# Influence of thyroid hormones on seasonal regulation of body weight, daily torpor and gene expression in Djungarian hamsters (*Phodopus sungorus*)

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*I applied my mind to study and to explore*
*by wisdom all that is done under the heavens.*
*What a heavy burden God has laid on mankind!*
*King Salomon of Israel (Ecclesiastes 1:13)*

# Abstract

## Introduction

Seasonal mammals live in environments with extreme annual changes in ambient temperature and food availability. In order to survive these energetically challenging conditions, they show massive physiological and morphological adaptations throughout the year that are driven by changes in day length. A well-studied seasonal animal model for seasonal adaptation is the Djungarian hamster (Phodopus sungorus). Hamsters cease reproduction, voluntarily reduce body weight and change fur well in advance of the coming winter season to reduce energy expenditure. During winter they express spontaneous daily torpor, a state of hypometabolism and hypothermia, to additionally reduce energy demands. These hamsters decode seasonal changes in day length by translating the photoperiodic signal into nocturnal melatonin secretion from the pineal gland. One target site of melatonin is the *pars tuberalis* where it inhibits TSH production and thereby indirectly influences thyroid hormone metabolism especially in the nearby hypothalamus. In the past 20 years several studies provided evidence that local thyroid hormone conversion in the hypothalamus plays a critical role during the transition from summer to winter. However, the link between thyroid hormones and regulation of body weight and torpor is not well understood.

## Methods

One experiment was performed to provide more information about the influence of photoperiodic changes on hypothalamic gene expression. After an initial collective short day adaptation different hamster groups were switched between long- and short photoperiod and gene expression was analyzed at several stages by *in situ* hybridization. Changes in gene expression were correlated with changes in body weight. Two further experiments were performed to reveal the acute influence of thyroid hormones on body weight, food intake and especially expression of torpor. Hamsters were made hyperthyroid by giving $T_4$ or $T_3$ via drinking water or hypothyroid by treatment with methimazole. Body weight and food intake were regularly weighted and torpor expression was monitored by continuous recording of body temperature. In a second approach hamsters were centrally treated with $T_3$ via microdialysis probes placed in close proximity to the hypothalamus. Both experiments were terminated after two weeks and effects of treatment on gene expression in hypothalamus, brown adipose tissue and skeletal muscle were analyzed by qPCR.

## Results

Alternations between photoperiods reveled that increasing body weight after the switch from SP to LP was linked to increasing expression of *dio2, vimentin, crbp1* and *gpr50* as well as reduced expression of *dio3, mct8* and *srif*. Hamsters were able to respond to SP a second switch after six and

14 weeks in LP. Small, but significant differences in hypothalamic gene expression showed, that transcription of deiodinases, responsible for $T_3$ metabolism, followed a fine tuned regulation, which seems to play a key role in body weight adaptation.

Systemic treatment with $T_3$ led to an increase of body weight after 10 days and more importantly to a direct inhibition of daily torpor. Contrarily hypothyroidism led to increased torpor frequency with prolonged and deeper torpor bouts. This showed that low $T_3$ availability seems to be a prerequisite for torpor induction. Central $T_3$ treatment led to an inhibition of torpor, too. Subtly nuanced differences between systemic and central treatment suggest that torpor is primarily regulated by the hypothalamus and subsequently by peripheral influences.

Gene expression analysis by quantitative PCR revealed that expression of *dio2* and uncoupling proteins was regulated in a treatment and tissue specific manner. Interestingly *dio2, ucp1* and *ucp3* were downregulated during torpor. This suggests low intracellular $T_3$ activation during torpor, which might have been be the reason for reduced *ucp1* and *ucp3* expression that might result in reduced thermogenesis during torpor.

### Conclusion

All together these experiments shed more light on long-term photoperiodic control of body weight and gene expression, especially genes related to thyroid hormone metabolism. Furthermore, results of this dissertation provide new evidence for the strong influence of thyroid hormones on torpor expression. Low intracellular $T_3$ availability in the hypothalamus is essential for seasonal body weight reduction and expression of daily torpor.

# Zusammenfassung

### Einleitung

Saisonale Säugetiere leben in einer Umwelt mit extremen, jahreszeitlichen Änderungen der Außentemperatur und Futterverfügbarkeit. Um diese energetische Herausforderung zu überleben, zeigen sie deutlich ausgeprägte physiologische und morphologische Anpassungen an die vorherrschende Jahreszeit, welche durch Änderungen der Tageslänge gesteuert werden. Ein gut erforschtes Tiermodell für saisonale Anpassung ist der Dsungarische Zwerghamster (*Phodopus sungorus*). Bereits vor Beginn der Winterjahreszeit stellen diese Hamster ihre Reproduktion ein, reduzieren freiwillig ihr Körpergewicht und wechseln das Fell um insgesamt den Energieverbrauch zu reduzieren. Um den Energiebedarf noch weiter zu senken, können sie während des Winters zusätzlich spontanen täglichen Torpor zeigen, welches ein hypometaboler und hypothermer Zustand ist. Die

Dsungarischen Zwerghamster nehmen jahreszeitliche Änderungen der Tageslänge wahr, indem sie das photoperiodische Signal in nächtliche Melatonin Ausschüttung aus der Epiphyse umwandeln. Eine Zielregion von Melatonin ist unter anderem die *Pars tuberalis* des Hypophysenvorderlappens, wo es die Produktion des Thyreoidea-stimulierenden Hormons hemmt. Dadurch beeinflusst Melatonin indirekt den Schilddrüsenhormonstoffwechsel, ganz besonders im Hypothalamus, welcher nahe an der *Pars tuberalis* liegt. In den letzten 20 Jahren haben mehrere Studien Hinweise geliefert, dass der lokale Schilddrüsenhormonstoffwechsel im Hypothalamus eine sehr wichtige Rolle bei der Anpassung vom Sommer zum Winter spielt. Allerdings ist der Zusammenhang zwischen Schilddrüsenhormonen und der Regulation von Körpergewicht und Torpor bisher noch weitestgehend unklar.

## Methoden

Das erste Experiment dieser Arbeit wurde durchgeführt, um mehr Informationen über den Einfluss von photoperiodischen Veränderungen auf die Genexpression im Hypothalamus zu gewinnen. Dafür wurden zunächst mehrere Hamstergruppen gemeinsamen an eine kurze Photoperiode adaptiert und anschließend gezielt zwischen langer- und kurzer Photoperiode transferiert. So konnte die Genexpression zu verschiedenen Zeitpunkten mit Hilfe von *in situ* Hybridisierung analysiert werden, um Veränderungen in der Transkription mit Änderungen des Körpergewichts zu korrelieren.

Zwei weitere Experimente wurden durchgeführt, um den direkten Einfluss von Schilddrüsenhormonen auf das Körpergewicht, die Futteraufnahme und besonders das Auftreten von Torpor deutlich zu machen. Dafür wurde bei einigen Hamstern durch die Gabe von $T_4$ oder $T_3$ über das Trinkwasser eine systemische Hyperthyreose induziert, während bei anderen Hamstern eine Schilddrüsenunterfunktion durch Methimazol erzeugt wurde. Das Körpergewicht und die Futteraufnahme wurden regelmäßig bestimmt und das Auftreten von Torpor wurde fortlaufend durch das Aufzeichnen der Köpertemperatur überwacht. Im folgenden Experiment wurde eine Mikrodialysesonde unmittelbar am Hypothalamus platziert und dadurch wurde den Hamstern $T_3$ nur zentral appliziert. Nach zwei Wochen wurden beide Experimente beendet und der Einfluss der Behandlung auf Änderungen in der Genexpression im Hypothalamus, braunem Fettgewebe und Skelettmuskulatur wurden mittels quantitativer PCR untersucht.

## Ergebnisse

Durch die gerichteten Wechsel zwischen den Photoperioden wurde offenbart, dass der Anstieg im Körpergewicht nach dem Wechsel von kurzer- zu langer Photoperiode mit einer Veränderung der Genexpression zusammen hing. Der Anstieg der Genexpression von *dio2, vimentin, crbp1* und *gpr50* sowie die Abnahme der Expression von *dio3, mct8* und *srif* waren mit der Gewichtszunahme verbunden. Hamster die anschließend für sechs bzw. vierzehn Wochen in einer langen Photoperiode

gehalten wurden, waren erstaunlicherweise in der Lage sich erneut an eine kurze Photoperiode anzupassen. Kleine, aber wesentliche Unterschiede in der hypothalamischen Genexpression zeigten, dass die Transkription von Dejodasen einer feinabgestimmten Steuerung unterliegt. Diese Enzyme sind für den intrazellulären Schilddrüsdenhormonestoffwechsel verantwortlich und spielen scheinbar eine Schlüsselrolle in der Körpergewichtsanpassung.

Die Systemische Behandlung mit $T_3$ im zweiten Experiment führte nach zehn Tagen zu einem Anstieg des Körpergewichts und zu einer direkten Hemmung des täglichen Torpors. Die induzierte Schilddrüsenunterfunktion führte hingegen zu einem häufigeren Auftreten von Torpor mit verlängerten und tieferen Torpor-Phasen. Dies deutet darauf hin, dass eine geringe $T_3$-Verfügbarkeit eine Voraussetzung für die Induktion von Torpor ist. Auch die zerebrale Behandlung führte zu einer Hemmung von Torpor. Feine Unterschiede zwischen systemischer- und zerebraler Behandlung legen nahe, dass Torpor primär durch den Hypothalamus gesteuert wird und erst nachfolgend durch somatische Einflüsse reguliert wird.

Genexpressionsanalyse durch qPCR offenbarte, dass die Expression von *dio2* und Entkopplerproteinen (*ucp*) in einer behandlungs- und gewebsspezifischen Weise gesteuert wurde. Interessanterweise waren *dio2, ucp1* und *ucp3* während des Torpors herunterreguliert. Dies ist ein Hinweis, dass während des Torpors wenig $T_3$ innerhalb von Zellen aktiviert wird. Dies kann ein Grund für die reduzierte *ucp1* und *ucp3* Expression sein, welches wiederum an der Reduzierung von Wärmebildung während des Torpors beteiligt sein könnte.

**Fazit**

Insgesamt geben alle Experimente meiner Arbeit mehr Aufschluss über die langfristige Steuerung von Körpergewicht und der beteiligten Genexpression. Besonders über Gene, die im Schilddrüsenhormonstoffwechsel involviert sind, wurden neue Erkenntnisse gewonnen. Zusätzlich geben die gewonnen Ergebnisse neue Hinweise auf einen starken Einfluss von Schilddrüsenhormonen auf das Auftreten von Torpor. Besonders eine geringe intrazelluläre $T_3$ Verfügbarkeit im Hypothalamus scheint entscheidend für die jahreszeitliche Körpergewichtsreduktion und das Auftreten von täglichem Torpor zu sein.

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# 1. Introduction

## 1.1 Annual changes in photoperiod and environment

Mammals in temperate or continental climate zones live in an environment with extreme changes in ambient temperature ($T_a$) and food availability. To adapt to these changes well in advance animals need a reliable environmental cue that triggers pronounced seasonal adaptations in morphology, physiology and behavior. The only consistent signal is day length (photoperiod), which changes in a predictable and constant manner every year. In Djungarian hamsters (*Phodopus sungorus*) critical day length to start winter adaptations is approximately 13 hours (Gorman and Zucker, 1995; Hoffmann, 1982). $T_a$ and food availability show high variability from year to year and play only a secondary and modularity role on seasonal adaptations. Mammals are not able to induce winter adaptations without the photoperiodic cue (Goldman, 2001; Paul et al., 2008).

The photoperiodic information is perceived via the eye and is integrated by a neuronal pathway in the brain. Retinal ganglion cells, which contain melanopsin, form the retino-hypothalamic tract and project to the suprachiasmatic nucleus (SCN) of the hypothalamus via the optic nerve and optic chiasm (Foster and Hankins, 2007). This pathway translates incoming exogenous information about day length into an endogenous signal. The SCN is the brain area, which is also known as the circadian clock, responsible for synchronization of circadian endocrine rhythms (Schwartz et al., 2001). Information from the SCN about day length are translated into nocturnal melatonin production in the pineal gland (Simonneaux and Ribelayga, 2003).

With seasonal changes in day length melatonin secretion during the night is proportionally adapted. Thus the duration of melatonin secretion provides an internal signal for the length of the night and thereby also for the time of year (Ebling, 2015; Wood and Loudon, 2014). The main site for melatonin action is the *pars tuberalis* (PT) of the pituitary gland that possesses a high density of melatonin receptors ($MT_1$) (Kell and Stehle, 2005; Morgan et al., 1994). Several genes regulated by melatonin have been identified in the PT (Wagner et al., 2007). One of these genes is the TSH subunit *tsh-ß*, which transcription seems to be inhibited by melatonin during winter (Böckers et al., 1997). TSH in turn has been shown to activate deiodinase type 2 (DIO2) in tanycytes, glial cells lining the third ventricle of the hypothalamus, in a season specific manner in birds and mammals (Nakao et al., 2008a; Revel et al., 2006; Watanabe et al., 2004; Yasuo et al., 2006; Yoshimura et al., 2003). DIO2 is an important enzyme involved in thyroid hormone metabolism (see chapter 1.3.2). Several studies have shown that thyroid hormone metabolism in the hypothalamus seems to be controlled by up- and downregulation of deiodinases in a photoperiodic manner and this has been suggested to be a critical step between rhythmic melatonin secretion and physiological seasonal adaptations (Barrett et

al., 2007; Hazlerigg and Wagner, 2006; Herwig et al., 2009; Herwig et al., 2013; Prendergast et al., 2002; Revel et al., 2006; Viguié et al., 1999; Watanabe et al., 2004). There is a lot of evidence that thyroid hormones play a critical role in seasonal adaptations, but many details of this complex correlation are not well understood.

## 1.2 Seasonal adaptations

All endothermic animals living in a seasonal environment face the challenge to keep their body temperature ($T_b$) at approximately 37°C, while being exposed to extreme changes in $T_a$. The big difference between $T_b$ and $T_a$ during winter leads to an increased energy demand to defend a constant high $T_b$. This energetic challenge is critical especially for small mammals. Due to their unfavorable surface to volume ratio they lose heat more rapidly. The greater heat production to compensate heat loss is concurrent with reduced food availability during cold winter. To overcome this paradoxical situation seasonal mammals are equipped with a wide range of adaptations to reduce energy requirements.

### 1.2.1 Djungarian hamster

A well-studied animal model for seasonal adaptations is the Djungarian hamster (*Phodopus sungorus*, also known as Siberian hamster) (Ebling, 1994; Flint, 1966). These hamsters originate from the steppes of Siberia and Kazakhstan, where they face extremely cold temperatures down to -40°C (Flint, 1966). Despite those cold temperatures they have been observed to be active during night at -34°C (Heldmaier and Steinlechner, 1981a). Djungarian hamsters are able to tolerate these low temperatures, because they increase their capacity for non-shivering thermogenesis (NST, see chapter 1.4.2) in brown adipose tissue (BAT) (Heldmaier et al., 1982a; Heldmaier et al., 1985; Rafael et al., 1985). Moderate cold exposure with $T_a$ below the hamsters' thermoneutral zone (ca. 20°C) is sufficient to increase NST capacity, which reaches its maximum at 10°C (Heldmaier et al., 1982b; Wiesinger et al., 1989). Increased NST capacity is mainly a mechanism to survive cold, but these animals possess more physiological adaptations to save energy during the winter season and to survive the extreme energetic challenge.

These adaptations are primarily driven by photoperiod, start well in advance of the coming winter and include quiescence of reproduction, increased fur insulation, reduction of body weight and expression of daily torpor (Scherbarth and Steinlechner, 2010). Reproduction is ceased during winter by significant reduction of gonads (Hoffmann, 1979a; Schlatt et al., 1993), because mating and rearing offspring would demand to much energy during winter. To reduce heat loss, hamsters change

their greyish brown winter fur gradually into a whitish winter fur (Figala et al., 1973). The winter fur has a high insulating function that allows hamsters to reduce their energy expenditure during winter (Kauffman et al., 2001). Reduction of body weight (see chapter 1.2.2) and expression of daily torpor (see chapter 1.2.3) lead to further reduction of energy requirements.

Under natural photoperiod hamsters start to increase body weight and gonads in late January (Figala et al., 1973). Experiments with artificial SP reveled that hamsters become spontaneously refractory to the melatonin signal between 18 and 30 weeks in SP, meaning that hamsters regain body weight an reverse all SP characteristics (Bittman, 1978b; Freeman and Zucker, 2001; Gorman and Zucker, 1995; Reiter, 1972). Before the onset of physiological changes due to refractoriness gene expression of seasonally regulated genes returns to LP-like expression patterns (Herwig et al., 2013). After more than 10 weeks in LP hamsters regain responsiveness to melatonin and are able to adapt to SP again (Reiter, 1972; Teubner et al., 2008).

### 1.2.2 Body weight

The seasonal body weight cycle of Djungarian hamsters is precisely regulated by photoperiod. Weight loss starts well in advance of the coming winter season and is no consequence of cold or reduced food availability. Hamsters reduce their body weight by up to 50% mainly by voluntary reduction of food intake (Figala et al., 1973; Wade and Bartness, 1984). This profound body weight loss requires several weeks to be completed and hamsters reach a stable low body weight after approximately ten weeks (Gorman and Zucker, 1995; Wade and Bartness, 1984). Reduced body weight leads to a reduced metabolic mass, which requires lower food intake to sustain high $T_b$. The combination of reduced body weight and low thermal conductance can decrease the energy demand by up to 37% (Heldmaier, 1989).

Body weight reduction can be induced by short photoperiod (SP) under laboratory conditions at any time of the year. The critical day length for Djungarian hamsters transferred from LP to SP is around 13 hours (Hoffmann, 1982). A photoperiod with shorter days than this critical length triggers the adaptation to winter season. The reduction in body weight can be reversed at any time by long photoperiod, which leads to an immediate increase in body weight (Hoffmann, 1979b). The gradual decrease in body weight is precisely regulated around a sliding set-point. When temporally food restricted, hamsters on their downwards curve during SP loose body weight faster, but return to their season specific set-point, when fed *ad libitum* (Mercer et al., 2001; Steinlechner et al., 1983). The loss of body mass is mainly a depletion of white adipose tissue, but also a reduction of gonads, organ- and bone mass (Bartness, 1996; Dumbell et al., 2015; Gorman and Zucker, 1995; Hoffmann, 1979a; Scherbarth et al., 2008; Wade and Bartness, 1984).

Reduction of adipose tissue causes a reduction of circulating leptin concentrations in the blood (Korhonen et al., 2008). Leptin is produced in adipocytes and gives a feedback about energy balance to the hypothalamus (Brennan and Mantzoros, 2006; Havel, 2000). The special seasonal body weight adaptation of Djungarian hamsters requires a particular molecular regulation. Interestingly LP hamsters have high leptin concentrations, but leptin does not induce a catabolic response, which would lead to a body weight reduction (Atcha et al., 2000; Klingenspor et al., 2000; Rousseau et al., 2002). Under SP conditions increased leptin sensitivity, triggered by the suppressor of cytokine signaling 3 in the hypothalamus, leads to an activation of anorexic and catabolic response and hamsters start to reduce body fat (Tups et al., 2004; Tups et al., 2006).

There is good evidence, that seasonal body weight regulation is controlled by the hypothalamus, but it is not clear which distinct areas and pathways are involved in this process. Different neuron populations in the arcuate nucleus (ARC) regulate food intake and energy expenditure in non-seasonal animal models such as rats and mice (Abizaid et al., 2006). Activation of neurons containing proopiomelanocortin (POMC) reduces food intake, whereas neuropeptide Y (NPY) producing neurons play the counterpart and stimulate food intake (Dietrich and Horvath, 2013; Herwig et al., 2008). However, no study could detect clear seasonal changes in expression of these genes. Hence there is no evidence, that these systems regulate the long-term change between an anabolic summer state and a catabolic winter state (Mercer et al., 1995; Reddy et al., 1999; Rousseau et al., 2002; Schuhler et al., 2003). Furthermore, lesion of the ARC in Djungarian hamsters could not prevent the normal photoperiodic response in reduced food intake and body weight (Ebling et al., 1998). Thus it has been proposed, that the ARC is responsible for regulating short-term energy balance, whereas long-term seasonal changes are regulated by a different, so far unknown, pathway (Ebling and Barrett, 2008).

### 1.2.3 Daily torpor

To save even more energy hamsters are able to enter spontaneous daily torpor, a hypometabolic and hypothermic state. After exposure to SP for 10-13 weeks, when gonads are regressed and body weight is close to its nadir, hamsters start to enter torpor spontaneously and unpredictably (Bartness et al., 1989; Diedrich et al., 2015b; Ruf et al., 1993). The ability to enter torpor is primarily triggered by photoperiod and hamsters even become torpid when kept at room temperature and with food *ad libitum* (Elliott et al., 1987; Heldmaier, 1989). Torpor expression is under circadian control and usually limited to the daily resting phase (Kirsch et al., 1991). Torpor entry (Figure 1) is characterized by a reduction in metabolic rate (MR) and heart rate before the $T_b$ declines (Elvert and Heldmaier, 2005;

Heldmaier et al., 2004; Morhardt, 1970). Djungarian hamsters can reduce their MR by up to 45%. This severe hypometabolism leads to reduced $T_b$ between 13°C and 32° (Heldmaier, 1989; Heldmaier and Ruf, 1992; Ruf et al., 1993).

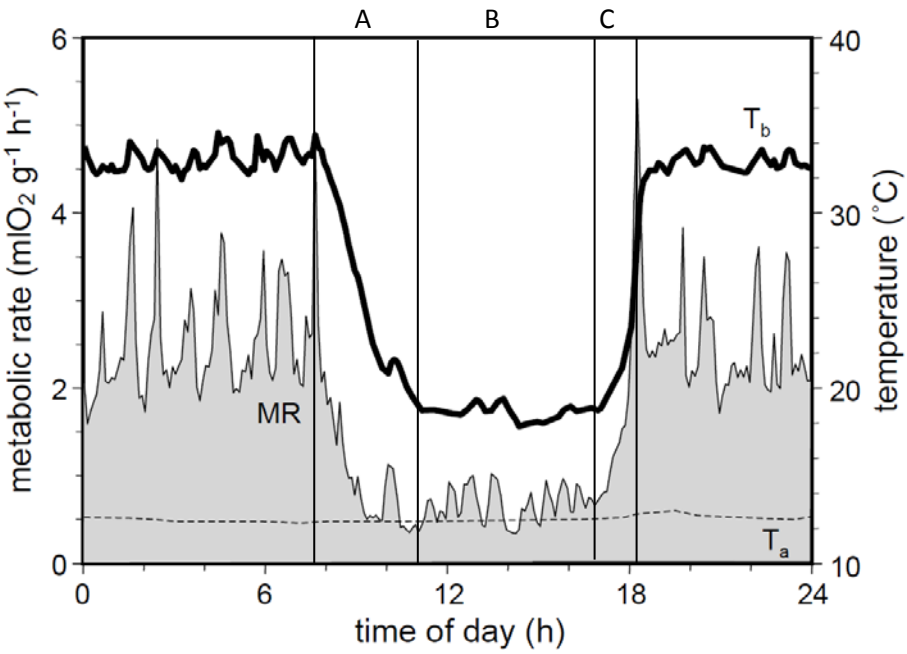


**Figure 1:**

**Exemplary torpor bout** (adapted from Heldmaier et al. 2004). Metabolic rate (MR) is reduced prior to the declining body temperature ($T_b$). A torpor bout always follows a similar course with entry- (A), maintenance- (B) and arousal -phase (C). During the arousal MR and $T_b$ return to normal levels. Longer torpor bouts have mainly an elongated maintenance phase.

Torpor bouts have an average duration of six to eight hours and can last up to 14 hours (Kirsch et al., 1991; Ruf et al., 1989; Ruf et al., 1993). Torpor is terminated by arousal and hamsters return to normothermia within 30 minutes (Heldmaier et al., 2004). With a regular use of daily torpor up to 67% of energy demands can be saved in the long-term (Heldmaier, 1989; Ruf and Heldmaier, 1992).

Torpor season is limited to a period of approximately ten weeks and ceases when hamsters become photorefractory and regain body and gonadal weight (Diedrich et al., 2015b; Kauffman et al., 2003; Lincoln et al., 2005; Ouarour et al., 1991).

The pathways underlying metabolic depression during torpor are only partially known and understood (Berriel Diaz et al., 2004; Heldmaier et al., 1999). Interestingly, an intact noradrenergic signaling of the sympathetic nervous system (SNS) is required for the expression of torpor and torpor frequency shows a positive correlation with increased capacity of NST (Braulke and Heldmaier, 2010; Jefimow et al., 2004). Gonadal regression with low androgen serum levels, low prolactin and leptin levels are also prerequisites for torpor expression, but the ultimate torpor inducing mechanism is still unknown (Elliott et al., 1987; Freeman et al., 2004; Ouarour et al., 1991; Vitale et al., 1985). The ARC seems to play a role in torpor regulation (Pelz et al., 2008), but the precise neuronal mechanisms and pathways of torpor regulation are a great mystery.

# 1.3 Thyroid hormones

## 1.3.1 Hypothalamic-Pituitary-Thyroid Axis

Thyroid hormones affect nearly every cell in the body of humans and mammals and regulate many essential physiological processes (see chapter 1.3.3). Under normal physiological conditions, the hypothalamic-pituitary-thyroid axis (HPT-axis) maintains stable thyroid hormone concentrations in blood serum. Neurons of the paraventricular nucleus (PVN) in the hypothalamus, which produce thyrotropin-releasing hormone (TRH), are the core of the HPT-axis (Figure 2). TRH defines the set point for thyroid hormone production in the thyroid gland by regulating the secretion of thyroid-stimulating hormone (TSH) in the pituitary gland (Harris et al., 1978; Lechan and Fekete, 2006; Nikrodhanond et al., 2006; Nillni, 2010; Persani, 1998). TSH is a glycoprotein and consisting of two subunits (α and β) and its synthesis is mediated by the TRH receptor 1 (Rabeler et al., 2004). The α-subunit is common for all glycoprotein hormones of the anterior pituitary, but the β-subunit is TSH-specific (Hashimoto et al., 2000).

Thyroid hormones are synthesized in the thyroid gland, which is an endocrine organ placed in the anterior neck consisting of two lobes. The prohormone L-thyroxine ($T_4$, 3,3',5,5'-Tetraiod-L-thyronine) is synthesized from the precursor protein thyroglobulin in follicular cells of the thyroid. Triiodothyronine ($T_3$, 3,3',5-Triiod-L-thyronine) is produced in the thyroid as well, but most $T_3$ is produced outside the thyroid by deiodination of $T_4$ (Bianco et al., 2014). $T_4$ and $T_3$ secreted from the thyroid into the blood stream are mostly bound to transport proteins like thyroxine-binding globulin, transthyretin or albumin. Less than 0.02% of $T_4$ and 0.3% of $T_3$ are unbound and considered as free $T_4$ or free $T_3$ respectively. Bound thyroid hormones are not biological active.

When circulating $T_3$ concentrations are elevated a feedback signal reduces the activity of the HPT-axis (Dyess et al., 1988). $T_3$ has the ability to directly suppress the transcription and posttranslational processing of TRH in the PVN (Perello et al., 2006; Segerson et al., 1987; Sugrue et al., 2010). Furthermore, secreted TRH can be deactivated in the median eminence (ME) by pyroglutamyl peptidase II (PPII), which is expressed in tanycytes (Charli et al., 1998; Sánchez et al., 2009). PPII expression can be upregulated by $T_3$ during hyperthyroidism (Marsili et al., 2011). Thyroid hormones can also exert a negative feedback on pituitary TSH secretion. $T_4$ itself does not downregulate TSH gene expression, but $T_4$ can be locally converted to $T_3$ in pituitary cells (Larsen et al., 1981; Visser et al., 1983). The feedback on TSH production is mainly executed by $T_3$ via thyroid receptor β2 (O'Shea and Williams, 2002; Weiss et al., 1997).

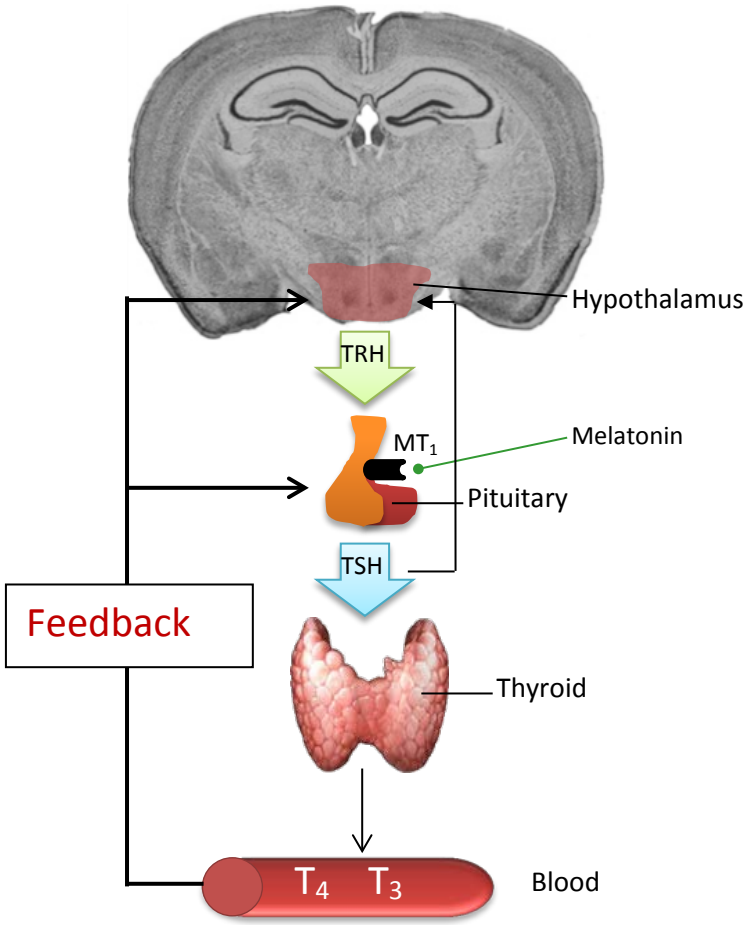


**Figure 2: Schematic summary of the Hypothalamic-Pituitary-Thyroid-axis:** TRH is synthesized and released from the hypothalamus. It stimulates the production of TSH in the pituitary gland. TSH itself controls the synthesis of thyroxin ($T_4$) in the thyroid gland. $T_4$ and small amounts of triiodothyronine ($T_3$) are released into the bloodstream and transported to target tissues. There $T_4$ can be converted into $T_3$ by deiodinases.

The concentrations of circulating thyroid hormones in the blood give a feedback to the pituitary and hypothalamus and increases or decreases the secretion of TSH as well as to a lesser extend the secretion of TRH. Melatonin can bind to $MT_1$ receptors in the *pars tuberalis* of the pituitary gland and increases the production of the TSH-ß subunit. Therefore, the HPT-axis is under photoperiodic control and the annual set point for thyroid hormone production can be changed.

Reduced food availability, decreased $T_a$ or diseases can change the demand of $T_3$ regulated by TRH production (Hollenberg, 2008). Decreased thyroid hormone concentrations in the blood below normal values, referred to as hypothyroid state, are sensed by the hypothalamus and TRH production changes the set point for thyroid hormone production. Currently, two hypothalamic subgroups (arcuate nucleus, dorsomedial nucleus) are known to give a feedback to TRH-neurons (Fekete and Lechan, 2007; Füzesi et al., 2009). This hypothyroid feedback leads to an increase of TRH production in the PVN and secretion into the portal blood to the anterior pituitary, where it stimulates TSH production (Dahl et al., 1994; Rondeel et al., 1992). The precise mechanisms are very complex and not fully known to date. Especially feedback mechanisms and local thyroid hormone metabolism in the brain are not well understood.

### 1.3.2 Thyroid hormone transport and metabolism

Despite being lipophilic, $T_4$ and $T_3$ have to be actively transported into target cells, where they can exert their biological functions. Several transport molecules, which are able to transport thyroid hormones to a different extent, have been identified. One important transporter with a high affinity for $T_4$ is the organic anion transporter polypeptide-related protein 5 (OATP1c1, Slco1c1). This protein is a transmembrane receptor that mediates the uptake of thyroid hormones into brain cells. A second important transport protein is the monocarboxylate transporter 8 (MCT8, Slc16a2), which has a higher affinity for $T_3$, but can also facilitate the transport of $T_4$, reverse $T_3$ ($rT_3$, 3,3'5'-triiodothyronine), and diiodothyronine ($T_2$, 3,3'-diiodothyronine). Furthermore, MCT8 plays an important role for thyroid hormone transport in the brain. So far two monocarboxylate transporters, seven organic anion transporters and two L-type amino acid transporters (LAT) with the potential to transport thyroid hormones have been identified in humans (Visser, 2000; Wirth et al., 2014), but only MCT8 has been found in Djungarian hamsters so far (Herwig et al., 2009).

Thyroid hormones can intracellularly be converted by deiodinase enzymes into different metabolites (Figure 3) (Gereben et al., 2008; Köhrle, 1999). Deiodinase type 1 (DIO1) is mainly expressed in thyroid, liver and kidney and is catalyzing both 5'-deiodination of the phenolic ring (activation by converting $T_4$ into $T_3$) and 5-deiodination of the tyrosyl ring (inactivation of T4 → rT3 and T3→ T2). However, the preferred substrates for DIO1 are $rT_3$ > $T_4$ > 3',5'-$T_2$ > 3,3'-$T_2$. Under normal physiological conditions DIO1 activates most of the circulating $T_3$ and its expression can be induced by thyroid hormones itself, but also by retinoic acid or cAMP in thyrocytes. For local metabolism of $T_3$ in other thyroid hormone target tissues, independent of circulating $T_3$, deiodinases type 2 (DIO2) and 3 (DIO3) are required. DIO2 is a membrane protein in the endoplasmatic reticulum (Baqui et al., 2000). It is mainly localized in the brain, pituitary gland, BAT, skeletal muscle, heart, placenta and skin.

The subcellular localization of DIO2 indicates that $T_3$ activation occurs in close proximity to the nucleus. Most effects exerted by $T_3$ are genomic actions via thyroid hormone-receptor (TR) mediated pathways in nuclear compartments involving modulation of gene transcription. The approximately 10-fold greater TR affinity for $T_3$ in comparison to $T_4$ is one reason for the higher biological activity of $T_3$ (Apriletti et al., 1981; Ichikawa et al., 1986; Samuels et al., 1979; Sandler et al., 2004). In addition to genomic actions, there are also some non-genomic effects driven by $T_3$, such as the direct modulation of protein or enzyme functions (Davis and Davis, 1996).

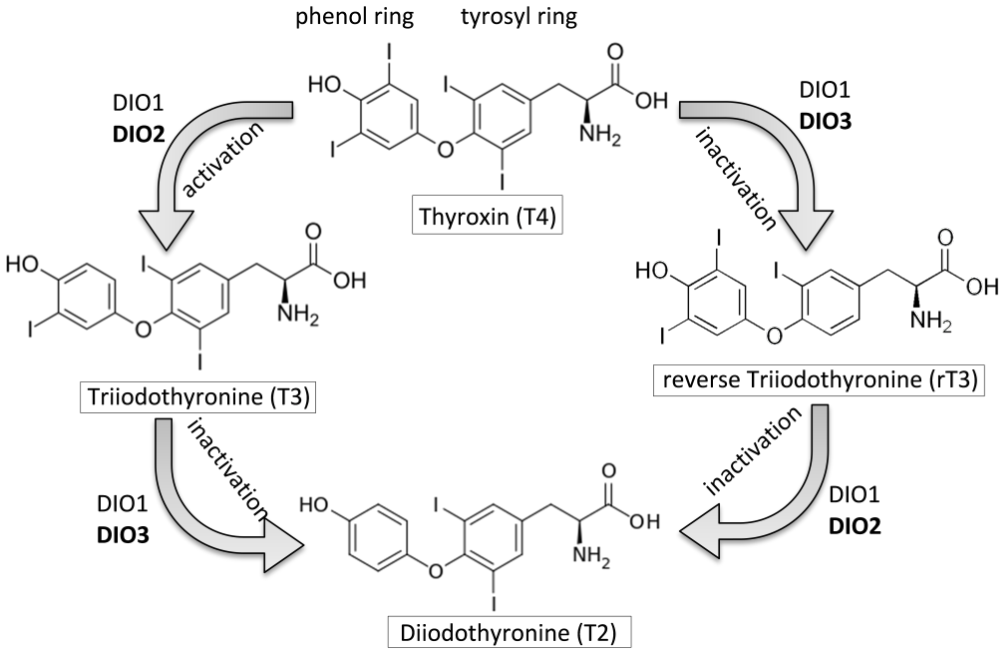


**Figure 3: Thyroid hormone metabolism by deiodinase enzymes.** DIO1 catalyzes the deiodination of $T_4$ at both phenol- and tyrosyl ring to the same extent, resulting in either generation of active $T_3$ or inactive $rT_3$. However, the preferred substrate for DIO1 is $rT_3$ and therefore, is also involved in the degradation of $rT_3$ and $T_3$. DIO2 is a phenol ring deiodinase enzyme and converts $T_4$ into $T_3$ or $rT_3$ into $T_2$. DIO3 almost exclusively catalyzes the conversion of $T_4$ to $rT_3$ and $T_3$ to $T_2$. Thus DIO3 is a thyroid hormone inactivating enzyme.

The action of DIO2 can be reduced by $T_4$, because it accelerates the inactivation of DIO2 by ubiquitination (Abdalla and Bianco, 2014; Gereben et al., 2008; Werneck de Castro et al., 2015). $T_3$ can be further metabolized and deactivated by DIO3, which removes iodide from the 5-position of the tyrosyl ring. In the same manner it is also able to convert $T_4$ into the inactive $rT_3$. The DIO3 enzyme is an integral membrane protein and located in many tissues like brain, skin and placenta, but is never co-expressed with DIO1 (Gereben et al., 2008). Overall, the complex regulation of thyroid hormone binding, transport, metabolism and action indicates a fine-tuned control mechanism.

### 1.3.3 Effects of thyroid hormones

Thyroid hormones are essential for humans and mammals and are necessarily involved in the control of metabolic rate, thermoregulation, protein synthesis, neuronal activity, growth, embryonal development (Brent, 2012; Cheng et al., 2010; Hollenberg, 2008; López et al., 2013; Silva, 2006). Importance of thyroid hormones is exemplified in patients or animals with thyroid dysfunction, which leads to severe alterations in energy metabolism and multiple diseases. Dysregulation of the thyroid axis and thyroid hormone production results in either hypothyroidism or hyperthyroidism with many different clinical syndromes. To understand the complex regulation of thyroid hormones is of prime

importance for treating dysregulation of metabolism, body temperature, body development and others.

To study physiological effects of thyroid hormones animals can either be directly treated with different thyroid hormones or rendered hypothyroid by drugs like methimazole (MMI), propylthiouracil (PTU) or sodium perchlorate (Groba et al., 2013; Marsili et al., 2010). MMI and PTU inhibit the enzyme thyroperoxidase in the thyroid, which is a critical step in the synthesis of thyroid hormones. Sodium perchlorate can additionally inhibit the activity of sodium-dependent iodide transporters, which reduces the availability of iodide for thyroid hormone synthesis in the thyroid gland. Another way to inhibit the activity of deiodinase enzymes is by iopanoic acid, flavonoids and others (Köhrle, 2000). However, selective inhibitors for the three different deiodinases are not available yet.

Many years physicians thought that thyroid hormones mainly exert their effects in peripheral tissues and that the brain has only regulatory function. However, the current understanding is that thyroid hormones act in tissues as well as directly in the brain (Herwig et al., 2008). The hypothalamus is the brain area, which is considered as the center of energy homeostasis and responsible for thermoregulation, food intake, energy expenditure and several other essential metabolic processes affected by thyroid hormones (reviewed by Münzberg et al., 2016). Therefore, this brain area came into focus of research on mechanisms of thyroid hormone action in the brain and several components of thyroid hormone metabolism have been found in the hypothalamus. Moreover, it has been shown that the hypothalamus can independently regulate its own thyroid hormone homeostasis (Lechan and Fekete, 2005). It is remarkable that in brain tissues $T_4$ and $T_3$ concentrations are in an equimolar range (Köhrle, 2000). $T_3$ concentrations are sometimes even higher than those of $T_4$, which is unusual for all other tissues than the brain. Moreover, the hypothalamus can change the set point for thyroid hormone production in the periphery (see chapter 1.3.1) and is the link between the neuronal- and endocrine system, because of its direct connection to the pituitary gland.

Thyroid hormone metabolism in the hypothalamus occurs in tanycytes, a unique glia-related cell type in this brain region. They consist of a cell body lining the third ventricle and have elongated single basal processes penetrating different areas of the hypothalamus (DMH, VMH, ARC, ME, see Figure 4) (Bolborea and Dale, 2013; Mathew, 2008). Like this, tanycytes build a morphological link between the cerebrospinal fluid (CSF) and discrete regions of the hypothalamus (Rodríguez et al., 2005). While the morphology of tanycytes is well described, their physiological role is poorly understood. Because of their strategic position, tanycytes can take up $T_4$ from the CSF or bloodstream through MCT8 (Friesema et al., 2006; Mayerl et al., 2014). Several studies could show the expression of deiodinases in tanycytes, which enables these cells to intracellularly activate or deactivate $T_3$ (Barrett et al., 2007; Bolborea and Dale, 2013; Bolborea et al., 2015; Samms et al., 2015). Therefore, an important

function of tanycytes is the local metabolism and supply of thyroid hormones to the hypothalamus. For example the PVN contains no *dio2* mRNA or DIO2 enzymes (Tu et al., 1997), therefore, is not capable to activate $T_3$ and thus TRH neurons in the PVN are dependent upon $T_3$ from surrounding hypothalamic structures.

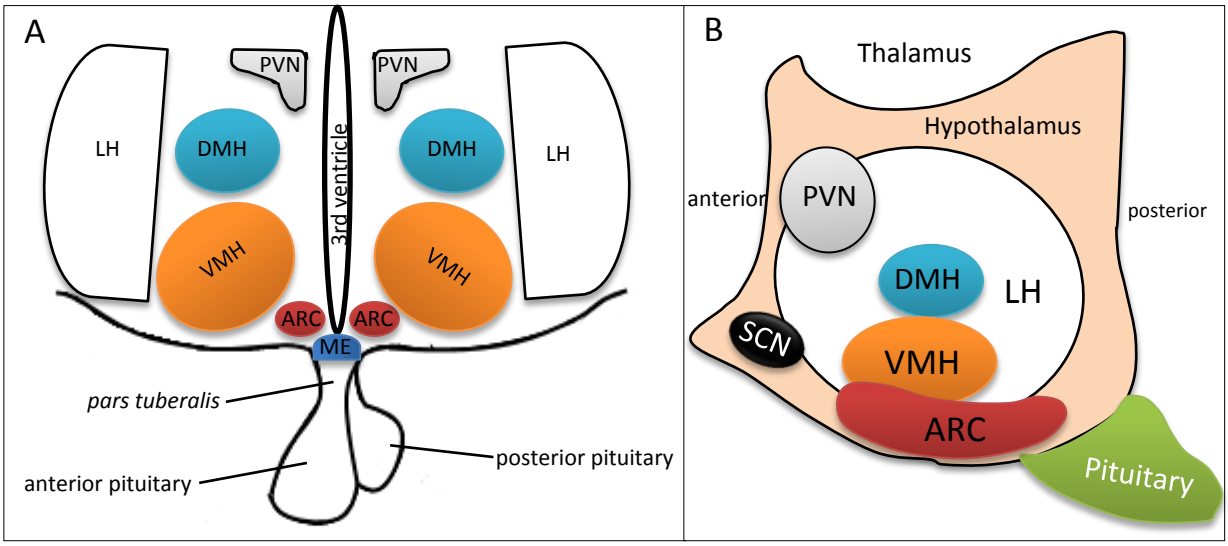


**Figure 4: Schematic coronal- (A) and lateral (B) view of hypothalamic nuclei.**
ARC= arcuate nucleus, DMH= dorsomedial nucleus, LH= lateral hypothalamus, ME= median eminence, PVN= paraventricular nucleus, SCN= suprachiasmatic nucleus

In the last decade it has been shown that the hypothalamus is essential to integrate photoperiodic changes and that several genes in this brain area are regulated in a seasonal manner. Seasonal changes in gene expression have been mainly found in the ARC of the hypothalamus and in the ventral ependymal layer lining the third ventricle. Tanycytes are an important cell type in this ependymal layer and adapt to photoperiodic changes dependent on melatonin secretion (Rodríguez et al., 2005). Djungarian hamsters exposed to SP showed lower expression of vimentin in tanycytes (Bolborea et al., 2011; Herwig et al., 2013; Kameda et al., 2003). Vimentin is an intermediate filament protein and as component of the cytoskeleton responsible for cell shape and –integrity. Hence tanycytes possess morphological plasticity and during SP the cell processes are significantly shortened (Kameda et al., 2003).

## 1.4 Seasonal regulation of gene expression

In Djungarian hamsters several genes have been identified that show an alternation after a switch between LP and SP. After the discovery of seasonally regulated clock genes responsible for photoperiodic time-measurement, genes involved in thyroid hormone metabolism became one focus of interest (chapter 1.4.1). Because of the close link between thyroid hormones and metabolism, this pathway seems to be promising to get a better understanding of seasonal regulation of body weight

and torpor expression. Therefore, thyroid hormone metabolism became the central topic of my studies. Also uncoupling proteins, which are well known $T_3$ target genes, are an interesting subject to understand thermogenesis and metabolic suppression during torpor. Subsequently I became interested in other genes that might play inferior roles in seasonal body weight regulation and are part of growth hormone synthesis (1.4.3), retinoic acid pathway (chapter 1.4.4), melatonin signaling (chapter 1.4.5) and histaminergic effects (chapter 1.4.6)

### 1.4.1 Deiodinases and thyroid hormone transporter

That thyroid hormones are essential for seasonal adaptation has first been shown in sheep, which failed to undergo seasonal reproduction after removal of the thyroid gland, hence the complete lack of thyroid hormones (Dahl et al., 1995; Parkinson and Follett, 1995; Webster et al., 1991). Some studies with sheep, Syrian- or Djungarian hamsters provided weak evidence that peripheral thyroid hormone concentrations are seasonally fluctuating (Champney, 2001; Masuda and Oishi, 1989; Seidel et al., 1987; Webster et al., 1991). However, it seems to be very unlikely that changes in serum thyroid hormone concentrations are the key to understand seasonal adaptations. Only in the last decade studies provided evidence that it appears to be local availability of $T_3$ in the hypothalamus controlled by deiodinases regulates reproduction, body weight and body temperature.

The role of DIO2 in the seasonal regulation of reproduction was first discovered in Japanese quails (*Coturnix coturnix japonica*) (Yoshimura et al., 2003). Later its role in reproduction was also discovered in different seasonal mammals (Revel et al., 2006; Watanabe et al., 2004; Yasuo et al., 2006). A key role for deiodinases in seasonal body weight regulation was later suggested by Barrett and colleagues (2007). So far seasonal expression of deiodinases follows a consistent pattern in different seasonal mammals. High expression of *dio2* and very low expression of *dio3* is typical for long summer-like photoperiods and reduced *dio2* expression and pronounced upregulation of *dio3* is characteristic for short winter-like photoperiods (Ebling, 2014). A known regulator of deiodinases is TSH produced by the PT of the anterior pituitary gland. TSH can act at TSH-receptors (TSH-r), expressed in the ependymal layer of the third ventricle, and increases the expression of *dio2* and inhibits the expression of *dio3* (Hanon et al., 2008; Klosen et al., 2013; Nakao et al., 2008a). To control this regulation of deiodinases the PT-hypothalamic pathway needs an endocrine signal, which is provided by melatonin (see chapter 1.2).

These photoperiod-dependent changes in deiodinase expression appear to regulate the bioavailability of $T_3$ in the hypothalamus. The general theory is that low $T_3$ concentrations in the hypothalamus are a prerequisite for winter adaptations (Barrett et al., 2007; Herwig et al., 2009;

Lechan and Fekete, 2005; Murphy et al., 2012). However, changes in *dio2* and *dio3* gene expression alone provide no ultimate evidence for changes in a complex thyroid hormone metabolism. Also thyroid hormone transporters MCT8 and OATP1c1 are seasonally regulated but it is not clear whether this is directly induced by photoperiodic changes or secondarily by compensatory responses to hypothyroidism in the hypothalamus.

Unfortunately, it is technically very difficult to directly measure picomolar concentrations of thyroid hormones in the hypothalamus. Only one study detected slightly higher $T_3$ concentrations in pooled samples of the hypothalamus of photoperiodic rats in LP compared to SP, but these results were not definite (Ross et al., 2011). However, experiments manipulating $T_3$ concentrations in the hypothalamus provided more insight into the importance of this hormone. $T_3$ releasing implants in the hypothalamus of Djungarian hamsters prevented adaptation to SP and immediately reversed short day adaptations in body weight when implanted during SP (Barrett et al., 2007; Murphy et al., 2012). Also the expression of daily torpor in hamsters was blocked by hypothalamic $T_3$ treatment (Murphy et al., 2012). Otherwise, systemic release of $T_3$ reversed reproduction of Djungarian hamsters in SP, but did not affect body weight (Freeman et al., 2007; Henson et al., 2013). This dissociating effect on body weight between central- and systemic treatment may indicate separate mechanism of thyroid hormone action in the hypothalamus and periphery. The central topic of my experiments was to gather more information about the influence of central and systemic thyroid hormone action on metabolic regulation (body weight and daily torpor) in Djungarian hamsters. Additionally photoperiodic regulation of deiodinases should provide more evidence for the connection between thyroid hormone metabolism and seasonal adaptations.

### 1.4.2 Thermogenesis and uncoupling proteins

Thyroid hormones play an important role in thermoregulation. Absence of thyroid hormones leads to a 30% reduction of basal metabolic rate, reduced cold tolerance and is associated with hypothermia (Silva, 2003). Animals and humans become quasi-poikilotherm and have problems to defend their $T_b$ in the absence of thyroid hormones.

Homoeothermic animals show ultradian variations in $T_b$ and are able to tightly regulate their $T_b$ between 35 and 38 °C despite highly variable $T_a$. To maintain a constant $T_b$ animals have to produce heat, also known as thermogenesis. Heat production can be divided into obligatory and facultative thermogenesis. Obligatory thermogenesis is an inevitable accompaniment and generated as by-product of all vital metabolic processes. Whenever energy is transformed or transferred, for example during ATP synthesis, some energy is released as heat (Silva, 2003). The thermoneutral zone is a

temperature range where obligatory thermogenesis of thermogenesis is sufficient *per se* to maintain $T_b$. This metabolic state of a resting and fasted adult animal in a thermoneutral environment is defined as basal metabolic rate (Kleiber, 1961).

Facultative thermogenesis summarizes different specific mechanisms and is superimposed on obligatory thermogenesis. It can be rapidly induced to produce additional heat in cold environments. The primary and most common form of facultative thermogenesis is muscle shivering, but it is very energy consuming and disruptive for activity and fur insulation. Therefore, it is not effective to survive prolonged cold exposure. A more efficient and long-lasting alternative is NST that uses pure metabolic processes to generate heat. The only organ for NST thermogenesis is BAT (Heldmaier and Buchberger, 1985).

In this specialized tissue uncoupling protein 1 (UCP1) is abundantly present in the inner mitochondrial membrane (Figure 5). Expression of UCP1 is limited to BAT and unique for mammals (Cannon and Nedergaard, 2004; Cannon et al., 1982). UCP1 creates a proton leak into the mitochondrial membrane, thus protons are bypassing the ATP synthase (ATPase) and energy is released as heat (Nicholls and Rial, 1999). In the resting state UCP1 is blocked by nucleotides and can be activated in response to cold stimulation by noradrenergic stimulation mediated by the sympathetic nervous system (SNS). The SNS itself is activated by the hypothalamus in response to temperature sensors in the skin. Cold exposure significantly increases the NST capacity of BAT and therefore, is an important adaptation to cold winter seasons (Heldmaier et al., 1982a; Rafael et al., 1985; Wiesinger et al., 1990).

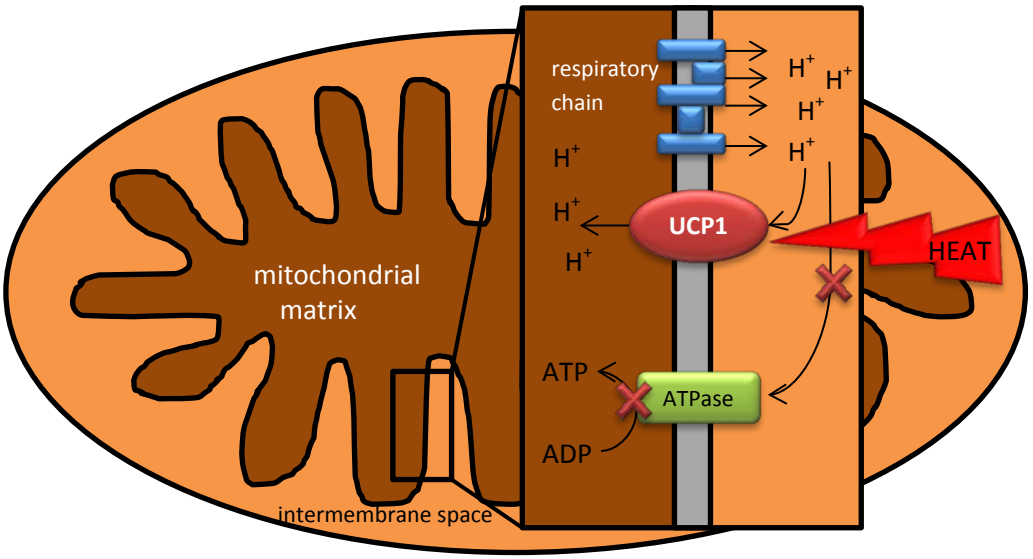


**Figure 5: Function of Uncoupling protein 1 (UCP1).** The respiratory chain transfers protons ($H^+$) across the membrane into the intermembrane space and thus creates a proton gradient. This usually drives synthesis of the ATP from ADP+P. The activation of UCP1, located at the inner mitochondrial membrane, uncouples the proton motive force from the ATPase by proton leak activity and energy is dissipated as heat.

Activation of UCP1 in BAT plays an important role in arousal from torpor, because it allows a rapid rewarming from cold $T_b$ (Janský, 1973; Kitao and Hashimoto, 2012; Oelkrug et al., 2011). Rewarming from torpor through mechanisms other than BAT UCP1-mediated thermogenesis is less efficient (shivering, passive rewarming).

The discovery of DIO2 in BAT and its activation by the SNS suggested a link between thyroid hormones and NST (Leonard et al., 1983; Silva and Larsen, 1983). Stimulation of DIO2 by the SNS leads to an increase of intracellular conversion of $T_4$ to $T_3$ in BAT and is essential for the full thermogenic response (Bianco and Silva, 1987a; Bianco and Silva, 1987b; Bianco et al., 1988; de Jesus et al., 2001). Additionally thyroid hormone response elements were identified upstream of the UCP1 gene and $T_3$ upregulates UCP1 expression (Rabelo et al., 1995). Other homologue genes of the UCP family have been identified later (Cioffi et al., 2009). UCP2 and UCP3 have a wider distribution, but their precise function is still under debate. High concentrations of *ucp2* mRNA has been found in heart, lung, BAT, testis and others, while low concentrations have been found in brain, liver and muscle (Pecqueur et al., 2001). UCP2 seems to interact with $T_3$ in the hypothalamus and might play a role in feeding behavior and local thermogenesis in the brain (Coppola et al., 2007; Horvath et al., 1999). UCP3 is mainly expressed in muscle as well as BAT and heart, but with lower concentrations (Boss et al., 1997; Vidal-Puig et al., 1997). Although its role in thermoregulation is not established, it might still be involved, because it is only increased during periods with high energy expenditure (de Lange et al., 2001; Hesselink and Schrauwen, 2005; Lanni et al., 1999; Larkin et al., 1997; Simonyan et al., 2001). Also UCP3 gene expression can be upregulated by $T_3$ (Larkin et al., 1997; Reitman et al., 1999).

### 1.4.3 Growth hormone pathway

Seasonal changes in body weight, including changes in adipose tissue, bone- and tissue mass, seem to correlate with circannual secretion of growth hormone (GH) (Dumbell et al., 2015; Petri et al., 2014; Vaughan et al., 1994). Growth hormone releasing hormone (GHRH) induces the production of GH in the pituitary gland and can be inhibited by somatotropin release inhibiting factor (SRIF, also known as Somatostatin), produced by neurons of the PVN and ARC (Atrens and Menéndez, 1993; Sawchenko et al., 1990; Spoudeas et al., 1992). Effects of GH in the periphery are primarily mediated by Insulin-like growth factor 1 (IGF-1) (Murray et al., 2015).

In Djungarian hamsters the expression of *srif* in the ARC is increased during short photoperiod, when body weight is low (Herwig et al., 2012; Herwig et al., 2013). It has been suggested, that *srif* is regulated downstream of the HPT-axis, because low *srif* expression correlates with high TSH

production during LP (Klosen 2013). The increase of *srif* expression in SP hamsters suppresses GH- and IGF-1 production and might regulate the body weight reduction. Contrarily in hamsters switched back to LP *srif* expression was reduced and IGF-1 serum concentrations as well as body mass increased (Dumbell 2015). Treatment with GH also led to an increase of body weight in SP hamsters (Dumbell et al., 2015).

Moreover pasireotide, a somatostatin receptor 5 agonist, suppressing the GH secretion in the pituitary, reduced body weight in LP hamster and retarded increase of body weight in SP hamsters. Interestingly pasireotide, mimicking the presence of somatostatin, also had a strong effect on torpor frequency and – duration (Scherbarth et al., 2015). Thus it was suggested, that specific activation of somatostatin receptor 5 might be critical for torpor induction or -modulation. Pasireotide also reduced IGF-1 serum concentration, but there was no correlation between IGF-1 and torpor expression. Therefore, it is questionable, if torpor expression is directly connected to the activity of the GH-axis. The exact function of somatostatin in the seasonal regulation of body weight and especially its effect on torpor is still unclear.

### 1.4.4 Retinoic acid pathway

Retinoic acid (RA), a metabolite of retinol (Vitamin A), is transcriptionally active and there seem to be strong parallels between thyroid hormone and RA synthesis and -signaling in the hypothalamus. The synthesis of RA includes two steps. First retinol is converted by retinol dehydrogenases to retinal and then converted by retinaldehyde dehydrogenases (RALDH) into RA. RALDH is expressed in extending processes of tanycytes in photoresponsive F344, which indicates RA metabolism in the hypothalamus (Shearer et al., 2010). Thus it seems to be likely that transcriptional processes in the hypothalamus are influenced by RA.

Retinol, the substrate for RA, is present in the CSF (Figure 6), where it is carried by retinol-binding protein (RBP) (Lane and Bailey, 2005). Transport into cells of the ependymal layer is facilitated by the membrane receptor Stra6 (Kawaguchi et al., 2007). After transport of retinol into tanycytes it is bound to cellular retinol-binding protein 1 (CRBP1) and directs the intracellular metabolism (Li and Norris, 1996). After conversion, RA is bound to cellular retinoic acid-binding protein 2 (CRABP2). Both CRBP1 and CRABP2 as well as nuclear retinoic acid receptor (RAR) and retinoid X receptor (RXR) are expressed in the hypothalamus of mice and rats (Shearer et al., 2010). RAR forms heterodimers with RXR, which bind to retinoic acid response elements (RAREs) and essentially function as transcription factors in the nucleus of neurons in the ARC and thus transduce the RA signal into genomic actions

(reviewed by (Lane and Bailey, 2005). Recent studies gave evidence that RAR can also execute non-genomic actions in the cytoplasm (Cañón et al., 2004; Maruvada et al., 2003).

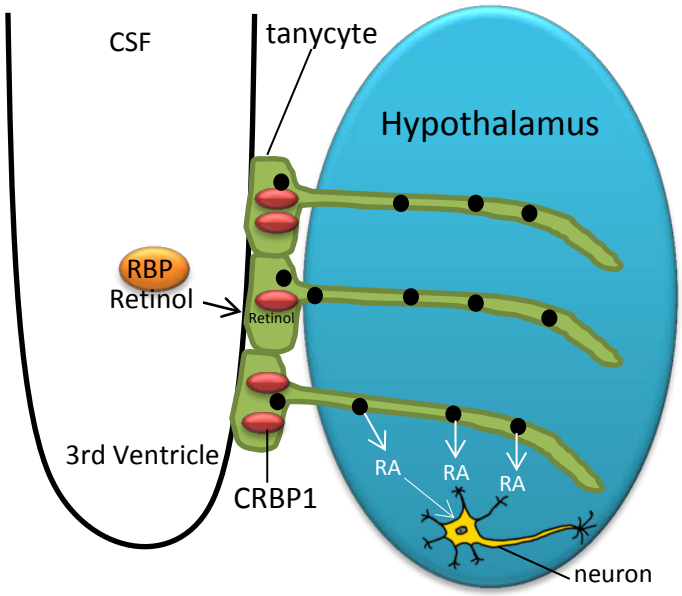


**Figure 6: Proposed model of the retinoic acid pathway in the hypothalamus.** (adapted from Shearer et al 2010)

Retinol is bound to retinol binding protein (RBP) and can be transported into tanycytes lining the 3rd ventricle. In tanycytes retinol is bound to cellular retinol binding protein 1 (CRBP1) and can be converted by RALDH (black dots) into retinoic acid (RA) in the long processes of tanycytes that penetrate subregions of the hypothalamus. RA is released from tanycytes and can bind to RA receptors in the nucleus of hypothalamic neurons and modifies gene expression.

The RA pathway seems to be under photoperiodic control with higher expression of several components during LP, which parallels with increased DIO2 expression under same conditions (Helfer et al., 2012; Ross et al., 2004; Ross et al., 2005; Shearer et al., 2010; Watanabe et al., 2004). In my thesis I investigated the regulation of CRBP1 in relation to body weight changes as important part of the retinoic pathway, which has the potential to influence indirectly gene transcription in the ARC involved in the regulation of feeding, drinking and body weight (Ebling, 2015).

### 1.4.5 G protein-coupled receptor 50

The G protein-coupled receptor 50 (GPR50) is an orphan of the melatonin receptor subfamily, because of its high sequence homology. However, it does not bind melatonin like the true melatonin receptors $MT_1$ and $MT_2$ (Drew et al., 1998; Reppert et al., 1996). Nonetheless GPR50 might be involved in the melatonin signaling by inhibiting the function of $MT_1$. In cells expressing both proteins they can form GPR50/$MT_1$ heterodimers, which leads to an inactivation of $MT_1$ (Levoye et al., 2006). Physiological consequences of $MT_1$/GPR50 heterodimers and tissues with co-expression of both proteins are unknown. To understand the physiological role of these heterodimers tissues and brain regions with both proteins co-expressed have to be identified. Unfortunately melatonin receptors are expressed at very low densities even in regions with high melatonin sensitivity and reliable

antibodies directed specifically against $MT_1$ and $MT_2$ are not available. However, $MT_1$ mRNA has been detected in the SCN and PT of rodents (Poirel et al., 2003; von Gall et al., 2002; von Gall et al., 2005).

GPR50 mRNA has been detected in the hypothalamus of humans, mice, rats and Djungarian hamsters (Barrett et al., 2006; Drew et al., 1998; Sidibe et al., 2010; Vassilatis et al., 2003). In the hypothalamus GPR50 protein is mainly located in neurons of the DMH region and in tanycytes. In tanycytes *gpr50* is down regulated under SP conditions in Djungarian hamsters, suggesting an involvement of this receptor in transducing photoperiodic changes (Barrett et al., 2006). To date no expression of melatonin receptors has been found in ependymal cells of the third ventricle (Schuster et al., 2000; Song and Bartness, 2001) and therefore, the function of GPR50 in these cells is not known. Mice lacking GPR50 have a disturbed circadian rhythm and an altered metabolism, suggesting a role of GPR50 in energy balance (Ivanova et al., 2008). Another role for GPR50 might be an influence on adaptive thermogenesis and torpor expression. GPR50-knockout mice had reduced metabolic rate and showed extended periods of fasting induced torpor (Bechtold et al., 2012). In these KO-mice reduced TRH expression in the PVN suggests a link between GPR50 and the HPT-axis. Altogether the function of GPR50 is still poorly understood, especially the link to thyroid hormones and energy balance.

### 1.4.6 Histamine receptor

Histamine has many important functions in most parts of the central nervous system (Panula et al., 1989). Neurons producing histamine are located in the tuberomammillary nucleus of the hypothalamus (Köhler et al., 1986). Histamine receptors mediate the action of this neurotransmitter at the cellular, synaptic and behavioral level.

In the hibernating ground squirrel histamine plays a central role of regulating $T_b$ and metabolism during long hibernation. In Djungarian hamsters little is known about the influence of histamine on daily torpor or seasonal adjustments of metabolism. The presynaptic histamine receptor 3 (H3R) was recognized as actively regulated in a photoperiod-dependent manner in this hamsters (Barrett et al., 2005; Barrett et al., 2005; Barrett et al., 2009). H3R is located in various neurons and activation causes autoinhibition of histamine synthesis and -release, but also regulates the release of other transmitters like noradrenaline and glutamate (Brown and Haas, 1999; Schlicker et al., 1992). During SP expression of *h3r* and neuronal activity in the dmpARC is reduced and it was suggested, that H3R plays a role in seasonal body weight regulation (Barrett et al., 2009; Song and Bartness, 2001). H3R seems to be involved in regulation of food intake and blocking this receptor leads to lower feeding (Doi et al., 1994; Jethwa et al., 2009; Ookuma et al., 1993; Sakata et al., 1991). An up regulation of

*h3r* during daily torpor in the ARC and DMH might play a role in torpor regulation (Herwig et al., 2007). The histaminergic system is very complex and its role especially in the regulation of body weight and daily torpor is not well understood.

## 1.5. Aim of the thesis

Understanding the regulation of energy balance is one of the main universal fields of scientific research. Many diseases result from obesity, which is a consequence of imbalanced metabolism and overnutrition. Thyroid hormones are involved in many metabolic processes, but after several decades of research many pathways underlying thyroid hormone action are not completely understood. In particular the actions in the hypothalamus, which is one main brain area for regulation of energy balance, remain unresolved.

Djungarian hamsters have extreme short- and long-term changes in metabolism, body weight and $T_b$. Thus they are an excellent animal model to study the link between thyroid hormones, body weight and $T_b$. After decades of research the seasonal regulation of body weight and expression of daily torpor is still a "black box". Many contents in this "black box" have been identified so far. The challenge today is to find the links between the different components and to identify functions and interactions of different pathways.

The first experiment of my thesis was designed to get a better understanding of gene expression linked to seasonal changes in body weight. Several genes have been identified to be under photoperiodic control, but their role and specific function is not well understood. To reveal connections between photo-responsive genes and body weight changes I exposed hamsters to multiple directional changes between SP and LP. Hamsters were sacrificed at different key points and hypothalamic gene expression was analyzed by in situ hybridization. Changes in gene expression were linked to increasing or decreasing body weight in response to alternation between long- and short photoperiod.

The second experiment focused on the systemic influence of thyroid hormones on torpor expression. Hamsters were made either hypothyroid with MMI and sodium perchlorate or hyperthyroid with $T_4$ or $T_3$. Previous studies suggested that low thyroid hormone concentrations are a prerequisite for torpor expression. Thus the hypothesis was that hypothyroidism promotes the expression of torpor and that systemic $T_3$ treatment inhibits torpor. Because $T_4$ has a low biological activity an effect of $T_4$ on torpor expression was not expected. Additionally gene expression of deiodinases, uncoupling proteins, somatostatin, NPY and POMC was analyzed by qPCR in hypothalamus, BAT and muscle. It

was expected that thyroid hormones influence gene expression and that genes are probably regulated during torpor because of hypometabolism and hypothermia.

The final experiment was preformed to shed more light on the central regulation of daily torpor. Many details of torpor expression are already known, but the final trigger for torpor initiation is still unclear. A hot candidate is $T_3$ action in the hypothalamus, but thyroid hormone metabolism and $T_3$ availability in hypothalamic tanycytes are not well understood. Thus hamsters were treated with $T_3$ by hypothalamic microdialysis. The hypothesis was that central $T_3$ release is sufficient to inhibit the expression of torpor, which would suggest a central pathway for torpor induction. In addition, potentially $T_3$-dependent gene regulation in the hypothalamus was analyzed by in situ hybridization. To date it is not clear, which photo-responsive genes are regulated by $T_3$ and therefore, dependent on seasonal expression of deiodinases.

# 2. Publications & Manuscripts

## 2.1 Influence of photoperiod on gene expression linked to body weight

The following chapter was written as a manuscript, but is unpublished so far. The author of this thesis was involved in the design of all experiments supported by Perry Barrett and Annika Herwig. All *in vivo* experiments were carried out by me. Gene expression analysis by *in situ* hybridization was carried out by me with the support of Dana Wilson and analyzed as well as interpreted by myself. Serum concentration analysis of thyroid hormones by radioimmune assays (RIA) was performed by Eddy Rijntjes. Manuscript was written by myself and revised by Annika Herwig, Eddy Rijntjes and Perry Barrett.

**Alternation between short- and long photoperiod reveal hypothalamic gene regulation linked to seasonal body weight changes in Djungarian hamster (Phodopus sungorus)**

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### 2.1.1 Abstract

Djungarian hamsters are able to reduce their body weight by more than 30% in anticipation of the winter season. This particular adaptation to extreme environmental conditions is primarily driven by natural changes in day length and conserved under laboratory conditions. We used this animal model to investigate hypothalamic gene expression linked to body weight regulation behind this physiological phenomenon. After an initial collective short day adaptation for 14 weeks hamsters were switched between long- (LP) and short photoperiod (SP). Our data showed that switch back from SP to LP led to an increase in body weight. In the hypothalamus *dio2*, *vimentin*, *crbp1* and *grp50* increased with increasing body weight, but expression of *dio3, mct8* and *srif* decreased. The changes in body weight and gene expression reversed after switching hamsters back to SP after 6 or 14 weeks in LP. Interestingly, body weight loss was more pronounced in six hamsters switched back from LP to SP after 14 weeks, while five hamsters did not respond to SP. In those that failed to reduce body weight a different gene expression pattern was manifested. All together we were able to shed more light on photoperiodic control of body weight and gene expression. Switchback hamsters revealed that body weight regulation seems to be tightly linked to expression of several genes in the hypothalamus involved in thyroid hormone metabolism (*dio2, dio3, mct8*) as well as growth-axis (*srif*) or other pathways (*crbp1, gpr50*).

## 2.1.2 Introduction

Seasonal mammals show a wide range of adaptations to cold winter seasons with short photoperiod and reduced food availability. Besides quiescence of reproduction, improved winter fur and optimized thermoregulation, body weight regulation is a very important component to survive winter (Figala et al., 1973; Heldmaier et al., 1989). Djungarian hamsters (*Phodopus sungorus*), also known as Siberian hamsters, have been used intensively as animal model for long-term changes in energy balance, because of their particular annual body weight cycle. They reduce body weight well in advance of the winter season and increase body weight in time for the next reproduction period in spring (Morgan and Mercer, 2001; Steinlechner et al., 1983). These changes are primarily driven by seasonal changes in photoperiod and can be easily induced under laboratory conditions by transferring hamsters between summer-like long photoperiod (LP) or winter-like short photoperiod (SP) (Steinlechner and Heldmaier, 1982; Vitale et al., 1985). Internal information about day length is provided by the duration of nocturnal pineal melatonin secretion (Illnerová et al., 1984). In the last decade several genes likely to be involved in seasonal adaptations have been discovered in the hypothalamus, but their specific role is not well understood.

The hypothalamus is the center for energy homeostasis and neuroendocrine regulator of many physiological processes with the ability to integrate photoperiodic signals. TSH-receptor (TSH-r) expressing cells in the hypothalamus respond to thyrotropin (TSH) produced by melatonin responsive cells in the *pars tuberalis* (PT) (Hanon et al., 2008). In long photoperiod TSH production in the PT is increased, which leads to an increased deiodinase 2 (DIO2) expression (Nakao et al., 2008a; Nakao et al., 2008b). Deiodinases, responsible for thyroid hormone metabolism, act as a gatekeeper in tanycytes, a special glial cell type in the ependymal layer of the third ventricle (Barrett et al., 2007). The reduction of the intermediate filament protein vimentin in the ependymal layer during SP exposure indicates structural changes of tanycytes in response to photoperiod (Bolborea et al., 2011; Kameda et al., 2003). Tanycytes operate at the interface between the cerebrospinal fluid (CSF) and neurons of the hypothalamus (Bolborea and Dale, 2013; Rodríguez et al., 2005). Thyroxin ($T_4$) can be transported via monocarboxylate transporter 8 (MCT8) into tanycytes. The phenolic ring of $T_4$ can be deiodinated by the enzyme DIO2 into biologically active 3,3',5-triiodothyronine ($T_3$) (Friesema et al., 2003; Herwig et al., 2014; Köhrle, 1999). Deiodinase type 3 (DIO3) acts as a counterpart and is responsible for the inactivation of $T_3$ by tyrosyl ring deiodination into 3,3'-diiodothyronine ($T_2$). Both enzymes are seasonally regulated and several studies have suggested that a low $T_3$ concentration in the hypothalamus, generated by low *dio2* expression and high *dio3* expression, is required for body mass reduction (Barrett et al., 2007; Herwig et al., 2009; Herwig et al., 2013; Murphy et al., 2012). However, *dio2* and *dio3* expression begin to return to LP levels after 14 weeks in SP, while body

weight remains at its nadir, before increasing again after more than 20 weeks in SP, when hamsters become refractory to the photoperiodic signal and spontaneously return to their summer phenotype (Barrett et al., 2007; Gorman and Zucker, 1995). Thus the regulation of deiodinases and the link to body weight is not fully understood.

In addition to genes involved in the thyroid hormone signaling pathway, several other genes regulated by photoperiod are expressed in the ependymal layer. Cellular Retinol-Binding Protein 1 (CRBP1) is a transport protein for retinol (vitamin A) and involved in the pathway for synthesizing retinoic acid (Lane and Bailey, 2005). *Crbp1* is down regulated during SP exposure and has been related to seasonal changes in body weight (Barrett et al., 2006; Helfer et al., 2012; Ross et al., 2004). Another protein expressed in the ependymal layer is GPR50 an orphan G-protein-coupled receptor, which inhibits melatonin receptor 1A ($MT_1$) through heterodimerization (Jockers et al., 2008). Despite its homology to melatonin receptors, it does not bind melatonin (Drew et al., 1998; Reppert et al., 1996). *Gpr50* is downregulated during SP and is a candidate of sensing components of the metabolic homeostasis in the CSF (Barrett et al., 2006). Somatostatin (SRIF) is produced in the hypothalamus amongst others and its gene expression in the arcuate nucleus (ARC) is upregulated during SP (Herwig et al., 2012; Herwig et al., 2013). A function of SRIF may be the inhibition of growth hormone and contributing to loss of body weight during the seasonal cycle of body weight regulation (Brazeau et al., 1973; Dumbell et al., 2015).

The aim of this study was to develop a better understanding of these photo-responsive genes by investigating their transcriptional plasticity to multiple directional changes between photoperiods. Hamsters switched between static photoperiods representing LP and SP have shown that physiological adaptation revert to LP phenotype within a period of six weeks after transfer from SP to LP (Dumbell et al., 2015; Ross et al., 2005). In this paradigm photoperiod responsive genes generally revert to LP expression after switch between static SP to LP (Ross et al., 2005). The hamsters continuously kept in SP become photorefractory to melatonin and automatically return back to their LP phenotype. Prior to the onset of physiological changes the expression of most genes reverts back to LP expression levels, with *dio2* expressed beyond the level found in hamsters constantly kept in LP (Herwig et al., 2013; Watanabe et al., 2007).

Although hypothalamic gene expression studies have been performed on LP to SP transition and a subsequent SP to LP transition, there is no further information on the plasticity of *dio2* and other critical components of the hypothalamic thyroid hormone pathway in response to a further directional change back to SP. Previously it has been found that responsiveness to melatonin or SP is not re-established before ten weeks in LP and hamsters are able to respond to SP again (Bittman, 1978a; Kauffman et al., 2003; Reiter, 1972). Understanding the plasticity of the hypothalamic thyroid

hormone pathway may be important to understand responsiveness to melatonin signaling following a photorefractory physiological recrudescence.

We hypothesize that hamsters are not able to respond to SP again after six weeks in LP. We assume that thyroid hormone metabolism generate higher hypothalamic $T_3$ concentrations after the switch back from SP to LP and causes a manifestation of the LP phenotype. With the second switchback after 14 weeks we hypothesize, that $T_3$ concentration in the hypothalamus is back to normal LP values and hamsters are able to respond to SP again. Furthermore, we assume that *gpr50* and *crbp1* gene expression increase with increasing body weight and decrease with decreasing body weight. For *srif* we expect a gene expression in the opposite direction. Our results reveal new insights in the photoperiodic regulation and plasticity of gene expression related to body weight regulation.

## 2.1.3 Material & Methods

### 2.1.3.1 Animals and experimental procedure

All experiments and procedures were approved by the local animal welfare authorities (Hamburg, Germany). Fifty-two Djungarian hamsters (Phodopus sungorus) of both sexes were bred and raised under artificial long photoperiod (LP; 16 h light : 8 h dark) as previously described (Bank et al., 2015, chapter 2.2.3.1). At an age of 3-4 month (=week 0) a cohort of six hamsters ($LP_0$) were sacrificed between Zeitgeber time (ZT) 4–5 by $CO_2$ inhalation and decapitation under LP conditions. The remaining 46 adult hamsters were transferred to an artificial short photoperiod (Figure 7, SP, 8 h light : 16 h dark). Hamsters were weighed once a week.

| Group | N | | | | | |
|---|---|---|---|---|---|---|
| $LP_0$ | 6 | **LP** | | | | |
| $SP_{14}$ | 6 | **LP** | **14 weeks SP** | | | |
| $SP_{14}LP_6$ | 6 | **LP** | **14 weeks SP** | **6 weeks LP** | | |
| $SP_{14}LP_{14}$ | 6 | **LP** | **14 weeks SP** | **14 weeks LP** | | |
| $SP_{14}LP_6SP_8$ | 6 | **LP** | **14 weeks SP** | **6 weeks LP** | **8 weeks SP** | |
| $SP_{14}LP_{22}$ | 11 | **LP** | **14 weeks SP** | **22 weeks LP** | | |
| $SP_{14}LP_{14}SP_8$ | 11 | **LP** | **14 weeks SP** | **14 weeks LP** | | **8 weeks SP** |

**Figure 7: Experimental schedule.** Hamsters were kept under long photoperiod LP or short photoperiod (SP) and killed at different time points.

After 14 weeks in SP a cohort of six hamsters ($SP_{14}$) was sacrificed and 40 hamsters were transferred back to LP. After six weeks in LP a further cohort of six hamsters ($SP_{14}LP_6$) was killed. At this time point a cohort of six hamsters was switched back to SP for a second time and killed after eight weeks

under SP conditions ($SP_{14}LP_6SP_8$). Six hamsters kept under LP for 14 weeks after the first switch from SP were killed together with the $SP_{14}LP_6SP_8$ group. The remaining 22 hamsters were split into two groups. Eleven hamsters stayed at LP for another eight weeks ($SP_{14}LP_{22}$) and 11 hamsters were switched back to SP for eight weeks ($SP_{14}LP_{14}SP_8$), too. Those hamsters were sacrificed at the end of the experiment. Blood of all hamsters was collected, serum extracted and total $T_4$ ($tT_4$) and total $T_3$ ($tT_3$) serum concentrations were analyzed by radio-immuno assays as described before (Bank et al., 2015, see chapter 2.2.3.3). The brains were removed, immediately frozen on dry ice and stored at -80°C until required for *in situ* hybridization.

### 2.1.3.2 Radioactive in situ hybridization

*In situ* hybridization was used to quantify expression of *dio2, dio3, mct8, tsh-r, crbp1, gpr50* and *vimentin* mRNA along the third ventricle of the hypothalamus and quantification of *srif* was restricted to the ARC region within the hypothalamus. The hypothalamic region of interest was between bregma -1.70 and -2.54 mm according to the Mouse Brain Atlas of Franklin & Paxinos (3rd ed., 2008). Frozen brains were cut with a cryostat into 16µm coronal sections, mounted onto polysine-coated slides and stored at -80°C until needed.

For *in situ* hybridization, brain sections were fixed in 4% paraformaldehyde, washed with 0.1 M PBS, incubated in 0.1M triethanolamine (pH 8) and acetylated with 0.25% acetic anhydride. Slides were washed again with 0.1 M PBS and subsequently dehydrated using an ascending ethanol series followed by vacuum drying. Riboprobes were synthesized as previously described from DNA fragments for *dio2, dio3, mct8, tsh-r, crbp1, gpr50*, vimentin and *srif*, using $^{35}$S-UTP with SP6 or T7 polymerases as appropriate (Barrett et al., 2006; Barrett et al., 2007; Herwig et al., 2009; Herwig et al., 2013; Ross et al., 2004; Ross et al., 2009). 70µl hybridization mixture (formamide, 0.3 M NaCl, 10 mM Tris-HCL (pH 8), 1 mM EDTA, 0.05% transfer RNA, 10 mM dithiothreitol,0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, and 10% dextran sulphate) containing the appropriate radioactive probes (ca. $10^6$ cpm) was applied per glass slide and sealed with DPX Mountant. Hybridization was carried out over night at 58°C. After hybridization, slides were washed in 4x SSC (Saline-Sodium Citrate), incubated with RNase A solution at 37°C for 30 minutes and washed in SSC solutions with decreasing concentrations (2x to 0.1x). Finally slides were dehydrated using an ascending ethanol series and air dried before exposed to Kodak BioMax MR Films (Sigma-Aldrich Company Ltd., Poole, Dorset, UK). Autoradiographic films were developed after 18-20h (*srif, vimentin*), 5 days (*mct8*), 6 days (*gpr50*), 7 days (*crbp1, tsh-r*) or 14 days (*dio2, dio3*). Autoradiographic films were scanned at 300 dpi and analysed using ImageJ 1.47v software. Integrated optical density (IOD) was obtained in two to three consecutive sections per animal by reference to a standard curve [y=a+b*ln(x-c)] generated from a $^{14}$C microscale, was measured. Values were averaged for each animal. Relative

gene expression was calculated by defining $SD_{14}$ as 100% for *dio2, dio3, mct8* and *srif* and $LD_0$ as 100% for *crbp1, tsh-r, gpr50* and *vimentin.*

### 2.1.3.3 Statistical analysis

Body weight is expressed as arithmetic mean values ± standard deviation (SD). Changes in body weight and differences between groups were tested by Two-way repeated measures ANOVA and Tukey post-hoc test. Differences in gene expression and serum concentrations between groups were analyzed by student's t-test (t-test, parametric) or Mann–Whitney-U test (U-test, non-parametric) as appropriate. P-Values *<0.05, **<0.01, *** <0.001 were considered as significant. Statistical analyses were performed with SigmaPlot™ 12 (Systat Software Inc).

## 2.1.4 Results

### 2.1.4.1 Body weight

Hamsters started with an initial average body weight (body weight) of 34.3 ± 4.8 g. During 14 weeks in SP the six groups of hamsters which were transferred to SP showed a significant body weight reduction of 8.8 ± 4.7 g from week 6 (Figure 8, ANOVA, $p<0.05$). After the switch back to LP the five remaining groups increased their body weight by 7.1 ± 3.3g. The increase was significant four weeks after the switch back to LP (ANOVA, $p<0.05$). The groups remaining in LP for 14 ($SP_{14}LP_{14}$) or 22 ($SP_{14}LP_{22}$) weeks reached a plateau after 12 weeks in LP.

Their body weight increases after the switchback were 18.1 ± 6.1 g after $LP_{14}$ and 18.9 ± 5.9 g after $LP_{22}$ respectively. The group switched back to SP for a second time after six weeks in LP stopped increasing their body weight (-1.9 ± 0.9, ANOVA, $p=0.11$) and had a significantly lower body weight compared to the corresponding $SP_{14}LP_{14}$ group (ANOVA, $p<0.5$). After a switchback to SP after hamsters had experienced 14 weeks in LP ($SP_{14}LP_{14}SP_8$) six hamsters reduced their body weight again by 6.5 ±4.4 g (ANOVA, $p<0.5$), whereas five hamsters did not respond again with a body weight loss (0.7 ±1.3 g). There was no significant difference between the nonresponding hamsters and the corresponding $SP_{14}LP_{22}$ group.

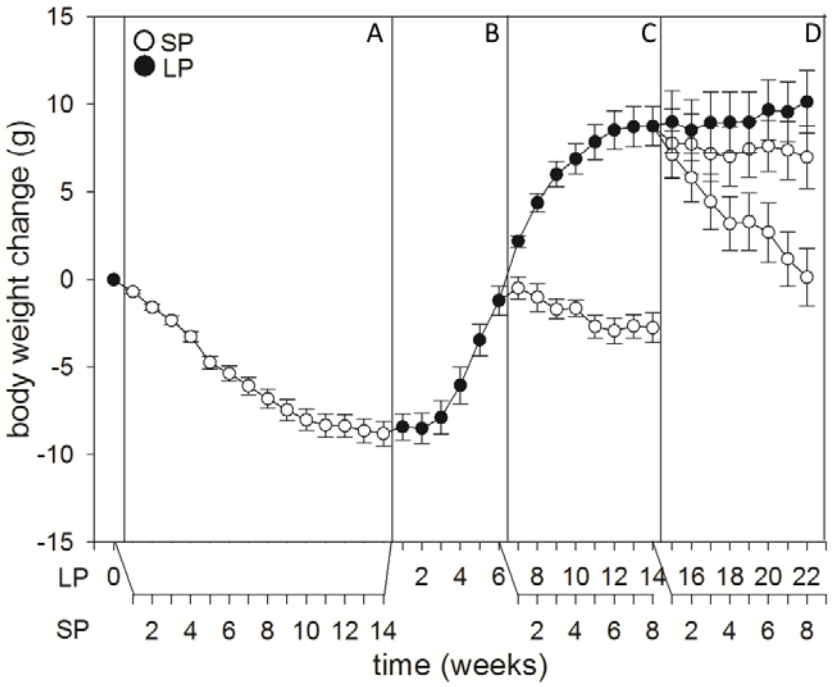


**Figure 8: BW changes are expressed as means (±SEM).** Animals were kept under short photoperiod (SP, white ○) or long photoperiod (LP, black ●). One cohort was sacrificed at time point A ($SP_{14}$), one cohort at B ($SP_{14}LP_6$), two cohorts at C ($SP_{14}LP_{14}$, $SP_{14}LP_6SP_8$) and two cohorts at D ($SP_{14}LP_{22}$, $SP_{14}LP_{14}SP_8$). The $SP_{14}LP_{14}SP_8$ (D) group was divided into hamsters that lost weight or maintained constant BW.

### 2.1.4.2 Serum thyroid hormone concentrations

One way ANOVA and t-test revealed no significant differences between all groups. Hamsters kept at LP for six weeks after switch back from SP ($SP_{14}LP_6$) showed a trend to an increase in total $T_4$ compared to $SP_{14}$ hamsters (see table 1; t-test, p=0.057). Serums concentrations in the group $SP_{14}LP_{14}SP_8$ between hamster which lost body weight and those that did not revealed no differences. This group collectively showed a statistical trend to an elevated total $T_4$ compared to the corresponding $SP_{14}LP_{22}$ group (t-test, 0.077).

**Table 1: Serum concentrations (mean ± SEM) of total $T_4$ and total $T_3$.**

| | $LP_0$ | $SP_{14}$ | $SP_{14}LP_6$ | $SP_{14}LP_{14}$ | $SP_{14}LP_6SP_8$ | $SP_{14}LP_{22}$ | $SP_{14}LP_{14}SP_8$ |
|---|---|---|---|---|---|---|---|
| Group size | n=6 | n=5 | n=6 | n=6 | n=6 | n=11 | n=11 |
| Total $T_4$ (nM) | 50 ± 3 | 42 ± 6 | 63 ± 6 | 55 ± 2 | 57 ± 2 | 44 ± 2 | 53 ± 14 |
| Total $T_3$ (nM) | 1.8 ± 0.1 | 1.8 ± 0.2 | 1.9 ± 0.1 | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.8 ± 0.1 | 2.0 ± 0.4 |

### 2.1.4.3 Hypothalamic gene expression

#### *2.1.4.3.1 Deiodinase 2*

*Dio2* expression was lower in hamsters that had experienced only LP (61.5 ± 11.9 %, t-test, p<0.03) as compared to $SP_{14}$ (Figure 9). After the switch from SP to LP *dio2* expression was increased by more than 6-fold after 6 weeks (630 ± 81%, U-test, p=0.002), before it declined in the following eight weeks, but still showing an elevated mRNA level after 14 weeks in LP (208 ± 17%, t-test, p<0.001). After 22 weeks in LP transcription was back to original $LP_0$ levels and below $SP_{14}$ values (44 ± 3%, t-test, p<0.001). Hamsters switched to SP for a second time after six weeks in LP ($SP_{14}LP_6SP_8$) had lower *dio2* levels (56 ± 5%, t-test, p=0.002) as compared to $SD_{14}$. Lower *dio2* expression than the $SP_{14}$ cohort (50 ± 8%, t-test, p=0.003) was also observed in hamsters switched back to SP for eight weeks after 14 weeks in LP ($SP_{14}LP_{14}SP_8$), but only in hamsters that had reduced their body weight. Hamsters that did not reduce body weight again, showed no differential *dio2* expression (73±13%) as compared to $SP_{14}$. However, the difference between hamsters that either lost or did not lose body weight was not great enough for a statistical difference (t-test, p=0.2).

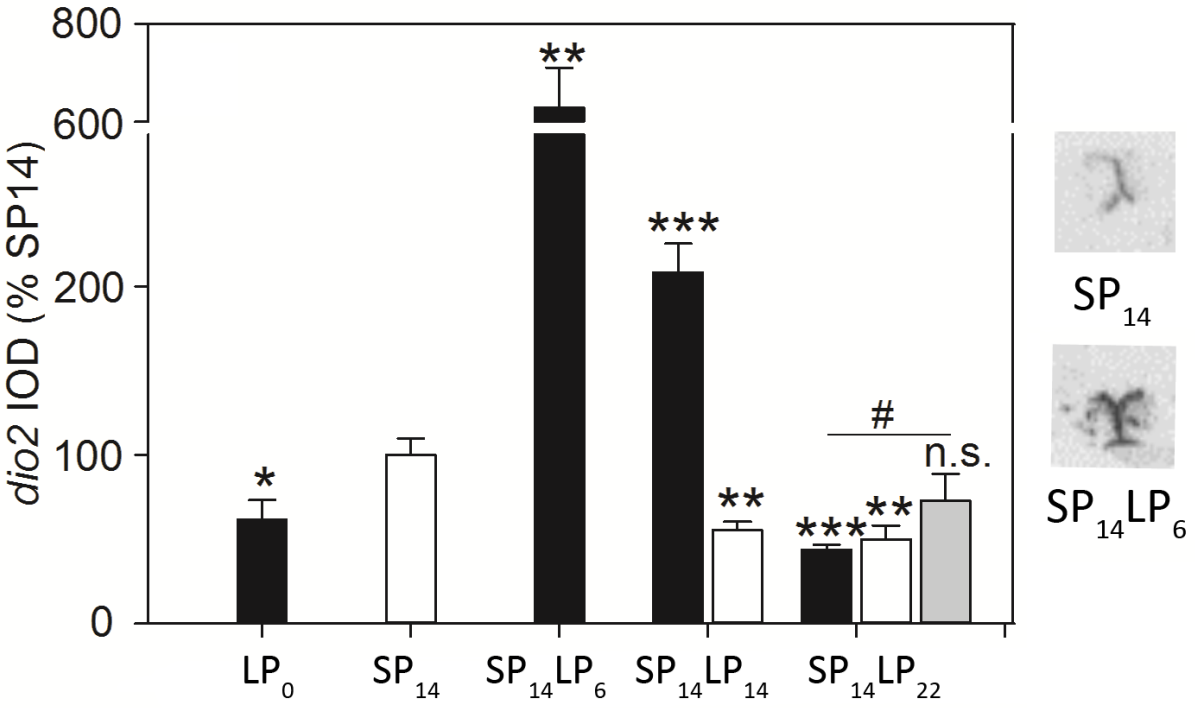


**Figure 9: *Dio2* gene expression in ventricular ependymal cells.** Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to $SP_{14}$. Hamsters were sacrificed in long photoperiod (LP, black) or short photoperiod (SP, white). White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. * significant difference to $SP_{14}$; # significant difference between other groups. Autoradiographs show representative brain sections with gene expression close to mean values.

*2.1.4.3.2 Deiodinase 3*

*Dio3* was not expressed in LP, but at different expression levels in all hamsters killed during SP (Figure 10). Within the $SP_{14}LP_6SP_8$ group (54 ± 8%, t-test, 0.184) hamsters showed a divided response with four animals showing no gene expression – similar to hamsters in LP-, two animals had increased gene expression similar to $SP_{14}$ hamsters (122% and 156%). In the $SP_{14}LP_{14}SP_8$ group all hamsters with a reduced body weight showed an increased *dio3* expression (184 ± 30%, t-test, p=0.04), but hamsters with a constant body weight showed almost no *dio3* expression (7 ± 4%, U-test, p=0.01).

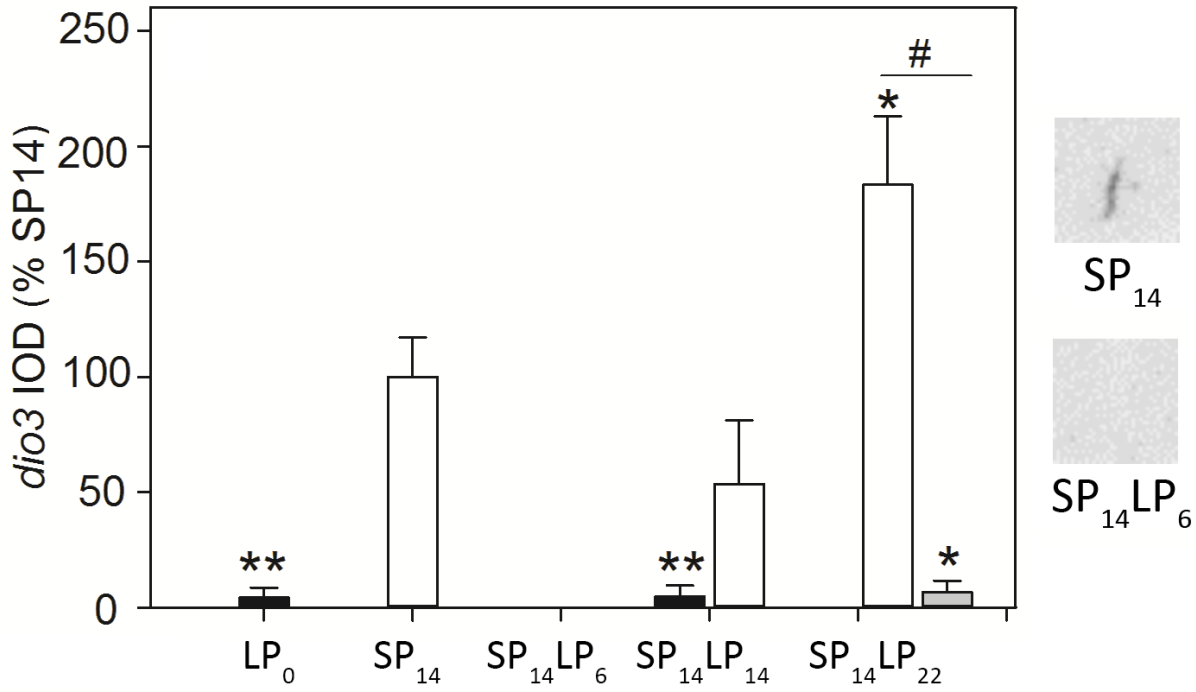


**Figure 10: *Dio3* gene expression in ventricular ependymal cells.** Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to $SP_{14}$. Hamsters were sacrificed in long photoperiod (LP, black) or short photoperiod (SP, white). White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. * significant difference to $SP_{14}$; # significant difference between other groups. Autoradiographs show representative brain sections with gene expression close to mean values.

*2.1.4.3.3 Monocarboxylate transporter 8*

After 14 weeks in SP ($SP_{14}$) *mct8* expression did not differ from $LP_0$ (87 ± 8%, Figure 11). After the switchback from SP to LP *mct8* expression was reduced in all LP groups. $SP_{14}LP_6$ (43 ± 5%, t-test, p<0.001) and $SP_{14}LP_{22}$ (51 ± 12%, t-test, p=0.006) showed lower expression in comparison to the $SP_{14}$ and $LP_0$ group, but the decrease in the $SP_{14}LP_{14}$ group did not reach significance (67 ± 9%, t-test, p=0.1). Hamsters of the $SP_{14}LP_6SP_8$ had higher *mct8* expression as compared to the parallel $SP_{14}LP_{14}$ group (t-test, p=0.003), but did not differ from the $SP_{14}$ group. The $SP_{14}LP_{14}SP_8$ group showed an interesting pattern. Here the gene expression of *mct8* in hamsters, which responded to SP with a body weight loss, was higher compared to $SP_{14}$ (129 ± 10%, t-test, p=0.003), $SP_{14}LP_{22}$ (51 ± 12%, t-test, <0.001) and hamsters with no response in body weight (86 ± 19% t-test, p=0.04). The non-responding hamsters had a *mct8* expression in between the parallel $SP_{14}LP_{22}$ group and hamsters with a response to SP ($SP_{14}LP_{14}SP_8$), but were not significantly different from the $SP_{14}$ group.

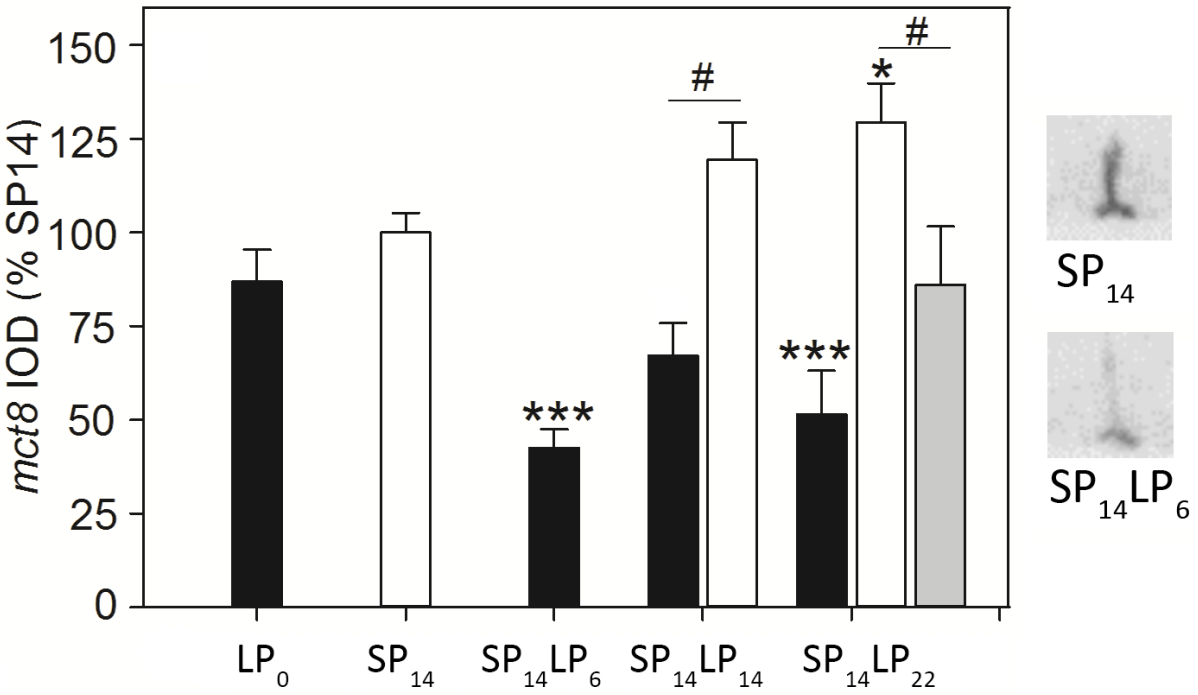


**Figure 11: *Mct8* gene expression in ventricular ependymal cells.** Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to $SP_{14}$. Hamsters were sacrificed in long photoperiod (LP, black) or short photoperiod (SP, white). White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. * significant difference to $SP_{14}$; # significant difference between other groups. Autoradiographs show representative brain sections with gene expression close to mean values.

*2.1.4.3.4 Thyrotropin receptor*

In situ hybridization of *tsh-r* mRNA revealed no generalized response caused by directional changes in photoperiod (Figure 12). The only significant difference appeared between $SP_{14}LP_{14}$ and $SP_{14}LP_6SP_8$ (116 ± 7% vs 85 ± 7%, t-test, p=0.01).

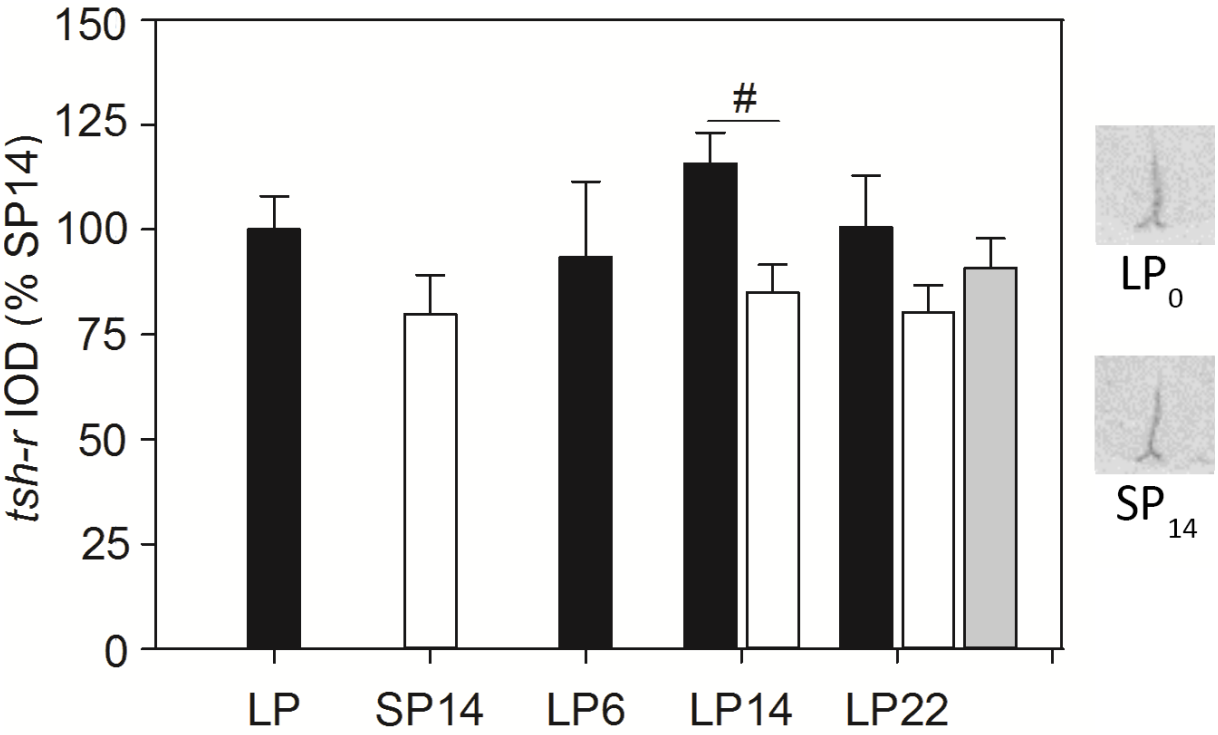


**Figure 12: *Tsh-receptor* gene expression in ventricular ependymal cells.** Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to $SP_{14}$. Hamsters were sacrificed in long photoperiod (LP, black) or short photoperiod (SP, white). White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. # significant difference between other groups.

*2.1.4.3.5 Vimentin*

The highest concentration of *vimentin* mRNA was found in $LP_0$ hamsters. *Vimentin* mRNA expression (Figure 13) was decreased after 14 weeks in SP (37 ± 2%, t-test, p>0.001). Six weeks after the switchback from $SP_{14}$ to LP *vimentin* expression was increased ($SP_{14}LP_6$, 75 ± 5%, U-test, 0.002) and continued to increase reaching 100 ± 6 % after eight more weeks in LP ($SP_{14}LP_{14}$). Hamsters from groups $SP_{14}LP_6SP_8$ (50 ± 3%) and $SP_{14}LP_{14}SP_8$ (43 ±5%) with reduced body weight had lower *vimentin* expression as compared to their parallel control LP groups, whereas hamsters that failed to reduce body weight again, had levels comparable to LP hamsters (75 ± 5%, t-test, p=0.8).

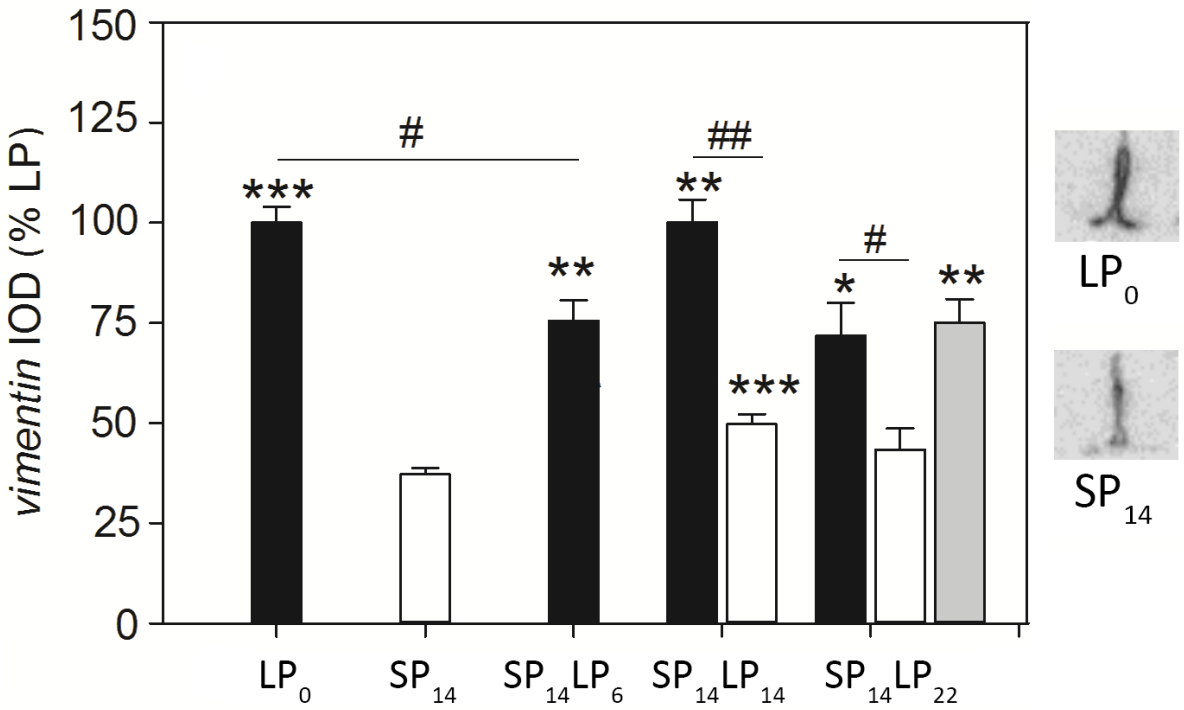


**Figure 13 V*imentin* gene expression in ventricular ependymal cells.** Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to $LP_0$. Hamsters were sacrificed in long photoperiod (LP, black) or short photoperiod (SP, white). White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. * significant difference to $SP_{14}$; # significant difference between other groups. Autoradiographs show representative brain sections with gene expression close to mean values.

*2.1.4.3.6 G protein-coupled receptor 50 (GPR50)*

Abundance of *gpr50* mRNA (Figure 14) showed clear differences between hamsters kept at LP or SP. After 14 weeks in SP *gpr50* expression was significantly reduced (22 ± 1%, U-test, p=0.002). Expression was partially restored six weeks after the switchback to LP (47 ± 6%, t-test, p=0.001) and regained original $LP_0$ expression after 14 weeks (103 ± 9%). All hamsters showed reduced *gpr50* expression after eight weeks SP when switched back a second time from LP after 6 weeks (34 ± 2%, U-test, p=0.002) or 14 weeks (37 ± 4%, U-test, p=0.001), except hamsters, that did not reduce the body weight (85 ± 13%). In the $SP_{14}LP_{14}SP_8$ group the difference between hamsters losing body weight and keeping body weight was significant (U-test, p=0.004).

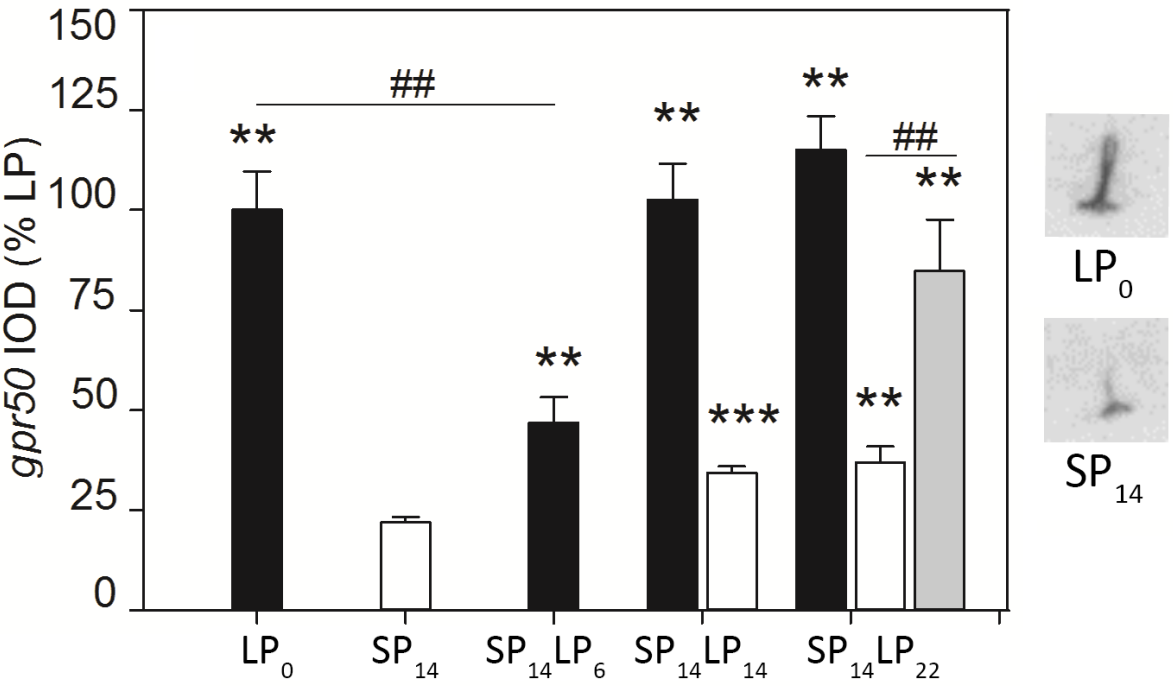


**Figure 14: *Gpr50* gene expression in ventricular ependymal cells.** Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to $LP_0$. Hamsters were sacrificed in long photoperiod (LP, black) or short photoperiod (SP, white). White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. * significant difference to $SP_{14}$; # significant difference between other groups. Autoradiographs show representative brain sections with gene expression close to mean values.

*2.1.4.3.7 Cellular Retinol-Binding Protein 1 (CRBP1)*

Relative to $LP_0$ expression was significantly reduced after 14 weeks in SP (11 ± 3%, U-test, 0.002) and increased again after switchback to LP ($SP_{14}LP_6$, Figure 15). After six weeks in LP ($SP_{14}LP_6$) the mRNA concentration increased to 64 ± 14% and was not significantly different to $LP_0$ (t-test, p=0.15). In the $SP_{14}LP_6SP_8$ group, gene expression returned to $SP_{14}$ levels (22 ± 5%, t-test, 0.08), whereas the $SP_{14}LP_{14}SP_8$ group had higher *crbp1* expression as compared to $SP_{14}$ (t-test, lost body weight: 30 ± 7%, p=0.049; no body weight loss: 50 ± 9%, p=0.004). However, compared to the $SP_{14}LP_{22}$ group hamsters with reduced body weight had significantly lower mRNA levels, but the difference in non-responsive hamsters with constant body weight was not significant (t-test, p=0.1).

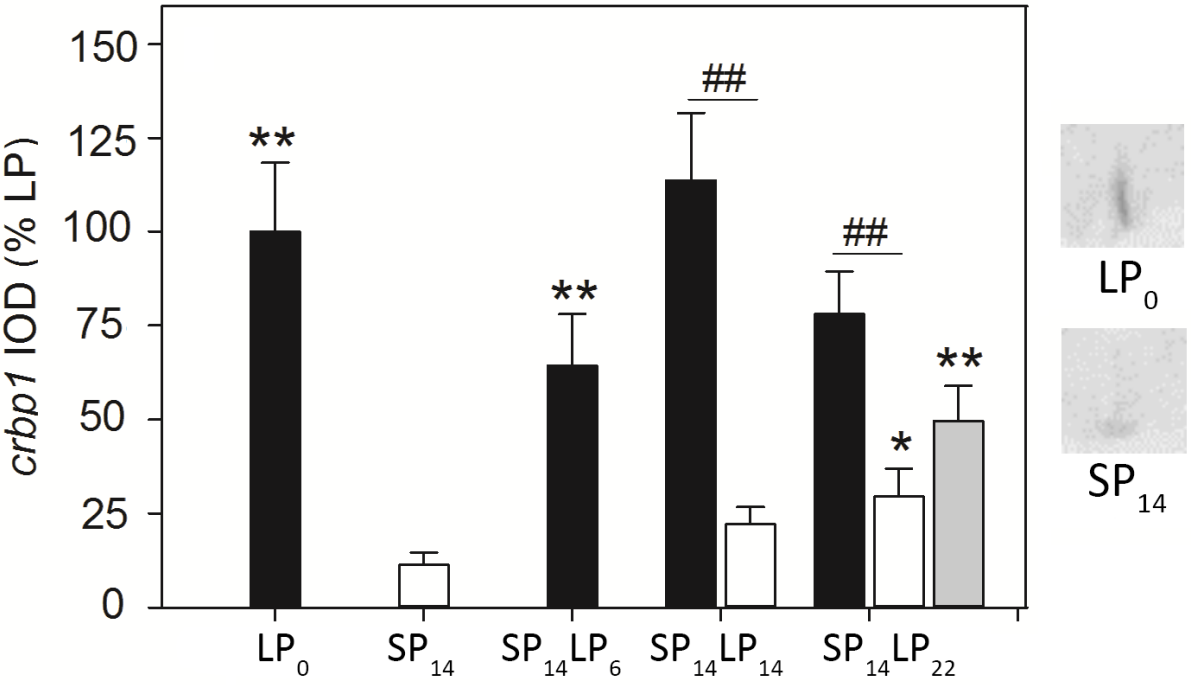


**Figure 15: C*rbp1* gene expression in ventricular ependymal cells.** Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to $LP_0$. Hamsters were sacrificed in long photoperiod (LP, black) or short photoperiod (SP, white). White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. * significant difference to $SP_{14}$; # significant difference between other groups. Autoradiographs show representative brain sections with gene expression close to mean values.

*2.1.4.3.8 Somatostatin*

Somatostatin (SRIF) was weakly expressed in all LP groups (Figure 16). After 14 weeks in SP somatostatin expression was approximately 5-fold increased relative to $LP_0$ (U-test, p=0.002). The switchback from $SP_{14}$ to LP, caused a decrease in *srif* expression to 7 ± 1% within six weeks ($SP_{14}LP_6$, U-test, p=0.002). Hamsters returned to SP for a further eight weeks, after six weeks in LP, showed a partial increase of *srif* expression to 36 ± 3% ($SP_{14}LP_6SP_8$, U-test, p=0.002), in contrast to a continuing low expression in hamsters maintained in LP ($SP_{14}LP_{14}$). After 14 weeks in LP and another eight weeks in SP ($SP_{14}LP_{14}SP_8$) *srif* showed increased expression to 60 ± 6% (U-test, p=0.01), but only in hamsters that reduced body weight. This partial increase was significantly lower compared to $SP_{14}$ (U-test, p=0.01). Hamsters with constant body weight still had low *srif* expression (28 ± 7%, t-test, p<0.001), which was not significantly different to hamsters that were maintained in LP for 22 weeks (t-test, p=0.54).

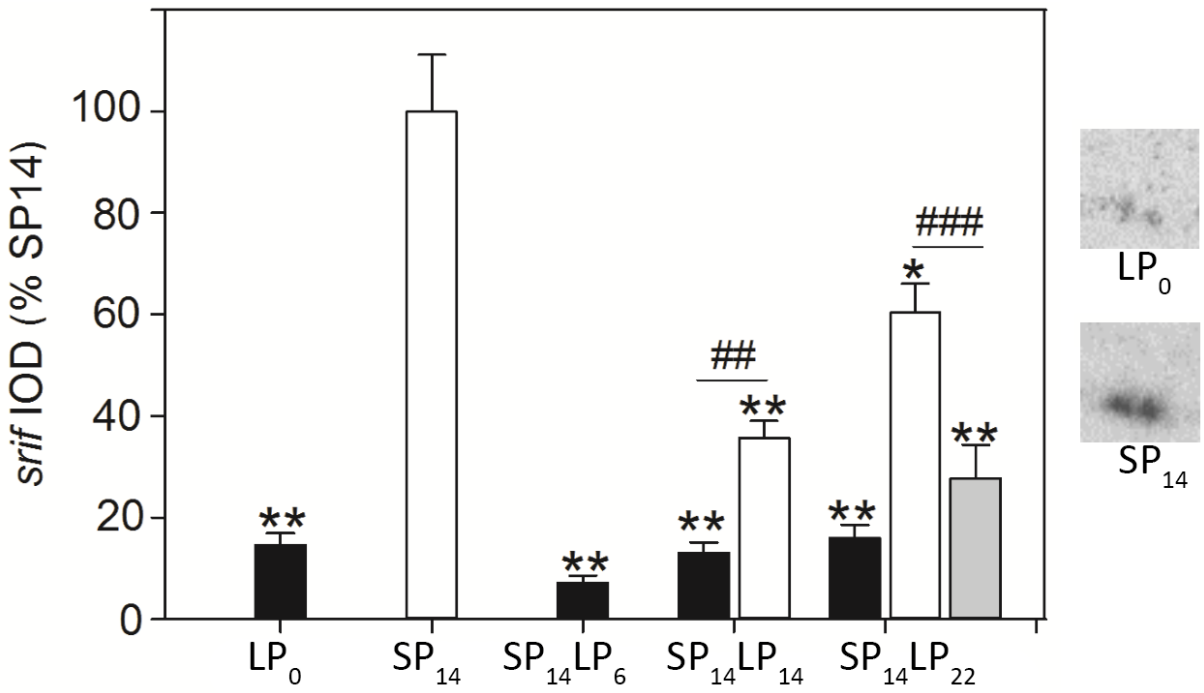


**Figure 16: Gene expression of *srif* in the arcuate nucleus.** Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to $SP_{14}$. Hamsters were kept in long photoperiod (LP, black) or short photoperiod (SP, white). White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. * significant difference to $SP_{14}$; # significant difference between other groups. Autoradiographs show representative brain sections with gene expression close to mean values.

## 2.1.5 Discussion

### 2.1.5.1 Switch from LP to SP

Hamsters reduced their body weight during exposure to SP for 14 weeks and gene expression showed significant changes to LP. Most seasonal changes of gene transcription were located in the ependymal layer of the third ventricle nearby the hypothalamus. Dependent on melatonin secretion Djungarian hamsters exposed to SP showed lower expression of *vimentin* in tanycytes, which is associated with structural changes in the hypothalamus (Bolborea et al., 2011; Herwig et al., 2013; Kameda et al., 2003). The exact function of vimentin in seasonal neuronal adaptation is not finally understood, but tanycytes play a pivotal role in seasonal adaptation of energy balance, metabolism and growth that all change with seasons (Bolborea and Dale, 2013; Ebling, 2015; Helfer and Tups, 2016; Nilaweera et al., 2011; Ojeda et al., 2008).

Thyroid hormone metabolism in the hypothalamus plays a central role in seasonal adaptation (Barrett et al., 2007; Ebling, 2015; Murphy et al., 2012). The current hypothesis is that changes in expression of *dio2* and *dio3* lead to low $T_3$ concentrations in the hypothalamus during SP exposure, which is considered to be a prerequisite for the catabolic response to SP (Barrett et al., 2007; Murphy et al., 2012). In the face of this hypothesis, it might be surprising that *dio2* expression was increased after 14 weeks in SP. However, earlier studies have already shown that *dio2* expression is lowest after 8 weeks in SP (Herwig et al., 2009), returns to LP levels after 14 weeks in SP (Barrett et al., 2007) and surpass LP with continuing time in SP (Herwig et al., 2013). It remains still unclear, how hamsters maintain a low body weight despite increasing *dio2* transcription. The earlier increase of *dio2* might be caused by a feedback mechanism, controlling central thyroid hormone metabolism, which tries to counteract ongoing low $T_3$ concentrations in the hypothalamus during SP.

In any case the increase of *dio2* seems to cause no increase of $T_3$ availability in the hypothalamus, because this would lead to an immediately increase of body weight (Murphy et al., 2012). Hence thyroid hormone metabolism always has to be considered in parallel with expression of *dio3*, which leads to catabolic inactivation of $T_3$. Consistent with previous studies, we found *dio3* abundantly expressed in SP, but not during LP (Barrett et al., 2007; Herwig et al., 2009; Herwig et al., 2012). In tanycytes, where *dio2* and *dio3* are probably co-expressed (Barrett et al., 2007; Bolborea et al., 2015; Samms et al., 2015) and DIO3 limits the availability of the pro-hormone $T_4$ by deiodinating it to inactive reverse $T_3$ (3,3',5'-triiodothyronine; $rT_3$) (Bianco et al., 2002). Despite high *dio2* expression it is likely that the presence of DIO3 prevents the accumulation of intracellular active $T_3$ and tanycytes remain hypothyroid. This could explain the plateau of low body weight despite increased *dio2* expression.

After 14 weeks in SP *mct8* expression did not differ from LP. A previous study showed that *mct8* is elevated after 18 weeks in SP (Herwig et al., 2013). Thus MCT8 might play no role during body weight adaptation, but could be important to maintain low thyroid hormone concentrations in the hypothalamus during continuing SP experience.

Furthermore, we analyzed expression of *tsh-r* in the ependymal layer as possible factor in the transduction from the photoperiodic signal, provided by melatonin, to genomic action. TSH is produced in the PT in a photoperiod-dependent manner and evidence emerged that TSH is involved in *dio2* transcription (Hanon et al., 2008; Nakao et al., 2008a; Ono et al., 2008). We found no differences in *tsh-r* mRNA concentration between LP and SP in contrast to a previous study, which showed a lower expression during SP (Herwig et al., 2013). Our new results hypothesize that *tsh-r* plays no critical role in mediating seasonal changes and that these changes depend more on TSH availability and not on presence of the receptor.

In accordance with previous studies (Barrett et al., 2006; Herwig et al., 2013) *gpr50* expression in the ependymal layer was low during SP. GPR50 is a melatonin- related receptor, which does not bind melatonin, but dimerizes with the melatonin receptor $MT_1$, which leads to an inactivation of $MT_1$ (Levoye et al., 2006). Physiological consequences of $MT_1$/GPR50 heterodimers and tissues with co-expression of both proteins are unknown. A down regulation of GPR50 in SP might reduce the inhibitory effect on $MT_1$ and therefore, increase melatonin efficiency during SP for example in the SCN and *pars tuberalis* were $MT_1$ receptors have been discovered (Lacoste et al., 2015). Recent studies with GPR50-KO-mice have also suggested an effect of GPR50 on metabolism (Ivanova et al., 2008; Jockers et al., 2008).

Similar to *gpr50*, gene expression of the cellular retinol-binding protein 1 in the ependymal layer was suppressed during SP. Barrett and colleagues (2006) showed, that transcription of *gpr50* and *crbp1* coincide with tanycytes near the hypothalamic arcuate nucleus, implicating a connection to these nearby neuronal structures. CRBP1 is a transport protein for retinol (vitamin A) the substrate for retinoic acid (RA) (Li and Norris, 1996). In mice and photoresponsive F344 rats retinaldehyde dehydrogenases, responsible for synthesis of retinoic acid (RA), is expressed in tanycytes with extended processes reaching into the arcuate nucleus (Shearer et al., 2010). A previous study showed that RA synthesizing and transducing elements of the RA signaling system are regulated in response to alterations in the photoperiod, with lower expression under SP conditions (Shearer et al., 2010). A down regulation of *crbp1* during SP might reduce the binding of retinol, which might lead to lower synthesis of RA in tanycytes. The retinoic pathway seem to have an important function in regulation gene transcription involved in the regulation of feeding, drinking and body weight (Ebling, 2015).

After 14 weeks in SP *srif* expression was highest. An increase *srif* expression has been observed after 18 weeks in SP (Herwig et al., 2013). Increased Somatostatin production seems to be important during SP. Somatostatin inhibits the production of GH (reviewed by Steyn et al., 2016). Reduced GH during SP is important to reduce body weight. It has been recently shown that reduced body weight includes reduction of lean mass and not only depletion of fat depots (Dumbell et al., 2015)

### 2.1.5.2 First Switchback from SP to LP

As expected, hamsters increased their body weight after the switchback from SP to LP, before reaching a plateau phase after 12 weeks. The final body weight after the switchback ($SP_{14}LP_{22}$) was higher than the initial body weight under LP conditions at week 0. A switchback from SP to LP causes a rapid reduction of melatonin production (Lerchl, 1995; Paul et al., 2008). Melatonin has been shown to act on $MT_1$ receptors in endocrine cells of the PT. An early response gene activated by LP is *tsh*-β in the PT. The two subunits TSH α and β produces TSH, which can act on TSH receptors at the ependymal layer of the third ventricle. Increase of TSH, induced by LP, causes a stimulation of *dio2* transcription in tanycytes (Bolborea et al., 2015; Hanon et al., 2008). We were unable to analyze *tsh*-β in our hamsters, because of fragmentary and incomplete sampling of the pituitary gland.

However, six weeks after the first switchback *dio2* expression in tanycytes was massively increased, probably caused by increased TSH concentrations, and at the same time point *dio3* was not expressed. Additionally expression of the thyroid hormone transporter *mct8* was reduced. This likely causes a lower influx $T_4$ influx into tanycytes, but also a lower efflux of $T_3$ (Heuer and Visser, 2009; Schweizer et al., 2014). This combination of high *dio2* and reduced *mct8* expression, should lead to high intracellular $T_3$ availability in the hypothalamus after the switchback. Murphy and colleagues (2012) showed that high $T_3$ availability in the hypothalamus lead to a rapid increase of body weight, mainly caused by an increase of food intake. While *dio3* was not expressed 6, 14 and 22 weeks after the switchback from SP, *dio2* expression slowly decreased to normal LP level. This suggests that high $T_3$ activation is only required during the time of body weight increase, but not to maintain the body weight during summer. Continuing hyperthyroidism in the hypothalamus might initiate a negative feedback loop via reduced TSH production in the PT resulting in decreasing *dio2* expression in tanycytes (Fonseca et al., 2013; Hanon et al., 2008; Pradet-Balade et al., 1997). Several studies showed that tanycytes build a morphological connection between the hypothalamus and PT and this connection seem to be very important for photoperiodic changes in physiology and behavior (Guerra et al., 2010; Kameda et al., 2003; Rodríguez et al., 1979).

Further changes in the ependymal layer were observed for *gpr50* and *crbp1* transcription after the switchback. However, their increase was slower and normal LP mRNA concentrations were not

reached after 6 weeks, but after 14 weeks. The function of GPR50 in seasonal adaptation is poorly understood. Most winter adaptations are driven by melatonin and these changes are reversed by refractoriness for melatonin or decreasing production caused by extended day length (Illnerová et al., 1984). A conceivable function of GPR50 could be inhibition melatonin-mediated effects by inactivation von $MT_1$ receptor through dimerization (Levoye et al., 2006). The slow increase of *crbp1* suggests that the retinoic acid pathway might play a minor or secondary role in body weight regulation and that this pathway might be under the control of an upstream regulatory process like thyroid hormone system (Helfer et al., 2012; Stoney et al., 2016). Interestingly retinoic acid is able to increase *mct8* expression in cell culture (Kogai et al., 2010). This might be another important cross-link between the RA pathway and thyroid hormones.

Beside seasonal changes in tanycytes we were interested in the ARC of the hypothalamus, which is considered as the center for regulation of energy balance. A fast-responding gene in the ARC is *srif*, which is involved in the seasonal regulation of the growth hormone somatotropin (GH) (Dumbell et al., 2015). In accordance to other studies, *srif* was higher expressed during SP (Dumbell et al., 2015; Herwig et al., 2013) and expression massively declined after the switchback to LD. It has been suggested that down regulation of *srif* is mediated downstream of thyroid hormone metabolism, because decreased *srif* expression is associated with higher TSH production during LP (Klosen et al., 2013). Low *srif* expression should enable GH production, which itself promotes production of insulin-like growth factor 1 (IGF-1) (Le Roith et al., 2001). Increase of IGF-1 concentrations linked to increased body weight has been shown in switchback hamsters (Dumbell et al., 2015). Our data support, that low *srif* expression is a requirement for body weight gain after the switchback from SP to LP.

#### 2.1.5.3 Second switchback from LP to SP (after 6 weeks)

Surprisingly all hamsters switched back a second time to SP after six weeks in LP stopped gaining body weight and few hamsters showed even a slight reduction of their body weight after eight weeks. Our initial hypothesis was that these hamsters are not able to respond to the SP signal again, after such a short time in LP, because they become insensitive to the SP signal. Several studies have shown that hamsters become refractory to the SP melatonin signal after about 18 to 24 weeks, despite continuous exposure to SP (Freeman and Zucker, 2001; Gorman and Zucker, 1995; Prendergast et al., 2000). To reverse refractoriness animals have to experience LP for at least 10 weeks, before they are able to respond to SP again (Bittman, 1978a; Kauffman et al., 2003; Reiter, 1972). Our data clearly show that hamsters have not become refractory to SP signals before the switchback. Thus our switchback hamsters are not comparable to photorefractory hamsters, where these mechanisms are irreversible.

Gene transcription initiated after the first switchback, which led to an increase of body weight, showed clearly reversed actions. After being a second time in SP for 8 weeks *dio2* expression was clearly reduced again and *mct8* increased to concentrations similar to those of the first time in SP. The response of *dio3* was not consistent, because only two hamsters showed an increase in response to SP. We cannot exclude that *dio3* was not expressed during the eight weeks after the switchback and downregulated again at the time point of sacrificing the animals, because deiodinases are able to quickly response to photoperiodic changes (Herwig et al., 2012). Despite partial changes in thyroid hormone metabolism, decrease of *dio2* suggest anew, but incomplete reduction of hypothalamic $T_3$ concentrations, which might explain the weaker body weight reduction. To reveal the coordination of *dio2* and *dio3* with the link to body weight a higher temporal resolution is necessary, otherwise fast shifts in gene expression might be overlooked.

*Vimentin, gpr50, crbp1* were considerably reduced again towards SP-like mRNA levels and clearly differed from LP groups. However, the reduction of *vimentin* and *gpr50* was less pronounced and gene expression was still higher compared to SP14. This might imply that these mechanisms were initiated later or the transition from LP to SP lasted longer. Also *srif* transcription reacted less pronounced, because the expression was only at 40% of SP14. This could mean that inhibition of GH was weaker, which could explain the minor body weight reduction. Maybe all these mechanisms are downstream of other pathways, like thyroid hormone homeostasis, thus changes are dependent and not quickly adjustable. It is conceivable that reversing the extreme expression of *dio2* might have worked as a molecular break. All changes after the early second switchback indicated that hamsters responded to the SP signal again, but reversed gene expression and body weight change was less pronounced.

#### 2.1.5.4 Second switchback from LP to SP (after 14 weeks)

The response to the second switchback after 14 weeks in LP was astonishing. While six hamsters showed an explicit reduction of body weight, which was stronger compared to the group with the earlier switch back, five hamsters maintained their high body weight. These hamsters did respond to the first switch to SP with a significant body weight reduction and therefore, are secondary non-responders. This different physiological response was manifested in a different response in gene expression, too. Hamsters with a reduced body weight showed a SP-like pattern in gene expression, whereas hamster with a constant body weight assigned more with LP groups. Therefore, we evaluated those hamsters as separate groups.

The expression of *dio2* did not reveal clear information, because *dio2* expression in hamsters kept at LP for 22 weeks after the first switchback returned to low concentrations - even lower than the $SP_{14}$

group. However, *dio2* expression was lower in hamsters with reduced body weight compared to $SP_{14}$, whereas hamster with a constant body weight did not differ from $SP_{14}$. This little variation might be enough to make a difference in hypothalamic $T_3$ concentrations. We assume that higher hypothalamic $T_3$ levels in non-responding hamsters prevented the body weight reduction. Fortunately *dio3* expression was more enlightening, because non-responding hamsters showed no *dio3* expression, but *dio3* transcription was remarkably increased in hamsters with reduced body weight. This clearly supports the general hypothesis of reduced $T_3$ concentrations in the hypothalamus as prerequisite for body weight reduction.

*Mct8* expression was higher in responding hamsters, indicating a higher thyroid hormone transport in tanycytes. However, the impact of higher thyroid hormone transport remains in the dark, because it could either mean a higher $T_4$ influx as compensatory mechanism for low intracellular $T_3$ concentrations or a higher efflux of $T_3$ to flush remaining $T_3$ out of the cells. At this point it is noteworthy that thyroid hormone concentrations in the blood showed no verifiable changes in response to photoperiodic alternations. Only circulating $tT_4$ concentrations after the first switch from LP to SP might be increased, which could be an evidence for higher thyroid hormone metabolism. However, the high variability between hamsters and small group size revealed no significant differences. For further studies we recommend larger groups to detect changes of thyroid hormones in the periphery in response to photoperiodic changes.

Back to the hypothalamic gene expression, *vimentin* showed a change only in hamsters of the $SP_{14}LP_{14}SP_8$ group with reduced body weight. Thus the cell structure in these hamsters was adapted to SP again and *vimentin* was reduced in all SP groups, when body weight was reduced. Also *gpr50* and *crbp1* were reduced in hamsters with a reduced body weight, which seem to be an indicator for SP adaptation. However, both genes expression was not as low as in $SP_{14}$ hamsters. Hamsters with body weight loss showed a transition of *crbp1* to SP levels, whereas hamster with constant body weight did not differ from the corresponding LP group. This might have been one reason, why *crbp1* transcription showed no significant difference between hamsters with and without body weight loss. As mentioned before, adaption of the retinoic pathway to alternating photoperiod seems to be a slow-going process, which might be an indicator for dependence on other upstream signaling pathways or a higher complexity.

Expression of *srif* clearly correlated to body weight loss. Hamsters with reduced body weight showed a clear increase of *srif*. This should increase somatostatin production, which inhibits release of growth hormone resulting in reduced body weight. Contrariwise body weight loss in the non-responding hamsters might have been prevented by absence of somatostatin, which probably

maintains higher GH production. These results manifest the assumption that somatostatin in the ARC plays a prominent role in seasonal body weight regulation.

Taken together all results from non-responding hamsters suggest that these animals seem to be refractory to SP. The question is why these hamsters did not respond to SP a second time while others were able to do so. Where these hamsters refractory to melatonin or did another mechanism, may be related to gonadal growth, prevent body weight reduction? To test the link between different molecular pathways and seasonal body weight adaptation further studies have to be done to shed more light on this highly complex regulation.

We conclude from these new results that deiodinases, *mct8, gpr50* and *srif* are regulated by photoperiodic alternations and linked to changes in body weight. Without changes in gene expression the body weight loss did not occur. We therefore suggest that these genes are involved in body weight regulation and did not follow changes in photoperiod. Crbp1 did follow the changes in photoperiod, but was not specifically linked to changes in body weight.

## 2.2. Influence of systemic thyroid hormone status on daily torpor and gene expression

The following chapter was published in the journal "Hormones and Behavior" in 2015. The original manuscript is included in this thesis, but figure numbers and references have been reformatted. The author of this thesis was involved in the design of all experiments supported by Annika Herwig and Eva Wirth. All *in vivo* experiments were carried out by me. Gene expression analysis by qPCR was carried out by Julia Kemmling and analyzed as well as interpreted by myself. Serum concentration analysis by radioimmune assays (RIA) was performed by Eddy Rijntjes and me. Manuscript was written by myself and revised by Annika Herwig, Eddy Rijntjes and Eva Wirth.

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**Thyroid hormone status affects expression of daily torpor and gene transcription in Djungarian hamsters (*Phodopus sungorus*)**

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### 2.2.1 Abstract

Thyroid hormones (thyroid hormone) play a key role in regulation of seasonal as well as acute changes in metabolism. Djungarian hamsters (*Phodopus sungorus*) adapt to winter by multiple changes in behavior and physiology including spontaneous daily torpor, a state of hypometabolism and hypothermia. We investigated effects of systemic thyroid hormone administration and -ablation on the torpor behavior in Djungarian hamsters adapted to short photoperiod. Hyperthyroidism was induced by giving $T_4$ or $T_3$ and hypothyroidism by giving methimazole (MMI) and sodium perchlorate via drinking water. $T_3$ treatment increased water-, food intake and body mass, whereas MMI had the opposite effect. Continuous recording of body temperature revealed that low $T_3$ serum concentrations increased torpor incidence, $T_b$ and duration, whereas high $T_3$ serum concentrations inhibited torpor expression. Gene expression of deiodinases (*dio*) and uncoupling proteins (*ucp*) were analyzed by qPCR in hypothalamus, brown adipose tissue (BAT) and skeletal muscle. Expression of *dio2,* the enzyme generating $T_3$ by deiodination of $T_4$, and *ucp*s, involved in thermoregulation, indicated a tissue specific response to treatment. Torpor per se decreased *dio*2 expression irrespective of treatment or tissue, suggesting low intracellular $T_3$ concentrations during torpor. Down regulation of *ucp1* and *ucp3* during torpor might be a factor for the inhibition of BAT thermogenesis. Hypothalamic gene expression of neuropeptide Y, propopiomelanocortin and somatostatin, involved in feeding behavior and energy balance, were not affected by treatment.

Taken together our data indicate a strong effect of thyroid hormones on torpor, suggesting that lowered intracellular $T_3$ concentrations in peripheral tissues promote torpor.

## 2.2.2 Introduction

Thyroid hormones (thyroid hormone) play an important role in the regulation of acute as well as seasonal changes in mammalian metabolism. They are produced in the thyroid gland, which mainly releases the prohormone thyroxine ($T_4$) and to a smaller extent the main bioactive form 3,3',5-triiodothyronine ($T_3$), into the bloodstream (Yen, 2001). Within the cell, three enzymes, deiodinases type 1, 2 and 3 (DIO1, DIO2, DIO3), are responsible for reductive removal of iodide atoms from thyroid hormone by phenolic ring deiodination (DIO1 and DIO2) and / or tyrosyl ring deiodination (DIO1 and DIO3). The intracellular $T_3$ concentrations are thus dependent on the tissue specific activity of deiodinases (Köhrle, 1999).

$T_3$ has been shown to affect metabolism and thermogenesis by peripheral and central mechanisms. In peripheral tissues such as brown adipose tissue and muscle $T_3$ is essential for facultative thermogenesis that can be rapidly induced or suppressed by the sympathetic nervous system (SNS) (Cannon and Nedergaard, 2004). It is well-known from humans and animals that hypothyroidism and hyperthyroidism are associated with considerable hypo- and hyperthermia, respectively (Silva, 2003). In both tissues uncoupling proteins (UCP), localized at the inner mitochondrial membrane, are responsible for heat dissipation. UCP1 is the most prominent protein of this family and is limited to BAT (Sell et al., 2004). UCP1 is primarily activated by noradrenergic stimulation mediated by the SNS, but T3 is involved as coactivator and is essential for the full thermogenic response of BAT (Bianco and Silva, 1987b; Silva and Larsen, 1986). UCP2 and UCP3 show a wider distribution, but their function is still under debate (Cioffi et al., 2009).

In the brain the hypothalamus is the most important area to regulate energy homeostasis. $T_3$ availability to the hypothalamus is regulated by transporters and deiodinase enzymes in the ependymal layer of the third ventricle and fenestrated capillaries in the median eminence (Herwig et al., 2009; Kalló et al., 2012; Watanabe et al., 2004). In the hypothalamus $T_3$ has been shown to be directly involved in the regulation of food intake and body weight by acting on neuropeptide Y (NPY) expressing neurons of the arcuate nucleus (ARC) (Coppola et al., 2007; Diano et al., 1998; Kong et al., 2004). The ARC regulates food intake and energy expenditure by two distinct neuronal populations (Abizaid et al., 2006). Activation of propopiomelanocortin (POMC) inhibits food intake, whereas neuropeptide Y (NPY) producing neurons play the counterpart and increase food intake (Dietrich and Horvath, 2013; Herwig et al., 2008).

Maintaining energy balance is a particular challenge for mammals living in a seasonally changing environment with low ambient temperatures ($T_a$) and reduced food availability during winter. A well-studied seasonal animal model is the Djungarian hamster (also known as Siberian hamster, *Phodopus sungorus,* that reduces energetic costs during winter by shutting down reproduction, decreasing body mass, increasing fur insulation and the capacity for non-shivering thermogenesis in BAT (Heldmaier and Steinlechner, 1981a; Kauffman et al., 2001; Rafael et al., 1985; Scherbarth and Steinlechner, 2010). $T_3$ availability to the hypothalamus has been shown to be a major driver of seasonal physiological adaptations (Ebling and Barrett, 2008; Hanon et al., 2008; Herwig et al., 2009; Herwig et al., 2013; Nakao et al., 2008b; Ross et al., 2011). Gene expression studies in the Djungarian hamster have shown a strong regulation of deiodinases in a time dependent manner and implicate that low $T_3$ concentrations specifically in the hypothalamus initiate the short day adaptations (Barrett et al., 2007; Herwig et al., 2009; Herwig et al., 2012; Herwig et al., 2013). It has been suggested that $T_3$ potentially regulates genes, such as somatostatin (sst), which are involved in this long term adjustment of energy balance (Dumbell et al., 2015; Herwig et al., 2012; Klosen et al., 2013).

Djungarian hamsters are able to express spontaneous daily torpor (SDT) as an ultimate mechanism to increase the energy saving capacity to between 44% and 63% (Heldmaier, 1989). Like all other seasonal adaptations in this species, torpor behavior is primarily driven by photoperiod and occurs after more than ten weeks of short photoperiod exposure under natural- or laboratory conditions (Elliott et al., 1987; Heldmaier and Steinlechner, 1981a; Ruf et al., 1989). SDT is a period of hypometabolism and hypothermia ($T_b$< 32°C) that occurs unpredictably during the light phase for 0.5-11 hours, even without obvious challenges like low $T_a$ or food restriction (Heldmaier and Ruf, 1992; Ruf et al., 1993). Although different hormonal systems (testosterone, leptin, somatostatin) have been shown to be involved in the expression of spontaneous torpor, the exact driving mechanisms for torpor are still unclear (Freeman et al., 2004; Scherbarth et al., 2015; Vitale et al., 1985).

Most studies so far have focused on the role of thyroid hormone in the regulation of body weight and reproduction in seasonal animal models, but despite its prominent role on metabolism and thermogenesis little attention has been paid to its role in the expression of torpor. Only one study directly showed an inhibitory effect of thyroid hormone on torpor after chronical release of $T_3$ into the hypothalamus (Murphy et al., 2012). Additionally the thyroid hormone derivate 3-iodothyronamine ($T_1$AM) is able to induce hypothermia in Djungarian hamsters and mice after i.p. injection (Braulke et al., 2008). Both these studies suggest that thyroid hormone and its further metabolites profoundly affect thermoregulation and torpor expression, however it is not clear whether and how these effects are mediated via central or peripheral mechanisms. The aim of this

study was to provide more detailed information about the role of thyroid hormones in torpor regulation. We examined the effects of systemically induced hypo- and hyperthyroidism on the torpor response of Djungarian hamsters. Moreover we provide information about the expression of deiodinases involved in $T_3$ metabolism in hypothalamus, BAT and skeletal muscle during normothermia and torpor as well as about potential $T_3$ target genes involved in thermogenesis (*ucp*), food intake (*npy, pomc*) and the seasonal control of growth and energy balance (*sst*).

## 2.2.3 Material & Methods

### 2.2.3.1 Animals and housing

Djungarian hamsters (*Phodopus sungorus*) were bred and raised in the Institute of Zoology at the University of Hamburg under artificial long photoperiod (LP; 16:8-h light : dark cycle) at 21°C ± 1°C ambient temperature ($T_a$). They were fed *ad libitum* with a hamster breeding diet (Altromin 7014, Germany) and had free access to water. At the age of 3-4 months, 56 hamsters of both sexes were transferred to a short photoperiod (SP; 8:16-h light : dark cycle) at 19°C ± 1°C $T_a$. The animals were single housed throughout the experiment and were weighed to ±0.1 g every other week during SP adaptation. All experiments and procedures were approved by the local animal welfare authorities (No. 83_13, Hamburg, Germany).

### 2.2.3.2 Experiment 1: *In vivo* experiment

For long-term recording of core body temperature ($T_b$) calibrated transmitters (DSI, Model TA-F10, St.Paul, MN, USA) were implanted i.p. in 24 hamsters in SP-week 12. The animals were anesthetized by inhalation of isoflurane (1.5-2.5%, Forene, Abott, Wiesbaden, Germany). Analgesia was maintained via s.c. injection of carprofen (5 mg/kg, Paracarp, IDT Biologika, Germany) which was administered before and 24h after surgery. Recording of $T_b$ in five minute intervals started in SP-week 13. Week 13 and 14 were defined as control weeks, during which none of the animals received any treatment. Torpor was defined as $T_b$ <32°C for more than 30 minutes. Mean torpor incidence was calculated as number of torpor bouts per 2 days within a treatment group. In SP-week 15 and 16 the animals were split into three groups (n=8) and were treated with $T_4$ (0.5 µg/ml L-Thyroxin; Sigma-Aldrich, Germany), $T_3$ (0.75 µg/ml 3,3',5-triiodothyronine; Sigma-Aldrich, Germany) or MMI (0.25 µg/ml Methimazole, Sigma-Aldrich, Germany), respectively via drinking water. Doses adapted from studies in mice and rats were tested in a pilot-study (Hamidi et al., 2010; Harun-Or-Rashid et al., 2010). $T_3$ and $T_4$ were dissolved in 0.025% sodium hydroxide (0.1N) and tap water. MMI treatment was administered with sodium perchlorate (10 µg/ml, Sigma-Aldrich, Germany) to amplify $T_4$ reduction and saccharin (3 µg/ml) was added for tastiness. During control as well as treatment

period, body mass, food- and water intake were monitored every other day at the end of the light phase. After SP-week 16 animals were sacrificed between Zeitgeber time (ZT) 4-5 by $CO_2$ and decapitation. Blood of all hamsters was collected for serum analysis. Serum was extracted from blood by centrifugation (3000 rpm for 15 min at 4°C) and stored at -21°C. Total $T_4$ ($tT_4$) and total $T_3$ ($tT_3$) concentrations were measured in serum samples by radio immune assays (DRG Instruments GmbH, Marburg, Germany) in duplicate determinations following manufacturer´s instruction. These assays contained anti-$T_4$ or anti-$T_3$ monoclonal antibodies, respectively. The functional sensitivity of the $T_4$ assay was 16.71 nmol/L, the intra-assay variation was ≤3.3% and the inter-assay variation was ≤ 7.5%. For the $T_3$ assay the functional sensitivity was 0.49 nmol/L, the intra-assay variation was ≤6.3% and the inter-assay variation was ≤ 7.7%. Cross-reactivity of $T_4$ assay with several related thyroid hormone metabolites was <0.1%. The $T_3$ assay had a cross-reactivity with most related molecules <0.1%, except 3,5-diiodo-L-thyronine (6.9%) and 3,3,5-triiodothyroacetic acid (TRIAC, 44.7%).

### 2.2.3.3 Experiment 2: Gene expression (qPCR)

A total of 32 hamsters were used for gene expression analysis without $T_b$ recording. In week 15 of SP adaptation they were split into 4 groups that received either no treatment (control n=9), $T_3$ (n=5), $T_4$ (n=9) or MMI/perchlorate (n=9) via drinking water for 14 days according to the treatment of experiment 1. In SP-week 16 thirteen hamsters (control n=4; $T_4$ n=4; MMI n=5) were killed between ZT4-5 during SDT. Torpor was defined by visual control and confirmed by a single $T_b$ measurement ($T_b$=23.9 ± 2.5°C) after decapitation. Hamsters treated with $T_3$ never entered torpor. Normothermic animals from each group (control n=5; T3 n=5; T4 n=5; MMI n=5) were killed between ZT4-5 in SP-week 16 and 17. Blood was sampled for serum analysis (see above), the brain was removed and frozen on dry ice, skeletal muscle (*M. gastrocnemius*) and intrascapular BAT were collected and snap frozen in liquid nitrogen. Tissues were stored at -80°C for qPCR analysis.

### 2.2.3.4 Quantitative real-time PCR (qPCR)

The hypothalamus, BAT and subsamples of skeletal muscle were homogenized in liquid nitrogen with a mortar and pestle to fine powder. Total RNA was extracted using 1 ml peqGOLD TriFast (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and 200 µl of chloroform. The RNA was purified with the Crystal RNA Mini Kit (Biolabproducts GmbH, Germany) following the manufacturer's instructions. Samples were treated with DNase (RNase-free DNase, Qiagen) and RNA quality was estimated by gel electrophoresis and quantity of total RNA was measured by spectrophotometric quantification (NanoDrop 1000, Thermo scientific, Germany). All samples had a ratio of absorbance (260nm/280nm) between 1.9 and 2.1 to verify the purity of RNA-extracts. Afterwards 1 µg of total

RNA was used to synthesize cDNA by using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) with oligo-(dT)-primer (0.5 µg/µl) in a final volume of 20 µl per reaction. Specific primers that targeted transcripts from following genes: *hprt, gapdh, 18s-rrna, dio2, dio3, ucp1, ucp2, ucp3, pomc, npy* and *sst* in *phodopus sungorus* were designed in conserved regions based on multiple sequence alignments from mouse, rat and Chinese hamster (*Cricetulus griseus)* sequences (GenBank). Melt temperature and dimer formation was checked using OligoAnalyzer online tool version 3.1 (http://eu.idtdna.com/calc/analyzer). Transcripts were amplified by PCR and products were cloned into standard cloning vector pGEM-T (Promega, Madison, USA) following manufacturer' s instructions and sequences were confirmed by a commercial service (GATC, Konstanz, Germany). Plasmids with the confirmed amplicon sequences were used for the standard curve generation in the qPCR. These sequences were also used to design specific qPCR primers used for expression studies (Table 2).

The synthesized total cDNA was used to estimate the expression of several genes by qPCR using the ABI Power SYBR Green master mix (Applied Biosystems, Darmstadt, Germany) and suitable primers (Table 2) in an ABI 7300 real-time PCR system. The qPCR conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 95°C for 15s and at 58°C (for: *18s, hprt and dio2*) or 60°C (for: *gapdh, dio3, ucp1, ucp2, ucp3, npy, pomc, sst*) respectively and 72°C for 30s at each step. Fluorescence was measured in the last step (72°C) of each cycle. All samples were analyzed in triplicates. The specificity of the amplification reaction was confirmed with melting point analysis ($T_m$) at the end of the qPCR by dissociation of the qPCR products (see Table 2).

**Table 2: *Phodopus sungorus* specific primer sequences used for qPCR.** *Gapdh* was used as reference gene in skeletal muscle, *hprt* in hypothalamus and *18s* in BAT. $T_m$ is the specific melting point temperature, at which qPCR products dissociate.

| Gene | Forward sequence | Reverse sequence | Amplicon length | Amplicon $T_m$ (°C) |
|---|---|---|---|---|
| *gapdh* | GACATCAAGAAGGTGGTGAAGCAGG | GTCAAAGGTGGAAGAGTGGGAGTCA | 120 bp | 83.5 |
| *hprt* | AGTCCCAGCGTCGTGATTAGTGATG | CGAGCAAGTCTTTCAGTCCTGTCCA | 141 bp | 76.7 |
| *18s* | GCTCCTCTCCTACTTGGATAACTGTG | CGGGTTGGTTTTGATCTGATAAATGCA | 111 bp | 80.9 |
| *dio2* | TGAAGAAACACAGGAGCCAAGAGGA | CATTATTGTCCATGCGGTCAGCCA | 111 bp | 82.6 |
| *dio3* | AGACTTCTTGTGCATCCGCAAGC | CACCTCCTCGCCTTCACTGTTG | 94 bp | 82.0 |
| *ucp1* | TCCAAGCACAGAGCCACCTA | GGGTTGTCCCTTTCCAGAGTGTTG | 114 bp | 79.7 |
| *ucp2* | AGAGCACTGTCGAAGCCTACAAGA | TCAGCACAGTTGACAATGGCGT | 109 bp | 81.6 |
| *ucp3* | ATGGATGCCTACAGAACCATCGC | GGTCACCATCTCAGCACAGTTGA | 111 bp | 80.7 |
| *pomc* | TGGAGAGCAGACAGTGTCAGGAC | TCTCGGTCAACGTCTGGTCGTC | 132 bp | 84.5 |
| *npy* | CCAGGCAGAGATACGGCAAGAGATC | CCATCACCACATGGAAGGGTCC | 119 bp | 79.2 |
| *sst* | GAAGTCTCTGGCGGCTGCTG | CAGCCTCATTTCATCCTGCTCCG | 145 bp | 84.4 |

Standard curves representing seven-point serial dilution (ranging from $10^{-2}$-$10^{-8}$) of plasmid cDNA were performed in each assay as duplicates and were used as calibrators for the relative quantification. Amplification efficiency (Eff%) of the primers was determined from the slope of the log-linear portion of the standard curve (Eff%=$10^{(-1/slope)}$-1). Eff% varied between 79% and 108% and the coefficient of determination ($r^2$) was greater than 0.98 for each essay. Relative gene expressions of products generated in the exponential phase of the amplification curve were calculated with the ΔΔCT-method. $C_t$ values >35 were excluded from the analysis. The reference genes were *hprt* for the hypothalamus, *18s* for BAT and *gapdh* for skeletal muscle. These genes were highly expressed in these tissues and the applied treatment as well as torpor had no influence on variance of expression in the appropriate tissue.

#### 2.2.3.5 Statistics

Body mass, food- and water intake values are expressed as mean values ± standard deviation (SD). All other data are shown as means ± standard error (SEM). Statistical analyses were performed with SigmaPlot™ 12 (Systat Software Inc). Data were tested for normal distribution using the Shapiro-Wilk-test. Analyses for parametric and dependent data were performed using paired student's t-test. Changes in body mass development were tested by Two-way repeated measures ANOVA and Tukey post-hoc test and effect size was calculated by eta-squared ($\eta^2$). Statistical analysis of qPCR was performed on normalized CT-values between two independent groups by student's t-test (parametric) or Mann-Whitney-U test (non-parametric) as appropriate depending on the compared groups. P-Values <0.05 were considered as significant. Effect size estimates for pair wise comparison were calculated with Cohen's d based on means, SDs and sample sizes.

### 2.2.4 Results

#### 2.2.4.1 Serum thyroid hormone concentration

Serum concentrations of $tT_3$ and $tT_4$ revealed no significant differences between untreated normothermic and torpid Djungarian hamsters (t-test, p=0.13, d=1.17). Therefore, all data of the same treatment group were pooled (Figure 17). $TT_3$ concentrations in untreated hamsters (control) was 1.46 ± 0.12 nM (n=9) and $tT_4$ was 59.5 ± 9.9 nM (n=8). $T_4$ treatment elevated $tT_3$ serum concentration to 2.26 ± 0.3 nM (n=11; Mann-Whitney-U, p=0.02, d=1.00) and $tT_4$ concentration to 209.94 ± 31.42 nM (n=12; Mann-Whitney-U, p<0.001, d=1.86). Hamsters treated with $T_3$ had a significant increase in $tT_3$ to 32.51 ± 5.55 nM (n=15; Mann-Whitney-U, p<0.001, d=1.85) and a decrease of $tT_4$ to 10.55 ± 0.42 nM (n=15; Mann-Whitney-U, p<0.001, d=4.20). Treatment with MMI

and perchlorate resulted in a significant decrease of $tT_3$ to 1.02 ± 0.06 nM (n=11; t-test, p<0.01, d=1.52) and $tT_4$ to 11 ± 0.42 nM (n=11; Mann-Whitney-U, p<0.001, d=3.84).

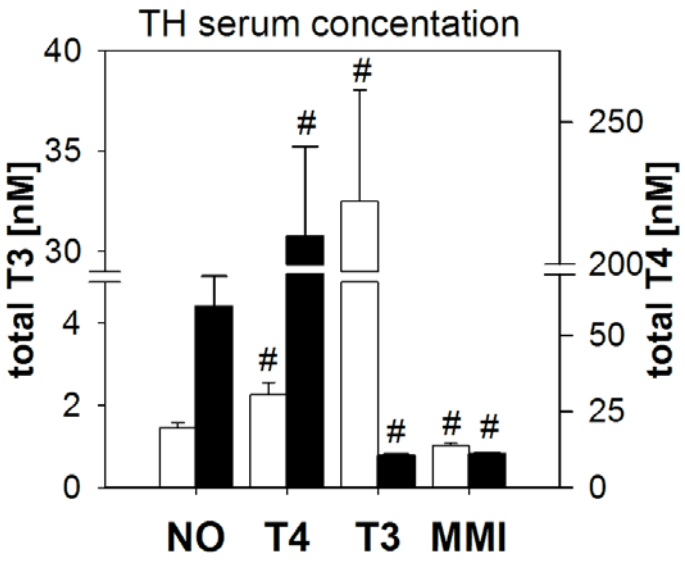


**Figure 17: Alteration of $tT_3$ (white bar) and $tT_4$ (black bar) serum concentrations after two weeks of treatment.** Significant differences (Mann-Whitney-U, p<0.05) in serum concentrations to the respective untreated hamster (NO) are marked with.

### 2.2.4.2 Body mass, food- and water intake

Mean ± SD body mass of all hamsters was 34.4 ± 4.68g in LP and decreased continuously to 26.4 ± 3.75g (-22.7 ± 8.5%) after 12 weeks in SP. $T_4$ treatment had no effect on mean water-, food intake or body mass (Figure 18). Daily $T_4$ uptake via drinking water was 2.2 ± 0.7 µg. $T_3$ treated hamsters increased their daily water intake from 4.2 ± 1.3g to 5.2 ± 2.1g (paired t-test, p=0.001, d=0.85) and their food intake from 3.2 ± 0.7g to 3.9 ± 0.8g (paired t-test, p=0.01, d=1.35 ) due to treatment. Daily $T_3$ uptake via drinking water was 3.9 ± 1.6 µg. Mean body mass during control weeks was 24.7 ± 0.5g and 25.2 ±1.1g during treatment weeks (paired t-test, p=0.20, d=0.17). The difference between the mean values was not significant, but the body mass increase was significantly higher from day 26 compared to the control weeks (Figure 19, Two-way-RM-ANOVA, p<0.05, $\eta^2$=0.08). Hamsters treated with MMI showed the opposite effect. They reduced their mean daily water intake from 4.2 ± 1.6g to 3.4 ± 1.1g (paired t-test, p=0.02, d= 1.30) and their food intake from 3.1 ± 0.6g to 2.6 ± 0.5g (paired t-test, p=0.01, d=1.36) due to treatment. Mean body mass was decreased from 26.5 ± 3.1g to 25.6 ± 3.0g (paired t-test, p<0.001, d=0.21). The body mass loss was higher at the beginning of the treatment and hamsters regained body mass in the second week of treatment (Figure 19). Two-way-RM-ANOVA found a significant difference only between the highest body mass of the control week and the lowest body mass of the treatment weeks (day 7 vs. day 16, p=0.04, $\eta^2$=0.06).

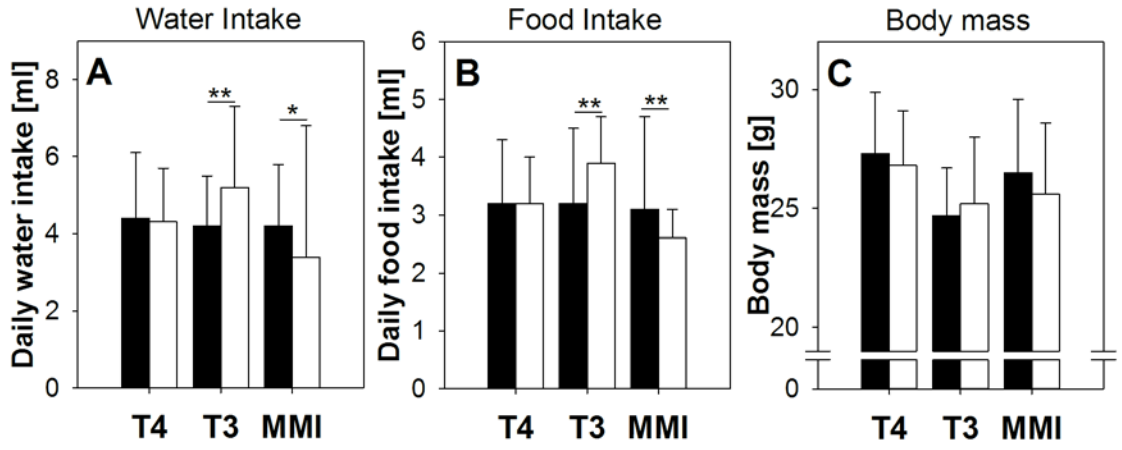


**Figure 18: Comparison of mean +SEM water intake (A), food intake (B) and body mass (C)** between two control weeks (black bar) and two treatment weeks (white bar). Significant differences (t-test) between control and treatment weeks are marked with * ($p<0.05$) or ** ($p<0.01$).

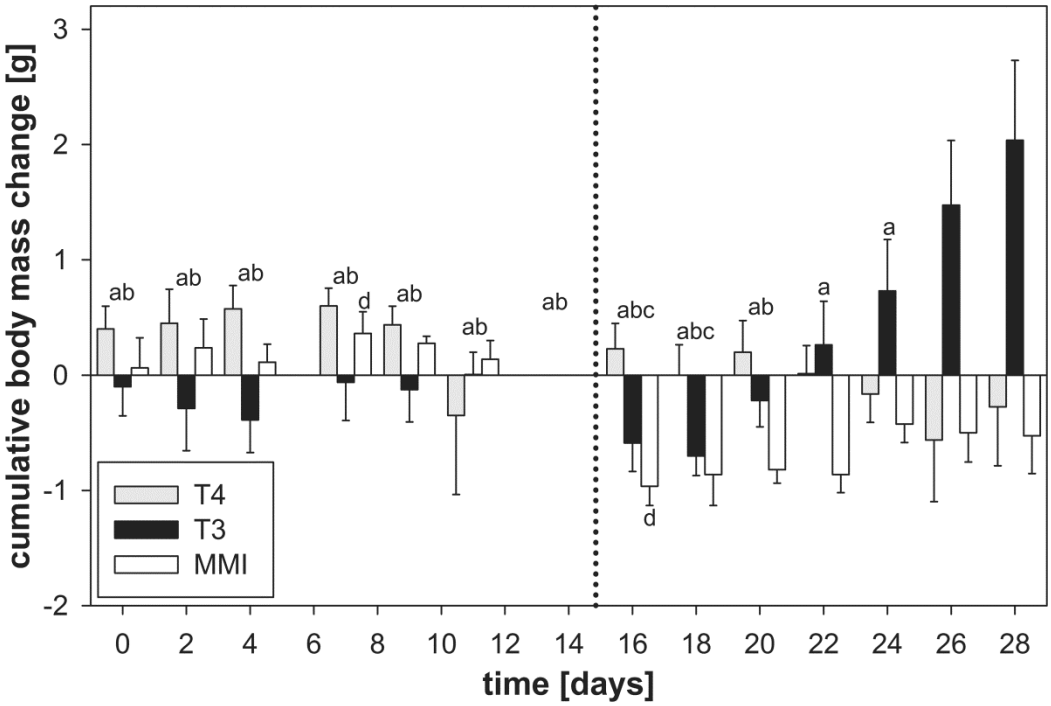


**Figure 19: Body mass change relative to day 14 (end control week).** Bars represent means ±SEM. Differences in the $T_3$-group are marked when significant to day 28 (a), day 26 (b), day 24 (c) and differences in the MMI-group were marked with (d) (Two-way-RM-ANOVA, $p<0.05$). In the $T_4$-group no significant changes were observed. The dotted line marks the start of the treatment.

### 2.2.4.3 Torpor expression

The different treatments had distinct effects on $T_b$ and torpor expression. Figure 20 shows representative individual $T_b$ recordings of hamsters treated with $T_4$, $T_3$ and MMI, respectively. Torpor incidence (Figure 20A) was not affected by $T_4$ treatment and mean ± SEM individual amount of torpor bouts was 5.8 ± 1.2 (control weeks) vs. 5.8 ± 1.3 (treatment weeks).

Mean ± SEM torpor duration was shortened from 270.0 ± 30.9 min to 218.2 ± 29.4 min (Paired t-test, p=0.05, d=0.64, Figure 20B) in $T_4$ treated animals. The mean $T_b$ during the dark period of these hamsters showed no changes. However, mean minimal $T_b$ during torpor, as indicator for the depth of torpor, was increased relative to the control weeks from 24.6 ± 0.43°C to 26.3 ± 0.46°C (Mann-Whitney-U, p<0.001, d=0.56) upon $T_4$ treatment.

$T_3$ treatment reduced torpor expression and increased $T_b$ (Figure 21B). After the start of $T_3$ treatment torpor incidence decreased rapidly within few days (Figure 20A) and mean ± SEM number of torpor bouts dropped from 7.5 ± 0.9 events during the control weeks to 1.5 ± 0.5 during treatment weeks (t-test, p<0.001, d=2.95). Mean ± SEM duration of torpor bouts decreased from 263.1 ± 38.3 min to 175.9 ± 48.0 min (paired t-test; p<0.01, d=0.79; Figure 20B). During the dark phase $T_b$ increased from 35.9 ± 0.06°C (control) to 36.4 ± 0.06°C (treatment; paired t-test, p<0.001, d=3.00). Mean minimal $T_b$ during torpor increased from 24.5 ± 0.36°C to 26.3 ± 0.73°C (Mann-Whitney-U, p=0.021, d=0.62 ) due to $T_3$ treatment.

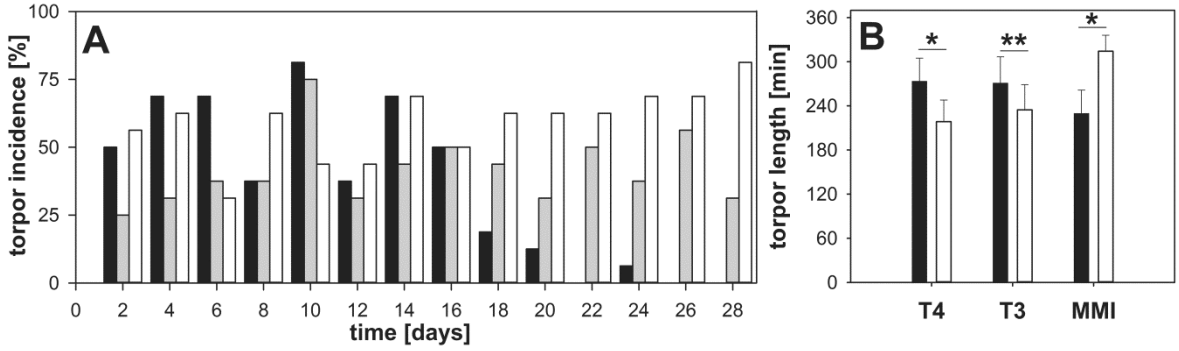


**Figure 20 Effect of treatment on torpor incidence and torpor length.** (A) Torpor incidence of the three groups (black= $T_3$, grey=$T_4$, white=MMI) are summarized in two day segments. (B) Mean torpor length of the control weeks (black) compared with the treatment weeks (white) and significant differences are marked with asterisks (paired t-test).

MMI had the opposite effect on torpor expression and $T_b$. Torpor incidence increased (Figure 20A) towards the end of the second treatment week. Thus the mean ± SEM total number of bouts increased from 7.4 ± 0.7 in two control weeks to 9.4 ± 1.1 in two MMI treatment weeks (t-test, p=0.087, d=0.74). The increased total number of torpor bouts indicated only a statistical trend due to a naturally occurring high variability in torpor frequency between individuals. In our study, one hamster showed a high torpor expression during the control weeks and did not further increase the expression due to MMI treatment. The mean torpor bout duration increased from 229.5 ± 31.9 min to 310.3 ± 23.9 min (paired-test, p=0.01, d=1.10; Figure 20B). Mean ± SEM $T_b$ during the dark phase was slightly reduced from 36.2 ± 0.06°C to 36.0 ± 0.09°C (paired t-test, p=0.02, d=0.75) and the $T_b$

amplitude was noticeably reduced in some hamsters (Figure 21C). MMI treatment reduced the mean minimal $T_b$ during torpor from 25.5 ± 0.35°C to 24.0 ± 0.33°C (Mann-Whitney-U, $p<0.001$, $d=0.52$).

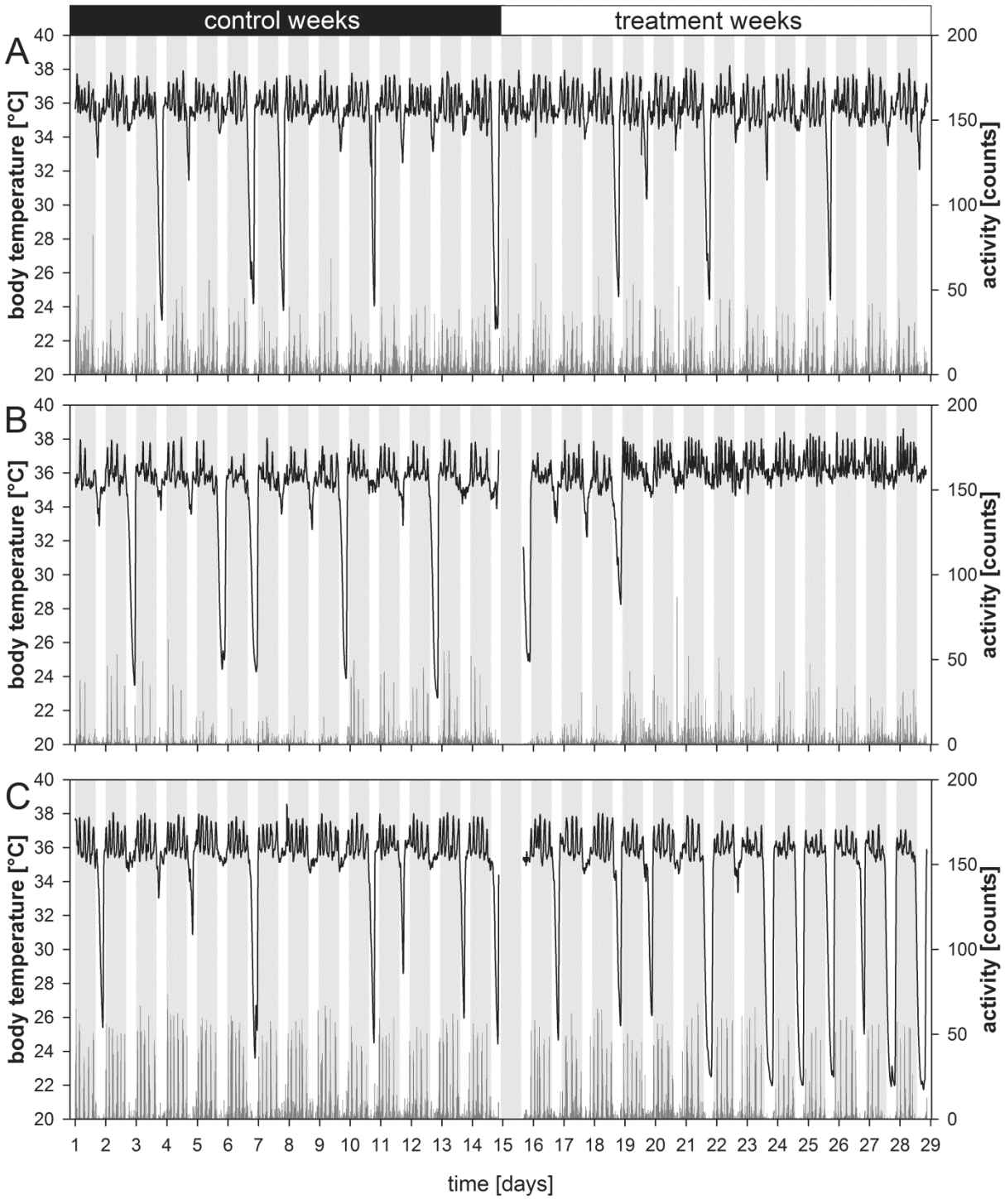


**Figure 21: Body temperature (black) and activity (dark grey) of exemplary hamsters treated with $T_4$ (A), $T_3$ (B) or MMI (C) respectively.** Hamsters were treated since day 15. Dark phase is highlighted in grey.

### 2.2.4.4 Gene expression

#### *2.2.4.4.1 Deiodinases*

*Dio2* was highly expressed in the hypothalamus and BAT, but less abundant in skeletal muscle. In the hypothalamus *dio2* was down regulated after $T_3$ treatment by 31% (Figure 22, t-test, p=0.01, d=2.02) relative to control animals, but unaffected by $T_4$ or MMI treatment in normothermic animals.

During torpor *dio2* expression decreased between 40 and 60% in all groups relative to their normothermic treatment group (Figure 22; t-test, control p=0.02, d= 3.62; $T_4$ p<0.01, d=3.72; MMI p=0.01, d=2.21). In BAT, $T_4$ provoked a down regulation of *dio2* by 70% in normothermic animals (t-test, p=0.02, d=1.94), but gene expression was unchanged after $T_3$ or MMI treatment. During torpor *dio2* expression in BAT was decreased by more than 94% in all hamsters (Figure 22, t-test; control, p<0.01, d=3.02; $T_4$, p<0.01, d= 3.00; MMI, p<0.01, d=3.18) compared to normothermic control hamsters. The difference to their normothermic control group was significant in MMI treated hamsters (t-test, p=0.03, d=1.74). *Dio2* down regulation in $T_4$ treated hamsters compared to their normothermic group during torpor was not significant. In skeletal muscle *dio2* expression was not regulated by treatment in normothermic animals. During torpor *dio2* expression was lowered by 52 to 98% in $T_4$ (t-test, p<0.001, d=6.65) and MMI (t-test, p=0.01, d=2.19) treated animals compared to their normothermic group, but not in untreated hamsters. The abundance of deiodinase type 3 (*dio3*) was marginal and close to the detection limit in either tissue and upon either treatment. Analysis of relative gene expression for *dio3* was inadequate.

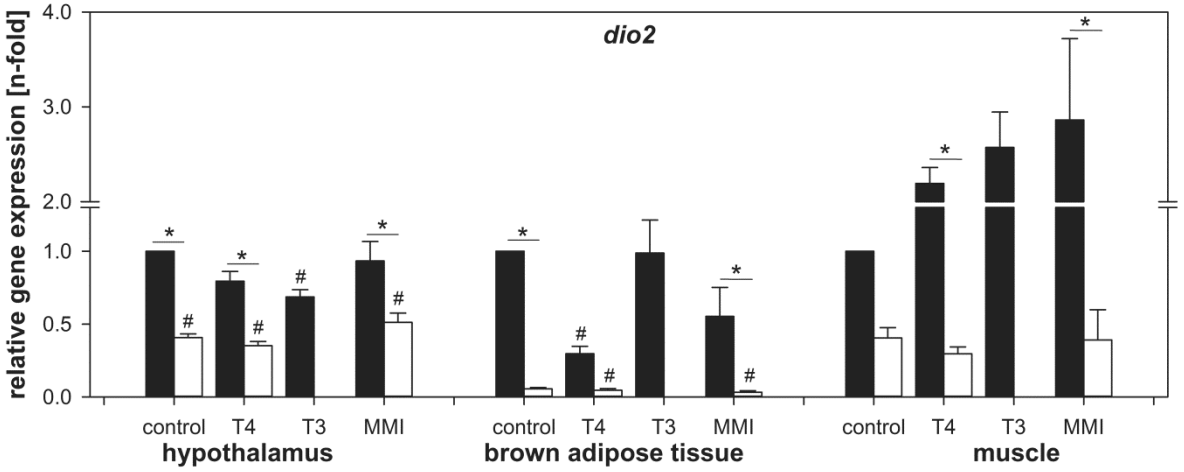


**Figure 22 Comparison of relative *dio2* gene expression** between normothermic (black) and torpid (white) hamsters. Significant differences (t-test) to untreated normothermic hamsters (control, black) are marked with # and differences between normothermic and torpid hamsters are marked with *

*2.2.4.4.2 Uncoupling proteins*

*Ucp1* mRNA was only detectable in BAT and none of the three treatments changed *ucp1* expression (Figure 23A). During torpor *ucp1* expression was reduced by 40-70% compared to non-torpid hamsters (t-test; control, p=0.03, d=2.04; $T_4$, p=0.03, d=1.60; MMI, p<0.01, d=2.82). Uncoupling protein 2 (*ucp2*) was detectable in all three tissues (Figure 23C), but predominantly in BAT.

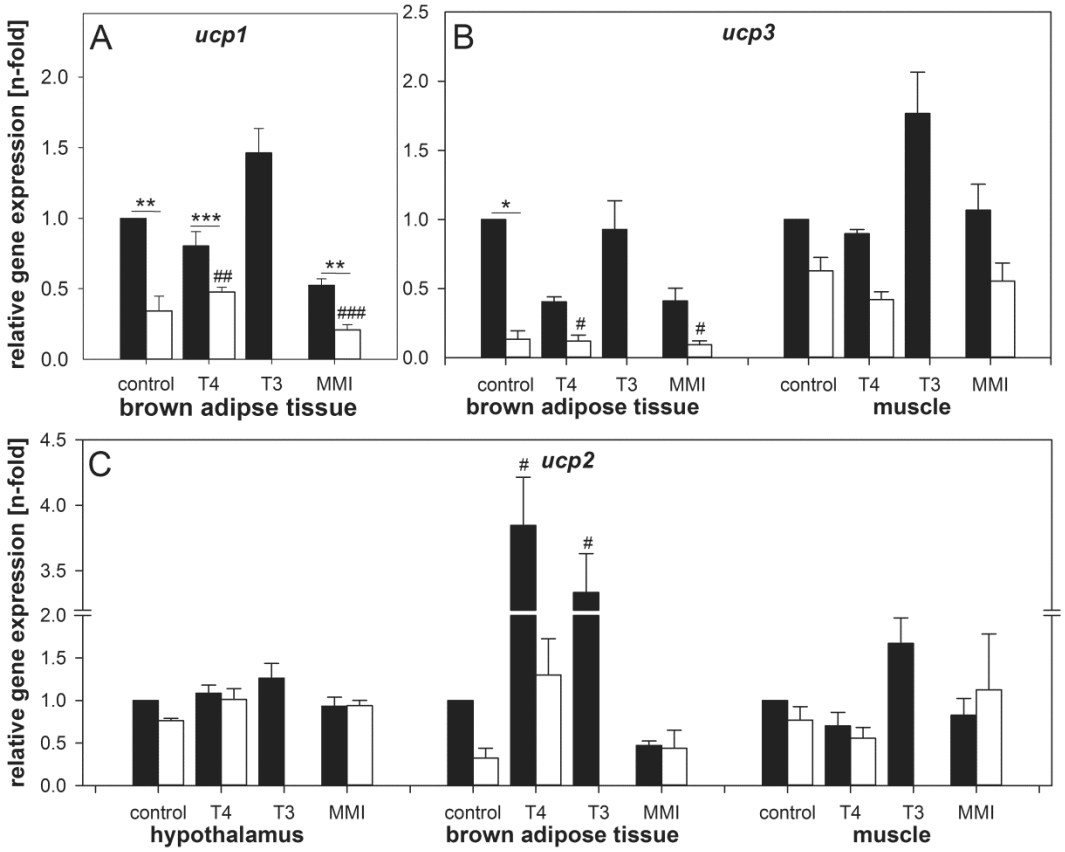


**Figure 23 : Comparison of relative gene expression of *ucp1* (A), *ucp3* (B) and *ucp2* (C)** between normothermic (black) and torpid (white) hamsters. Significant differences to untreated normothermic hamsters (control, black) are marked with # (t-test) and differences between normothermic and torpid hamsters are marked with * (t-test).

In BAT, *ucp2* expression was increased by 234% after $T_3$ (t-test, p=0.01, d=2.09) and by 285% after $T_4$ (t-test, p=0.01, d=2.16) treatment in normothermic hamsters. No treatment effect of MMI was detected. Torpor did not affect *ucp2* expression in BAT. In hypothalamus and skeletal muscle the expression of *ucp2* was unaffected by either treatment and was not regulated during torpor. Expression of uncoupling protein 3 (*ucp3*) in the hypothalamus was close to the detection limit (data not shown). However, *ucp3* was highly expressed in BAT and expressed lower in the skeletal muscle. In BAT *ucp3* was not affected by treatment in normothermic animals but gene expression during torpor was down regulated by more than 87% compared to normothermic control hamsters

independent of treatment (Figure 23B; t-test, control p=0.04, d=1.88; $T_4$ p=0.02, d=2.09; MMI p<0.01, d=2.49). In skeletal muscle none of the treatments affected *ucp3* neither in normothermic nor in torpid hamsters (Figure 23B).

#### *2.2.4.4.3 Expression of hypothalamic genes involved in energy balance*

*Npy* and *pomc* were noticeably expressed in the hypothalamus (Figure 24). Neither treatment nor torpor had an impact on the expression of *npy.* After treatment with $T_4$, *pomc* expression was 8-fold higher in normothermic hamsters and 10-fold higher in torpid hamster, but there was no significant difference between normothermia and torpor. The only change of gene expression during torpor was present in the MMI group with an up regulation of 400% (t-test, 0.01, d=2.09). *Srif* was abundantly present in the hypothalamus. It was neither regulated by treatment nor by hypothermia (data not shown).

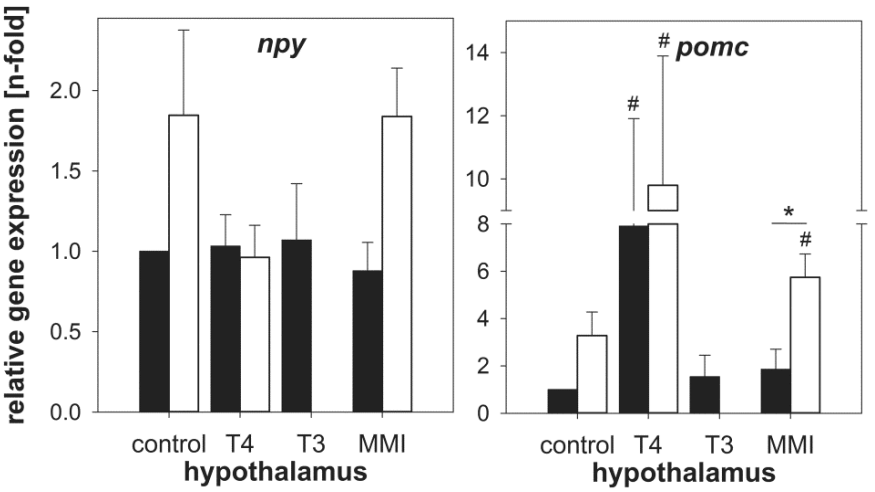


**Figure 24 Comparison of relative gene expression of *npy* and *pomc* in the hypothalamus** between normothermic (black) and torpid (white) hamsters. Significant differences to untreated normothermic hamsters (control, black) are marked with # (t-test) and differences between normothermic and torpid hamsters are marked with * (t-test).

## 2.2.5 Discussion

Thyroid hormones have long been known for their potent effects on food intake, body mass and thermogenesis. More recent studies have demonstrated that thyroid hormones also play a pivotal role in the seasonal regulation of energy balance in mammals (Ebling, 2015). Mostly these studies have focused on long-term adaptations of body mass and reproduction, but only two studies so far have described a direct effect of thyroid hormones on torpor expression (Braulke et al., 2008; Murphy et al., 2012). Here we provide novel data about different systemic thyroid hormone short-

term manipulations on torpor expression in the Djungarian hamster. Our results confirm that thyroid hormones profoundly affect torpor behavior. Increased $T_3$ concentrations disabled torpor expression, whereas hypothyroidism favored torpor. Gene expression in hypothalamus, BAT and skeletal muscle revealed tissue specific responses to thyroid hormone. During torpor expression of some genes involved in thyroid hormone activation and BAT thermogenesis were suppressed.

Thyroid hormone treatment via drinking water was sufficient to cause pronounced changes in serum thyroid hormone concentrations. The downside of this treatment was that thyroid hormone uptake was inconstant and animals probably never reached a stable thyroid hormone serum level, but injections of thyroid hormone or implantation of minipumps would have been an unwanted disturbing factor for torpor. After treatment with $T_4$ and $T_3$, blood serum concentrations of $tT_4$ or $tT_3$, were increased above the normal physiological concentrations respectively. Hamsters treated with $T_4$ had increased $tT_3$ serum concentration (+56%), indicating a higher turnover from $T_4$ into $T_3$. In Djungarian hamsters the circulating $tT_3$ and $tT_4$ serum levels naturally underlie seasonal changes and are elevated during the winter (Herwig et al., 2009; Seidel et al., 1987). It seems that slight additional increase of active $T_3$ in serum of $T_4$ treated SP hamsters was not sufficient enough to cause obvious changes in body mass and food intake. However, our $T_3$ treatment resulted in a 22-fold increase of $tT_3$ serum concentrations and caused an increased food- and water intake resulting in an increased body mass.

The increasing water intake consequently increased the daily $T_3$ uptake and a steady state was not reached within two weeks. The increase of food intake and body mass are in accordance with the study by Murphy et al. 2012 that have shown the same effect in Djungarian hamsters after central $T_3$ administration. This contrasts with studies of peripheral administration of $T_3$ in mice and rat that have shown a decrease in body mass, because the energy expenditure during hyperthyroidism exceeded the energy intake (López et al., 2010; Luo and MacLean, 2003). We assume that hyperthyroid hamsters can avoid a negative energy balance, due to their improved fur insulation in SP, which could reduce heat loss (Kauffman et al., 2001). Accordingly, this could lead to a positive energy balance in hamsters.

MMI treated hamsters had lowered $tT_3$ (-30%) and $tT_4$ (-80%) serum concentrations, but a complete thyroid hormone ablation was not possible. A partial reduction of thyroid hormones with MMI has also been reported for mice (Braun et al., 2011; Groba et al., 2013; Marsili et al., 2010). Despite incomplete ablation of thyroid hormone serum concentrations, MMI treatment had clear physiological effects opposing those of $T_3$ treated hamsters. Food- and water intake were reduced and resulted in a body mass loss. That MMI treated hamsters showed a body mass loss could be a short-term treatment effect and is agreement to studies with mice and rats (Alva-Sánchez et al.,

2012; Groba et al., 2013), but contradicts the clinical symptoms of humans. Typical clinical symptoms of hypothyroidism are a low basal metabolic rate, loss of appetite, but an increasing body mass due to the decreased energy expenditure (Chang et al., 2014; Laurberg et al., 2012). Over the entire treatment period of our study, water intake was still high enough to ensure drug uptake and food intake was still above 80% of the normal food intake to exclude food restriction, which could have been a factor for torpor increased expression (Diedrich et al., 2015a; Ruf et al., 1993). That MMI treated hamsters were not fasted was supported by unchanged *npy* mRNA expression. Fasting would have led to increased *npy* expression (Coppola et al., 2007; Herwig et al., 2009).

Thyroid hormones had considerable effects on body temperature and torpor expression. Firstly, $T_4$ had no influence on $T_b$ and number of torpor bouts, confirming the low biological activity of $T_4$ itself. However, the duration and depth of torpor bouts were reduced after $T_4$ treatment. This effect might have been caused rather by the slightly increased serum $T_3$ concentration upon $T_4$ treatment than by the increased $T_4$ concentration. $T_4$ treatment in normothermic hamsters only led to limited gene expression changes in BAT. Here, *dio2* was down regulated whereas *ucp2* was upregulated. Less *dio2* should lead to a reduced intracellular turnover from $T_4$ to $T_3$. $T_4$ treated hamsters had slightly higher $tT_3$ serum concentration and the down regulation of *dio2* in BAT might be a counter reaction to this higher $tT_3$ level to normalize $T_3$ concentration in adipocytes. It is surprising, that *dio2* was not down regulated in the hypothalamus or skeletal muscle and could lead to the assumption, that different tissues have a different sensitivity to $T_3$. In hypothalamus, BAT and muscle *dio1* was not expressed (data not shown) and is not involved in the inactivation of $T_3$ in these tissues.

Secondly $T_3$ had obvious effects on $T_b$ and torpor and gene expression. Expression of torpor was immediately absent or ceased after few days of treatment. If torpor events occurred during treatment, these torpor bouts were shorter and shallower. Again these findings are in accordance with those reported by Murphy and colleagues (2012) who showed that the central administration of $T_3$ prevents torpor. The administration via drinking water was sufficient to cause the same effect. Our data point to a differential response to $T_3$ in central and peripheral tissues. Surprisingly *dio2* transcription was only down regulated in the hypothalamus, likely compensating for the increased serum $T_3$ concentrations. That *dio2* was not clearly regulated in the muscle, indicating a low thyroid hormone turnover, might be a consequence of a 10-fold lower mRNA content compared to hypothalamus and BAT.

However, no regulation on the transcription level in BAT and muscle does not exclude a lower DIO2 activity in these tissues. $T_3$ could also have posttranscriptional regulatory effects at the protein level. In rat cell cultures high doses of $T_3$ reduced DIO2 activity, but not *dio2* transcription (Martinez de Mena et al., 2010). Surprisingly *dio3* was nearly undetectable in all three tissues, and intracellular

deactivation of $T_3$ via DIO3 seems to play no role. It has previously been shown that *dio3* is highly expressed in the hypothalamus of Djungarian hamsters after a photoperiodic switch from LP to SP (Barrett et al., 2007; Herwig et al., 2009). However, after 8 weeks in SP *dio3* mRNA content started to decrease again and reached low levels after 14 weeks in SP (Barrett et al., 2007). Since our samples were taken after 16 weeks of SP exposure, it is possible, that *dio3* mRNA content was down to an almost undetectable level. It is surprising though that *dio3* expression was not increased after $T_3$ treatment to counteract excess of $T_3$. In rats *dio3* expression is strongly switched on after a single i.p. injection of $T_3$ (Herwig et al., 2014). It must be taken into account that our samples were only taken after 10 days of $T_3$ treatment. Perhaps *dio3* is only up regulated immediately at the start of $T_3$ treatment to counter short-term changes, but the up regulation is not sustained and therefore, not involved in long-term thyroid hormone regulation.

Overall we found an increased $T_b$ during $T_3$ treatment, which is in conformity with previous studies and indicates a generally increased metabolism. It has been known for many years, that $T_3$ stimulates thermogenesis resulting in an increasing $T_b$ (Silva, 2003). Heat production can be divided into obligatory and facultative thermogenesis and is essential for endothermic animals to maintain a high $T_b$. Obligatory thermogenesis is an inevitable accompaniment of all metabolic processes in all organs and is generally named basal metabolic rate. Facultative thermogenesis is superimposed on obligatory thermogenesis, can be rapidly induced or suppressed by the sympathetic nervous system (SNS) and is restricted to brown adipose tissue (BAT) and skeletal muscle (Cannon and Nedergaard, 2004). The modulation of thermogenesis by thyroid hormones is typically linked to the uncoupling of cellular metabolism from ATP synthesis in peripheral tissues such as BAT and skeletal muscle. Moreover central pathways are involved in thermogenesis and the most significant interaction is between thyroid hormone regulation and adrenergic signaling (Cannon and Nedergaard, 2010; López et al., 2013). It has been shown that the genes of UCPs are directly regulated by $T_3$ (Bianco et al., 1988; Rabelo et al., 1995; Silva and Rabelo, 1997).

Surprisingly we found no significant effect on *ucp1*, the most important protein for facultative thermogenesis. The lack of effect of $T_3$ on *ucp1* in BAT might be explained by the fact, that *ucp1* is already highly expressed during short day acclimation (Demas et al., 2002). Furthermore, $T_3$ does not have exclusively genomic effects and also acts additionally as coactivator of UCP1. Activation of UCP1 is primarily under control of the SNS, but activation of facultative thermogenesis in BAT is dependent on locally generated $T_3$ (Bianco and Silva, 1987b). Increased $T_b$ after $T_3$ treatment results from increased obligatory thermogenesis, but the possible involvement of BAT UCP1 in this type of thermogenesis is not clear (Kim, 2008). It is more likely that $T_3$ increases general $T_b$ via skeletal muscle-mediated thermogenesis. In the skeletal muscle UCP3 is a candidate for thermogenic effects

given its highly $T_3$-responsive gene expression (de Lange et al., 2001), although the exact function of UCP3 in thermoregulation is still not established (Hesselink and Schrauwen, 2005). An up regulation of *ucp3* by $T_3$, observed in rats (Larkin et al., 1997; Reitman et al., 1999), was not significant in our Djungarian hamsters. UCP3 was also highly expressed in BAT, but gene expression was not affected by $T_3$ treatment. Because gene expression is not affected it has to be proven, if obligatory thermogenesis is activated by $T_3$ on protein level.

Although the main function of UCP2 is still unclear, previous studies indicate at least a special role in the family of UCPs. Expression of *ucp2* is increased in situations with increased energy expenditure, but the capacity to uncouple is still a matter of debate (Cioffi et al., 2009). A specific role of *ucp2* in the hypothalamus in response to $T_3$ was proposed by (Coppola et al., 2007). UCP2 is co-expressed in NPY/AgRP-neurons in the hypothalamic arcuate nucleus and tanycytes directly contact with these neurons. These tanycytes express *dio2* and regulate the local hypothalamic production of T3 and increasing $T_3$ levels stimulate *ucp2* transcription (Coppola et al., 2007). In our study an increase of hypothalamic *ucp2* expression by $T_3$ was not observed. The reduced *dio2* expression after $T_3$ might have been sufficient to prevent an increase of $T_3$ in the hypothalamus. However, our data support a particular role of UCP2, because only *ucp2* and neither *ucp1* nor *ucp3* expression were elevated in BAT after $T_3$. This effect was also observed in studies with rats (Masaki et al., 1997). An effect of $T_3$ on expression of *npy* or *pomc* was not observed in this study and did not reflect the increased food intake. However, genes which are involved in food intake are linked to a circadian rhythm and to feeding behavior and might cover effects linked to the thyroid hormone treatment (Stütz et al., 2007).

Finally the importance of $T_3$ on thermogenesis also became obvious in hypothyroid hamsters. Reduced $T_3$ levels were sufficient to provoke a negative effect on thermoregulation, probably by lower thermogenesis in BAT and skeletal muscle. Hypothyroid hamsters had a reduced $T_b$ and were more prone to enter torpor. Most hamsters entered torpor more often and torpor bouts were longer and deeper. The effect of MMI seems to be stronger after few days of treatment, because longer torpor bouts occurred more towards the end of the experiment. Delayed effects seem to be reasonable, because MMI is an inhibitor of thyroid hormone production and does not directly act on the thyroid hormone level in the blood. Hypothyroidism had no effect on gene transcription in normothermic hamsters. It was surprising that *dio2* was not up regulated to compensate low $T_3$ levels. MMI treatment had no influence on *dio2* mRNA levels in mice either (Groba et al., 2013). However, serum $T_3$ was only decreased by 30% in methimazole treated hamsters and $T_3$ levels seem to be sufficient to maintain the expression of the analyzed target genes. The lowered $T_3$ levels had no significant negative effect on the gene expression of UCPs, but *ucp1* and *ucp3* showed a tendency of

being down regulated. If these UCPs are down regulated during hypothyroidism, this could be a reason for a lower $T_b$ and might improve the preconditions for torpor. Taken together we found pronounced effects of thyroid hormone manipulation on gene regulation in peripheral tissues, but none of the investigated $T_3$ target genes (*npy, sst*) in the hypothalamus was regulated by thyroid hormone treatment. This is surprising, because $T_3$ administration specifically to the hypothalamus has been shown to regulate long and short-term energy balance (Barrett et al., 2007; Kong et al., 2004). However, the mechanisms triggered by $T_3$ in the hypothalamus are not well studied. A recent study showed a considerable effect of pasireotide, a somatostatin receptor agonists, on torpor behavior (Scherbarth et al., 2015). However, this seems to be a peripheral effect and does not contradict of our finding, that SST in the hypothalamus is not regulated during torpor.

During torpor the gene expression seems to be specifically regulated independent of treatment, but transcription is not generally reduced during torpor as suggested by (Berriel Diaz et al., 2004), because gene regulation of UCPs in the hypothalamus and skeletal muscle were unchanged. *Dio2* was massively down regulated in hypothalamus, BAT and skeletal muscle and could have short-term effects on the DIO2 protein level due to its short half-life of 2h (Baqui et al., 2003). The classical reason for *dio2* down regulation is a high intracellular $T_3$ concentration (Croteau et al., 1996). This would contradict the fact that $T_3$ inhibits torpor and that transcription of $T_3$ target genes *ucp1* and *ucp3* was inhibited in BAT of our hamsters.

Therefore, we suppose that decrease of *dio2* during torpor might have been regulated via a different mechanism such as a cAMP-mediated pathway. *Dio2 expression* in BAT can be regulated by cAMP, which is involved in the activation of UCP1 (Lowell and Spiegelman, 2000; Watanabe et al., 2006). A rapid decrease of *dio2* would cause an acute reduction of T3 levels during torpor in these tissues. This lack of $T_3$ could explain the inhibited expression of *ucp1* and *ucp3* in BAT during torpor. That $T_3$ plays a key role in the regulation of non-shivering thermogenesis in BAT mediated by UCP1 is established (Silva, 1995). Although the exact function of UCP3 is still unknown, a minor role in thermoregulation and energy regulation is probable (Cioffi et al., 2009; Flandin et al., 2009). The reduced transcription of both UCPs together with an inhibited activation by $T_3$ would cause less energy dissipation in form of heat by BAT. A shut down of thermogenesis is a prerequisite for torpor. However, the trigger for this cascade remains unclear. Further remaining questions are how the lower transcription affects the protein level of DIO2 and UCPs during torpor and how thyroid hormone affects non-genomic actions during the course of daily torpor. Lower $T_3$ levels in hypothyroid hamsters seem to promote torpor, but do not induce torpor on a daily and predictable level.

## Conclusion

Taken together our data indicate that thyroid hormone metabolism plays an important role in the regulation of torpor. Our gene expression data during deep torpor suggest that genes linked to thyroid hormone seem to be involved in the shutdown of thermoregulation during torpor. Further genes involved in obligatory thermogenesis and torpor as well as other $T_3$ target genes in the brain and in the periphery needs to be investigated to uncover signaling pathways. Transcriptomic and proteomic studies in different tissues and torpor states might provide further insights in regulatory mechanism of torpor induction, maintenance and arousal.

## 2.3. Influence of hypothalamic $T_3$ microdialysis on torpor and gene expression

The following chapter was written as a manuscript, but is unpublished so far. 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 *in situ* hybridization was carried out by me with the support of Dana Wilson and analyzed as well as interpreted by myself. Serum concentration analysis by radioimmune assays (RIA) was performed by Eddy Rijntjes. Manuscript was written by myself and revised by Annika Herwig, Eddy Rijntjes and Perry Barrett.

**Effects of hypothalamic $T_3$ microdialysis on spontaneous daily torpor and gene expression in Djungarian hamsters (*Phodopus sungorus*)**

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### 2.3.1 Abstract

Thyroid hormones play an important role in regulating seasonal adaptations of mammals. Several studies suggested that reduced availability of 3,3',5-triiodothyronine ($T_3$) in the hypothalamus is required for physiological adaptation to winter in Djungarian hamsters. We have previously shown that $T_3$ is also involved in the regulation of daily torpor, but it is still unclear, whether $T_3$ affects torpor by central or peripheral mechanisms. In this study we investigated the effects of hypothalamic $T_3$ microdialysis on torpor frequency, -duration and -depth in short day adapted hamsters. Increased $T_3$ concentrations in the hypothalamus reduced expression of torpor as well as torpor bout duration. Analysis of gene expression in the ependymal layer of the third ventricle showed clear upregulation of deiodinase type 3 but no regulation of type 2. Neither transcription of thyroid hormone transporters *mct8* and *oatp1c1* nor TSH-receptor and *gpr50* in the ependymal wall were affected by $T_3$ treatment. This was also true for somatostatin gene expression in the arcuate nucleus. Interestingly $T_3$ microdialysis seems to cause locally limited induction of *oatp1c1, crbp1, h3r* and *tsh-r* around the probe, which might indicate inflammatory mechanisms due to damage of brain tissue. All gene expression data provide new insight of the regulatory potential of $T_3$. Additionally reduced serum concentration of total thyroxine ($tT_4$) revealed that central $T_3$ microdialysis altered the hypothalamic-pituitary-thyroid-axis. Altogether our results provide a deeper understanding of hypothalamic $T_3$ action and provide new evidence that torpor is primarily regulated in the hypothalamus.

## 2.3.2 Introduction

In mammalian brain the hypothalamus is the center for energy homeostasis and involved in regulation of food intake, energy expenditure and body temperature ($T_b$). Seasonal animals have distinct abilities to adapt their energy balance to extreme environmental changes and make them a good animal model to investigate hypothalamic mechanisms of these physiological processes. Our animal model, the Djungarian hamster (*Phodopus sungorus*), reduces food intake and body weight to reduce energy demands well in advance of winter (Heldmaier and Steinlechner, 1981a; Knopper and Boily, 2000). Another remarkable adaptation is the expression of spontaneous daily torpor. Torpor in Djungarian hamsters is a state of hypometabolism combined with a reduction of $T_b$ below 32°C, that is spontaneously expressed after 10-12 weeks in artificial short photoperiod (SP) (Heldmaier and Ruf, 1992; Heldmaier et al., 1999; Ruf et al., 1989; Ruf et al., 1991). Torpor is regulated by the circadian clock and limited to the daily resting phase, which allows searching for food during the night (Diedrich et al., 2015b; Heldmaier and Steinlechner, 1981b). Overall up to 65% of daily energy demands can be saved when torpor is used regularly as compared to normothermia (Heldmaier et al., 2004; Ruf and Heldmaier, 1992). The hypothalamus is involved in energy sensing and integrating as well as regulation of non-shivering thermogenesis, which is a mechanism to produce heat during the arousal from torpor in brown adipose tissue (BAT) (Himms-Hagen, 1984). The connection between the hypothalamus and BAT seem to be important for thermogenesis and the expression of torpor (Braulke and Heldmaier, 2010; Contreras et al., 2015).

Several studies indicated that availability of 3,3',5-triiodothyronine ($T_3$) in the hypothalamus plays a central role in seasonal adaptation of body weight and reproduction as well as expression of torpor (Barrett et al., 2007; Hanon et al., 2008; Herwig et al., 2009; Herwig et al., 2013; Murphy et al., 2012; Watanabe et al., 2004). Thyroid hormones are produced in the thyroid gland, mainly as the prohormone $T_4$. $T_4$ and $T_3$ are transported via the blood while mostly bound to binding proteins and can be taken up into cells of target tissues by transporters such as monocarboxylate transporters (MCT8), organic anion-transporting polypeptide (OATP1c1) and others (reviewed by Wirth et al., 2014). Intracellular deiodinase enzymes can locally convert $T_4$ to the active form $T_3$ by phenolic ring deiodination (DIO1 and DIO2) and deactivate $T_3$ by tyrosyl ring deiodination into 3,3'-diiodothyronine (DIO3) (Bianco et al., 2002; Köhrle, 1999). So far only DIO2 and DIO3 have been detected in the brain (Schroeder and Privalsky, 2014). Experiments with Djungarian hamsters have already revealed that hypothalamic gene expression of *dio2* and *dio3* is seasonally regulated (Barrett et al., 2007; Watanabe et al., 2004). Furthermore, chronic $T_3$ treatment with silastic implants starting at the beginning of torpor season seems to prevent torpor in castrated hamsters (Murphy et al., 2012). However, the acute influence of $T_3$ in the hypothalamus on torpor during torpor season is unknown.

The previous experiment has shown that systemic $T_3$ administration has an acute inhibitory effect on torpor expression (2.2.4.3). It remains unclear, whether this effect is mediated by a peripheral or central mechanism. Therefore, we investigated the effect of central $T_3$ administration on spontaneous daily torpor. Furthermore, we provide data of hypothalamic gene transcription after nine days of central $T_3$ administration of *dio2, dio3, mct8, oatp1c1, vimentin, gpr50, crbp1, h3r* and *srif*, all of which have been linked to seasonal adaptation and thyroid hormone metabolism or uptake.

## 2.3.3 Material & Methods

### 2.3.3.1 Animal housing

All experiments and procedures were performed in accordance with German animal ethics legislation and were approved by the local animal welfare authorities (No. 83_13, Hamburg, Germany). Djungarian hamsters (*Phodopus sungorus*) were bred at the Institute of Zoology at the University of Hamburg and raised under artificial long photoperiod (LP, 16h light : 8h dark) at 20 ± 2°C. Hamsters were weaned at an age of three weeks and housed individually with *ad libitum* access to hamster breeding diet (Altomin) and water. At an age of 3-4 month, 20 male hamsters were transferred to an artificial short photoperiod (SP, 8h light : 16h dark) with an ambient temperature of 18 ± 2°C.

### 2.3.3.2 Surgical procedure and treatment

Eighteen hamsters showed clear adaptations to SP after ten weeks in body weight and fur color, while two non-responding hamsters had to be excluded from the experiment. Six hamsters were used as untreated control group and did not undergo surgery to receive a cannula or dialysis probe. Twelve animals were used for the microdialysis experiment. These were anaesthetized by inhalation of isoflurane (1.5–2.5%, Forene, Abott, Wiesbaden, Germany) and analgesia was provided by s.c. injection of carprofen (5 µg/g, Paracarp, IDT Biologika, Germany) prior to surgery. Hamsters were implanted with a DSI transmitter in the abdominal cavity for $T_b$ measurements as previously described (see chapter 2.2.3.2). Then hamsters were fixed in a stereotactic apparatus for implantation of a guide cannula. The scalp was removed and the scull was locally anaesthetized with Lidocain (Xylocain, Astra Zeneca GmbH). Two fitting holes for anchor screws were drilled in the anterior part of the scull. A 2 mm hole was unilaterally drilled at the stereotactic coordinates Bregma -1.6 mm (anterior-posterior) and -0.5 mm (medio-lateral). A guide cannula (CMA/7 Microdialysis AB, Solma, Sweden) was vertically attached to the stereotactic apparatus and slowly inserted into the brain with a depth of 5 mm. The guide cannula and a magnetic head block (CMA) were attached to the anchor screws with dental cement. After the surgery hamsters received a s.c. buprenorphine

injection (0.03 µg/g) for analgesia and were closely monitored for 3-5 hours. Hamsters were placed in microdialysis cages (CMA 120 plastic bowl). Health status was monitored daily and hamster could recover from surgery for at least one week. Recording of $T_b$ in 1 min intervals started after 12 weeks in SP. $T_b$ was recorded with only the guide cannula in place for the first 10 days of the experiment (days 1-10). On day 11, Cuprophane microdialysis probes (CMA/7) were inserted into the guide cannula under short isoflurane anesthesia. The microdialysis probes were 7 mm long, with a membrane diameter of 0.26 mm and an active dialysis area of 2 mm with a 6000 kDa molecular weight cut-off. Probes were connected with FEP Teflon tubing (Agn Tho's AB, Lidingö, Sweden) to a dual channel swivel (CMA 120) attached to a counterbalance lever. The swivel inlets were connected with Tygon tubing (VWR international GmbH, Randor, PA, USA) to 2 ml plastic syringes in a microinjection pump (PHD 2000, Harvard Apparatus GmbH, Germany). This setup allowed the hamster to move freely, while being permanently connected to the dialysis system. One hamster had to be killed after insertion of the microdialysis probe according to our termination criteria.

The microdialysis system was perfused with Ringer's solution ($Na^+$: 131 mM, $K^+$: 5.36 mM, $Ca^{++}$: 1.84mM, $Cl^-$: 112 mM, Lactate: 28.3 mM) at a flow rate of 1µl/min for three days in all animals (days 11-13). While three control hamsters were maintained on Ringer´s perfusion for 12 days (days 11-22), eight hamsters received 10 µg/ml T3 solved in Ringer's solution from day 14 to day 22 for a further nine days. All solutions were exchanged daily to maintain concentrations. At the end of the experiment all hamsters were killed by $CO_2$ and decapitation at Zeitgeber Time (ZT) 4-6 in a normothermic state. The six untreated control hamsters were killed normothermic after 14 weeks in SP (SP14). Hamsters were weighted after the experiment and compared to the weight before insertion of the microdialysis probe.

Brains were removed and directly frozen on dry ice. Blood was sampled for serum analysis and total $T_4$ and total $T_3$ concentrations were determined by radioimmunoassay as previously described (see chapter 2.2.3.2). Skeletal muscle and BAT were collected and snap frozen in liquid nitrogen for qPCR analysis. Brain, BAT and muscle were stored at -80°C until needed. Position of the microdialysis probe (Bregma -2.30 to -2.70) was determined after the brains were cut for *in situ* hybridization. Two hamsters had to be excluded from further analysis, because the probe was not placed in the posterior hypothalamic area.

### 2.3.3.3 *In situ* hybridization

Frozen brains were cut into 16µm coronal sections on a cryostat, cutting approximately from Bregma -1.70 to -2.54 mm (Franklin and Paxinos, 2008) covering the hypothalamus, and directly mounted onto polysine-coated slides. Riboprobes were synthesized, using $^{35}$S-UTP with SP6, T3 or T7 polymerases as appropriate, from DNA fragments for *dio2, dio3, mct8, oatp1c1, h3r, tsh-r, crbp1,*

*gpr50*, *vimentin* and *srif. In situ* hybridization was carried out as previously described (see chapter 2.1.3). Briefly, brain sections were fixated in 4% PFA, afterwards washed in 0.1 M PBS, incubated with 0.25% acetic anhydrate, dissolved in 0.1 M triethanolamine, washed again in PBS and dehydrated with an ascending ethanol series followed by vacuum drying. Appropriate radioactive probes were applied to slides in 70 µl hybridization mixture (see chapter 2.1.3). Hybridization was carried out at 58°C over night. Finally slides were washed in 4x SSC and incubated with ribonuclease A at 37°C before being washed with SSC solutions with decreasing concentrations and dehydrated using graded ethanol. Dried slides were exposed to Kodak BioMax MR Films (Sigma-Aldrich Company Ltd., Poole, Dorset, UK). Autoradiographic films were developed after 18-20h (*srif, vimentin*), 5 days (*mct8*), 6 days (*gpr50*), 7 days (*oatp1c1*, *h3r*, *crbp1, tsh-r*) or 14 days (*dio2, dio3*). Autoradiographic films were scanned at 300 dpi and analyzed with ImageJ 1.47v software. Integrated optical density, obtained by reference to a standard curve [y=a+b*ln(x-c)] generated from the $^{14}C$ microscale, was measured in two to three consecutive sections per animal. Values were averaged for each animal. Gene expression of hamsters with a microdialysis probe was compared relative to untreated SP14 hamsters.

#### 2.3.3.4 Quantitative real-time PCR (qPCR)

Gene expression of *dio2* and *dio3* in BAT and skeletal muscle was analyzed by qPCR as described before (see chapter 2.2.3.4). Briefly, total RNA from BAT and muscle was extracted using peqGOLD TriFast and chloroform. 1 µg of total RNA was used to synthesize cDNA by using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) with oligo-(dT)-primer (0.5 µg/µl). The synthesized total cDNA was used to estimate the expression by qPCR using the ABI Power SYBR Green master mix (Applied Biosystems, Darmstadt, Germany) and suitable primers for *Ph. sungorus* in an ABI 7300 real-time PCR system. Standard curves of plasmid cDNA were used as calibrators for relative quantification. Gene expression was calculated with the ΔΔCT-method relative to untreated control hamsters from a the previous experiment (see chapter 2.2.3.4) with *18s* mRNA as reference gene.

#### 2.3.3.5 Statistical analysis

Data are expressed as arithmetic mean ± standard error of the mean (SEM). Differences between groups in torpor expression, gene expression and serum $T_4$ and $T_3$ concentrations were analyzed by student's t-test (t-test, parametric) or Mann–Whitney-U test (U-test, non-parametric) as appropriate. P-Values <0.05 (*), <0.01 (**), <0.001 (***) were considered as significant. Statistical analyses were performed with SigmaPlot™ 12 (Systat Software Inc).

## 2.3.4 Results

### 2.3.4.1 Body weight

Hamsters treated with Ringer's solution had a body weight of 34.6 ± 2.0 g, when the probe was inserted and at the end of the experiment the body weight was 33.6 ± 2.3 g. The difference of was not significant. Furthermore, the body weight in $T_3$ treated hamsters was unchanged with 33.0 ± 1.2 g before the treatment and 32.7 ± 1.5 g after the treatment.

### 2.3.4.2 Torpor expression

Torpor was defined as $T_b$ <32°C for more than 30 minutes. $T_b$ rhythm and torpor expression were reinstated few days after surgery. Two hamsters treated with $T_3$ showed no torpor at all. Insertion of the microdialysis probe at day 10 and rinsing with Ringer's solution the following days had no influence on torpor expression (Figure 25 and 26). Addition of $T_3$ into the Ringer's solution reduced torpor frequency from 25 ± 12% to 11± 4% (Figure 25A), but did not reach significance (t-test, p= 0.254). However, over all animals only six torpor bouts were recorded during $T_3$ treatment, while a total of 15 torpor bouts were recorded in the same animals before the start of $T_3$ treatment. All six torpor bouts during $T_3$ treatment occurred during the first 6 days of treatment. Furthermore, torpor duration (Figure 25B) was significantly reduced from 275 ± 29 min to 138 ± 30 min (t-test, p=0.01) and minimal $T_b$ during torpor (Figure 25C) increased clearly from 25.3 ± 0.5°C to 28.8 ± 0.5°C (t-test, p<0.001) due to $T_3$ treatment. The shortening of torpor bouts and increase of minimal $T_b$ can clearly be observed in Figure 26C. Treatment with Ringer's solution had no influence on torpor frequency, torpor duration or minimal $T_b$ during torpor.

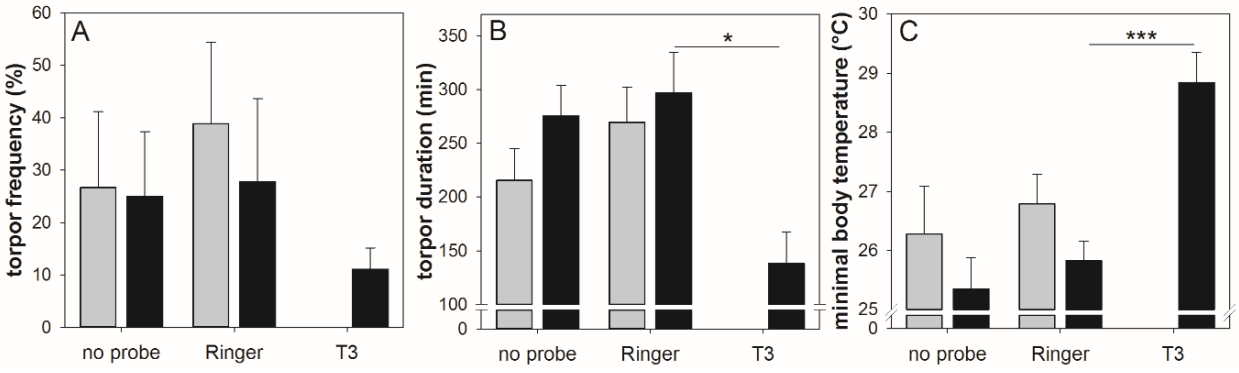


**Figure 25: Mean + SEM torpor frequency (A), - duration (B) and minimal body temperature during torpor (C).** Grey bars represent three hamsters from the Ringer's-group and black bars six hamsters from the $T_3$ treated group. Hamsters had no probe for 10 days before the implantation of dialysis probes (left bars). The Ringer's-group was dialyzed with Ringer's solution for 12 days (middle grey bar), while the $T_3$-group received Ringer's solution for 3 days (middle black bar) and were subsequently dialyzed with $T_3$ for further 9 days (right black bar).

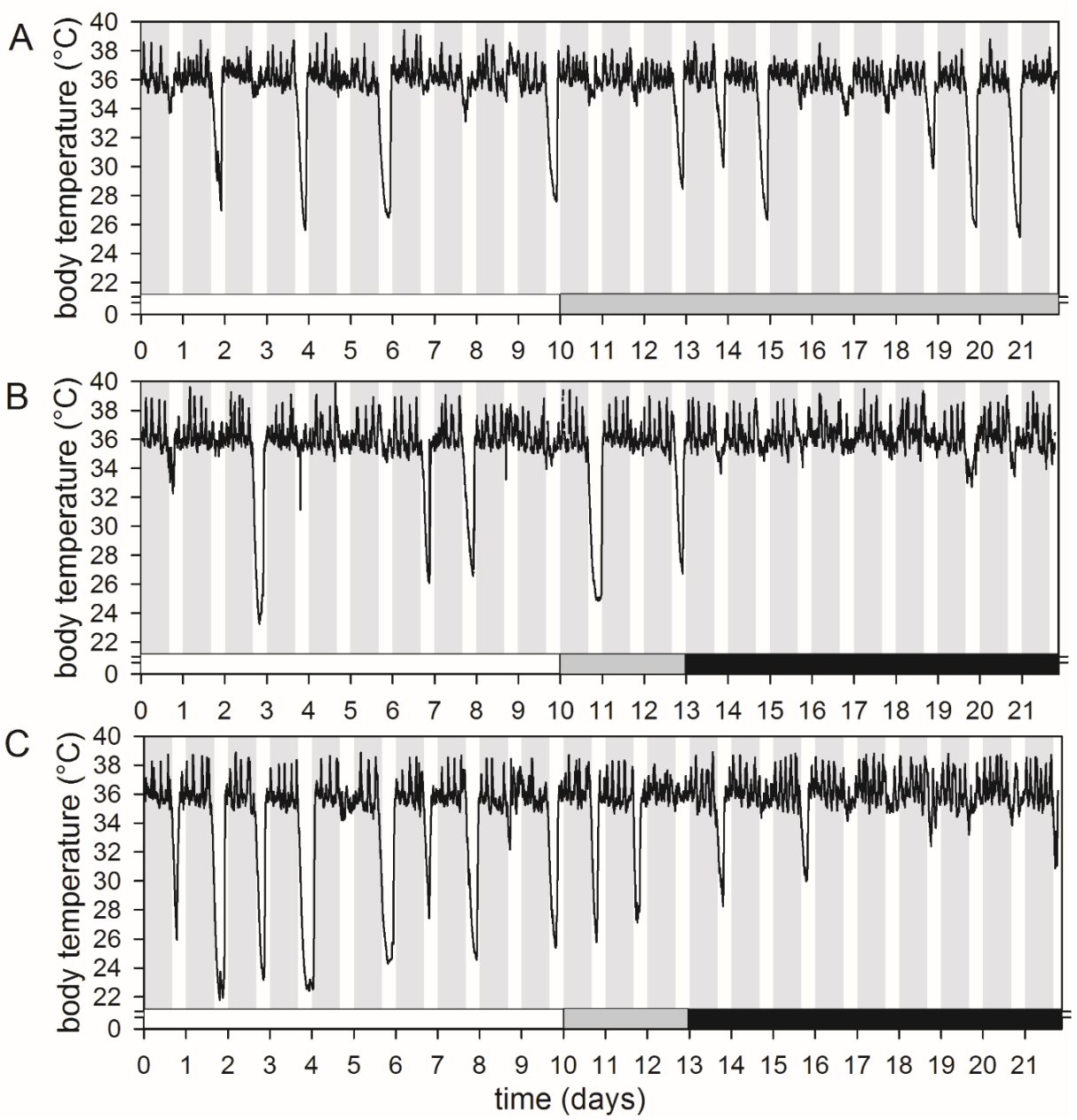


**Figure 26: Body temperature recordings of three exemplary hamsters.** Hamsters received no dialysis in the first 10 days after the guide cannula was inserted. Microdialysis probe was inserted at day 10. Grey bar indicates dialysis with Ringer's-Solution (A, B, C) and black bar dialysis with additionally $T_3$ (B, C). Dark phases are highlighted in grey.

### 2.3.4.3 Gene expression

Gene expression was analyzed in the ependymal layer at the third ventricle and in the ARC of the hypothalamus (Figure 27) by *in situ* hybridization relative to untreated control hamsters. *Dio2, mct8, tsh-r, vimentin* and *gpr50* showed no significant changes after $T_3$ treatment (Figure 27). *Srif* was only expressed in the arcuate nucleus (ARC) and did not respond to $T_3$ treatment (Figure 27). *Dio3* showed a 13.4-fold increase after $T_3$ treatment (U-test, p=0.02). *H3r* in the dmpARC region showed an increase of 77.7 ± 32% (U-test, p=0.017) after $T_3$ release (Figure 27). *Crbp1* and *Oatp1c1* mRNA were not detectable in the ependymal layer, but interestingly there was an expression around the

microdialysis probe in $T_3$ treated animals (Figure 28 I, K). In this area around the probe also mRNA of *vimentin, tsh-r* and *h3r* was detectable. Only vimentin was detectable in hamsters treated with Ringer's solution (Figure 28B), while all other genes were only visible in $T_3$ treated animals (Figure 28 I-L).

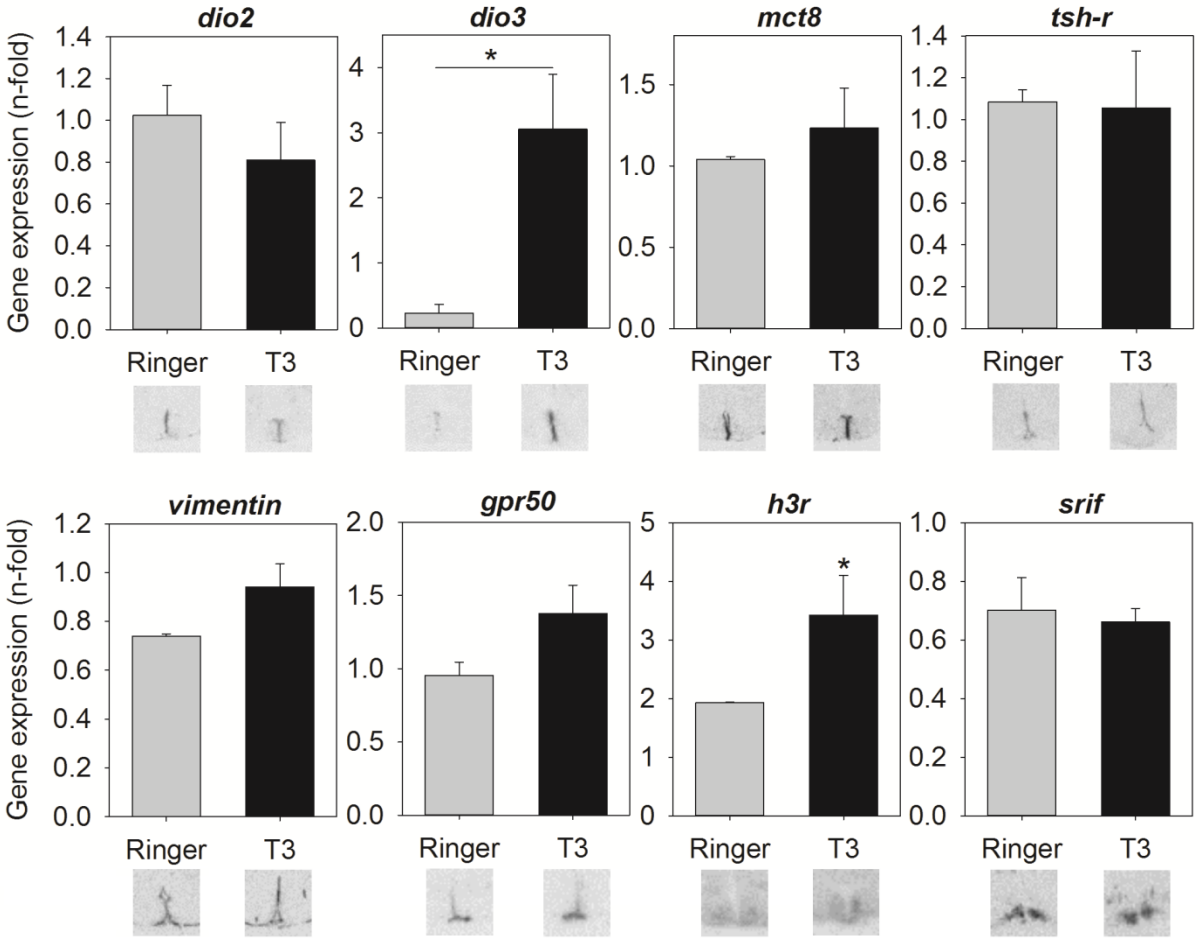


**Figure 27: Gene expression (mean + SEM), relative to untreated control hamsters, of *dio2*, *dio3, mct8, tsh-r, vimentin* and *gpr50* in the ependymal layer of the hypothalamus as well as *h3r* and *srif* in the ARC.** Hamsters were treated with either Ringer's solution (grey) or $T_3$ (black). Autoradiographs show representative brain sections with gene expression close to mean values. Significant differences are marked with *

Gene expression in BAT and skeletal muscle of deiodinases was analyzed by qPCR. Hamsters treated only with Ringer's solution showed no significant changes in gene expression compared to untreated SP14 control hamsters. In BAT and muscle *dio2* expression was not affected by hypothalamic $T_3$.

*Dio3* gene expression in untreated- and Ringer's-microdialysis hamsters was close to detection limit, thus the amount of mRNA copies in untreated hamsters was close to zero and it was not feasible to calculate an exact fold change for T3 treated hamsters. However, *dio3* mRNA in BAT was clearly

increased after central $T_3$ administration (U-test, p=0.004). Expression of *dio3* in muscle was close to detection limit in all groups and $T_3$ treatment had no effect.

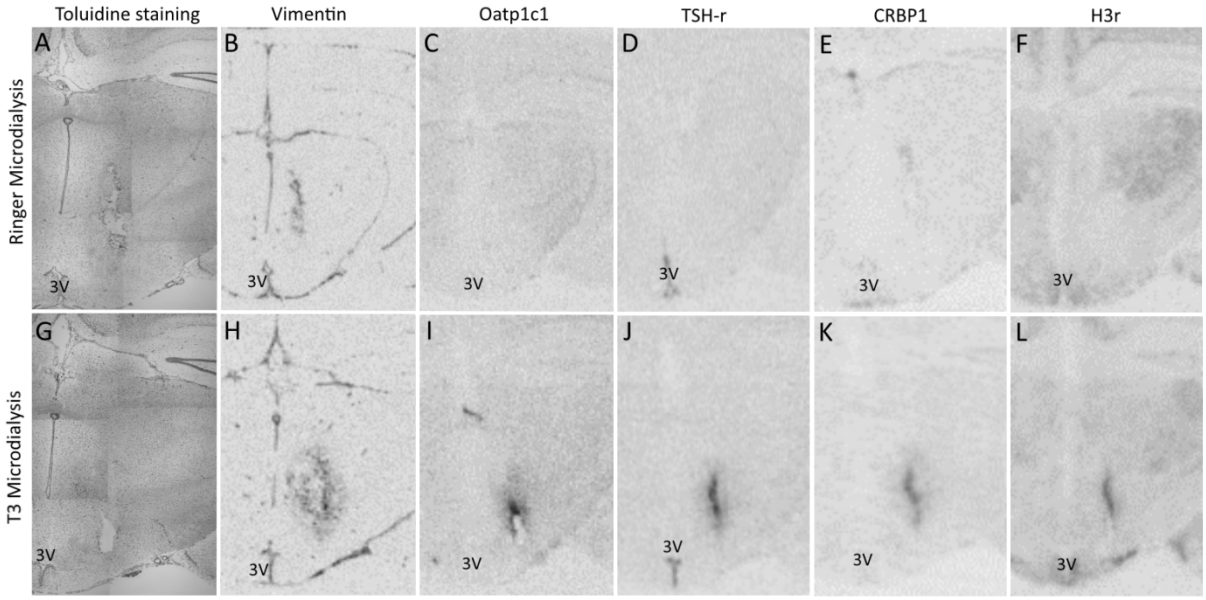


**Figure 28: Exemplary brain sections of two hamsters treated with Ringer's solution (A-F) or $T_3$ (G-L).** A and D show sections stained with toluidine blue, the others show autoradiographic section with radioactive labelling against vimentin (B, H), Oatp1c1 (C, I), TSH-r (D, J), CRBP1 (E, K) and H3R (F, L). The third ventricle (3V) is indicated for orientation.

### 2.3.4.4 Serum analysis

Thyroid hormone serum concentration in hamsters with a microdialysis probe was compared to untreated control hamsters (Figure 29). Hypothalamic $T_3$ release caused a 72 ± 10% reduction of total $T_4$ serum concentration compared to hamsters with hypothalamic Ringer treatment (Figure 29, t-test, p>0.001). The increase of total $T_3$ serum concentration from 1.5 ± 0.1 nM to 4.1 ± 1.3 nM was not significant (t-test, p=0.2). Dialysis with Ringer's solution had no effect on total $T_4$ or $T_3$ concentrations in serum.

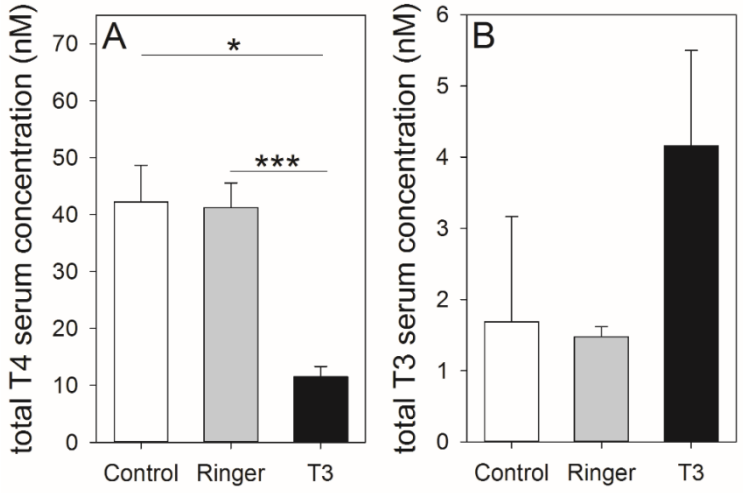


**Figure 29: Mean + SEM serum concentration of total $T_4$ (A) and total $T_3$ (B).** Control hamsters were not operated and hamsters with a microdialysis probe in the hypothalamus received either Ringer-Solution or Ringer-Solution with $T_3$. Significant differences between groups were marked with * p<0.05 (U-test) or *** p<0.001 (t-test).

## 2.3.5 Discussion

In our present study we provide new data, which strengthen the evidence that T3 availability in the hypothalamus plays a key role in the regulation of spontaneous daily torpor. Unilateral release of $T_3$ was sufficient to reduce the expression of torpor. Two hamsters stopped showing torpor one day after the $T_3$ dialysis started and two hamsters still showed shallow torpor bouts, which were characterised by shorter torpor duration and higher minimal $T_b$. The heterogeneous response to $T_3$ dialysis suggests that complete absence of torpor only occurs above a critical threshold for $T_3$ concentration in the hypothalamus. Below this threshold $T_3$ might just limit duration and depth of torpor, before an intracellular accumulation of $T_3$ completely prevents torpor. An explanation for different hypothalamic $T_3$ concentrations could be varying positions of the microdialysis probe. Differences of few micrometres could delay or reduce the diffusion or transport of $T_3$ into the hypothalamus. Our aim was to place probe adjacent to the posterior part of the hypothalamus rather than directly into the hypothalamus to maintain its functional integrity. Inhibition of torpor, reduced serum $T_4$ and increased gene expression of *dio3* (Zhang et al., 2016) provide good evidence for increased $T_3$ concentrations in the hypothalamus.

In the previous experiment (see chapter 2.2.4.3) a reduction of torpor expression after systemic $T_3$ treatment has been observed. In the present study two hamsters also had increased $T_3$ serum concentrations after hypothalamic $T_3$ administration. However, this was no prerequisite for torpor inhibition, indicating a central effect of $T_3$ on torpor regulation. The other hamsters stopped expressing torpor without elevated $T_3$ serum concentrations. Therefore, torpor inhibition by systemic $T_3$ treatment might cause a secondary inhibition of torpor through a hypothalamic pathway, because is probable that systemic treatment also elevated $T_3$ concentrations in the hypothalamus. Furthermore, torpor inhibition by $T_3$ administration via drinking water was a result of 21-fold higher $T_3$ serum concentrations (chapter 2.3.4), which facilitates a strong torpor inhibition and made a differentiation between central and peripheral effects difficult.

A central $T_3$ effect on peripheral thyroid hormone homeostasis was visible in reduced total $T_4$ serum concentrations. This strong reduction of $T_4$ was comparable to $T_4$ reduction after systemic $T_3$ administration (see chapter 2.2.4.3). This suggests $T_3$ is present in sufficient concentration in the region of the paraventricular nucleus (PVN) to invoke the inhibitory action of $T_3$ on peripheral $T_4$ levels. It has been shown that the hypothalamus plays a unique role in thyroid hormone metabolism and can independently regulate its own thyroid hormone homeostasis and thus change the set point for thyroid hormone production in the periphery (Lechan and Fekete, 2005). Hence a hypothalamic hyperthyroid state is expected to reduce systemic thyroid hormone production and metabolism as a result of negative feedback mechanism regulating the hypothalamic-pituitary-thyroid axis (Sánchez

et al., 2009). The cause for increased $T_3$ serum concentration in two hamsters is unclear, but one explanation could be higher $T_4$ conversion to $T_3$ by DIO1 or DIO2 in the periphery. To understand the detailed chronological order of feedback mechanisms between the hypothalamus and the periphery more studies needs to be done.

Analysis of deiodinases in BAT and muscle only revealed an upregulation of *dio3* in BAT. This change was unlikely to be caused by circulating thyroid hormone in the blood, since systemic *in vivo* treatment with $T_3$ produced no clear upregulation of *dio3* and photoperiodic upregulation could be excluded as well (see chapter 2.2.4.4.1). This may indicate signalling of the hypothalamic hyperthyroid state via a hypothalamic-BAT pathway regulating thyroid hormone metabolism in BAT, which has been proposed before in the context of sleep and thermoregulation (Contreras et al., 2015; Rodrigues et al., 2015).

Increased transcription of *dio3* in hypothalamic tanycytes along the third ventricle provides clear evidence of increased $T_3$ concentrations in the hypothalamus. This gene encodes for the enzyme that catalyses the deactivation of $T_3$ (Bianco et al., 2002; Köhrle, 1999). Transcription of *dio3* in hypothalamic tanycytes is sensitive to locally increased $T_3$ concentrations, which has been recently shown in rats (Zhang et al., 2016). In our experiment the increased *dio3* expression along the third ventricle provides evidence of increased $T_3$ concentrations in the hypothalamus as a result of the microdialysis procedure. Furthermore, *dio2* expression was not affected by $T_3$ microdialysis, which is consistent with previous studies (Werneck de Castro et al., 2015). Additionally DIO2 can be post-translational deactivated, which might be an adequate mechanism during hypothyroidism (Martinez de Mena et al., 2010).

*Mct8* was not regulated by $T_3$ administration. Previous studies had shown that *mct8* is regulated by photoperiod in seasonal animals and it was suggested, that this is dependent on $T_3$ metabolism (Herwig et al., 2013; Ross et al., 2011). However, our new data provide suggest that *mct8* may not be directly regulated in a $T_3$ dependent manner. Maximum expression of this thyroid hormone transporter might be a mechanism to increase the efflux of $T_3$ to deal with excessive $T_3$ concentrations in tanycytes. However, other thyroid hormone transporters besides MCT8 and OATP1c1 might be activated during hypothalamic hyperthyroidism (Wirth et al., 2014).

Previous studies suggested that several genes involved in seasonal adaptation might be linked to $T_3$ metabolism in tanycytes. *Gpr50*, an orphan G-protein-coupled receptor, expressed in the ependymal wall of the third ventricle, is regulated by photoperiod and seems to be involved in the expression of torpor (Barrett et al., 2006; Bechtold et al., 2012). However, in our study g*pr50* transcription was not altered by hypothalamic $T_3$ dialysis.

Somatostatin receptor activation most likely at the level of the pituitary has been shown to be involved in torpor regulation (Scherbarth et al., 2015). In our study *srif* in the ARC was not decreased in response to $T_3$ microdialysis and may suggest that somatostatin of arcuate nucleus origin plays no primary role in the control of torpor. This supports the idea that torpor is directly inhibited by $T_3$ and not blocked by activation of the growth hormone axis with increasing body weight. However, $T_3$ may also be acting on other thermoregulatory areas in the hypothalamus and may override a neuroendocrine control.

Besides *dio3*, only *h3r* was slightly upregulated in the ARC of the hypothalamus after $T_3$ treatment. In SP *h3r* is usually lower expressed than in LP (Barrett et al., 2005; Ross et al., 2005). The role of *h3r* in seasonal hamster is not well understood, but *h3r* transcription has been shown to increase with rising *dio2* levels in photorefractory animals (Herwig et al., 2013; Ross et al., 2005) indicating a link between histamine and thyroid hormone system. Our data support the idea, that *h3r* could be upregulated by $T_3$.

*Crbp1*, a retinol binding protein, which shows a reduced level of expression in the ependymal layer during SP, was not influenced by $T_3$ release along the third ventricle. However, *crbp1* as well as *vimentin*, *oatp1c1*, *tsh-r* and *h3r* mRNA were detected around the microdialysis probe.

Specific induction of gene transcription around the probe might be an evidence for neuroinflammatory processes. Increased expression of genes seemed to be $T_3$ dependent, because they were not expressed in Ringer treated hamsters. The only exception was vimentin, which was also expressed in the Ringer group. The physical insertion of the microdialysis probe caused a local brain tissue injury. The insertion of the probe caused no obvious effects on the behaviour of the hamsters. Also $T_b$ showed no fever reaction after the surgery (data not shown). Moreover, the expression of torpor few days after the surgery is a clear indicator for well-being of the animals.

The brain tissue damage probably initiated a cascade of progressive inflammatory tissue response (Kozai et al., 2015). This response includes recruitment and activation of microglia and astrocytes. It is known that the intermediate filament vimentin is an important part of astrocytes cell structure (Pekny et al., 2007). In our study vimentin was expressed in all hamsters with a microdialysis probe, independent of $T_3$. On the other side the increase of *tsh-r* and *oatp1c1* mRNA were probably initiated by $T_3$. It has been shown that *tsh-r* is expressed in astrocytes (Crisanti et al., 2001). A known function of TSH in astrocytes is the regulation of deiodinases (Saunier et al., 1993), however, an expression of *dio2* or *dio3* surrounding the microdialysis probe was not observed in our study. The expression of the thyroid hormone transporter *oatp1c1,* which is especially competent to transport $T_4$ into the brain, has been detected in different rodents (Ross et al., 2011; Werneck de Castro et al., 2015; Wirth

et al., 2014), but not previously in Djungarian hamsters. *Oatp1c1* was not found to be expressed in the ependymal layer but after $T_3$ release expression was observed adjacent to the probe. Expression of *oatp1c1* linked to inflammation has been shown in astrocytes of mice and rats (Wittmann et al., 2015). Furthermore, the retinol transport protein *crbp1* was expressed in the area of injured brain tissue. This might be an evidence for presence of retinol (vitamin A). It has been reported that retinoic acid, a retinol derivate, has an anti-inflammatory role in astrocytes (Choi et al., 2005). Therefore, we assume that CRBP1 as part of retinol pathway is involved during inflammation, too. Another candidate with a potent role in microglia-mediated neuroinflammation is histamine and is able to upregulate *h3r* (Dong et al., 2014). This upregulation of *h3r* in our study was only observed in $T_3$ treated hamsters. The activation of several genes by $T_3$ provides evidence that $T_3$ might be involved during inflammation.

### Summary and outlook

We could show that hypothalamic $T_3$ microdialysis is sufficient to reduce torpor expression. This provides further evidence that low $T_3$ concentrations in the hypothalamus are required to spontaneously express torpor in Djungarian hamsters. However, the fine-tuned regulation of torpor expression is very complex and needs further studies to reveal more details. Our data provide new evidence, that torpor is primarily regulated by central pathways, but we cannot finally exclude peripheral thyroid hormone related mechanisms in torpor control. In the future it might be useful to use $T_3$ microdialysis in hypothyroid animals to elucidate the interaction between the hypothalamus and peripheral organs in torpor regulation. Accordingly, more components of the thyroid hormone pathway in the hypothalamus need to be analysed especially during the expression of torpor. *In situ* hybridisation revealed that only *dio3* and *h3r* in the hypothalamus responded to local hyperthyroidism. Some other genes regulated by photoperiodic changes including *dio2, mct8, tsh-r, vimentin, gpr50* in the ependymal layer and *srif* in the ARC did not change after $T_3$ treatment in these two brain regions. The specific expression of *oatp1c1, tsh-r*, *crbp1* and *h3r* around the microdialysis probe suggests that $T_3$ seems to play a role during inflammation.

# 3. Collective and concluding discussion

Thyroid hormones have been known as potent candidates to regulate body mass and thermogenesis. In the last decade several studies have shown that thyroid hormones play a central role in the seasonal adaptation of energy balance in mammals living in environments with extreme annual climate changes. In my thesis I provide novel data about the influence of thyroid hormones on short-term and long-term body weight regulation as well as direct effects on daily torpor.

## 3.1 Physiological effects

### 3.1.1 Serum concentrations

In the switchback experiment $tT_4$ and $tT_3$ serum concentrations showed no significant differences in response to alternating photoperiods (2.1.4.2). Changes in thyroid hormone concentrations showed a high variance between different hamsters, which might have overridden potential differences caused by photoperiodic changes. One study with larger cohorts was able to measure seasonal fluctuations in serum concentrations (Seidel et al., 1987). Only the increase in total $T_4$ concentrations six weeks after the switchback from SP to LP was noticeable and might have been significant with a larger group size. This would be an evidence for systemically increased thyroid hormone metabolism necessary for gaining body weight and changing back to the morphological summer type. However, intracellular levels of $T_3$, controlled by deiodinases, seem to be more important than circulating serum concentrations to influence metabolism (see chapter 3.2.1).

To date, measuring tissue specific thyroid hormone concentrations is technically not possible. We tried to extract thyroid hormones in vivo via microdialysis from the hypothalamus, but quantitative and qualitative analysis of picomolar concentrations was technically not possible. Therefore, manipulating systemic or hypothalamic thyroid hormone concentrations was an appropriate method to investigate effects of thyroid hormones. Systemic treatment with either $T_4$ or $T_3$ via drinking water successfully caused pronounced changes in serum concentrations. Treatment with $T_4$ elevated $tT_4$ and $tT_3$ levels above normal values, whereas $T_3$ increased only $tT_3$ concentrations and reduced $tT_4$ levels to a minimum (2.2.4.1). This confirms that $T_3$ feeds back to the HPT-axis and reduces thyroid hormone production in the thyroid gland, probably via reduced TSH production in the pituitary gland (Dyess et al., 1988). Unfortunately it was impossible to additionally measure TSH concentration in the same serum samples, because hamsters have a limited amount of blood serum (50-200 µl). $T_3$ microdialysis confirmed, that hyperthyroidism in the hypothalamus reduces the production of $T_4$ in the periphery (see chapter 2.3.4.4). $T_3$ in the serum, after hypothalamic $T_3$ release, was noticeably

elevated in only two hamsters. One plausible explanation could be a higher conversion of $T_4$ to $T_3$ by DIO1 and DIO3 in the periphery. Another possibility could be diffusion into the blood and transport from the brain into the periphery, where the capacity for $T_3$ inactivation is higher with the presence of DIO1 in the liver (Köhrle, 1999).

Systemic MMI treatment caused severe hypothyroidism with lowered $tT_4$ and $tT_3$ concentrations (2.2.4.1). This indicated that thyroid hormone production in the thyroid was inhibited by MMI. This is in agreement to studies performed with mice (Braun et al., 2011; Groba et al., 2013; Marsili et al., 2010). A complete ablation of thyroid hormones would be lethal for hamsters, but reduced concentrations as obtained in our study had clear physiological effects opposing those of $T_3$ treated hamsters.

Altogether altered thyroid hormone concentrations in the serum confirmed the success of systemic of cerebral treatment. It was apparent that the HPT axis tried to counterbalance hyperthyroidism by decreasing $T_4$ production in the thyroid gland. However the system was not able to remove pathological treatment doses hence elevated $T_3$ or $T_4$ were measurable in the blood. These non-physiological doses caused remarkable physiological effect, but hamsters did not show signs of sickness (e.g. fever) at any time of the experiments.

## 3.1.2 Body weight

### 3.1.2.1 Long-term adaptation

Hamsters in all three experiments reduced their body weight between 15% and 35% in 14 weeks of SP adaptation. Compared to previous studies this is slightly below the maximum possible body weight reduction (reviewed by Scherbarth and Steinlechner, 2010). The first reason is that hamsters are still able to reduce their body weight after 14 weeks and former studies referred to the nadir, the lowest point of body weight during the winter season. Genetic factors as well as starting body weight $T_a$ can have an influence on body weight reduction. Nonetheless, only hamsters with sufficient body weight reduction and clear evidence for a change to winter fur were used for the experiments.

Hamsters switched back from SP to LP showed a fast increase of body weight. The increase was steeper compared to the body weight decrease. Most hamsters reached their original body weight after only six weeks in LP. Hamsters also exceeded their original body weight and reached a plateau phase after approximately 12 weeks. Ten more weeks in LP had no significant effect on body weight.

It was surprising that switchback hamsters responded to SP again after only six weeks in LP. All hamsters stopped gaining body weight and some animals clearly started to reduce their weight

again. This contradicts the initial hypothesis that these hamsters become photorefractory and are not able to respond to the SP signal after six weeks. Our results show that Hamsters did neither become refractory during the initial 14 weeks in SP nor developed refractoriness after the switchback to LP. We initially hypothesized that only the latter group switched back after 14 weeks in LP would be able to respond to SP again, because it has been shown that hamsters need at least ten weeks to reverse photorefractoriness (Bittman, 1978a; Kauffman et al., 2003; Reiter, 1972).

However results from the present experiments clearly show, that hamster did not become photorefractory at any time. However, the response in some hamsters switched back after 14 weeks in LP was stronger compared to the early switch back group, because these hamsters showed a significant body weight reduction. Thus there seems to be an effect of time spent in LP after the first switch from SP to LP. One explanation could be elevated hypothalamic intracellular $T_3$ concentrations, because of high *dio2* expression after the switchback (more details in chapter 3.2.1). Therefore, high $T_3$ concentrations in the hypothalamus after the switchback could work as a molecular break, which slowed down the response after the early second switchback. These hamsters probably just showed a delayed response in body weight and would have lost more body weight when kept in SP for more than eight weeks. *Dio2* expression decreased with time in LP after the switchback and was back to initial LP values 22 weeks after the switchback. We conclude that hypothalamic $T_3$ concentrations decreased, thus hamsters, switched back after 14 weeks, were able to directly initiate body weight reduction.

Surprisingly, five hamsters did not respond to the second switch to SP. These hamsters were no general non-responders, because they showed a normal SP adaptation after the initial switch from LP to SP for 14 weeks. The question is why these hamsters did not respond again and how the physiological effects were linked to gene expression. Interestingly, this different physiological response was also manifested in hypothalamic gene expression (see chapter 3.2.1). In these hamsters the gene expression pattern assigned more with the LP groups, while hamsters with reduced body weight showed a SP-like gene expression. Additionally, it is possible that changes in gene expression were just delayed in these hamsters and that the time in SP was not long enough for those to start SP adaptation. Nonetheless the cause for the divergent response in this group remains a mystery. It remains unclear, if gene expression was the cause or the consequence of failed body weight reduction. Experiments with a higher temporal resolution are necessary to understand the long-term regulation of body weight and associated gene expression.

### 3.1.2.2 Short-term adjustment

With systemic or central $T_3$ treatment the short-term effect of $T_3$ on body weight were analyzed. Systemic $T_3$ treatment caused a significant increase of body weight after 12 days of treatment (2.2.4.2). Probably hamsters would have gained more weight with extended $T_3$ treatment, but the experiment was terminated after 14 days to analyze gene expression at this stage. Hamsters gained weight because of increased food intake. The increasing body weight contradicts studies with mice, which lost body weight after peripheral $T_3$ administration (López et al., 2010; Luo and MacLean, 2003). Mice probably lost weight, because hyperthyroidism caused an increased metabolism which resulted in increased energy expenditure that exceeded energy intake. Hamsters seem to be able to better deal with a negative energy balance and increased energy demands. One explanation could be, that they can control the energy expenditure better with an improved fur insulation during SP (Kauffman et al., 2001). Winter fur lowers heat loss and therefore, energy demands can be reduced.

The results from hamsters also contradict reports from humans, where clinical hyperthyroidism like Grave's disease can lead to weight loss, despite good appetite and normal food intake (Bossowski et al., 2007; Dale et al., 2001). However, it is also known, that in obese humans thyroid hormone levels are elevated, which might be a long-term adaptive process to increase resting energy expenditure to counter overweight (Reinehr, 2010). With reduced weight thyroid hormone concentrations are normalized again. This antagonism in humans shows, that thyroid hormone seem to play a crucial role in body weight adaptation, but the underlying pathways are not fully understood.

Release of $T_3$ in the hypothalamus had no short-term effect on body weight (2.3.4.1). However, long-term treatment showed that $T_3$ caused an increase of body weight in SP hamsters, which led to termination of winter adaptation (Murphy et al., 2012). The present data indicate that peripheral $T_3$ treatment might lead to a faster increase of body weight compared to central treatment. Therefore, short-term adjustments might be regulated on a peripheral level and not on a central level. However, an involvement of the hypothalamus cannot be finally excluded, because the $T_3$ uptake via drinking water was probably higher compared to the $T_3$ release via microdialysis. Insertion of the microdialysis probe in the hypothalamus also possibly disturbed regulatory mechanisms for a few days, which could delay the body weight response.

Hamsters treated with MMI, which caused severe hypothyroidism, showed effects opposing those of $T_3$ (2.2.4.2). They had an initial reduced body weight, but this might be just a short-term effect like in previous studies with mice (Alva-Sánchez et al., 2012; Groba et al., 2013). Body weight of MMI treated hamsters was back to the initial body weight after 14 days. Hypothyroidism usually leads to reduced metabolism with reduced energy demands, because of low $T_3$ availability. MMI treated hamsters showed a significant reduction of food intake and were able to adapt to changed energy

balance. This is usually not true for humans, who have an increasing body weight during hypothyroidism, because food intake exceeds reduced energy expenditure (Chang et al., 2014; Laurberg et al., 2012). Hamsters were able to keep their food intake in check and seem to be able to control reduction of energy balance without signs of starvation. Unchanged expression of *npy* in the hypothalamus indicated that hamsters were not starved because of voluntarily reduced food intake. NPY is normally increased during starvation to increase food intake (Coppola et al., 2007; Herwig et al., 2009).

In conclusion $T_3$ activation by DIO2 in the hypothalamus seems to play an important role in long-term regulation of body weight. Deactivation of hypothalamic $T_3$ is a prerequisite for seasonal body weight reduction, because decreased *dio2* and increased *dio3* expression were necessary for weight loss. Systemic $T_3$ treatment led to an increase of body weight, whereas central $T_3$ microdialysis had no significant effect on short-term body weight regulation.

### 3.1.3 Torpor

Thyroid hormones play a critical role for expression of daily torpor. $T_4$ treatment was used to confirm the lower biological activity of $T_4$. Elevated $tT_4$ concentrations were not sufficient to suppress expression of torpor. Amount of torpor bouts was not affected as well as effects on $T_b$ were not observed, whereas systemic treatment with $T_3$ had obvious effects on torpor expression (2.2.4.3). Torpor was immediately blocked or ceased after a few days. Hamsters, that initially continued expressing torpor, showed severely reduced depth and duration of torpor bouts. The effects after central $T_3$ microdialysis were similar (2.3.4.2). Unilateral release of $T_3$ was sufficient to inhibit torpor. Murphy and colleagues (2012) had previously shown that implants chronically releasing $T_3$ into the hypothalamus blocked torpor expression. However, their study was performed in castrated hamsters and focused on long-term effects on torpor and body weight. Thus in their study it was not clear, if either $T_3$ had a direct effect on torpor or if torpor was blocked by increasing body weight.

Data of the present experiments provide clear evidence that $T_3$ had an immediate effect on torpor expression. Central $T_3$ release led to remarkable increase of serum $T_3$ concentrations in two hamsters. However, this was no prerequisite for torpor inhibition, because central $T_3$ release completely blocked torpor in hamsters with normal circulating $T_3$ serum concentrations. This provides new evidence that torpor is primarily controlled by the hypothalamus and that increasing hypothalamic $T_3$ concentrations lead to an inhibition of torpor. Besides, torpor inhibition by systemic $T_3$ treatment was also obtained by much higher $T_3$ levels. These pathological concentrations probably increased $T_3$ concentrations in the brain, which than inhibited torpor expression via a hypothalamic

pathway. Therefore, inhibition of torpor by peripheral treatment seems to be more a secondary effect and that $T_3$ concentrations above physiological levels are necessary to block torpor. It would be interesting to see, which $T_3$ doses are sufficient to block torpor expression. Intraperitoneal release of $T_3$ via osmotic minipumps might be an option, without disturbing torpor expression via injections.

$T_4$ treated animals had also slightly increased $T_3$ serum concentrations, but this was not sufficient to block torpor expression. However, this might have been the reason for shorter and shallower torpor bouts in these hamsters (2.2.4.3). This provides evidence, that torpor expression does not follow an all-or-nothing principle. Regulation of torpor is a fine-tuned process, probably dependent on $T_3$, which seems to be able to modulate occurrence and progress of torpor bouts.

The importance of $T_3$ absence was underlined by our results in hypothyroid hamsters (2.2.4.3). Treatment with MMI caused severe hypothyroidism and hamsters were more prone to enter torpor. Most hamsters had a higher torpor frequency and torpor bouts were longer with lower $T_b$. Increasing torpor bout duration showed that the effect of MMI seems to be stronger at the end of the experiment. This delayed effect is reasonable, given that MMI does not directly affect circulating thyroid hormone concentrations in the blood. MMI is inhibiting thyroid hormone production in the thyroid, which needs some time to be fully reduced. Hypothyroidism also had a noticeable effect on $T_b$ and reduced $T_b$ amplitude indicated a limited thermoregulation. Reduced $T_3$ concentrations probably caused limited thermogenesis, because $T_3$ is essential for full thermogenic activity in the BAT (Bianco and Silva, 1987a). Furthermore, reduced $T_3$ availability limits many metabolic processes, which reduces basal metabolic rate (Soboll, 1993).

Hypothyroidism might lead to reduced $T_3$ concentrations in the hypothalamus as result of systemic shortage of thyroid hormones. However, the hypothalamus has the ability to independently regulate its own $T_3$ homeostasis by deiodinases (Köhrle, 2000). Nevertheless an increase of hypothalamic *dio2* expression was not observed to compensate reduced $T_3$ availability (see chapter 2.3.4.3). Under natural conditions it is imaginable that deiodinases reduce $T_3$ availability in the hypothalamus before torpor, which could be a central mechanism for torpor induction. Unfortunately to date it is not possible to specifically inhibit deiodinases or somehow pharmacologically reduce $T_3$ specifically in the hypothalamus. Currently the one possibility to reduce hypothalamic $T_3$ might be a molecular approach by overexpressing *dio3*. Furthermore, the local inhibition of thyroid hormone transporters is a conceivable option to reduce thyroid hormone availability in the hypothalamus (Braun et al., 2012) Pharmacological inhibition of deiodinases or transporters in the hypothalamus might lead to induction of torpor. It would be also interesting to see the long-term effect of hypothyroidism on torpor expression. Though it has to be considered that thyroid hormones are essential for many

metabolic processes and a long-lasting inhibition could be lethal for animals. Thus at best an inhibition should be reversible.

### 3.1.4 Intermediate conclusion for physiological effects

Thyroid hormone treatment in hamsters showed that $T_3$ - and not $T_4$ - was responsible for all monitored physiological effects. Treatment with $T_4$ caused no increase of body weight, food- or water intake in hamsters. This confirms that $T_4$ has a lower biological activity, which has been described for many years (reviewed by Cheng et al., 2010). One reason for the lower activity is a 10-fold lower affinity to thyroid hormone-receptors (Apriletti et al., 1981; Ichikawa et al., 1986; Samuels et al., 1979; Sandler et al., 2004). Thyroid hormones binding these receptors in the cell nucleus enable binding to TRE regions on the DNA and thus modulate gene expression with downstream effects on physiology and energy balance. $T_4$ has also the highest affinity to all thyroid hormone transport proteins (Köhrle, 1999). Therefore, most $T_4$ is bound and not easily accessible. Another factor is the possible intracellular inactivation of $T_4$ by DIO1 or DIO3 through conversion into inactive $rT_3$. Altogether, the characteristics of $T_3$ make this hormone more active despite lower concentrations, but possible biological effects of $T_4$ are still under debate (Cheng et al., 2010; Schroeder and Privalsky, 2014)

## 3.2 Gene expression

### 3.2.1 Deiodinases

#### 3.2.1.1 Effect of alternating photoperiod

Thyroid hormone availability in the hypothalamus is regulated by deiodinases, which play a central role in seasonal adaptation (Barrett et al., 2007; Ebling, 2015; Murphy et al., 2012). It has been accepted that during SP *dio2* is down regulated and *dio3* up regulated, which leads to reduced $T_3$ availability in the hypothalamus. The present experiments confirm that low $T_3$ availability is a prerequisite for seasonal body weight reduction and torpor expression. However, some studies pointed out, that this paradigm is not as simple as initially thought. *Dio2* expression was lowest after eight weeks in SP (Herwig et al., 2009), returned to LP expression after 14 weeks in SP (Barrett et al., 2007) and surpassed LP levels with continuing time in SP (Herwig et al., 2013). However, body weight remains low until at least week 18 in SP even with already increasing *dio2* expression (Herwig et al., 2013). This already increased hypothalamic *dio2* expression after 14 weeks in SP was confirmed in the first experiment (chapter 2.1.4.3.1). The reason for an early increase of *dio2* might be a feedback

mechanism, which tries to counteract hypothyroidism in the hypothalamus. However, increase of *dio2* expression seems to cause no increase of $T_3$ availability in the hypothalamus, because this would lead to an immediate increase of body weight (Murphy et al., 2012).

In any case *dio2* expression must always be considered in combination with *dio3* expression, which deactivates $T_3$ and $T_4$. In Djungarian hamsters *dio3* expression in the hypothalamus is unique for SP. *In situ* hybridization revealed that *dio3* is only expressed during SP, but not during LP. This pattern has been observed in previous studies (Barrett et al., 2007; Herwig et al., 2009; Herwig et al., 2012) and was confirmed in our experiments. *Dio2* and *dio3* are probably co-expressed in hypothalamic tanycytes (Barrett et al., 2007; Bolborea et al., 2015; Samms et al., 2015). While DIO2 is localized in close proximity to the nucleus, DIO3 is localized at the cell membrane. Therefore, DIO3 can convert $T_4$ into $rT_3$, before it reaches DIO2, and additionally inactivates available $T_3$. Despite high DIO2 concentrations it is conceivable that the presence of DIO3 prevents the accumulation of intracellular active $T_3$ and tanycytes remain hypothyroid. This could explain the low body weight after 14 weeks in SP despite increased *dio2* expression.

Switching hamsters back from SP to LP caused a massive increase in *dio2* expression. This was probably caused by increasing TSH production in the pituitary due to reduced melatonin production in LP. Unfortunately it was not possible to measure *tsh-ß* expression in the pituitary or TSH levels in the blood serum or specific tissues. From previous studies it is known, that TSH is stronger expressed under LP conditions and stimulates expression of *dio2* in tanycytes (Bolborea et al., 2015; Hanon et al., 2008). A seasonal regulation of TSH receptors in the ependymal layer was not found. Thus the regulation must happen on the level of TSH production and deiodinase expression. After the switch from SP to LP *dio3* mRNA was not detectable in the hypothalamus, therefore, $T_3$ inactivation was disabled. Thus it is likely that high turnover of $T_4$ to $T_3$ by DIO2 created high intracellular $T_3$ concentrations in the hypothalamus, which led to the fast increase of body weight. Six weeks after the switch back from LP *dio2* expression slowly decreased to initial LP values. This suggests that high $T_3$ concentrations are only required during the time of body weight increase, but not to maintain body weight during summer. The reason for decreasing *dio2* expression is probably a feedback mechanism triggered by hyperthyroidism in the hypothalamus. This negative feedback might reduce TSH production in the PT resulting in decreasing *dio2* expression in tanycytes (Fonseca et al., 2013; Hanon et al., 2008; Pradet-Balade et al., 1997). It is known that tanycytes build a morphological connection between the PT and the hypothalamus, which supports the idea of this feedback loop (Guerra et al., 2010; Kameda et al., 2003; Rodríguez et al., 1979).

Interestingly another switch from LP to SP reversed gene transcription of *dio2* and *dio3*. *Dio2* was clearly reduced, which was not expected. It was hypothesized that hamsters are not able to reverse the massive *dio2* expression after six weeks in LP and that high $T_3$ concentrations would work as

molecular break preventing another SP adaptation. *Dio3* showed only a partial response after the early second switchback, but a strong increase after the later second switchback. It seems to matter how long the hamsters were in LP after the first switchback from SP. Hamsters switched back later showed a more distinct reverse of gene transcription. However, the later switchback group suggests, that an exact interaction of *dio2* and *dio3* is necessary to reduce body weight. *Dio2* transcription had to be reduced by the time of the switchback and *dio3* up regulation was necessary for loss of body weight. This is in accordance to the hypothesis that low hypothalamic $T_3$ concentrations are a requirement for body weight reduction. Hamsters with an incomplete adaptation of gene expression to SP might have failed to reduce body weight again, because of too high $T_3$ availability in the hypothalamus.

Thus the body weight seems to follow a distinct pattern of deiodinase expression and less actual photoperiod. However, the expression of deiodinases is indirectly controlled by the photoperiod. It remains unclear, why some hamsters adapted their gene expression to SP again and other did not. Under natural conditions a repeated experience of SP within 2-3 month is not intended. We investigated a complex mechanism under artificial conditions to reveal the flexibility and constraints of photoperiodic regulation of thyroid hormone metabolism and linked physiological adaptations. We discovered that the translation of photoperiod to gene expression is very complex and probably more regulators of deiodinases have to be identified. To understand a highly complex thyroid hormone metabolism in the hypothalamus and the consequences for body weight more studies need to be done to reveal the complete pathway. This would improve the understanding of diseases linked to thyroid hormone and obesity.

#### 3.2.1.2 Effect of thyroid hormones

Systemic treatment with $T_3$ caused a reduction of *dio2* in the hypothalamus (2.2.4.4.1). This was not observed after central $T_3$ release (2.3.4.2). Therefore, only increased circulating $T_3$ concentrations appear to feed back to the hypothalamus to decrease central *dio2* expression and encounter the systemic hyperthyroid state. In BAT and muscle $T_3$ had no influence on *dio2* expression, which points to a central regulation of thyroid hormone metabolism during hyperthyroidism. However, most DIO2 is probably deactivated at the protein level without changes in transcription. In rat cell cultures high doses of $T_3$ reduced DIO2 activity, but not *dio2* transcription (Martinez de Mena et al., 2010).

Compared to $T_3$, hyperthyroidism caused by $T_4$ treatment had an effect on *dio2* expression in BAT, but not in hypothalamus and muscle (2.2.4.4.1). $T_4$ is known to be an adequate inhibitor for *dio2* (Köhrle, 2000). However, inhibition by $T_4$ is probably restricted to the post-translational level and it is little known about direct inhibition on transcription. $T_3$ might have regulatory effects at the protein

level of deiodinases, too, but little is known about the inhibitory function of $T_3$. Altogether deiodinases are regulated in a tissue specific manner. This suggests that different tissues can operate differently with elevated thyroid hormone concentrations.

Surprisingly *dio2* was not upregulated during hypothyroidism induced by MMI to compensate for intracellular $T_3$ deficiency. In hypothalamus and BAT *dio2* mRNA concentrations were nearly unchanged. In muscle expression was higher, but this change was not significant, because of a high variance in the control group. This indicates that additional factors might influence *dio2* expression, which superimposes effects directly triggered by changed thyroid hormone concentrations in the blood.

The role of *dio3* is still very indefinite. After systemic $T_3$ treatment *dio3* was close to the detection limit and not upregulated (2.2.4.4.1). This was surprising, because it was expected that *dio3* is upregulated to inactivate excessive supply of $T_3$. This was the case after central $T_3$ treatment, which led to a significant increase of *dio3* in the hypothalamus (2.3.4.3). This was a good indicator for hyperthyroidism in the hypothalamus and has been recently shown in rats (Zhang 2016). DIO3 is definitely important for long-term body weight regulation and is under photoperiodic control. However, the short-term impact is still unclear. DIO3 might play a critical role during spontaneous and local hyperthyroidism.

Regulation of deiodinase gene expression and *in vivo* protein activity is still poorly understood. Transcription factors for *dio2* and *dio*3 are unknown and also the initial trigger for specific and photoperiodic deiodinase regulation. Changed deiodinase expression could be a compensatory effect during hypo- or hyperthyroidism to counterbalance low or high $T_3$ concentrations. On the other side it could also be under physiological control and help to adapt intracellular $T_3$ availability to changed energy requirements. In the present experiments it became clear, that genes are differently regulated in hypothalamus, BAT and muscle in response to hyper- or hypothyroidism. This has to be considered in further studies, when investigating treatment effects on gene expression in different tissues. Furthermore, the effect of changed deiodinase expression on *in vivo* protein activity needs to be analyzed.

### 3.2.1.3 Torpor

During daily torpor *dio2* expression was specifically downregulated in hypothalamus, BAT and muscle independent of treatment. This regulation was specific and not just a temperature- or hypometabolic effect, because not all investigated genes were down regulated during torpor. This observation speaks against a general suppression of transcription as mechanism to reduce metabolism as suggested before (Berriel Diaz et al., 2004). It is conceivable that down regulation of *dio2* also effects protein concentrations, which could result in reduced intracellular $T_3$ activation during torpor. DIO2

has a relatively short half-life of approximately 2h (Baqui et al., 2003) and degradation can be accelerated under certain conditions. It is probable that DIO2 can be also inhibited without degradation, but the deactivation of deiodinases is not well understood. *Dio3* was not increased during torpor to amplify $T_3$ deactivation. Despite this fact it is imaginable that intracellular $T_3$ availability might be reduced by increased DIO3 concentrations before torpor entrance and might be a key factor for torpor induction. More research is needed to understand the dynamic of deiodinases and resulting intracellular thyroid hormone concentrations during torpor, but this complex physiological phenomenon is difficult to unscramble. Gene expression analysis is limited to the *post mortem in situ* state and *in vivo* measurement of tissue specific thyroid hormone concentrations is not possible to date. However, specific regulation of genes during hypothermia opens a new field for molecular research. In the future it could be possible to specifically and locally regulate genes involved in thyroid hormone metabolism to manipulate the physiological output.

### 3.2.2 Thyroid hormone transporter

Thyroid hormone transporters MCT8 and OATP1c1 are expressed in the brain of humans and rodents. So far only *mct8* expression has been shown in Djungarian hamsters (Herwig et al., 2013). MCT8 is under photoperiodic control, but changes are not obvious as compared to deiodinases. During SP *mct8* expression is slightly increased (Herwig 2013, Ross 2009). After switchback from SP to LP *mct8* expression showed a regression to approximately 50% and increased again after a second switch back to SP. In photorefractory hamsters *mct8* declined after more than 37 weeks in SP (Herwig et al., 2013). *Mct8* expression in hypothalamus, BAT and muscle was neither affected by any systemic (unpublished data) nor by central $T_3$ treatment (2.3.4.3). It has to be considered that *mct8* expression might be at its maximum during SP and further increased in response to thyroid hormone treatment was not possible.

Thyroid hormone transporters have at least two binding sites, while most other cellular transporters have usually one binding site (Braun et al., 2013). One binding site is accessible from the exterior site of the cell and the other one accessible from the interior of the cell (Braun et al., 2013). MCT8 is the only known transporter, which is highly specific for thyroid hormone and no other substrates. Therefore, this transporter is considered to be the most important transporter for $T_4$ uptake into tanycytes and important for thyroid hormone supply in the hypothalamus (Visser, 2000; Wirth et al., 2014).

The exact transport mechanism of MCT8 is still unclear. High *mct8* expression could imply a high cellular influx of $T_4$, but also a high efflux of $T_3$. Therefore, it is not clear, whether the role of MCT8 is

to compensate low intracellular $T_3$ levels or to flush out $T_3$ from cells during hyperthyroidism. MCT8 might play a role in maintaining low intracellular $T_3$ concentrations in the hypothalamus during SP. On the other side reduced *mct8* expression during LP could reduce the efflux of $T_3$ to maintain higher $T_3$ concentrations in the hypothalamus, which seems to be necessary for the anabolic summer state.
Little is known about the molecular regulation of *mct8* expression. From the present studies it seems to be unlikely, that thyroid hormones availability directly regulate this transporter. Especially the photoperiodic control of *mct8* seems to be independent of $T_3$. One known regulator of MCT8 is retinoic acid, which is able to increase expression in cell culture (Kogai et al., 2010). However, it seems to be unlikely, that RA is responsible for the photoperiodic translation and regulation of *mct8*, because several components of the RA pathway are down regulated during SP, when *mct8* expression is highest. TSH might be a candidate to regulate MCT8, because it is a transmitter of photoperiodic information from the pituitary gland to the hypothalamus.

The other transporter OATP1c1 was not expressed in the ependymal layer of the third ventricle in Djungarian hamsters like in photoresponsive F344 rats (Ross 2009). However, we could show for the first time that Djungarian hamsters are able to express this transporter (2.3.4.3). A specific expression around the microdialysis probe was detectable after $T_3$ release into the hypothalamus. Thus OATP1c1 seems to have a different role in thyroid hormone transport and might be expressed in hamsters only under specific conditions like neuroinflammation.
Several other thyroid hormone transporters had been identified in the brains of humans and mice (Wirth 2014). However, these transporters are usually expressed in different brain areas and thus MCT8 deficiency or down regulation cannot be fully compensated by another thyroid hormone transporter (Wirth 2009). The mechanism of MCT8 function is of interest for further studies. Blocking this specific transporter might be interesting to cause local intracellular hypothyroidism in the hypothalamus, which could induce torpor. Finally, revealing the pathway behind photoperiodic regulation of *mct8* would help to get a deeper understanding of MCT8, because MCT8 deficiency is the cause for Allan–Herndon–Dudley syndrome (Schweizer and Köhrle, 2013).

## 3.2.3 Uncoupling proteins

### 3.2.3.1 UCP1

UCP1 is uniquely expressed in BAT and essential for NST (Cannon and Nedergaard, 2004; Cannon et al., 1982). Thus the activation of facultative thermogenesis in response to an adrenergic stimulus or cold is typically linked to uncoupling of the proton motive force from the ATP synthesis in mitochondria of BAT (Cannon and Nedergaard, 2004). In this case, energy is released as heat instead

of ATP. Thyroid hormones play an important role in activation of facultative thermogenesis. $T_3$ amplifies the noradrenergic stimulus of BAT and the absence of $T_3$ reduces the capacity of heat production (Bianco and Silva, 1987a; Bianco and Silva, 1987b; Bianco et al., 1988). The source of $T_3$ in BAT is also the conversion of $T_4$ into $T_3$ by DIO2, which can be stimulated by the SNS (Leonard et al., 1983; Silva and Larsen, 1983).

It has been shown that expression of UCP1 is directly regulated by $T_3$ (Bianco et al., 1988; Rabelo et al., 1995; Silva and Rabelo, 1997). Therefore, it was surprising that *ucp1* in SP hamsters was not upregulated after systemic $T_3$ treatment (2.2.4.4.2). Central $T_3$ microdialysis had no effect on *ucp1* expression in BAT, too (*unpublished data*). The reason for an unchanged *ucp1* expression in Djungarian hamsters might be that *ucp1* is already highly expressed during SP acclimation (Demas et al., 2002). Another explanation could be the small sample size. The 1.5 fold difference after $T_3$ and also the 0.5-fold change after MMI treatment were not significant, but this might be different larger experimental groups.

The increased $T_b$ after $T_3$ treatment might be caused by an increased total metabolism, therefore, an increased obligatory thermogenesis and not by an activation of UCP1 mediated facultative thermogenesis. Interestingly *ucp1* transcription was down regulated during daily torpor and correlated with the down regulation of *dio2*. Thus reduced *ucp1* expression might be a consequence of low intracellular $T_3$ concentrations during torpor. Lower *ucp1* transcription combined with an inhibited UCP1 activation by $T_3$ could be a reason for reduced thermogenesis during torpor and reduced $T_b$. However, the activator for this cascade remains unclear. It is also unclear at which time point expression of *dio2* and *ucp1* is reduced and when expression returns so normal expression. Torpor is usually under circadian control and most hamsters enter torpor with the beginning of the light phase (Kirsch et al., 1991). Gene expression was analyzed approximately four hours after “lights on”, when $T_b$ is close to its nadir. To find torpor induction mechanisms gene expression has to be analyzed before expression of torpor. Understandably, with *post mortem* analysis this is impossible, because natural daily torpor occurs in an unpredictable manner.

A further remaining question is how low gene expression affects protein level of DIO2 and UCP1 during torpor. Nothing is known about degradation, inactivation, protein content and activity of UCP1 during the course of torpor. However, it is known, that hamsters use UCP1 mediated NST to rewarm quickly from torpor (Kitao and Hashimoto, 2012). Therefore, it would be interesting to know, if hamsters activate *ucp1* transcription before they arousal from torpor of if the content of UCP1 is not affected by reduced gene expression. However, UCP1 availability is no prerequisite for the expression of hypothermia, because UCP1-KO mice are able to express torpor, but with a slower

rewarming from hypothermia (Oelkrug et al., 2011). Despite the unique function of UCP1 its role and activity during daily torpor in hamsters is not well investigated and understood.

#### 3.2.3.2 UCP3

In BAT *ucp3* showed a similar expression compared to *ucp1* (2.2.4.4.2). Expression was not affected by any systemic or central treatment. It has been recently shown in mice that expression of *ucp3* directly correlates with *ucp1* expression in BAT (Hilse et al., 2016). *Ucp3* was also expressed in skeletal muscle. An effect of $T_3$ on *ucp3* has been shown in rats (Larkin et al., 1997; Reitman et al., 1999), but the increase in Djungarian hamsters was not significant. Central $T_3$ release had no effect on *ucp3* expression in muscle, too (*unpublished data*). The exact function of UCP3 is still under debate (Cioffi et al., 2009; Hesselink and Schrauwen, 2005; Jezek, 2002; Nedergaard et al., 2005). It is considered as candidate involved in local thermogenesis and is probably a $T_3$-responive gene (de Lange et al., 2001; Flandin et al., 2009).

However, UCP3 cannot replace the lack of UCP1 and UCP3-KO mice have a normal metabolism and thermogenic response to cold (Hilse et al., 2016; Vidal-Puig et al., 2000). *Ucp3* was down regulated during torpor expression, too, which gives new evidence for a thermogenic role. After MMI treatment *ucp1* and *ucp3* showed a tendency of being down regulated. A lower expression of these UCPs during hypothyroidism could contribute to the reduced $T_b$ and limited thermoregulation. It might improve the preconditions for torpor, which could explain the higher torpor incidence in hypothyroid hamsters.

#### 3.2.3.3 UCP2

*Ucp2* gene expression pattern differed from the other two UCPs (2.2.4.4.2). Previous studies already provided evidence that UCP2 has a special role in the UCP family. *Ucp2* gene expression was increased in situations with increased energy expenditure (Cioffi et al., 2009). However, UCP2 showed a remarkable discrepancy between gene- and protein expression, while UCP1 and UCP3 show a tight link between mRNA and protein occurrence (Cioffi et al., 2009; Vidal-Puig et al., 1997). Despite abundance of *ucp2* mRNA in BAT, muscle and hypothalamus, its protein has been identified only in the hypothalamus so far (Nedergaard et al., 2005). Therefore, a role in local brain thermogenesis and protection against oxidative stress has been suggested (Andrews et al., 2005; Arsenijevic et al., 2000; Ricquier and Bouillaud, 2000). A special role of UCP2, co-expressed with NPY in glial cells of the ARC, in response to $T_3$ has been proposed by Coppola et al. (2007). Increased $T_3$ concentrations caused by increased *dio2* expression were linked to a stimulation of *ucp2* transcription (Coppola et al., 2007). This interaction between *dio2* and *ucp2* was critical for increased activation of orexigenic NPY-AgRP neurons following food restriction.

In the present study systemic treatment with $T_3$ led to a reduced hypothalamic *dio2* expression in hamsters, but an unchanged expression of *ucp2*. However, *ucp2* in BAT was increased after systemic $T_4$ and $T_3$ treatment. Central $T_3$ microdialysis caused an upregulation of *ucp2* in BAT, too. An interplay between hypothalamus and BAT has been recently proposed in the context of sleep and thermoregulation (Contreras et al., 2015; Rodrigues et al., 2015). However, a function of *ucp2* mRNA in BAT without the presence of its protein in this tissue is still unclear. During hypothyroidism and torpor *ucp2* expression was unchanged, which supports a particular role of UCP2 in comparison to UCP1 and UCP3.

### 3.2.4 Somatostatin

The function of somatostatin during winter is to suppress the production of GH. Strong srif expression in the hypothalamus was observed after 14 weeks in SP (2.1.4.3.8). Under LP conditions increased TSH production is associated with decreased *srif* expression in the hypothalamus (Klosen et al., 2013), which is probably reversed under SP conditions. Thus during SP with decreasing TSH production inhibition of *srif* is reduced and *srif* expression should increase. Therefore, TSH is considered as potential inhibitor of *srif*. However, it is not clear if TSH directly regulates hypothalamic *srif* transcription or indirectly via deiodinases and $T_3$. It has been shown that TSH regulates deiodinases in the ependymal layer of the third ventricle (Nakao et al., 2008a; Wood and Loudon, 2014). In the hypothalamus somatostatin can be produced by neurons of the PVN and ARC (Atrens and Menéndez, 1993; Sawchenko et al., 1990; Spoudeas et al., 1992.) However, somatostatin producing ARC neurons were not sensitive for $T_3$ in our hypothalamic microdialysis experiment (2.34.2). Therefore, $T_3$ might control *srif* production only in the PVN, which is then responsible for inhibition of the GH-axis. Unfortunately the PVN region was not covered by our *in situ* hybridization and further studies need to identify the possible influence of the PVN and the link between somatostatin and thyroid hormone metabolism.

After the switch from $SP_{14}$ back to LP *srif* expression was massively reduced and body weight increased (2.1.4.3.8). This confirms that low *srif* expression is required for body weight gain. The fast gain of body weight is probably caused by secretion of GH in the pituitary. Also the hypothalamus is involved in this pathway, by releasing GHRH in the ARC, which is inhibited by somatostatin during SP (Dumbell et al., 2015; Minami et al., 1998). The analysis of GH is difficult because of its pulsatile release, but increased IGF-1 concentrations in the blood are an indirect sign for GH release (Chomczynski et al., 1988; Le Roith et al., 2001). High IGF-1 concentrations with increasing body weight were detected in switch back hamsters (Dumbell et al., 2015). Altogether the GH-axis is

involved in seasonal body weight regulation and probably linked to thyroid hormone metabolism in the hypothalamus.

It has been recently suggested that somatostatin receptor 5 activation is involved in torpor expression (Scherbarth et al., 2015). Our experiments showed no increase of *srif* expression in the hypothalamus during torpor (2.2.4.3). Therefore, somatostatin might play a role in torpor regulation in the periphery, but might not act on a central level during torpor induction. However, we assume that torpor is primarily controlled on a central level (see chapter 3.2.1.3). Effects on torpor induced by the somatostatin receptor 5 agonist pasireotide might be artificial and be induced only by pharmacological substances. We suggest that a regulation of somatostatin production is not involved in regulation of torpor, but specific activation of somatostatin receptors is an interesting topic for further studies.

### 3.2.5 Cellular retinol-binding protein

In my experiments *crbp1* gene expression was analyzed linked to seasonal body weight regulation and hypothalamic $T_3$ microdialysis. CRBP1 was first discovered as photoperiod-responsive gene during a microarray expression analysis and *in situ* hybridization revealed further retinoid-signaling genes responding to photoperiodic changes (Ross et al., 2004). Decreasing *crpb1* mRNA concentrations were also linked to decreasing body weight during SP. CRBP1 is responsible for the intracellular transport of retinol, the substrate for RA (Li and Norris, 1996). All components necessary for RA synthesis have been identified in tanycytes penetrating the hypothalamus (Barrett et al., 2006; Shearer et al., 2010). A down regulation of *crbp1* in the hypothalamus during SP was confirmed in my study (2.1.4.3.7). This might lead to a reduced availability of retinol in tanycytes and the consequence could be a reduced synthesis of RA.

Switchback of hamsters from SP to LP induced a slow reversing of *crbp1* gene expression. Initial LP mRNA concentrations were not reached after 6 weeks in LP, but after 14 weeks. This might indicate that *crbp1* expression depends on other mechanisms and that the transition last longer or was initiated later. This slow response of *crbp1* has been shown in a previous study, whereas *crabp2*, transporting RA, showed a quick increase after the transfer from SP to LP (Ross et al., 2004). *Crbp1* remained low in photorefractory hamsters, while other genes and body weight already start to increase again (Ross et al., 2004). Therefore, CRBP1 seems to play either a minor or no role during seasonal body weight regulation. Interestingly *crbp1* responded again to the second switchback from LP to SP after six and 14 weeks in LP. However, *crbp1* was stronger down regulated in hamster with reduced body weight compared to non-responsive hamsters in the later switchback group. *Crbp1*

gene expression does not clearly follow the body weight changes. In the $SP_{14}LP_6SP_8$ group with minor body weight loss *crbp1* was lower compared to hamsters of the $SP_{14}LP_{14}SP_8$ group with a significant body weight reduction. Hamsters of this later group with constant body weight had reduced *crbp1* levels, but they did not reach $SP_{14}$ levels again.

The gene expression pattern of *crbp1* and the slow response after the switchback from SP to LP might suggest that this transcription depends on hypothalamic $T_3$, which is probably low during SP and increases after the switchback. However, our $T_3$ microdialysis experiment did not elevate *crbp1* expression in the ependymal layer of the third ventricle within two weeks (2.3.4.3). On the other hand $T_3$ seems to induce *crbp1* expression around the microdialysis probe in the area of injured brain tissue. This might be an evidence for the presence of retinol during neuroinflammation. An anti-inflammatory role of RA in astrocytes has been shown before (Choi et al., 2005). Thus *crbp1* gene expression might be regulated by $T_3$, but induction of *crbp1* transcription in the ependymal layer seems to be inhibited during SP in Djungarian hamsters. The mechanism behind this blocking of *crbp1* and the seasonal regulation of this gene remains unclear. The switch back experiment suggests that CRBP1 plays no primary role in seasonal body weigh regulation.

### 3.2.6 G protein-coupled receptor 50

This G protein-coupled receptor has a close sequence homology with melatonin receptors (Reppert et al., 1996). However, it does not bind melatonin and remains an orphan receptor with no known ligands, but it can deactivate $MT_1$ receptors by forming $MT_1$/GPR50 heterodimers. GPR50 is also expressed in tanycytes of the hypothalamus, where many seasonal changes take place (Barrett et al., 2006; Bechtold et al., 2012). Therefore, the function and regulation of *gpr50* is of interest for research in the context of seasonal adaptations. In agreement with previous studies (Barrett et al., 2006; Herwig et al., 2013) *gpr50* was down regulated during SP in Djungarian hamsters. The low expression of *grp50* during SP probably reduces $MT_1$/GPR50 heterodimers and thus increases the effect of melatonin at $MT_1$ receptors. This receptor has been detected in the SCN and PT, which both play a crucial role in transducing the photoperiodic into endocrine signals.

After the switchback from SP to LP *gpr50* expression increased, but initial LP level was reached after 14 weeks in LP (2.1.4.3.6). It is imaginable, that GPR50 supports the inhibition of melatonin effects during the transition from SP to LP to restore the summer-type of hamsters (Levoye et al., 2006). Most winter adaptations are mainly reversed by decreasing melatonin concentrations or refractoriness to melatonin (Illnerová et al., 1984). GPR50 needs the change from SP to LP, because it does not spontaneously increase in photorefractory hamsters under constant SP conditions (Herwig

et al., 2013). This indicates that an abrupt switch between static SP and LP differs from a natural transition from winter to summer including melatonin refractoriness. After the second switch back to SP after 6 or 14 weeks in LP, *gpr50* was reduced again, but hamsters of the $SP_{14}LP_{14}SP_8$ that did not respond to SP again with reduced body weight, had unchanged *gpr50* expression. While *crbp1* seems to be reduced in these hamsters, *gpr50* expression remained at LP-like levels. Therefore, *gpr50* might be involved in seasonal body weight regulation and could be one reason for failed body weight reduction under SP conditions.

Interestingly GPR50-KO mice had increased *dio2* expression in tanycytes, hence might be involved in the modulation of $T_3$ availability in the hypothalamus (Bechtold et al., 2012). However, the link between *dio2* and *gpr50* in Djungarian hamsters seems to be more complex, because *dio2* expression is low during SP, when *gpr50* is low, too. After the switchback from SP to LP *dio2* increased faster than *gpr50*, but the delayed increase of *gpr50* might inhibit *dio2* expression and caused declining *dio2* expression after more than six weeks in LP.

Hypothalamic treatment with $T_3$ had no significant influence on *gpr50* expression in the ependymal layer of the third ventricle (2.3.4.3). This would suggest that seasonal *gpr50* expression is independent on hypothalamic $T_3$ availability. On the other hand two weeks of treatment might not be enough to see changes in *gpr50* expression. The switchback experiment gave evidence that *gpr50* is a slowly responding gene. G*pr50* was not expressed around the microdialysis probe after $T_3$ release, so $T_3$ does not seem to acutely regulate *gpr50*.

Recently a link between *gpr50* and leptin was discovered (Bechtold et al., 2012). Leptin is involved in regulation of energy balance and is responsible to induce satiety (Brennan and Mantzoros, 2006). It binds to NPY neurons in the ARC and inhibits their activity and thus reduces food intake. GPR50 seem to be essential for leptin signaling in the hypothalamus (Bechtold et al., 2012). Circulating leptin concentrations and –signaling is altered between SP and LP with lower leptin levels but higher sensitivity during winter (Klingenspor et al., 2000; Tups et al., 2004). Thus increasing *gpr50* expression after the switch from SP to LP might be important for the transition of leptin signaling between winter and summer.

Since the discovery of GPR50 20 years ago some interesting effect of this receptor has been discovered, but little is known about the intracellular downstream signaling pathway (Khan et al., 2016). Future research has to reveal the significance of GPR50 in seasonality, adaptive thermogenesis, body weight regulation, leptin signaling and possibly a role during fasting induced torpor (Bechtold et al., 2012)

### 3.2.7 Histamine receptor 3

This histamine receptor has been proposed to be involved in body weight regulation (Hancock, 2003; Takahashi et al., 2002). H3R controls the release of histamine (Arrang et al., 1987), which regulates food intake (Sakata et al., 1997; Yoshimatsu et al., 1999; Yoshimatsu et al., 2002). However, histamine synthetizing neurons are not present in the dmpARC, which led to the assumption that H3R is more a signalling molecule acting as heteroreceptor and regulates the release of other compounds (Barrett et al., 2005; Haas and Panula, 2003).

Barrett and colleagues showed in 2005 that *h3r* in the dmpARC was significantly down regulated after 14 weeks in SP. H3R expression was inversely related to *c-fos* expression, a marker for neuronal activity, which suggests that with low *h3r* expression during SP neuronal activity increased (Barrett et al., 2009). This correlation was reversed after a switch back to LP. In the present switchback experiment, hamsters had high *h3r* expression after the switch back from SP to LP, too (unpublished data, Figure 28). However, *h3r* expression was not reduced after 14 weeks SP in my experiment. The increase of *h3r* after the switchback might reflect decreasing melatonin production, because melatonin seems to inhibit *h3r* expression (Barrett et al., 2005). Given that melatonin receptors are not expressed in the dmpARC this effect must be mediated by an upstream brain region, probably the SCN (Bartness et al., 2002). Increasing *h3r* expression after the switchback might be induced by increasing $T_3$ concentrations, because *h3r* in the dmpARC was increased after hypothalamic $T_3$ microdialysis (2.3.4.2). Previous studies already showed a link between *h3r* and *dio2* transcription in photorefractory hamsters (Herwig et al., 2013; Ross et al., 2005).

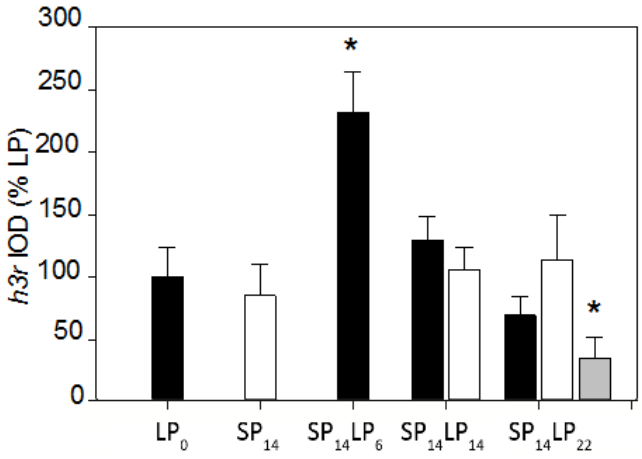


**Figure 30: Gene expression of *h3r* in the hypothalamus.** Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to long photoperiod. Hamsters were kept in long photoperiod (LP, black) or short photoperiod (SP, white). White bar at LP14 represents group SP-LP6-SP8 and at LP22 it represents group SP-LP14-SP8. The grey bar represents hamsters from the SP-LP14-SP6 group that did not reduce body weight. * significant difference to SP14; # significant difference between other groups

## 3.3 Perspectives

In my study I investigated the link between gene expression and physiological changes in body weight and torpor expression. Thyroid hormones emerged as a promising candidate for regulation of energy balance and metabolic suppression. In the next step translation from gene expression to protein activity is necessary to get further insight into the molecular pathways underlying body weight regulation and torpor induction. However, specific antibodies for deiodinases are not available. Also deiodinase activity can be studied only *post mortem* in cell culture systems. To understand the complex system behind body weight regulation and especially torpor expression *in vivo* experiments are indispensable.

For *in vivo* experiments pharmacological manipulation is still the best method of choice. Further experiments could provide more details between the interaction of the hypothalamus and somatic tissues in regards to thyroid hormone status. Central manipulation of $T_3$ in hypothyroid hamsters would be an interesting design to reveal hypothalamic pathways regulating energy balance and metabolism in the periphery. Inhibition of deiodinases or thyroid hormone transporters via microdialysis might be a promising approach to induce local hypothyroidism in the hypothalamus or other target tissues. The ultimate aim is still to directly measure local thyroid hormone concentrations in different tissues. *In vivo* microdialysis is a tool, which theoretically allows extracting molecules from the hypothalamus. However, to date the analysis of dialysis samples is not established. Probably larger hibernating animals should be used to study effects of thyroid hormones on hypothermia, because they have a higher total abundance of thyroid hormones in tissues and blood. In the future, local genetic manipulation via a CRISPR/CAS approach could be a useful tool to specifically overexpress or inhibit gene expression of deiodinases. This could reveal further information about the influence on gene expression on seasonal regulation of body weight and torpor.

# 4. List of abbreviations

| | |
|---|---|
| ADP | **A**denosine **Di**ph**o**sphate |
| AgRP | **Ag**outi-**R**elated **P**eptide |
| ANOVA | **An**alysis **o**f **Va**riance |
| ARC | **Arc**uate nucleus |
| ATP | **A**denosine **T**ri**p**hosphate |
| BAT | **B**rown **A**dipose **T**issue |
| BSA | **B**ovine **S**erum **A**lbumin |
| $C_T$ | **C**ycle **T**hreshold |
| $CO_2$ | **C**arbon **d**ioxide |
| CRBP | **C**ellular **R**etinol **B**inding **P**rotein |
| CRABP | **C**ellular **R**etinoic **A**cid **B**inding **P**rotein |
| CSF | **C**erebro**s**pinal **F**luid |
| DIO | **D**e**io**dinase |
| DMH | **D**orso**m**edial **H**ypothalamus |
| DNA | **D**eoxyribonucleic **a**cid |
| dmpARC | **D**orso**m**edial **p**osterior **arc**uate nucleus |
| EDTA | **E**thylene**d**iamine**t**etraacetic **a**cid |
| GAPDH | **G**lycer**a**ldehyde 3-**p**hosphate **d**e**h**ydrogenase |
| GH | **G**rowth **H**ormone |
| GHRH | **G**rowth **H**ormone **R**eleasing **H**ormone |
| GPR50 | **G P**rotein-Coupled **R**eceptor **50** |
| $H^+$ | Proton |
| HPRT | **H**ypoxanthine **P**hospho**r**ibosyl**t**ransferase |
| HPT | **H**ypothalamus-**P**ituitary-**T**hyroid |
| IGF | **I**nsulin-like **G**rowth **F**actor |
| IOD | **I**ntegrated **O**ptical **D**ensity |
| LAT | **L**-type **A**mino acid **T**ransporters |
| LH | **L**ateral **H**ypothalamus |
| LP | **L**ong **P**hotoperiod |
| MCT | **M**ono**c**arboxylate **T**ransporter |
| ME | **M**edian **E**minence |
| MMI | **M**ethimazole |
| MR | **M**etabolic **R**ate |
| MT | **M**ela**t**onin receptor |
| mRNA | **m**essenger **R**ibo**n**ucleic **A**cid |
| NPY | **N**europeptide **Y** |
| NST | **N**on-**S**hivering **T**hermogenesis |
| OATP | **O**rganic **A**nion **T**ransporter **P**olypeptide-related protein |
| PBS | **P**hosphate-**b**uffered **s**aline |
| PFA | **P**ara**f**orm**a**ldehyde |
| POMC | **P**ro**o**pio**m**elano**c**ortin |
| PPII | **P**yroglutamyl **P**eptidase **2** |

| | |
|---|---|
| PT | **P**ars **T**uberalis |
| PTU | **P**ropyl**t**hio**u**racil |
| PVN | **P**ara**v**entricular **N**ucleus |
| qPCR | **q**uantitative **P**olymerase **C**hain **R**eaction |
| RNA | **R**ibo**n**ucleic **a**cid |
| $rT_3$ | **R**everse **Tri**iodo**t**hyronine |
| SCN | **S**uprachiasmatic **N**ucleus |
| SD | **S**tandard **D**eviation |
| SDT | **S**pontaneous **D**aily **T**orpor |
| SEM | **S**tandard **E**rror of the **M**ean |
| SNS | **S**ympathetic **N**ervous **S**ystem |
| SP | **S**hort **P**hotoperiod |
| SRIF | **S**omatotropin **R**elease–**I**nhibiting **F**actor (a.k.a. SST) |
| SSC | **S**aline **S**odium **C**itrate |
| SST | **S**omato**st**atin |
| RALDH | **R**etin**al**dehyde **D**e**h**ydrogenases |
| RA | **R**etinoic **A**cid |
| RAR | **R**etinoic **A**cid **R**eceptor |
| RARE | **R**etinoic **A**cid **R**esponse **E**lement |
| RBP | **R**etinol **B**inding **P**rotein |
| RXR | **R**etinoid **X** **R**eceptor |
| $T_2$ | **Di**iodo**t**hyronine |
| $T_3$ | **Tri**iodo**t**hyronine |
| $T_4$ | **T**hyroxin |
| $T_a$ | **T**emperature$_{\textbf{a}\text{mbient}}$ |
| $T_b$ | **T**emperature$_{\textbf{b}\text{ody}}$ |
| $tT_3$ | **t**otal **Tri**iodo**t**hyronine |
| $tT_4$ | **t**otal **T**hyroxin |
| TR | **T**hyroid Hormone **R**eceptor |
| TRH | **T**hyrotropin-**R**eleasing **H**ormone |
| TSH | **T**hyroid-**S**timulating **H**ormone |
| UCP | **U**n**c**oupling **P**rotein |
| UTP | **U**ridine-5'-**t**riphos**p**hate |
| VMH | **V**entromedial **H**ypothalamus |
| ZT | **Z**eitgeber **T**ime |

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

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# Eidesstattliche Versicherung

Hiermit erkläre ich, dass ich die vorliegende Dissertation zum erlangen des Doktortitels (Dr. rer.nat) mit dem Thema

*Influence of thyroid hormones on seasonal regulation of body weight, daily torpor and gene expression in Djungarian hamster (Phodopus sungorus)*

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 27.06.2016 _____

Jonathan Bank

## Bestätigung der Korrektheit der englischen Sprache

Hiermit bestätige ich, Tracey Williams-Zinn geboren am 23. Juli 1966 in New York City, New York (USA), dass die Dissertation von Jonathan Bank mit dem Titel "Influence of thyroid hormones on seasonal regulation of body weight, daily torpor and gene expression in Djungarian hamsters (*Phodopus sungorus*)" in einem korrekten Englisch verfasst wurde.

22.06.16 Hamburg — Datum, Ort

Tracey McM... — Unterschrift

*What does a man get for all of his hard work on earth?*

*What does he get for all of his worries?*

*As long as he lives, his work is nothing but pain and sorrow.*

*Even at night his mind can't rest. That doesn't have any meaning either.*

*A man can't do anything better than eat and drink and be satisfied with his work.*

*I'm finally seeing that those things also come from the hand of God.*

*Without his help, who can eat or find pleasure?*

*God gives wisdom, knowledge and happiness to a man who pleases him.*

*But to a sinner he gives the task of gathering and storing up wealth.*

*Then the sinner must hand it over to the one who pleases God.*

*That doesn't have any meaning either.*

*It's like chasing the wind.*

*Ecclesiastes 2:22-26*