steinlechner, 2012). Thus, the inhibition of thermogenesis to maintain reduced T_bs, possibly provoked by reduced insulin and leptin, is not needed.

Since our results did not reach significance, the here discussed alterations of glucose, insulin and leptin clearly need to be verified in further studies. A new method offers the potential to continuously measure glucose *in vivo*. *In vivo* glucose telemetry provides the opportunity for more detailed analysis of glucose alterations by conducting continuous measurements over a 28 days in the same animal (Brockway *et al.*, 2015). Furthermore, this method facilitates the monitoring of alterations directly before a hamster enters the torpid state. The biggest issue in investigating torpor initiation mechanisms is the unpredictability of torpor occurrence. It cannot be predicted whether a hamster is entering torpor on a particular day or not until T_b and metabolic rate start to decrease. Thus, this new method would also shed light on the question whether a lowered glucose availability is the cause for or the consequence of the metabolic depression by determining the precise time point when serum glucose starts to decrease.

To date, there is no method available for *in vivo* real time measurements of insulin and leptin. Also the collection of repeated blood samples from the same animal by cannulation could strengthen our results, but this method is limited to larger species and not suitable for Djungarian hamsters. To minimize the standard error of means in the investigated groups, a larger number of animals and high frequency sampling is required to profile the complex regulation pattern of insulin and leptin secretion during physiological changes in torpid animals.

Relative expression analysis of *OB-Rb* and *Socs3*, as components of the hypothalamic leptin signaling pathway, showed elevated *Socs3* mRNA expression of summer adapted *ad libitum* fed non-torpid hamsters compared to winter adapted *ad libitum* fed non-torpid hamsters. This indicates reduced inhibition of the leptin receptor during winter and is in accordance with earlier studies which also

demonstrated reduced *Socs3* expression in winter hamsters resulting in season-dependent enhanced leptin sensitivity. During summer, elevated *Socs3* mRNA expression increases inhibition of leptin sensitivity. Also SOCS3 protein levels have been shown to be up regulated throughout the summer months, leading to the known leptin resistance of Djungarian hamsters (Tups *et al.*, 2004).

OB-Rb mRNA expression did not significantly differ, but was slightly higher in summer adapted than in winter adapted hamsters. Indeed, it has been shown that in Djungarian hamsters the expression of *OB-Rb* is down regulated during the winter season (Mercer *et al.*, 2001). Decreased *OB-Rb* mRNA levels would suggest reduced leptin sensitivity. Nevertheless, Djungarian hamsters are clearly leptin sensitive during winter. Thus, it seems like down regulated expression of the leptin sensitivity inhibitor *Socs3* alone is sufficient to initiate leptin sensitivity in this species.

Earlier investigations using *in situ* hybridization showed that besides *Socs3* also *Ptpn1* mRNA expression, as component of the hypothalamic insulin signaling pathway, is down regulated in winter adapted *ad libitum* fed hamsters resulting in insulin sensitivity (Tups *et al.*, 2006).

In our study however, we were not able to verify this result since relative expression analysis of *Ptpn1* did not differ between summer and winter adapted *ad libitum* fed non-torpid hamsters. This might be caused by the fact that we investigated gene expression within the whole hypothalamus by using RT-qPCR instead of distinct hypothalamic nuclei by using *in situ* hybridization.

During SDT as well as FIT-SD, analysis of relative *OB-Rb* mRNA expression over the course of a torpor bout showed no alterations of the investigated torpor states relative to the NT-SD group. Also *Socs3* mRNA expression did not differ over the course of a torpor bout during SDT or FIT-SD. However, in the post torpid state of the SDT group mRNA expression of *OB-Rb* was decreased. This suggests reduced leptin signaling after a day with torpor, when hamsters are active again at night. The FIT-SD group did not show *OB-Rb* decreased expression, but here *Socs3* expression was increased in the post torpid state. Thus, also in the FIT-SD group leptin signaling seems to be disturbed at night after a day with torpor. The lowered leptin sensitivity after a day with torpor might be a consequence of torpor. These results indicate that a torpor dependent alteration of hypothalamic leptin sensitivity is unlikely to act as acute regulatory mechanism in the control of torpor.

The common prerequisite of severe body weight reduction before SDT as well as FIT start to occur might be reflected in hypothalamic leptin sensitivity. However, during FIT-LD *Socs3* expression was elevated compared to NT-SD. This was true for all investigated torpor states. Concurrently, *OB-Rb* expression was decreased at least during torpor entrance and mid torpor. Taken together, this suggests a lowered leptin sensitivity of hamsters undergoing FIT-LD. This however, is rather likely caused by the animals' seasonal state than by the type of torpor. As already mentioned, Djungarian hamsters are leptin resistant during summer which most likely is caused by the existence of a high SOCS3 protein level inhibiting the leptin receptor. Even though, food restricted LD hamsters showed

severe body weight reduction, tracking that of SD hamsters with reduced serum leptin concentrations, caused by the depletion of internal fat stores, the mRNA expression value of *Socs3* seemed to be unaffected. Tups *et al*. (2004) showed that LD hamsters are able to develop leptin sensitiveness after 48 hours of food deprivation possibly as consequence of the abrupt drop in circulating leptin. However, the same study showed that a gradual decrease of serum leptin induced by chronic long-term food restriction instead of complete food deprivation did not alter *Socs3* mRNA levels despite low serum leptin. The latter is in accordance with the results of our study, indicating no development of leptin sensitivity in food restricted LD hamsters either.

Our results provide evidence for a season dependent adjustment of leptin sensitivity rather than an adjustment based on low circulating leptin. Additionally, the development of leptin sensitivity does not seem to be a crucial factor for the occurrence of torpor since the FIT-LD group was able to use FIT although they appeared to remain leptin resistant.

No circadian regulation of *OB-Rb* and *Socs3* for hamsters held under SD conditions was found. This is in accordance with a study which investigated 24 hour expression profiles of *OB-Rb* and *Socs3* in adult male Djungarian hamsters. In this study no diurnal variation was found in winter adapted hamsters either, but circadian rhythmicity of *OB-Rb* expression was shown for summer adapted hamsters (Ellis *et al.*, 2008).

Relative expression analysis of *Ptpn1* showed differences in its expression level in SDT compared to both, FIT-SD as well as FIT-LD, with lowered mRNA expression in FIT groups during torpor entrance and mid torpor. Also circadian rhythmicity of *Ptpn1* mRNA expression differed between SDT and FIT-SD. Here, the circadian regulation of SDT was comparable to those of NT-SD with a higher expression value at ZT1 gradually decreasing over the course of the day, whereas in FIT-SD the expression of *Ptpn1* was lowest at ZT1 and gradually increased over the course of a day. These data might indicate a torpor dependent reduction of insulin inhibitory action and thus possibly elevated insulin sensitivity during torpor entrance and mid torpor in FIT using hamsters. This alteration seems to be specific for FIT and independent from the animals' seasonal state. In contrast, the SDT group showed no torpor dependent alterations in insulin sensitivity since *Ptpn1* expression pattern was comparable to the pattern seen for NT-SD for all investigated torpor states.

Unfortunately, we were not able to additionally investigate the insulin receptor. To definitely determine whether insulin sensitivity is adjusted during FIT or not also expression pattern of the insulin receptor itself needs to be investigated because insulin sensitivity or insulin resistance is affected by the interaction of both constituents (Tups *et al.*, 2006).

It is commonly assumed that SDT is entered from an energetically balanced state without any anticipatory development of starvation symptoms (Heldmaier *et al.*, 1999; Stamper *et al.*, 1999; Morgan *et al.*, 2003; Diedrich *et al.*, 2015). Therefore, we did not expect elevated *Npy* and *Agrp*

mRNA expression values during SDT. Accordingly, our study revealed that the mRNA expression of these two orexigenic neuropeptides was unaltered and remained comparable to those of the NT-SD group throughout all torpid states. Even though circulating insulin and leptin concentration appeared to be decreased during mid torpor, *Npy* and *Agrp* mRNA expression remained low. This indicates that during SDT a balanced energy homeostasis is still maintained when hamsters are torpid and at night after a day with torpor when hamsters are active again. Thus, the initiation of SDT is triggered independent from any energy deficit symptoms.

In contrast, FIT is considered to act in case of emergency to improve the negative energy balance caused by food scarcity (Diedrich & Steinlechner, 2012; Diedrich *et al.*, 2015). In the FIT-SD group, *Agrp* mRNA expression was increased during mid torpor and also *Npy* expression was up regulated during this state, but did not reach statistical significance. These elevated levels of *Agrp* and *Npy* mRNA might be caused by the slightly decreased serum insulin and leptin concentrations during mid torpor. Up regulated values of these two orexigenic neuropeptides indicate an acute negative energy state of FIT using animals. The increased expression level of both genes promotes the increase of food intake and decrease of energy expenditure to counter the energetic deficit caused by food restriction. Surprisingly, we did not detect an overall up regulated expression of *Npy* and *Agrp*. The alterations found in hypothalamic *Agrp* and *Npy* expression during FIT-SD and especially the differences between SDT and FIT were smaller than expected. It has already been demonstrated that food restriction leads to increased expression of orexigenic peptides and that FIT is entered from lipid based metabolism (Mercer *et al.*, 2001; Rousseau *et al.*, 2002; Diedrich *et al.*, 2015). But it has to be considered that we investigated relative mRNA expression of *Agrp* and *Npy* while animals are torpid. Earlier studies, showing increased *Agrp* and *Npy* expression, investigated the influence of food restriction on *Agrp* and *Npy* expression while animals were active. This might result in diverging mRNA expression patterns caused by the animals' metabolic state. It has been shown that food restriction leads to enhanced torpor frequency in SD adapted hamsters to maintain energy balance (Diedrich *et al.*, 2015). Since hamsters were food restricted for at least for days before sampling it could be possible that an adjustment in torpor frequency already took place in investigated FIT-SD animals to counter the negative energy state. This might cause the rather unaltered *Agrp* and *Npy* mRNA level.

During FIT-LD, no differences were shown throughout all investigated torpor states, neither in *Agrp* nor in *Npy* mRNA expression. The unaltered expression of *Npy* was consistent with other studies showing unaffected hypothalamic *Npy* expression when Djungarian hamsters held under LD are food deprived for 24 and 48 hours (Mercer *et al.*, 2000; Mercer *et al.*, 2001). However, the same studies also showed up regulated *Agrp* mRNA expression when hamsters were fasted. In contrast to the chronic fasting period of over one month (providing 60% of individual daily food intake) conducted in

our study, the other studies conducted acute food deprivation for a short time period. The difference of acute food deprivation and chronic food restriction might lead to differential regulation of *Agrp* expression. It is likely that the long food restriction period and the thereby caused depletion of body weight led to reduced energy requirements of investigated Djungarian hamsters. It has been shown that *Agrp* expression is elevated in acute food deprived rats, but is unaffected by chronic food restriction (Bi *et al.*, 2003). It might be possible that the chronically provided 40% reduced daily food portion together with the use of FIT was sufficient to maintain energy homeostasis so that an adjustment of orexigenic neuropeptide gene expression was not required.

5.7. Thyroid hormone system

Thyroid hormones are well known for their function in regulating metabolic rate and thermogenesis and have been shown to be involved in the expression of SDT (Bianco *et al.*, 2005; Herwig *et al.*, 2008; Murphy *et al.*, 2012; Bank *et al.*, 2015; Bank *et al.*, 2017a). To uncover the hypothalamic thyroid hormone state related to different phases of torpor in SDT and FIT expressing Djungarian hamsters, we investigated relative mRNA expression of *Dio2* and *Mct8*. *Dio2* and *Mct8* expression levels may indicate the local T3 availability. *Dio2* is responsible for the conversion of T4 into the active metabolite T3 whereas *Mct8* is a thyroid hormone transporter, identified in tanycytes of the third ventricle of Djungarian hamsters, which facilitates the transport of thyroid hormones to the hypothalamus (Kohrle, 1999; Friesema *et al.*, 2003; Herwig *et al.*, 2009).

In the present thesis, *Dio2* mRNA expression was down regulated throughout all investigated torpor states of fasted hamsters undergoing FIT-SD as well as FIT-LD. During mid torpor in animals undergoing SDT, *Dio2* expression was down regulated without reaching statistical significance. This was most likely due to the relatively high standard error of the NT-SD group which might be lowered by enhancing sample size. The decreased expression found for all three torpor groups provides evidence for an overall suppressed activation of thyroid hormone by a less pronounced conversion of T4 into T3 during torpor. This would result in a lowered hypothalamic T3 availability and is congruent with other studies which clearly demonstrated that local as well as peripheral high T3 concentrations are able to affect torpor behavior. Pharmacological manipulations of the thyroid hormone state demonstrated that high T3 concentrations result in an inhibition of SDT, whereas an elevated level of the inactive metabolite T4 had no effect on torpor. Apparently, T3 availability in the hypothalamus rather than in the periphery plays a major role since central T3 administration sufficiently blocks torpor, even if T3 serum concentrations are not increased. In contrast, low T3 levels have been shown to promote torpor by increasing torpor frequency, depth and duration (Murphy *et al.*, 2012; Bank *et al.*, 2015; Bank *et al.*, 2017a). With our experiment, we confirm that a decrease in

hypothalamic T3 availability by lowered *Dio2* mRNA expression is an important factor for the occurrence of SDT as well as FIT-SD and FIT-LD. A previous study, which investigated the effect of food deprivation on components of the thyroid hormone system, demonstrated increased *Dio2* expression in hypothalamic tanycytes of winter acclimatized Djungarian hamsters (Herwig *et al.*, 2009). However, we clearly detected decreased *Dio2* levels in fasted winter acclimatized hamsters showing FIT compared to non-torpid SD hamsters. The discrepancy might be caused by differences of the experimental setup between the studies. The hamsters of the present thesis were fasted but still received 60% of their daily food consumption and samples were collected during the torpid state whereas hamsters of the other study were starved for 48 hours and samples were collected while animals were active. Based on the hypothesis that high T3 availability suppresses torpor, an elevated *Dio2* mRNA level in fasted animals appears to be counteractive during FIT. However, it is not clear whether high hypothalamic T3 concentrations also prevent the occurrence of FIT. It might be interesting to proof the influence of hyper- and hypothyroidism on Djungarian hamsters undergoing FIT.

It has been shown that *Mct8* mRNA expression is increased in winter adapted hamsters compared to hamsters held under LD conditions (Herwig *et al.*, 2009; Petri *et al.*, 2016). These observations are in accordance with our results showing an overall decreased *Mct8* expression of the FIT-LD group compared to the NT-SD group. This indicates decreased thyroid hormone transport in these hamsters due to their physiological summer phenotype. Interestingly, in SDT *Mct8* expression was down regulated during mid torpor and in the post torpid state, whereas mRNA expression of *Mct8* remained unaltered during FIT-SD in all four investigated torpor states. This suggests a torpor dependent alteration of the hypothalamic thyroid hormone transport, which seems to be specific for SDT. MCT8 is a bidirectional transporter with binding sites at the exterior and at the interior site of the cell (Braun *et al.*, 2013). Therefore, it is not clear whether lowered expression level during mid torpor and post torpor results in reduced T4 uptake into tanycytes and thus reduced supply to the hypothalamus or in reduced efflux of T3.

5.8. Conclusion

The aim of this thesis was to uncover regulatory mechanisms within the hypothalamus, which might play a role in the regulation of torpor in Djungarian hamsters. It has to be considered that most of the results discussed here reflect regulatory mechanisms on gene expression level, which do not necessarily reflect the torpor dependent regulation on protein level. Therefore, further studies comprising also protein based experiments are needed.

In summary, our data provide evidence for enhanced synaptic plasticity, elevated transport of biomolecules and readjustment of blood clotting during torpor initiation. These adjustments might play a role in defending the brain against cellular damage while being torpid. Additionally, investigation of genes associated with hypothalamic orexigenic, circadian and thyroid hormone systems indicated that SDT and FIT are just partly regulated by differing molecular mechanisms within the hypothalamus. Thus, our findings do not entirely support the hypothesis that SDT and FIT are two distinct types of torpor. Furthermore, we detected slight alterations in circulating glucose, insulin and leptin concentrations over the course of a torpor bout. This might indicate a role of glucose in torpor initiation mechanisms and of insulin and leptin during the mid torpor state potentially regulating reduced T_b values during torpor maintenance. But it has to be noted that alterations in glucose, insulin and leptin were not statistically significant. Thus, further experiments are needed to proof this hypothesis.

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Abbreviations

Actb	Actin beta
Agrp	Agouti-related protein
ANOVA	Analysis of variance
ARC	Arcuate nucleus
BAT	Brown adipose tissue
Bmal1	Brain and muscle Arnt-like protein-1
bp	Base pair
cDNA	Complementary desoxyribonucleic acid
CLOCK	Circadian locomotor output cycles kaput
col	Collagen
CRY	Cryprochrome
DGE	Digital gene expression
Dio2	Iodothyronine deiodinase 2
Dio3	Iodothyronine deiodinase 3
DNA	Desoxyribonucleic acid
Dnah2	Dynein, axonemal, heavy chain 2
FDR	False discovery rate
FIT	Fasting-induced torpor
FoxO1	Forkhead box protein O1
Hprt	Hypoxanthine phosphoribosyltransferase
IR	Insulin receptor
JAK	Janus kinase
LD	Long day
Mct8	Monocarboxylate transporter 8
MPOA	Median preoptic area
mRNA	Messenger ribonucleic acid
Myo15a	Myosin XVA
NGS	Next generation sequencing
Npy	Neuropeptide Y
NT	Non-torpid
OB-Rb	Leptin receptor isoform b
PER	Period

PI3K	Phosphatidylinositol-3-kinase
Pomc	Proopiomelanocortin
Ptpn1	Protein tyrosine phosphatase, non-receptor type 1
PVN	Paraventricular nucleus
Rn18s	18S ribosomal RNA
RNA	Ribonucleic acid
RPKM	Reads per kilobase per million mapped reads
Rplp0	Ribosomal protein lateral stalk subunit P0
RQ	Respiratory quotient
RT-qPCR	Real-time quantitative polymerase chain reaction
SCN	Suprachiasmatic nuclei
SD	Short day
SDT	Spontaneous daily torpor
SEM	Standard error of the mean
Socs3	Suppressor of cytokine signaling 3
SON	Supraoptic nucleus
STAT3	Signal transducer and activator of transcription 3
T3	Triiodothyronine
T4	L-thyroxin
T_a	Ambient temperature
T_b	Body temperature
U-test	Mann Whitney Rank Sum test
Vwf	von Willebrand factor
ZT	Zeitgeber time

Abbr. in CAPITAL letters = Protein
Abbr. in *italic* letters = mRNA

Danksagung

An erster Stelle gilt mein größter Dank Prof. Dr. Annika Herwig, die es mir ermöglicht hat in diesem spannenden Forschungsgebiet zu arbeiten und meine Dissertation zu verfassen. Deine eigene Begeisterung für die Forschung ist regelrecht ansteckend und hat mich von Anfang an mitgerissen. Du hast stets an mein Können geglaubt, auch wenn mir dann und wann mal Zweifel kamen. Auch möchte ich dir für allerlei Denkanstöße, Anregungen und die vielen Stunden Korrekturlesen danken. Du hast es stets geschafft, mich zu motivieren über meine eigenen Grenzen hinauszuwachsen und mich jeder neuen Herausforderung erfolgreich zu stellen.

Ebenso möchte ich Prof. Dr. Thorsten Burmester einen großen Dank aussprechen. Dies soll nicht nur der provisorische Dank für die Übernahme des Zweitgutachtens sein, denn du hast mich darüber hinaus während meiner gesamten akademischen Ausbildung begleitet. Ich danke dir für deine stetige Unterstützung, die weit über das übliche Maß hinausging.

Ich möchte auch meiner Arbeitsgruppe für die wunderschöne Zeit und die vielen gemeinsam gesammelten Erfahrungen danken. Hanna und Julia gilt ein ganz besonderer Dank für die tatkräftige Unterstützung im Labor und Hamsterstall und die unzähligen Stunden, die ihr unermüdlich damit zugebracht habt 96-well Platten zu bestücken. Ohne euch beide wäre diese Arbeit im vorliegenden Umfang nicht möglich gewesen.

Für die vielen tollen Tage (und auch so manche Abende) und die stete Hilfsbereitschaft möchte ich nicht nur meiner eigenen Arbeitsgruppe, sondern auch der gesamten AG Burmester danken. Dank euch allen ist die Zeit am Institut nie langweilig geworden. An dieser Stelle hervorheben mochte ich Andrej Fabrizius, dessen vielseitige Hilfestellungen und Denkanstöße mir bei der Lösung so mancher Probleme geholfen haben.

Zum Abschluss möchte ich mich bei meiner über alles geliebten Familie, meinem Freund und meiner besten Freundin bedanken. Ihr habt immer fest an mich geglaubt und mir geholfen in schwierigen Momenten den Durchblick zu behalten. Besonders in kritischen Phasen habt ihr mich geerdet und mich gelehrt Arbeit auch mal Arbeit sein zu lassen. Insbesondere meiner Mutter und meinem Vater danke ich dafür, dass ihr mich in allen Lebenslagen unterstützt habt.
Es ist schön euch in meinem Leben zu haben und zu wissen, dass ich mich in jeder nur erdenklichen Hinsicht voll auf euch verlassen kann.

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe verfasst, ganz oder in Teilen noch nicht als Prüfungsleistung vorgelegt und keine anderen als die angegebenen Hilfsmittel benutzt habe. Die Stellen der Arbeit, die anderen Quellen im Wortlaut oder dem Sinn nach entnommen wurden, sind durch Angabe der Herkunft kenntlich gemacht. Dies gilt ebenso für Abbildungen. Abbildungen die mit keiner Quelle vermerkt wurden, wurden von mir persönlich erstellt.

Ich habe zur Kenntnis genommen, dass ich bei nachgewiesenem Betrugsfall die eventuell entstehenden Kosten eines Rechtsstreites zu übernehmen habe und mit weiteren Sanktionen rechnen muss.

Hamburg, den _____

Ceyda Cubuk