Effects of photoperiod and voluntary exercise on the hypothalamic energy balance circuitry and physiological traits in the seasonal Djungarian hamster (*Phodopus sungorus*)

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ZUSAMMENFASSUNG

Dsungarische Zwerghamster (*Phodopus sungorus*) weisen physiologische Anpassungen auf, um in dem extremen kontinentalen Klima ihres natürlichen Lebensraums überleben zu können. Besonders im Winter, wenn die Umgebungstemperatur weit unter -40 °C fallen kann und die Futterverfügbarkeit eingeschränkt ist, sind Mechanismen zur Energieeinsparung überlebenswichtig. Ausgelöst durch abnehmende Tageslängen färben Dsungarische Zwerghamster in ein gut isolierendes weißliches Winterfell um, sie zeigen täglichen Torpor (ein Zustand charakterisiert durch Hypometabolismus und Hypothermie), Hodenregression und eine Körpergewichtsabnahme. Vorausgehende Studien haben jedoch gezeigt, dass freiwillige Laufaktivität einige dieser Winter-Anpassungen beeinflusst. Hamster mit Zugang zu einem Laufrad sind nur selten torpid, sie zeigen eine verzögerte Hodenregression und eine Körpergewichtszunahme.
Ergänzend zu den bereits bekannten Einflüssen konnten wir zeigen, dass Laufradaktivität eine erfolgreiche Reproduktion negativ beeinflusst. Wir fanden eine reduzierte Anzahl an Trächtigkeiten und vermehrten Infantizid, sowohl bei freiwillig laufenden Zuchtpaaren als auch bei einzeln gehaltenen Weibchen. Folglich hat die zusätzliche energetische Herausforderung der Laufradaktivität das Gleichgewicht zwischen mütterlicher Investition in die Jungtiere und eigener Versorgung eindeutig auf Kosten des Fortpflanzungserfolgs verschoben. Um weitere, durch Laufaktivität induzierte Veränderungen zu ermitteln, die dem Gewichtsanstieg von Winter-angepassten Hamstern unterliegen könnten, haben wir den Einfluss von Laufradaktivität auf die Genexpression im hypothalamischen Nukleus arcuatus (ARC) untersucht. Der ARC ist ein Gehirnzentrum, das an der Regulation des energetischen Gleichgewichts beteiligt ist. Wir konnten zeigen, dass weder die Expression von orexigenen noch anorexigenen Genen durch Laufaktivität im Kurztag verändert wurde. Der Melanocortin Signalweg und sekretorische Prozesse in einem Unterbereich des ARC (dmpARC) scheinen jedoch stimuliert zu werden. Zudem haben wir den Nachweis erbracht, dass durch Laufaktivität induziertes Wachstum zu dem Anstieg im Körpergewicht beiträgt. Die Quantifizierung der Genexpression im Schilddrüsensystem hingegen zeigte, dass die zentrale Wahrnehmung der Photoperiode nicht beeinflusst wurde. Daraufhin haben wir in einer nachfolgenden Studie untersucht, ob durch Laufaktivität induzierte Signale aus der Peripherie die Mechanismen beeinträchtigen könnten, die der saisonalen Körpergewichtsregulation unterliegen. Folglich haben wir die Phosphorylierung von Enzymen, die in den Stoffwechsel der Myozyten involviert sind, im Musculus gastrocnemius analysiert. Außerdem wurden die Konzentrationen von Insulin und dem Insulinähnlichen Wachstumsfaktor-1 im Serum bestimmt. In einer weiteren Studie wurde zum ersten Mal die zeitliche Abfolge hypothalamisch exprimierter Gene, von denen angenommen wird, dass sie an der Körpergewichtsregulation beteiligt sind, in Hamstern, die ein Jahr lang in natürlicher Photoperiode und Umgebungstemperatur gehalten wurden, untersucht. Da sich die Genexpressionen, wie z.B. von type 2 deiodinase (*Dio2)*, monocarboxylate transporter 8 (*Mct8*) und somatotropin release-inhibiting factor (*Srif*) vor oder parallel zu dem Körpergewicht änderten, konnte bestätigt werden, dass sie an der Regulation des saisonalen Körpergewichtszyklus beteiligt sind.
Insgesamt nehmen wir an, dass mehrere Signalwege, die in die Regulation des energetischen Gleichgewichts involviert sind, durch freiwillige Laufradaktivität beeinflusst werden. Dabei könnten sich bereits kleine Veränderungen in einzelnen dieser Signalwege aufsummieren und somit einen Gewichtsanstieg in Winter-angepassten Dsungarischen Zwerghamstern ermöglichen.

Energetisches Gleichgewicht · Saisonale Anpassung · Freiwillige Laufaktivität

SUMMARY

To be able to survive the extreme continental climate in their natural habitat, Djungarian hamsters (*Phodopus sungorus*) exhibit physiological adaptations. Particularly in winter, when the ambient temperature may drop far below -40 °C and food availability is restricted, mechanisms to save energy are essential. Triggered by decreasing day lengths, Djungarian hamsters moult into a well-insulating whitish winter fur, they show daily torpor (a state of hypometabolism and hypothermia), testes regression and a reduction in body mass. However, in previous studies, voluntary exercise in short days has been shown to affect some of these winter-acclimatizations. Hamsters with access to a running wheel are rarely torpid, they show a delayed testes regression and a body weight gain.

In addition to the already known influences we could show that wheel-running activity negatively affects successful reproduction. We found a decreased number of pregnancies and increased infanticide in voluntarily exercising breeding pairs and singly kept females. Thus, the additional energetic challenge due to wheel-running activity clearly shifted the balance between maternal investment into the offspring and self-maintenance at the expense of reproductive success. To further determine exercise-induced changes that might underlie the weight gain in winter-adapted hamsters, we investigated the influence of wheel running on gene expression in the hypothalamic arcuate nucleus (ARC). The ARC is a brain centre that is involved in energy balance regulation. We could show that the expression of neither orexigenic nor anorexigenic genes was changed due to exercise in short days. However, the melanocortin pathway and secretory processes in a sub-region of the ARC (dmpARC) seem to be stimulated. Moreover, we provide evidence that exercise-induced growth contributes to the increase in body mass. Quantification of gene expression in the thyroid system on the other hand indicated that the central perception of photoperiod was not affected. We thereupon investigated in a subsequent study, whether exercise-induced signals from the periphery might affect the mechanisms underlying seasonal body weight regulation. Therefore, the phosphorylation of enzymes involved in myocyte metabolism in the gastrocnemius muscle was analysed. In addition, serum concentrations of insulin and insulin-like-growth factor 1 were determined. In another study, the temporal sequence of hypothalamic expression for genes, assumed to be involved in body weight regulation, was investigated for the first time in hamsters kept in natural photoperiod and ambient temperature for one year. Since gene expression, such as type 2 deiodinase (*Dio2)*, monocarboxylate transporter 8 (*Mct8*) and somatotropin release-inhibiting factor (*Srif*) changed prior to or in parallel to the body mass, they could be confirmed to be involved in the regulation of the seasonal body weight cycle.

Altogether, we assume that several pathways involved in energy balance regulation are affected by voluntary wheel-running activity. Thereby, already small changes in some of these pathways might sum up, thus allowing a weight gain in winter-acclimatized exercising Djungarian hamsters.

Energy balance · Seasonal adaptation · Voluntary exercise

CONTENTS

ABBREVIATIONS

AA-NAT	arylalkylamine-N-acetyltransferase
ACC	acetyl CoA carboxylase
AGRP	agouti-related protein
AICAR	5-amino 4-imidazolecarboxamide riboside
Akt	serine/threonine kinase or protein kinase B
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
α-MSH	α-melanocyte-stimulating hormone
ANOVA	analysis of variance
ARC	arcuate nucleus
ATP	adenosine triphosphate
BAT	brown adipose tissue
BSA	bovine serum albumin
C	control
CART	cocaine- and amphetamine-regulated transcript
cDNA	complementary deoxyribonucleic acid
CPT-1	carnitine palmitoyl transferase 1
CRABP-2	cellular retinoic acid binding protein 2
CRBP-1	cellular retinol binding protein 1
CSF	cerebrospinal fluid
DEXA	Dual-Energy X-ray Absorptiometry
DIO2	type 2 deiodinase
DIO3	type 3 deiodinase
dmpARC	dorsal medial posterior arcuate nucleus
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ERK-1/2	extracellular signal-regulated kinase 1/2 or p44/42 MAPK
FSH	follicle stimulating hormone
GABA	γ-aminobuyric acid
GH	growth hormone
GHRH	growth hormone-releasing hormone

GLUT-4	glucose transporter 4
GnRH	gonadotropin-releasing hormone
GPR50	G-protein-coupled receptor 50
h	hour(s)
H3R	histamine 3 receptor
HRP	horseradish peroxidase
5-HT-2A/7	serotonin receptor 2A/7
i.c.v.	intracerebroventricular
IGF-1	insulin-like-growth factor 1
IGFR-1	insulin-like-growth factor receptor 1
IL-6	interleukin 6
JAK	janus kinase
LD	long day length (i.e. summer)
LH	luteinising hormone
MAPK	mitogen-activated protein kinase
MC3/4	melanocortin-3/4
MCT8	monocarboxylate transporter 8
ME	median eminence
min	minute(s)
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
PBN	parabrachial nucleus
PBS	phosphate buffered saline
PC	personal computer
PC-2	prohormone convertase 2
PFA	paraformaldehyde
PGC-1	peroxisome-proliferator-activated receptor γ co-activator-1
PI3K	phosphatidylinositol 3-kinase
POMC	proopiomelanocortin
PT	pars tuberalis
PVDF	polyvinylidene difluoride
PVN	paraventricular nuclei

RAR	retinoic acid receptor
rev.	revolutions
RNA	ribonucleic acid
rT_3	reverse thyroid hormone (inactive)
RT-PCR	reverse transcriptase polymerase chain reaction
RW	running wheel
RXRγ	retinoid X receptor γ
SCN	suprachiasmatic nuclei
SD	short day length (i.e. winter)
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SgIII/VI	secretogranin III/VI
SOCS3	suppressor of cytokine signalling 3
SRIF	somatotropin release-inhibiting factor
SS	summer solstice
SSC	standard saline citrate
STAT	signal transducer and activator of transcription
T_2	diiodothyronine (inactive)
T_3	active thyroid hormone (triiodothyronine)
T_4	thyroid prohormone (thyroxine)
T_a	ambient temperature
TBS	Tris buffered saline
TEA	triethanolamine
TLQP-21	(nonacronymic)
TRH	thyrotropin-releasing hormone
tRNA	transfer ribonucleic acid
TSH	thyroid stimulating hormone
VGF	(nonacronymic)
WS	winter solstice

CHAPTER 1

GENERAL INTRODUCTION

One of the most serious public health problems worldwide is obesity, with increasing prevalence in the 21st century. Obesity is associated with various sequels (cardiovascular diseases, type 2 diabetes, cancer etc.), whereby life expectancy is reduced. The cause of this disease is excess energy consumption in combination with a lack of energy expenditure through metabolism or exercise. Developing therapies to help the increasing number of diseased people is the main focus of recent science in this field. Therefore, basic research in energy homeostasis regulation and body weight control ranks first. A suitable animal model for this kind of investigations is the Djungarian hamster (*Phodopus sungorus*; also known as Siberian hamster). This small mammal naturally shows a pronounced seasonal cycle in body mass, which is associated with seasonal adiposity (Wade and Bartness 1984). Alterations in body and fat mass can easily be examined by transferring the photoperiodic hamster species to different light regimes.

The following sections further introduce the Djungarian hamster as an animal model and deliver insights into established pathways in the brain and the periphery that are known to be involved in the regulation of body mass. Besides photoperiod, another 'tool', namely wheel-running activity in the Djungarian hamster, is demonstrated to be suitable to challenge and investigate the mechanisms underlying seasonal body weight regulation.

The Djungarian hamster and seasonal adaptations

Seasons derive from to the yearly rotation of the earth around the sun and the tilt of the earth's axis. At latitudes above and below the equator, considerable changes in climate and day lengths occur in the course of one year, as both hemispheres are illuminated and heated by the sun with changing duration. During evolution, strategies to adapt to seasonal changing conditions were beneficial for survival in local animals that were not able to escape if environmental conditions (temperature, precipitation and food availability) became temporarily unfavourable. Thus, many species that do not exhibit the preconditions to migrate in winter are seasonal in their behaviour and physiology themselves. The most reliable factor, changing consistently in the course of one year, is photoperiod. In mammals, the perception of this environmental signal results in neuroendocrine changes leading to seasonal adaptations (for review, see Scherbarth and Steinlechner 2010).

Djungarian hamsters are native to an area including the steppes of northern Kazakhstan and China, Mongolia and southern Siberia (Flint 1966), approximately between 47°N and 57°N.

According to the continental climate, environmental temperatures may range between 40° C in summer and -72 °C in winter. To survive this extreme annual amplitude in climate and ensuing food availability (seeds and insects), Djungarian hamsters adapt physiologically and show annual cycles in gonadal size and function, torpor occurrence, pelage colour and body weight, all of which are induced by photoperiod (Figala et al. 1973, Hoffmann 1973). The hamsters are long-day (LD) breeders, showing fully developed gonads and an associated period of reproduction during spring and summer. Regression of the gonads takes place during the transition from summer to winter, as day lengths decline, leading to reproductive quiescence (Figala et al. 1973, Hoffmann 1973). Additionally, individuals moult from a greyish-brown summer fur into a whitish winter fur, which shows improved properties in thermal insulation (Heldmaier and Steinlechner 1981a). In winter, the animals show an increased fur depth and a higher proportion of short wool hair (Kuhlmann et al. 2003). The change back to the summer fur takes place between late winter and early spring (Figala et al. 1973, Hoffmann 1973). Together with a decreased heat loss because of the better insulating winter fur, short phases of spontaneous daily torpor lead to a reduction in total energy requirements (Heldmaier and Steinlechner 1981b, Heldmaier et al. 1982). Daily torpor is characterized as a state of reduced metabolic rate and decreased body temperature not below 14 °C (Figala et al. 1973, Körtner and Geiser 2000). The hypometabolic and hypothermic state usually occurs the first time after 12-13 weeks under short-day (SD) conditions (Elliott et al. 1987, Ruf et al. 1993). Torpor bouts may last up to eight hours and occur during the light phase (i.e. the inactive phase of the hamsters). Furthermore, in response to decreasing photoperiod in autumn, Djungarian hamsters spontaneously reduce food intake and body mass (Knopper and Boily 2000). Dependent on sex, they reach a minimum weight of about 25-30 g in winter, compared to a maximum of 40-45 g in summer (Figala et al. 1973, Hoffmann 1973). The advantages of this adaptation are further decreased energy requirements of a smaller hamster in winter (Steinlechner et al. 1983), facilitating survival during this unfavourable time of the year.

After prolonged exposure to SD, the photoneuroendocrine system develops refractoriness. This lack of response to the inhibitory SD signal induces the hamsters' reversion from the winter phenotype to the summer state in anticipation of the favourable time for reproduction in spring (Hoffmann 1978, 1979, Schlatt et al. 1993). Thus, spontaneous recrudescence, as well as the increase in body mass and the moult to the summer fur are induced despite the short photoperiod. Subsequently, hamsters require a period of about 10-15 weeks in LD to re-sensitize the neuroendocrine system to the inhibitory SD signal again (Bittman 1978,

Kauffman et al. 2003, Reiter 1972, Stetson et al. 1977). However, the mechanisms involved in the development of photorefractoriness remain to be elucidated. Up to now, it is assumed that refractoriness occurs due to an inability to read the SD melatonin signal.

In mammals, the perception of photoperiodic information is linked to the eyes. Photoreceptors of the retina are stimulated by light and transmit information via the retinohypothalamic tract to the hypothalamic suprachiasmatic nuclei (SCN) (Larsen et al. 1998) where the circadian clock resides. Its rhythmic output entrains metabolism, physiology and behaviour to the 24-hour-period of a day (for review, see Welsh et al. 2010). Across the paraventricular nuclei (PVN), the clock's output is conveyed to the pineal gland via a multisynaptic pathway and the sympathetic nervous system (Larsen et al. 1998). Within the pineal gland, neural information is transformed into an endocrine signal in the form of rhythmic secretion of melatonin into the blood stream and cerebrospinal fluid (CSF). The rate limiting enzyme for the synthesis of melatonin in the pinealocytes is arylalkylamine-N-acetyltransferase (AA-NAT) that catalyses the conversion of serotonin into a melatonin precursor. Since AA-NAT is active only during night (Klein and Weller 1970), the duration of the melatonin peak is positively correlated with the night length. Subsequently, the hormonal signal acts in the brain and in tissues where melatonin receptors are expressed. There is accumulating evidence that the pars tuberalis (PT) of the pituitary gland is one target site for the action of melatonin. The PT in turn transduces photoperiodic changes into seasonally changing patterns of prolactin secretion by the pituitary gland, which triggers a cascade of processes leading to seasonal changes in physiological traits (Duncan and Goldman 1984, Dupré et al. 2008, Hazlerigg et al. 1996, Wagner et al. 2007). However, further research is necessary to identify other brain areas that are possibly involved in melatonin-mediated signalling.

Seasonality and exercise

Hamsters are known for their intense and voluntary wheel-running activity, which is feasible for continuous long-term recordings. Registration of wheel running is primarily used in biological rhythms research, where the hamsters' endogenous rhythm is assumed to be reflected by the day-night activity patterns. However, the nature of voluntary wheel-running activity in captive rodents is discussed controversially (for review, see Sherwin 1998). It remains unclear, whether wheel running in small laboratory cages is a reflection of the locomotor activity that would also occur in the natural habitat, or whether it is an artefact of

captive environments or of the running wheel (RW) itself. One study revealed that even cage enrichment has only very small effects on the pattern and amount of running-wheel activity in Syrian hamsters (*Mesocricetus auratus*) (Reebs and Maillet 2003). This finding supports the hypothesis that voluntary wheel-running activity might be self-reinforcing (Sherwin 1998).

In further studies, wheel-running activity has been shown to have various effects in rodents. For example, spatial learning in rats is improved through physical exercise (Fordyce and Farrar 1991). Furthermore, the proliferation of precursor cells, cell survival and neurogenesis is increased in the hippocampus of exercising mice (van Praag et al. 1999). In addition, physiological and morphological changes were found in voluntarily exercising Syrian and Djungarian hamsters. Like Djungarian hamsters, Syrian hamsters show seasonal changes in gonadal size and body weight but they increase body mass in response to shortening photoperiods (i.e. pre-hibernation fattening). Several studies showed both incomplete gonadal regression and inhibited hibernation in this hamster species caused by wheel-running activity in SD (Gibbs and Petterborg 1986, Menet et al. 2003). Furthermore, prolonged exercise in Syrian hamsters evoked an increase in body mass (Borer and Kaplan 1977, Borer and Kooi 1975, Gattermann et al. 2004) due to exercise-induced growth (Borer and Kelch 1978, Borer and Kuhns 1977). However, the perception of photoperiodic information was not impaired in hamsters with access to a RW (Menet et al. 2005).

Studies in the Djungarian hamster revealed similar results concerning the influence of wheel-running activity on seasonal acclimatizations (Scherbarth et al. 2007, 2008). In an experiment under natural photoperiod and natural ambient temperature (T_a), Scherbarth and coworkers (2007) found the seasonal cycle in body weight and adiposity to be affected by wheel-running activity. Instead of the typical SD-induced decrease in body mass, the animals increased their body mass and remained heavy. Furthermore, the results indicated that mainly lean mass was responsible for the exercise-induced increase in body mass.

In the present study, we thereupon investigated signalling pathways in skeletal muscle of exercising hamsters, as muscles are the main component of lean mass. We hypothesized that altered signalling from peripheral skeletal muscle to the brain might be involved in affecting the mechanisms that regulate the seasonal body weight cycle in Djungarian hamsters (see chapter 4).

Like in Syrian hamsters, a growth-stimulating effect of wheel-running activity has also been demonstrated in Djungarian hamsters. Under SD conditions, Scherbarth and coworkers (2008) found elongated femora in exercising hamsters compared to controls. Besides body mass, voluntary exercise also affected torpor and the gonadal cycle. Torpor was inhibited and

testicular recrudescence was advanced in hamsters with access to a RW. Likewise, in a following study it was shown that testes regression in SD is delayed due to wheel-running activity (Scherbarth et al. 2008).

In the present study, we investigated the influence of voluntary exercise on reproduction to further shed light on the challenge of high energetic costs for the females during reproduction and additionally increased energy expenditure due to wheel-running activity (see chapter 2).

However, from all examined seasonal traits, only the moult to the whitish winter fur was not affected by wheel-running activity in Djungarian hamsters (Scherbarth et al. 2007, 2008). That provides evidence that the perception of photoperiod is not impaired by exercise, as it was shown for Syrian hamsters (Menet et al. 2005).

Mechanisms for short-term and long-term energy balance regulation in the hypothalamus

The arcuate nucleus (ARC) in the hypothalamus is an important brain centre that maintains energy homeostasis by integrating peripheral metabolic and nutritional signals (for review, see Kalra et al. 1999), such as leptin and insulin (see *Peripheral nutritional hormones*). The ARC resides at the base of the hypothalamus on either side of the CSF-filled 3rd ventricle and close to the hypothalamo-hypophyseal portal system that links the hypothalamus and the anterior pituitary with the brain. The anterior pituitary receives signal molecules through the blood of the portal capillaries and is involved in the control of other endocrine glands. Facilitating the linking role with the hypothalamus, the median eminence (ME) at the base of the 3rd ventricle does not form a blood brain barrier. However, a barrier (through tight junctions) (Mullier et al. 2010) as well as a link (through transcytosis) between CSF, brain and portal blood supply to the pituitary gland is formed by tanycytes (for review, see Rodriguez et al. 2005). Tanycytes are elongated bipolar glial cells, which are located in the basolateral walls of the ependymal layer of the 3rd ventricle. Their cell bodies are part of the ependymal layer and the processes proceed through the ME to the portal capillaries (Figure 1). Through this design, the ME bathes in the 3rd ventricular and subarachnoidal CSF and is accessible by circulating factors. However, substances in the portal capillary spaces are not able to enter the 3rd ventricular CSF or the intercellular space of the ARC. But the other way around, neurohormones of the ARC can reach the portal capillaries by axonal transport through tanycytes.

The homeostatic system in the ARC that regulates short-term energy balance by affecting

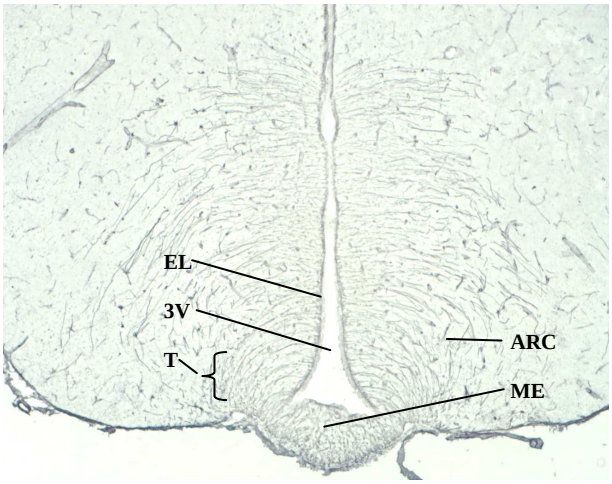

Figure 1: Detail of a coronal brain section (*Phodopus sungorus*) in the region of the arcuate nucleus (ARC), stained via immunohistochemistry for vimentin protein (magnification: x 50). EL: ependymal layer of the 3rd ventricle, 3V: ventral 3rd ventricle, T: tanycytes, ARC: area of the arcuate nucleus, ME: median eminence

food intake and energy expenditure involves two antagonistically acting populations of neurons (for review, see Sainsbury and Zhang 2010) (Figure 2). Neurohormones that are expressed and secreted from these neurons can modulate the activity or response of target neurons in other brain nuclei. During food deprivation, the expression of orexigenic neuropeptides such as neuropeptide Y (NPY) and agouti-related protein (AGRP) increases, thus stimulating food intake and reducing energy expenditure to compensate for the energy deficit. Simultaneously, the expression and secretion of satiety-inducing anorexigenic neuropeptides [proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART)] is inhibited. POMC is the precursor of α-melanocyte stimulating hormone (α-MSH) that mediates its anorexigenic effects by binding to the melanocortin-4 (MC4) receptor in the PVN. The PVN is located on either side of the roof of the 3rd ventricle and several neurons originating in the ARC project to this area. In addition to MC4 receptors, receptors for NPY (Y receptors) are found to be accumulated in this brain region (for review, see Sainsbury and Zhang 2010).

The main mechanism to regulate energy expenditure involves the hypothalamo-pituitary-thyroid gland axis. An increase in NPY or AGRP, in response to an energy deficit, inhibits the expression of thyrotropin-releasing hormone (TRH) in the PVN and thus reduces the activity of this axis. Hence, the secretion of thyroid prohormone (T_4; thyroxine) and active thyroid hormone (T_3; triiodothyronine) from the thyroid gland into the blood is limited, leading to a reduced metabolic rate. Thus, the PVN as well as the ARC, seem to be involved in the hypothalamic control of appetite and energy expenditure.

However, in Djungarian hamsters the expression of the homeostatic genes (*Npy*, *Agrp*, *Pomc*, *Cart*) does not show seasonal cycles (Mercer et al. 2000, Reddy et al. 1999). In addition, lesions of the ARC do not disrupt seasonal changes in pelage colour, reproduction, food

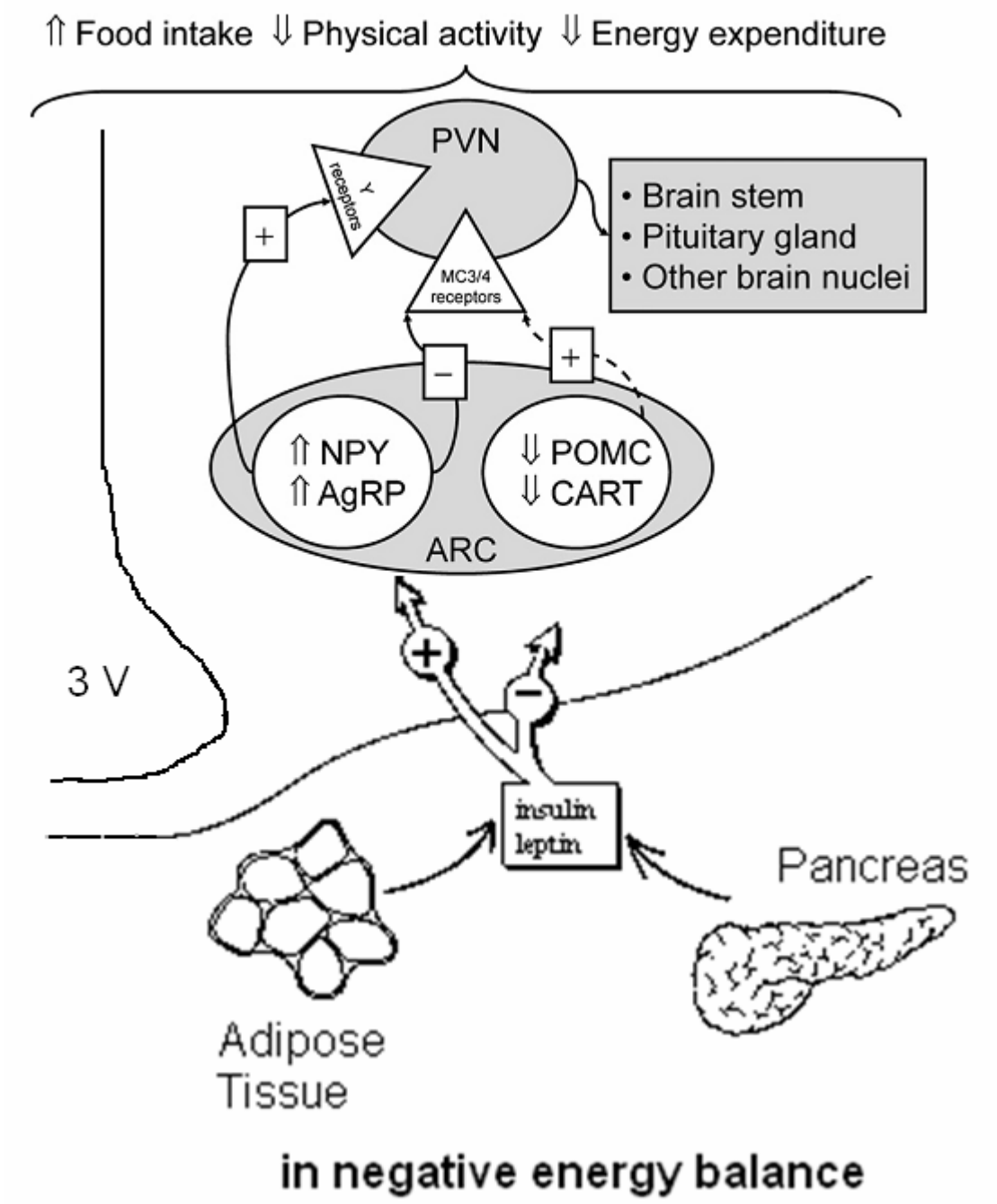

Figure 2: Insulin and leptin are secreted into the bloodstream and interact with target neurons in the ARC. In negative energy balance, insulin and leptin activate the orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) expressing neurons, whereas the anorexigenic POMC and CART expressing neurons are inhibited. These neurons project to the paraventricular nucleus (PVN). Through agonistic (NPY) and antagonistic (AgRP) receptor binding of secreted orexigenic peptides, anabolic mechanisms for the maintenance of energy homeostasis are initiated, involving the brain stem, the pituitary gland and other brain nuclei (modified after Niswender and Schwartz 2003 and Sainsbury and Zhang 2010).

intake and body weight (Ebling et al. 1998). These findings indicate that two different mechanisms are involved to differentiate between short-term (food availability) and long-term (seasonal) effects on body mass. At present, only POMC is considered to participate in both mechanisms as MC4 receptors were found to be photoperiodically regulated in the brainstem, which is suggested to be involved in the long-term regulation of body mass (Helwig et al. 2009).

To identify the mechanisms underlying the seasonal regulation of body weight, several studies pursued the identification of photoperiodically regulated genes in the brain of Djungarian hamsters (Barrett et al. 2005, 2006, 2007, 2009, Herwig et al. 2009, Nilaweera et al. 2009, Ross et al. 2004, 2005). A recently re-identified subregion of the ARC is the dorsal medial posterior arcuate nucleus (dmpARC), which was initially described as the dorsal tuberomamillary nucleus (Barrett et al. 2005). Quantification of gene expression in the dmpARC showed a photoperiodic regulation of histamine 3 receptor (*H3R*), the retinoid-binding proteins cellular retinol binding protein 1 (*Crbp-1*) and cellular retinoic acid binding protein 2 (*Crabp-2*), as well as the nuclear retinoic acid receptors retinoid X receptor γ (*Rxrγ*) and retinoic acid receptor (*Rar*) (Barrett et al. 2005, 2009, Ross et al. 2004, 2005).

Histamine and H3R are suggested to be involved in the regulation of food intake and body weight since histaminergic neurons project to, and H3Rs are expressed in, the ARC (Inagaki et al. 1988, Pillot et al. 2002). Studies that dealt with the role of the histaminergic system in Djungarian hamsters showed a SD-induced downregulation of *H3R* gene expression in the dmpARC (Barrett et al. 2005). Presumably, this leads to a reduced secretion of inhibitory neurotransmitters such as γ-aminobutyric acid (GABA), thereby activating dmpARC neurons. These data are in agreement with a study of Nilaweera and coworkers (2009), who found an increase in secretory and intracellular signalling pathways in the dmpARC of Djungarian hamsters in SD compared to LD. They quantified the gene expression of secretogranin III (*SgIII*) and *SgVI*, which both are translated into proteins contributing to the formation of secretory granules, as well as melanocortin-3 receptors (*Mc3-R*) and serotonin receptors 2A (*5-HT-2A*) and *5-HT-7*, which are involved in the signalling pathway of the dmpARC. Furthermore, neurons of the dmpARC are involved in the innervation and regulation of the sympathetic nervous system input to white adipose tissue (Bamshad et al. 1998). Thus, an activation within the dmpARC in SD might contribute to the seasonal loss in fat mass. Additional four genes that are components of the retinoic acid signalling pathway were found to be photoperiodically regulated in the dmpARC. They include genes encoding the retinoid binding and transport proteins CRBP-1 and CRABP-2 and the retinoic acid receptors RXRγ

and RAR (Ross et al. 2004, 2005). *Crbp-1* was found to be photoperiodically expressed in the ependymal layer of the 3rd ventricle as well (Barrett et al. 2006). Although several functions of retinoic acid are known, its targets and the precise involvement in regulating seasonal adaptations in Djungarian hamsters remain to be clarified.

Gene expressions of *Vgf* (nonacronymic) and G-protein-coupled receptor 50 (*Gpr50*), as well as gene expression in tanycytes and both the thyroid and growth axis are also regulated seasonally in the hypothalamus of Djungarian hamsters. The role of these genes in seasonality and the hypothalamic energy balance circuitry is explained in detail in the introduction of the according chapters (3 and 5).

Peripheral nutritional hormones

Peripheral hormones are assumed to have an impact on energy homeostasis by stimulating or inhibiting the activity of orexigenic and anorexigenic peptide-secreting neurones. To date, only leptin and insulin have been identified as afferent adiposity signals (for review, see Niswender and Schwartz 2003). These hormones circulate in the blood stream, enter the hypothalamus and act on the energy balance system via the regulation of food intake. Thus, they present a negative-feedback signal from body fuel stores in the periphery to the hypothalamic ARC (Figure 2).

Leptin is a well described hormone that is expressed and secreted by adipocytes, whereby its concentration in the blood changes in relation to body fat stores. Leptin receptors were found in the ARC, signalling through the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. The expression of target genes is regulated by translocation of activated STAT3 to the cell nucleus. Through leptin signalling, mRNA expression levels of anorexigenic *Pomc* and *Cart*, that mediate reduced appetite and increased energy expenditure, are up-regulated. However, exogenous leptin turned out not to be an effective therapy against obesity, as neuronal leptin resistance is common among obese individuals. They do not show reduced food consumption and increased energy expenditure despite high levels of plasma leptin. Investigations in Djungarian hamsters revealed results that are in line with these findings. Authors of previous studies reported that leptin levels changed in parallel to the seasonal cycle of body weight (fat). A decreased leptin gene expression in white adipose tissue and low serum leptin concentrations were found in hamsters in winter or under artificial SD conditions, in contrast to high leptin levels in summer or LD (Klingenspor et al. 1996,

2000). Gene expression of the leptin receptor (*Ob-Rb*) in the ARC was also down-regulated in SD compared to LD (Mercer et al. 2000). Hence, the body mass of Djungarian hamsters increases in the course from SD to LD despite increasing plasma leptin concentrations and up-regulated leptin receptor expression in the ARC. Furthermore, leptin injections in SD decreased body fat mass to a larger amount compared to exogenous leptin administration in LD (Klingenspor et al. 2000). Therefore, leptin resistance in 'obese' LD hamsters was strongly suggested. The cause of leptin resistance in LD is still unclear but might involve increased signalling of the suppressor of cytokine signalling 3 (SOCS3). *Socs3* is a gene, whose expression is regulated through the JAK/STAT pathway distal from the leptin receptor and SOCS3 inhibits leptin receptor signalling. Quantification of photoperiodically regulated *Socs3* mRNA expression in the ARC of Djungarian hamsters (in advance of changes in body mass) revealed an up-regulated gene expression in LD compared to SD (Tups et al. 2004, 2006a). This indicated inhibited leptin signalling in LD, probably causing leptin resistance.

Plasma leptin levels were also measured in reference to wheel-running activity in Djungarian hamsters (Scherbarth et al. 2007). The study revealed similar leptin levels in hamsters with and without access to a RW in December, despite the significant difference in body mass. Hence, similar amounts of fat mass in both groups were suggested. In December, when control hamsters reached their body mass nadir, leptin treatment via implanted minipumps caused a decrease in body mass only in the RW group. Whether this was due to an already increased SOCS3 level in the control hamsters, which might cause leptin resistance, or due to the fact that these animals already reached their body mass nadir, remains open.

In the present study we focussed on the measurement of plasma insulin concentration in Djungarian hamsters (see chapter 4) as up to now, studies analysing the involvement of insulin signalling in seasonal body weight regulation of the Djungarian hamster are scarce. In one study that sought to investigate this very issue, the authors caused diabetes in Djungarian hamsters via injection of streptozotocin (a pancreatic β cell toxin) (Bartness et al. 1991). As this treatment revealed a highly adverse effect on the animals' state of health, even up to death and, additionally, subsequent essential insulin replacement therapy was carried out, the results have to be regarded cautiously.

Insulin is secreted into the blood from pancreatic β cells and through the blood brain barrier it enters the ARC where insulin receptors are present. However, little is known about insulin signalling pathways in the hypothalamus. Contrasting to the assumed catabolic properties of the insulin pathway, Tups and colleagues (2006b) found insulin receptor mRNA expression to be down-regulated in SD. Likewise, another study revealed that plasma insulin concentrations

were low in SD compared to LD in Campbelli hamsters (*Phodopus campbelli*) (Mercer et al. 1995), which are closely related to the Djungarian hamster. Conceivably, increased anorexigenic leptin signalling due to an increased leptin sensitivity in SD may down-regulate insulin signalling to prevent catabolic overdrive. However, the implicated mechanisms of a cross-talk between insulin and leptin receptor signalling still need to be elucidated.

Aims and scope of the present study

In chapter 2 we challenged Djungarian hamster breeding pairs and singly kept females energetically with voluntary wheel-running activity and concurrent reproduction. Thus, we were able to further investigate potential impacts on the hamsters' high motivation to run.
In another study (chapter 3), we investigated exercise-induced changes in hypothalamic gene expression of hamsters kept in SD and LD. Thereby, we clarified whether mechanisms at the level of the hypothalamus might be involved in the weight gain of individuals with access to a RW.
Furthermore, we analysed signalling pathways in skeletal muscle of exercising Djungarian hamsters as we hypothesized that muscle-derived peripheral signals might feed back to the hypothalamus and thus might have an impact on the regulation of energy homeostasis and body weight (chapter 4).
In the last chapter (5) we verified the involvement of photoperiodically expressed genes in the seasonal body weight cycle of Djungarian hamsters. Therefore, we performed an experiment in the course of one year in Hannover (52°N), under natural T_a and natural photoperiod with its gradual transitions from summer to winter and vice versa.

CHAPTER 2

Voluntary exercise at the expense of reproductive success in Djungarian hamsters (*Phodopus sungorus*)

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Abstract

Energy demands of gestation and lactation represent a severe challenge for small mammals. Therefore, additional energetic burdens may compromise successful breeding. In small rodents, food restriction, cold exposure (also in combination) and wheel running to obtain food have been shown to diminish reproductive outcome. Although exhibited responses such as lower incidence of pregnancy, extended lactation periods and maternal infanticide were species-dependent, their common function is to adjust energetic costs to the metabolic state reflecting the trade-off between maternal investment and self-maintenance. In the present study, we sought to examine whether voluntary exercise affects reproduction in Djungarian hamsters (*Phodopus sungorus*), which are known for their high motivation to run in a wheel. Voluntary exercise resulted in two different effects on reproduction; in addition to increased infanticide and cannibalism, which was evident across all experiments, the results of one experiment provided evidence that free access to a running wheel may prevent successful pregnancy. It seems likely that the impact of voluntary wheel running on reproduction was associated with a reduction of internal energy resources evoked by extensive exercise. Since the hamsters were neither food-restricted nor forced to run in the present study, an energetic deficit as reason for infanticide in exercising dams would emphasise the particularly high motivation to run in a wheel.

Keywords: reproduction, pup mortality, cannibalism, wheel-running activity

Introduction

Mammalian reproduction is associated with high energetic costs, representing a severe challenge especially for small species. In fact, lactation is the most energy-demanding time for female small mammals (Bronson 1985; Speakman 2008). In animals that exhibit postpartum estrous such as mice (*Mus musculus*), rats (*Rattus norvegicus*) and Djungarian hamsters (*Phodopus sungorus*; also known as Siberian hamster), lactation is even likely to coincide with gestation. Different strategies of feeding and allocation of energy resources have evolved to cope with elevated energy demands during reproduction. On the one hand, physiological adaptations are important that counteract and, thereby, attenuate the rise in energy expenditure during reproduction. For example, a decrease in brown adipose tissue

(BAT) activity saves energy by reducing heat production, which is known for several rodent species (Frontera et al. 2005; Martin et al. 1989; Schneider and Wade 1987; Trayhurn 1983; Wade et al. 1986). On the other hand, increased calorie intake is essential to avoid severe energy deficits during reproduction.

In Djungarian hamsters, food consumption rises considerably during lactation but only slightly increases during pregnancy (Bartness 1997; Schneider and Wade 1987; Weiner 1987). As a consequence, females exhibit a striking loss of body fat (~50%) before lactation (Schneider and Wade 1987), which is comparable to findings in Syrian hamsters (*Mesocricetus auratus*; Bhatia and Wade 1993; Wade et al. 1986). Hence, instead of increasing food intake appropriately to prevent depletion of fat stores, pregnant hamsters tolerate the negative energy balance. This counterintuitive strategy has been related to the hamsters' specific ingestive behaviour; the fact that food hoarding is increased in pregnant and lactating Djungarian hamsters suggests that external energy resources might be involved in energy balance regulation (Bartness 1997; Keen-Rhinehart et al. 2010). Thus, energy reserves might be considered as being composed not only of body fat but also of hoarded food, which is easily available.

In several rodent species, it has been demonstrated that an additional energetic burden such as food restriction, cold exposure and increased locomotor activity during pregnancy and/or lactation may compromise successful reproduction (Bronson and Marsteller 1985; Johnson and Speakman 2001; Labov et al. 1986; Marsteller and Lynch 1983, 1987a,b; McClure 1981). Interestingly, responses of reproductive females to these additional energetic challenges vary between rodent species. For example, when house mice (*Mus musculus*) were forced to run more than a certain number of wheel turns to receive a food pellet during lactation, they routinely cannibalised young (Perrigo 1987). In contrast, in the same study, deer mice (*Peromyscus maniculatus*) did not eliminate pups to support their own energy balance, but instead increased wheel running and thus feeding effort. The percentage of pregnant females, however, considerably decreased with increasing numbers of revolutions required to obtain food, indicating an 'all-or-nothing' response in deer mice. In Djungarian hamsters, cold exposure impaired both weight gain and survival of pups (Paul et al. 2010) although dams increased food intake at low ambient temperature (T_a) compared to moderate conditions.

With regard to increased locomotor activity, both food intake and oxygen uptake also increased in non-reproductive Djungarian hamsters (Bartness and Wade 1985, Scherbarth et al. 2008). Moreover, we could show that running exercise affected the hamsters' body composition (Scherbarth et al. 2007). The findings indicated a growth-promoting effect

(increased lean mass) associated with a reduction in relative fat mass. This anabolic effect of voluntary wheel running together with the rise in energy expenditure is likely to be an energetic challenge for pregnant and lactating females. For that reason, the hamsters in our experiments were not forced to run for food. Instead, we sought to examine whether voluntary wheel running affects reproduction. Indeed, in an earlier study, no differences in reproductive outcome were found between singly kept female hamsters either with or without access to a running wheel (RW) (Scribner and Wynne-Edwards, 1994a). However, females obtained RW access only one week before parturition, and they gave birth to merely one litter, i.e. without the burden of lactation and concurrent gestation. In the present study, therefore, we allowed free RW access from the time of mating until hamsters had produced several litters.

Methods

Animals

Hamsters (*Phodopus sungorus*) were reared either outdoors under a natural photoperiod (NP; Hannover, ~52°N latitude) with natural T_a (*experiment 1*) or in a temperature-controlled chamber (23 ± 1°C) with an artificial light-dark cycle of 16 h of light and 8 h of darkness (LD 16:8; *experiment 2* and *3*). Until the beginning of the experiments, hamsters were kept singly in polycarbonate cages (20.7 x 14 x 26.5 cm) and supplied with breeding diet (Altromin 7014) and tap water *ad libitum*, supplemented by a slice of apple once a week. During experimental procedures, breeding pairs and solitary dams were housed in bigger cages (26.5 x 18 x 42 cm) and received oat flakes, sunflower seeds and curd cheese (20% fat in dry matter) twice a week in addition to the common food (pellets and apple).

Experimental design

Initially, reproduction was compared between two parallel groups (RW vs. sedentary) of breeding pairs kept under natural lighting and temperature conditions. In the second experiment, we sought to examine whether running exercise has a lasting effect on reproduction and therefore chose a crossover trial. Because of the long duration, we had to carry out the experiment indoors using artificial lighting. Finally, in the third experiment, females were kept without male after mating. This enabled us to determine the females' amount of exercise.

Experiment 1

From 17 March until 9 June (2006), twelve virgin females (10-12 months old) and twelve males (6-12 months old) were kept outdoors in pairs and were provided with a wooden nest box and tissue for nesting material. T_a (mean: 12.2°C; range: -2 to 28°C) was measured at intervals of one hour with a temperature logger (DS1921L, iButton, Maxim Integrated Products, Inc., Sunnyvale, CA). For the duration of the experiment (12 weeks) six breeding pairs had free access to a running wheel (RW; ~14.5 cm in diameter). Commercial metal wheels were improved by a continuous running tread to avoid leg injuries. Wheel revolutions were registered continuously with a reed contact on the wire lid and a magnet attached to the wheel. Data were stored at 6-min intervals on a personal computer. To determine the individual contribution of female and male hamsters to the registered number of wheel turns, two breeding pairs were monitored with an infrared video camera for one night. Recordings were analysed by visual inspection (PC software Noldus) for the hamsters' stay in the RW. For each interval (3 min), it was determined whether the female or male hamster, or both, used the RW. All hamsters were weighed once a week. For determination of reproductive success all pups were considered that were born within the twelve weeks and successfully weaned. Breeding pairs with pups younger than 21 days of age at the end of the twelve weeks were kept under the experimental conditions until weaning.

Experiment 2

Animals were kept in an artificial light-dark cycle (LD) of 16 hours of light and 8 hours of darkness (LD 16:8) at 22 ± 2°C. Virgin females (3 months old) and males (3-6 months old) were paired and provided with tissue for building nests and a RW (~14.5 cm in diameter) that either was locked (2a) or released (2b) (N = 5, each) for the first twelve weeks. Subsequently, for the second twelve weeks, locked RWs were released and vice versa (Fig. 2). Since wheel revolutions were not registered, breeding pairs were repeatedly monitored with an infrared video camera during the dark phase to verify their use of the RW. Adult hamsters were weighed twice a week to achieve body weight courses with higher resolution compared to experiment 1. Overall litter weights (including all pups of each litter) were determined each day until weaning (21st day). In case of new offspring, older siblings were removed from the cage. Two of five breeding pairs in experiment 2b did not reproduce at all. Staining of the uteri (according to Kopf et al. 1964) revealed that implantation scars were lacking. A low percentage of motile sperm found in the epididymis of both males indicated infertility.

Therefore, these breeding pairs were excluded from the results (N = 3). In experiment 2a, all litters that were born during the first twelve weeks were considered for calculations of mean litter size and litter frequency. However, only pups that were also successfully weaned within this period were included into calculation of weaned litter size. This applies also to the second section (RW locked) of experiment 2b.

Experiment 3

Female hamsters were kept in LD 16:8 at 20 ± 1°C. Before the beginning of the experiment, 13 females were housed each with a male for five days (covering an estrous cycle), and were separated subsequently into two experimental groups. Pregnant (P) hamsters of one group had free access to a RW (P_{RW}; N = 6) unlike the sedentary pregnant controls (P_{SED}; N = 7). Females were 14 months old and had already produced four to six litters. Female hamsters with proven fertility were used to make sure that they will give birth to another litter during the experiment. A third group of females (unmated; 4-5 months old; N = 6) served as control group for wheel-running behaviour (C_{RW}). Wheel revolutions were measured continuously as described in experiment 1. Body mass of the females was determined each day. For later analysis, data were aligned to the day of parturition.

Statistics

Results are given as mean values and SEM. Differences were considered significant if $P < 0.05$. For comparison of two or three unpaired samples, t-test or one-way ANOVA were used, respectively. Paired samples were compared by paired t-test or repeated measures ANOVA. Statistical procedures were carried out using Statistica 6 (StatSoft, Tulsa, OK).

Results

Experiment 1

Within the twelve weeks of the experiment (outdoors), all control breeding pairs (C; N = 6), i.e. without RW, produced offspring 2-3 times (Table 1). In the RW group, two female hamsters had no offspring at all and were excluded from analyses. Remaining females (RW; N = 4) had 1-4 litters. However, three litters (sizes not known) were cannibalised within two days after birth in the RW group. Cannibalised litters were the first and first two litters of breeding pairs. Subsequently, both pairs bred successfully.

Table 1

Different reproductive parameters for exercising and sedentary hamsters compared within the respective experiment.

			born		weaned	
Exp.	RW access	*N*	litter size	litters/female	litter size	pups/pair
1	no	6	–[§]	2.5 ± 0.2	5.9 ± 0.5	14.3 ± 1.0*****
	yes	4	–[§]	2.3 ± 0.6	6.0 ± 0	9.0 ± 1.7
2a	no	5	5.4 ± 0.6	3.2 ± 0.4******	3.6 ± 0.5******	7.4 ± 1.3******
	yes		4.0 ± 1.0	0.6 ± 0.2	0	0
2b	yes	3	3.0	0.3 ± 0.3	3.0	0.3 ± 0.3
	no		6.1 ± 0.6	2.3 ± 0.3	3.3 ± 1.2	4.7 ± 2.0
3	no	7	5.0 ± 0.6	1[#]	4.3 ± 0.5	–[+]
	yes	6	4.8 ± 0.9	1[#]	2.2 ± 1.1	–[+]

Results are given as mean ± SE; * significantly different to RW breeding pairs ($P < 0.05$; t-test); ** significantly different to experimental phase with released RW ($P < 0.01$; paired t-test); [§] litter sizes were not always determined at the day of parturition; [#] experiment was limited to one litter; [+] number of weaned pups/pair is equivalent to weaned litter size

The number of weaned pups per breeding pair was significantly reduced in the RW group compared to controls (Table 1). In contrast, weaned litter size (without cannibalised litters; see above) was similar in both groups. Litter sizes ranged from 2-9 (C) and 2-10 pups (RW). The small number of two pups occurred only once per group, and it was the first litter of each female.

Male RW hamsters significantly increased their body mass (39.6 ± 1.3 g vs. 47.6 ± 1.0 g;

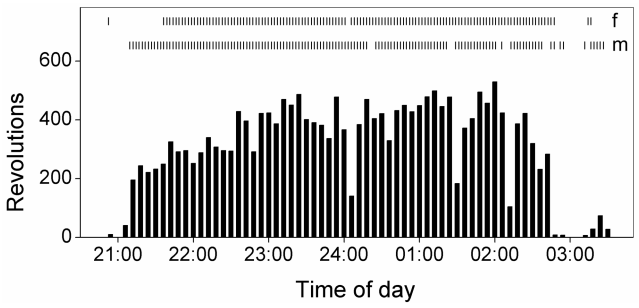

Figure 1: Wheel-running activity (6-min intervals) of a breeding pair kept outdoors under natural lighting conditions. One night after about 10 weeks of free RW access is depicted. The upper part of the figure shows the intervals (3 min) when the female (f) and/or male (m) used the RW. Both the total number of revolutions (20,594) and the pattern of running exercise (restricted to the nighttime) is representative. Sunset and sunrise were at 20:30 and 04:05 h, respectively.

paired t-test; $P = 0.01$) unlike male sedentary controls (36.6 ± 1.5 vs. 38.9 ± 2.1 g; $P = 0.17$). In contrast to female controls, body weight courses of RW females showed a tendency of increasing body weights over the time of the experiment, too. However, the females' body weight at the end of the experiment was variably affected by the individual stage of reproduction, which prevented a meaningful calculation.

Video analysis revealed that both male and female hamsters of the two monitored breeding pairs extensively used the RW (Fig. 1). As expected, wheel-running activity in general was almost completely restricted to the night, i.e. from sunset to sunrise (activity records not shown). On average each breeding pair ($N = 4$) produced 33,272 ± 4,092 revolutions per day.

Experiment 2

2a)

During the first twelve weeks of the experiment (RW locked) females gave birth to offspring 2-4 times (Table 1; Fig. 2). A total of 83 pups were born (range of litter size: 1-8), however,

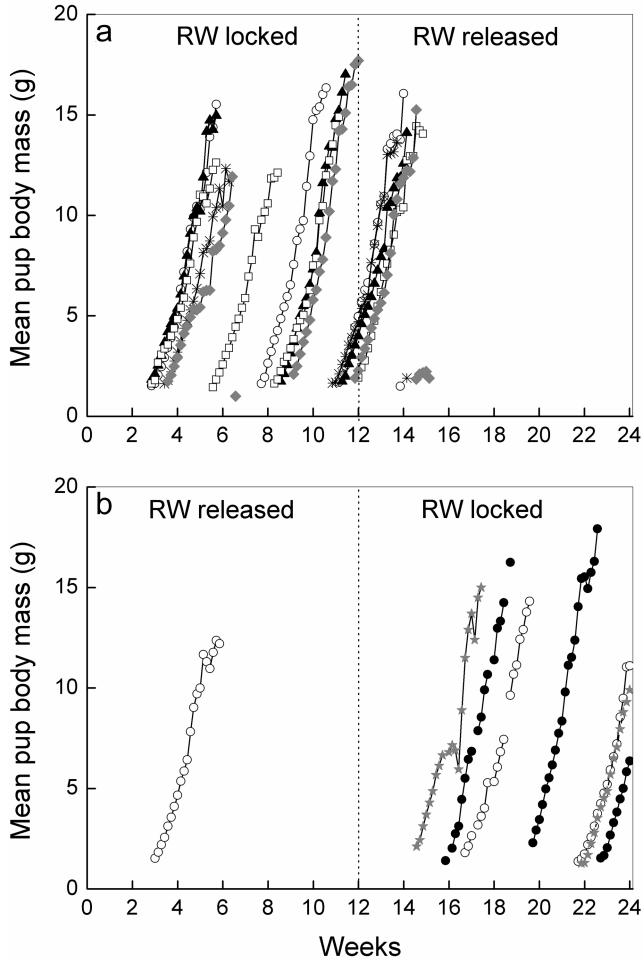

Fig. 2 Mean pup body mass until weaning (or death) and litter frequency of breeding pairs in experiment 2a (a; $N = 5$) and 2b (b; $N = 3$). Different symbols represent litters of different breeding pairs

only 52 pups were born early enough to be weaned before experimental conditions changed (RW locked → RW released). Thirty-seven (71.2%) of these 52 pups survived until weaning (21st day). Only one pup was cannibalised out of the 31 pups that were born within the first 12 weeks but were weaned during the following experimental section (RW released).

While RWs were released, only three out of five breeding pairs produced offspring (a total of 12 pups; range of litter size: 3-6) but none of them survived. Conceptions for the first two litters occurred during the first twelve weeks when RWs were still locked. Mean litter sizes did not differ significantly between both experimental conditions.

2b)

Only one litter was born and weaned while RWs were released (Table 1, Fig. 2). During the second period (locked RWs), 43 pups were born (range of litter size: 4-8). Females gave birth to offspring 2-3 times. In accordance with experiment 2a, only pups that were born early enough to be weaned within the twelve weeks (24) were included into calculation of weaned litter size. Actually, fourteen of these 24 pups were weaned (58.3%).

Males and females of all breeding pairs in experiment 2 showed an increase in body weight after RWs had been released (Fig. 3).

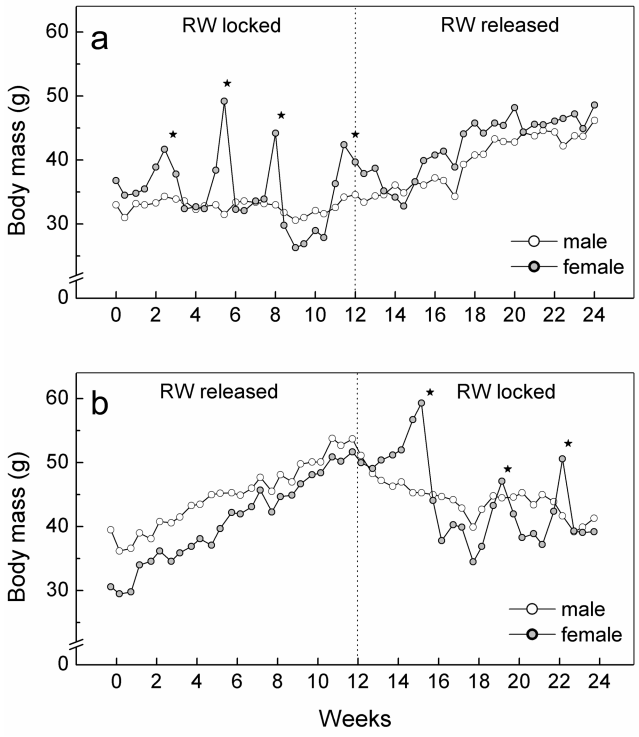

Fig. 3 Body weight development of a breeding pair in experiment 2a (a) and 2b (b). Both females and males exhibited the expected exercising-induced weight gain. Asterisks indicate time of parturitions

Experiment 3

Litter sizes of P_{SED} and P_{RW} females were similar (Table 1) with ranges of 2-7 (P_{SED}) and 1-7 pups (P_{RW}). The difference in weaned litter size did not reach significance. However, the percentage of offspring that survived until weaning was significantly reduced in P_{RW} compared to P_{SED} (36.2 ± 17.3% vs. 87.8 ± 6.3%; P = 0.013; t-test). This was due to three females in the P_{RW} group that killed all their pups. Accordingly, these litters were not considered for calculation of weaned litter size. Activity records of these females showed only a slight decrease in wheel running at the day of parturition (Fig. 4b). In comparison, dams that weaned at least some of their pups, exhibited a striking decline in wheel-running activity at the day of parturition (about –80%) and recovered to prepartum levels about two weeks later (Fig. 4c). Analysis of wheel running during the last 8 days of pregnancy (day of parturition excluded) revealed no difference in the mean number of revolutions/day between females that killed all pups and females that weaned at least some of their pups (30,448 ± 2,344 vs. 23,665 ± 3,938). Similarly, wheel-running activity of unmated controls (25,983 ± 566; mean for the whole experiment) did not significantly differ from both other groups during pregnancy.

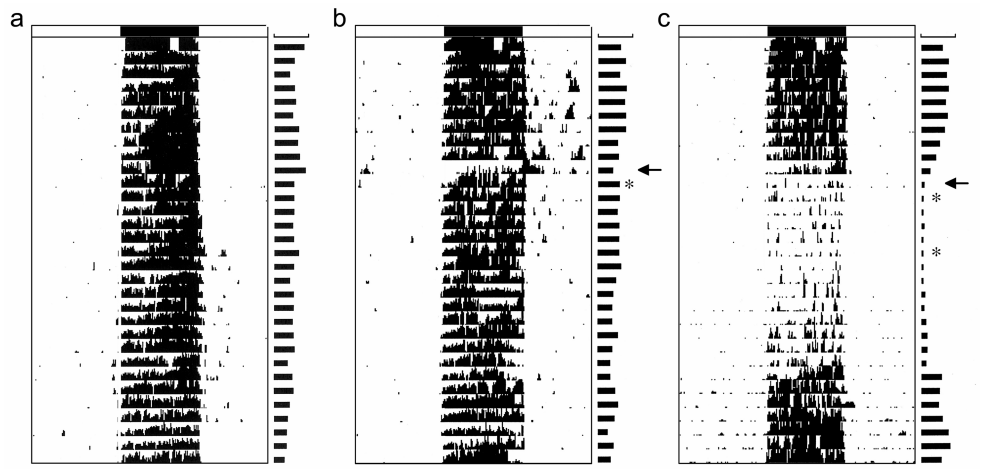

Fig. 4 Wheel-running activity of (a) a non-reproductive female, (b) a dam that cannibalised all young within 24 hours, and (c) a dam that weaned 4 out of 7 pups. Consecutive days (24 h) are depicted one below the other. The black bar at the top represents the dark phase. Days of parturition are indicated by an arrow. Occurrence of cannibalism is shown by asterisks

Discussion

The present findings show a remarkable effect of free RW access on reproduction in Djungarian hamsters. In both breeding pairs and singly kept dams with RW access, reproductive outcome was reduced irrespective of lighting conditions (natural or artificial long photoperiods) and T_a (natural or constant). Litter sizes at birth were not affected compared to sedentary controls, but exercising hamsters exhibited a clear tendency towards infanticide. Furthermore, the present results strongly indicate that free access to a RW may prevent reproduction.

It is important to note that the incidence of infanticide was not entirely restricted to RW hamsters. Cannibalism of single pups occurred also in breeding pairs without access to a RW, even though much more scarcely compared to exercising animals. Moreover, only RW hamsters cannibalised entire litters (exp. 1, 2a and 3). A similar behaviour has been observed in house mice that were forced to run for food (Perrigo 1987); whether female mice cannibalised entire litters soon after parturition or only reduced the number of pups was dependent on energetic demands of increased 'foraging' (rev./pellet). In the third experiment of this study, females that killed the whole litter after parturition did not run significantly more during the last half of pregnancy compared to females that weaned at least some of their offspring. This might have been due to individually different susceptibility to wheel running-induced effects as already indicated by previous studies (Scherbarth et al. 2007, 2008).

These previous examinations revealed that running exercise induces weight gain (lean mass) and skeletal growth. In accordance with the augmentation of lean mass, elevated body weight of exercising hamsters was not associated with an increase in the plasma leptin concentration (Scherbarth et al. 2007), which is known to be positively correlated with the amount of fat stores (Klingenspor et al. 2000). Thus, in the present study, the exercise-induced anabolic effect together with a decrease in the proportion of body fat might have contributed to unfavourable energetic preconditions for successful breeding. Furthermore, the adipose-derived hormone leptin represents an important metabolic signal to the reproductive system. Since leptin stimulates hypothalamic secretion of gonadotropin-releasing hormone (GnRH), a decrease in circulating leptin and thereby GnRH secretion might have inhibited reproductive function (for review, see Popovic and Casanueva 2002). Speculation about leptin being involved in observed effects of wheel running on reproduction is strongly supported by recent observations in Djungarian hamsters (French et al. 2009), these providing convincing evidence that maternal investment is closely connected with the concentration of circulating

leptin. Leptin treatment in pregnant hamsters resulted in larger litters and suppressed maternal infanticide compared to vehicle-treated pregnant controls. Thus, leptin appears to be a crucial factor for energy allocation in favour of reproduction. Future examinations, therefore, should include measurement of blood leptin concentrations.

Surprisingly, RW pairs in experiment 1 (natural photoperiod) gave birth to and weaned more pups compared to breeding pairs with a released RW in experiment 2 (artificial long photoperiod). This might be explained by additional stress, since offspring and adult hamsters were weighed much more frequently in experiment 2. Furthermore, only hamsters kept outdoors had access to a wooden nest box, which is likely to be a stress-reducing factor for burrow-dwelling hamsters.

Analysis of wheel-running activity in singly kept females (experiment 3) revealed that the number of rev./day was similar in unmated controls and mated animals during pregnancy. However, compared to a slight decrease in wheel running of females that cannibalised their pups, the dams that successfully weaned some offspring had shown a striking drop in the number of rev./day (by about 80%) at the day of parturition. Lactating dams gradually increased rev./day afterwards and returned to prepartum levels around day 14 (postpartum), when the young were not completely dependent on milk anymore. This described pattern of wheel-running activity is very well in accordance with observations in a previous study (Scribner and Wynne-Edwards 1994b), where female hamsters not only consistently exhibited the temporary reduction in wheel running, but also invariably weaned their offspring. That indicates that the reduction in voluntary exercise is of energetic relevance for successful breeding. However, the reason for the different maternal behaviour of three females in our study (cannibalism of the whole litter and high wheel-running activity) remains unclear.

Collectively, wheel-running behaviour considerably impaired reproductive outcome in Djungarian hamsters. Exercising animals, i.e., breeding pairs as well as solely kept dams, showed a clear tendency towards a higher incidence of cannibalism compared to sedentary controls. The findings suggest infanticide to be an energetic adjustment of the females, which has been demonstrated before in other rodent species. According to this, maternal responses to an additional energetic burden during reproduction in seasonal Djungarian hamsters appear to be comparable to those of house mice. However, the hamsters were neither food-restricted nor forced to run in the present study. Thus, an energetic deficit as reason for maternal infanticide in exercising females would highlight the pronounced motivation to run in a wheel. A high motivation, in turn, is well in accordance with the hypothesis that wheel-running activity is self-reinforcing and perceived by animals as ‘important’ (Sherwin 1998). Nevertheless, it is

still not known whether wheel running is merely an artefact of captive environments or of the RW itself, or whether it represents a natural behaviour, even though considerably enhanced.

Acknowledgments

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Ethical standards

All experiments were in accordance with the German Animal Welfare Act and approved by the district government of Lower Saxony (ref. no. 09/1739).

Conflict of interest

The authors declare that they have no conflict of interest.

CHAPTER 3

Voluntary exercise and photoresponsiveness at the hypothalamic level

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Abstract

The Djungarian hamster is a seasonal mammal that, driven by changing photoperiod, adapts physiologically to changing environmental conditions. Previous studies revealed that voluntary exercise in this species seems to interfere with the mechanisms that regulate the seasonal body weight cycle and energy expenditure. In short days (SD), access to a running wheel (RW) reverses the seasonally programmed decrease in body mass and hamsters gain weight. In this study, we investigated the influence of wheel-running activity on the expression of photoperiodically regulated genes in the hypothalamic arcuate nucleus (ARC), which is known to be involved in energy balance regulation. We could show that the expression of neither orexigenic [neuropeptide Y (*Npy*) and agouti-related protein (*Agrp*)] nor anorexigenic [proopiomelanocortin (*Pomc*) and cocaine- and amphetamine- regulated transcript (*Cart*)] genes seems to be involved in the body weight gain of exercising hamsters. However, there are some hints indicating that exercise in SD might stimulate the melanocortin pathway via POMC-derived α-melanocyte-stimulating hormone (α-MSH). Additionally, we found increased secretory processes in the dorsal medial posterior ARC (dmpARC) of exercising hamsters, indicated by *Vgf* (nonacronymic) gene expression. Furthermore, via quantification of somatotropin release-inhibiting factor (*Srif*) gene expression we provide evidence that exercise-induced growth contributes to the increase in body mass. However, quantification of photoperiodically regulated gene expression of G-protein-coupled receptor 50 (*Gpr50*) and genes of the thyroid system revealed that the central perception of photoperiod is likely to be unaffected by voluntary exercise. Thus, further mechanisms that are involved in the weight gain of SD-RW hamsters remain to be elucidated.

Introduction

In the course of a year, photoperiod changes gradually in latitudes above and below the equator; from long day lengths (LD) in summer to SD in winter. This robust environmental signal allows animals to anticipate the forthcoming season and to undergo seasonal acclimatizations to ensure survival.

In mammals, photoperiodic information is perceived by the retina that sends information via the retinohypothalamic tract to the suprachiasmatic nuclei (SCN). The rhythmic output of this circadian oscillator is conveyed to the hypothalamic paraventricular nuclei (PVN), which

transfer the information via a multisynaptic pathway and the sympathetic nervous system to the pineal gland (for review, see Bartness and Wade 1985b). Within the pineal gland, neural information is transformed into a hormonal signal. During the night, the hormone melatonin is synthesized and secreted into the blood and cerebrospinal fluid (CSF) as light inhibits hormone synthesis in pinealocytes. Consequently, the melatonin peak is positively correlated with the night length. The endocrine signal acts in the brain and in tissues where melatonin receptors are expressed and induces a cascade of processes leading to seasonal physiological alterations (for review, see Bartness et al. 2002).

A robust animal model to study seasonal physiology is the Djungarian hamster (*Phodopus sungorus*; also known as Siberian hamster) a native species to western Siberia and eastern Kazakhstan where large seasonal changes in food availability and temperature occur. This species undergoes a number of physiological adaptations to survive the harsh conditions of winter it experiences in its natural habitat. These include a moult to a whitish and well-insulating winter pelage (Heldmaier and Steinlechner 1981a, Kuhlmann et al. 2003), shallow daily torpor (a state of hypometabolism and hypothermia) (Heldmaier and Steinlechner 1981b), gonadal regression and a reduction of body mass over a 12 to 16 week period during the autumn-winter period (Hoffmann 1973, Steinlechner et al. 1983). These SD-induced responses result in decreased energy expenditure, reducing the requirement for food intake in winter when many food sources are restricted (Heldmaier et al. 1982, Heldmaier and Steinlechner 1981a, b, Knopper and Boily 2000).

The mechanisms underpinning appetite, energy metabolism and body weight regulation in seasonal mammals is not yet understood. However, food intake and energy balance mechanism are regulated centrally by the brain. A well described brain area, known to be involved in appetite and energy balance regulation is the hypothalamic arcuate nucleus (ARC), whose major function is the integration of peripheral nutritional signals in the homeostatic regulation of body weight. The principal neuropeptides involved in appetite and energy balance homeostasis are expressed and secreted by orexigenic (hunger inducing; NPY and AGRP) and anorexigenic (satiety inducing; POMC and CART) neurons. Changes in gene expression and balance between these neuropeptides is a key element involved in maintaining an appropriate body weight at which these peptides are viewed as components of a compensatory system (for review, see Morgan and Mercer 2001). Therefore, as a result of starvation the orexigenic peptides NPY and AGRP increase, whilst anorexigenic POMC decreases (Mercer et al. 1995, 2000).

Furthermore, several scientists have pursued the identification of photoperiodically regulated genes in the hypothalamus of Djungarian hamsters. Recently, a sub-region of the ARC with a photoperiodically differential gene expression of *Vgf* (nonacronymic) has been identified (dmpARC) (Barrett et al. 2005). VGF is assumed to be involved in the regulation of energy expenditure and reproduction and is activated in the dmpARC in SD (Hahm et al. 1999, 2002, Salton et al. 2000).

Another important regulatory circuit of body weight in the brain includes thyroid hormone (T_3/T_4) that is known to regulate seasonal energy expenditure in Djungarian hamsters (Barrett et al. 2007). The production of thyroid prohormone (T_4; thyroxine) in the thyroid gland is controlled by thyrotropin-releasing hormone (TRH) produced in the PVN. TRH neurons project to the median eminence (ME) wherefrom TRH reaches the anterior pituitary gland. Here, TRH induces the secretion of thyroid stimulating hormone (TSH), which, released into the blood, stimulates T_4 production in the thyroid gland. T_4 enters the brain across the blood-brain barrier and through the CSF and is transported in the ME and ARC via astrocytes and tanycytes lining the 3rd ventricle. In the latter glial cells, T_4 is activated by type 2 deiodinase (DIO2) which converts T_4 to active T_3. T_3 uptake into cells of the ependymal layer of the 3rd ventricle is facilitated by the monocarboxylate transporter 8 (MCT8) (Friesema et al. 2003, Heuer et al. 2005, Visser et al. 2008). Its gene expression is regulated photoperiodically and is increased in SD leading to an increased T_3 transport into the brain (Herwig et al. 2009). The actual level of active T_3 in the hypothalamus is regulated by the intracellularly localized enzyme type 3 deiodinase (DIO3) in the ependymal layer, which converts T_4 to inactive reverse T_3 (rT_3), or T_3 to T_2. Gene expression of *Dio3* is also regulated photoperiodically, showing an upregulation in SD and thus leading to an overall decrease in hypothalamic T_3 concentrations, which is important for initiation of the SD catabolic state (for review, see Herwig et al. 2008).

Another gene, whose expression is regulated photoperiodically in the ependymal layer of the 3rd ventricle in the Djungarian hamster is the orphan G-protein-coupled receptor (*Gpr50*). *Gpr50* mRNA expression is down-regulated in SD hamsters compared to LD (Barrett et al. 2006). It belongs to the melatonin receptor subfamily, although it does not bind melatonin (Reppert et al. 1996). This receptor is localised in tanycytes lining the 3rd ventricle of the hypothalamus, at the border between the ventricular CSF, portal blood system of the ME and the hypothalamic neuropil, suggesting a role in the regulation of the hypothalamo-pituitary axis (Drew et al. 2001, Sidibe et al. 2010).

Furthermore, growth is regulated by two important hormones that are both expressed in the ARC. The stimulatory growth hormone-releasing hormone (GHRH) and the inhibitory somatotropin release-inhibiting factor (SRIF) reciprocally regulate the release of growth hormone (GH) from the anterior pituitary (for review, see Müller et al. 1999). To date, only a few studies examined the influence of photoperiod on growth in juvenile Djungarian hamsters. Based on low body mass and fat mass, accompanied by reduced gonadal weight in SD compared to LD hamsters, the authors assumed that this species inhibits growth in winter (Adam et al. 2000, Ebling 1994).

However, the above described photoperiodically regulated and balanced circuits seem to be disturbed once Djungarian hamsters have free access to a running wheel (RW). They increase body weight independent of photoperiod and the seasonal cycle of body weight regulation is apparently lost. Furthermore, regression of the testes was shown to be impeded and torpor did not occur in voluntarily exercising Djungarian hamsters (Scherbarth et al. 2007, 2008).

The aim of the present study was to investigate at a molecular level, whether voluntary wheel-running activity modulates the perception of the photoperiod or whether it overrides mechanisms for the initiation of the SD catabolic state in Djungarian hamsters. Therefore, we examined the expression of several genes involved in short-term regulation of energy balance and long-term seasonal body weight adaptation in the hypothalamus of hamsters kept in LD and SD with and without access to a RW.

Materials and Methods

Animals and tissue collection

Djungarian hamsters were bred and raised under a natural photoperiod and natural ambient temperatures in Hannover, Germany (52°N latitude). Water and food (hamster breeding diet, Altromin 7014, Lage) were available *ad libitum*, supplemented weekly by a piece of apple before the start of the experiments. Three experiments were conducted consecutively on adult male hamsters that were divided into four weight-matched groups. The experiments lasted either eight or twelve weeks. Hamsters kept in LD photoperiod were exposed to a light-dark cycle of 16 h of light and 8 h of darkness and hamsters in SD photoperiod were exposed to a light-dark cycle of 8 h of light and 16 h of darkness. Dim red light (< 5 lx) was provided during the dark phase in both photoperiods. Irrespective of the photoperiod, animals were kept at 21 ± 1 °C. In each experiment, six hamsters received a RW (Ø 14.5 cm) and were kept in

LD (LD-RW group). Further six animals stayed in LD without a RW (LD-C). Twelve hamsters were transferred to SD. Six of them received a RW (SD-RW) and six represented the sedentary control group without a RW (SD-C). Voluntary wheel-running behaviour in both RW groups was monitored continuously and stored every 6 min on a PC. Body weight was registered twice a week in all experiments. Food intake was measured in the 12 weeks experiment by weighing the rack weekly for calculation of differences in the amount of food. Animals were culled with carbon dioxide at the end of the experiments, 3-4 h after lights went on. Brains were immediately dissected, frozen on dry ice and stored at -80 °C for later procedure of *in situ* hybridizations. Whole animal bodies (without brains) of the first 8 weeks experiment were stored at -80 °C and later, thawed hamster carcasses were individually scanned by magnetic resonance imaging (MRI) (Echo MRI ™, Whole Body Composition Analyser, Echo Medical Systems, Houston, Texas). Body composition data were obtained as grams fat or lean tissue.

Riboprobes

Riboprobes complementary to fragments of the required DNA sequences were generated from Djungarian hamster, mouse or rat brain cDNAs by RT-PCR as described previously [Adam et al. 2000 (*Cart*), Barrett et al. 2005 (*Vgf*), Barrett et al. 2007 (*Dio2, Dio3*), Drew et al. 2001 (*Gpr50*), Ebling et al. 2008 (*Trh*), Herwig et al. 2009 (*Mct8*), Mercer et al. 1995 (*Npy*), Mercer et al. 2000 (*Agrp, Pomc*), Ross et al. 2009 (*Srif*)]. Templates for riboprobe synthesis were generated by PCR amplification of the insert from plasmid DNA. M13 forward and reverse primers which spans both insert and polymerase transcription binding and initiation sites in the host vectors were used. One hundred µg of PCR product were used in an *in vitro* transcription reaction with T7, T3 or SP6 polymerases as appropriate in the presence of ^{35}S-uridine 5-triphosphate (Perkin-Elmer, Buckinghamshire, UK) for radioactive *in situ* hybridization.

In situ hybridization

Coronal sections of the hypothalamus (14 µm thick) were collected onto two sets of 12 glass slides for the ARC and PVN region, respectively. Adjacent sections were mounted on consecutively numbered slides, permitting a number of mRNAs to be localised and quantified in each brain.

In situ hybridization was carried out as described previously (Morgan et al. 1996).

Briefly, frozen slides were fixed in 4% PFA in 0.1 m PBS, acetylated in 0.25% acetic anhydride in 0.1 m TEA, pH 8. Radioactive probes (approximately 10^6 cpm) were applied to the slides in 70 µl hybridization buffer containing 0.3 M NaCl, 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.05% tRNA, 10 mM DTT, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA and 10% dextran sulfate. Hybridization was performed overnight at 58 °C. Following hybridization, slides were washed in 4 x SSC (1 x SSC is 0.15 M NaCl, 15mM sodium citrate), then treated with ribonuclease A (20 µg/µl) at 37 °C and finally washed in 0.1 x SSC at 60 °C. Slides were dried and apposed to autoradiographic Biomax MR film (Kodak, Rochester, New York) for several hours to days.

In this study, we focus on analysis of mRNA expressions in the 8 weeks experiments, since a previous study showed that *Dio3* mRNA expression peaks at this time before a subsequent decline (Barrett et al. 2007). Only *Dio2* gene expression was analysed from the second 8 weeks experiment (for body mass, see Figure 1 C). However, results of the first 8 weeks experiment (for body mass, see Figure 1 B) were compared with those of the 12 weeks experiment, where we analysed the same gene expression in both experiments.

Image analysis

Films were scanned at 600 dpi on an Umax scanner and quantification was carried out using Image J 1.37v software (Wayne Rasband, National Institutes of Health, USA). For each probe, three sections spanning a selected region of the hypothalamus were chosen for image analysis. Integrated optical density for each selected region was obtained by reference to a standard curve generated from the autoradiographic ^{14}C microscale (Amersham). An average (with SEM) for the integrated optical densities for all sections of one animal and for all animals in one group was calculated. The LD-C value was set to 100% expression and other treatment values were calculated accordingly.

Statistical analysis

Statistical tests applied in this study were two-way ANOVA with photoperiod and activity as factors. Differences revealed by two-way ANOVA were tested with Student-Newman-Keuls post-hoc test for multiple comparisons as appropriate. SigmaStat statistical software (Jandel) was used, values are expressed as mean + SEM and differences were considered significant if $P < 0.05$.

Results

Body mass

In the course of the 12 weeks experiment, body mass of the SD-C group decreased and showed a trajectory that significantly differed from that of the SD-RW group after 8.5 weeks (N = 6 in each group; 33.1 ± 2.9 g vs. 41.5 ± 2.3 g; two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$). Since then, two-way ANOVA showed an overall effect of activity on body mass in this experiment (two-way ANOVA; $F = 4.47$; $P < 0.05$). After 11 weeks, the body weight of the SD-C group was significantly different compared to all other three groups (two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$). Comparison of week 0 and

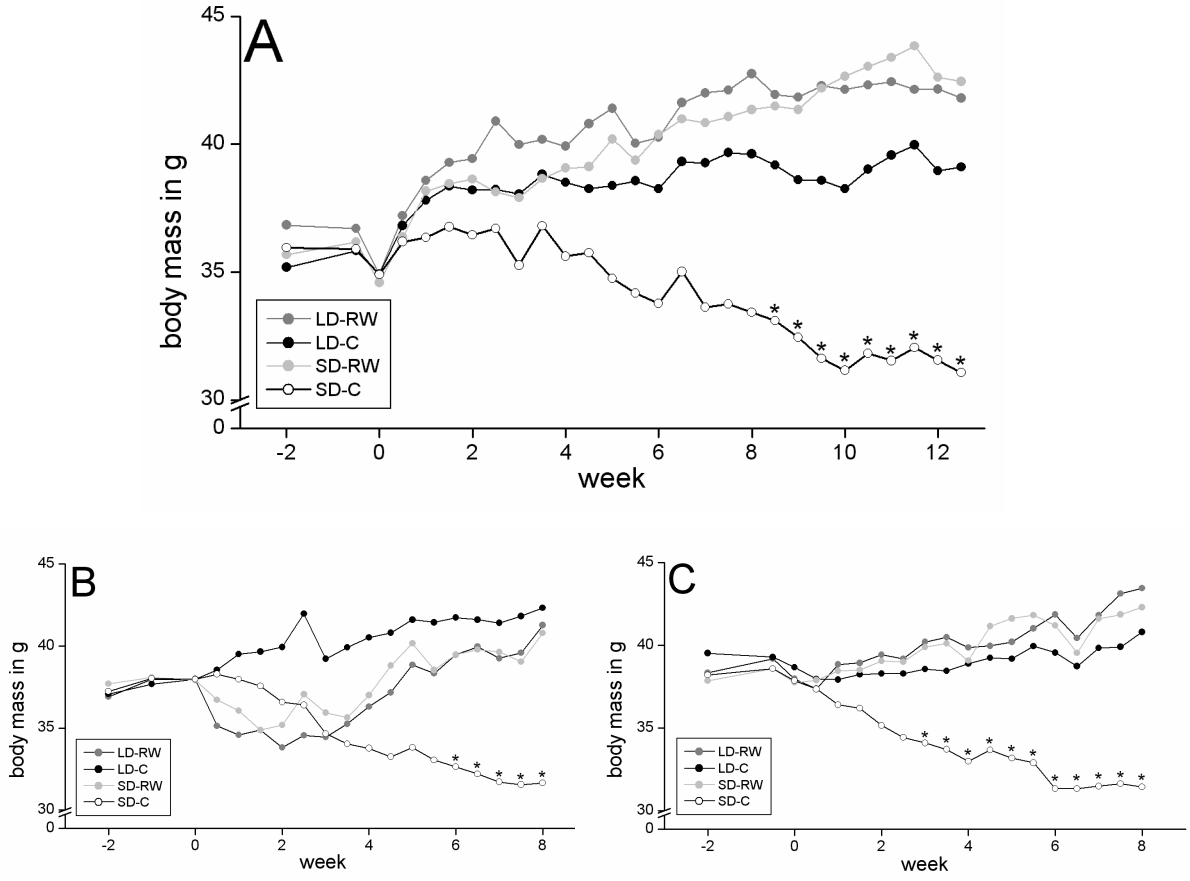

Figure 1: Mean body mass (g) of adult male Djungarian hamsters during a (A) 12 weeks or (B and C) 8 weeks exposure to short day (SD) photoperiod (8:16 h light-dark cycle) or long day (LD) photoperiod (16:8 h light-dark cycle) with or without access to a running wheel (RW) (N = 6 (A and B); N = 7 (C) in each group). *, SD-C significantly different vs. SD-RW ($P < 0.05$).

week 12 revealed that the SD-C group lost ~11% of body mass, in contrast to a weight gain of ~23%, ~12% and ~20% in the SD-RW, LD-C and LD-RW group, respectively (Figure 1 A). Comparable results on body mass were found in both 8 weeks experiments. In the first experiment (Figure 1 B), the SD-C group was significantly different compared to all other three groups in week 6 (N = 6 in each group; two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$) and there was an overall effect of activity in week 8 (two-way ANOVA; $F = 4.471$; $P < 0.05$). Compared to the start of the experiment (week 0), the SD-C group lost ~17% of body mass, whereas the other three groups gained weight (SD-RW ~8%, LD-C ~11% and LD-RW ~9%) in week 8. In the second 8 weeks experiment (Figure 1 C), the SD-C group significantly lost weight compared to all other three groups from week 3 onwards (N = 7 in each group; two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$) and since then there was also an effect of activity on body mass (two-way ANOVA; $F = 7.446$; $P < 0.05$). In week 8, the SD-C group lost ~17% of body mass compared to week 0, whereas the other three groups gained weight (SD-RW ~12%, LD-C ~6% and LD-RW ~14%).

Food intake

Added up over 12 weeks, hamsters in both RW groups show a significantly increased food intake compared to the SD-C group (LD-RW 66.0 ± 4.4 g, LD-C 55.8 ± 1.3, SD-RW 65.9 ± 4.0 g, SD-C 50.6 ± 2.6 g, two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$).

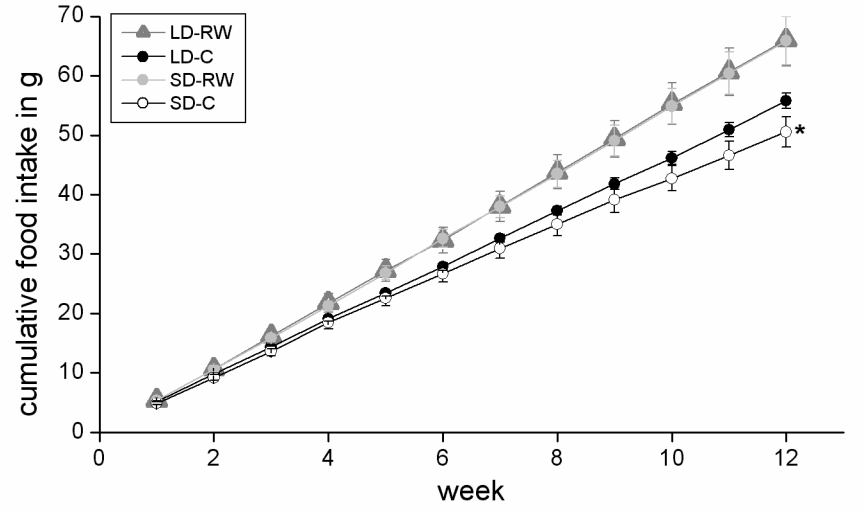

Figure 2: Mean cumulative food intake (g) ± SEM of adult male Djungarian hamsters during a 12 weeks exposure to short day (SD) photoperiod (8:16 h light-dark cycle) or long day (LD) photoperiod (16:8 h light-dark cycle) with or without access to a running wheel (RW) (N = 6 in each group). *, $P < 0.05$ compared to both RW groups.

There was also an effect of activity on cumulated food intake after 12 weeks (two-way ANOVA; F = 14.999; P < 0.001) (Figure 2).

Body composition

After 8 weeks in SD, hamsters in the SD-C group significantly lost fat mass (~53%) compared to the LD-C group (two-way ANOVA with Student-Newman-Keuls test; P < 0.01) and there was an effect of photoperiod on fat mass (two-way ANOVA; F = 6.356; P < 0.05). Both RW groups showed values in-between the LD-C and SD-C values and did not differ significantly from these two groups or from each other (Figure 3 A). Hamsters in both RW groups slightly increased lean mass (LD-RW ~0.9%, SD-RW ~1.2%) compared to LD-C (effect of activity; two-way ANOVA; F = 6.934; P < 0.05) (Figure 3 B). There was also an effect of photoperiod on lean mass (two-way ANOVA; F = 5.274; P < 0.05) with the SD-C group significantly decreasing lean mass (~17%) compared to the other three groups (two-way ANOVA with Student-Newman-Keuls test; P < 0.01).

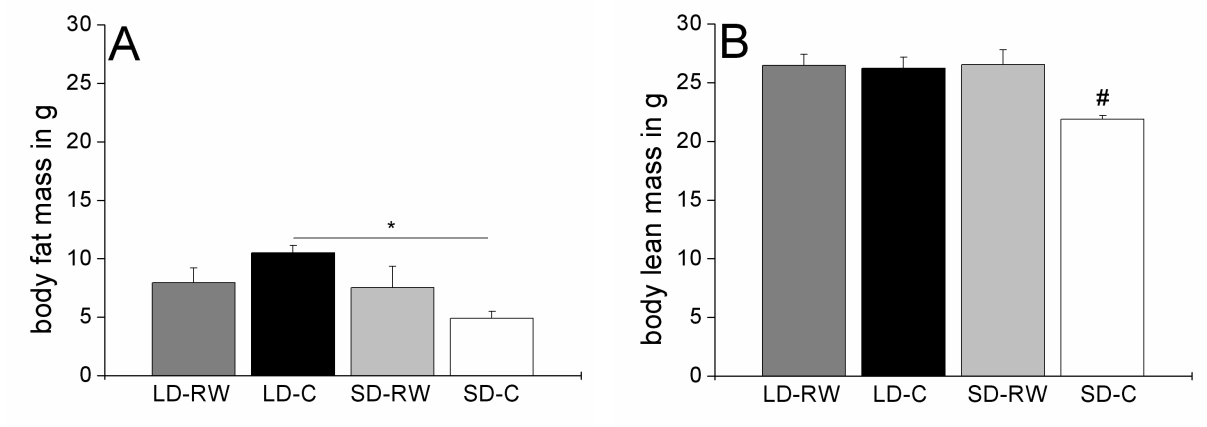

Figure 3: MRI scan results for (A) fat and (B) lean mass (g) of adult male Djungarian hamsters after 8 weeks exposure to short day (SD) photoperiod (8:16 h light-dark cycle) or long day (LD) photoperiod (16:8 h light-dark cycle) with or without access to a running wheel (RW) (N = 6 in each group). Results show means + SEM. *, P < 0.05; #, P < 0.05 compared to other three groups.

Orexigenic/anorexigenic gene expression

Agrp, *Npy*, *Cart* and *Pomc* mRNA expressions were assessed by *in situ* hybridization. These genes were expressed in the ARC of the hypothalamus. There was an effect of photoperiod on *Agrp* gene expression (two-way ANOVA; F = 11.025; P < 0.01) after 8 weeks due to the

significantly increased gene expression in the LD-RW group (two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$). On the other hand, comparison of the LD-C and SD-C group revealed no significant effect of photoperiod. An increase in the LD-RW group was not apparent after 12 weeks, with no significant differences between any groups (data not shown).

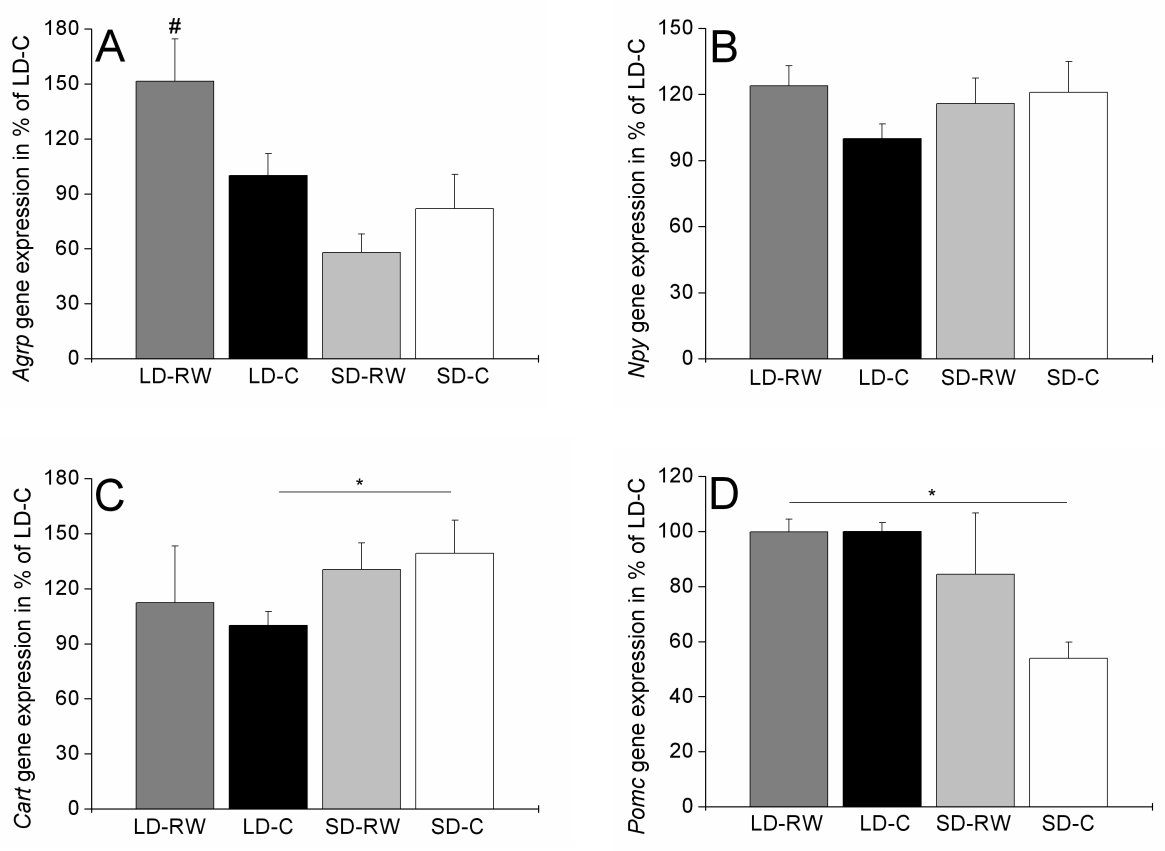

Figure 4: Quantification of (A) agouti-related protein (*Agrp*), (B) neuropeptide Y (*Npy*), (C) cocaine- and amphetamine- regulated transcript (*Cart*) and (D) proopiomelanocortin (*Pomc*) mRNA expression in the hypothalamic arcuate nucleus (ARC) of adult male Djungarian hamsters after 8 weeks exposure to short day (SD) photoperiod (8:16 h light-dark cycle) or long day (LD) photoperiod (16:8 h light-dark cycle) with or without access to a running wheel (RW) (N = 6 in each group). Results show means + SEM. The LD-C group was set to 100% expression value and other treatment values were calculated accordingly. *, $P < 0.05$; #, $P < 0.05$ compared to other three groups.

Neither photoperiod nor activity affected *Npy* gene expression in the 8 weeks experiment (Figure 4 B), which could be confirmed after 12 weeks (data not shown). *Cart* mRNA showed a small but significant increase in SD (effect of photoperiod; two-way ANOVA; $F = 4.76$; $P <$

0.05) and the same result was found after 12 weeks (data not shown). The LD-C group differed significantly from SD-C after 8 weeks (two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$) (Figure 4 C). Photoperiod affected *Pomc* gene expression after 8 weeks (two-way ANOVA; $F = 7.364$; $P < 0.05$), with the SD-C group being significantly different compared to LD-RW (two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$) (Figure 4 D). *Pomc* gene expression in the SD-C group was further decreased after 12 weeks leading to a significant difference compared to the other three groups (two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$; data not shown).

There was no effect of activity on any of the analysed gene expression for orexigenic and anorexigenic peptides.

Photoperiodically regulated Srif, Vgf and Gpr50 gene expression

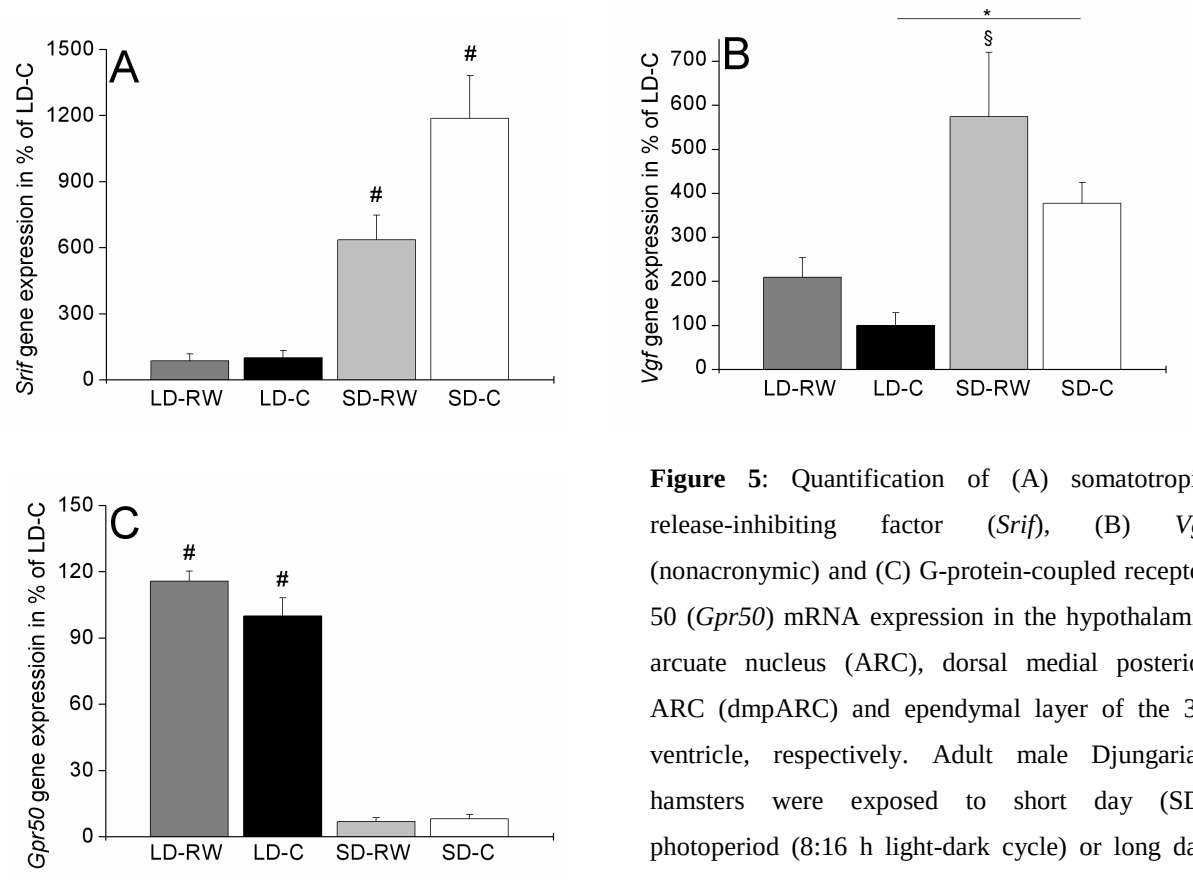

Figure 5: Quantification of (A) somatotropin release-inhibiting factor (*Srif*), (B) *Vgf* (nonacronymic) and (C) G-protein-coupled receptor 50 (*Gpr50*) mRNA expression in the hypothalamic arcuate nucleus (ARC), dorsal medial posterior ARC (dmpARC) and ependymal layer of the 3rd ventricle, respectively. Adult male Djungarian hamsters were exposed to short day (SD) photoperiod (8:16 h light-dark cycle) or long day (LD) photoperiod (16:8 h light-dark cycle) with or without access to a running wheel (RW) for 8 weeks (N = 6 in each group). Results show means + SEM. The LD-C group was set to 100% expression value and other treatment values were calculated accordingly. *, $P < 0.05$; #, $P < 0.05$ compared to other three groups; §, $P < 0.05$ compared to both LD groups.

Srif gene expression was measured in the ARC of the hypothalamus. Two-way ANOVA revealed an effect of photoperiod ($F = 50.7$; $P < 0.001$) and activity ($F = 6.1$; $P < 0.05$) on *Srif* gene expression after 8 weeks. Both SD groups were significantly different from each other and the LD groups (two-way ANOVA with Student-Newman-Keuls test; $P < 0.01$). The LD-RW and LD-C group did not differ from each other. The same result was found after 12 weeks but there was only a trend for an effect of activity on gene expression (two-way ANOVA; $F = 3.904$; $P = 0.062$; data not shown). *Vgf* was quantified in the dmpARC. After 8 weeks there was an effect of photoperiod (two-way ANOVA; $F = 20.383$; $P < 0.001$) with the gene expression being increased in both SD groups. There was also an effect of activity on *Vgf* gene expression (two-way ANOVA; $F = 4.655$; $P < 0.05$), which we did not find in the 12 weeks experiment (data not shown). In the 8 weeks, as well as in the 12 weeks experiment, gene expression in the SD-C group was significantly up-regulated compared to LD-C (two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$, for both experiments). G-protein-coupled receptor 50 (*Gpr50*) mRNA expression in the ependymal layer of the 3rd ventricle was down-regulated in both SD groups compared to the LD groups after 8 weeks (effect of photoperiod, two-way ANOVA; $F = 423.88$; $P < 0.001$). Furthermore, gene expression in the LD-RW group was significantly up-regulated compared to LD-C (two-way ANOVA with Student-Newman-Keuls test; $P < 0.05$). We did not find a difference in *Gpr50* mRNA expression between the LD-C and LD-RW group after 12 weeks, but also an effect of photoperiod (data not shown).

Gene expression in the thyroid system

Trh mRNA was expressed in the PVN. Photoperiod affected gene expression after 8 weeks with an increased expression in both SD groups (two-way ANOVA; $F = 11.578$; $P < 0.01$). *Trh* gene expression in the SD-C group increased by ~32% compared to LD-C (Figure 6 A). After 12 weeks, there was no difference between the groups (data not shown). Gene expression of *Mct8* was affected by photoperiod (two-way ANOVA; $F = 105.548$; $P < 0.001$) but not by activity. *Mct8* gene expression in SD was increased by ~150% compared to LD. *Dio2* and *Dio3* mRNAs were expressed in tanycytes of the ependymal layer lining the 3rd ventricle. *Dio2* gene expression was not different between any of the groups after 8 weeks and *Dio3* mRNA expression was only present in both SD groups (effect of photoperiod; two-way ANOVA; $F = 94.65$; $P < 0.001$), which did not differ significantly from each other. Activity did not influence any of the four examined genes of the thyroid system.

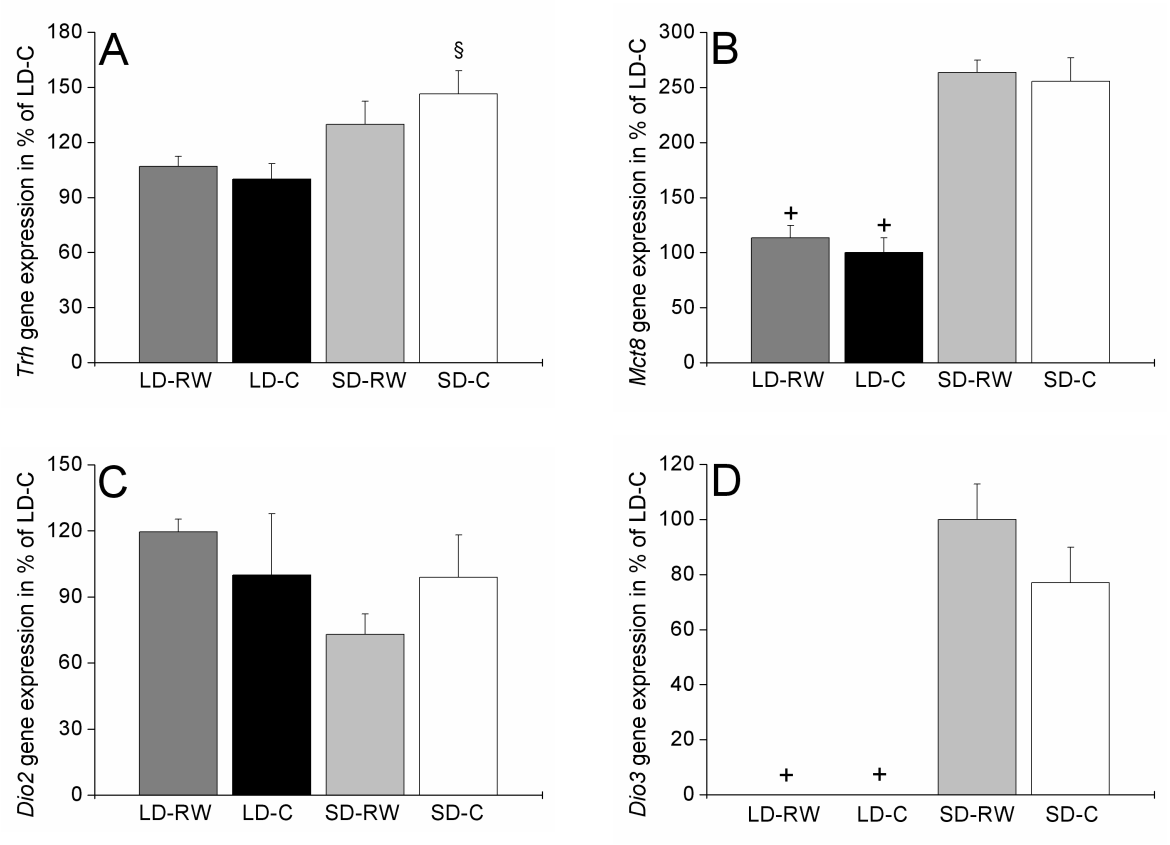

Figure 6: Quantification of (A) thyroid releasing hormone (*Trh*) mRNA expression in the paraventricular nucleus (PVN) and (B) monocarboxylate transporter 8 (*Mct8*), (C) type 2 and (D) type 3 deiodinase (*Dio2* and *Dio3*) mRNA expression in the 3rd ventricular tanycyte layer of adult Djungarian hamsters. Hamsters were kept 8 weeks in short day (SD) photoperiod (8:16 h light-dark cycle) or long day (LD) photoperiod (16:8 h light-dark cycle) with or without access to a running wheel (RW) (N = 6 in each group). Results show means + SEM. The LD-C group was set to 100% expression value and other treatment values were calculated accordingly. §, $P < 0.05$ compared to both LD groups; +, $P < 0.001$ compared to both SD groups.

Discussion

Seasonal mRNA expression of orexigenic and anorexigenic peptides in adult Djungarian hamsters was subject of several previous studies (Adam and Mercer 2001, 2004, Jethwa et al. 2010, Mercer et al. 2000, 2001, Mercer and Tups 2003, Reddy et al. 1999). Our findings in LD-C and SD-C groups are in agreement with their results. Photoperiod did not affect *Npy* and *Agrp* mRNA expressions, whereas *Pomc* decreased and *Cart* mRNA expression increased in SD (Figure 4). Former investigations revealed that seasonal regulation of body weight in Djungarian hamsters is anticipatory and not associated with compensatory changes in mRNA

expression for orexigenic and anorexigenic peptides. Hence, hamsters remain in energy balance despite seasonal changes in food intake and body mass. In fact, orexigenic and anorexigenic peptides seem to be involved in the regulation of short-term energy homeostasis, like in defence of weight loss due to food deprivation. Thus, the hypothalamus seems to be able to differentiate between short-term and seasonally programmed body weight changes (Adam and Mercer 2004, Jethwa et al. 2010, Mercer et al. 2001, Reddy et al. 1999).

Significant energy expenditure will be required to maintain continuous wheel-running activity during the dark phase of the light cycle. Furthermore, additional energy intake will be required to meet the long-term increase in body mass these hamsters undergo over the prolonged period of wheel-running activity. We therefore hypothesized that neuropeptides involved in the homeostatic regulation of appetite and energy balance would change to facilitate an increased food intake. However, we did not find an effect of wheel-running activity on orexigenic and anorexigenic gene expression in this study, which might indicate that activity does not cause a short-term effect. But this conclusion has to be considered cautiously as the time point for brain collection was 3-4 hours after the lights went on, while hamsters have been active and were feeding during the dark phase. Ellis and coworkers (2008) demonstrated a lack of a diurnal gene expression profile for orexigenic and anorexigenic genes in the ARC of Djungarian hamsters, corresponding with the recent finding that Djungarian hamsters do not display a significant nocturnal increase in total food intake (Warner et al. 2010), which was misleadingly assumed before. Thus, the time point of sampling might not have affected the level of mRNA expression in our LD-C and SD-C groups. However, due to the 3-4 hours time delay to the activity phase we might have missed any acute effect of wheel-running activity on mRNA expression levels.

Pomc mRNA expression was slightly increased in the SD-RW group compared to SD-C after 8 weeks (Figure 4 D) and this effect was significant in the 12 weeks experiment (data not shown). At first sight, downregulation of *Pomc* mRNA expression in the SD control group, which would imply a decreased anorexigenic action, is counterintuitive and therefore this phenomenon was further analysed. POMC is a neuropeptide precursor that undergoes enzymatic post-translational processing. Helwig and coworkers (2006) showed an increase of prohormone convertase 2 (PC-2) in SD, through which more precursor POMC is cleaved to the active peptide α-MSH, which then acts anorexigenic at the melanocortin-4 receptor. In animals of the SD-RW group in our study, an increased expression of precursor POMC might lead to a further increase of the mature cleaving product α-MSH, which subsequently may cause a higher activity of the melanocortin system with its catabolic actions. This molecular

action remains speculative as we did not measure levels of PC-2 in this study. Furthermore, this finding seems to be paradoxical, because SD-RW animals significantly increased body mass already after 8 weeks (~8-12%) and even more after 12 weeks (~23%) (Figure 1).

However, another gene expression that changed counterintuitively by trend in response to activity in this study is *Vgf* (Figure 5 B). Gene expression of *Vgf* has been shown to be up-regulated in the dmpARC of Djungarian hamsters in SD, in advance of seasonal changes in physiology (Barrett et al. 2005, Nilaweera et al. 2009, Ross et al. 2005). Furthermore, previous studies revealed that i.c.v. administration of a VGF-derived peptide (TLQP-21) decreased food intake by stimulating satiety (Jethwa et al. 2007). Herwig and coworkers (2009) showed that *Vgf* gene expression does not react to the short-term signal of starvation. Therefore, VGF was assumed to contribute to the catabolic state that induces the long-term weight loss in SD. We could show an effect of activity on *Vgf* mRNA expression after 8 weeks with an upregulation in both RW groups, but it did not occur after 12 weeks. This fact might demonstrate a time-dependent regulation of *Vgf* mRNA in response to exercise. Together with *Pomc*, the upregulation (temporarily for *Vgf*) of these coexpressed genes (Hahm et al. 2002) might present a compensatory response of the energy balance system to antagonise the increased body mass that deviates from the seasonal set point in RW hamsters. However, the signal seems to be not strong enough to influence body mass but it might influence food intake. Interestingly, we found an effect of activity on cumulative food intake in both RW groups (Figure 2), although the absolute food intake in g per day in the SD-RW group increased by only ~2% and not at all in the LD-RW group (week 1 compared to week 12) (data not shown). In addition, comparison of relative food intake [(g/d)/body mass] at the end of the 12 weeks experiment (data not shown) showed no difference between all four groups. Altogether, hamsters in the RW groups did not increase their food intake as much as one would expect in view of their body mass trajectory. A similar discrepancy has been reported by Scherbarth and coworkers (2008) comparing LD-RW hamsters with controls in relative daily O_2 uptake (~27% increase) and relative daily food intake (~14% increase). They concluded that exercising animals might improve utilization of nutrients compared to controls and thus would be able to cope with less food. But comparison of the effectiveness of digestion between exercising hamsters and controls in LD and SD revealed no difference (data not shown). Thus, the phenomenon concerning food intake still has to be resolved. However, our study provides a hint that the low relative food intake in exercising animals compared to controls might be mediated by increased anorexigenic action of the melanocortin system, and increased VGF secretion of the dmpARC. The fact that *Pomc* gene expression in

the LD-RW group did not increase further might indicate a maximum gene expression in the ARC and/or the edge of measurable signal by *in situ* hybridization.

We measured a significantly increased *Agrp* mRNA expression in the LD-RW group compared to LD-C after 8 weeks (Figure 4 A), but this effect was not existent after 12 weeks (data not shown). This might reflect different responses of body mass to wheel-running activity in both experiments. Body mass of the LD-RW group shows an increasing trajectory at the end of the 8 weeks experiment (Figure 1 B), whereas it reached a plateau after 12 weeks (Figure 1 A). On the other hand, we did not find an associated upregulation of *Agrp* mRNA in the SD-RW group compared to SD-C after 8 weeks. In accordance with a possible role of AGRP in affecting food intake and body mass, we would expect an upregulation of *Agrp* gene expression in both RW groups. The difference in *Agrp* mRNA expression between LD-RW and LD-C might also reflect an inaccuracy in the process of quantification. Mercer and coworkers (2000) found an increase in *Agrp* gene expression in SD only in the rostral ARC, whereas gene expression in other regions of the ARC was not influenced by photoperiod. Thus, the level of *Agrp* gene expression seems to be dependent on the respective ARC region.

In the absence of an effect of wheel-running activity on homeostatic gene expression, our second hypothesis proposed that wheel-running activity may affect gene expressions involved in the photoperiodical regulation of body weight, particularly to facilitate a weight gain in SD-RW hamsters.

Ebling (1994) and Adam and coworkers (2000) suggested a cessation of growth in SD adapted Djungarian hamsters. We can confirm this result as we did not only find an effect of photoperiod on fat mass (Figure 3 A), but also on lean mass (Figure 3 B). Lean mass mainly consists of bone and muscles and thus, the increase in lean mass in LD-C hamsters compared to SD-C can be attributed to growth. In 2007, Scherbarth and colleagues investigated the influence of wheel-running activity on body composition in the Djungarian hamster. Dual-Energy X-ray Absorptiometry (DEXA) measurement in hamsters kept outdoors under natural T_a and photoperiod revealed no difference in fat mass between RW and control hamsters in February (SD) despite the significant difference in body mass. After 8 weeks exposure to artificial SD, we also did not find a difference in fat mass between hamsters in the SD-RW and SD-C group in this study (Figure 3 A). However, lean mass in the study of Scherbarth and colleagues (2007) was increased in the RW group, which is also in accordance with our study (~17% increase in the SD-RW group compared to SD-C; Figure 3 B). Furthermore, on radiographs of RW hamsters in the study of Scherbarth and coworkers (2007), the vertebral columns between head and pelvis were significantly longer compared to controls. In a

following study (Scherbarth et al. 2008) elongated femora in exercising animals were found, giving another hint to exercise-induced growth. Growth is regulated in the hypothalamus by two contrarily acting hormones that stimulate (GHRH) or inhibit (SRIF) synthesis and pulsatile release of GH from the anterior pituitary gland. We found an increased mRNA expression of *Srif* in hamsters of the SD-C group compared to both LD groups (Figure 5 A). Thus, secretion of GH is inhibited in the SD-C group, whereas decreased inhibition in both LD groups might allow growth. If *Srif* gene expression in LD was at the lowest level, it would explain why exercise (LD-RW group) did not result in a further decrease. These results support the suggestion of growth being cessated in SD. In the SD-RW group, *Srif* gene expression was down-regulated compared to SD-C with values lying inbetween the SD-C and LD groups. Therefore, growth was significantly less inhibited in the SD-RW group compared to SD-C. Hence, on a molecular level, our results substantiate the hypothesis of a growth-promoting effect through wheel running, which was assumed in previous studies for two hamster species (Borer 1980, Borer and Kaplan 1977, Borer and Kelch 1978 in the Syrian hamster; Scherbarth et al. 2007, 2008 in the Djungarian hamster).

The present results confirm an earlier report on photoperiodic regulation of *Gpr50* mRNA expression in the Djungarian hamster (Barrett et al. 2006). We also found decreased gene expression in SD compared to LD. After 8 weeks, *Gpr50* expression was significantly up-regulated in the LD-RW group compared to LD-C, which was not existent in the 12 weeks experiment. In addition, *Gpr50* mRNA was not up-regulated in SD-RW compared to SD-C. Therefore, we assume that the significant difference between both LD groups might be an artefact of expression analysis. Consequently, we presume that *Gpr50* mRNA expression seems not to be affected by wheel-running activity, similarly to the thyroid system.

Transport and activation of thyroid hormone in the hypothalamus were not impaired by exercise. Accordingly, we concluded that photoperiod perception is not affected in wheel-running hamsters. We did not find an effect of photoperiod on the hamsters' *Dio2* gene expression, which is in accordance with previous findings (Barrett et al. 2007). On the other hand, Herwig and coworkers (2009) found an increase of *Dio2* mRNA expression in LD and a decrease in SD after 8 weeks. They suggested a time-dependent change of *Dio2* mRNA to explain the difference to the previous study. However, our study also lasted for 8 weeks and we did not find a difference in *Dio2* mRNA expression. In contrast, two studies showed differing results in *Dio2* gene expression in Djungarian hamsters (Watanabe et al. 2004, 2007). The authors found an induction of *Dio2* gene expression in hamsters in LD compared to SD. However, in these studies hamsters were kept in SD after weaning and for the LD

group, hamsters were transferred to LD for two weeks. In a reversed experimental set-up (i.e. hamsters were kept in LD and were transferred to SD for the SD group), *Dio2* expression was also not affected by photoperiod. In fact, concerning the light regime, we designed our experiments comparable to that of Herwig and colleagues (2009) (i.e. hamsters were kept in LD and were transferred to SD for several weeks in the SD group). Therefore, we assume that the discrepancy in our results might present a difference between the breeding colonies in Hannover and Aberdeen. This assumption is supported by our results for *Trh* mRNA expression. We found an effect of photoperiod on *Trh* gene expression, being up-regulated in SD compared to LD after 8 weeks (Figure 6 A). Studies of Ebling and coworkers (2008) and Herwig and colleagues (2009) revealed no photoperiodical regulation of *Trh* gene expression in Djungarian hamsters of their breeding colony but they could show a role of TRH in short-term homeostatic control of appetite and energy expenditure. However, the increase in *Trh* gene expression in SD in our study might contribute to decreased food intake and increased catabolism of fat stores in SD.

Summarised, we are able to confirm exercise-induced growth in Djungarian hamsters at a molecular level, probably contributing to the increase in lean mass and body mass in exercising hamsters. Furthermore, there are some hints indicating that exercise might stimulate the melanocortin pathway and secretory processes in the dmpARC to compensate for the increased body mass. However, via analysis of the gene expression of orexigenic peptides, *Cart*, the photoperiodically regulated genes *Gpr50* and genes in the thyroid system, we could show that central perception of photoperiod seems not to be affected by voluntary exercise. This result would be in line with the finding that the change in fur colouration, which is based on the level of the pituitary hormone prolactin (Duncan and Goldman 1984, Lincoln and Clarke 1994, Niklowitz and Hoffmann 1988), was similar in animals with and without a RW (Scherbarth et al. 2007, 2008). Studies in sheep indicated that prolactin secretion from the pituitary might be independent of the hypothalamus but controlled by the pars tuberalis (Hazlerigg et al. 1996, Lincoln and Clarke 1994). That would imply different pathways for fur colour and energy balance regulation, but a recent study revealed that there might be a role for the hypothalamus in fur colouration in the Djungarian hamster (Dodge and Badura 2004). However, these processes need further research in our animal model. Collectively, further causes for the body weight increase in SD-RW hamsters remain to be discovered. Consequently, in future studies, we will focus on peripherally derived signals (for example from muscle or liver) that might feed back to the brain, potentially overriding

mechanisms for the initiation of SD traits and thus allowing the weight gain in winter-adapted Djungarian hamsters.

CHAPTER 4

Effects of photoperiod and voluntary exercise on skeletal muscle metabolism

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Abstract

Djungarian hamsters (*Phodopus sungorus*) with access to a running wheel (RW) increase their body mass in winter-like short days (SD), instead of exhibiting the seasonally programmed weight loss. Analysis of body composition in a previous study revealed an effect of photoperiod and activity on lean mass. Lean mass decreased in SD control animals compared to long day (LD) controls, but increased in SD-RW hamsters. In the present study we examined whether exercise-induced signals from peripheral skeletal muscle might affect the mechanisms underlying seasonal body weight regulation in Djungarian hamsters. Male hamsters were kept with or without access to a RW under SD or LD conditions. After 12 weeks, the gastrocnemius muscle of the calf was analysed for phosphorylation (i.e. activation/inactivation) of AMP-activated protein kinase, acetyl CoA carboxylase, serine/threonine kinase and p44/42 MAPK, which all are involved in myocyte metabolism. In addition, serum concentrations of insulin and insulin-like-growth factor 1 (IGF-1) were determined. Body mass increased in RW hamsters compared to the controls, whereas hamsters in the SD-C group reduced body mass compared to LD-C. However, the results of phosphorylated muscle enzymes and serum levels of insulin and IGF-1 revealed neither an impact of photoperiod nor activity. This might have been due to the fact that the time point of killing in this study (3-4 hours after the lights went on) was not close enough to the nocturnal exercise bout and thus, enzyme and hormonal concentrations probably recovered already to baseline levels. Furthermore, activity intensity and food intake were not standardized, leading to a large individual variability in measured parameters. Further investigations are necessary to clarify the hypothesis that wheel-running activity and photoperiod might influence muscle metabolism and thus provide signals that might interact with the mechanisms regulating the seasonal body weight cycle in Djungarian hamsters.

Introduction

To survive in winter, when many food sources are restricted, Djungarian hamsters (*Phodopus sungorus*) need to save energy. Amongst others, this is achieved by a reduction in food intake and body mass in response to the shortening photoperiod from summer to winter, resulting in decreased energy expenditure (Heldmaier and Steinlechner 1981a). However, in hamsters with access to a RW, the mechanisms underlying the body mass decline in SD seem to be

disturbed. In contrast to SD controls, voluntarily exercising hamsters in SD gain weight (Scherbarth et al. 2007, 2008). In accordance with a study of Klingenspor and coworkers (2000), MRI scan data in a previous study (unpublished data, see chapter 3) revealed not only a decrease in body fat mass in SD control animals compared to LD controls, but also a significant reduction of lean mass in SD. Moreover, lean mass increased in hamsters with access to a RW in SD, thus contributing to their weight gain. Lean mass mainly consists of skeletal muscle that shows high plasticity to adapt to changing functional demands. During exercise, activity of metabolic enzymes, transcription, translation and post-translational modification of proteins in myocytes can be modulated. Altogether, skeletal muscle seems to be an important organ to induce metabolic signals that might trigger physiological adaptations at the whole body level (for review, see Pedersen and Febbraio 2008).

During exercise, a pivotal enzyme that elicits fundamental adaptations of metabolism is adenosine monophosphate (AMP)-activated protein kinase (AMPK) (for review, see Hardie et al. 1998). The AMPK complex contains 3 subunits, with the α subunit being catalytic and the β and γ subunits being essential for forming the functional complex. The enzyme is a highly conserved sensor of the cellular energy status and plays a role in systemic energy balance by inhibiting anabolic adenosine triphosphat (ATP) consuming processes (synthesis of fatty acids, glycogen and proteins) while activating catabolic pathways that are crucial for generation of ATP (fatty acid oxidation, glycolysis, glucose uptake).

Activation of AMPK in skeletal muscle takes place in response to muscle contraction (Hayashi et al. 1998, Hutber et al. 1997, Vavvas et al. 1997, Winder and Hardie 1996) and an associated increased cellular metabolic stress, whereupon the AMP/ATP ratio increases (Corton et al. 1994, Hutber et al. 1997). In addition to direct binding of AMP, AMPK is activated via phosphorylation by an upstream kinase (AMPK kinase). Important for maintaining energy balance not only at the cellular, but also at the whole body level appears to be the involvement of several hormones in AMPK regulation (for review, see Towler and Hardie 2007 and Velloso 2008). The white adipose tissue-derived hormones leptin and adiponectin, that are known to play a critical role in regulating energy balance, can also phosphorylate and thus activate the AMPK system in skeletal muscle (Minokoshi et al. 2002, Tomas et al. 2002, Yamauchi et al. 2002). Furthermore, hormones like insulin and IGF-1 activate a pathway that can interact with AMPK (described more in detail below).

In the signal transduction pathway of activated AMPK, PGC-1 [peroxisome-proliferator-activated receptor γ (PPAR γ) co-activator-1] is activated. PGC-1 regulates transcription factors that are involved in controlling the expression of metabolic and mitochondrial genes

and thus stimulates long-term effects on gene and protein expression in mitochondrial oxidative metabolism (for review, see Handschin and Spiegelman 2006).

Furthermore, AMPK phosphorylates acetyl CoA carboxylase (ACC) and thus inhibits its activity (Vavvas et al. 1997, Winder and Hardie 1996). This results in decreased malonyl CoA levels, disinhibiting carnitine palmitoyl transferase 1 (CPT-1) that thereupon increases uptake and β–oxidation of fatty acids in muscle mitochondria (for review, see Ruderman et al. 1999).

AMPK also enhances skeletal muscle glucose uptake (Ploug et al. 1984) by increasing expression and translocation of the glucose transporter GLUT-4 to the sarcolemma (Fryer et al. 2002, Kurth-Kraczek et al. 1999, Ojuka et al. 2000).

A different pathway that responds to exercise and interacts with AMPK is the insulin/IGF-1 pathway. The liver is the primary source of circulating IGF-1 that mediates the effects of pituitary growth hormone (GH). During and after exercise, GH release from the pituitary increases (Borer and Kelch 1978, Hartley et al. 1972) which, in turn, increases the release of hepatic IGF-1 and also induces the synthesis of IGF-1 in other tissues such as muscles (DeVol et al. 1990, Turner et al. 1988). IGF-1 binds to its receptor (IGFR-1) that signals through the phosphatidylinositol 3-kinase (PI3K)/Akt (serine/threonine kinase or protein kinase B) pathway. Insulin also binds to the IGFR-1 and, conversely, IGF-1 binds to the insulin receptor indicating shared activities. However, the plasma insulin level decreases during exercise while insulin sensitivity in muscle increases (Berger et al. 1979, Hartley et al. 1972).

The insulin/IGF-1 pathway induces glucose uptake via increased expression of GLUT-4 (Hayashi et al. 1998) and activates glycogen and protein syntheses (Cross et al. 1995, Gingras et al. 1998) leading to muscle hypertrophy.

Through exercise and the receptor binding of insulin, the mitogen-activated protein kinase (MAPK) signalling cascades are activated, too. They include the extracellular signal-regulated kinase 1 and 2 (ERK-1/2 or p44/42 MAPK), that are assumed to regulate glucose transport, protein synthesis and gene expression by phosphorylation of transcription factors in response to exercise (Goodyear et al. 1996).

As protein synthesis in muscle, which is stimulated via the insulin/IGF-1 pathway is an ATP-consuming process, it is also negatively regulated by AMPK, probably by suppressing the activity of Akt (Bolster et al. 2002).

On the one hand, AMPK and insulin/IGF-1 act via different pathways, but on the other hand, both increase glucose uptake in muscle cells (Ploug et al. 1984). However, both pathways have distinct long-term effects on skeletal muscle glucose: the release of insulin and IGF-1 increases storage of glucose (glycogen synthesis; anabolic), whereas glucose is oxidated

through activation of AMPK (glycolysis; catabolic). Thus, the net outcome in protein and glucose synthesis or degradation depends on the balance of both pathways (Towler and Hardie 2007).

The aim of this study was to examine the phosphorylation of enzymes and serum hormone levels involved in skeletal muscle metabolism in exercising and control hamsters to achieve further hints for signals being involved in the reversal of the body mass decrease of SD-RW hamsters. In addition, our experimental set-up allowed us to analyse the influence of photoperiod on these parameters, as MRI scan data showed that muscle mass was affected by photoperiod, too.

Materials and Methods

Animals and tissue collection

Twenty-four male Djungarian hamsters (*Phodopus sungorus*) were bred and raised under natural photoperiod and ambient temperature in Hannover, Germany (52°N latitude). Water and food (hamster breeding diet, Altromin 7014, Lage) were available *ad libitum*, supplemented weekly by a piece of apple before the start of the experiment. At the vernal equinox (23 March) all animals were transferred to artificial LD photoperiod with a light/dark cycle of 16 h light/8 h darkness. At the age of 5-14 months the animals were divided into four weight-matched groups of six animals each. Two groups stayed in LD and one group received a RW (Ø 14.5 cm) (LD-RW), the second group stayed without a RW (LD-C). Remaining two groups were transferred to SD photoperiod with a light/dark cycle of 8 h light/16 h darkness. Like in LD, one group received a RW (SD-RW) and the other one represented the sedentary control group without a RW (SD-C). Hamsters were weighed twice a week and voluntary wheel-running behaviour in both RW groups was monitored by recording wheel revolutions on a PC.

Twelve weeks later, arranged over two days, the animals were killed 3-4 h after the lights went on. Blood samples were taken and the gastrocnemius muscle was dissected and frozen in liquid nitrogen for subsequent Western blotting.

Muscle protein extraction

An electrical homogenizer was used to break down the gastrocnemius muscle tissue mechanically in 0.3 ml homogenisation buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA,

1% Triton X-100) on ice. Protease and phosphatase inhibitors were added to the homogenisation buffer to prevent digestion of the sample by its own enzymes. The tissue homogenate was then shaken for 1 h at 4 °C and afterwards centrifuged at 13,000 g and 4 °C for 10 min. For determination of the total protein amount, the supernatant was used to carry out a Bradford assay.

Western blotting

Primary (AMPK α, Phospho-AMPK α, AMPK β, Phospho-AMPK β, ACC, Phospho-ACC, Akt, Phospho-Akt) and secondary [horseradish peroxidase (HRP)-conjugated] antibodies were purchased from Cell signalling technology.

For gel electrophoresis, samples were diluted to 900 µg protein/30 µl homogenisation buffer including 10 µl 3x gel loading buffer (3.75 M Tris-HCl pH 6.8, 6% SDS, 0.003% bromophenol blue, 30% glycerol, 15% β-mercaptoethanol). Samples were boiled for 5 min and electrophoresed through a 4% stacking gel and a 10% separating gel (7.5% separating gel for ACC and Phospho-ACC). A protein molecular weight marker was electrophoresed at the same time in the outer lane. To check for loading differences, one test gel was stained with Brilliant blue (20% methanol, 0.5% acetic acid, 0,2% Brilliant Blue) and destained with 30% methanol. In order to make separated proteins accessible to antibody detection, they were electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF, Millipore Corporation, Bedford, MA, USA) in transfer buffer (25 mM Tris base, 192 mM glycine, 0.05% SDS in 20% methanol). After transfer, blocking of non-specific binding was achieved by incubating the membrane for 1 h at room temperature in blocking buffer [5% non-fat dry milk in TBS (20 mM Tris, 140 mM NaCl, 0.1% Tween 20)] and afterwards the primary antibody (1:1000) was incubated at 4 °C overnight with gentle agitation. After that, the membrane was incubated with the HRP-conjugated secondary antibody (1:2000 in blocking buffer) for 1 h and finally the membrane was incubated with chemiluminescent substrate (LumiGlo Reagent and Peroxide, Cell signalling technology). The light emission was captured by exposure of the membrane to autoradiographic Biomax MR film (Kodak, Rochester, New York).

Image analysis

Films were scanned at 600 dpi on an Umax scanner and quantification was carried out using Image J 1.37v software (Wayne Rasband, National Institutes of Health, USA). The integrated optical density for each blot was obtained by reference to a standard curve and all values were

corrected for protein loading differences. To correct for the amount of total protein on the PVDF membrane, the ratio of phosphorylated protein to total protein was calculated.

IGF-1 and insulin assay

Blood serum was obtained by centrifugation of clotted blood samples (10 min at 9000 g at room temperature) and collecting of the supernatant. The circulating hormones IGF-1 and insulin were measured using a mouse/rat specific radioimmunoassay kit (R&D Systems, Quantikine, mouse/rat IGF-1; Mercodia, Rat insulin ELISA). Serum was diluted 1:1000 for IGF-1 and was used undiluted for insulin. Both kits were validated for hamster serum by applying a hamster serum dilution series, whose values ran parallel to the standard curve.

Statistical analysis

Statistical tests applied in this study were two-way ANOVA with photoperiod and activity as factors. Differences revealed by two-way ANOVA were tested with Student-Newman-Keuls post-hoc test for multiple comparisons. SigmaStat statistical software (Jandel) was used, values are expressed as mean ± SEM and differences were considered significant if $P < 0.05$.

Results

Body mass

After 8.5 weeks, there was an effect of activity on body mass, with RW hamsters significantly increasing their body mass compared to controls (LD-RW 41.9 ± 3.5 g; LD-C 39.2 ± 1.4 g; SD-RW 41.5 ± 2.3 g; SD-C 33.1 ± 2.9 g; two way ANOVA; $F = 4.47$; $P < 0.05$). Since week 11, the SD-C group significantly lost weight compared to LD-C (SD-C 31.5 ± 2.9 g; LD-C 39.6 ± 1.3 g; two way ANOVA with Student-Newman-Keuls test; $P < 0.05$).

Phosphorylation of AMPK α, ACC and Akt

Phosphorylation of AMPK α did not differ significantly between the four experimental groups and we could not detect any phosphorylation for AMPK β. There was a tendency for an effect of photoperiod on ACC phosphorylation (two way ANOVA; $F = 4.08$; $P = 0.057$), but groups did not differ significantly. No difference between the groups was found for Akt phosphorylation.

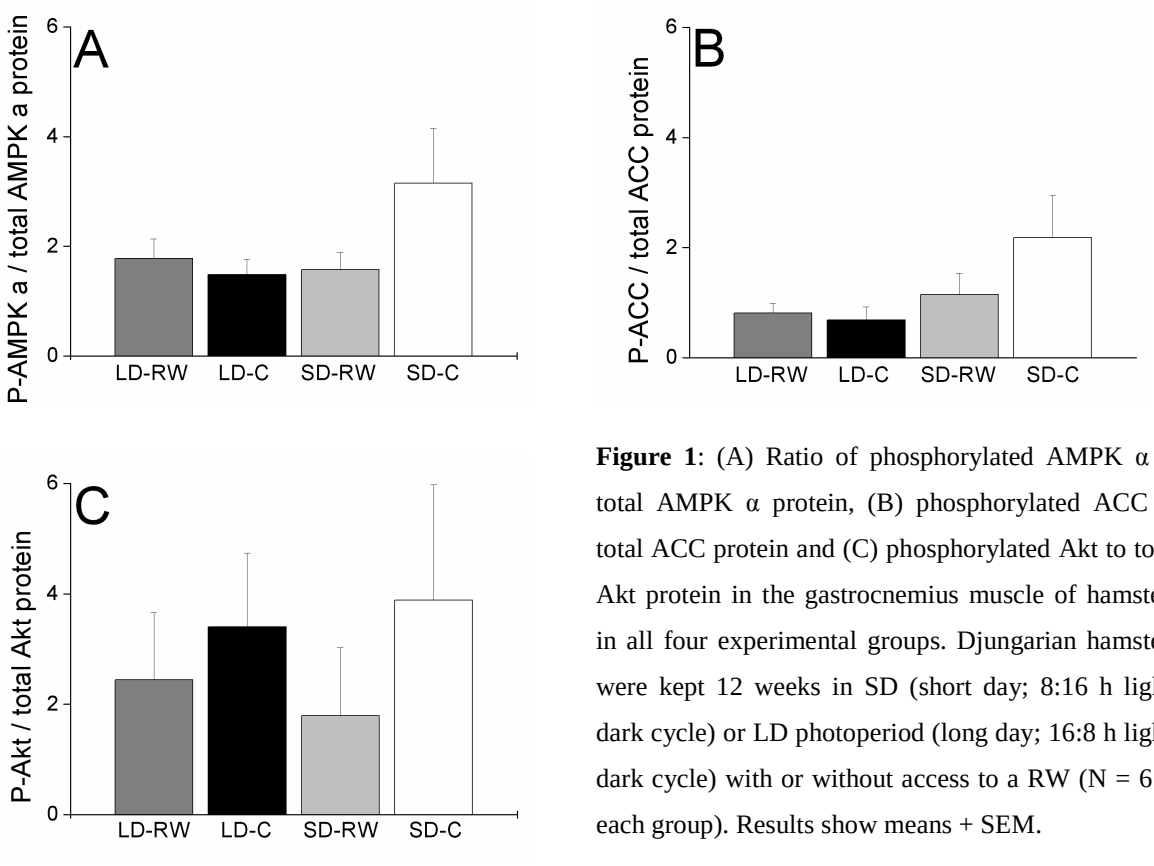

Figure 1: (A) Ratio of phosphorylated AMPK α to total AMPK α protein, (B) phosphorylated ACC to total ACC protein and (C) phosphorylated Akt to total Akt protein in the gastrocnemius muscle of hamsters in all four experimental groups. Djungarian hamsters were kept 12 weeks in SD (short day; 8:16 h light-dark cycle) or LD photoperiod (long day; 16:8 h light-dark cycle) with or without access to a RW (N = 6 in each group). Results show means + SEM.

p44/42 MAPK

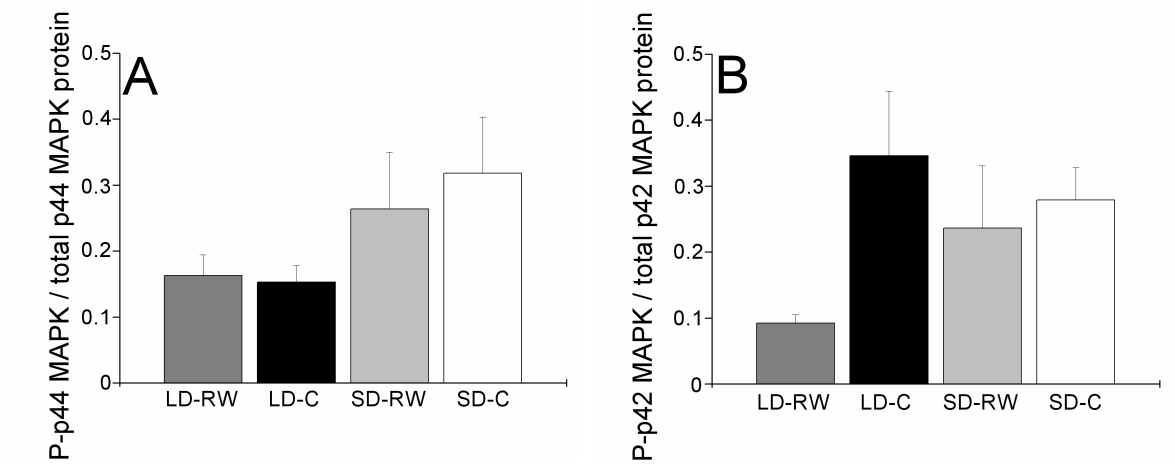

Figure 2: (A) Ratio of phosphorylated p44 to total p44 protein and (B) phosphorylated p42 to total p42 protein in the gastrocnemius muscle of hamsters in all four experimental groups. Djungarian hamsters were kept 12 weeks in SD (short day; 8:16 h light-dark cycle) or LD photoperiod (long day; 16:8 h light-dark cycle) with or without access to a RW (N = 6 in each group). Results show means + SEM.

Two way ANOVA revealed an effect of photoperiod on the p44 MAPK ratio ($F = 4.38$; $P < 0.05$), but groups did not differ significantly. There was a tendency for an effect of activity on the p42 MAPK ratio (two way ANOVA; $F = 4.15$; $P = 0.055$), however, the four groups were not significantly different.

Serum insulin and IGF-1 concentration

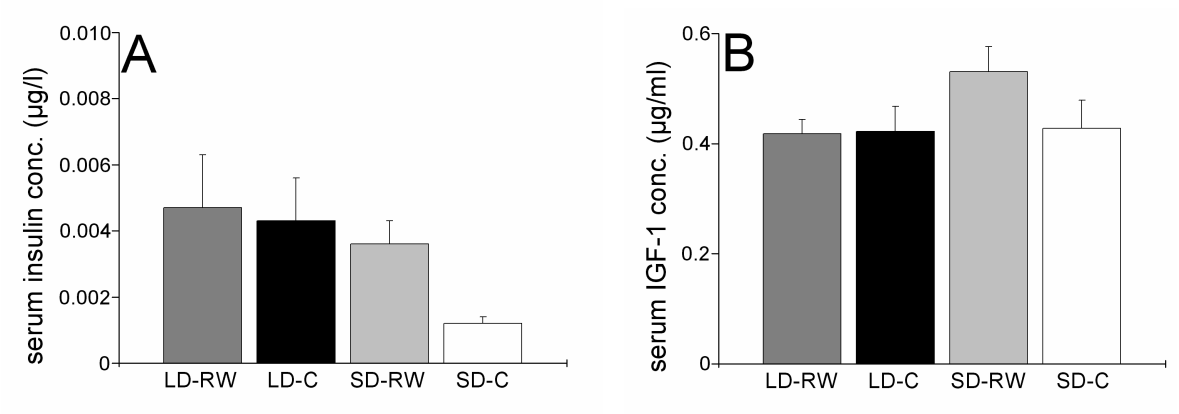

Figure 3: (A) Serum insulin (µg/l) and (B) IGF-1 levels (µg/ml) in all four experimental groups. Djungarian hamsters were kept 12 weeks in SD (short day; 8:16 h light-dark cycle) or LD photoperiod (long day; 16:8 h light-dark cycle) with or without access to a RW (N = 6 in each group). Results show means + SEM.

After 12 weeks, neither serum insulin levels nor IGF-1 levels differed significantly between the four groups.

Discussion

At the time of killing in our study, 3-4 hours after the light went on and thus during the resting phase of nocturnal hamsters, we were not able to detect any effect of wheel-running activity on the level of phosphorylated enzymes in the gastrocnemius muscle. This was probably due to the fact that enzyme phosphorylation was back to resting level already.

To metabolically adapt to exercise, phosphorylation (activation/inactivation) of enzymes in skeletal muscle occurs very fast. For example, AMPK is activated in the contracting muscle within 5 minutes of the beginning of exercise (Winder and Hardie 1996), after 15 minutes

with incubation of adiponectin (Tomas et al. 2002) or within 10-20 minutes after electrical stimulation of the ischiatic nerve (Hutber et al 1997). For ACC, Tomas and coworkers (2002) showed that phosphorylation significantly increased after 30 minutes of adiponectin incubation and in the study of Hutber and colleagues (1997) ACC activity decreased after 2-5 minutes of nerve stimulation.

However, enzyme phosphorylation levels return to baseline level very rapidly after exercise, when contraction-induced signals decrease and dephosphorylation takes place. Tomas and coworkers (2002) revealed that the level of activated AMPK in muscle strips returned to baseline after 1 hour of incubation with adiponectin. However, temporal resolution in this study was rough, so an earlier decrease of AMPK activity, between 30-60 minutes of incubation, cannot be ruled out. Therefore, in other studies, the level of activated/ phosphorylated enzymes in muscle was measured rapidly after an exercise bout (Rasmussen and Winder 1997, Winder and Hardie 1996), or directly after *in vitro* stimulation of muscle cells via an AMPK activator [5-amino 4-imidazolecarboxamide riboside (AICAR)] or via electrodes (Bolster et al. 2002, Hayashi et al. 1998, Hutber et al. 1997, Ihlemann et al. 1999, Kurth-Kraczek et al. 1999, Tomas et al. 2002, Vavvas et al. 1997).

For a general effect of photoperiod (LD vs. SD) on the level of phosphorylated muscle enzymes, the time point of killing might not have been as important as for the detection of an effect of activity. Nevertheless, enzyme levels are strongly dependent on the activity level right before killing as short activity bouts of several minutes might be sufficient to affect the results (Winder and Hardie 1996). Unfortunately, in this study we did not record the activity level of hamsters right before killing and thus individuals might have been very active or, on the contrary, asleep.

Another important factor that is known to influence muscle enzyme phosphorylation is exercise intensity. The greater the running speed or the force production generated by contraction, the greater the activation of AMPK (Ihlemann et al. 1999, Rasmussen and Winder 1997). Hamsters in our study used their RW voluntarily and the analysis of recorded revolutions revealed a large variation in wheel running intensity between the individuals.

All the above mentioned factors might explain the large individual variation in the results of our experimental groups. Thus, to improve experimental conditions in future studies, the time point of killing should be assessed directly after exercise. Furthermore, exercise intensity should be controlled, e.g. by placing hamsters into a closed RW that rotates with defined speed so that the hamsters are forced to run a defined amount of revolutions per time. In

addition, sedentary control hamsters without access to a RW should be observed by infrared motion detectors to be able to compare their individual activity quantitatively.

For analysis of serum insulin concentrations the energetical state before and during exercise is important as insulin levels change according to blood glucose levels. In our experiment, the hamsters received food *ad libitum* until directly before killing. Therefore, a schedule for food intake should be used in future studies to standardize the time-lag between the last meal and the time point of sampling. Nevertheless, results might hint at slightly increased insulin levels in the SD-RW group compared to SD-C, although this finding was not significant. In obese mammals that developed insulin resistance, elevated plasma insulin levels can be found as well. In these individuals, insulin-stimulated glucose transport and uptake is diminished in glucose absorbing organs. As a consequence, the blood glucose level is elevated and the pancreas increases insulin secretion. But previous studies showed that exercising hamsters increase their body mass without increasing fat mass (Scherbarth et al. 2007). Furthermore, it was shown that exercise counteracts insulin resistance. An important role in this process plays AMPK. In animal models of insulin resistance, AMPK activity was found to be low (for review, see Winder and Hardie 1999). However, activation of AMPK via AICAR or through exercise training has been shown to effectively counteract insulin resistance via improved glucose transport and fatty acid oxidation (Brandt et al. 2010, Buhl et al. 2002, Iglesias et al. 2002, Kraegen et al. 1989). Thus, the development of insulin resistance in exercising hamsters seems to be unlikely.

A previous study found decreased serum IGF-1 levels in trained rats that voluntarily used a RW for 12 weeks, compared to untrained controls (Matsakas et al. 2004). However, exercising individuals decreased their body mass and thus the authors suggested that wheel running may diminish anabolic stimuli in rats. This is in contrast to our findings in the hamster where anabolic stimuli are induced through wheel-running activity, demonstrated by an increased body mass. Thus, we would expect an elevated IGF-1 plasma level in our study. However, the above mentioned unregulated experimental parameters might have contributed to the absence of an effect of wheel-running activity on serum IGF-1 levels. Like with insulin, there might at best be a faint hint to slightly elevated IGF-1 concentrations in the SD-RW group compared to SD-C.

In conclusion, the results of this study do not allow a statement about how wheel-running activity and photoperiod might influence phosphorylation of enzymes involved in muscle metabolism and concentrations of plasma insulin and IGF-1. However, we could show that

Western blotting, using above-mentioned antibodies, seems to be an appropriate method to detect defined enzymes in the gastrocnemius muscle of Djungarian hamsters.

In future studies, another factor that could be analysed is interleukin 6 (IL-6). IL-6 is a cytokine secreted by skeletal muscle ("myokine") that initiates a signalling cascade similar to that of leptin (for review, see Pedersen and Febbraio 2008). With highest increases of plasma IL-6 in response to running, the peak occurs at the end or shortly after exercise, when muscle glycogen stores are depleted. IL-6 activates AMPK (Kelly et al. 2004), p44/42 MAPK and PI3K (Al-Khalili et al. 2006) to increase glucose uptake and fatty acid oxidation. Therefore, IL-6 might represent another systemic signal to trigger physiological adaptations at the whole body level and thus it might be involved in the body mass response to photoperiod and wheel-running activity in Djungarian hamsters.

CHAPTER 5

Seasonal changes in hypothalamic gene expression in relation to the body weight cycle

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Abstract

The Djungarian hamster (*Phodopus sungorus*; also known as Siberian hamster) is a seasonal mammal, exhibiting annual cycles of reproduction, fur colouration and body mass to adapt to changing environmental conditions during the course of a year. Central mechanisms, induced by changes in photoperiod-driven gene expression in the brain, are expected to underpin the seasonal physiological responses. Several previous studies have investigated the temporal expression of hypothalamic genes in hamsters after the transfer from long to short days (SD) and the transfer back to long days (LD). Genes were suggested to be involved in the seasonal cycle of body weight if their expression was consistent with physiological responses. However, the temporal sequence of gene expression changes has not been previously investigated in Djungarian hamsters held in natural photoperiod over the course of one year. In this study, the pattern of hypothalamic expression for known genes proposed to be involved in body weight regulation of the Djungarian hamster [*nestin*, *vimentin*, type 2 deiodinase (*Dio2*), type 3 deiodinase (*Dio3*), monocarboxylate transporter 8 (*Mct8*), *Vgf* (nonacronymic), somatotropin release-inhibiting factor (*Srif*), G-protein-coupled receptor 50 (*Gpr50*) and cellular retinoic acid-binding protein 1 (*Crbp-1*)] were investigated in hamsters held in natural photoperiod and ambient temperature (T_a) (Hannover, Germany; 52°N latitude) for 12 months, spanning both winter and summer seasons. Changes in gene expression were related to body mass determined by weight measurements, and body composition determined via magnetic resonance imaging (MRI) scanning. MRI scan data revealed that primarily a change in fat mass, but also in lean mass contributes to the seasonal cycle in body mass. Gene expression data support the view that *nestin*, *vimentin*, *Dio2*, *Dio3*, *Mct8*, *Srif*, *Gpr50* and *Crbp-1* genes are involved in the SD-mediated loss in body mass as their expression changes prior to or in parallel to the body mass. The data are also consistent with the view that *Dio2*, *Mct8*, *Vgf* and *Srif* genes might be involved in the mechanism of photorefractoriness that underlies the increase in body weight from late winter and early spring on. Furthermore, this result indicates that these gene expressions may be independent of photoperiodically regulated morphological changes of tanycytes, since expression of genes for filament proteins including nestin and vimentin were delayed compared to the increase in body mass.

Introduction

Most species living at higher latitudes reproduce seasonally to adjust birth and rearing of offspring to optimal environmental conditions. Particularly a favourable climate and sufficient food supply are important for survival of the young. A well known seasonal animal is the Djungarian hamster *(Phodopus sungorus*) that not only reproduces seasonally [long day (LD) breeder; spring and summer], but also shows a seasonal cycle in body mass and pelage colour (Figala et al. 1973, Hoffmann 1973, Schlatt et al. 1993). The mediator of season is the humoral melatonin signal from the pineal gland. Melatonin is synthesized and secreted only during the night (Klein and Weller 1970). After the summer solstice, when days are getting shorter, the gradual prolongation of melatonin secretion induces the moult to a whitish winter fur, the decrease in body mass and gonadal regression in photosensitive Djungarian hamsters (Darrow and Goldman 1985, Hoffmann 1979, Hoffmann et al. 1986, Steinlechner et al. 1987). However, the seasonal cycle of physiological adaptations is not entirely in parallel to the annual cycle of photoperiod. The Djungarian hamster is able to anticipate the reproductive season in spring by developing photorefractoriness to short days (SD; winter) (for review, see Herbert 1989 and Prendergast 2005). Photorefractoriness in this species describes a spontaneous loss of responsiveness to the long melatonin signal in SD, the primary environmental factor inhibiting reproduction. When hamsters become photorefractory, they exhibit spontaneous recrudescence (Hoffmann 1978, 1979, Prendergast et al. 2006, Schlatt et al. 1993) whereupon gonadal maturation starts independently and in advance of the lengthening photoperiod. The increase in body mass and the moult to brown summer fur are closely connected with spontaneous recrudescence in Djungarian hamsters.

In experiments under natural photoperiod (Scherbarth et al. 2007, 2008), an increase in the hamsters' body mass is already observable three weeks after the winter solstice. At this time of the year, day length increased by about 32 minutes (~52°N). Furthermore, the weight gain appeared 19-22 weeks after the body mass peak in mid-August. Under artificial lighting conditions, photorefractoriness occurs after a similar time interval in SD (18-20 weeks) (Gorman and Zucker 1995, Hoffmann 1978, 1979, Kauffman et al. 2003, Prendergast et al. 2000, Schlatt et al. 1993, Teubner et al. 2008, Tups et al. 2006a). These findings indicate that not only under constant artificial lighting conditions but also under natural photoperiodic conditions photorefractoriness is likely to underlie the increase in body mass. However, in experiments under natural photoperiod a modulation of the body weight gain due to the slightly increasing day length cannot be ruled out.

After spontaneous recrudescence, the reproductively active state remains indefinitely, if there is no subsequent change in photoperiod. Hamsters require a period of about 10-15 weeks in LD to re-sensitize the neuroendocrine system, to be able to adapt to SD again (Bittman 1978, Kauffman et al. 2003, Reiter 1972, Stetson et al. 1977).

The mechanisms involved in the development of refractoriness are unknown yet. Nevertheless, it is assumed that the body weight gain, change to summer fur and recrudescence in spring occur as a result of the inability to read the SD melatonin signal, either through a de-sensitization of the melatonin receptor or a downstream signalling event (Bittman 1978, Reiter 1972).

A major brain centre involved in the regulation of body weight is the hypothalamic arcuate nucleus (ARC) and a number of photoperiodically regulated genes in the ARC of Djungarian hamsters have been identified in previous studies (Barrett et al. 2005, 2006, 2007, Drew et al. 2001, Herwig et al. 2009, Ross et al. 2004, 2005). In these experiments, hamsters experienced artificial photoperiod transitions from summer-like LD to winter-like SD photoperiod and subsequently back to LD. Gene expression in the ARC was investigated after 8-14 weeks in SD when body mass had decreased and at 2, 4 and 6 weeks after returning to LD by which time an increase in body mass and testicular recrudescence had occurred. If differential gene expression between both groups occurred ahead or in parallel with the weight change, the corresponding gene was suggested to be involved in the mechanism underlying the photoperiod-mediated change in body weight.

In this study, two groups of male Djungarian hamsters experienced the transition from winter to summer and vice versa under natural photoperiod and ambient temperature (T_a). In the course of one year, we examined the hypothalamic expression of photoperiodically regulated genes that are assumed to be involved in the regulation of the seasonal body weight cycle. Via comparison of the seasonal body mass cycle with gene expression in the winter to summer transition (photorefractory hamsters) and summer to winter transition (photosensitive hamsters), we are able to provide further support for gene expression changes underlying the seasonal cycle in body weight.

Materials and Methods

Animals and tissue collection

Djungarian hamsters (*Phodopus sungorus*) were reared under natural photoperiod and natural T_a (Hannover, Germany; ~52°N latitude). They were kept singly in polycarbonate cages (20.7 x 14 x 26.5 cm) and supplied with breeding diet (Altromin 7014) and tap water *ad libitum*, supplemented by a slice of apple once a week. Cages were provided with wood shavings and tissue for nest building.

This study contains two experimental parts. In one part, hamsters were sacrificed in the course of one year from winter (January, SD) to summer (June, LD). A total of 62 male hamsters, born under natural photoperiod and T_a from March to July 2008, were killed every 2-4 weeks (N = 6-7) starting from January 2009. Correspondingly, these animals had experienced one winter and were at the age of 9-13 months at the respective time point of killing. In the second experimental part, 42 male hamsters were sacrificed during the transition from summer (June, LD) to winter (December, SD). All animals were born outside, from March to April 2009. Seven animals were killed every 4-6 weeks starting in June 2009 until December. They were 3-9 months of age when they were culled 3-4 h relative to sunrise. All animals were weighed weekly throughout the experiment.

For experimental controls, twelve male hamsters, born outside from March to July 2008, were transferred to an artificial light-dark cycle of 16 h of light and 8 h of darkness [LD; dim red light (5 lux) during the dark phase] at 20 ± 2 °C at the equinox in March 2009. Thus, these hamsters had experienced one winter outside. Eight weeks later, six hamsters were transferred to an artificial light-dark cycle of 8 h of light and 16 h of darkness (SD; SD-C). Remaining six individuals stayed in LD (LD-C). After twelve weeks, at the age of 11-17 months, all hamsters were killed 3-4 h after lights on.

Hamsters were killed with carbon dioxide. Brains were immediately dissected, frozen on dry ice and then stored at -80 °C for later procedure of *in situ* hybridizations. Whole animal bodies (minus the head) were sealed in plastic bags and also stored at -80 °C. Later, sealed carcasses were thawed and heated to 37 °C before being individually scanned by magnetic resonance imaging (MRI) (Echo MRI ™, Whole Body Composition Analyser, Echo Medical Systems, Houston, Texas).

Riboprobes

Riboprobes complementary to fragments of the required DNA sequences were generated from Djungarian hamster, mouse or rat brain cDNAs by RT-PCR as described previously [Drew et al. 2001 (*Gpr50*), Ross et al. 2004 (*Crbp-1*), Barrett et al. 2005 (*Vgf*), Barrett et al. 2006 (*nestin*), Barrett et al. 2007 (*Dio2, Dio3*), Ross et al. 2009 (*Srif*), Herwig et al. 2009 (*Mct8*, *vimentin*)]. Templates for riboprobe synthesis were generated by PCR amplification of the insert from plasmid DNA. M13 forward and reverse primers which spans both insert and polymerase transcription binding and initiation sites in the host vectors were used. One hundred µg of PCR product were used in an *in vitro* transcription reaction with T7, T3 or SP6 polymerases as appropriate in the presence of ^{35}S-uridine 5-triphosphate (Perkin-Elmer, Buckinghamshire, UK) for radioactive *in situ* hybridization.

In situ hybridization

Coronal sections of the hypothalamic ARC region (14 µm thick) were collected onto a set of 12 glass slides. Adjacent sections were mounted on consecutively numbered slides, permitting a number of mRNAs to be localised and quantified in each brain.

In situ hybridization was carried out as described previously (Morgan et al. 1996).

Briefly, frozen slides were fixed in 4% PFA in 0.1 m PBS, acetylated in 0.25% acetic anhydride in 0.1 m TEA, pH 8. Radioactive probes (approximately 10^6 cpm) were applied to the slides in 70 µl hybridization buffer containing 0.3 M NaCl, 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.05% tRNA, 10 mM DTT, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA and 10% dextran sulfate. Hybridization was performed overnight at 58 °C. Following hybridization, slides were washed in 4 x SSC (1 x SSC is 0.15 M NaCl, 15mM sodium citrate), then treated with ribonuclease A (20 µg/µl) at 37 °C and finally washed in 0.1 x SSC at 60 °C. Slides were dried and apposed to autoradiographic Biomax MR film (Kodak, Rochester, New York) for several hours to days.

Image analysis

Films were scanned at 600 dpi on an Umax scanner and quantification was carried out using Image J 1.37v software (Wayne Rasband, National Institutes of Health, USA). For each probe, three sections spanning a selected region of the hypothalamus were chosen for image analysis. Integrated optical density for each selected region was obtained by reference to a standard curve generated from the autoradiographic ^{14}C microscale (Amersham). An average (with SEM) for the integrated optical densities for all sections of one animal and for all

animals in one group was calculated. The highest value of one group in an assay was set to 100% expression value, and other treatment values were calculated accordingly.

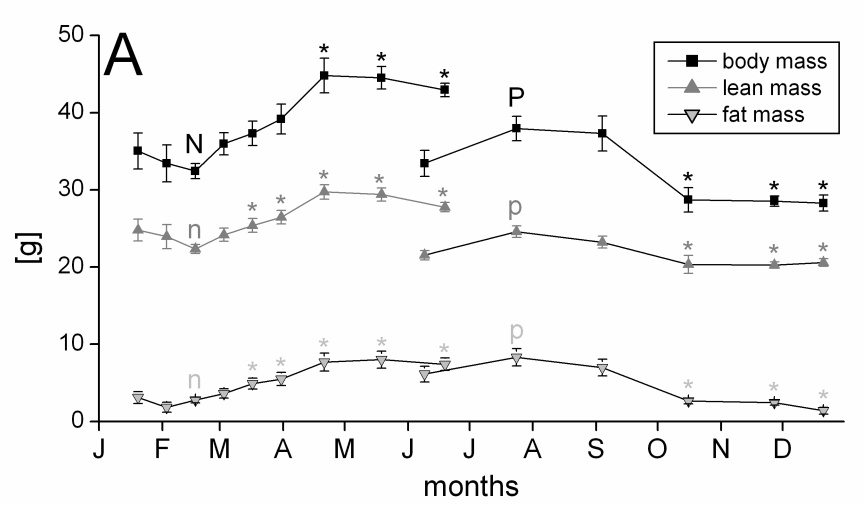
Statistical analysis

The body mass peak (P) in the transition from winter to summer was defined as the highest group average value and body mass nadir (N) in the transition from summer to winter as the lowest group average value. These values were used to compare body weights at other time points for the determination of body weight change. For lean mass, fat mass and gene expression, points in time corresponding to P or N were indicated as p and n, respectively. Similarly, all other values determined for body composition and gene expressions were compared with these defined reference points. Using this method we were able to determine whether lean and fat mass or gene expressions changed prior to or later than total body mass. Statistical tests applied in this study were one-way ANOVA with Student-Newman-Keuls post-hoc test for multiple comparisons where appropriate. Furthermore, for the comparison of gene expression between both control groups we applied t-test or, where normality test failed, Mann-Whitney-U-test. SigmaStat statistical software (Jandel) was applied, values are expressed as mean ± SEM and differences were considered significant if $P < 0.05$.

Results

Body composition

Djungarian hamsters significantly increased body mass during the course of the transition from winter to summer, reaching statistical significance from April onwards [week 17 post winter solstice (WS)] relative to the body weight nadir (*N*; 32.4 ± 1.0 g) in February (week 8 post WS) (one-way ANOVA, $P < 0.05$) (Figure 1A). Lean and fat mass significantly increased in March (week 12 post WS) relative to *n* (February) (one-way ANOVA, $P < 0.05$ for lean and fat mass). That is 5 weeks prior to the increase in body mass. Regarding the transition from summer to winter, body mass was significantly lower from October onwards [week 17 post summer solstice (SS)] relative to the body weight peak (*P*; 38.0 ± 1.6 g) in July (week 5 post SS) (one-way ANOVA, $P < 0.05$) (Figure 1A). Lean and fat mass decreased in parallel to the body mass and were significantly different to *p* (July) from October as well (one-way ANOVA, $P < 0.05$ for lean and fat mass).



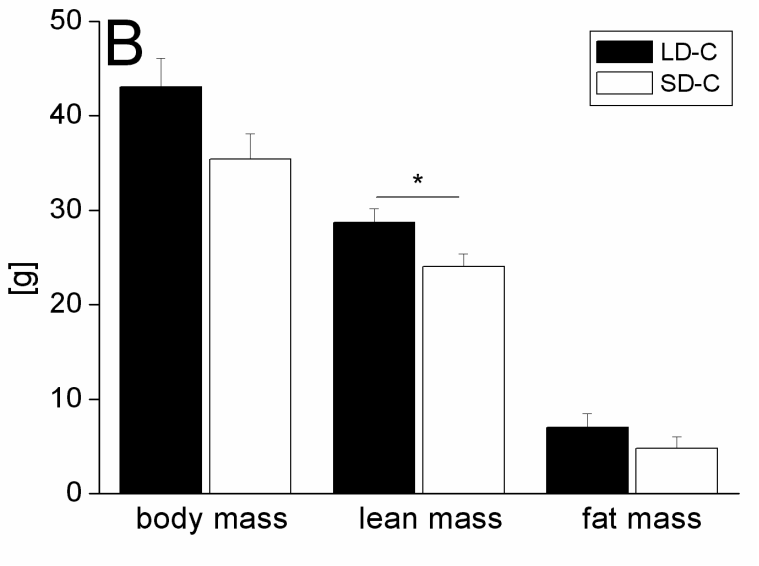


Figure 1: (A) Body mass of male Djungarian hamsters and corresponding lean and fat mass determined by MRI scan. Two groups of animals were kept outside under natural photoperiod and natural T_a (Hannover, Germany, 52°N latitude) and were killed in the course of one year (January to June and June to December, N = 6-7). *P*; body mass peak, all other body weights were compared with this value, *p*; time point corresponding to *P*, all other lean and fat mass values were compared with this value. *N*; body mass nadir and *n*; time point corresponding to *N*, following the same procedure as for *P* and *p*, *; P < 0.05 compared to *P*, *N*, *p* or *n*, respectively.

(B) Body mass and corresponding lean and fat mass determined by MRI scan in two groups of male Djungarian hamsters, kept in artificial LD (16 h of light and 8 h of darkness; LD-C) or SD (8 h of light and 16 h of darkness; SD-C) conditions at 20 ± 2 °C for 12 weeks. *; P < 0.05.

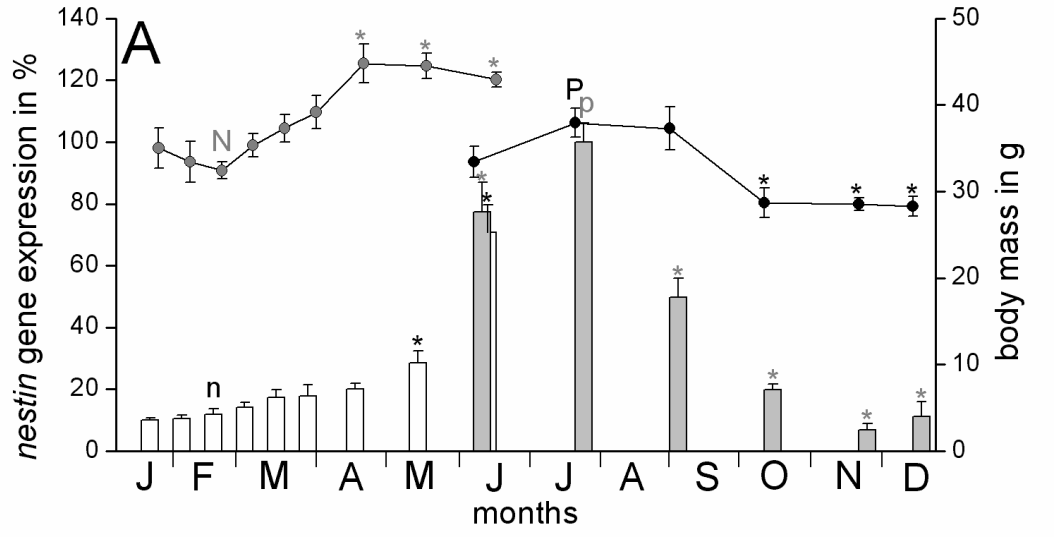
Body mass between the two experimental groups was significantly different in June (~1 week before the SS) (winter to summer vs. summer to winter, 42.9 ± 0.9 g vs. 33.4 ± 1.7 g; t-test; P < 0.01) as well as absolute lean mass (winter to summer vs. summer to winter, 27.8 ± 0.6 g vs. 21.5 ± 0.6 g; t-test; P < 0.01). Only absolute fat mass was similar in both groups in June (winter to summer vs. summer to winter, 7.4 ± 0.8 g vs. 6.1 ± 1.0 g). Body composition in percentage of total body mass was not significantly different between both groups at this time

(winter to summer: lean mass ~65%, fat mass ~17%; summer to winter: lean mass ~65%, fat mass ~18%).

Body mass and absolute fat mass in the LD-C and SD-C group did not differ significantly after 12 weeks (body mass 43.1 ± 3.0 g vs. 35.4 ± 2.7 g; fat mass 7.0 ± 1.4 vs. 4.8 ± 1.2 g) (Figure 1B), whereas absolute lean mass was significantly increased in LD-C compared to SD-C (28.7 ± 1.4 vs. 24.1. ± 1.3 g; t-test; $P < 0.05$). Body composition in% of total body mass did not differ between both groups (LD-C: lean mass ~67%, fat mass ~16%; SD-C: lean mass ~69%, fat mass ~13%).

Gene expression in tanycytes

Nestin and *vimentin* mRNA expressions were measured in tanycytes lining the 3rd ventricle in the hypothalamus of male Djungarian hamsters. During the transition from winter to summer, both gene expressions increased and were significantly up-regulated in May (week 21 post WS) relative to *n* (February) (one-way ANOVA, $P < 0.05$) (Figure 2A and B). Thus, both gene expressions increased 4 weeks later than body mass. Regarding the transition from summer to winter, *nestin* mRNA expression significantly decreased in September (weeks 11 post SS) relative to *p* (July) (one-way ANOVA, $P < 0.05$), which is 6 weeks prior to the significant decrease in body mass. Gene expression of *vimentin* decreased in parallel to the decrease in body mass and was significantly different to *p* (July) in October (one-way ANOVA, $P < 0.05$).



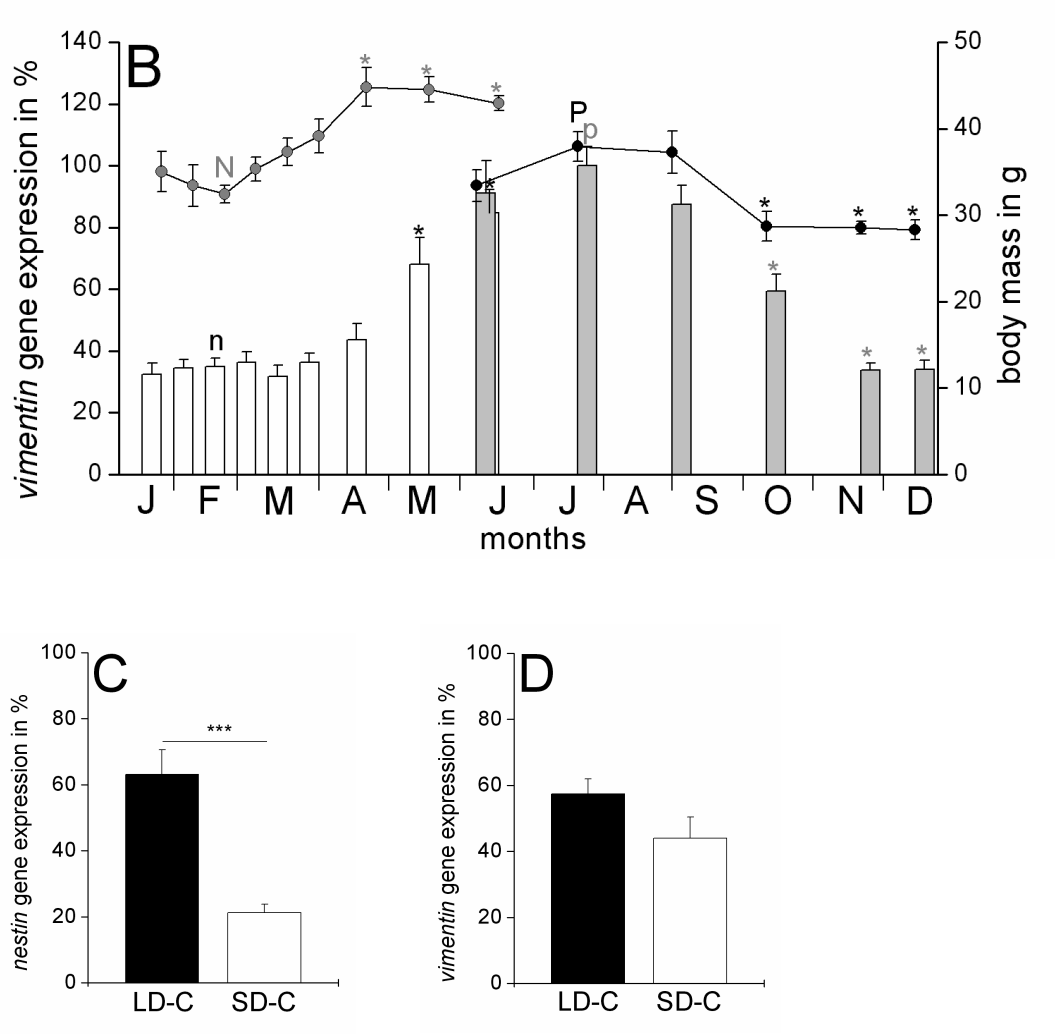
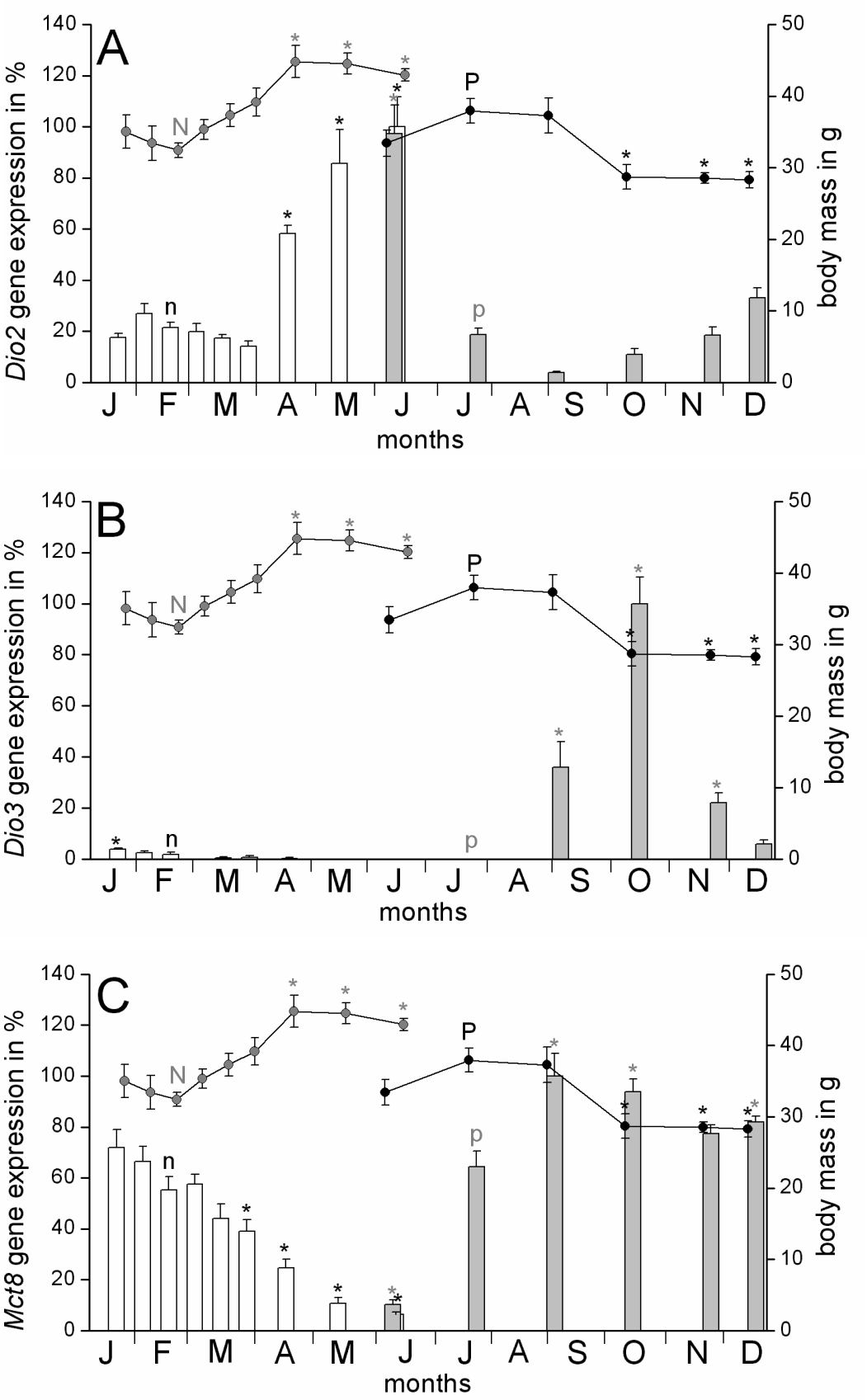


Figure 2: (A) *Nestin* and (B) *vimentin* mRNA expression in tanycytes lining the 3rd ventricle (columns) and corresponding body mass (dots) in male Djungarian hamsters. Two groups of animals were kept outside under natural photoperiod and T_a (Hannover, Germany, ~52°N latitude) and were killed in the course of one year (January to June and June to December, N = 6-7). *P*; body mass peak, all other body weights were compared with this value, *p*; time point corresponding to *P*, all other gene expression values were compared with this value. *N*; body mass nadir and *n*; time point corresponding to *N*, following the same procedure as for *P* and *p*, *; $P < 0.05$ compared to *P*, *N*, *p* or *n*, respectively.

(C) *Nestin* and (D) *vimentin* mRNA expression in two groups of male Djungarian hamsters, kept in artificial LD (16 h of light and 8 h of darkness; LD-C) or SD (8 h of light and 16 h of darkness; SD-C) conditions at 20 ± 2 °C for 12 weeks. ***; $P < 0.001$.

After 12 weeks under artificial lighting and T_a, *nestin* mRNA expression was significantly higher in hamsters of the LD-C group compared to SD-C (t-test, $P < 0.001$) (Figure 2C) but there was no difference in *vimentin* gene expression at this time point (Figure 2D).

Gene expression in the thyroid hormone system



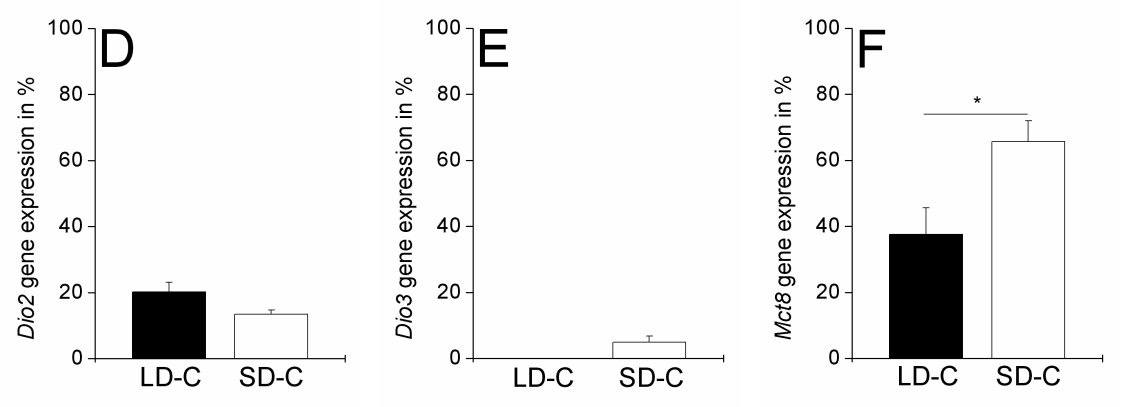


Figure 3: (A) Type 2, (B) type 3 deiodinase (*Dio2* and *Dio3*) and (C) monocarboxylate transporter 8 (*Mct8*) mRNA expressions in the 3rd ventricular tanycyte layer (columns) and corresponding body mass (dots) in male Djungarian hamsters. Two groups of animals were kept outside under natural photoperiod and natural T_a (Hannover, Germany, ~52°N latitude) and were killed in the course of one year (January to June and June to December, N = 6-7). *P*; body mass peak, all other body weights were compared with this value, *p*; time point corresponding to *P*, all other gene expression values were compared with this value. *N*; body mass nadir and *n*; time point corresponding to *N*, following the same procedure as for *P* and *p*, *; $P < 0.05$ compared to *P*, *N*, *p* or *n*, respectively.

(D) *Dio2*, (E) *Dio3* and (F) *Mct8* mRNA expression in two groups of male Djungarian hamsters, kept in artificial LD (16 h of light and 8 h of darkness; LD-C) or SD (8 h of light and 16 h of darkness; SD-C) conditions at 20 ± 2 °C for 12 weeks. *; $P < 0.05$.

Type 2 (*Dio2*) and type 3 (*Dio3*) deiodinase and monocarboxylate transporter 8 (*Mct8*) mRNA expression was measured in the 3rd ventricular tanycyte layer of the hypothalamus. *Dio2* gene expression was up-regulated in parallel to the increase in body mass during the transition from winter to summer, reaching significance from April onwards (week 17 post WS) relative to *n* (February) (one-way ANOVA, $P < 0.05$) (Figure 3A). During the transition from summer to winter, the *Dio2* mRNA expression level was higher in June (1 week before the SS) relative to p (July) (one-way, $P < 0.05$). Afterwards, gene expression decreased and stayed low during the rest of the year. Thus, *Dio2* gene expression decreased 12 weeks prior to the significant decrease in body mass (October).

Dio3 mRNA expression was very low in hamsters that underwent the transition from the winter to the summer phenotype (Figure 3B). Gene expression was highest in January (week 4 post WS) relative to *n* (February) (one-way ANOVA, $P < 0.05$) and was then almost undetectable until September. From September (week 11 post SS) to November (week 23 post SS), *Dio3* gene expression was significantly up-regulated relative to *p* (July) (one-way

ANOVA, $P < 0.05$) but in December (at the WS) *Dio3* gene expression decreased again and was not significantly different to *p* anymore. Relative to the increase in body mass, *Dio3* mRNA expression increased 6 weeks earlier.

In the course from winter to summer, *Mct8* mRNA expression decreased significantly relative to *n* (February) at the end of March (week 14 post WS) (one-way ANOVA, $P < 0.05$) (Figure 3C). That is 3 weeks prior to the significant change in body mass in April. During the transition from summer to winter, *Mct8* gene expression was significantly up-regulated in September (week 11 post SS) relative to *p* (July) (one-way ANOVA, $P < 0.05$). Thus, *Mct8* gene expression changed 6 weeks prior to the change in body mass.

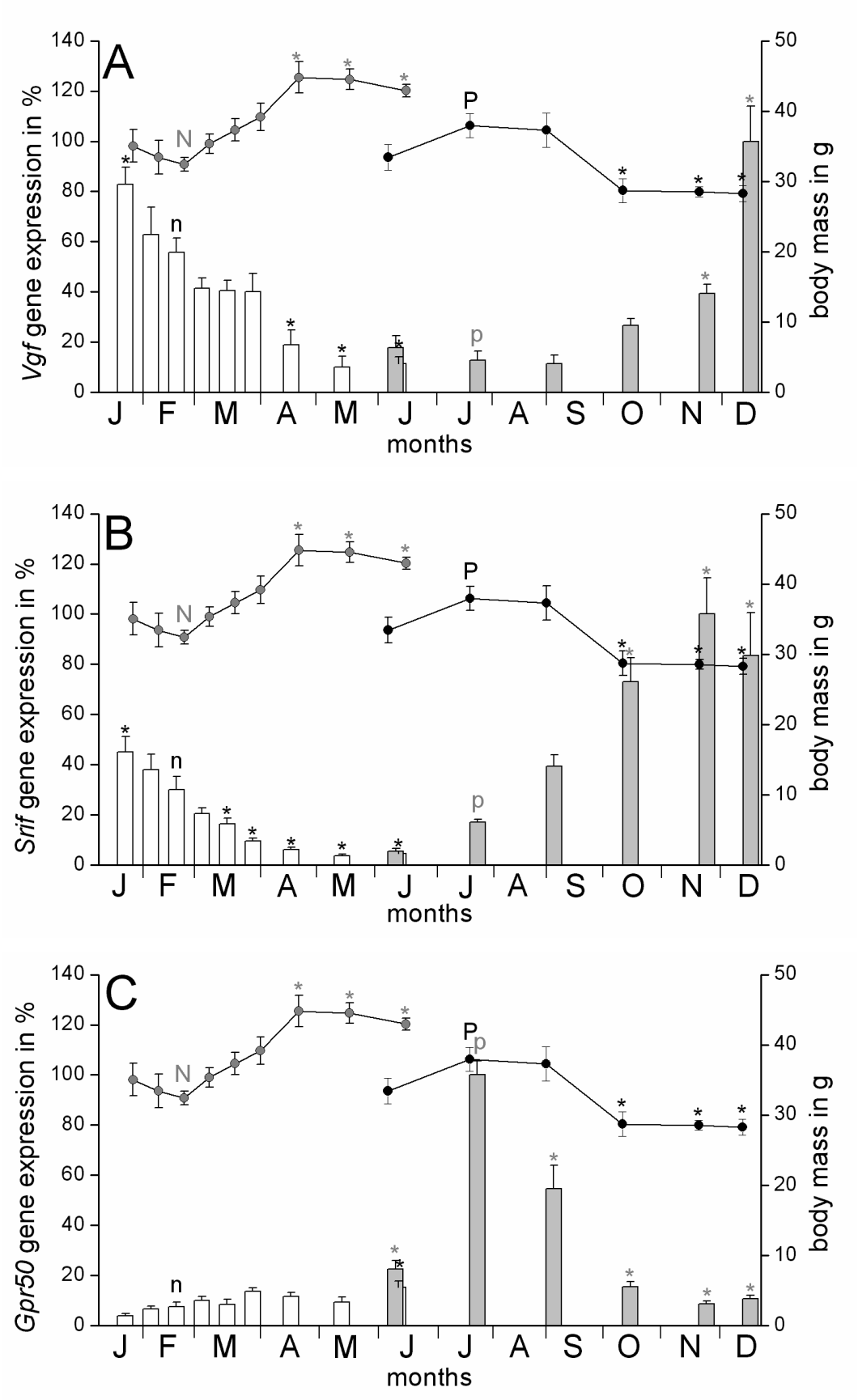
After 12 weeks, there was no difference in *Dio2* and *Dio3* mRNA expression in hamsters kept in artificial LD compared to SD (Figure 3D and E), whereas *Mct8* gene expression was significantly up-regulated in SD (t-test, $P < 0.05$) (Figure 3F).

Expression of seasonally regulated genes (*Vgf, Srif, Gpr50, Crbp-1)*

Vgf mRNA expression was quantified in the hypothalamic dorsal medial posterior arcuate nucleus (dmpARC). In hamsters that underwent the transition from winter to summer, *Vgf* mRNA expression was significantly up-regulated in January (week 4 post WS) and significantly down-regulated from April onwards (week 17 post WS) relative to *n* (February) (one-way, $P < 0.05$) (Figure 4A). Thus, *Vgf* gene expression decreased prior to the increase in body mass. In the course from summer to winter, *Vgf* gene expression was significantly up-regulated in November (week 23 post SS) relative to *p* (July) (one-way ANOVA, $P < 0.05$), which is 6 weeks later compared to the change in body mass.

Srif mRNA expression, measured in the ARC, decreased from summer to winter (Figure 4B). It was significantly up-regulated in January (week 4 post WS) and significantly down-regulated from March onwards (week 12 post WS) relative to *n* (February) (one-way ANOVA, $P < 0.05$) and, hence, decreased prior to the increase in body mass. During the second part of the experiment, *Srif* gene expression was up-regulated while the body mass decreased. From October onwards (week 17 post SS) there was a significant difference in *Srif* mRNA expression compared to *p* (July) (one-way ANOVA, $P < 0.05$).

In the transition from winter to summer, *Gpr50* mRNA expression in the ependymal layer of the 3rd ventricle significantly increased in June (1 week before the SS) relative to *n* (February) (one-way ANOVA, $P < 0.05$) (Figure 4C) and thus 8 weeks later than the first significant


A
Vgf gene expression in %
body mass in g
months
N
n
P
p
J F M A M J J A S O N D
B
Srif gene expression in %
body mass in g
months
C
Gpr50 gene expression in %
body mass in g
months

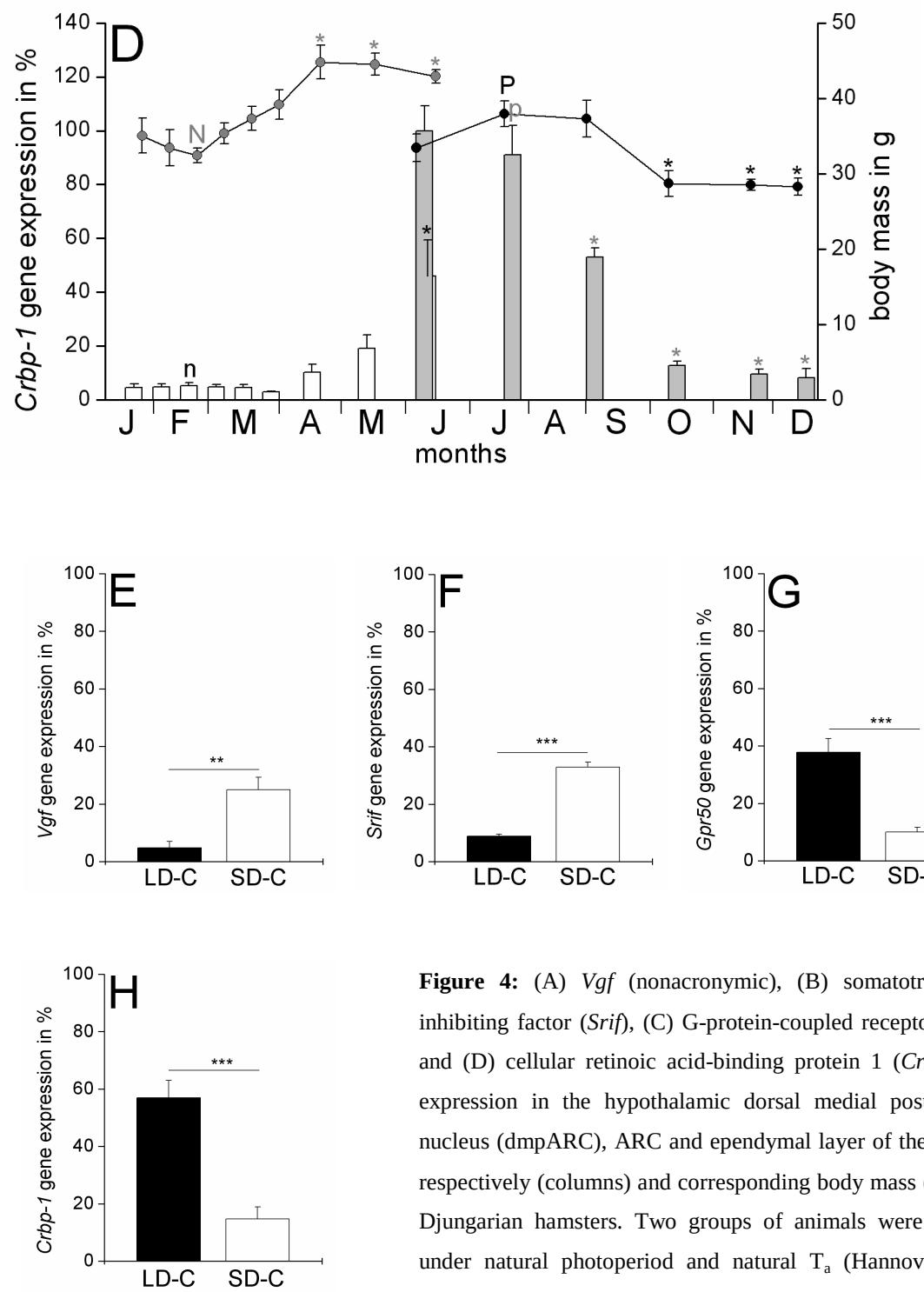


Figure 4: (A) *Vgf* (nonacronymic), (B) somatotropin release-inhibiting factor (*Srif*), (C) G-protein-coupled receptor 50 (*Gpr50*) and (D) cellular retinoic acid-binding protein 1 (*Crbp-1*) mRNA expression in the hypothalamic dorsal medial posterior arcuate nucleus (dmpARC), ARC and ependymal layer of the 3rd ventricle, respectively (columns) and corresponding body mass (dots) in male Djungarian hamsters. Two groups of animals were kept outside under natural photoperiod and natural T_a (Hannover, Germany, ~52°N latitude) and were killed in the course of one year (January to June and June to December; N = 6-7). *P*; body mass peak, all other body weights were compared with this value, *p*; time point corresponding to *P*, all other gene expression values were compared with this value. *N*; body mass nadir and *n*; time point corresponding to *N*, following the same procedure as for *P* and *p*, *; $P < 0.05$ compared to *P*, *N*, *p* or *n*, respectively.

(E) *Vgf*, (F) *Srif*, (G) *Gpr50* and (H) *Crbp-1* mRNA expression in two groups of male Djungarian hamsters, kept in artificial LD (16 h of light and 8 h of darkness; LD-C) or SD (8 h of light and 16 h of darkness; SD-C) conditions at 20 ± 2 °C for 12 weeks. **; $P < 0.01$, ***; $P < 0.001$.

difference in body mass (April). During the transition from summer to winter, *Gpr50* gene expression was at a peak in July (*p*; week 5 post SS) relative to June (1 week before the SS). The expression showed an increase between June and July, but then decreased once more from September (week 11 post SS) (one-way ANOVA, $P < 0.05$). Thus, *Gpr50* mRNA expression decreased 6 weeks prior to the body mass (October).

In hamsters that underwent the transition from winter to summer, *Crbp-1* mRNA expression in the ependymal layer of the 3rd ventricle was significantly up-regulated in June (1 week before the SS) relative to *n* (February) (one-way ANOVA, $P < 0.05$) (Figure 4D). Thus, *Crbp-1* gene expression increased 8 weeks later than body mass. In the transition from summer to winter, *Crbp-1* gene expression was significantly down-regulated in September (week 11 post SS) relative to *p* (July) (one-way ANOVA, $P < 0.05$), which is 6 weeks prior to the change in body mass.

After 12 weeks under an artificial LD or SD light regimen, *Vgf* and *Srif* mRNA expression were significantly higher in the SD-C group compared to LD-C (t-test, $P < 0.01$ and $P < 0.001$, respectively) (Figure 4E and F). On the contrary, *Gpr50* and *Crbp-1* mRNAs were significantly higher in the LD-C group compared to SD-C (t-test, $P < 0.001$ for both gene expressions) (Figure 4E and F).

Discussion

This is the first study to investigate and relate changes in body mass with gene expression changes in the hypothalamus of the Djungarian hamster over the course of one year covering transitions from summer to winter and winter to summer. At ~52°N latitude (Hannover), at the SS, day length is 16 hours and 48 minutes from sunrise to sunset and at the WS day length is only 7 hours and 41 minutes. Hannover is located at a latitude that is within the geographical range of the natural distribution area of Djungarian hamsters (Western Siberia and Eastern Kazakhstan). Therefore the data gathered in this study is likely to reflect similar changes that occur in wild populations of Djungarian hamsters.

To date, several genes, such as *nestin*, *vimentin*, *Dio2*, *Dio3*, *Mct8*, *Vgf*, *Srif*, *Gpr50* and *Crbp-1* are suggested to be involved in the Djungarian hamsters' seasonal regulation of body mass, which is associated with a seasonal cycle of reproduction and fur colouration (Figala et al. 1973, Hoffmann 1973, Schlatt et al. 1993). Consistent with the change in body mass, the

expression of these genes has been shown to be regulated by photoperiod in the hypothalamic ARC after defined periods (8-14 weeks) under artificial LD or SD conditions (Barrett et al. 2005, 2006, 2007, Drew et al. 2001, Herwig et al. 2009, Ross et al. 2004, 2009). In our study, male Djungarian hamsters were kept outside under natural T_a and photoperiod to track gene expression changes in the course of one year with its inherent gradual change in photoperiod. In photosensitive hamsters that experienced the transition from summer (LD) to winter (SD), we could show a seasonal mRNA expression of *nestin*, *vimentin*, *Dio2*, *Dio3*, *Mct8*, *Srif*, *Gpr50* and *Crbp-1* (Figure 2, 3 and 4). As expressions changed in parallel to or prior to the decrease in body mass, the data confirm the assumption that these genes might be involved in the mechanisms underlying the annual decrease in body mass in autumn. The only gene, whose expression was significantly up-regulated one month later compared to the decrease in body mass was *Vgf* (Figure 4A). However, this result has to be considered cautiously as *Vgf* was the only gene that was quantified in the dmpARC. Since the dmpARC is a rather small hypothalamic area, only very few brain slices per animal could be analysed. Some animals even had to be excluded from analyses as the dmpARC region was not detectable on the slices. Thus, without reduced sample size, significance of *Vgf* from values at *n* or *p* (corresponding to the time point of the nadir or peak in body mass), may have occurred earlier than indicated where significance was achieved.

Circulating thyroid hormone (triiodothyronine; T_3) is known to regulate energy expenditure and body weight in mammals (for review, see Herwig et al. 2008). An important enzyme that regulates the availability of T_3 to the brain is DIO3. It is located in the ependymal layer of the 3rd ventricle and converts T_3 to inactive diiodothyronine (T_2). In the present study, *Dio3* expression was detectable for the first time in September, i.e. after eleven weeks in decreasing photoperiod. *Dio3* mRNA expression reached a peak in October (17 weeks after the SS) and then declined again despite the ongoing decrease in photoperiod. Expression of *Dio3* remained low thereafter. This indicates that *Dio3* gene expression might have become refractory to the SD signal. An early decline in *Dio3* gene expression under SD conditions might contribute to an early increase of T_3 availability in the brain, like under LD conditions. Thus, *Dio3* refractoriness to SD might present a mechanism underlying the overall process of photorefractoriness, thereby triggering LD physiology. Studies of Barrett (2007) and Watanabe (2007), performed under artificial photoperiod, observed a peak in *Dio3* mRNA expression after 6-8 weeks in SD. Subsequently, the expression decreased reaching only 40% of peak value by 14 weeks in SD and was undetectable after 27 weeks in SD, when animals were then photorefractory. This result concerning photorefractory hamsters is in accordance

with the findings in hamsters that experienced the transition from winter to summer in our study. *Dio3* expression was very low in January and February and it was undetectable since then.

Several studies that analysed *Dio2* gene expression in Djungarian hamsters under artificial lighting conditions revealed inconsistent results (Barrett et al. 2007, Herwig et al. 2009, Watanabe et al. 2004, 2007). To explain this, the authors suggested a time-dependent effect and an influence of the photoperiodic history of the animals on *Dio2* gene expression. Like DIO3, DIO2 is located in the ependymal layer of the 3rd ventricle but in contrast to DIO3, it activates the thyroid prohormone T_4 by conversion to T_3 (for review, see Herwig et al. 2008).

Watanabe et al. (2004, 2007) found an induction of *Dio2* expression in juvenile hamsters that were transferred to SD after weaning until 7 weeks of age, then maintained in SD or were transferred to LD for further 2 weeks. However, in hamsters kept in LD after weaning and then transferred to SD, the expression of *Dio2* decreased in the LD and SD group at a similar rate. This decline in both groups might reflect an age related change and the hamsters mature. Further changes then seem not to occur in SD when *Dio2* levels are established. Consequently, there was no difference in *Dio2* mRNA expression between both groups after 6 weeks, or after 27 weeks in SD when hamsters will have become photorefractory to SD. Consistently, in a study of Barrett and coworkers (2007) where hamsters were also transferred from LD to SD, they did not find a difference in *Dio2* gene expression between the LD and SD group after 14 weeks. This is also in accordance with the result of both control groups in this study. In our study, after 12 weeks in artificial photoperiod there was no difference between LD-C and SD-C (Figure 3D). However, Herwig and coworkers (2009) found a difference in *Dio2* expression between LD and SD animals after 8 weeks, although animals in the SD group were transferred from LD to SD and thus treated similar to those in the study of Barrett and coworkers (2007) and animals in the control groups of our study. The authors suggested a time-dependent change of *Dio2* gene expression. However, in another study under similar conditions, we could not find a difference in *Dio2* gene expression after 8 weeks (see chapter 3).

Altogether, in our experiment under natural photoperiod and T_a *Dio2* and *Dio3* mRNA expression seemed to be inversely regulated. We found significantly elevated *Dio2* mRNA expression levels in June in both experimental groups, independent of their photoperiodic history (transition from natural SD to LD or LD to SD). Watanabe and coworkers (2007) found a low mRNA expression of *Dio2* in SD refractory hamsters that were kept in SD for 27 weeks. However, in our study *Dio2* expression increased in hamsters that became

photorefractory (transition from winter to summer). We cannot rule out an effect of T_a on *Dio2* expression in our experiment. Collectively, the new findings indicate that further research is necessary to characterize seasonal *Dio2* gene expression and the underlying mechanisms.

In photorefractory hamsters that experienced the natural transition from winter to summer, we found that *Dio2*, *Mct8*, *Vgf* and *Srif* mRNA levels changed either in parallel or prior to the change in body mass. Therefore, we suggest that these genes might be involved in the mechanism underlying the increase in body weight in photorefractory Djungarian hamsters. However, the change in these mRNA expressions might occur independently of the morphological state in the tanycytes. The up-regulation of both *nestin* and *vimentin* mRNA, which are markers for tanycytes in the ependymal layer, was delayed relative to the increase in body mass. Nestin and vimentin are intermediate filaments that constitute an element of the cytoskeletal architecture. They are involved in axonal growth and may have a role to play in tanycyte morphology. *Vimentin* has previously been shown to be down-regulated in SD in the Djungarian hamster, consistent with a retraction of tanycyte end feet from basal epithelium of the brain (Kameda et al. 2003). This potentially facilitates access of neuronal axons to the portal blood system of the ME. The findings that both, morphological changes of tanycytes and *vimentin* and *nestin* expression in tanycytes are photoperiodically regulated in the Djungarian hamster indicate that tanycytes and the ependymal layer might play a role in seasonal responsiveness (Barrett et al. 2006, Herwig et al 2009, Kameda et al. 2003, Xu et al. 2005). Tanycytes may be involved in the mechanism associated with the seasonal body mass decline in photosensitive hamsters, as *nestin* and *vimentin* expression decrease prior to the decrease in body mass (Figure 2A and B). However, our results show that *vimentin* and *nestin* gene expression is delayed relative to the change in body mass in the transition from winter to spring. This finding indicates that morphological changes of tanycytes may not be involved in the physiological adaptations occurring during spring.

Gpr50 and *Crbp-1* mRNA expression significantly increased in June, which is eight weeks later compared to the significant increase in body mass in April in photorefractory hamsters (Figure 4C and D). CRBP-1 is a retinol transport protein that was reported to be photoperiodically regulated in the Djungarian hamster (Barrett et al. 2006, Ross et al. 2004). GPR50 is an orphan G-protein-coupled receptor, but its localisation in tanycytes and its regulation by photoperiod in the Djungarian hamster suggest a role in communication between the hypothalamus and the pituitary gland (Barrett et al. 2006, Drew et al. 2001, Sidibe et al. 2010). However, like *vimentin* and *nestin*, *Gpr50* and *Crbp-1* gene expression do

not seem to be essentially involved in the mechanism leading to the body mass increase in spring.

Lean und fat mass significantly increased five weeks prior to the significant increase in total body mass during the natural transition from SD to LD (Figure 1A). This was probably due to the different methods of measurement we applied to determine total body mass and body composition. We excluded body water from analyses, as hamsters lost variable amounts of blood at killing, due to brain and organ dissection. Furthermore, carcasses have been frozen initially and were thawed later for carrying out the MRI scan. Both situations might have caused individual differences in body water content that cannot be distinguished when measuring total body mass.

In our experiment we could show that in addition to the seasonal cycle in fat mass, a seasonal cycle in lean mass contributed to the cycle in total body mass. In animals exposed to the transition from winter to summer, fat mass increased 2.8-fold and lean mass increased 1.3-fold from February (*n*) to April, where body mass was significantly increased compared to *N* (Figure 1A). During winter acclimatization, fat mass decreased 3.1-fold and lean mass 1.2-fold between July (*p*) and October, where body mass was significantly lower compared to *P*. In a previous study the authors stated that the decrease in body mass in SD in Djungarian hamsters was almost entirely due to a reduction in fat mass (Wade and Bartness 1984). However, measurement of body composition in a study of Klingenspor and coworkers (2000) revealed that the SD-mediated decrease of body mass is equally due to a reduction of fat mass and fat-free mass. Our study indicates that the contribution of fat mass to the seasonal cycle in body mass might reside in between the suggestions of both studies.

In the study of Klingenspor and colleagues (2000), they found a 50% decrease of fat mass in SD control hamsters compared to LD controls, which we did also find in another study (see chapter 3) but not in the control hamsters of this study (Figure 1B). In addition, there was no significant difference in total body mass after 12 weeks. This might indicate that the control hamsters of our study were unsuitable to get reliable data of gene expression and body composition comparable to other studies. An explanation might be the old-age (11-17 months) of our control hamsters at the time points of sampling together with the fact that hamsters of both control groups had experienced one winter outside before they were transferred to artificial LD and finally to SD again. However, 12 weeks under increasing natural photoperiod and subsequently 8 weeks under artificial LD should have been sufficient to re-sensitize the neuroendocrine system, to be able to adapt to SD again (Bittman 1978, Kauffman et al. 2003, Reiter 1972, Stetson et al. 1977). Nevertheless, Figala and coworkers

(1973) discovered that most of the hamsters experiencing autumn/winter conditions for the second time in their short life, either develop considerably reduced SD responses or do not respond at all.

This might also explain the lacking difference in *Dio3* mRNA expression between the LD and SD-C group after 12 weeks (Figure 3E). The results indicate that the amplitude of seasonal *Dio3* gene expression might be strongly dampened in the control hamsters of our study, hence not reaching statistical significance. The same might be true for the *vimentin* mRNA expression. Generally, we noticed that the expression level of genes in both control groups under artificial lighting conditions was lower compared to animals under natural photoperiod and T_a. These hamsters have been 9-13 months of age at the time point of sampling. Furthermore, we cannot rule out an effect of constant vs. changing temperature and photoperiod on the processes of seasonal acclimatization. However, for *Crbp-1, Gpr50, nestin, Mct8* and *Vgf* genes we found photoperiod-dependent mRNA expression levels in our control hamsters consistent with previous studies (Barrett et al. 2005, 2006, Herwig et al. 2009).

In summary, we demonstrate a possible involvement of *nestin*, *vimentin*, *Dio2*, *Dio3*, *Mct8*, *Srif, Gpr50* and *Crbp-1* genes in the seasonal body mass regulation in photosensitive Djungarian hamsters. In photorefractory hamsters, only *Dio2, Mct8, Vgf* and *Srif* mRNA expressions were found to change prior to the increase in body mass. Thus, only these genes might be involved in the mechanisms leading to the weight gain in late winter/spring. In contrast, *nestin* and *vimentin* gene expression in tanycytes was delayed relative to the body mass increase. However, future studies should further investigate the varying gene expression of *Dio2*. Furthermore, an increase in sampling points during the course of one year and the measurement of the gonadal cycle in addition to the body mass cycle would increase precision and significance of follow-up studies.

CHAPTER 6

GENERAL DISCUSSION

Djungarian hamsters are known for their high motivation to run in a wheel but the origin of this behaviour remains speculative. However, voluntary running is clearly associated with increased energy expenditure. Similarly, reproduction that is essential for the survival of the species is also associated with high energetic costs, especially for the females. Thus, in our first study (chapter 2) we investigated the challenge and influence of voluntary wheel-running activity on reproductive success. The outcome was an increased prevalence of infanticide and cannibalism in exercising breeding pairs and singly kept females, presumably to compensate for the energy deficit caused by wheel-running activity. Only a few females reduced the amount of wheel revolutions after parturition and weaned several pups. This difference between individuals might be explained by a differing propensity to wheel-running activity as it has been shown in previous studies (Scherbarth et al. 2007, 2008). Another result was that wheel-running activity seemed to prevent successful pregnancy. We hypothesized that this might have been due to unfavourable energetical preconditions for reproduction. In previous studies it was shown that exercising hamsters increased body mass without increasing plasma leptin levels (Scherbarth et al. 2007). This could be explained by the fact that the increase in body mass was due to an increase in lean but not fat mass and leptin is known to be adipocyte-derived (for review, see Ahima and Flier 2000). Leptin stimulates hypothalamic gonadotropin-releasing hormone (GnRH) release and via a subsequent signalling cascade including the release of follicle stimulating hormone (FSH) and luteinising hormone (LH) from the pituitary, GnRH regulates the oestrus cycle (for review, see Popovic and Casanueva 2002). In exercising females, an impaired oestrus cycle due to low plasma leptin concentrations might have prevented pregnancy (similar to exercise-induced amenorrhea in women). Furthermore, leptin plays a role in maternal investment and was shown to reduce infanticide in Djungarian hamsters (French et al. 2009). From our data we do not know whether pups that were born by an exercising female were fed directly after birth or whether they died through starvation or hypothermia due to negligence of the parents prior to cannibalization. Thus, in future studies video recordings should track the behaviour of exercising breeding pairs directly after parturition. Thereby, further insight into behavioural changes, such as maternal care, in wheel-running hamsters could be gained. In addition, plasma leptin levels of the females should be determined as well as other hormonal concentrations, such as prolactin that is involved in male and female fertility, pup-induced maternal behaviour and the control of lactation (for review, see Bachelot and Binart 2007).

In the experiment described in chapter 3, we observed a weight gain in hamsters with access to a RW, independent of the LD or SD photoperiod, as shown in previous studies (Scherbarth

et al. 2007, 2008 and chapter 2). Although energy expenditure and cumulative food intake increased in hamsters that voluntarily ran in a wheel for 12 weeks, orexigenic and anorexigenic gene expressions in the hypothalamic ARC that regulate energy homeostasis were not affected. One exception was *Pomc* whose expression significantly increased in the SD-RW group compared to SD-C after 12 weeks. Likewise, we found an up-regulated *Vgf* gene expression in the dmpARC of both RW groups. These increases in gene expression are counterintuitive since on the one hand, POMC- and VGF-derived peptides are known to act catabolic (for reviews, see Jethwa and Ebling 2008, Mountjoy 2010) but on the other hand, both RW groups increased body mass compared to the controls. However, we found absolute food intake of exercising hamsters to be almost unaltered after 12 weeks compared to the first week of the experiment. Thus, increased levels of POMC and VGF, probably triggered by the increased body mass that deviates from the seasonal set point, might counteract an increase in food intake. Nevertheless, the activated melanocortin pathway and increased secretory and signalling activity of the dmpARC seem not to be strong enough to affect body mass. A possible explanation could be that both catabolic systems might be overridden by anabolic mechanisms like growth, whose involvement in the increase in body mass of exercising hamsters could be confirmed in this study via determination of *Srif* gene expression. Future studies might additionally analyse the impact of voluntary exercise on gene expression of the melanocortin system in the brainstem [parabrachial nucleus (PBN) and nucleus of the solitary tract (NTS)]. Both brain nuclei are involved in appetite and feeding in rodents (for review, see Scott and Small 2009) and many neuropeptides and receptors involved in the regulation of energy balance in the hypothalamus are also present in this area (Joseph et al. 1983). A previous study in rats revealed that activation of the melanocortin system by *Pomc* gene transfer to the NTS counteracts diet-induced obesity. However, an identical treatment in the ARC failed to do so (Zhang et al. 2010). Furthermore, the expression of MC4 receptors in this brain area was shown to be regulated by photoperiod in the Djungarian hamster (Helwig et al. 2009).

To further investigate a potentially increased secretory activity of the dmpARC in exercising hamsters, gene expression of the secretogranins *SgIII* and *VI* that are involved in secretory processes (Nilaweera et al. 2009) should be quantified. In addition, gene expression of other seasonally expressed neuropeptides in the dmpARC, such as MC3 receptor and serotonin receptors 5-HT-2A and 5-HT-7 might be determined. If actually, under the influence of wheel-running activity the intracellular signalling pathways in the dmpARC are activated, these gene expressions might be up-regulated.

Our hypothesis that wheel-running activity may affect photoperiod-driven gene expressions involved in the regulation of body weight to facilitate a weight gain in RW hamsters (especially in SD) seems not to include the thyroid system. This system is known to be important for the regulation of energy expenditure (for review, see Herwig et al. 2008). However, there was no difference between the RW groups and their respective controls in any of the investigated gene expression. Based on this results, we could show that the perception of photoperiod at the level of the hypothalamus was not affected by voluntary wheel running. The same was shown in the golden hamster (*Mesocricetus auratus*) by demonstrating that the ability of the SCN to integrate the photoperiodic change from LD to SD was not modified in exercising individuals (Menet et al. 2005). As hypothesized in their study, we then suggested that downstream physiological and metabolic responses might be involved in the exercise-induced weight gain.

Therefore, in the next study (chapter 4) we analysed skeletal muscle metabolism based on enzyme phosphorylation levels in the gastrocnemius muscle. Since peripheral skeletal muscles show a high plasticity to adapt to exercise training, we hypothesized that they might secrete factors that feed back to the brain and thus are involved in the mechanisms overriding the seasonal processes of body weight regulation. However, our results revealed neither an impact of photoperiod nor activity on enzyme phosphorylation. This might have been due to the inappropriately chosen sampling point 3-4 hours after the lights went on and thus not close enough to the nocturnal exercise bout. Furthermore, activity intensity and food intake were not standardized in our experiment, leading to a large individual variability in the measured parameters.

In previous studies it was shown that besides fat mass, lean mass (mainly muscle mass) contributes to the seasonal cycle in body weight as well (Klingenspor et al. 2000 and chapter 3 and 5). However, to date only few studies addressed seasonal changes in muscle morphology and metabolism in the Djungarian hamster (Braulke et al. 2010). Thus, the endocrine control of the photoperiod-driven change in muscle mass remains to be elucidated and more studies in this field are required.

We measured serum concentrations of insulin and IGF-1 in this study as well. The results are comparably affected by the above mentioned factors. Although not significant, results might hint at slightly increased insulin levels in the SD-RW group compared to SD-C. Elevated insulin levels are found in mammals that developed insulin resistance, predominantly caused by obesity. Initially, insulin is produced in sufficient quantities in these animals but insulin-stimulated glucose transport and uptake is diminished in glucose absorbing organs such as

muscle. Thus, the blood glucose level increases and the pancreas in turn compensates with increased insulin secretion. However, an elevated insulin secretion cannot be sustained over a long period of time. As a consequence of insulin deficiency in a later stage, type 2 diabetes may develop. But exercising hamsters increase their body mass without increasing fat mass (Scherbarth et al. 2007) and thus, they cannot be described as 'obese'. Furthermore, exercise does counteract insulin resistance. An important role in this process plays the enzyme AMPK in muscle. AMPK is involved in regulating energy balance at the whole body level via inhibition of anabolic pathways while activating catabolic processes (for review, see Hardie et al. 1998). In animal models of insulin resistance, AMPK activity was found to be low due to defect or disuse of the AMPK signalling system (for review, see Winder and Hardie 1999). Activity of AMPK can be pharmacologically stimulated by AICAR (Buhl et al. 2002, Iglesias et al. 2002) or directly through exercise training (Brandt et al. 2010, Kraegen et al. 1989). Both procedures have been shown to effectively counteract insulin resistance via improved glucose transport and fatty acid oxidation. Thus, the development of insulin resistance in exercising hamsters seems not to be very likely.

One study on voluntarily exercising and thus trained rats revealed low levels of plasma IGF-1 compared to controls (Matsakas et al. 2004). However, exercising rats had decreased their body mass after 12 weeks and thus it was suggested that wheel running may diminish anabolic stimuli in this species. This is in contrast to our findings in the hamster where anabolic stimuli are induced through wheel-running activity. Thus, we would expect a rather elevated IGF-1 plasma level in our study, but in contrast to our expectations, we did not find any significant differences between exercising hamsters and controls. However, like with insulin, there might at best be a faint hint to slightly elevated IGF-1 serum concentrations in the SD-RW group compared to SD-C. Altogether, the discussion points out that the experiment should be repeated under standardized conditions with an appropriate time point for muscle and blood sampling. Consequently, we then might be able to shed light on the involvement of insulin, IGF-1 and the muscle metabolism in seasonal body weight regulation as well as the impact of wheel-running activity on it.

Considering the present results of all our studies, the question remains how the exercise-induced increase in body mass can develop without an increase in food intake. Moreover, we found that the effectiveness of digestion seems not to be altered by wheel-running activity. Analysis of the energy content in faeces via a bomb calorimeter showed no difference between hamsters with or without access to a RW in either photoperiod (data not shown). Hence, we conclude that several factors such as growth, altered insulin and IGF-1 levels, a

conversion of body fat to body lean mass and probably a lot of other still unknown factors, might sum up and contribute to the exercise-induced weight gain.

The last experiment (chapter 5) is the first study to investigate the temporal sequence of gene expression changes in the ARC of the Djungarian hamster over the course of one year under natural T_a and photoperiod. Thereby, the gradual transitions from summer to winter and winter to summer were covered (Hannover, Germany; 52°N latitude). These gene expressions have previously been investigated only under artificial lighting and temperature conditions. By relating changes in body mass with gene expression changes, we were able to confirm several genes to be involved in body mass regulation.

During the course of the year, *Srif* gene expression was in antiphase with the body mass cycle, changing prior to or in parallel with the body mass. As SRIF inhibits the release of GH from the pituitary, the result indicates that growth seems to be inhibited in SD and allowed in LD. This result is in line with our finding under artificial conditions, as described in chapter 3.

In previous studies under artificial lighting conditions, *Dio3* gene expression in Djungarian hamsters was shown to be present only in SD, reaching a peak after 6-8 weeks (Barrett et al. 2007, Herwig et al. 2009, Watanabe et al. 2007). DIO3 is located in the ependymal layer of the 3rd ventricle and converts T_3 to inactive T_2. Thus, in SD, T_3 availability in the brain decreases, thereby decreasing energy expenditure which is an important adaptation to save energy in winter. On the other hand, the hypothalamic T_3 availability seems to be high in LD due to the lack in *Dio3* gene expression (Freeman et al. 2007). After the peak in *Dio3* gene expression, previous studies showed a rapid decline, indicating that the *Dio3* expression might have become refractory to the SD signal. An early decline in *Dio3* gene expression under SD conditions might contribute to an early change in physiology, thereby anticipating LD conditions. Thus, *Dio3* refractoriness to SD might present a mechanism underlying the overall process of photorefractoriness. The gene expression of *Dio3* in our experiment under natural photoperiod and T_a shows a comparable pattern. *Dio3* mRNA expression appeared for the first time in September (week 11 post SS) and reached a peak in October (week 17 post SS). Then it decreased again between October and November (week 23 post SS) and was almost undetectable during the rest of the year. After 27 weeks in SD, when hamsters have been photorefractory, Watanabe and coworkers (2007) found *Dio3* gene expression to be undetectable. This is also in accordance with the findings in photorefractory hamsters that experienced the transition from winter to summer in our study. *Dio3* expression was very low in January and February and it was undetectable since then.

Studies determining *Dio2* gene expression in Djungarian hamsters revealed inconsistent results so far and the authors suggested a time-dependent effect and an influence of the photoperiodic history of the animals on *Dio2* gene expression (Barrett et al. 2007, Herwig et al. 2009, Watanabe et al. 2004, 2007). The outcome of our experiment suggests another influence on *Dio2* gene expression, namely T_a, as we found significantly elevated *Dio2* mRNA expression levels from April (week 17 post WS) to June (~1 week before the SS). Concerning photorefractory hamsters, Watanabe and coworkers (2007) found a low mRNA expression of *Dio2* in hamsters after 27 weeks in SD. However, in our study *Dio2* expression increased in hamsters that became photorefractory (transition from winter to summer). Interestingly, in our experiment under natural photoperiod and T_a *Dio2* and *Dio3* mRNA expression seemed to be inversely regulated.

Nestin and vimentin are intermediate filaments that constitute the cytoskeleton and thus they might be involved in tanycyte morphology. In previous studies on the Djungarian hamster, *Vimentin* mRNA expression has been shown to be decreased in SD. Simultaneously, tanycytes retracted from the basal epithelium of the hypothalamus in SD (Kameda et al. 2003). The findings that both, morphological changes of tanycytes and *vimentin* and *nestin* expression in tanycytes are photoperiodically regulated in the Djungarian hamster indicate that tanycytes and the ependymal layer might play a role in seasonal responsiveness (Barrett et al. 2006, Herwig et al 2009, Kameda et al. 2003, Xu et al. 2005). The results of our study suggest that tanycytes may be involved in the mechanism underlying the seasonal body mass decline, as *nestin* and *vimentin* expression decreased prior to the decrease in body mass. However, the up-regulation of both genes was delayed relative to the increase in body mass in photorefractory hamsters (in the transition from winter to spring). This finding indicates that morphological changes of tanycytes may not be involved in the physiological adaptations occurring during spring.

A subsequent experiment under natural conditions should include an increase in sampling frequency and a determination of the testes cycle in addition to the body weight cycle. Besides the verification of the pattern of *Dio2* gene expression, the increasing number of genes, whose expression is found to be regulated by photoperiod in the hypothalamus of Djungarian hamsters kept under artificial conditions, could be analysed and related to the seasonal testes cycle and cycle in body mass.

Altogether, this study confirms wheel-running activity as a useful tool in the Djungarian hamster to manipulate and challenge the neuroendocrine energy balance system and the

mechanisms underlying seasonal acclimatization. However, further research in this field is necessary to understand the interaction of the complex mechanisms in the brain and periphery that mediate the divergence from the natural seasonal cycle of physiological adaptations due to voluntary exercise. From the data achieved in the context of this thesis we assume that there should be several factors that are affected by wheel-running activity. Thus, even small influences on these factors, all acting in the same direction, might sum up to induce the exercise-induced weight gain.

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