Molecular and Neuroendocrine Determinants of Seasonal Body Weight Regulation

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Contents

Glossary of terms

AGRP	agouti-related protein
AKT	derived from murine retrovirus AKT; synonym: protein-kinase B
α-MSH	α-melanocyte-stimulating hormone
ARC	arcuate nucleus
CART	cocaine-and amphetamine-regulated transcript
GHSR	growth hormone secretagogue receptor
GRB2	growth factor receptor binding protein 2
ERK	extracellular-regulated kinase
ICV	intra-cerebroventricular
IR	insulin receptor
ir	immunoreactivity
IRS	insulin receptor substrates
JAK2	januskinase 2
LD	long day-length
LHA	lateral hypothalamic area
LRb	full length isoform of the leptin receptor
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
PFA	peri-fornical area
Pi3K	phosphatidylinositol 3-kinase
POMC	proopiomelanocortin
PT	pars tuberalis
PTP1B	protein tyrosine phosphatase 1B
PVN	paraventricular nucleus
SCN	suprachiasmatic nucleus
SD	short day-length
SHP2	SH2-containing tyrosine phosphatase 2

SOCS3	suppressor of cytokine signalling 3
STAT3	signal transducer and activator of transcription 3
Tyr^{985}	tyrosine residue associated with the leptin receptor
Tyr^{1138}	tyrosine residue associated with the leptin receptor
VMH	ventromedial hypothalamus

Introduction

Humoral signals maintain energy homeostasis

The availability of sufficient energy is one prerequisite for all biological processes; and animals obtain this required energy by feeding regularly. However, amount and composition, and thus energy content, of the ingested diet can differ considerably from meal to meal. Nevertheless, most organisms can regulate their body weight within certain limits, i.e. control the increase or decrease of their body weight. This indicates the existence of a precise mechanism to achieve a balanced energy budget (energy homoeostasis).

The causal relationship between food intake and the amount of body fat is well documented. However, comparatively little is known about the mechanisms controlling fat storage. According to the lipostatic theory (1), “adiposity signals” convey information about the status of body fat stores to the brain. Such signals circulate in proportion to body fat mass and act in the central nervous system to reduce food intake. To date only two molecules have been identified - leptin and insulin - that meet the criteria proposed for these “adiposity signals” (2). These hormones secreted by adipocytes or by the pancreas respectively, circulate in proportion to body fat mass and are transported into the brain via the blood stream where they display their catabolic action. Both humoral signals are able to reduce food intake upon central administration and, in addition, reduced neuronal signalling by either hormone results in hyperphagia and obesity (3-7). Insulin was the first putative “adiposity signal” to be described and albeit insulin levels vary significantly on a meal-to-meal basis, fasting and 24h integrated insulin levels clearly reflect body fat mass (8). However, after the landmark discovery of the second “adiposity signal” leptin (derived from the greek word leptos = thin) by positional cloning in 1994 (9), a more potent weight reducing effect of this hormone compared to insulin was very soon identified. Conclusively, deficiency of either leptin (*ob/ob* mouse) or its receptor (*db/db* mouse) leads to extreme hyperphagia and obesity.

In 1999 with the outstanding discovery of the hormone ghrelin (derived from the Proto-Indo-European root of the word 'grow') it emerged that maintenance of energy homoeostasis does not only require anorexigenic (food intake inhibiting) circulating factors (10). Because ghrelin which is secreted by the stomach is in contrast to “adiposity signals” such as leptin and insulin the only hormone identified so far which exhibits orexigenic (food intake stimulatory) actions. Ghrelin plays an essential role in meal initiation and circulating plasma ghrelin concentrations are dynamically related to feeding state (11,12). In humans it has been demonstrated that circulating ghrelin levels are decreased in chronic (obesity) and acute (feeding) states of positive energy balance. In contrast, plasma ghrelin levels are increased by fasting and in patients with anorexia nervosa (12-15).

Central signalling pathways

Despite the obvious heterogeneity of these hormones in terms of their origin and their body weight regulatory effects one common feature is striking: all three humoral signals exert their central effects mainly by conveying their energy homeostasis encoding information on neurons in the hypothalamus. In this context a crucial event is binding of these hormones to their receptors which are located in a key neuronal centre for the regulation of body weight, the hypothalamic arcuate nucleus (ARC). Subsequent transduction of these peripheral signals leads to integration into neuronal responses by altering neuronal firing, as well as by affecting gene transcription and possibly post-translational processing of a set of orexigenic and anorexigenic downstream effectors with autocrine and paracrine action. Among these so called neuropeptides cocaine-and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC) are representatives for the most potent anorexigenic-, whereas neuropeptide Y (NPY) and agouti related protein (AGRP) represent the most potent orexigenic neuropeptides. CART and POMC are colocalised in the “catabolic” neurons whereas the “anabolic” neuron colocalises NPY and AGRP. These neurons which are located

in the ARC innervate other brain areas such as the paraventricular nucleus (PVN) where second-order neurons in the energy homeostasis circuit are located. From here anabolic and catabolic pathways project to the hindbrain where they are synchronised with afferent input from the vagus nerve in the nucleus of the solitary tract (NTS) which acts as a satiety centre. Despite the common feature of hypothalamic central signal transduction exhibited by leptin, insulin and ghrelin, the mechanisms underlying receptor signalling are diverse. The neuroendocrine pathways involving body weight regulatory hormones and the key neuronal centres on which they act are illustrated in Fig. 1.

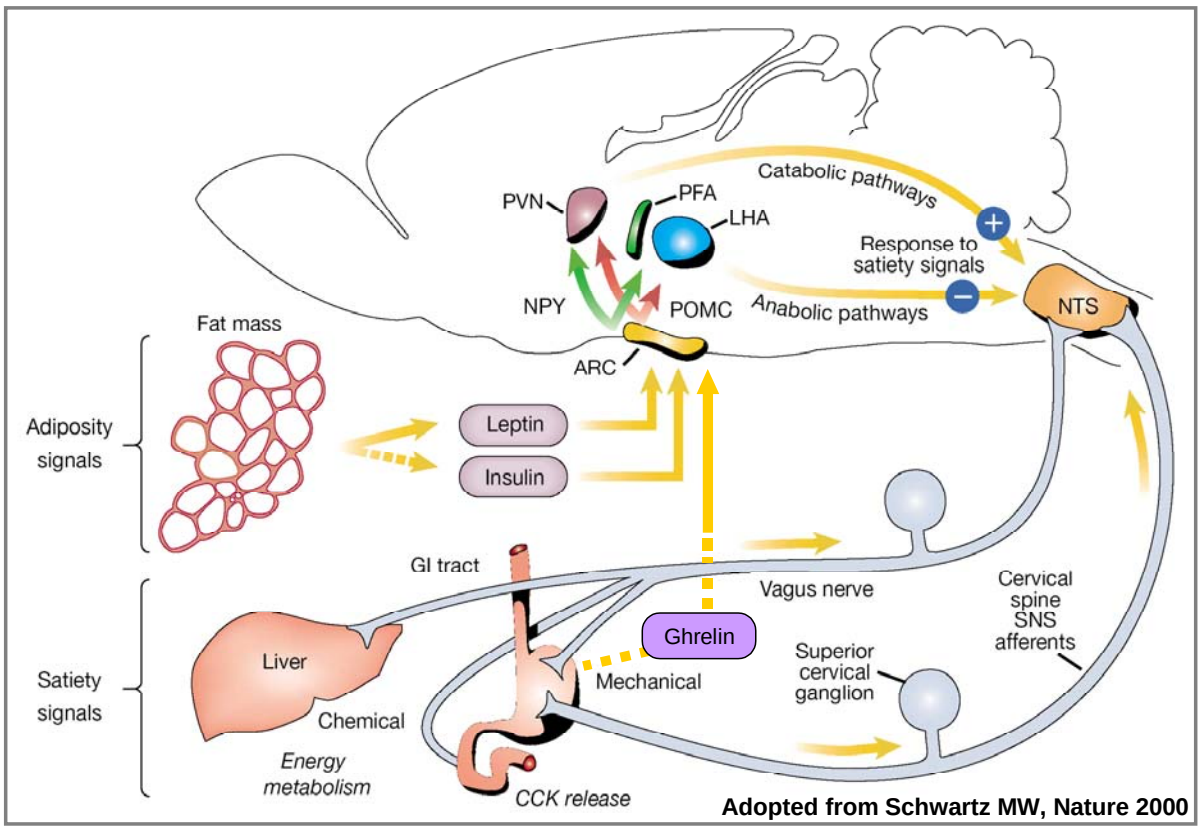

Fig. 1: Neuroanatomical model of pathways by which humoral signals, leptin (secreted by adipocytes), insulin (secreted by the pancreas in proportion to adiposity) and ghrelin (secreted by the stomach), interact with central autonomic circuits regulating meal size. Catabolic- (POMC/CART neurons) or anabolic- (NPY/AGRP neurons) secondary pathways are altered in the ARC, the neuroanatomical determinant for integration of body weight regulatory signals. These pathways project to the PVN and LHA/PFA, where they establish connections to hindbrain autonomic centres that process satiety signals. Afferent input related to satiety from the liver, or gastrointestinal tract are transmitted through the vagus nerve and sympathetic fibres to the nucleus of the solitary tract (NTS), where they are integrated with descending hypothalamic input.

The insulin receptor (IR) which consists of a disulfide bonded dimer of an α and β subunit is associated to the class of (enzyme linked) tyrosine-kinase receptors (16,17). The leptin receptor, which is not related to the insulin receptor belongs to the cytokine family (class 1) and coexists as several isoforms (17,18). However, only the full length isoform (LRb) is considered to possess full signalling capacity. This variant which in contrast to IR does not possess intrinsic tyrosine kinase becomes activated by extrinsic Januskinase 2 (JAK2). The structure of the ghrelin receptor (due to the first identified ligands called the growth hormone secretagogue receptor; GHSR), however, totally differs from the one of IR and LRb since GHSR belongs to the class of G-protein-coupled receptors (19).

Potential crosstalk of central leptin- and insulin signalling

The cascade of intracellular signalling events utilized by IR and LRb exhibits conspicuous similarities (19) whereas evidence for parallels in intracellular signalling events initiated by ghrelin is lacking (Fig. 2).

Leptin's anorexigenic action is mediated via intracellular transduction distal to LRb by three signal transduction pathways involving certain tyrosine residues and associated JAK2 (17,18). The first, best characterised pathway is the JAK-STAT pathway in which the key transcription factor, signal transducer and activator of transcription 3 (STAT3), becomes transactivated (phosphorylated) by Tyr^{1138} (18,20,21). The second pathway involves the extracellular-regulated kinase (ERK) signalling cascade whose activation is mainly critically dependent on the association of SH2-containing tyrosine phosphatase (SHP2) and the growth factor receptor binding protein (GRB2), mediated via Tyr^{985} (18,22,23). However, 30% of transactivation of ERK are considered to be attributable directly upon phosphorylation by JAK2. The third pathway, ultimately is mediated via the enzyme phosphatidylinositol 3-kinase (Pi3K) which activates the key downstream target AKT [also known as protein kinase B; (18,22)].

Intracellular signalling of insulin, despite its enormous complexity is thought to interact with leptin signalling on the level of Pi3K since this pathway is activated by the insulin receptor substrates (IRS) proteins, pivotal insulin signalling molecules (24). However, at present possible convergence of IR- and LRb signalling facilitated by the ERK- or the JAK-STAT pathway cannot be excluded.

The central role of inhibitory molecules such as SOCS3 and PTP1B

Of particular interest is the control of both leptin and insulin intracellular signalling events by inhibitory molecules. Here the leptin-induced target gene, the suppressor of cytokine signalling 3 (SOCS3); and the protein tyrosine phosphatase 1B (PTP1B) play crucial roles. Neuronal deficiency of SOCS3 in mice leads to enhanced leptin-induced hypothalamic STAT3 tyrosine phosphorylation, greater body weight loss and suppression of food intake. Furthermore, SOCS3-deficient mice exhibit resistance to diet-induced obesity and retain insulin sensitivity (25,26). PTP1B deficiency in mice and diabetic rodents, however, leads to enhanced central and peripheral insulin sensitivity (27-30). These animals maintain euglycemia (in the fed state) with one-half the level of insulin observed in wild-type littermates, and surprisingly are resistant to diet-induced obesity.

The molecular mechanisms, utilised by these molecules, leading to inhibition of both insulin and leptin signalling are not completely unravelled. However, accumulating evidence suggest convergence in their central effects upon signalling of either hormone (2,31,32). SOCS3 and PTP1B may be responsible for deactivating IRS proteins, leading to attenuated insulin signalling, whereas their inhibitory effect upon leptin signalling is considered to be based on discrete (yet to be identified) mechanisms leading to inhibition of STAT3 and possibly ERK resulting in impaired target gene transcription.

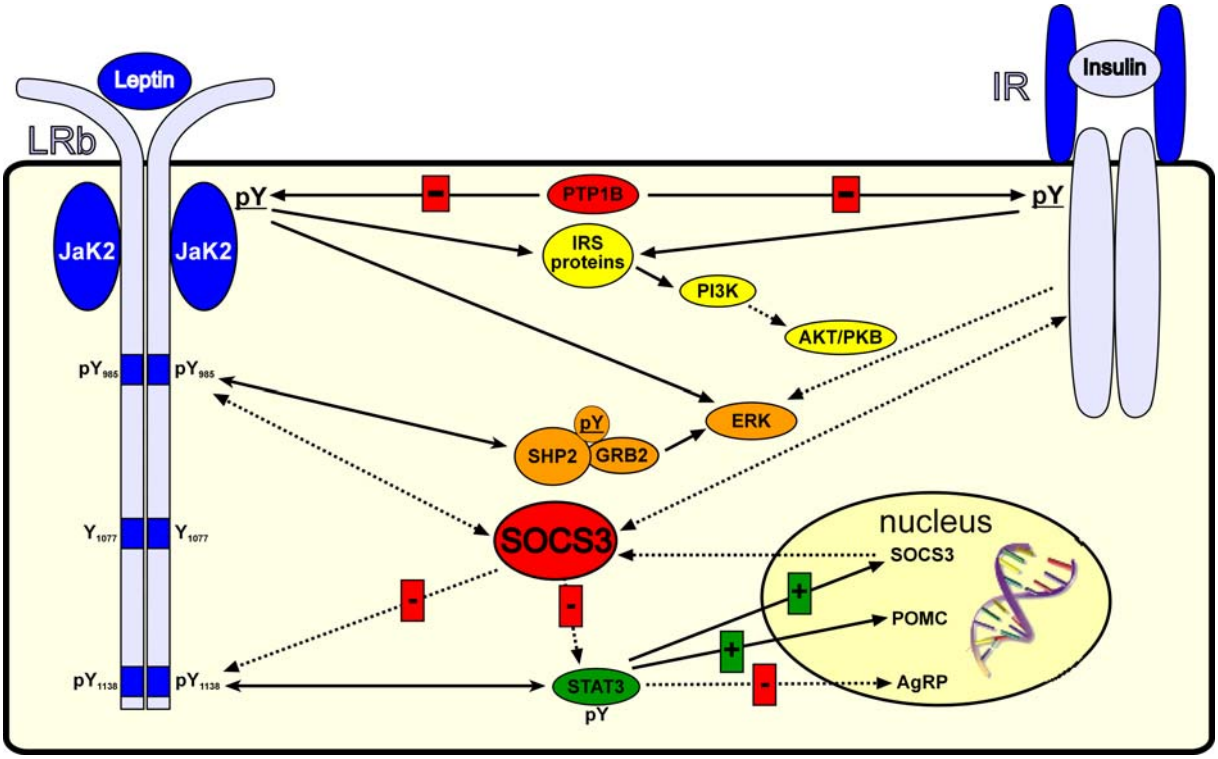

Fig. 2.: Intracellular signalling by LRb and IR and potential "crosstalk" of signalling events. Leptin binding initiates activation of the extrinsic JAK2 tyrosine kinase that associates with LRb whereas insulin provokes IR intrinsic tyrosine phosphorylation. JAK2 tyrosine autophosphorylates and transphosphorylates Tyr^{985} as well as Tyr^{1138} on the intracellular domain of LRb. Phosphorylated Tyr^{1138} binds and mediates the phosphorylation-dependent activation of STAT3, which in cell culture activates transcription of SOCS3 (feedback inhibitor of LRb and IR signalling) and POMC (anorexigenic neuropeptide), whereas it possibly inhibits the transcription of AGRP (orexigenic neuropeptide). JAK2 as well as phosphorylated IR discretely activate IRS proteins part of the PI3K pathway in which a key downstream component is the proteinkinase B (AKT). A third signalling pathway, the ERK pathway, is activated via transphosphorylated Tyr^{985} (70 %) and directly via JAK2 (30 %). Tyr^{985} recruits SHP2 and binds GRB2 which is crucial for Tyr^{985}-dependent ERK activation. Tyr^{985} also mediates binding to SOCS3 (after prolonged exposure). The proteintyrosine phosphatase 1B (PTP1B) acts upstream of JAK2 and is capable of inhibiting both IR and LRb mediated downstream signalling events. Possible interactions of IR with the JAK-STAT or ERK signalling pathway are indicated by dotted arrows.

The phenomenon of leptin resistance and the seasonal mammal *Phodopus sungorus*

Despite the outstanding anorexigenic potential of leptin, catabolic effects are not always apparent. Since circulating leptin levels are proportionate to body fat stores obesity in humans as well as in rodents is very often associated with a phenomenon called leptin resistance, and this is even often claimed as the key event for the onset of perturbed energy homeostasis (33,34).

Insensitivity to leptin could be the result of changes at a number of levels in the signalling pathway from molecule to post-receptor signal transduction. The rate of entry of

leptin into the brain across the blood-brain barrier could be reduced. Alternatively, there could be a reduction in the availability of leptin receptors on the cell surface, although several studies make this appear unlikely (35-38). The third and most plausible mechanism modulating leptin sensitivity is modification of intracellular signal transduction distal to the leptin receptor involving the signal transduction pathways elaborated above.

Over the last couple of years the considerable therapeutic potential underlying treatment of leptin resistance led to an explosion of interest to unravel the molecular identity of this phenomenon. However, much of our knowledge about intracellular leptin signalling and the role of distinct components involved in this process has derived from studies of genetically obese rodents or from models of imposed negative energy balance. Our knowledge about the mechanisms underlying precise adjustments in dynamic long-term body weight regulation and leptin sensitivity remains limited.

A fascinating and powerful model for studies in this field is presented by the seasonal Siberian hamster (also known as the Djungarian hamster), *Phodopus sungorus*, which exhibits a remarkable natural body weight cycle, accompanied by a biannual reversible switch in leptin sensitivity, mediated by the environmental cue photoperiod. The neuroendocrine transducer of photoperiod information is the pineal hormone melatonin which acts on its receptor predominantly expressed in the pituitary pars tuberalis (PT) and the hypothalamic suprachiasmatic nucleus [SCN; ((39)]. Siberian hamsters synchronise their physiology and behaviour to the seasonally programmed signal imposed by photoperiod, which is dramatically exemplified by an increase of body weight in a summer like photoperiod as compared to reduction of body weight in a winter like photoperiod. The changes induced by natural, and gradually changing photoperiod cues can be replicated in the laboratory by a simple square-wave switch from long day length (LD; 16:8h light-dark) to short day length

(SD; 8:16h light-dark). Here, different functional *in vivo* studies investigating effects of exogenously applied leptin revealed a transition from leptin sensitivity in SD to leptin resistance in LD (40-42).

Methods

In this thesis the following methods were deployed:

- Serum blood concentrations of leptin and ghrelin were determined by **radio-immunoassay**.

- **Standard molecular cloning techniques** were utilised to generate homologues riboprobes for SOCS3, STAT3, SHP2, IR, Pi3K, PTP1B from hamster cDNA. The heterologues riboprobe for GHSR was generated from rat cDNA (kindly provided by Dr. Zoe Archer).

- Hypothalamic gene expression was detected with neuroanatomical precision by **in situ hybridisation** on coronal cryo-brainsections with radio-labelled riboprobes. This very sensitive method enables quantification of subtle differences in gene expression within specific hypothalamic nuclei.

- Hypothalamic concentration of phosphorylated ERK and AKT- as well as total GRB2 protein was determined by standard **immunoblotting** techniques.

- By **immunohistochemistry** phosphorylated STAT3, ERK and AKT- as well as total GRB2 protein were localised with neuraonatomical precision on free-floating cryo-brainsections. The number of *phospho*-STAT3 immunoreactive (ir) nuclei as well as *phospho*-ERK ir cells was counted.

Controls

Complementary sense riboprobes were generated for each investigated signalling component. They generated a low intensity non-specific signal.

The utilised antibodies detected only a single band by immunoblotting (except for ERK which detected ERK 1 and 2) and immunoreactivity (ir) was conspicuously confined to the hypothalamus. This in addition to the consistency with numerous other published studies performed with this antibodies confirms the specificity of these peptides.

Specific aims

The specific aim of this thesis was to unravel the neuroendocrine pathways modulating seasonal body weight and leptin sensitivity in the Siberian hamster, *Phodopus sungorus.*

Therefore the following questions ought to be answered:

1. What are the molecular mechanisms underlying the biannual switch in leptin sensitivity?

2. Is the seasonal body weight cycle associated with alterations in central insulin signalling?

3. Does convergence of hypothalamic leptin and insulin signalling represent a likely mechanism for adjustments in seasonal energy homeostasis?

4. Is ghrelin and its hypothalamic transduction via GHSR implicated in seasonal body weight regulation and may ghrelin act as an antagonist to leptin?

Results and Discussion

The molecular mechanisms underlying the biannual switch in leptin sensitivity

The central role of SOCS3 in mediating seasonal changes in leptin sensitivity

The results of this PhD thesis provide substantial evidence for an involvement of central leptin signalling distal to its receptor in mediating the biannual switch in leptin sensitivity. In this context it emerged very soon that SOCS3 may act as a central player. Therefore, subsequently a series of experiments with hamsters subjected to energetic and hormonal challenges (leptin injections) were designed to scrutinize the potential key role of this inhibitory factor in mediating seasonal changes in leptin sensitivity. The initial results which revealed, amongst others, a marked differential in arcuate nucleus SOCS3 gene expression with increased levels in LD compared to SD, as well as acute stimulation of SOCS3 mRNA by leptin restricted to SDs, are reviewed in Chapter I (Mercer J.G and **Tups A**., 2003; "European Journal of Pharmacology"). In this chapter the initial findings are described comprehensively, related to behavioural and physiological anticipatory changes underlying seasonal body weight regulation in *Phodopus sungorus*.

Further analyses substantiated that the development of leptin resistance in LD-acclimated hamsters involves SOCS3-mediated suppression of leptin signalling in the arcuate nucleus. Moreover, it emerged that photoperiod alone is able to trigger the biannual reversible switch in leptin sensitivity independent of body fat and endogenous serum leptin levels. These results are presented in chapter II (**Tups A.** *et al.* 2004; Endocrinology).

SOCS3 as a potential molecular determinant of seasonal body weight changes

Since we demonstrated that photoperiod is the environmental cue triggering seasonal changes in leptin sensitivity and that the establishment of a differential in SOCS3 mRNA by transition

of hamsters from LD to SD clearly preceded body weight changes, we scrutinized whether SOCS3 may further act as a pivotal modulator of the seasonal body weight cycle.

Therefore a series of experiments were conducted in which the second aspect of the seasonal body weight cycle, namely the positive body weight trajectory, triggered by SD-LD transition or by spontaneous photorefractoriness, was substantially highlighted. Furthermore, this study addressed whether the photoperiod-induced changes in SOCS3 gene expression are secondarily due to gonadal regression in SD.

Indeed, we were able to demonstrate that SOCS3 may be one critical modulator of seasonal body weight changes, and the observed alterations in arcuate nucleus SOCS3 gene expression seem to be independent of reproductive activity. These and the previous findings suggest a model by which a seasonally appropriate body weight may be rheostatically[1] adjusted by dynamic modulation of leptin sensitivity via SOCS3. The obtained data and the suggested model are illustrated in Chapter III (**Tups A.,** *et al.*; submitted to "Journal of Neuroendocrinology").

The dual role of LRb associated Tyr^{985} in mediating seasonal leptin sensitivity

Since SOCS3 acts as a target gene of STAT3 *in vitro* and SOCS3 attenuates LRb-mediated signalling *in vitro* and *in vivo* we posit that the inhibitory feedback action of the LRb-SOCS3 pathway may explain the different reading of the leptin signal in LD and SD. Therefore comprehensive studies were designed to scrutinise the molecular identity underlying inhibitory feedback of SOCS3 in the state of leptin resistance exhibited by hamsters acclimated to LD (Chapter IV, **Tups A.**, *et al.*; in preparation for "Neuroscience"). Here of particular interest was the key question whether altered SOCS3 gene expression may be associated with impairment of the three distinct LRb signalling pathways, JAK-STAT, ERK

[1] Rheostasis: 'condition or state in which homeostatic defenses are still present but over a span of time there is a change in the regulated level (43)'

or Pi3K. To answer this question we investigated whether post-translational modifications (phosphorylation) of key components within these pathways may be perturbed in leptin resistant LD hamsters. Intriguingly, leptin-induced phosphorylation of the transcription factor STAT3 (critical for its activation) was dramatically reduced in LD compared to SD suggesting that the inhibitory feedback of increased SOCS3 in LD may be based on deactivation of this transcription factor. Since the utilised time frame of 40 min post leptin injection was sufficient for leptin-induced phosphorylation of STAT3 which is followed by a subsequent rise in SOCS3 gene expression (a time course-study revealed that leptin needs 1h to induce SOCS3 gene transcription), STAT3 mediated transcription of the SOCS3 gene *in vivo* is plausible.

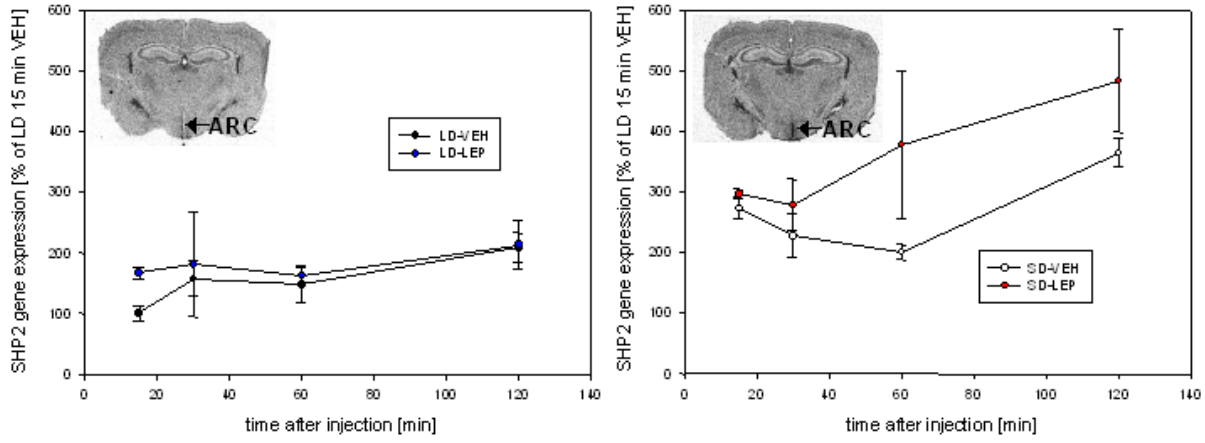

Fig.3: Leptin time course study depicting SHP2 mRNA in the arcuate nucleus (ARC) of juvenile female hamsters (8 weeks post weaning). Animals received an intraperitoneal injection of either leptin (LEP) or vehicle (VEH) and were sacrificed 15, 30, 60 or 120 min later (n=3 in each group). Notably, SHP2 mRNA is markedly increased in SD-VEH compared to LD-VEH throughout the timecourse of injection. Leptin rapidly stimulates SHP2 mRNA in SD but not in LD. Autoradiographs illustrate SHP2 gene expression in a coronal hamster brain section. (Tups A. unpublished results)

Phosphorylation of other key components in the hypothalamus reflecting the activity of the ERK- and Pi3K signalling pathways was not altered by leptin [see Chapter IV for ERK, preliminary data for Pi3K need further to be substantiated (data not shown)] and either not affected by photoperiod (ERK) or counter-intuitively downregulated in SD (*phospho*-AKT, reflecting Pi3K activity). These findings suggest that SOCS3 may not negatively feed back on activation of these denominators of alternative leptin signalling pathways. Interestingly,

however, we assessed a marked SD induced increase of the signalling components SHP2 (Fig. 3) and GRB2 which are crucial for LRb associated Tyr^{985} mediated signalling. Since this distinct tyrosine residue is considered to possess a dual role in LRb signalling – binding SHP2 but also providing an important site of interaction for SOCS3 – we postulate a hypothetical negative feedback loop responsible for the precise adjustments in seasonal leptin sensitivity (Fig. 4): Leptin resistance revealed by LD acclimated hamsters could be based on high expression of arcuate nucleus SOCS3 which may lead to competitive suppression of SHP2 binding to Tyr^{985} and association with GRB2. Subsequently this may result in inhibition of JAK2 (enhanced by increased PTP1B) which in turn leads to reduced signalling via Tyr^{1138} followed by diminished STAT3 activation. In states of increased leptin sensitivity (SD acclimated hamsters), however, low levels of arcuate nucleus SOCS3 expression would result in competitive inhibition of SOCS3 binding to Tyr^{985} enhanced by the observed high levels of SHP2 and GRB2. If this model holds true it seems paradoxical that high levels of the leptin responsive target gene SOCS3 in LD are maintained despite postulated reduction in LRb mediated signalling. However, increased levels of SOCS3 in LD may be dissociated from transactivation by leptin. Substantial support for this hypothesis is provided by the fact that a dramatic decline of circulating leptin levels induced by chronic food restriction does not affect arcuate nucleus SOCS3 gene expression The basis for this hypothetical model is further provided by the dramatic effects demonstrated by exogenous administration of supraphysiological doses of leptin. Nevertheless, at endogenously altered levels of leptin (between LD and SD) phosphorylation of hypothalamic STAT3 was not dramatically different. A twofold higher concentration of *phospho*-STAT3 positive cells in SD, however, could be observed in the basomedial part of the hypothalamus, a region in close proximity to the median eminence and third ventricle which probably lacks a blood-brain barrier and thus may possess a key function in integrating peripheral signals (44,45). Although this differential was not significant it is plausible that subtle changes in the cell number integrating the leptin

signal in this region may have major impact on overall energy homeostasis. This is also supported by the finding that both SHP2 mRNA and GRB2 protein changes were most dramatic in this region. Interestingly, a twofold higher number in *phospho*-STAT3 positive cells could be observed despite leptin levels in SD are just half of those in LD, supporting a substantial rise of leptin sensitivity in SD on a molecular level.

Taken together these findings strongly imply that the different reading of the leptin signal in LD and SD may be explained by the proposed mechanism involving the dual function of Tyr^{985} which represents the first, molecular site-, whereas the mediobasal hypothalamus represents the second, neuroanatomical site-, of the fascinating story of seasonal leptin resistance.

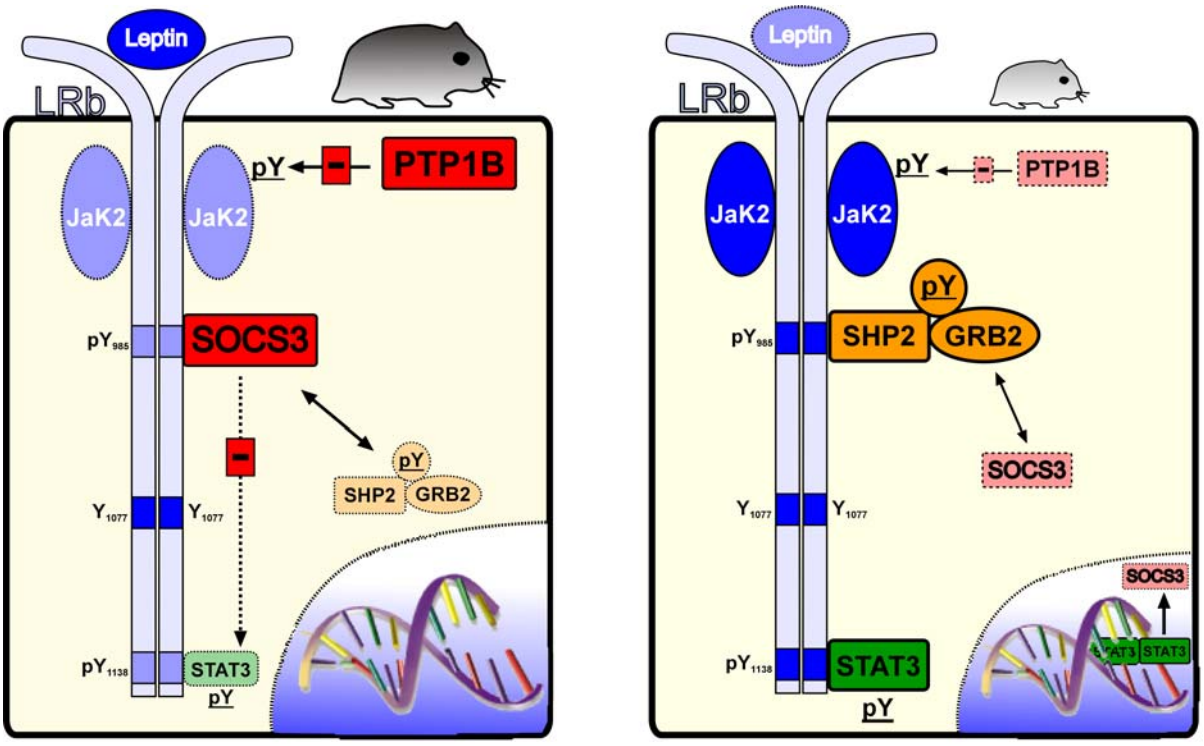

Fig. 4: Model proposing the molecular identity for the biannual switch in leptin sensitivity revealed by *Phodopus sungorus*. High circulating leptin levels in LD (left panel) are associated with increased gene expression of the inhibitory molecules SOCS3 and PTP1B. PTP1B deactivates JAK2 resulting in diminished phosphorylation of LRb associated Tyr^{985} and Tyr^{1138}. SOCS3 binds to Tyr^{985} and competitively displaces SHP2 which is associated with GRB2 (both factors are downregulated in LD). This may further reduce the already reduced phosphorylation of Tyr^{1138} resulting in attenuated activation of STAT3. In SD low levels of leptin (right panel) are associated with reduced PTP1B mRNA resulting in maximal phosphorylation of the two intrinsic tyrosine residues. In this state elevated SHP2 and GRB2 may bind to Tyr^{985} and competitively displace SOCS3. SOCS3 which is already substantially decreased compared to LD fails to exhibit its inhibitory function. Conclusively, STAT3 phosphorylation is augmented leading to dimerisation and translocation to the nucleus where target gene transcription (e.g. SOCS3) becomes initiated. In LD a marked drop of circulating leptin induced by food restriction below the levels observed in SD is not associated with reduced SOCS3 mRNA suggesting that in this photoperiod high levels of SOCS3 are sustained by other mechanisms than the JAK-STAT pathway. This model suggests a crucial role for the dual function of Tyr^{985} in mediating seasonal changes in leptin sensitivity. Noteworthy, phosphorylation of STAT3 in LD is reduced to up to 50% of SD levels despite leptin levels are 2-4 fold increased implying severe endogenous leptin resistance. (Implication of Tyr^{1077} in leptin signalling has not yet been satisfactorily resolved).

Hypothalamic insulin signalling and its implication in seasonal body weight regulation

Much of our knowledge about insulin signalling primarily arises from studies in peripheral tissues and cell culture. However, in these signalling models a phenomenon referred to as "cross-talk" between insulin and leptin signalling has been demonstrated. Hence in Chapter V (**Tups A**. *et al*.; in preparation for "American Journal of Physiology") we tested a possible involvement of central insulin signalling in the mediation of seasonal body weight regulation.

Indeed, we assessed a marked photoperiod-induced regulation of IR mRNA, *phospho*-AKT protein and PTP1B mRNA within the hypothalamus. However, regarding the fundamental central catabolic action of insulin (i.e. reducing food intake and body weight) the finding that both IR gene expression and insulin signalling via *phospho*-AKT are downregulated in SD hamsters appears to be counterintuitive. Conceivably, reduced insulin signalling in SD may be the **result,** rather than the trigger of an increased catabolic tone in SD. As elaborated above SD hamsters exhibit increased leptin sensitivity as compared to LD, whose imposed catabolic drive ultimately leads to mobilisation of body fat stores. As a consequence thereof circulating levels of insulin, as an "adiposity signal", would expected to be reduced in this photoperiod. Indeed, in the related species *Phodopus campbelli* exactly this phenomenon has been reported (46). Assumed that insulin levels are also reduced in *Phodopus sungorus* in SD it is imaginable that this may lead to reduced central IR-signalling. Since additive anorexigenic effects of central insulin and leptin were reported in rats (47), it is conceivable that the at first glance expected SD-induced increase in IR signalling would significantly enhance the already by augmented leptin signalling imposed catabolic drive. This, in turn, would lead to a further mobilisation of body fat stores alongside with a drop in both circulating leptin and insulin levels ultimately increasing the anabolic tone relieved by the absence of these hormones, which may act as a feedback loop to guarantee the survival of the Siberian hamster in harsh winter conditions. Downregulation of central IR signalling in SD may function as a control mechanism by which a catabolic overdrive induced by increased leptin signalling may be prevented. Stated differently central IR-signalling may anticipate disproportional sensitisation of LRb-mediated signalling in SD acclimated hamsters.

It has to be mentioned that the hypothetical mechanisms elaborated above are related to the central action of insulin. Peripheral and central actions of this hormone are dissociated, since insulin in the periphery acts anabolic (i.e. increases energy storage), whereas its function in the CNS is catabolic. Assumed it proves true that circulating insulin levels are elevated in

LD compared to SD, the increased anabolic drive in the periphery would ultimately induce body weight gain and would trigger the maintenance of a comparatively high body weight in LD. However, despite the apparently contrary actions of insulin, the peripheral and central functions of this hormone are balanced and are consistent with an endocrine feedback loop. Presumably, a postprandial rise in circulating insulin first induces the anabolic drive in the periphery (energy storage), whereas its central catabolic (primarily anorexigenic) tone may be temporally delayed since insulin entry into the brain may depend on a regulated transport mechanism (48).

Potential convergence of hypothalamic leptin and insulin signalling in the Siberian hamster

The likelihood of cross-talk of hypothalamic leptin and insulin signalling represents a key therapeutic target for treatment of the worldwide obesity pandemic, which ultimately led to an explosion of studies during the last two years investigating the underlying mechanisms. However, despite still limited knowledge about the details of central insulin signal transduction, it is emerging that the PI3K pathway may play a central role for the affiliation of both leptin and insulin signalling events. This idea arose from very recent findings, which demonstrated that signalling through Pi3K may be critical for leptin's and insulin's additive effects on membrane potential and firing rate in a specific subset of hypothalamic neurons (31,49). Other studies have addressed the role of hypothalamic Pi3K signalling in the regulation of food intake (31,50,51).

The findings reported in this thesis, however, do not support the idea of possible synergistic effects of leptin and insulin signalling in the hypothalamus of Siberian hamsters via the Pi3K pathway. Although we did not functionally test whether both leptin and insulin affect the activity of the Pi3K, the downstream target *phospho*-AKT was significantly downregulated in leptin sensitive SD hamsters. Downregulation of *phospho*-AKT is

counterintuitive because it implies a partial compensation of the established increase leptin signalling via JAK-STAT in SD. Therefore, it seems plausible that the decrease in hypothalamic *phospho*-AKT can be attributed to reduced insulin signalling mediated by the reduction in IR mRNA rather than being associated to LRb signalling. This hypothesis is supported by preliminary results which demonstrate that leptin fails to induce phosphorylation of AKT protein within the hypothalamus (Tups A. unpublished data). A very recent study performed by Carvalheira et al. (47), indicated that leptin- and insulin- induced signalling events downstream of Pi3K may diverge. Pi3K activates two discrete kinases, PDK1 and PDK2, which phosphorylate AKT on Thr^{308} or Ser^{473}, respectively. Although in this study both intra-cerebroventricular (ICV) leptin and insulin alter hypothalamic Pi3K activity in rats, only insulin is being capable of phosphorylating AKT on Ser473. Since we also investigated serine-phosphorylation of AKT our preliminary data are substantiated by this study. (To the authors knowledge a leptin- or insulin-induced threonine-phosphorylation of AKT is unknown and needs to be investigated in future studies).

However, since photoperiod co-regulates arcuate nucleus gene expression of the potent inhibitors SOCS3 and PTP1B, synergistic deactivatory effects on signalling pathways other than Pi3K (i.e. ERK, or JAK-STAT) utilised by both leptin and insulin is plausible. From the teleological perspective, the comprehensively elaborated concept of a complex circuitry of signalling events with various control mechanisms utilised by both adiposity signals make it appear unlikely that life maintaining energy homeostasis may be critically dependent on only a single biochemical pathway.

Clearly it remains a promising target for future functional studies to unravel the potential of cross-talk via these alternative pathways.

Ghrelin and its central processing seems not to be implicated in seasonal body weight cycles

In Chapter VI (**Tups A**. *et al.*, "Journal of Neuroendocrinology 2004"), we investigated, whether the orexigenic hormone ghrelin may be involved in the seasonal body weight cycle in order to complement leptin's and insulin's catabolic action by inverse regulation. However, our results do not substantiate a primary implication of ghrelin in the modulation of seasonal body weight cycles. This is being due to the fact that neither circulating ghrelin levels nor hypothalamic GHSR mRNA is affected by transition of hamsters from LD to SD. Nevertheless, a marked increase in both ghrelin and GHSR gene expression in the ARC and VMH was induced by starvation for 48h. These findings strongly imply that ghrelin may be involved in short-term regulation of appetite and body weight. Only chronic food restriction for 12 weeks imposed to match SD body weight trajectory in LD hamsters led to a slight decrease in GHSR gene expression within the ARC. This manipulation is a crucial event since it markedly dissociates LD hamsters from their desired body weight "set point". This implies that only these extreme catabolic conditions cause a reduction of central ghrelin signalling resulting in an attenuated ghrelin-mediated orexigenic drive which may alert the animal to alter behaviour and physiology appropriately.

Circulating leptin, LRb mRNA and LRb-mediated signalling are affected by long-term body weight changes driven by photoperiod, whereas circulating ghrelin and GHSR are not. This in addition to the fact that intraperitoneal leptin injections did not affect central GHSR mRNA, implies, that ghrelin seems not to counteract leptin. These findings shed a new light on the central action of ghrelin related to the available literature.

Consolidated intersections of the neuroendocrine circuits utilised by ghrelin, leptin and insulin require further investigations and currently remain an interesting enigma in body weight regulation.

Outlook

This PhD thesis aimed to unravel the molecular mechanisms underlying the remarkable seasonal cycle in body weight and leptin sensitivity exhibited by *Phodopus sungorus*. It provides novel insight into the role of hypothalamic signalling events for the integration of peripheral body weight regulatory hormones into central responses, which are responsible for the maintenance of energy balance. However, several important questions remain unanswered. First, what is the consequence of photoperiod-driven alterations in hypothalamic signalling pathways? Since the action of neither leptin, insulin nor ghrelin on body weight are direct, i. e. these signals modulate neuronal firing rate, and gene transcription of downstream effectors (neuropeptides), which then, in turn, are part of a complex network regulating energy homeostasis, it is important to establish the molecular fate of these neuropeptides. Among the prominent neuropeptides only for CART photoperiod-induced changes in arcuate nucleus mRNA were in the direction that would be anticipated for a catabolic peptide (see Chapter I). However, neither for POMC, NPY, AGRP nor for LRb itself observed photoperiod-induced changes were conclusive. The study presented in Chapter VII (Helwig, M. et al., submitted to “Journal of Neuroendocrinolgy”), paves the way to unravel the neuroendocrine feedback loops initiated by hormonal signal transduction. This study focussed on the photoperiod-mediated fate of the neuropeptide precursor POMC, whose endoproteolytic processing results in the release of prominent bioactive anorexigenic neuropeptides. Intriguingly, despite counterintuitive SD-induced downregulation of the precursor the cleaved products α-MSH and β-endorphin were increased in key hypothalamic centres of hamsters acclimated to SD. Alongside, arcuate nucleus mRNA of an enzyme (prohormone convertase 2) responsible for proteolytic cleavage of POMC was augmented. This study may represent a vital step forward to better understand the complex neuroendocrine circuits underlying seasonal body weight regulation.

A further fascinating enigma remains the identification of the missing link between the signal encoding the photoperiod (substantial evidence support that the pineal hormone triggers photoperiod-induced adaptations) and the imposed alterations in hypothalamic signalling events and energy balance. However, in Chapter IV our data suggest a potential novel mechanism in which the melatonin signal may be processed in a photoperiod dependent manner via ERK phosphorylation within the pituitary pars tuberalis.

Finally, it has to be mentioned that the ARC on itself, is not able to regulate energy balance. It is part of complex neuroendocrine projections that begin in this key neuronal centre and terminate in other hypothalamic and extra-hypothalamic nuclei. Among the extra-hypothalamic regions the nucleus of the solitary tract (NTS), situated in the caudal hindbrain, may act as a secondary key centre in integrating afferent hormonal input. This inspired us to initiate experiments in order to investigate the role of this hindbrain region in seasonal body weight regulation. Interestingly, phosphorylated protein of the key signalling components STAT3, ERK and AKT was conspicuously localised in the NTS (Tups A., unpublished data), suggesting a key role of this second integrative centre in triggering seasonal body weight cycles.

The key target, however, remains to develop evermore sophisticated experimental approaches in model systems such as the seasonal Siberian hamster in addition to rodent species with imposed diet- or genetic- induced body weight perturbations to improve our understanding of the pathogenesis of obesity.

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Neuropeptides and anticipatory changes in behaviour and physiology: seasonal body weight regulation in the Siberian hamster

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Abstract

The Siberian hamster, *Phodopus sungorus*, is a powerful model of physiological body weight regulation. This seasonal model offers the potential to distinguish between the compensatory neuroendocrine systems that defend body weight against imposed negative energy balance, and those that are involved in the programming of the level of body weight that will be defended—a seasonally appropriate body weight. Of the known, studied, components of the hypothalamic energy balance system, the anorexogenic peptide, cocaine- and amphetamine-regulated transcript (CART), is the only candidate where gene expression changes in a manner consistent with a role in initiating or sustaining photoperiod-induced differences in body weight trajectory. Siberian hamsters effect a reversible biannual switch in leptin sensitivity in which only short day (SD)-acclimated hamsters that have undergone a reduction in body weight, adiposity and plasma leptin are sensitive to peripheral exogenous leptin. The suppressor of cytokine signalling protein, SOCS3, appears to be the molecular correlate of this seasonal sensitivity.

Keywords: Melatonin; Photoperiod; *Phodopus*; Leptin; SOCS3; CART

1. Anticipating a predictably hostile environment

A significant proportion of mammalian species lives out their lives in environments that not only vary from day-to-day and within each 24-h period, but which also have a pronounced seasonal cycle. Among the primary environmental variables to which animals have to adapt are day length, temperature, and climatic conditions such as rainfall and windspeed. These primary variables in turn have a major influence on food supply, energy expenditure and thus on the probability of successful breeding outcome. Consequently, animals that live in temperate latitudes are profoundly seasonal, an attribute perhaps best exemplified by the compression of reproductive and breeding behaviour and physiology into particular parts of the year. Since, in temperate latitudes, the seasons are predictable in occurrence, if not entirely in severity or duration, the possibility is opened up for animals to prepare their physiological processes for the coming challenges. By undertaking anticipatory changes in behaviour and physiology, animals are less likely to be caught out by seasonal changes in climate that might affect their own survival, and are less likely to invest costly effort in gestating and rearing young whose own survival prospects are limited by the environment into which they are born.

In order to be able to alter behaviour and physiology in anticipation of seasonal change, suitable environmental cues must be perceived and correctly interpreted before the animal commits itself to potentially costly adaptations. The environmental cue that is most predictable, and least likely to be subject to short-term or year-on-year variation, is day length or photoperiod, whereas another environmental cue, ambient temperature, is relatively undamped in terms of its day-to-day variability. Using the photoperiod signal, seasonal mammals synchronise a number of different key physiologies to the seasonal cycle in climate and thus in availability of nutrients. Characteristically, mating is timed in coordination with the gestation period of the species in question in order that offspring are born into a favourable

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environment where food is plentiful. However, in addition to seasonal reproductive cycles, such species frequently display additional physiological adaptations to the environment. Of particular interest in the context of this review are the annual cycles of food intake and body weight (Mercer, 1998). All animals consume food to satisfy their requirement for energy and nutrients. These requirements are determined by the physiological state of the animal at any given time, but, by the same token, the availability of food is a major determinant of the physiological state that the animal can afford to maintain. Mammals that live in temperate latitudes are variously confronted by a shortage or complete absence of food during the winter months, and have adapted their physiology and behaviour accordingly. These animals clearly *anticipate* the change in food availability and adopt strategies to cope with and survive this challenge. A familiar example of this is hibernation, wherein fat stores are laid down during times of plenty, and the animal lives off this stored energy during the winter months, when food is essentially unavailable, at least in the environment outside the overwinter retreat. Less familiar, but nevertheless with proven evolutionary value, is the strategy of voluntarily reducing food intake and body weight during the transition between summer and winter photoperiods. The strength of this drive to adhere to a seasonally appropriate body weight trajectory is illustrated by two rather different mammalian species, the Siberian hamster, *Phodopus sungorus*, also known as the Djungarian hamster, and the sheep, both of which, despite the provision of food in excess in the laboratory throughout the year, increase food intake and body weight in long photoperiods, and decrease food intake and body weight in short photoperiods.

2. Seasonal body weight regulation in the Siberian hamster

The changes induced by natural, and gradually changing photoperiod cues can be replicated in the laboratory by a simple square-wave switch in photoperiod. Many small seasonal mammals such as the Siberian hamster exhibit profound anticipatory changes in food intake, body weight and adiposity in response such simple changes in photoperiod (Wade and Bartness, 1984; Morgan and Mercer, 2001). Thus, transfer of laboratory-reared adult male hamsters from long day (LD) photoperiod (16 h light/8 h dark) to short day (SD) photoperiod (8 h light/16 h dark) can induce weight loss that may average 30–40% over a 12–18-week period (Fig. 1; Mercer et al., 2000, 2001). These large amplitude changes in body weight are reversible, either spontaneously following the development of a refractory state in hamsters held in SDs for prolonged periods, or following transfer back to LDs. Similarly, juvenile hamsters are also sensitive to photoperiod; transfer to SDs at weaning giving rise to restricted growth, low body fat and delayed pubertal development (Adam et al., 2000). Over the last two decades, and particularly since the landmark cloning of the leptin gene, rapid progress has been made in identifying components of the hypothalamic energy balance circuitry and peripheral feedback to these circuits (Schwartz et al., 2000; Woods et al., 1998) in defining the involvement of molecular components in the compensatory response of rodents to imposed challenges such as negative energy balance. The hamster model serves to emphasise the difference between compensatory and programmed body weight regulation, the former being essentially a defence mechanism, whereas the latter provides a means of effecting advantageous long-term changes in body weight, and moreover in the level of body weight that will be defended (Morgan and Mercer, 2001; Mercer and Speakman, 2001). By contrast to the compensatory systems, the regulation of programmed or anticipatory body weight change, such as that exhibited by seasonal mammals, remains largely unresolved, and elucidation of the mechanisms by which 'defended body weight' is adjusted is a research objective of considerable significance.

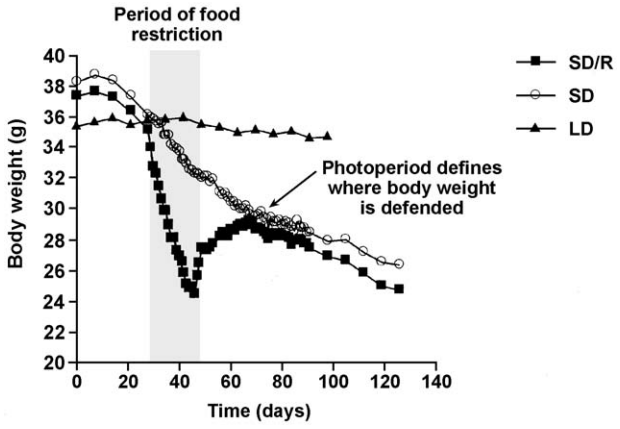

Fig. 1. Body weight of male Siberian hamsters fed ad libitum in short day length (SD) for 126 days, or held in short day length with restricted food (60% of ad libitum intake) between days 28 and 46 (SD/R). Shaded area represents food restriction period. For comparison, a typical body weight trajectory of hamsters fed ad libitum in long day length is shown (LD).

The power of the Siberian hamster as a model in which to address the issues outlined above is exemplified by a comparatively simple experiment that provides some of the best evidence that mammals directly regulate their body weight, and also provides some insight into how different levels of body weight regulation might function. The characteristics of body weight regulation in this species suggest the existence of a comparator system whereby actual body composition is assessed against encoded seasonally appropriate 'target' parameters. The behaviour of this system is defined in experiments first described over 20 years ago, where food restriction was superimposed on weight loss induced by a natural shortening photoperiod (Steinlechner et al., 1983), causing body weight to fall below a seasonally appropriate level. We

have recently replicated this experiment in the laboratory with a square-wave photoperiod transformation, with essentially the same outcome (Mercer et al., 2001). When restriction is lifted, body weight increases but only to the point where it approximates to the declining weight of control animals fed ad libitum in SDs throughout. The previously restricted animals then adopt a weight trajectory that parallels that of the control group (Fig. 1). Thus, the system behaves in a manner consistent with the seasonal timekeeping mechanism continuing to operate, and to adjust the encoded appropriate body weight, even when animals are prevented from maintaining their desired body weight (Bartness et al., 1989). There are several lines of evidence to suggest that melatonin signal 'accumulates', presumably at a brain site; the entry of animals into a refractory state appears to be determined by the number of days during which the nocturnal melatonin secretion profile exceeds a certain threshold, while the restriction experiment depicted in Fig. 1 is suggestive of incremental changes in appropriate body weight according to the accumulating photoperiodic history of the animal.

3. Central and peripheral energy balance systems

The maintenance of an appropriate body weight involves interactions between a network of central and peripheral signalling systems focussed on critical integratory centres in the hypothalamus (Kalra et al., 1999). The cloning of the leptin gene in 1994 (Zhang et al., 1994) has been the catalyst for increased activity in the field of energy balance, and several new candidate hypothalamic neuropeptide and receptor systems have been implicated in the regulation of food intake and body weight. A primary role for leptin is in the communication of information about adipose tissue energy stores and energy flux, providing prompt feedback to brain centres involved in the regulation of energy balance (Ahima and Flier, 2000). Leptin is generally present in the circulation in proportion to body adiposity, and exogenously administered leptin reduces food intake and body weight. The primary brain target of the leptin signal appears to be the hypothalamic arcuate nucleus, although other hypothalamic structures such as the dorsomedial nucleus, the lateral hypothalamus and the paraventricular hypothalamic nucleus also express the leptin receptor (Mercer et al., 1996). It is now recognised that the arcuate nucleus contains complementary orexigenic (e.g. neuropeptide Y and agouti-related protein [AGRP]; Ollmann et al., 1997) and anorexigenic (e.g. proopiomelanocortin [POMC] and cocaine- and amphetamine-regulated transcript [CART]) neurones that target the paraventricular hypothalamic nucleus. The lateral hypothalamus, which contains the cell bodies of the melanin concentrating hormone (MCH; Qu et al., 1996) and orexin (de Lecca et al., 1998; Sakurai et al., 1998) orexigenic systems, has connections to and from the arcuate, and has long been considered an important site in energy balance. Elucidation of the interactions of different components of the signalling array in the context of physiological body weight regulation has not been well studied, and may provide insight into the longer-term regulation of body weight in the normal animal. The majority of the information that we possess about the above systems relates to their involvement in the 'defence' of body weight against energy deficit. By contrast, little is known of the signalling framework underlying the encoding of an 'appropriate' body weight, i.e. the determination of the level at which body weight will be defended. Experimental evidence and mammalian life histories indicate that body weight regulation does indeed function at different levels (Morgan and Mercer, 2001; Mercer and Speakman, 2001). These can be broadly categorised as 'compensatory' weight change (i.e. acting to reverse an imposed perturbation) and 'programmed' long-term weight control, including anticipatory weight change.

Seasonal body weight trajectories have the appearance of being tightly controlled. There are a number of plausible routes through which photoperiod and the pineal hormone, melatonin, could effect this regulation (Fig. 2). Photoperiod could alter the tone of orexigenic and/or anorexigenic drive within the hypothalamus, modulate sensitivity to peripheral hormonal inputs, and in particular sensitivity to leptin, or it could impact upon as yet unknown regulatory systems that are relatively elevated in the hierarchy of energy balance signalling, i.e. that bridge the gap in our knowledge between the durational melatonin signal and the compensatory hypothalamic systems. In surveying known pathways for evidence of involvement in seasonal body weight trajectories or in attempts to define novel components of the regulatory system, we could be looking for either gradual, incremental changes in the activity of a signalling system that leads body weight along an appropriate course, or for a more abrupt switch in activity that effectively pushes body weight along. In examining neuroendocrine systems that are involved in short-term 'compensatory' regulation, and that are perturbed by imposed energetic manipulations, we should anticipate that many of these systems will not change activity in response to seasonally appropriate body weight change, and it will be important to distinguish neuroendo-

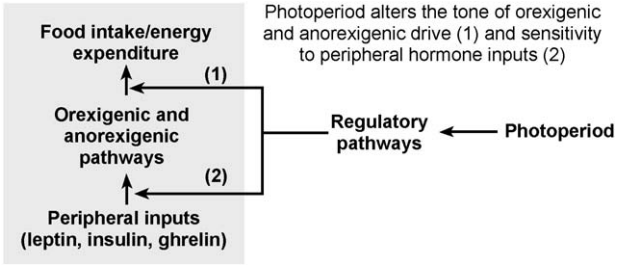

Fig. 2. Schematic showing possible mechanisms of seasonal body weight regulation in the Siberian hamster.

crine changes that drive the dynamic weight change from those that are secondary either to this change or to other co-incident physiological changes. In studying the molecular mechanisms underlying seasonal weight change, the majority of studies have understandably concentrated on animals with established body weight differentials. Latterly, however, with the identification of some potential mediators of seasonal weight change, attention has turned to the more dynamic phase in the seasonal cycle as body weight trajectories begin to diverge.

4. Hypothalamic neuropeptides in the Siberian hamster

Studies in a number of laboratories carrying out research with the Siberian hamster have identified several neuropeptide and receptor genes where levels of expression change following photoperiod manipulation. Significantly, compared with their respective controls, profiles of hypothalamic gene expression in adult male hamsters exhibiting sustained SD weight loss were very similar to those of SD juvenile females with growth restriction (Mercer et al., 2000, 2001; Adam et al., 2000). Changes are centred on the arcuate nucleus. The common denominator in these studies is POMC, the mRNA of which is consistently down-regulated after exposure to SDs of a duration sufficient to induce significant weight differential (Reddy et al., 1999; Mercer et al., 2000, 2001; Adam et al., 2000; Rousseau et al., 2002). This down-regulation is observed in both adult and juvenile animals after prolonged SD exposure but not after exposures of 3 weeks or less (Mercer et al., 2003). The counter-intuitive down-regulation of POMC gene expression, which would presumably result in reduced catabolic drive through the melanocortin MC_4 receptor in the paraventricular hypothalamic nucleus, has been shown to be photoperiodically driven and not to be influenced by declining gonadal steroid in elegant steroid clamped castrate experiments carried out by Rousseau et al. (2002). This is important since one component of the physiological response of the Siberian hamster to short photoperiod is the regression of the reproductive system and an accompanying fall in gonadal steroid synthesis. The reduction in gonadal steroid feedback will be responsible for part of the weight loss observed on transfer to SDs since surgical gonadectomy reduces body weight in LD hamsters independent of any photoperiod change (Wade and Bartness, 1984; Mercer et al., 1997). Other neuropeptide genes, which have shown altered expression following photoperiod manipulation, include AGRP and CART. Significantly, neuropeptide Y gene expression has been consistently demonstrated not to be affected by the prevailing photoperiod even when hamsters at their SD body weight nadir are compared to maximum weight LD animals (Reddy et al., 1999; Mercer et al., 2000, 2001; Adam et al., 2000). The neuropeptide Y gene has been well characterised as one of the orexigenic systems that are up-regulated in negative energy balance states such as food deprivation and its stability throughout photoperiod-induced weight change is interpreted as recognition of an appropriate state of energy balance despite a net catabolic state, i.e. seasonally appropriate weight change. The changes observed in AGRP gene expression in adult males (Mercer et al., 2000) were again counter-intuitive in the context of weight loss - elevated expression being likely, if translated into peptide synthesis, to increase antagonist availability at the melanocortin MC_4 receptor and thus further reduce catabolic drive. However, trends in the AGRP data from juvenile female hamsters were in the opposite direction (Adam et al., 2000), so the significance of these findings is unclear. Nevertheless, overall the changes in POMC and AGRP gene expression were not consistent with a critical role for signalling through the melanocortin MC_4 receptor in photoperiod-induced body weight trajectories, since their summated effect would be to reduce the negative drive on energy balance and presumably oppose the programmed catabolic state that exists in the SD hamster. Of relevance, although a melanocortin MC_4 receptor agonist administered into the cerebral ventricle of the brain inhibits food intake in Siberian hamsters, there was no effect of photoperiod on sensitivity to this compound (Schuhler et al., 2003). Neuropeptide Y is similarly equipotent in LD and SD hamsters (Boss-Williams and Bartness, 1996).

In the case of CART, observed changes in SDs were in the direction that would be anticipated for a catabolic peptide (Kristensen et al., 1998) that was involved in the establishment of a body weight differential. In our laboratory, and in animals drawn from the Rowett breeding colony, the up-regulation of CART gene expression in the arcuate nucleus is very consistent, being observed following a number of different manipulations of adult and juvenile hamsters of either sex, while, most significantly, these changes were observed as early as 2 weeks into SD exposure and prior to the development of a significant LD–SD weight differential (Adam et al., 2000; Mercer et al., 2003). This suggests not only a role for this catabolic/anorexigenic peptide in SD weight loss or growth restriction, but also that a change in activity of the CART system could be involved in driving seasonal body weight regulation, as opposed to being a secondary response to that regulation. However, other laboratories have been unable to substantiate these findings. Possible explanations for this situation have been discussed in a number of recent publications (Rousseau et al., 2002; Robson et al., 2002; Mercer et al., 2003), without resolution, although from comparison of the studies of Rousseau et al. (2002) and Robson et al. (2002), gonadal steroid status appears unlikely to provide an explanation. Technical differences between the studies could be important, but these could be difficult to unravel.

In order to distinguish the effect of photoperiod on hypothalamic gene expression from the effect of the weight loss that accompanies prolonged SD exposure, i.e. to differentiate between hypothalamic changes that

induce weight change and those that are secondary, consequential, events, hamsters held in LDs were food restricted to mimic the body weight trajectory of SD hamsters (Mercer et al., 2001). Although an equivalent degree of weight loss could be achieved through complete food deprivation, this is an inappropriate manipulation for comparison with short photoperiod-induced weight reduction. The more subtle manipulation of food intake through long-term restriction is likely to be more informative. The response of adult male hamsters to this imposed negative energy balance was largely as anticipated from studies of other rodents, with orexigenic systems and anorexigenic systems being, respectively, up- and down-regulated, consistent with their involvement in body weight defence or compensatory processes. Two of the genes examined were regulated in opposite directions by a similar degree of weight loss achieved through either imposed or programmed weight change achieved through energy restriction or photoperiod (Fig. 3). These genes were CART and the leptin receptor long form. CART gene expression, predictably, was down-regulated by imposed negative energy balance, but as discussed above was up-regulated in SDs. The opposite effects were observed for leptin receptor long form; low leptin levels induced by food restriction in LDs resulted in an up-regulation of receptor expression, in line with predictions from non-seasonal rodents (Baskin et al., 1998), whereas in SDs, leptin receptor long form gene expression was reduced compared to LD controls, as reported previously (Mercer et al., 2000). These findings clearly suggest some modulation of the way the leptin signal is read and integrated into the hypothalamus, according to the photoperiodic history of the animal, facilitating the recognition of the difference between weight change brought about by imposed negative energy balance and seasonal, and thus by definition appropriate, body weight change.

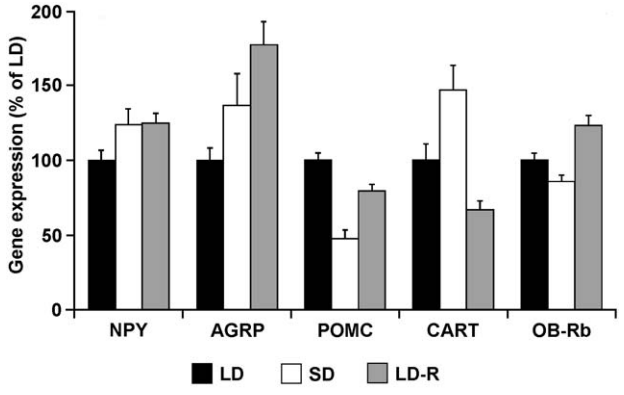

Fig. 3. Neuropeptide and leptin receptor gene expression in the hypothalamic arcuate nucleus of adult male Siberian hamsters ($n=9$ or 10) fed ad libitum in long (LD) or short day length (SD) for 84 days, or held in long day length with restricted food from day 14 onwards to mimic short day length body weight trajectory (LD-R). Values are expressed as percentages of values in LD-ADLIB hamsters. Means ± S.E.M. Abbreviations: neuropeptide Y, NPY; agouti-related protein, AGRP; proopiomelanocortin, POMC; cocaine and amphetamine-regulated transcript, CART; leptin receptor long form, OB-Rb.

5. Leptin signalling in the Siberian hamster

The concept of differential integration of the leptin signal into the hypothalamic circuitry was identified earlier as one of the routes through which mammals might effect changes in behaviour and physiology in response to a changing environment (Fig. 2), i.e. through adjustments in sensitivity to peripheral hormonal feedback. In the adult male Siberian hamster, a significant proportion of the body weight lost during the transition from an obese, LD phenotype to a lean, SD phenotype is adipose tissue. It is now well established that mobilisation of adipose tissue in SD hamsters reduces leptin gene expression and blood leptin concentrations (Klingenspor et al., 1996, 2000). The paradox in this feedback, then, is why, given the known effects of leptin on energy balance, the declining leptin signal during this transition does not act to reverse the changes programmed by photoperiod. The explanation of this paradox is now emerging from the data generated in a number of functional and molecular studies, although precisely what information is communicated in the leptin signal in seasonal mammals is still a matter for debate. For example, feedback on the levels of body fat storage is likely to be read quite differently to acute changes that result from energetic challenges, to which the lean SD hamster is likely to be more alert.

A number of studies of exogenous leptin administration to Siberian hamsters either by injection or by infusion with osmotic minipumps provide support for seasonal changes in sensitivity to leptin. Thus, LD and SD male hamsters had reduced food intake and body weight in response to peripheral injections of leptin over a 10-day period, but the effect on body weight was more substantial in SDs (Klingenspor et al., 2000). Using 14-day continuous infusions of recombinant leptin into male and ovariectomised oestradiol-replaced female hamsters, Atcha et al. (2000) observed reductions in body weight and adiposity in SD hamsters, but not in LD controls. There was no effect of leptin on food intake in either photoperiod. These seasonal changes in sensitivity to leptin, comparing relatively obese, LD hamsters with lean, SD hamsters are reminiscent of leptin resistance in age-related and diet-induced obesity (Scarpace et al., 2002), where experimentally reversing the trend towards increasing adiposity and hyperleptinaemia overcomes the insensitive state. However, experimental evidence suggests that leptin insensitivity in the LD hamster is not driven directly through body fat and circulating leptin, since LD hamsters that were food restricted prior to leptin infusion and thus had low plasma leptin and depleted fat depots remained insensitive, whereas SD animals with similar body composition and circu-

lating leptin lost further body weight and fat (Rousseau et al., 2002). These data clearly imply that it is photoperiod rather than nutritional or leptin status that is the key regulator of leptin sensitivity in the seasonal model. Regulated sensitivity to leptin feedback may be critical for the maintenance of seasonal bodyweight.

Insensitivity to leptin in LD Siberian hamsters could be the result of changes at a number of levels in the signalling pathway from molecule to post-receptor signal transduction. The rate of entry of leptin into the brain across the blood–brain barrier could be reduced. Alternatively, there could be a reduction in the availability of leptin receptors on the cell surface, although the elevated mRNA levels in LD hamsters compared to SD hamsters makes this appear unlikely (Fig. 3). More plausibly, there may be down-regulation of intracellular signal transduction distal to the leptin receptor. The JAK/STAT signalling pathway plays a critical role in mediating the effects of leptin on intracellular signalling (Sweeney, 2002). The Janus-family tyrosine kinases (JAKs) phosphorylate STAT proteins (signal transducers and activators of transcription), specifically STAT3, which then facilitates transcription of target genes. One class of target genes is the suppressors of cytokine signalling (SOCS), including SOCS3, which inhibits JAK/STAT activity and subsequent signal transduction. Thus, leptin activates SOCS3, which then reduces intracellular signalling by inhibiting the JAK/STAT pathway. This signalling system thus possesses powerful feedback loops, changes in the activity of which could well underlie observed changes in leptin responsiveness in the seasonal hamster. Such regulation may underlie leptin resistance in ageing-related obesity (Scarpace and Turner, 2001).

Our preliminary data from analysis of SOCS3 gene expression in the Siberian hamster do indeed suggest a role for this peptide in the reversible changes in sensitivity to leptin. SOCS3 in the arcuate nucleus is up-regulated in LDs compared to SDs (Tups, A., Ellis, C., Mercer, J.G., unpublished results). In juvenile female hamsters, this gene expression differential is apparent quite rapidly after photoperiod manipulation, and this appears to be a photoperiod effect rather than one that is dependent upon changes in body adiposity or leptin levels, thereby providing clear parallels with the in vivo functional data described above. Furthermore, SOCS3 mRNA in the arcuate nucleus was acutely stimulated by peripheral leptin injection only in SDs and not in LDs. Thus SOCS3 constitutes a molecular correlate of whole animal leptin sensitivity. These findings support the notion that reduced SOCS3 activity contributes to the increased sensitivity to leptin in SDs and, conversely, that increased SOCS3 activity results in the relative leptin insensitivity seen in LD.

One interpretation of these data is that the changing photoperiod primes arcuate nucleus sensitivity to circulating leptin, again suggestive of a direct interaction between the leptin and melatonin systems. This provides an explanation for how broadly equivalent long-term changes in the circulating leptin signal may be read differently according to the manipulation employed in their generation. The apparent early change in sensitivity at least at a molecular level could be interpreted as the leptin signal contributing to the maintenance of an appropriate body weight trajectory in SDs.

6. The role of the hypothalamic arcuate nucleus

The arcuate nucleus is a common denominator for the two genes, CART and SOCS3, that exhibit changes in gene expression prior to or simultaneous with the divergence of body weight trajectories in LDs and SDs. However, doubts exist surrounding the likely role of the arcuate nucleus in seasonal body weight cycles in the light of recent studies of hamsters bearing monosodium glutamate lesions (Ebling et al., 1998). Monosodium glutamate-lesioned Siberian hamsters, although growing more slowly than controls, retained their ability to regulate body weight to a seasonally appropriate level, despite the loss of 74% of neuropeptide Y gene expression in the arcuate nucleus in adult life, as a consequence of neonatal monosodium glutamate treatment. Although this is a major lesion, and one that also affects other brain regions that are considered to be outside the blood–brain barrier, it is possible that surviving neuropeptide Y-ergic cells still possess regulatory potential. The effect of monosodium glutamate treatment on other neuronal phenotypes in these animals is unknown. However, caution should be exercised before the potential role of a hypothalamic structure with such a pivotal function in peripheral-brain communication is discounted. Changes in the arcuate nucleus could be involved in effecting the adjustments in body composition that are required for the maintenance of an appropriate body weight, the level of which is likely to be encoded in a brain centre that is either directly or indirectly sensitive to seasonal inputs, photoperiod and the melatonin signal.

7. Resetting a seasonally appropriate body weight

The behaviour of the seasonal body weight regulatory process in Siberian hamsters is indicative of a brain system that somehow encodes a target body weight or body composition, and a comparator that allows actual body weight to be compared to this target. The accumulating melatonin signal then effects adjustments in this target that are appropriate to the perceived season. The known regulators of energy balance may be involved in executing adjustments in actual body weight but seem unlikely to be directly involved in the progressive resetting of the target or defended body weight. It is likely that components of the regulatory system are still to be discovered, particularly those that are relatively elevated in the hierarchy of signalling and which may be close to the integration of

the melatonin signal into the regulatory process. It is reasonable to assume that genes that are involved in determining the seasonal target body weight will exhibit maximal differences in expression at the extremes of body weight in LDs and SDs. Using this rationale, we have employed subtractive hybridisation techniques and gene arrays to identify genes that change in expression in response to photoperiod manipulation. The task for the future is to demonstrate that leads so generated are causative of weight change rather than secondary to that change. Identification of key components of a system that determines the level of body weight that will be defended, as opposed to the system that is involved in that defence, would open up a new class of targets for pharmacological manipulation. There is clear potential for such genes as drug targets in human obesity, where elevated body weight is strongly defended against conventional attempts at weight loss.

8. Summary

Although we understand some of the details of the pathways that are activated to defend body weight against imposed negative energy balance, or which are perturbed in obesity, the detailed mechanistic underpinnings of physiological body weight regulation are poorly understood. The accumulating data suggest that changes in signal transduction through the leptin receptor, mediated by SOCS3, and in the activity of the anorexigenic hypothalamic peptide, CART may be involved in the chain of events that leads to physiological changes in body weight in the Siberian hamster induced by photoperiod manipulation. Indeed CART is the only candidate mediator of SD weight loss whose activity changed in a manner consistent with the observed catabolic weight loss or growth restriction state, and which exhibited changes at or prior to the divergence of body weight trajectories in different photoperiods. Even so, changes in CART gene expression could clearly be responsive rather than inductive. Establishing a direct causative role in seasonal body weight regulation for any putative component of the signalling system represents a substantial challenge. Siberian hamsters are able to encode and defend a shifting seasonally appropriate body weight from further imposed change. The mechanisms controlling these processes are likely to be applicable in most mammalian species, including man, although not in a seasonal context. For example, the means by which chronic incremental changes in leptin signalling are integrated into hypothalamic regulatory systems may provide insight into the development of leptin resistance in human obesity. Similarly, the gradual resetting in an upward direction of the body weight that will be defended may underlie the difficulty experienced by many individuals in sustaining weight loss achieved by dieting. Seasonal animal models such as the Siberian hamster, where defended body weight is readily manipulated by photoperiod, provide a route through to these mechanisms.

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Photoperiodic Regulation of Leptin Sensitivity in the Siberian Hamster, *Phodopus sungorus*, Is Reflected in Arcuate Nucleus SOCS-3 (Suppressor of Cytokine Signaling) Gene Expression

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We present the first evidence that suppressor of cytokine signaling-3 (SOCS3), a protein inhibiting Janus kinase/signal transducer and activator of transcription (STAT) signaling distal of the leptin receptor, conveys seasonal changes in leptin sensitivity in the Siberian hamster. Food deprivation (48 h) reduced SOCS3 gene expression in hamsters acclimated to either long (LD) or short (SD) photoperiods, suggesting that leptin signals acute starvation regardless of photoperiod. However, SOCS3 mRNA levels were substantially lower in the hypothalamic arcuate nucleus of hamsters acclimated to SD than in those raised in LD. In juveniles raised in LD, a rapid increase in SOCS3 mRNA was observed within 4 d of weaning, which was completely prevented by transfer to SD on the day of weaning. The early increase in SOCS3 gene expression in juvenile hamsters in LD clearly preceded the establishment of different body weight trajectories in LD and SD. In adult LD hamsters, SOCS3 mRNA was maintained at an elevated level despite the chronic food restriction imposed to lower body weight and serum leptin to or even below SD levels. A single injection of leptin in SD hamsters elevated SOCS3 mRNA to LD levels, whereas leptin treatment had no effect on SOCS3 gene expression in LD hamsters. Our results suggest that the development of leptin resistance in LD-acclimated hamsters involves SOCS3-mediated suppression of leptin signaling in the arcuate nucleus. Increased SOCS3 expression in LD hamsters is independent of body fat and serum leptin levels, suggesting that the photoperiod is able to trigger the biannual reversible switch in leptin sensitivity. (*Endocrinology* 145: 1185–1193, 2004)

MUCH OF OUR knowledge about the roles of leptin and other peripheral and central signals involved in the regulation of body weight has come from studies of genetically obese mice and rats or from models of imposed negative energy balance. However, our knowledge of the dynamic long-term regulation of body weight remains limited. A fascinating model for studies in this field is presented by the seasonal mammal, *Phodopus sungorus*, which shows a remarkable natural body weight cycle determined by the prevailing photoperiod. Short day exposure (SD), either as a gradual change (natural conditions) or as an abrupt change (laboratory conditions), leads to a progressive reduction in body weight. Over an 18-wk period under these conditions, the Siberian (also known as Djungarian) hamster can lose 30–40% of its initial body weight, more than half of this loss being due to a reduction of adipose tissue mass (1). Associated with the loss in body fat is a decreased level of leptin gene expression in fat (2, 3) and lowered serum leptin concentration (1). A paradoxical situation is thus evident, where long day (LD)-acclimated hamsters with comparatively high endogenous leptin levels show a higher level of food intake (1) despite the purported anorexigenic leptin signal. This implies some resistance to the biological effects of this hormone in a LD summer photoperiod. Furthermore, Klingenspor *et al.* (1) demonstrated that twice daily leptin injections led to a more substantial reduction in body fat mass in SD than in LD animals. However, the neuroendocrine basis of photoperiod-induced changes in leptin sensitivity is not known.

The arcuate nucleus of the hypothalamus (ARC) is probably the most important integrative center for mediation of the leptin signal (4). Central transduction of the leptin signal is mediated by the long form of the leptin receptor (LRb) (5), activation of which results in autophosphorylation of the associated Janus kinase type 2 tyrosine kinase and the transmission of downstream phosphotyrosine-dependent signals. The transcription factor, signal transducer and activator of transcription-3 (STAT3), is the most potent intracellular mediator of the leptin signal. Once activated, STAT3 regulates the transcription of leptin-responsive target genes. One particular target gene is SOCS3 (suppressor of cytokine signaling), a broadly acting suppressor of cytokine signaling (6) that suppresses signaling downstream of the receptor by

Abbreviations: ARC, Arcuate nucleus; DMH, dorsomedial nucleus; IWAT, inguinal white adipose tissue; LD, long day photoperiod; PVN, paraventricular nucleus; RTWAT, reproductive tract white adipose tissue; RWAT, retroperitoneal white adipose tissue; SCN, suprachiasmatic nucleus; SD, short day photoperiod; SOCS3, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; VMH, ventromedial nucleus.

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inhibition of STAT3 phosphorylation. SOCS3 has been identified as a potential mediator of central leptin resistance (7, 8).

The hypothesis underlying the present study is that changes in leptin signaling pathways mediated by the inhibitory peptide SOCS3 may be critical to the physiological changes in body weight in the Siberian hamster. We hypothesize that photoperiod has a direct effect on the leptin signaling system at the level of signal transduction, whereby reduced arcuate nucleus SOCS3 expression in SD hamsters activates leptin signaling. Lower SOCS3 expression as an early response to SD could lead to an increased anorexigenic action of leptin levels and trigger the weight loss or growth restriction induced by SD. Here, we address this hypothesis in a series of experiments with hamsters subjected to energetic and hormonal challenges to characterize the impact of energy balance, photoperiod, and leptin on SOCS3 mRNA.

Materials and Methods

Animals

All procedures involving animals were licensed under the Animals (Scientific Procedures) Act of 1986 and received approval from the ethical review committee at the Rowett Research Institute. All experimental animals were drawn from the Rowett breeding colony of Siberian hamsters (9–11) and were gestated and suckled in an LD photoperiod (16-h light, 8-h dark cycle). All hamsters were weaned at 3 wk of age and were individually housed either at weaning or, in the case of adult animals, at least 2 wk before photoperiod manipulation. Where specified, hamsters were maintained in a SD photoperiod (8-h light, 16-h dark cycle), but with all other environmental conditions unaltered. Food (Labsure pelleted diet, Special Diet Services, Witham, UK) and water were available *ad libitum* unless specified otherwise, and rooms were maintained at 22 C. All animals were killed by cervical dislocation in the middle of the light phase, and brains were rapidly removed and frozen on dry ice.

Experimental protocols

Experiment 1: effect of extended SD exposure and food deprivation in juvenile female hamsters. Twenty-four female hamsters were divided into two groups of 12 at weaning, one of which was transferred to SD. After 8 wk, half the animals in each photoperiod group (n = 6) were deprived of food for 48 h, and the remainder continued to feed *ad libitum*.

Experiment 2: SOCS3 mRNA changes during the time course of SD acclimation. Archived brain sections were used from juvenile female hamsters killed at weaning or 4, 7, 14, or 21 d postweaning in either photoperiod (11). As before, all animals were gestated and weaned in LD, with transfer to SD at weaning, as appropriate.

Experiment 3: effect of extended SD exposure and food restriction in juvenile female hamsters. The protocol employed was similar to that previously described for adult males (10), except that the restriction periods were of either 6-wk (experiment 3a) or 12-wk (experiment 3b) duration. For each study duration, hamsters were allocated to one of three groups. One group remained in LD and fed *ad libitum* throughout the 6-wk (n = 12) or 12-wk (n = 12) experiment (LD-ADLIB). A second group was transferred to SD and was fed *ad libitum* throughout (SD-ADLIB; n = 12). The third group remained in LD, but received a restricted ration of food (LD-REST; n = 12), such that the group mean body weight tracked that of the SD hamsters. Body weight and food intake were measured daily for all animals during the restriction period. The degree of food restriction imposed on the LD-REST group did not exceed 65% of the LD-ADLIB intake at any point during the study. After 6 wk (experiment 3a) or 12 wk (experiment 3b), hamsters were killed, trunk blood was collected in lithium-heparin tubes, and brains were removed and frozen on dry ice. Selected white and brown adipose tissue depots were excised, among them retroperitoneal white adipose tissue (RWAT), inguinal white adipose tissue (IWAT), reproductive tract white adipose tissue (RTWAT), and interscapular brown adipose tissue (IBAT). The total mass of the dissected white adipose tissue depots was taken as a measure of body adiposity.

Experiment 4: duration-dependent effect of leptin injection in juvenile female hamsters. Forty-eight juvenile female hamsters were allocated to two weight-matched groups of 24 animals, one of which was transferred to SD for 8 wk. They were subdivided in each photoperiod, with half being injected ip with recombinant mouse leptin (2 mg/kg), and the other half with vehicle. Animals were killed 15, 30, 60, or 120 min after injection with leptin or vehicle (n = 3/group).

Experiment 5: effect of extended SD exposure and leptin injection in adult male hamsters. Twenty-four adult male hamsters, 4–6 months of age, were allocated to two weight-matched groups of 12 animals, one of which was transferred into SD for 12 wk. These groups were then subdivided so that one group in each photoperiod (n = 6/group) received an ip injection of recombinant mouse leptin (2 mg/kg body weight; R&D Systems, Minneapolis, MN) 1 h before death, and the other group received a control injection of vehicle (15 mM sterile HCl and 7.5 mM sterile NaOH) at the same time point.

RIA

Serum concentrations of leptin were measured using the Linco Multispecies kit (Biogenesis, Poole, UK) according to the manufacturer's instructions and as validated previously for use with hamster serum (10).

Hypothalamic gene expression

mRNA levels were quantified by *in situ* hybridization in 20-μm coronal hypothalamic sections using techniques described in detail previously (10). A riboprobe complementary to the suppressor of cytokine signaling-3 (SOCS3) was generated from cloned cDNA from the hypothalamus of the Siberian hamster. cDNA synthesis was performed using the cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The 465-bp SOCS3 fragment was amplified by PCR with 35 cycles of 94 C for 1 min, 59 C for 1 min and 40 sec, and 72 C for 2 min, then finally one cycle at 72 C for 10 min. The amplification was performed using the following primers: 5′-ACACCAGCCTGCGCCTCAAGACCT-3′ and 5′-TCGCCCCCAGAATAGATGTAGTAA-3′. The DNA fragment was ligated into pGEM-T-Easy, transformed into *Escherichia coli* DH5α, and sequenced. For cRNA synthesis by *in vitro* transcription, the SOCS3 cDNA fragment was subcloned into pBluescript II SK$^-$.

As previously described (10), forebrain sections (20 μm) were collected throughout the extent of the ARC onto a set of eight slides, with six or seven sections mounted on each slide. Accordingly, slides spanned the hypothalamic region approximating -2.7 to -1.46 mm relative to Bregma according to the atlas of the mouse brain (11a). One slide from each animal was hybridized. Briefly, slides were fixed, acetylated, and hybridized overnight at 58 C using ^{35}S-labeled cRNA probes (1–2 $\times$ 10^7 cpm/ml). Slides were treated with ribonuclease A, desalted, with a final high stringency wash (30 min) in 0.1$\times$ standard saline citrate at 60 C, dried, and apposed to Kodak Biomax MR Film (Eastman Kodak Co., Rochester, NY). Autoradiographic images were quantified using the Image-Pro Plus system (version 4.5.1, Media Cybernetics, Inc., Silver Spring, MD). Equivalent sections of individual animals were matched according to the atlas of the mouse brain. Four sections from the ARC of each animal spanning from -2.54 to -1.94 mm relative to Bregma were analyzed. Data were manipulated using a standard curve generated from ^{14}C autoradiographic microscales (Amersham Pharmacia Biotech, Arlington Heights, IL), and the integrated intensity of the hybridization signal was computed.

Statistical analysis

Data were analyzed by *t* test, one- or two-way ANOVA, followed by Student-Newman-Keuls multiple comparison test, as appropriate, using SigmaStat statistical software (Jandel Corp., Erkrath, Germany). Where data failed equal variance or normality tests, they were analyzed by Mann-Whitney rank-sum test or one-way ANOVA on ranks, followed

by Dunn's multiple comparison test. Results are presented as the mean ± SEM, and differences were considered significant at $P < 0.05$.

Results

Localization of SOCS3 mRNA expression in hamster hypothalamus

The species-specific probe to SOCS3 mRNA had an identity of 94% in nucleic acid sequence to *Mus musculus* (accession no. 14335396). Within the hypothalamus of the Siberian hamster, the probe to SOCS3 hybridized to the ARC, ventromedial nucleus (VMH), dorsomedial nucleus (DMH), paraventricular nucleus (PVN) and suprachiasmatic nucleus (SCN; Fig. 1). In addition to the hypothalamus, SOCS3 mRNA was detected in the pyriform cortex and the CA 1–3 region of the hippocampus (Fig. 1). Cross-hybridization of the hamster SOCS3 fragment with other members of the SOCS family is unlikely, as sequence comparison revealed low identities with other SOCS cDNAs; the highest identity was 25.5% to SOCS 1 of *M. musculus*. A sense probe synthesized from the cloned cDNA generated a low intensity, nonspecific signal.

Experiment 1: effect of extended SD exposure and food deprivation in juvenile female hamsters

Food deprivation has been reported to down-regulate SOCS3 in nonseasonal rodent species (12). We examined the effect of this manipulation in hamsters acclimated to either LD or SD (8 wk), thereby simultaneously assessing whether seasonal changes in photoperiod affect SOCS3 mRNA expression. The body weight gain of LD and SD hamsters started to diverge after 3 wk, attaining a statistically significant difference ($P < 0.05$) from 5 wk onward (Fig. 2A). Over the 8-wk period, SD hamsters gained 10.9 ± 1.0 g, whereas hamsters in LD gained 16.8 ± 0.8 g. Animals maintained in LD had significantly ($P < 0.05$) higher reproductive tract weights than animals in SD, independent of feeding status (LD-ADLIB, 260.6 ± 16.4; SD-ADLIB, 146.8 ± 16.6; LD-FD, 269.5 ± 16.0; SD-FD, 155.3 ± 13.2). Food deprivation for 48 h led to a loss in body weight of 13.4 ± 2.3% in LD hamsters and 17.9 ± 2.3% in SD hamsters. There were significant differences in serum leptin concentration between the groups. Both short photoperiod (by two-way ANOVA: $F = 46.20$; $P < 0.001$) and food deprivation (by two-way ANOVA: $F = 21.92$; $P < 0.001$) suppressed serum leptin concentrations (Fig. 2B). A highly significant reduction of SOCS3 mRNA levels was observed in the ARC (Fig. 2B; see also Fig. 1) in response to SD acclimation (by two-way ANOVA: $F = 91.96$; $P < 0.001$) as well as in response to food deprivation (by two-way ANOVA: $F = 19.40$; $P < 0.001$; Fig. 2C). However, there were no marked differences in SOCS3 gene expression in other hypothalamic nuclei (Fig. 1; data not shown).

Experiment 2: SOCS3 mRNA changes during the time course of SD acclimation

In view of the effect of 8-wk acclimation to SDs on SOCS3 gene expression, the time course of this regulation was examined in hypothalamic sections from an experiment in which, after weaning, juvenile female hamsters were maintained in either LD or SD for 4, 7, 14, or 21 d. There was no significant effect of photoperiod on body mass over this time course (11). Nevertheless, consistent differences in SOCS3 mRNA expression were observed between LD and SD hamsters (Fig. 3); in comparison with the weaning control group, SOCS3 mRNA expression in SD was maintained at a level similar to that observed at weaning, whereas LD hamsters had elevated SOCS3 mRNA expression. There was a significant effect of postweaning photoperiod ($F = 26.95$; $P < 0.001$). The differences in SOCS3 mRNA expression between LD and SD at individual time points attained significance ($P < 0.01$) on d 14 and 21 postweaning. Furthermore, after

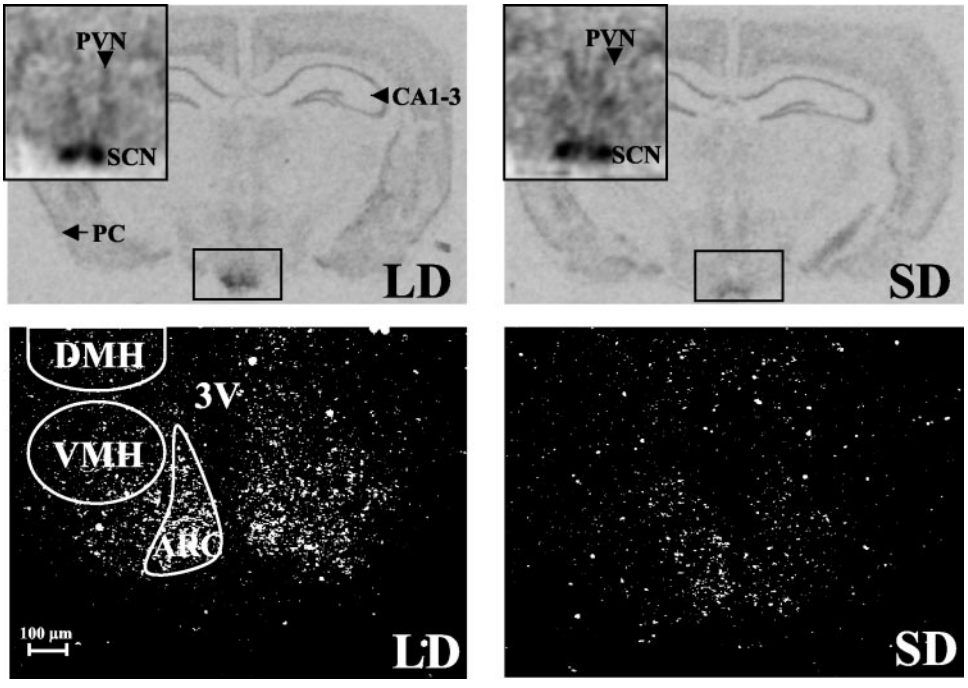

FIG. 1. *Upper panel*, Autoradiographs of LD or SD female Siberian hamster brain sections (20-μm coronal sections; 8 wk postweaning) after *in situ* hybridization to an antisense ^{35}S-labeled riboprobe to SOCS3 mRNA (*insets* in the *upper panel* depict hybridization to SCN and PVN). Shown are representative sections of animals from each photoperiod. *Lower panel*, Darkfield photomicrographs showing high resolution images of the respective hypothalamic regions (*boxed regions* in *upper panel*) in both photoperiods. CA 1–3, CA 1–3 region; PC, pyriform cortex; 3V, third ventricle.

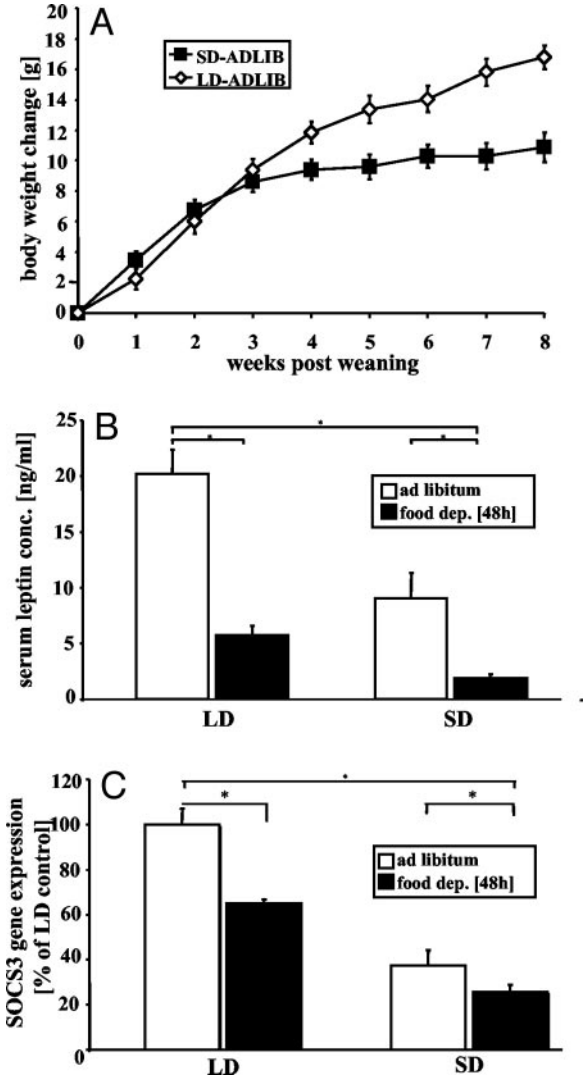

FIG. 2. Body weight change during 8 wk postweaning (A), serum leptin concentrations (B), and SOCS3 gene expression (C) of juvenile female Siberian hamsters fed *ad libitum* or food deprived for 48 h (n = 6) in LD or SD (n = 6). The body weight change gains significance from wk 5 onward ($P < 0.05$). The gene expression values are expressed as percentages of values in LD-ADLIB hamsters (mean ± SEM). *, $P < 0.05$.

only 4 d, LD animals revealed a significant increase in SOCS3 expression compared with the weaning group on LD ($P < 0.05$).

Experiment 3: effect of food restriction in LDs to mimic SD body weight changes in juvenile female hamsters

We then tested whether gradual reductions in food intake, body mass, or body fatness contributed to decreased SOCS3 mRNA levels in SD. Differential postweaning body weight gains were achieved over 6 wk (Fig. 4A) and 12 wk (Fig. 4B) for hamsters maintained in either LD or SD (LD-ADLIB and SD-ADLIB groups). The body weight gains of the SD group were mimicked by gradual incremental food intake restriction in LDs (LD-REST). At the end of the restriction periods, body masses of SD-ADLIB and LD-REST hamsters were 76.9% and 71.9% of those in respective LD-ADLIB controls at

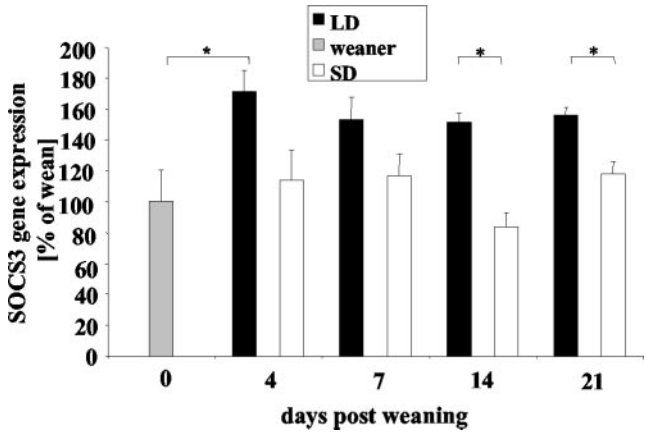

FIG. 3. SOCS3 gene expression in the hypothalamic ARC of juvenile female Siberian hamsters held in either LD or SD for 4, 7, 14, or 21 d postweaning (n = 4–8) and an LD weaning control group. Values are expressed as percentages of values in hamsters at weaning (mean ± SEM). *, $P < 0.05$.

6 and 12 wk postweaning. Body mass differentials were accompanied by differences in weight of adipose tissue (Table 1). There were linear correlations between body mass and adipose tissue mass among animals of the same age (6 wk: $r_s = 0.879$; $P < 0.0001$; 12 wk: $r_s = 0.942$, $P < 0.0001$). Hamsters at 6 wk postweaning had a higher body fat content at the same body weight than hamsters at 12 wk postweaning (Fig. 4C). Differences between LD-REST and SD-ADLIB hamsters were restricted to the mass of the reproductive tract (Table 1), although LD-REST hamsters had slightly more adipose tissue than SD-ADLIB at both 6 and 12 wk postweaning at a similar body weight. However, although LD-REST had an intermediate serum leptin level at 6 wk (LD-ADLIB > SD-ADLIB = LD-REST; $P \leq 0.05$; Fig. 4A), the 12 wk group had serum leptin concentrations below the level observed in SD-ADLIB animals (LD-ADLIB > SD-ADLIB > LD-REST; $P \leq 0.05$; Fig. 4, B and D). There was a significant correlation between adipose tissue weight and serum leptin concentration (Fig. 4D). Food restriction for either 6 or 12 wk had no effect on SOCS3 mRNA expression; there were no differences between LD-ADLIB and LD-REST hamsters (Fig. 4, E and F). Significant differences were observed between LD-ADLIB and SD-ADLIB groups (6 wk, F = 6.28; 12 wk, F = 25.9; $P < 0.05$). After 6- and 12-wk SD exposure, SOCS3 mRNA expression was suppressed 42% (Fig. 4E) and 52%, respectively (Fig. 4F) relative to that in LD-ADLIB hamsters.

Experiment 4: duration-dependent effect of leptin injections on SOCS3 mRNA expression in juvenile female hamsters

In this time-course experiment, we investigated the durational effect of leptin injections on SOCS3 mRNA expression in juvenile female hamsters. In all animals, an increase in serum leptin levels was observed after leptin injection (Table 2). Leptin only had an effect on SOCS3 mRNA expression in SD-acclimated hamsters. One hour after leptin injection, a clear increase in SOCS3 mRNA expression was observed (Fig. 5A). This effect was still apparent after 2 h. In contrast, LD-acclimated hamsters did not exhibit marked changes in SOCS3 mRNA expression at either 1 or 2 h after

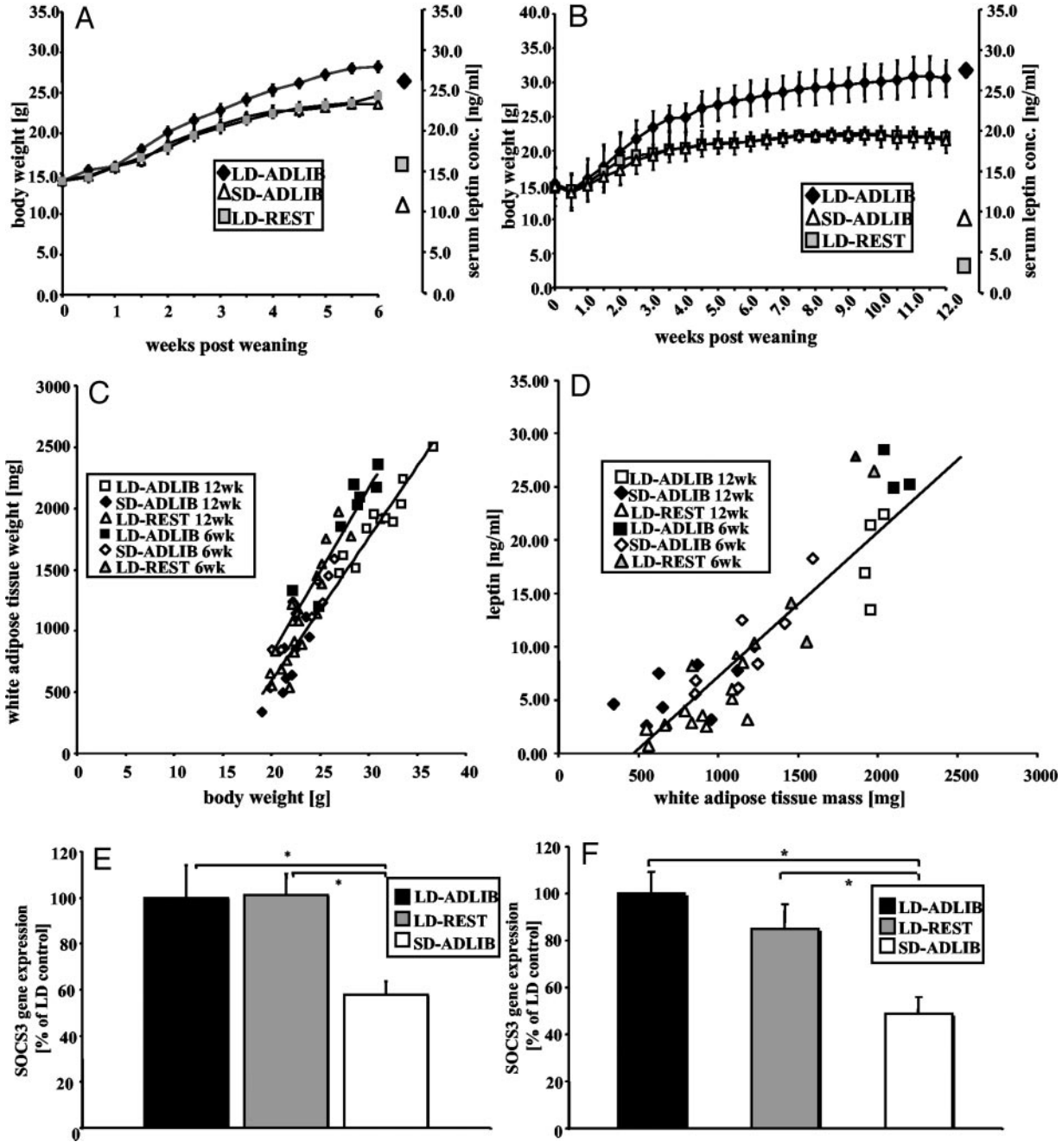

FIG. 4. Juvenile female Siberian hamsters (n = 12) were fed *ad libitum* in LD (LD-ADLIB) or SD (SD-ADLIB) for either 6 or 12 wk or were held in LD with restricted food from d 0 postweaning onward to mimic SD body weight trajectory (LD-REST). Figures show body weight trajectories and serum leptin concentrations (nanograms per milliliter; mean ± SEM) at 6 wk (A) or 12 wk (B) postweaning, correlation between body weight and pooled white adipose tissue weight (C; IWAT, RWAT, and RTWAT), correlation between pooled white adipose tissue weight (D; IWAT, RWAT, and RTWAT) and serum leptin ($y = 0{,}0135x - 5.9285$; $r^2 = 0.8236$), and SOCS3 gene expression (expressed as a percentage of LD-ADLIB; mean ± SEM) in the ARC after 6 wk (E) or 12 wk (F). *, $P < 0.001$.

leptin injection (Fig. 5, A and B). The animals that received a control injection exhibited higher SOCS3 gene expression in LD than in SD (by one-way ANOVA: $F = 45.3$; $P < 0.001$).

Experiment 5: effect of extended SD exposure and leptin injection in adult male hamsters

This experiment was designed to substantiate the effect of leptin injection on SOCS3 expression in a second paradigm, the adult male hamster. It also served to substantiate the effect of photoperiod on basal SOCS3 gene expression. Adult male hamsters were held in SD for 12 wk, by which time their mean body weight had fallen to 74.8% of the LD control group. LD hamsters had a final body weight of 39.3 ± 1.7 g, whereas SD hamsters decreased their body weight to 29.4 ± 1.2 g. Each photoperiod group was then divided into two weight-matched subgroups that received either leptin or ve-

TABLE 1. Tissue weights (milligrams) from hamsters maintained in LD or SD on *ad libitum* feeding (LD-ADLIB and SD-ADLIB, respectively) or subjected to food restriction to match SD body weight trajectory for either 6 or 12 wk (LD-REST, experiment 3)

	LD-ADLIB 6 wk	SD-ADLIB 6 wk	LD-REST 6 wk	LD-ADLIB 12 wk	SD-ADLIB 12 wk	LD-REST 12 wk
Reproductive tract	78.9 ± 8.2^a	36.3 ± 1.8^b	92.6 ± 4.7^a	99.5 ± 12.9^a	37.4 ± 2.0^b	85.7 ± 4.6^a
RWAT	267.9 ± 26.9^a	169.1 ± 12.7^b	197.7 ± 18.1^b	259.4 ± 14.5^a	110.8 ± 17.3^b	113.8 ± 8.8^b
IWAT	1365.6 ± 100^a	852.8 ± 62.8^b	1050.0 ± 76^b	1314.3 ± 71^a	532.3 ± 56.3^b	597.2 ± 45.9^b
IBAT	356.0 ± 29.3^a	240.1 ± 21.2^b	280.9 ± 27.8^b	393.9 ± 24.4^a	164.9 ± 17.0^b	179.9 ± 12.8^b
RWTAT	272.1 ± 31.1^a	183.1 ± 18.0^b	211.8 ± 29.6^b	323.5 ± 22.6^a	115.9 ± 17.8^b	146.1 ± 17.2^b
Total WAT	1905.6 ± 149.6^a	1205.0 ± 84^b	1459.4 ± 115.6^b	1897.1 ± 91.3^a	759.0 ± 88.6^b	857.1 ± 65.3^b

Values are the mean ± SEM. *Different letter superscripts* indicate statistically significant differences between groups ($P < 0.001$) compared with SD-ADLIB and LD-REST animals.

TABLE 2. Leptin concentration postinjection in juvenile female hamsters acclimatized to LD and SD for 8 wk postweaning (experiment 4)

Photoperiod	Treatment	15 min postinjection	30 min postinjection	60 min postinjection	120 min postinjection
LD	Vehicle	22.9 ± 2.8	18.4 ± 2.6	13.6 ± 2.9	16.9 ± 1.7
SD	Vehicle	3.5 ± 2.2	2.2 ± 0.3	9.1 ± 1.4	10.8 ± 8.8
LD	Leptin	(274.6 ± 13.9)	(273.3 ± 17.8)	(132.3 ± 99.9)	(262.6 ± 23.0)
SD	Leptin	(156.4 ± 15.0)	(121.6 ± 1.6)	(143.4 ± 3.5)	(142.9 ± 3.2)

Leptin concentration is presented as nanograms per milliliter. Values in *parentheses* are extrapolated because they are above the detection limit of the assay. Values are the mean ± SEM.

hicle injection. The serum leptin concentration in leptin-injected hamsters was increased compared with that in vehicle-injected animals in SD (SD vehicle, 3.9 ± 0.5; SD leptin, 184.1 ± 37.5 ng/ml) as well as in LD (LD vehicle, 14.6 ± 4.8; LD leptin, 143.2 ± 13.7 ng/ml). The values for the leptin-treated animals were extrapolated because they were above the detection limit of the assay.

Adult male hamsters exhibited a highly significant effect of photoperiod on SOCS3 expression (by two-way ANOVA: $F = 22.36$; $P < 0.001$; Fig. 5C), with a reduction to 36.6% in the SD compared with the LD control group. Consistent with experiment 4, 1 h after leptin injection, SOCS3 mRNA expression was significantly increased in SD hamsters to 94.0% of the LD control value (by two-way ANOVA: $F = 16.09$; $P < 0.001$), whereas no significant effect was observed in LD (Fig. 5C).

Discussion

Resistance to leptin is regarded as a significant potential factor in the development of obesity. Despite intensive research, the molecular mechanisms for generating leptin resistance are still largely unknown. Different functional *in vivo* studies that explored the effect of exogenous leptin demonstrated photoperiodic changes in leptin sensitivity in the seasonal mammal, *P. sungorus* (1, 13). Due to the reversibility of these effects in *P. sungorus*, this animal may contribute to a better understanding of the neurochemical basis of leptin resistance. Here we present strong evidence for central mediation of leptin resistance via the leptin signaling pathway distal to the leptin receptor within the hypothalamus. Our findings support a central role of SOCS3 in the seasonal changes in leptin sensitivity. However, it is important to note that sensitivity to centrally administered leptin has not been examined in the Siberian hamster.

The distribution of SOCS3 mRNA in the hypothalamus of the Siberian hamster was similar to that in other mammals (14) with the most intense hybridization in the ARC, an important integrative center in body weight regulation, and less intense, but distinct, hybridization to the VMH and DMH. The present study focuses on SOCS3 mRNA expression in the ARC, a nucleus that is assumed to play the most important role in transduction of the leptin signal into a neuronal response (14, 15) and where SOCS3 mRNA is co-expressed with NPY and POMC mRNA (Tups, A., unpublished observations). The localization of SOCS3 mRNA within the hamster brain is consistent with the distribution of leptin receptor mRNA (3) and the leptin-responsive transcription factor, STAT3 (Tups, A., unpublished observations). This implies that SOCS3 is involved in transduction of the leptin signal within the hamster hypothalamus, and differential SOCS3 mRNA expression may thus represent a marker for changes in leptin sensitivity.

In the present study we focused on hypothalamic SOCS3 expression in juvenile female hamsters in which photoperiod manipulations were performed at weaning. The duration of acclimation to opposite photoperiods was varied to allow determination of changes in SOCS3 mRNA expression longitudinally throughout SD acclimation. This facilitated investigation of both the molecular basis of catabolic drive early in SD photoperiod in animals whose body weight trajectories had not begun to diverge (4–21 d postweaning) and the importance of leptin signal transduction to the maintenance of SD adaptations over longer periods during which SD hamsters gained less weight than LD animals. The effect of leptin injection in female *P. sungorus* was substantiated in adult male hamsters, suggesting that the leptin signal is regulated similarly in adult and prepubertal hamsters of either sex.

It is known that food deprivation for 48 h down-regulates SOCS3 gene expression in the ARC of nonseasonal rodent species [*e.g.* rats (14)]. The present study confirms this effect for the first time in the seasonal animal, *Phodopus sungorus*. The decline in SOCS3 mRNA levels mediated by acute catabolic intervention is independent of the photoperiod in

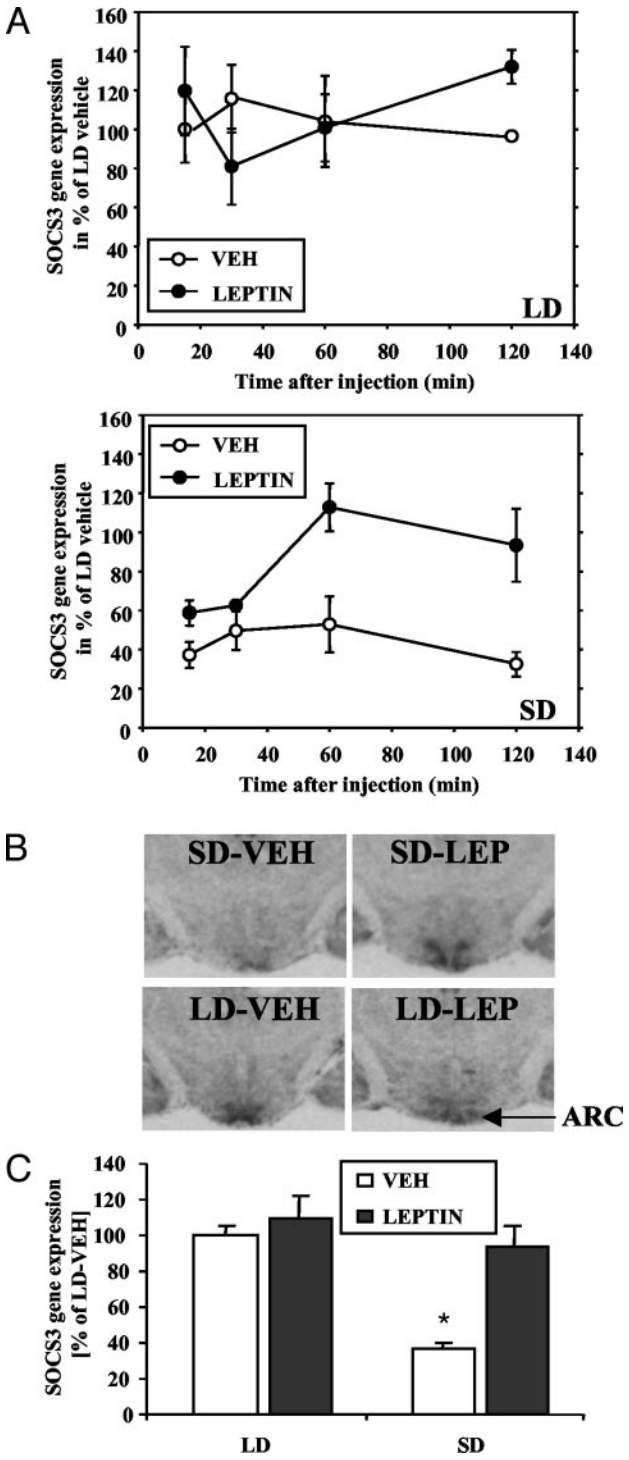

FIG. 5. Time-dependent effect (A) of leptin injection on SOCS3 gene expression in the hypothalamic ARC of juvenile female hamsters held in LD or transferred to SD for 8 wk (n = 3) and typical autoradiographic images (B) 1 h after injection [recombinant mouse leptin was injected ip at different time points (15, 30, 60, and 120 min) before preparation of the brains]. Values are expressed as percentages of values in LD 15 min vehicle-treated animals (mean ± SEM). C, SOCS3 gene expression in the ARC of adult male Siberian hamsters in LD and SD (n = 6) 1 h after leptin injection. Values are expressed as percentages of values in LD animals injected with vehicle (mean ± SEM). *, $P < 0.001$.

which the hamster is maintained and may be the consequence of the abrupt fall in circulating leptin in response to acute food deprivation. However, in contrast to food deprivation, more gradual changes in serum leptin resulting from chronic food restriction do not affect SOCS3 mRNA levels. In these studies and in states of energy balance, photoperiod is the prime modulator of SOCS3 gene expression.

We determined elevated expression of SOCS3 mRNA in hamsters maintained in LD compared with animals kept in SD. This photoperiodic difference is present in hamsters of both sexes and becomes manifest after limited exposure to different photoperiods; the maximum difference occurs between 8 and 12 wk of exposure. Our results support the hypothesis that SOCS3, as a potential inhibitor of leptin signaling, may contribute to the reduction in leptin sensitivity in hamsters acclimated to a LD photoperiod, as reported previously (1). Comparatively high intracellular SOCS3 mRNA levels, which most likely result in elevated SOCS3 protein concentrations, may suppress the anorectic action of the leptin signal in LD by inhibiting phosphorylation of the transcription factor, STAT3. The finding that SOCS3 mRNA expression in SD juvenile female hamsters over the period up to 3 wk postweaning remains close to the level observed at weaning, whereas gene expression in LD is already augmented after 4 d postweaning, suggests that exposure to LD is accompanied by a gain of leptin resistance, whereas SD exposure does not change leptin sensitivity compared with that of animals at weaning. However, body weight trajectories for animals in opposite photoperiods did not start to diverge within the first 3 wk postweaning. Thus, changes in SOCS3 mRNA preceded body weight changes, raising the possibility that SOCS3 may be involved in the induction of these changes. Disinhibition of the leptin signal, represented by decreased SOCS3 mRNA expression in SD, may enhance the anorectic action of leptin in SD. In juvenile females, the early blockade of SOCS3 up-regulation observed in LD by SD exposure suggests that SOCS3 may be involved in the induction and maintenance of an appropriate body weight trajectory.

The study revealed differential SOCS3 expression independent of body weight change in young female hamsters immediately postweaning (*i.e.* 4–21 d). This suggests that body weight is unlikely to play a substantial role in SOCS3 regulation. To investigate the relationship between body weight and SOCS3 expression, we analyzed LD animals that were food restricted to mimic SD weight trajectory. Chronic food-restricted LD hamsters exhibited SOCS3 expression levels that were unaffected by this long-term catabolic intervention. The fact that LD-REST hamsters had significantly reduced adipose tissue mass and serum leptin compared with their *ad libitum*-fed conspecifics indicates that leptin plays a minor role in mediating SOCS3 mRNA expression in this photoperiodic state, as substantiated by the serum leptin levels recorded in this restriction experiment. In contrast to *ad libitum*-fed hamsters, in which serum leptin levels were proportional to adipose tissue mass, in the 12-wk LD-REST group, leptin levels were lower than expected from adipose tissue mass, indicating altered leptin secretion or turnover in response to long-term food restriction. To our knowledge this is the first indication that leptin resistance, as indicated

by high expression of SOCS3, can also be associated with low endogenous leptin levels. These findings imply that SOCS3 in LD is expressed constitutively and is unaffected by chronic changes in serum leptin levels; thus, SOCS3 is still expressed at a high level despite a low serum leptin concentration due to food restriction. A functional central leptin resistance in LD could be mediated by constitutive inhibition of the anorexigenic action of leptin. This implies that either the low leptin concentration is sufficient for activating SOCS3 mRNA expression, or degradation of SOCS3 mRNA is reduced, which may lead to decreased turnover of this inhibitory peptide. Furthermore, up-regulation of LRb, as observed in male hamsters after food restriction in LD (10), provides a possible mechanism for compensating for declining leptin levels to keep SOCS3 mRNA expression on a high constitutive level.

Our findings imply that aside from acute energetic challenges, photoperiod is a major parameter triggering adjustments in leptin sensitivity in *P. sungorus.* The molecular transducer of photoperiodic information is the pineal hormone, melatonin, and interaction between photoperiod, melatonin, and the leptin system may occur, but there is also evidence for photoperiodic responses not mediated by melatonin (16). However, the importance of photoperiod, rather than leptin, as a key regulator of leptin sensitivity in the seasonal hamster was also supported by a functional study by Rousseau *et al.* (17). In this experiment, chronic peripheral leptin infusion was given to hamsters with low body weight, fat reserves, and circulating leptin, brought about by either SD exposure or imposed food restriction in LD. This treatment caused body weight and fat loss in SD, but had no such effects in LD. Furthermore, by performing studies in ovariectomized, steroid-clamped hamsters, the Rousseau study also strongly suggested that whole body and hypothalamic responses to leptin are primarily induced by photoperiod rather than by seasonal changes in sex steroids.

SOCS3 gene expression is not exclusively regulated by leptin. It is known, for example, that insulin can induce phosphorylation of SOCS3 through Janus-activated kinase (18). Nevertheless it is very unlikely that photoperiodic regulation of the SOCS3 gene is mediated via insulin; the current experiments demonstrate the direct induction of SOCS3 mRNA by exogenous leptin and continuing elevated SOCS3 gene expression in LD-REST hamsters, which, due to their negative energy balance, are presumably hypoinsulinemic.

SOCS3 mRNA expression in the ARC of SD hamsters was increased 1 h after leptin injection. This was demonstrated in adult male hamsters as well as in juvenile female hamsters, with maximum induction after injection occurring at 30–60 min. These data are consistent with SOCS3 induction in cell culture. Auernhammer *et al.* (19) demonstrated that SOCS3 gene expression was induced several-fold by the ligand leukemia inhibitory factor within 30 min. This finding can be compared with SOCS3 activation by leptin, because both ligands use the same Janus kinase-STAT pathway. Our data are the first to provide a time scale for SOCS3 gene activation by leptin *in vivo.* However, central signaling induced by exogenous leptin seems to be processed differently in long and short photoperiods, with further indication that the SOCS3 gene may be activated constitutively in LD. Even leptin concentrations well above the physiological range (and consistently higher in LD compared with SD) had only a minor effect on SOCS3 mRNA expression in LD, providing further evidence of leptin resistance in LD.

Intriguingly, the present study provides evidence that seasonal leptin resistance, as represented by *P. sungorus*, seems not to be associated with obesity as such. The significant positive correlation between body weight and total adipose tissue weight represented by IWAT, RWAT, and RTAT indicates that LD hamsters are appropriately fat for their body mass, suggesting that increased leptin resistance in LD is not a result of a disproportional elevation in adiposity.

In summary, our studies suggest that changes in SOCS3 mRNA within the ARC contribute to adjustments in leptin sensitivity, leading to a different reading of the leptin signal in LD and SD hamsters. These observed changes in SOCS3 gene expression are informative of the way in which the animal uses the leptin signal. The LD-acclimated and thus leptin-resistant hamster does not regulate SOCS3 gene expression via leptin under all circumstances; even a substantial decline in endogenous leptin levels after chronic food restriction did not affect SOCS3 mRNA expression. However, an abrupt decline in leptin levels caused by complete food deprivation decreases SOCS3 gene expression in both photoperiods, suggesting that leptin acts as a starvation signal regardless of photoperiod. This finding also suggests that manipulations leading to such acute reductions in blood leptin may effectively resensitize the brain to the leptin signal. In contrast, our data imply that the reading of gradual changes in circulating leptin is photoperiod dependent. This suggests that there may be an interaction between the leptin and melatonin signaling systems, with melatonin being elevated within the hierarchy of signaling. However, as some studies have demonstrated photoperiodic responses not mediated by melatonin (16), future experiments should address a potential role of melatonin in the control of SOCS3 expression. Nevertheless, in the seasonal animal, SOCS3 may be an early mediator of an appropriate body weight trajectory. The inhibition of leptin signal transduction by SOCS3 is regarded as a highly conserved mechanism. Thus, the biannual switch from leptin sensitivity in SD to leptin resistance in LD, which is manifested by ARC SOCS3 gene expression in *P. sungorus*, provides a basis for elucidating mechanisms of human leptin resistance and ways of manipulating the leptin system to overcome this central resistance.

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The suppressor of cytokine signalling 3, SOCS3, may be one critical modulator of seasonal body weight changes in the Siberian hamster, *P. sungorus*

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Abstract:

The Siberian hamster, *Phodopus sungorus*, exhibits a remarkable cycle of body weight, reproduction and leptin sensitivity in response to seasonal change in photoperiod. Here we scrutinise our hypothesis that the suppressor of cytokine signalling 3 (SOCS3) plays a critical role in the regulation of the seasonal body weight cycle. We analysed arcuate nucleus SOCS3 gene expression in SD (SD; 8:16-h light-dark) acclimated Siberian hamsters that were transferred back to LD (LD; 16:8h light-dark) and in hamsters that spontaneously became photorefractory to SD induced by prolonged exposure. SD acclimated hamsters that were transferred back to LD for 1, 2, 3, 4 or 6 weeks, increased arcuate nucleus SOCS3 gene expression to the LD level within 2 weeks, and maintained this higher level thereafter. The early increase of SOCS3 gene expression preceded the LD induced rise in body weight by approximately 3 weeks. Hamsters kept in SD for an extended period (25 weeks), began to become refractory to SD and to increase body weight. By this time point there was no difference in level of SOCS3 gene expression between LD and SD photoperiods, although body weight was still suppressed in SD hamsters. Finally we addressed whether SOCS3 gene expression is related to SD-induced gonadal regression or to body weight decrease by comparing Siberian hamsters with Syrian hamsters, the latter exhibiting substantial SD induced gonadal regression but only limited seasonal changes in body weight. Here acclimation to either LD or SD for 14 weeks had no effect on SOCS3 gene expression. This implies that arcuate nucleus SOCS3 gene expression is unlikely to be related to seasonal cycles in reproductive activity. Taken together the findings further strengthen our hypothesis that SOCS3 may be one molecular trigger of seasonal cycles in body weight.

Introduction:

Some small seasonal mammals endemic in habitats with extreme climate conditions, especially harsh winters, exhibit extraordinary physiological adaptations to their environment to increase their chance of survival. One representative species revealing extreme adaptations is *P. sungorus* (also known as the Siberian or Djungarian hamster). This species inhabits the dry winter climates in the arid Asian steppes, in Eastern and Northern Kazakhstan and South-Western Siberia (1) and must cope with extremely harsh winter conditions. *P. sungorus* is responsive to changes in photoperiod, which trigger a range of physiological adaptations, including a change in fur colour from greyish-brown to white, reproductive quiescence, and a remarkable progressive loss of body weight until a nadir corresponding to 60-70 % of the body weight in summer is attained. Much of this weight loss is due to depletion of body fat stores (2-4). Since these changes are directly induced by photoperiod they can be reproduced under artificial laboratory conditions providing a convenient model to investigate the molecular neuroendocrine basis of long-term body weight changes. The loss of body weight after switching hamsters from long day length (LD,16:8h light-dark cycle) to short day length (SD,8:16h light-dark cycle) is fully reversible as hamsters regain weight after transfer back from SD to LD. When hamsters are kept in SD for an extended period (more than 20 weeks) they exhibit photorefractoriness, meaning that they spontaneously and progressively regain body weight and reverse other SD characteristics until they attain a typical LD body weight although still being exposed to SD (3,5-8).

The seasonal body weight cycle of *P. sungorus* is closely correlated with circulating leptin concentrations and it has been demonstrated that hamsters in LD, which exhibit comparatively high leptin levels, are resistant to the catabolic effects of this hormone (4,9-11). Twice daily leptin injections at supraphysiological doses or chronic leptin infusion at physiological doses either caused a more substantial reduction in body fat in SD than in LD or

failed to induce a catabolic response in LD hamsters, respectively (4,9,10). The molecular identity of this seasonal switch in leptin sensitivity was not known. However, recently we demonstrated that the suppressor of cytokine signalling 3 (SOCS3), commonly regarded as one of the key factors in leptin signal transduction, is expressed at an elevated level in the arcuate nucleus of LD hamsters in comparison to SD hamsters. Furthermore, intraperitoneal leptin injection induced SOCS3 mRNA in SD hamsters but not in LD hamsters, suggesting that SOCS3 is a potential mediator of seasonal leptin sensitivity (11). The functional role of SOCS3 as a critical determinant of leptin sensitivity *in vivo* was recently substantiated by studies conducted in mice (12,13). Either heterozygous SOCS3 deficiency or neural cell-specific SOCS3 conditional knockout led to enhanced leptin sensitivity, weight loss and increased leptin receptor signalling in response to exogenous leptin administration.

Based on our previous studies we propose a model for the rheostatic[1] adjustment of the seasonally appropriate body weight by dynamic modulation of leptin sensitivity via SOCS3. We propose that a rapid downregulation of SOCS3 causing increased leptin sensitivity occurs after transition from LD to SD, well before body weight and circulating leptin levels decrease. Indeed, we observed a very rapid establishment of a SOCS3 gene expression differential as early as 4 days after transfer from LD to SD (11). Thus, in hamsters sensitised by SD exposure the high 'LD-like leptin levels' are translated into anorexigenic and catabolic responses mediated by enhanced central leptin signal transduction. Diminished SOCS3 expression could be one molecular trigger for the progressive decrease of body weight in SD photoperiod which proceeds until a new state of energy balance is achieved.

The present experiments were designed to assess whether the model proposed above can be applied to the second aspect of the seasonal body weight cycle, namely the positive body weight trajectory, which is presumed to be associated with the development of leptin

[1] Rheostasis: 'condition or state in which homeostatic defenses are still present but over a span of time there is a change in the regulated level (14)'

resistance, triggered either by SD-LD transition or by spontaneous photorefractoriness to SD. In these two situations we investigated the relationship of temporal changes in SOCS3 gene expression and body weight. Our model would require that body weight gain induced by LD or by photorefractoriness is preceded by an early increase in SOCS3 gene expression.

In addition, we verified whether SOCS3 gene expression is exclusively related to the SD induced body weight cycle or also reflects the seasonal cycle in reproductive status by comparing two seasonal hamster species. Other than *P. sungorus*, in the Syrian hamsters (*Mesocricetus auratus*), SD induces reproductive quiescence, but only modestly affects body weight (15,16).

Materials and Methods:

Animals

All procedures involving animals were licensed under the Animals (Scientific Procedures) Act of 1986 and received approval from the Ethical Review Committee at the Rowett Research Institute. Male Siberian hamsters were drawn from the Rowett breeding colony (17-19), and were gestated and suckled in LD. All hamsters were weaned at 3 weeks of age and were maintained in LD into adult life (4-6 months of age). Where specified, hamsters were transferred to SD, but with all other environmental conditions unaltered. Food (Labsure pelleted diet; Special Diet Services, Witham, Essex, UK) and water were available *ad libitum*, rooms were maintained at 22^{o}C, and animals were weighed every week. All animals were killed by cervical dislocation 3h after light on (ZT3), and brains were rapidly removed and frozen on dry ice.

Adult male Syrian hamsters were purchased from Harlan UK (Shaw's Farm, Blackthorn, Bicester, Oxon, UK) and housed individually in LD before photoperiod manipulation, whereupon these animals were either maintained in LD or transferred to SD for 14 wk.

Experimental Procedure

Experiment 1: Transfer of SD hamsters back to LD

To assess SOCS3 gene expression in SD hamsters transferred back to LD, two separate timecourse studies were performed to investigate early and late responses. In both cases groups of 40 adult male hamsters were divided into two weight matched groups, one of which was maintained in LD throughout whereas the other was transferred to SD. Starting body weights were very similar in each study. The difference between the two studies was the duration of the period of re-exposure to LD. In the first study after acclimation to SD for 15 weeks (timepoint = 0 weeks), hamsters were transferred back to LD for up to 3 weeks (SD to LD). Subgroups of hamsters were killed (n=5/group) at 3 timepoints: 1, 2 or 3 weeks after transfer back to LD. In the second study, 16 week SD acclimated hamsters were transferred back to LD for up to 6 weeks (20). In this experiment, in addition to the control animals (0 weeks), hamsters were killed (n=5 in each group) at 2, 4 or 6 weeks. In both studies, LD hamsters were killed as controls at each time-point.

Experiment 2: Spontaneous photorefractoriness

Archived brain sections were used from two groups of 14 adult male Siberian hamsters that were exposed to either LD or to SD for 25 weeks, as described previously (20).

Experiment 3: Cross-species comparison of SOCS3 gene expression

Two groups of 6 adult male Syrian hamsters were exposed to either LD or SD for 14 weeks. Brains were rapidly removed and frozen on dry ice, testes were excised and weighed.

Hypothalamic gene expression

Messenger RNA levels were quantified by *in situ* hybridization in 15 µm coronal hypothalamic sections, using techniques described in detail elsewhere (21). Sections were collected throughout the extent of the ARC onto a set of twelve slides with six or seven sections mounted on each slide. Accordingly, slides spanned the hypothalamic region approximating from -2.7mm to -1.46mm relative to Bregma according to the atlas of the mouse brain (22). One slide from each animal was hybridised with a Siberian hamster specific SOCS3 riboprobe cloned from cDNA, as described previously (11). Briefly, slides were fixed, acetylated, and hybridised overnight at 58^{o}C using [^{35}S]-labelled cRNA probes (1-2 x 10^{7} cpm/ml). Slides were treated with RNase A, desalted, with a final high stringency wash (30 min) in 0.1 x SSC at 60^{o}C, dried and apposed to Kodak Biomax MR Film (Kodak, Rochester, New York, USA). The integrated optical densities of autoradiographic images were quantified using the Image-Pro Plus system (MediaCybernetics). Equivalent sections of individual animals were matched according to the atlas of the mouse brain. By selection of four region matched sections spanning from –2.54mm to –1.94mm relative to Bregma the medial and most of the caudal part of the arcuate nucleus which revealed the strongest density in SOCS3 gene expression was covered. This alignment guaranteed the analysis of equivalent sections of each animal. In *Experiment 1,* where hamsters were transferred back to LD, sections of animals from both studies (1-3 weeks and 2-6 weeks) were hybridised in the same assay and analysed together. A standard curve was generated from ^{14}C autoradiographic micro-scales (Amersham Pharmacia Biotech), and the integrated intensities of the hybridisation signals computed.

Statistical analysis

Data were analysed by t-test, One or Two Way Analysis of Variance followed by Student-Newman-Keuls multiple comparison test, as appropriate, using SigmaStat statistical software (Jandel Corp., Erkrath, Germany). Where data failed equal variance or normality tests they were normalised by log transformation. If normality test was not passed after this transformation, data were analysed by Mann Whitney Rank Sum test or One Way Analysis of Variance on Ranks followed by Dunn's multiple comparison test. Results are presented as means ± SEM, and differences considered significant if $P<0.05$.

Results:

Localisation of SOCS3 mRNA

As previously described, the probe for SOCS3 hybridised within the investigated hypothalamic region of the Siberian hamster to the arcuate, ventromedial (VMH), dorsomedial (DMH), as well as to the paraventricular nuclei (11). In addition to the hypothalamus, SOCS3 mRNA was detected in the piriform cortex, in the CA 1-3 region of the hippocampus and in the dentate gyrus. The expression pattern of the SOCS3 gene in coronal forebrain sections of Syrian hamsters was very similar to that observed in *P. sungorus*. In all of the experiments reported below, distinct quantifiable differences in SOCS3 gene expression were only detected in the arcuate nucleus. In other hypothalamic regions SOCS3 gene expression was near the detection limit of the assay and thus not further analysed.

Experiment1:

In both studies, acclimation of adult male hamsters to SD for 15-16 weeks led to a well established body weight differential in comparison to the respective LD control group (LD 40.1 ± 0.9 g vs 15 wk SD 29.1 ± 0.6 g; LD 40.6 ± 1.0 g vs 16 wk SD 31.7 ± 1.1 g). A statistical significant reduction in body weight induced by SD occurred after 5 weeks

(compared to LD group *P< 0.05*; data not shown). Consistently in both studies, SD acclimated hamsters transferred back to LD maintained their low SD-induced body weight for 3 weeks (Fig. 1a,b). In the second study, body weights of these animals increased rapidly after 4 weeks and had regained LD levels within 5-6 weeks (Fig. 1b). Due to the very similar body weight parameters in each of the two studies, SOCS3 gene expression was assayed simultaneously for all animals in both studies, and data pooled for analysis.

There was a significant effect of both photoperiod (*Two way ANOVA: F=19.37; P< 0.001*) and the duration of time following transfer back to LD, on SOCS3 gene expression (*Two way ANOVA: F=4.00; P< 0.01*) but no interaction. Pair wise multiple comparison analysis exhibited a marked down-regulation *(P< 0.05)* of arcuate nucleus SOCS3 mRNA to 40% of the LD control group after acclimation to SD for a period of 15-16 weeks (Fig. 1c). This effect was still apparent between groups one week after transfer back to LD (*P< 0.05*). Two weeks after transfer of SD hamsters back to LD, an expression level typical of LDs was re-established, and maintained thereafter (no differences between groups, Fig. 1c).

Experiment2:

The body weight data for this experiment have been published previously (20). However, to illustrate the temporal heterogeneity in development of the photorefractory state, the weight trajectories of individual animals are shown in Fig. 2a. At the end of the experiment (week 25), a few animals were still on a SD body weight trajectory, some had attained body weights that were similar to controls held in LDs, whereas the majority had gained some weight but were still below the level of LD controls (20). Paired testes weight was also still lower in the SD photorefractory hamsters (LD: 456.7 ± 32.9; SD: 217.1 ± 27.8; *F=30.9; P<0.001*).

After 25 weeks of SD exposure, arcuate nucleus SOCS3 gene expression of all investigated hamsters was similar to that of LD controls (Fig. 2b). There was no correlation between individual body weights and SOCS3 gene expression (*P> 0.05,* data not shown).

However, for further analysis SOCS3 gene expression values of hamsters in both photoperiods were divided into two subgroups by body weight, one representing the lower 50 %, whereas the other the higher 50 %. There was a trend towards increased SOCS3 gene expression in SD photorefactory hamsters allocated to the 50 % higher body weight group (*Two way ANOVA: F=3.60; P = 0.07*). However, in LD such a trend was absent (Fig. 2c).

Experiment 3:

In contrast to Siberian hamsters, effects of photoperiod on body weight in Syrian hamsters were only observed after prolonged exposure with lower body weights in SD at weeks 13 and 14 (Fig. 3a; $P<0.05$) Acclimation to SD led to a substantial reduction in paired testes weights (Fig. 3b; $F=617.1; P<0.001$). There was no effect of photoperiod on hypothalamic arcuate nucleus SOCS3 gene expression (Fig. 3c).

Discussion:

Despite intensive research over the last decade we are still unravelling details of the pathways that are activated to defend body weight against imposed negative energy balance, or that are perturbed in obesity. In contrast to the investigation of these 'compensatory' mechanisms, research conducted in seasonal animals facilitates the decoding of the molecular identity of those mechanisms mediating a programmed or 'anticipatory' body weight change (23).

Seasonal cycles in body weight and body fat are closely correlated to changes in serum leptin concentration and a photoperiod-inducible and reversible switch in leptin sensitivity has been reported in two representatives of seasonal mammals, the Siberian hamster, *P. sungorus* (4,9,11) and the field vole, *Microtus agrestis* (E. Krol, personal communication). In a recent study, we demonstrated that SOCS3, as a molecular mediator, may convey seasonal changes in central leptin sensitivity in the Siberian hamster (11). This study revealed a significant reduction in SOCS3 mRNA levels in the hypothalamic arcuate nucleus of hamsters

acclimated to SD as compared to LD. In juvenile female hamsters this change clearly preceded observed changes in body weight trajectory. These previous findings suggest a model in which SOCS3 may be an important mediator adjusting sensitivity to circulating leptin to allow the establishment of the appropriate seasonally altered body weight. The current studies were performed to scrutinize this model and to verify a potential role of SOCS3 as one molecular trigger of seasonally programmed body weight cycles. Furthermore, cross-species comparison of photoperiod responses enabled us to examine the possibility that changes in SOCS3 gene expression are secondary to SD-induced reproductive quiescence.

The first experiment substantiated the results obtained from juvenile female hamsters (11), where significantly lower SOCS3 mRNA levels in response to SD were observed 2-3 weeks prior to a decrease in body weight. Here, in adult male SD hamsters transferred back to LD, SOCS3 mRNA attained a typical LD level within 2 weeks, and clearly preceded the change in body weight evident 3 weeks later. Also, in photorefractory hamsters, which were killed at a time when their mean body weight was on a positive trajectory but had not fully regained a LD level, SOCS3 gene expression was indistinguishable from that of LD controls. Further analysis by separating the photorefractory hamsters into two subgroups of the 50 % lower vs. 50 % higher body weights even revealed a trend towards increased SOCS3 gene expression only in the latter group which consisted of animals that had initiated a body weight gain towards LD levels.

Taken together the fact that in these two studies a rise in SOCS3 gene expression preceded either the body weight gain or the complete reestablishment of LD levels further verifies our proposed model. In this case increased SOCS3 gene expression may desensitise the neuronal network against leptin and induce a temporally delayed progressive body weight gain. Thus SOCS3 also meets the criteria of a potential modulator for the second aspect of seasonal body weight regulation, that being the positive body weight trajectory, induced either by switch back from SD to LD, or by photorefractoriness. An extremely interesting research

approach which may further validate our proposed model remains the determination of changes in leptin sensitivity during the entire seasonal body weight cycle. Specifying the temporal onset of leptin resistance (e.g. by investigating physiological effects of exogenously applied leptin) when hamsters become photorefractory may help to identify the physiological relevance of altered sensitivity against leptin in mediating seasonal body weight cycles.

Recent studies conducted in genetically modified mice with targeted disruption of SOCS3 reported a more pronounced leptin-induced weight loss compared to WT mice associated with resistance to diet-induced obesity (12,13). However, intriguingly, neither of these studies reported differences in body weight between SOCS3-deficient mice and their WT littermates when both were fed standard chow *ad libitum*. This contrasts with our observations where seasonally altered body weight is preceded by changes in SOCS3 gene expression. Thus action of SOCS3 in mediating body weight change may be specific for seasonal species. Although mechanisms regulating the spontaneous increase in SOCS3 gene expression and body weight in hamsters transferred from SD back to LD or photorefractory hamsters are presently still unknown, it seems to be very likely that melatonin may drive those changes because this pineal hormone is well known as the neuroendocrine cue regulating seasonal changes in physiology and behaviour in *Phodopus sungorus* (24).

SOCS3 is not the only gene that is up-regulated in the hypothalamus of *P. sungorus* in advance of overt changes in physiology. CRABP2 (cellular retinoic acid binding protein 2) and H3R (histamine H3 receptor) mRNAs are increased 2 weeks after SD hamsters have been transferred back to LD, with CRABP2 levels even exceeding the respective LD level. However, an interaction of these molecules with SOCS3 seems to be unlikely as differential expression of CRABP2 and H3R is restricted to a defined subregion of the arcuate nucleus, the dmp-arcuate, which we have proposed as a modulator of seasonal body weight (16,20,25). In contrast, SOCS3 mRNA extends throughout the entire arcuate nucleus where the leptin

receptor is also expressed in both Siberian and Syrian hamsters (26,27). The fact that the leptin receptor and SOCS3 mRNA are codistributed and that peripheral leptin administration activates arcuate nucleus SOCS3 gene expression in SD hamsters indicates that SOCS3 is largely involved in leptin signal transduction (11). However, it is important to note that SOCS3 has also been implicated in insulin signalling (28-32) and very recently we discovered a pattern of hypothalamic insulin receptor gene expression in the Siberian hamster which is strikingly similar to the distribution of SOCS3 mRNA in this species (A.Tups, unpublished data). Currently we cannot rule out the possibility that insulin also contributes partly to the modulation of photoperiod-induced changes in SOCS3 gene expression.

As SOCS3 sequesters the transcription factor STAT3, the body weight regulatory potential of SOCS3 may be based on impairment of the JAK-STAT pathway, and thus leptin-responsive target genes. A potential candidate downstream of the leptin receptor which may be a key player in regulating seasonal changes in body weight is the anorexigenic neuropeptide, cocaine- and amphetamine-regulated transcript (CART), for which SD induced up-regulation was reported as early as 14 days after SD exposure in juvenile female hamsters (19). This is consistent with the time course of change in our SOCS3 data (11). Early reduction in SOCS3 gene expression may trigger the increase of CART gene expression by reduced inhibition of leptin signalling on the level of transactivation of STAT3.

The gene expression of other candidate genes involved in the regulation of energy balance such as NPY, POMC, orexin, melanin-concentrating hormone and agouti related peptide are unlikely to be modulated by the inhibitory factor, SOCS3, in the context of seasonally programmed changes as none of them has been clearly implicated in seasonal body weight regulation (10,33,34). However, it is conceivable that post translational processing of these genes may be seasonally programmed.

Leptin has been reported to function as a gatekeeper of reproductive activity. In rodent models, as well as in man, mutations of the leptin gene or the leptin receptor lead to infertility, and leptin may have an important role in modulating the onset of puberty. Juvenile female hamsters acclimated to SD directly post-weaning fail to attain puberty and remain reproductively quiescent (17,35). Consequently, photoperiod-dependent alterations in leptin sensitivity and SOCS3 gene expression may be a secondary feature of reduced sex steroid secretion in SD. Atcha et al. (9) demonstrated that in ovarioectomized and steroid-clamped Siberian hamsters, leptin fails to act on either pituitary LH concentration or testicular size and testosterone levels in males suggesting that the catabolic effects of leptin can be dissociated from the sexual steroid axis.

Our results provide clear evidence that the observed changes in arcuate nucleus SOCS3 gene expression are primarily and exclusively induced by photoperiod, the only environmental parameter altered in our experiments. In hamsters transferred back from SD to LD, paired testes weights 2 weeks after transfer back to LD were still at reduced SD levels (20). In addition, in photorefractory hamsters, SOCS3 gene expression was already elevated to LD at a time when paired testes weights were still at a low SD level. The fact that Syrian hamsters which only exhibit limited seasonal changes in body weight but substantial SD induced gonadal regression fail to exhibit photoperiod-induced alterations in arcuate nucleus SOCS3 gene expression on the one hand validates the independence of observed changes in SOCS3 gene expression from photoperiod mediated gonadal regression, and on the other hand substantiates the role of SOCS3 as one potential trigger of seasonal body weight changes. However, a field for future research studies remains the determination of the exact physiological role of this factor in mediating seasonal body weight cycles by molecular manipulation of the SOCS3 gene in a seasonal species.

In summary, our data provide additional evidence for SOCS3 not only as a modulator of seasonal changes in leptin sensitivity, but also as a critical molecular factor in driving and

timing the programmed seasonal body weight cycle. Seasonal alterations in SOCS3 gene expression are independent of reproductive background and are primarily and exclusively induced by photoperiod.

Acknowledgements

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Figure legends

Fig. 1: Body weights of hamsters after transfer back from SD to LD compared with LD controls in the 3-week (a) and 6-week (b) studies in Experiment 1, and pooled data (c) showing SOCS3 gene expression in the hypothalamic arcuate nucleus of hamsters sacrificed after 15 or 16 weeks of SD acclimation (0 weeks), and 1, 2, 3, 4 or 6 weeks after transfer back to LD, and LD controls (0 and 2 weeks: n=7-10, otherwise n=4-5). The gene expression values are expressed as percentages of values in LD hamsters at 0 weeks. Means ± SEM,* $P < 0.05$, *** $P < 0.001$

Fig. 2: Body weight trajectories of individual hamsters held in SDs for 25 weeks and group means of LD controls (a), and arcuate nucleus SOCS3 gene expression of photorefractory hamsters as well as LD controls after 25 weeks photoperiod acclimation [n=13-14; Experiment 2 (b)]. Arcuate nucleus SOCS3 gene expression as shown in (b) but divided into the values of hamsters in both photoperiods that exhibited the 50 % lower body weights compared to the 50 % higher body weights [LD, lower 50 %: 35.5 ± 1.0 g; LD, higher 50 %: 41.6 ± 1.0 g; SD, lower 50 %: 26.2 ± 0.6 g; SD higher 50 %: 33.8 ± 1.3 g, (c)]. The gene expression values are expressed as percentages of values in LD hamsters. Means ± SEM

Fig. 3: Body weight (a), paired testes weight (b) and arcuate nucleus SOCS3 gene expression (c) of adult male Syrian hamsters acclimated to either LD or SD for 14 weeks (n=6; Experiment 3). The gene expression values are expressed as percentages of values in LD hamsters. Means ± SEM,* $P < 0.05$, *** $P < 0.001$

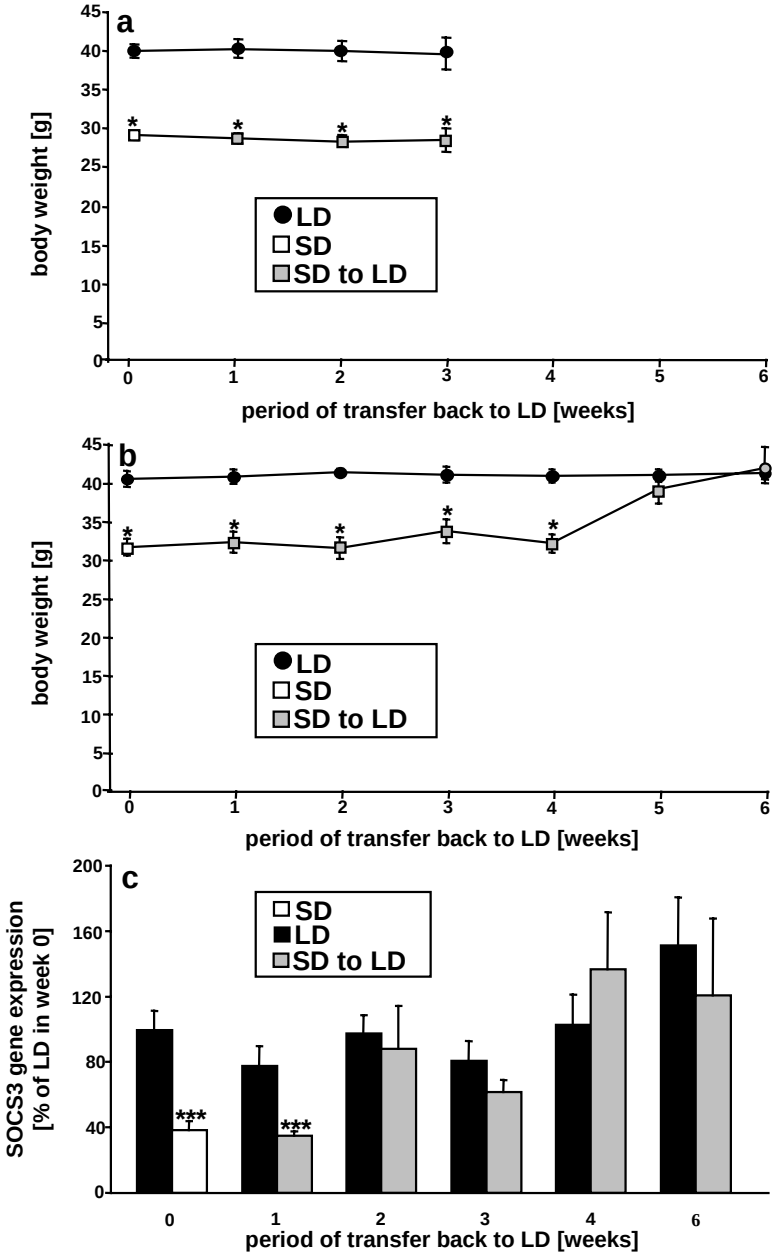
a
body weight [g]
LD
SD
SD to LD
period of transfer back to LD [weeks]
b
body weight [g]
LD
SD
SD to LD
period of transfer back to LD [weeks]
c
SOCS3 gene expression
[% of LD in week 0]
SD
LD
SD to LD
***
***
period of transfer back to LD [weeks]

Fig.1

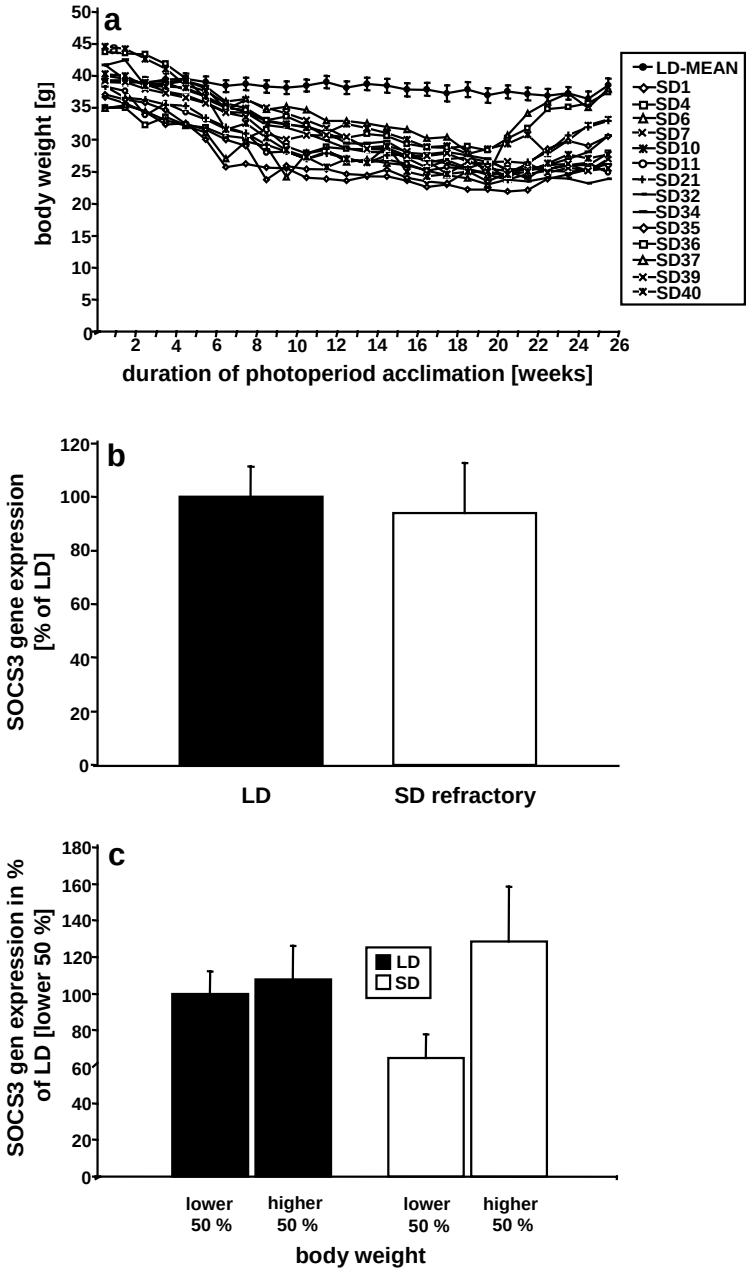
a
body weight [g]
duration of photoperiod acclimation [weeks]
LD-MEAN
SD1
SD4
SD6
SD7
SD10
SD11
SD21
SD32
SD34
SD35
SD36
SD37
SD39
SD40
b
SOCS3 gene expression [% of LD]
LD
SD refractory
c
SOCS3 gen expression in % of LD [lower 50 %]
LD
SD
lower 50 %
higher 50 %
lower 50 %
higher 50 %
body weight

Fig.2

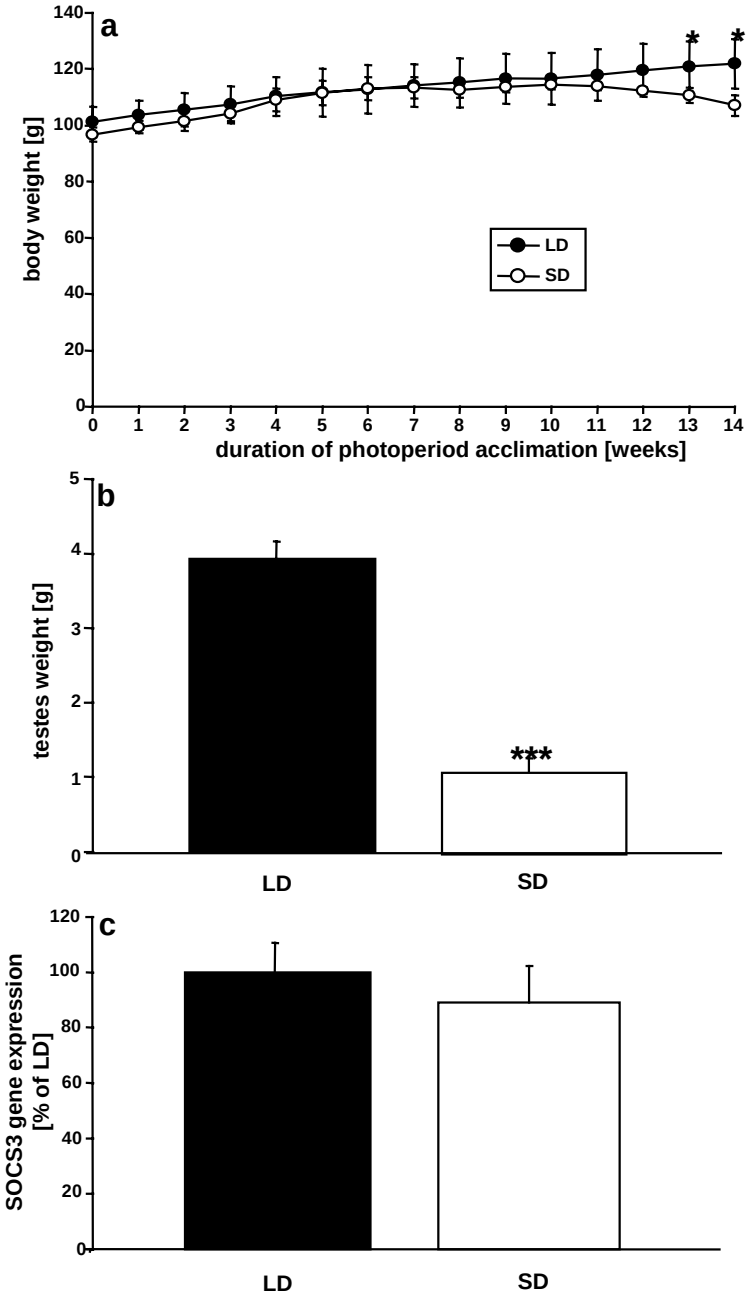
a
body weight [g]
duration of photoperiod acclimation [weeks]
LD
SD
b
testes weight [g]
***
LD
SD
c
SOCS3 gene expression [% of LD]
LD
SD

Fig.3

The JAK-STAT and ERK signalling pathways and their implication in seasonal body weight regulation of the Siberian hamster, *Phodopus sungorus*

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Abstract

It is well established that the striking seasonal cycle of leptin sensitivity and body weight revealed by the Siberian hamster, *Phodopus sungorus*, is strongly associated with dramatic alterations of arcuate nucleus suppressor of cytokine signalling 3 (SOCS3) gene expression. SOCS3 is known to inhibit phosphorylation of the signal transducer and activator of transcription 3 (STAT3). Here we demonstrate that nuclear translocation of STAT3 in the hypothalamus is rapidly stimulated by leptin throughout its rostro-caudal extension of hamsters held in long-daylength (LD) or subjected to short daylength (SD) for 10 weeks. However, in the hypothalamus of LD hamsters that are resistant to exogenous leptin and which exhibit increased SOCS3 mRNA, leptin-induced translocation of STAT3 was markedly reduced compared to hamsters in SD. Effects of leptin on STAT3 appeared to be limited on nuclear translocation of *phospho*-STAT3 associated with the cell surface rather than transphosphorylation of STAT3. In contrast to STAT3, the number of *phospho*- extracellular signal-regulated kinase (ERK) cells within the hypothalamus was not affected by either photoperiod or leptin. However, the pituitary pars tuberalis which plays a crucial role in melatonin (the neuroendocrine encoder of photoperiod information) signalling, revealed marked SD-induced increase in *phospho*-ERK cell numbers suggesting a hypothetical novel mechanism by which ERK signalling may integrate the signal encoding photoperiod with seasonal alterations in energy homeostasis. Proximal of ERK phosphorylation, hypothalamic SH2-containing tyrosine phosphatase (SHP2) and the small growth factor receptor binding protein (GRB2), which act as competitive negative modulators on binding of SOCS3 to LRb associated Tyr^{985}, were increased in SD compared to LD. Our findings suggest that activation of STAT3 by leptin may be dependent on interaction of stimulatory SHP2/GRB2 as well as inhibitory SOCS3 on the level of competitive binding to LRb associated Tyr^{985}. This hypothetical mechanism may represent the molecular identity of seasonally induced adjustments in leptin sensitivity.

Introduction

The seasonal Siberian hamster, *Phodopus sungorus* (also known as the Djungarian hamster), undergoes an anticipatory seasonal cycle in energy balance and body weight in response to the annual change in photoperiod. Acclimation to short daylength (SD) induces alterations in physiology such as reductions in food intake, energy expenditure and body weight that are reversible after transfer back to long daylength [LD; (1)]. Intriguingly, this cycle is closely correlated with circulating leptin concentrations and it has been demonstrated that hamsters in LD, which exhibit comparatively high leptin levels, are resistant to the catabolic effects of this hormone (2-5). Twice daily leptin injections at supraphysiological doses or chronic leptin infusion at physiological doses either caused a more substantial reduction in body fat in SD than in LD or failed to induce a catabolic response in LD hamsters, respectively (2-4). These features of the seasonal rodent provide a powerful means of investigating the dynamic long term mechanisms leading to the onset of leptin resistance, which is regarded as a key event in development of obesity and Type II diabetes.

The adipose tissue derived hormone, leptin, transported via the blood stream binds to its receptor, a member of the class 1 cytokine receptor family, expressed in the hypothalamus. Only the long form of the leptin receptor (LRb) possesses full signal transduction capacity, which can be initiated by three intracellular signal transduction mechanisms (6,7). The first mechanism involves the tyrosine residue Tyr^{985} located in the intracellular domain of LRb which recruits SH2-containing tyrosine phosphatase [SHP2; (6,8,9)]. The second is facilitated by LRb associated Tyr^{1138} which activates the signal transducer and activator of transcription 3 [STAT3; (6,10,11)]. The third is mediated via the Janus kinase 2 (JAK2), which is associated with the leptin receptor and, beside recruitment of numerous undefined signalling proteins, accounts in part (about 30%) for activation of the extracellular signal-regulated kinase [ERK; (6,8)]. However, major activation (about 70%) of ERK is considered to occur downstream of Tyr^{985}. Here, the small SH2 domain-containing adaptor, the growth factor

receptor binding protein (GRB2), plays a crucial role because of its ability to facilitate phosphorylation of ERK after binding to the Tyr^{985} activated SHP2 (6,8).

Recently we observed that differential expression of the suppressor of cytokine signalling 3 (SOCS3) within the arcuate nucleus may modify seasonal leptin sensitivity, leading to differential reading of the leptin signal in LD and SD hamsters (5). The inhibitory effect of SOCS3 upon leptin signalling is considered to be mediated by deactivation of STAT3 (9,12). Furthermore, it was reported that SOCS3 inhibits ERK phosphorylation, possibly via competitive binding to Tyr^{985} (13).

Therefore, in the present study we hypothesise that seasonal regulation of SOCS3 may simultaneously counteract activation of STAT3. We systematically examined the effect of leptin and photoperiod on STAT3 mRNA by *in situ* hybridisation and additionally assessed the physiological relevance of STAT3 phosphorylation by analysis of immunoreactivity (ir) at a cellular level throughout the entire hypothalamus. Moreover, we tested the hypothesis whether ERK, whose leptin-induced phosphorylation largely represents activity of the two STAT3-independent signal transduction mechanisms associated with LRb, may in part contribute to modulation of seasonal leptin sensitivity. Therefore, we analysed the effect of leptin on ERK activation on a post-translational level, by analysing *phospho*-ERK semi-quantitatively by immunoblotting as well as with neuroanatomical resolution by immunohistochemistry. Employing these powerful proteo-analytical methods, a possible photoperiod-induced modulation of total GRB2 protein was also assessed, which may be critical for ERK activation. Overall this study aims to further scrutinize the complex molecular mechanisms beyond SOCS3 that may modulate seasonal leptin sensitivity and energy homeostasis.

Materials and Methods

All procedures involving animals were licensed under the Animals (Scientific Procedures) Act of 1986 and received approval from the Ethical Review Committee at the Rowett Research Institute. Siberian hamsters were drawn from the Rowett breeding colony (14-16), and were gestated and suckled in LD. Juvenile female Siberian hamsters were weaned at three weeks of age and were individually housed at weaning whereas adult male animals were group housed until 4-6 months of age, then individually housed for 2-4 weeks before photoperiod manipulation. Half of the hamsters were transferred into SD, but with all other environmental conditions unaltered; rooms were maintained at 22°C. Food (Labsure pelleted diet; Special Diet Services, Witham, Essex, UK) and water were available *ad libitum*. Where specified, hamsters received a single intraperitoneal injection of recombinant mouse leptin (5mg/kg body weight; R&D Systems, USA) or vehicle (saline) 40 min before death. All animals were killed in the middle of the light phase by either cervical dislocation (for *in situ* hybridisation and immunoblotting) or by transcardial perfusion with 4 % paraformaldehyde under deep sodium pentabarbitone anaesthesia (for immunohistochemistry). Brains were conserved by methods described below appropriate for subsequent analysis.

Experimental procedure

Two experiments were performed to characterise the impact of photoperiod and/ or hormonal challenges on various levels of gene regulation of leptin signalling components. For the first experiment (immunohistochemical analysis of *phospho*-STAT3 and *phospho*-ERK), 16 adult male hamsters were employed and divided into two groups, one of which was transferred to SD. After ten weeks half of the animals in each photoperiod received a single injection of leptin or vehicle 40 min before being killed by transcardial perfusion. The second experiment (analysis of *phospho*-ERK by immunoblotting and investigation of GRB2 by immunoblotting and immunohistochemistry) consisted of juvenile female hamsters, which were either

maintained in LD for eight weeks (n=7) or transferred to SD for the same period (n=7) post weaning.

STAT3 mRNA was analysed by utilising archived brain sections of adult male hamsters kept in LD or SD for 12 weeks (5).

Hypothalamic gene expression

For *in situ* hybridisation, brains were rapidly removed and frozen on dry ice. Messenger RNA levels were quantified in 20 μm coronal hypothalamic sections, using techniques described in detail elsewhere (15). A riboprobe complementary to STAT3 was generated from cloned cDNA from the hypothalamus of the Siberian hamster. cDNA synthesis was performed using the cDNA synthesis Kit (Invitrogen, USA), according to the manufacturer's instructions. The 435-bp STAT3 fragment was amplified by PCR with 35 cycles of 94°C for 1 min, 57°C for 1 min 40 s, and 72°C for 2 min, then finally one cycle at 72°C for 10 min. The amplification was performed using the following primers: 5'-CCCCCGGGCACCTTCCTACT-3' and 5'-GGGCTCAGCACCTTCACCGTTATT-3'. The DNA fragment was ligated into pGEM-T-Easy, transformed into E. coli DH5α and sequenced. For cRNA synthesis by in vitro transcription the SOCS3 cDNA fragment was subcloned into pBluescript II SK-.

As previously described (15), 20μm forebrain sections were collected throughout the extent of the ARC onto a set of eight slides with six or seven sections mounted on each slide. Accordingly, slides spanned the hypothalamic region approximating from -2.7mm to-1.46mm relative to Bregma according to the atlas of the mouse brain (17). One slide from each animal was hybridised. Briefly, sections were fixed, acetylated, and hybridised overnight at 58°C using [^{35}S]-labelled cRNA probes (1-2 x 10^7 cpm/ml). Slides were treated with RNase A, desalted, with a final high stringency wash (30 min) in 0.1 x SSC at 60°C, dried and apposed

to Kodak Biomax MR Film (Kodak, Rochester, New York, USA). Autoradiographic images were quantified using the Image-Pro Plus system (MediaCybernetics). Corresponding sections of individual animals were matched according to the atlas of the mouse brain. Three-four sections from the ARC of each animal spanning from –2.54mm to –1.94mm relative to Bregma were analysed. Data were manipulated using a standard curve generated from ^{14}C autoradiographic micro-scales (Amersham Pharmacia Biotech), and the integrated intensities of the hybridisation signals were computed. Where appropriate, slides were coated with LM-1 film emulsion (Amersham Bioscience UK Limited, Buckinghamshire, UK) followed by counterstaining with cresylviolet dye.

Immunohistochemistry

For immunohistochemistry, animals were anesthetised with Euthatal (Rhone Merieux, Harlow, UK), transcardially perfused with 0.9% saline containing heparin (1000 U/litre) followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed and postfixed in the same fixative overnight at 4°C. On the next morning brains were transferred to 30% sucrose in 0.1M phosphate buffer for dehydration and cryoprotection. When brains had sunk they were frozen in isopentane cooled on dry ice for 1 min and sectioned coronally at 35 µm throughout the extent of the hypothalamus on a freezing microtome, collected in four series and stored in cryoprotectant at 4°C. For detection of phospho-ERK and GRB2 ir the following protocol was employed: Free-floating sections were incubated in 10% H_2O_2, 10% Methanol diluted in H_2O for 15 min to quench endogenous peroxide followed by incubation in blocking solution [5% bovine serum albumin (BSA), 0.5% Triton X-100 in phosphate buffer] for 45 min. Sections were incubated overnight at 4°C with anti-phospho-STAT3, anti-phospho-ERK or anti-GRB2 primary rabbit antibodies respectively [Cell Signaling Technology, USA; phospho-STAT3 (Tyr705): # 9131, 1:3000; phospho-ERK (Thr202/Tyr204; the antibody detects phospho-ERK 1 and 2 with similar

specificity), 1:100; GRB2: # 3972, 1:200] diluted in blocking solution. On the next day, sections treated with GRB2 or phospho-STAT3 primary antibodies were incubated with a biotinylated secondary goat anti-rabbit antibody for 1 h (1:1000, in blocking solution containing 3% BSA), and then treated with ABC (Vector Laboratories, Inc., Burlingame, CA) solution for 2 h. For detection of phospho-ERK ir utilization of the ABC method for signal enhancement was not necessary. Thus sections incubated with phospho-ERK primary antibody were treated with a horseradish peroxidase-conjugated (HRPO) goat-anti-rabbit antibody (Jackson Immunoresearch, 111-035-144) diluted 1:500 for 1h. Between steps, sections were washed in phosphate buffer containing 0.25% Triton X-100. Finally, the signal was developed by Nickel-DAB solution (Vector Laboratories, Inc., Burlingame, CA), giving a grey/black precipitate. For detection of phospho-STAT3 ir a slightly modified protocol was utilised. Endogenous peroxide was quenched in 1% NaOH and 1% H_2O_2 for 20 min. Before the subsequent steps of the protocol described above were deployed, two additional incubation steps were performed involving immersion in 0.3% Glycine for 10 min followed by a treatment with 0.03% SDS solution. Section images were captured using a Polaroid DMCe digital camera mounted on a Zeiss Axioskop (Jena, Germany).

Cell counting and quantification

For analysis of *phospho*-STAT3 and *phospho*-ERK, ir nuclei or cells, respectively, were counted in every fourth brain section. Sections were organised in a rostro-caudal manner and exactly region-matched between individual animals so that a longitudinal profile of ir throughout the entire hypothalamus was achieved. Since one of the bilateral halves of the hypothalamus was counted from each section, all numbers were multiplied by two to estimate the total number of nuclei or cells.

Immunoblotting

For immunoblotting, hypothalami were immediately excised with anatomical precision from freshly prepared brains, weighed, and rapidly frozen in liquid nitrogen. Using a glass homogenizer, tissues were homogenised in buffer containing phosphatase- and protease-inhibitors (10mM Hepes pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5 mM PMSF, 20mM NaF and 1mM Na_3V0_4) and incubated on ice for 10 min. For detection of GRB2 and *phospho*-ERK protein, the cytoplasmic fraction was separated from the nuclear fraction by centrifugation for 15 min at 3300g. The protein content of the supernatant containing the cytoplasmic fraction was determined by the Bradford assay, and equal amounts of protein were loaded into each lane. Immunoblotting-analysis was performed by standard method (18). Samples were separated by 12.5% SDS-PAGE and, after transfer to nitrocellulose membrane, stained with red Ponceau dye to ensure accurate protein loading and transfer. GRB2- and *phospsho*-ERK- protein were detected with the same polyclonal rabbit antibodies described under immunohistochemistry. Goat anti-rabbit-IgG-HRPO conjugate (Dako-Cytomation, Glostrup, Denmark) was used as secondary antibody (1:10000). Immunodetection was performed by enhanced chemiluminescence using ECL-reagent. Autoradiographs were quantified densitometrically using Scion-Image (Scion-Corporation, Maryland, USA) software.

Statistical analysis

Data were analysed by Two Way Analysis of Variance followed by Student-Newman-Keuls multiple comparison test, or repeated measurements ANOVA as appropriate, using SigmaStat (Jandel Corp., Erkrath, Germany) or SPSS Version 12.0 statistical software (SPSS Inc., Chicago, USA). Results are presented as means ± SEM, and differences considered significant if $P<0.05$.

Results

Effect of photoperiod on body weight in juvenile female and adult male hamsters

Acclimation of juvenile female or adult male hamsters to LD or SD for eight and ten weeks, respectively, led to similar body weight differentials determined in earlier studies [data not shown, (5)].

Localization of STAT3 mRNA expression in the hamster brain

The species-specific probe to STAT3 mRNA had an identity of 94% in nucleic acid sequence to *Rattus norvegicus* (Access. Nr. 6981591). Within the investigated region of the hypothalamus of the Siberian hamster, the probe to STAT3 hybridized to the ARC and ventromedial nucleus (VMH), as well as to the zona incerta (ZI; Fig. 1a-c). In addition to the hypothalamus, STAT3 mRNA was detected in the pyriform cortex, in the CA 1-3 region and dentate gyrus (DG) of the hippocampus (Fig. 1a,d,e). Cross-hybridization of the hamster STAT3 fragment with other members of the STAT family is unlikely, as sequence comparison revealed low identities with other STAT cDNAs, the highest identity found to be 37.1% to STAT4 of *Mus musculus*. A sense probe synthesised from the cloned cDNA generated a low intensity non-specific signal (data not shown).

Effect of photoperiod and leptin treatment on arcuate nucleus STAT3 gene expression

In adult male hamsters, exposure to SD led to a substantial reduction in body weight and serum leptin concentration (5).

Acclimation to SD induced a slight downnregulation of arcuate nucleus STAT3 gene expression to up to 80 % of the level observed LD. This effect was significant as revealed by *Two way ANOVA* ($F=8.595$; $P< 0.01$) and was restricted to the vehicle treated group (pair wise multiple comparison). There was no significant effect of leptin treatment or interaction of photoperiod and leptin treatment on STAT3 mRNA.

Effect of photoperiod and leptin treatment on hypothalamic content of phospho-STAT3 protein

Phosphorylation of STAT3 is a crucial event enabling this transcription factor to display its transcriptional activity. In order to investigate the role of STAT3 in seasonal leptin signal transduction we also analysed the hypothalamic content of total and *phospho*-STAT3 protein by immunohistochemistry.

The neuroanatomical distribution pattern of total STAT3 protein (which was equally distributed within individual cells) was identical to the localization of STAT3 mRNA and not different between LD and SD or leptin vs. vehicle treatment (determined by immunohistochemistry and immunoblotting; data not shown). Intriguingly, conspicuous regional differences in *phospho*-STAT3 ir occurred within individual cells. In vehicle-injected hamsters high resolution imaging revealed that most hypothalamic cells exhibited *phospho*-STAT3 ir confined to a structure very likely representing the cell surface with no conspicuous differences between SD and LD (Fig. 3a,c). Only a small subpopulation of hypothalamic cells revealed dense nuclear *phospho*-STAT3 ir. This cell population, belonging to the medial arcuate nucleus, was adjacent to the third ventricle or median eminence (Fig. 2d central panel).

Phospho-STAT3 ir was observed from –0.46mm to –2.70mm relative to Bregma across the hypothalamus but was absent more rostrally i.e from –0.46mm relative to Bregma. Leptin injection led to dense nuclear staining of *phospho*-STAT3, with simultaneous disappearance of *phospho*-STAT3 ir on the cell surface (Fig. 3b,d), and a striking increase in the number of *phospho*-STAT3 positive nuclei. This increase occurred in both photoperiods but notably was much more distinctive in SD, reaching a maximum in the mediobasal hypothalamus (Fig. 2a-e). The regional distribution of *phospho*-STAT3 positive nuclei was mainly confined to the

ARC, VMH and DMH but was not clearly associated with individual nuclei leading to the analysis of total nuclei numbers across the rostro-caudal extension of the hypothalamus.

Repeated measurements ANOVA of positive nuclei through the rostro-caudal extent of the hypothalamus revealed a highly significant effect of photoperiod ($F=20.459;\ P< 0.001$), treatment (leptin vs. vehicle, $F=98.577;\ P< 0.001$), and an interaction of both terms ($F=15.454;\ P<0.01$).

Localization of phospho-ERK protein in the hamster hypothalamus

Notably, the neuroanatomical localization of *phospho*-ERK protein within the investigated area of the hamster brain was conspicuously confined to the hypothalamus (Fig. 4a). *Phospho*-ERK protein was detectable in fibres and cell-bodies belonging to the ARC, VMH and DMH (Fig. 4a) as well as the SCN (here ir appeared to be only present within fibres, Fig. 4d). Within the hypothalamus the strongest density of *phospho*-ERK positive cell-bodies and fibres was detected in the VMH (Fig. 4b).

Outwith the hypothalamus immunohistochemical staining was restricted to the pituitary pars tuberalis (PT; Fig. 4c), the piriform cortex (Pir; Fig. 4e), cerebral cortex (data not shown) and the amygdala in which signals agglomerated in the posteroventral medial part (MePV; Fig. 4f).

Quantification of hypothalamic phospho-ERK protein by immunoblotting in LD and SD hamsters

In hypothalamic lysates of juvenile female hamsters two distinct bands were detected representing phosphorylated ERK 1 and 2 protein at 44 and 42 kDa, respectively. Notably, signal intensity was consistently increased in SD (Fig. 5a) compared to LD as substantiated by densitometric analysis (Fig. 5b). In order to assign these global hypothalamic changes in

phospho-ERK protein content to distinct hypothalamic nuclei we performed immunohistochemistry in the adult male hamster.

Effect of photoperiod and leptin treatment on hypothalamic phospho-ERK positive cells

Similar to *phospho*-STAT3, *phospho*-ERK ir was localized throughout the extent of the ARC, VMH and DMH without clear separations between respective nuclei (Fig. 4a, 6b-e). Thus we determined the total cell number throughout the entire rostro-caudal extent of the hypothalamus. This analysis revealed *phospho*-ERK positive cells extending from –0.46mm to –2.70mm relative to Bregma across the hypothalamus with a clear peak around the medial part of the ARC (Fig. 6a). However, analysis of these data by *repeated measurements ANOVA* did not reveal significant effects of photoperiod, leptin treatment or interaction of these terms.

Effect of photoperiod and leptin treatment on phospho-ERK ir within the pars tuberalis

Sections employed in the analysis of hypothalamic *phospho*-ERK neurons, were also surveyed for regional differences in *phospho*-ERK ir outwith the hypothalamus. The strongest signal intensity was observed in the pars tuberalis of the pituitary gland (PT) (Fig. 4c). Intriguingly, this dense *phospho*-ERK ir was only observed in SD hamsters (Fig. 7a); there was a highly significant increase in *phospho*-ERK ir cells in SD compared to LD [(Fig. 7b) *Two way ANOVA ($F=155.5$; $P< 0.01$)*]. However, there was no significant effect of leptin treatment or interaction of these terms.

Effect of photoperiod on hypothalamic content of GRB2 protein

Since GRB2 largely facilitates activation of ERK, we investigated whether the SD induced increase of *phospho*-ERK is reflected in the level of total GRB2 protein. Therefore, we analysed GRB2 protein quantitatively by immunoblotting as well as at the cellular level by immunohistochemistry in the juvenile female hamster.

A conspicuous and highly specific band of the expected size (25 kDa), representing total GRB2 protein, was detected in hypothalamic lysates of Siberian hamsters (Fig. 8a). Hamsters acclimated to SD exhibited increased signal intensity as compared to LD (confirmed by quantitative analysis, Fig. 8b). This SD induced rise of hypothalamic GRB2 protein content was confirmed by immunohistochemistry in four additional juvenile female hamsters (Fig. 8c-f). Within the investigated brain region, GRB2 protein was exclusively detectable in the VMH and the ARC of the hypothalamus (Fig. 8c,d). Here of particular interest was the strong density of GRB2 ir cell bodies and fibres within the ependymal layer separating the basomedial ARC from the third ventricle (Fig. 8e,f). The SD induced increase in GRB2 ir was consistently observed within the ARC and VMH (n=4 in each group). However, precise quantification of GRB2 ir was not satisfactorily accomplishable due to the heterogeneity of the ir signal; a clear separation of cell bodies from fibres was impossible). Notably, GRB2 ir was absent in the PT in either photoperiod.

Discussion

The phenomenon of leptin resistance has been well characterized in the Siberian hamster over the last five years (2-4). Dissection of hypothalamic leptin signalling pathways is crucial in order to better understand the molecular mechanism underlying dynamic long-term alterations in leptin sensitivity revealed by the seasonal hamster. Recently we reported that SOCS3 may be a key molecule mediating seasonal changes in leptin sensitivity. Acclimation to SD led to a rapid down-regulation of arcuate nucleus SOCS3 mRNA and intraperitoneal leptin injection induced SOCS3 mRNA only in SDs with expression levels being induced to a level similar to that observed in LDs. These findings substantiate leptin resistance at a molecular level in LDs (5). However, the biological relevance of this seasonal differential expression of SOCS3 SOCS3 and the molecular mechanism by which SOCS3 is altered with photoperiod remained unclear.

We now demonstrate that this inhibitory potential of SOCS3 is likely to be transmitted by STAT3 activation. Although in the present study arcuate nucleus STAT3 mRNA was only marginally affected by photoperiod and leptin treatment paradoxically failed to induce STAT3 gene expression in both photoperiods, *phospho*-STAT3 positive nuclei, representing the active variant of STAT3, were consistently induced by leptin in both LD and SD with a markedly greater elevation in the latter photoperiod. This leptin induced increase in *phospho*-STAT3 ir spanned the rostro-caudal extent of the hypothalamus and was apparent in the arcuate nucleus, VMH and to a lesser extent in the DMH. The maximal number of ir nuclei was detected in the mediobasal hypothalamus in both vehicle and leptin treated animals suggesting that this area plays a pivotal role in transduction of the leptin signal. This is consistent with studies performed in mice and rats. Furthermore, these findings may provide a link to the SOCS3 data. Here leptin induced elevation of SOCS3 mRNA in SD occurred 1h after intraperitoneal administration of the hormone and was maximal within the mediobasal hypothalamus, although restricted to the arcuate nucleus. Since cell culture studies demonstrated that STAT3 is a positive regulator of the SOCS3 gene (13), it is plausible that the leptin induced increase in SOCS3 mRNA may be explained by this mechanism especially given the temporal anticipation of the increase in STAT3 activation, the rise in the number of *phospho*-STAT3 nuclei precedes the increase in SOCS3 gene expression by 20 min. Regulation of leptin signal transduction *in vivo* by an oscillating negative feedback mechanism involving these two factors is theoretically conceivable. However, in our study hamsters were challenged with supraphysiological doses of leptin and it is questionable whether this hypothetical mechanism is of importance for the longterm dynamic regulation of leptin sensitivity at endogenous leptin levels. Hence two findings conflict with this hypothetical scenario: First, we did not detect a significant difference in the number of *phospho*-STAT3 positive nuclei between hamsters acclimated to either LD or SD (albeit there was a trend towards a slight increase in SD) despite marked reduction of SOCS3 mRNA in

the latter photoperiod. Second, as described above in control hamsters *phospho*-STAT3 ir nuclei were conspicuously confined to a small subpopulation of arcuate nucleus cells located adjacent to the third ventricle and median eminence whereas SOCS3 mRNA is expressed abundantly throughout the arcuate nucleus. Although the low number together with the limited topological extension of *phospho*-STAT3 positive nuclei in control hamsters may raise further doubts for the significance of this transcription factor as a gatekeeper of leptin sensitivity paradoxically exactly this finding might be of particular interest. Namely, the close proximity of *phospho*-STAT3 ir nuclei to the median eminence may imply a special function of these cells. The fact that the median eminence as a circumventricular organ is not protected from the blood brain barrier raises the possibility that the adjacent selected neurons may possess a special regulatory function due to the direct exposition to circulating factors. Hence it is conceivable that access of circulating leptin may be improved to these cells implying that enhanced *phospho*-STAT3 ir especially at "the gate to the arcuate nucleus" may be of special relevance for the transduction of the leptin signal into neuronal responses.

The findings discussed above are based on the absolute number of *phospho*-STAT3 ir nuclei. However, interestingly *phospho*-STAT3 ir was not restricted to this cellular structure. High resolution imaging of immunostaining revealed that most of *phospho*-STAT3 ir was present in a microstructure which we interpreted as the cell surface. This pattern was only observed within the hypothalamus and was absent outwith the hypothalamus substantiating the high specificity of this signal. This protein distribution pattern was present in leptin treated hamsters in both LD and SD and to a similar extent in control hamsters of either photoperiod. These observations strongly suggest that phosphorylated STAT3 protein may be associated with leptin receptors located on the cell surface. Furthermore, they imply that in contrast to studies performed in cultured cells, *in vivo* leptin may not induce the phosphorylation of STAT3 but rather may be responsible for the release of *phospho*-STAT3 from the receptor

leading to subsequent transduction to the nucleus where it displays its regulatory potential on target gene transcription. This hypothesis is supported by the fact that the signal on the cell surface was absent in cells that revealed intense nuclear immunostaining. At present we cannot rule out the possibility of subtle differences in total cellular *phospho*-STAT3 protein content between LD and SD. Quantification of absolute hypothalamic *phospho*-STAT3 protein content by immunoblotting would measure entire cellular *phospho*-STAT3 and thus represents a strategy to identify these potential differences. However, unfortunately at present none of the commercially available antibodies for *phospho*-STAT3 is compatible with this assay in the hamster.

Despite the solid evidence in the literature about leptin-dependent induction of STAT3 phosphorylation in other rodents species (19-22), equivalent studies of translocation phenomena are lacking. Double labelling strategies with markers specific for the nucleus and cell surface are planned in order to further substantiate these initial observations.

Nevertheless, by comparing our data with the literature describing phosphorylation of STAT3 as a marker for leptin sensitivity, striking similarities are apparent. Two studies dealing with alternative models of leptin sensitivity are particularly relevent. The first study performed by Münzberg *et al.* reported hypothalamic nucleus selective induction of STAT3 phosphorylation in mice fed a high fat diet vs. mice on low fat diet (22). In this model of diet induced obesity (DIO), mice which had been on high fat diet for 16 wk, 4 wk or only 6 days revealed reduced leptin-induced *phospho*-STAT3 within the ARC whereas other hypothalamic nuclei remained leptin sensitive. The second study performed by Ladyman *et al.* dealing with pregnancy associated leptin resistance, identified *phospho*-STAT3 as a possible regulatory element for this phenomenon (19). Here leptin-induced phosphorylation was reduced in the ARC and VMH in pregnant- compared to control rats. Although both studies describe leptin resistance initiated by totally different cues as pregnancy, diet or photoperiod (as it is the case for Siberian hamsters), the underlying molecular mechanisms

mediating this phenomenon seem to be similar. These findings strongly imply that the state of leptin resistance induced by e.g. high fat diet is very similar to that one induced by LD exposition in Siberian hamsters. Thus, future studies ought to focus on the advantage provided by Siberian hamsters, namely the possibility of completely reversing characteristics associated with leptin resistance followed by total regain of leptin sensitivity.

Since the JAK-STAT pathway is not the only mechanism by which the leptin signal can be transduced and seems not to account entirely for the marked regulation of SOCS3 gene expression by photoperiod, we analysed *phospho*-ERK since phosphorylation of ERK is critical for the activation of the Ras → Raf → ERK → c-fos signalling cascade and can be inhibited by SOCS3 *in vitro* (8,13). The ERK signalling cascade represents a broadly utilised signalling pathway where leptin is only one of numerous ligands displaying the potential of transactivation. Hence it is more than surprising that *phospho*-ERK ir within the brain of the Siberian hamster was largely confined to the hypothalamus extending through the ARC, DMH and VMH, with strongest intrahypothalamic density of ir fibres and cell bodies in the latter. Although SD induced elevation of phospho-ERK (1 and 2) was detected in hypothalamic lysates by immunoblotting in juvenile female hamsters, this effect could not be confirmed by comparing the number of *phospho*-ERK ir cells in adult male hamsters acclimated to LD or SD and which received either leptin or vehicle treatment. Since earlier studies revealed identical effects of leptin and photoperiod on SOCS3 gene expression in our two paradigms, the juvenile female and the adult male hamster (5), it seems unlikely that this discrepancy is due to functional differences between these two systems. Despite the fact that the number of *phospho*-ERK ir cells was not affected by either photoperiod or leptin treatment the conspicuous confinement of ir to hypothalamic body weight regulatory key centres strongly implies involvement of this signalling component in maintaining energy homeostasis. In contrast to our data, in *ob/ob* mice leptin-dependent phosphorylation of ERK was reported by immunoblotting 20 min after intraperitoneal injection (9). This discrepancy

could be related to methodological differences, or be due to the absence of circulating leptin in *ob/ob* mice hypersensitizing the signalling pathways and leading to hyperactivation of ERK in these animals. Thus the observed differences may reflect the functional distinctions between animals lacking endogenous leptin due to genetic defects and animals exhibiting intact energy homeostasis. However, the lack of leptin-induced phosphorylation of ERK does not explain the lack of photoperiod impact on this event. In this context our data question whether phosphorylation of ERK is a critical step for mediating long-term (seasonal) changes in leptin sensitivity. There was a dramatic heterogeneity of *phospho*-ERK ir within animals belonging to same treatment groups. These differences were up to 12-fold between region matched sections of individual hamsters. These findings imply that parameters other than photoperiod regulate phosphorylation of ERK within the hypothalamus. We hypothesise that one of these parameters may be the feeding status of the animal. Phosphorylation of ERK may depend on whether the animal just initiated or terminated a meal. Evidence for this hypothesis is provided by a recent study performed by Morikawa et. al (23). Here the number of *phospho*-ERK ir cells within the hypothalamus of mice was induced by fasting (48h). Additionally this effect was opposed in *ob/ob* mice further indicating perturbations of leptin signalling in these animals.

At first glance SD induced ERK phosphorylation revealed by immunoblotting clearly contradicts the density of phospho-ERK ir cells within the hypothalamus of SD and LD hamsters. However, on closer examination this apparent discrepancy may be explained by a very interesting finding. Within the brain sections we investigated, strongest *phospho*-ERK ir was exhibited by the PT. During brain preparation, major parts of the PT of the pituitary remain attached to the hypothalamus, and consequently the SD-induced increase in immunoblotting signal may be the result of cross-contamination of hypothalamic lysates with PT cells. Analysis of *phospho*-ERK ir within the PT revealed a striking increase in the number of positive cells in SD compared to LD. Since the PT has a very high density of

melatonin receptors, differential *phospho*-ERK ir within the PT may provide the molecular link between the pineal hormone melatonin, as the neuroendocrine transducer of the photoperiod signal, on the one hand and signalling mechanisms triggering seasonal changes in energy balance on the other hand. Several studies described in the literature support the idea of a role for PT *phospho*-ERK in transduction of the melatonin signal. For example, studies *in vitro* and *in vivo* indicate a critical role of ERK in melatonin signal transduction mediating the input in the circadian clock located in the SCN (24,25). Second, a dramatic impact of photoperiod on melatonin receptor 1 gene expression (which is assigned as the subtype with the dominant functional role (26)) towards elevated levels in SD within the PT was described in the seasonal European hamster (*Cricetus cricetus*) suggesting a highly functional role of this neuroanatomic structure in melatonin signal transduction (27). Further dissection of shared intracellular signalling pathways is required to further scrutinize the fascinating mechanisms by which melatonin may mediate seasonal changes in energy balance.

Since ERK is largely transactivated by the crucial small adaptor protein GRB2, we tested whether a change in photoperiod may affect GRB2 protein in the hypothalamus and the PT. By both immunoblotting and immunohistochemistry a consistent SD-induced increase in hypothalamic protein content of this factor was determined. The latter method also revealed, as for *phospho*-ERK, that GRB2 protein was confined to the hypothalamus and in particular to the ARC and VMH. GRB2 protein was detected in cell bodies and fibres with the strongest photoperiod-induced differences within the ependymal layer in close proximity to the third ventricle as well as the median eminence. Here striking similarities to *phospho*-STAT3 are inflicting suggesting similar underlying reasons. The SD-induced increase in hypothalamic GRB2 implies enhanced signalling via Tyr^{985}, which may lead to alterations in leptin signalling. Enhanced signalling via Tyr^{985} in SD is supported by preliminary data revealing

that SHP2, which binds GRB2, is also increased at the mRNA level. Neuroanatomical localisation of SHP2 mRNA by *in situ* hybridisation revealed striking similarities in distribution with GRB2. Since SHP2 and the heterodimerisation with GRB2 are considered as indirect positive regulators of STAT3 signalling via competitive inhibition of SOCS3 binding to Tyr^{985} (9,13), increase in both proteins in SD may account for enhanced leptin signalling via STAT3 and reduced gene expression of SOCS3. The absence of seasonal regulation of ERK phosphorylation within the hypothalamus may indicate that modulations in seasonal leptin signalling are more likely to depend on competitive binding of these factors to Tyr^{985} rather than on downstream signalling of this tyrosine residue via activation of ERK.

In conclusion our findings suggest that seasonal changes in leptin sensitivity may largely be mediated via hypothalamic alterations in LRb associated Tyr^{1138} signalling, at the level of transactivation of STAT3, rather than being conveyed by ERK signalling via Tyr^{985}. We suggest that the latter tyrosine residue might be crucial for keeping a balance between competitive binding of SOCS3 with subsequent inhibition of STAT3 transactivation on the one hand and binding to SHP2 and GRB2 on the other hand, which may lead to enhanced activity of the JAK-STAT pathway. In addition our data propose that photoperiod- induced differences in *phospho*-ERK may be involved in transducing the signal reflecting the photoperiod, melatonin, into neuronal responses altering energy balance.

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Figure legends

Fig.1: STAT3 gene expression detected by *in situ* hybridisation with an antisense ^{35}S-labelled riboprobe in adult male Siberian hamsters. Photomicrographs showing high resolution images of the respective hypothalamic regions in a dark-field emulsion-autoradiograph (a,c-e) and a bright-field film-autoradiograph (b) of representative brain sections. Panel c-e depicts higher magnification images of brain regions assigned in (a). Bar chart (f) represents quantified STAT3 mRNA in the arcuate nucleus of adult male Siberian hamsters held in LD or SD for 12 weeks (n=6, means ± SEM), 1h after intraperitoneal leptin (LEP) or vehicle injection (VEH). Values are expressed as percentages of values of LD animals injected with vehicle. Means ± SEM; ** P< 0.01; ARC: arcuate nucleus; CA 1-3: Cornu ammonis 1-3 region; DG: dentate gyrus; Pir: piriform cortex; VMH: ventromedial hypothalamus; ZI: zona incerta.

Fig. 2: Leptin stimulates hypothalamic nuclear *phospho*-STAT3 ir in a photoperiod-dependant manner in adult male Siberian hamsters. Hamsters were held in long daylength (LD) or transferred to short daylength (SD) for 10 weeks. Coronal brain sections were subjected to immunohistochemistry using a *phospho*-specific-(Tyr705)-STAT3 antibody. Rostro-caudal extension profile (a) of *phospho*-STAT3 ir nuclei extending throughout the arcuate nucleus, ventromedial- and dorsomedial hypothalamus in LD (b,c) and SD (d,e) hamsters (n=3-4 in

each group) injected with recombinant mouse leptin (c,e) or vehicle (b,d) 40 min before death. Photomicrographs depict *phospho*-STAT3 ir nuclei within the rostral- (rARC), medial- (mARC), and caudal arcuate nucleus (cARC) of representative sections. Insets represent an overview of *phospho*-STAT3 ir in the entire hypothalamus (from which nuclei countings were obtained).

Fig. 3: Leptin stimulates translocation of *phospho*-STAT3 to the nucleus. High resolution photomicrographs illustrate hypothalamic *phospho*-STAT3 ir associated with a cell surface-like microstructure in neurons of hamsters which received injections with vehicle (a,c) acclimated to long- (LD; a) or short daylength (c) for 10 weeks. Leptin induced rapid translocation of *phospho*-STAT3 ir with simultaneous disappearance of signals on the cell surface in both LD (b) and SD (d). Dotted lines indicate cellular outlines whereas insets reveal high resolution of cellular structures.

Fig. 4: Localisation of *phospho*-ERK ir within the hamster brain. *phospho*-ERK ir is confined to the hypothalamus with staining within the arcuate nucleus (ARC), ventromedial- (VMH) and dorsomedial- hypothalamus (DMH). Shown is a representative coronal section from a short daylength (SD) acclimated hamster (a). High resolution imaging revealed dense hypothalamic *phospho*-ERK ir within cell bodies and fibres of the VMH (b) and intense fibre staining within the suprachiasmatic nucleus (SCN; d). Outwith the hypothalamus *phospho*-ERK ir was detected within the pars tuberalis (PT; c; this region revealed the strongest signal of all investigated brain regions), the piriform Cortex (Pir; e) and the posteroventral medial part of the amygdala (MePV; f).

Fig. 5: Short daylength (SD) induces total hypothalamic phosphorylation of ERK. Juvenile female hamsters were held in long daylength (LD) or were subjected to SD for eight weeks.

Immunoblotting using a *phospho*-specific-(Thr202/Tyr204)-ERK 1 and 2 antibody depicts two confined bands specific for *phospho*-ERK 1 (at 44 kDa) and 2 (at 42 kDa). SD induced increase in *phospho*-ERK 1 and 2 is illustrated by a representative immunoblot analysing 3 animals from each photoperiod (a) and confirmed by densitometric analysis (b), Means ± SEM

Fig. 6: Neither photoperiod nor leptin alters hypothalamic *phospho*-ERK ir in rostro-caudal extension in adult male hamsters. Hamsters were held in long- (LD) or transferred to short daylength (SD) for 10 weeks. Coronal brain sections were subjected to immunohistochemistry using a *phospho*-specific-(Thr202/Tyr204)-ERK 1 and 2 antibody. A rostro-caudal extension profile (a) of *phospho*-ERK ir cells was obtained by determining the total number of hypothalamic *phospho*-ERK ir cells extending throughout the arcuate nucleus, ventromedial- and dorsomedial hypothalamus in LD (b,c) and SD (d,e) hamsters (n=4 in each group) injected with recombinant mouse leptin (c,e) or vehicle (b,d) 40 min before death. Photomicrographs depict *phospho*-ERK ir nuclei within the rostral- (rARC), medial- (mARC), and caudal arcuate nucleus (cARC) of representative sections. Insets represent an overview of *phospho*-ERK ir in the entire hypothalamus (from which cell countings were obtained).

Fig.7: Acclimation to short daylength (SD) is associated with a marked increase in *phospho*-ERK ir within the pituitary pars tuberalis (PT). The same adult male animals which did not reveal photoperiod-induced differences in hypothalamic *phospho*-ERK exhibited a marked increase in *phospho*-ERK ir in SD (c,d) compared to long daylength (LD; a,b) independent of vehicle (a,c; VEH) or leptin treatment (b,d; LEP). e: Results from counts of all sections (n=4 apart from SD-LEP n=2) revealing *phospho*-ERK ir cells within the PT. Means ± SEM; ***P< 0.001; 3V: third ventricle

Fig. 8: Short daylength (SD) increases GRB2 protein concentration in the hypothalamus of juvenile female hamsters acclimated to long- (LD) or SD for eight weeks. Immunoblotting using a total GRB2 protein specific antibody revealed a confined band of the expected molecular weight (25 kDa). SD induced increase in hypothalamic GRB2 protein is illustrated by a representative immunoblot analysing 3 animals from each photoperiod (a) and confirmed by densitometric analysis (b). GRB2 in the arcuate nucleus (ARC) and ventromedial hypothalamus (VMH) detected by immunohistochemistry in LD (c) and SD (d). Shown are microphotographs of coronal sections of one (from a group of four animals in each photoperiod) representative hamster in each photoperiod. e-f: High resolution images depicting dense GRB2 immunostaining within the ependymal layer of the ARC; Means ± SEM; 3V: third ventricle

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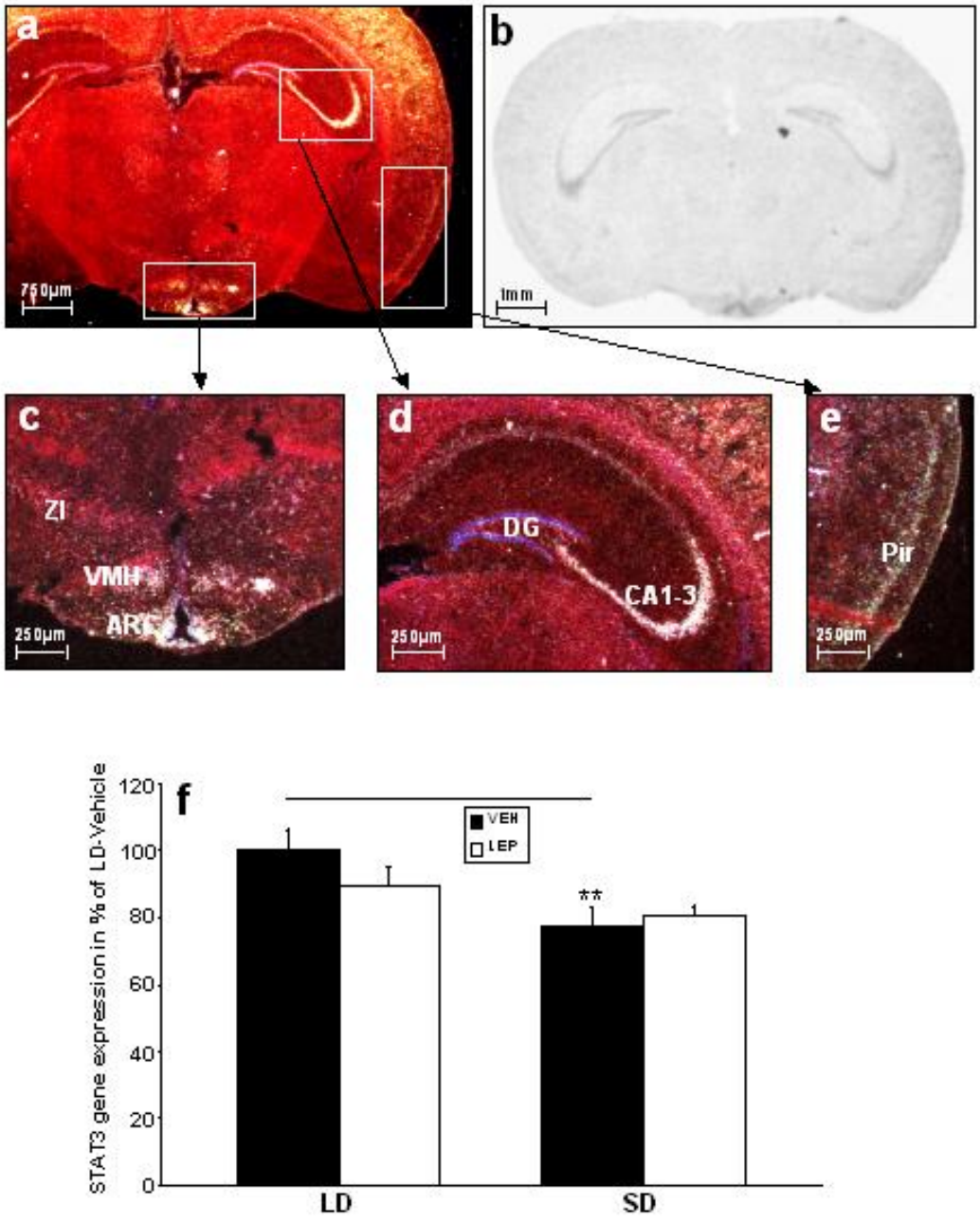

a
b
c
d
e
f
750µm
1mm
ZI
VMH
ARC
250µm
DG
CA1-3
Pir
STAT3 gene expression in % of LD-Vehicle
120
100
80
60
40
20
0
VEH
LEP
**
LD
SD

Fig. 1

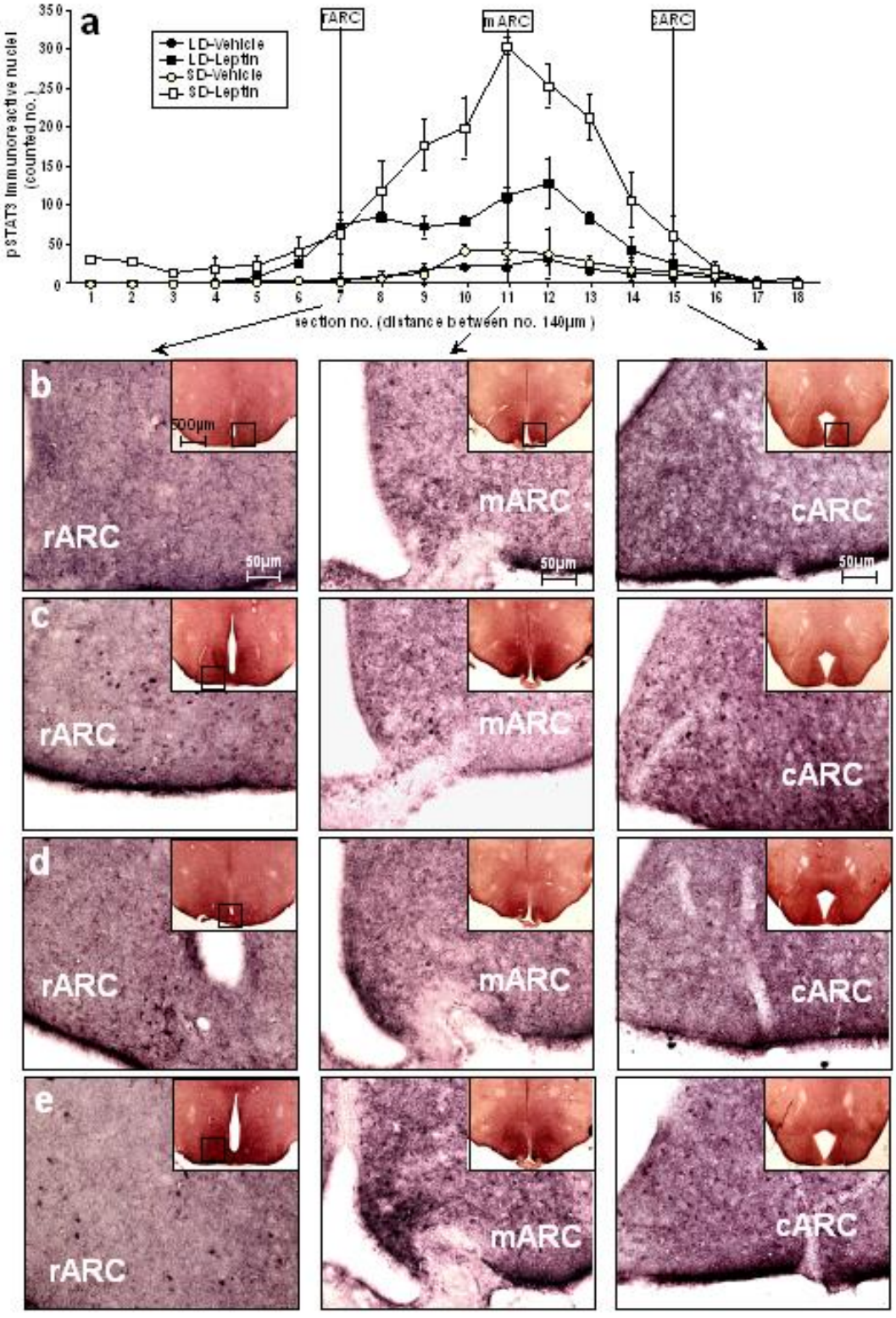

a
LD-Vehicle
LD-Leptin
SD-Vehicle
SD-Leptin
rARC
mARC
cARC
pSTAT3 immunoreactive nuclei (counted no.)
section no. (distance between no. 140µm)
b
c
d
e
500µm
50µm
rARC
mARC
cARC

Fig. 2

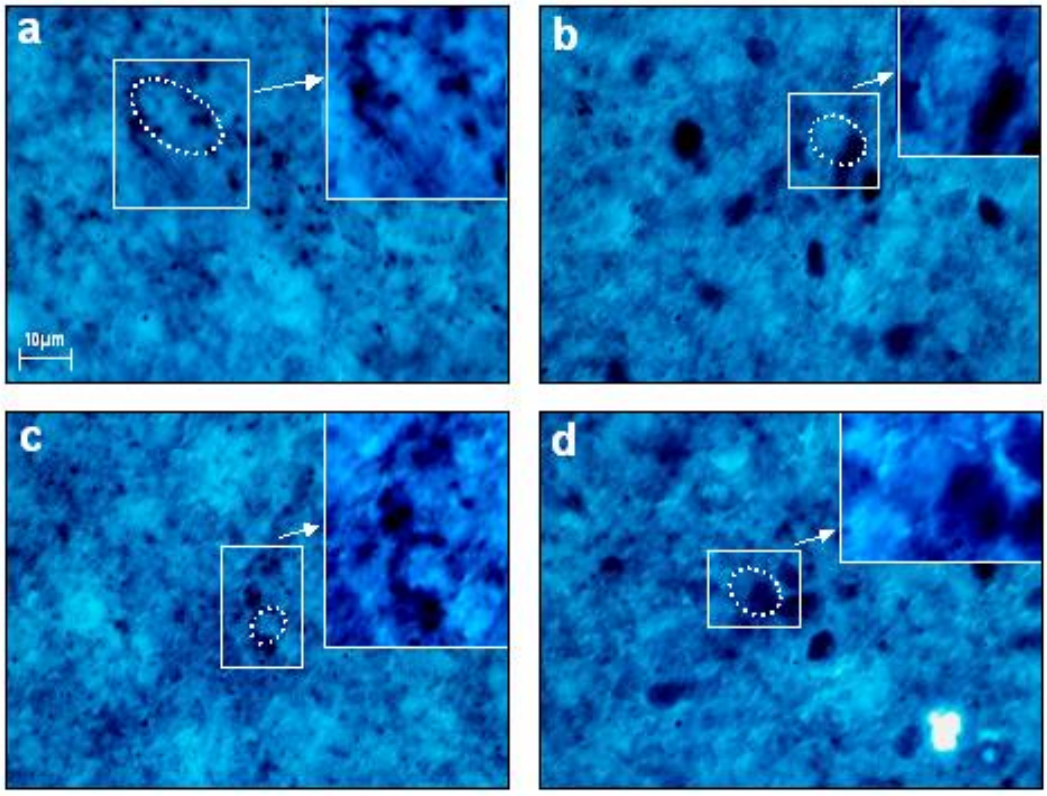


Fig. 3

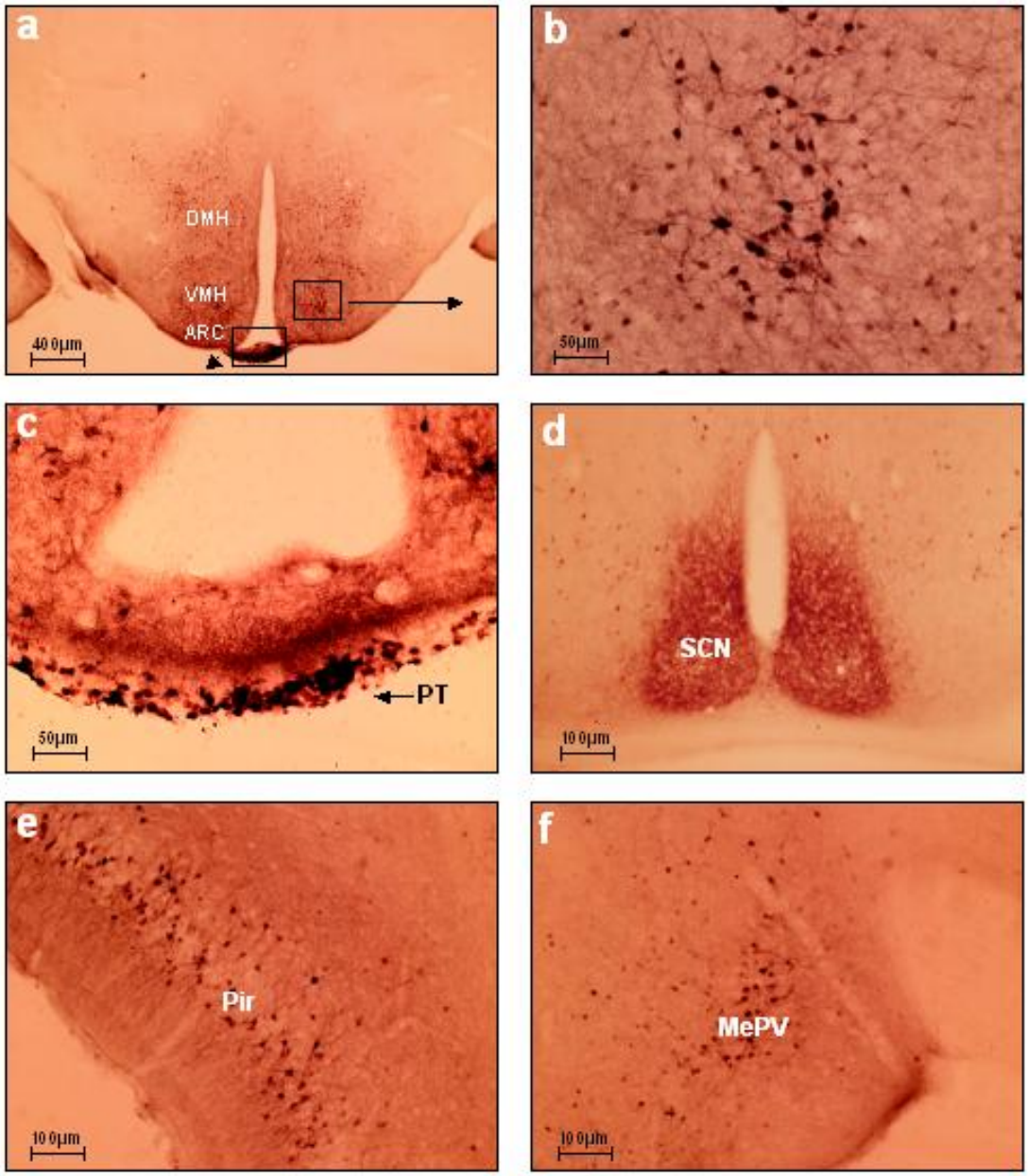

a
DMH
VMH
ARC
400µm
b
50µm
c
PT
50µm
d
SCN
100µm
e
Pir
100µm
f
MePV
100µm

Fig. 4

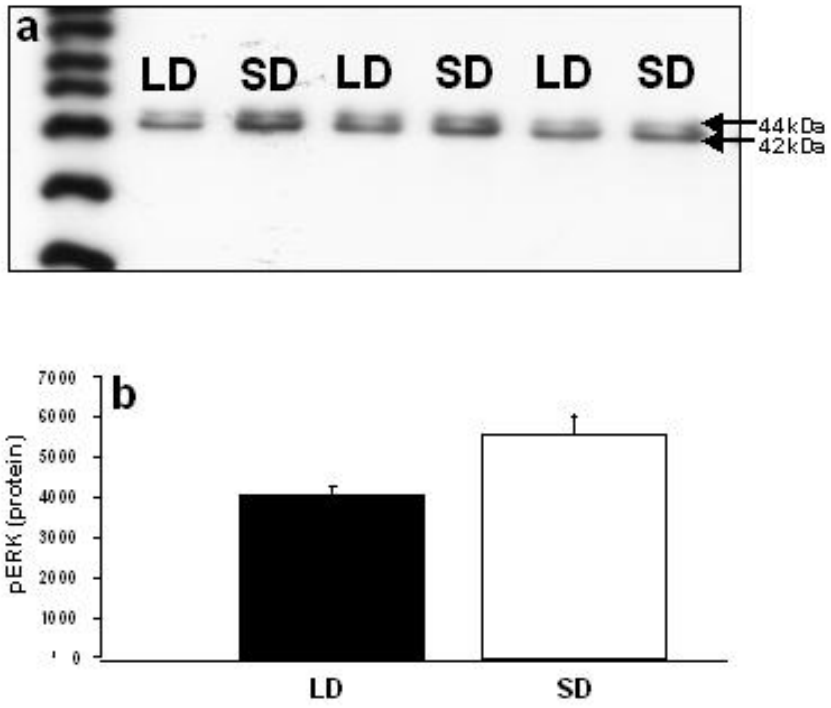

a
LD
SD
LD
SD
LD
SD
44kDa
42kDa
b
pERK (protein)
7000
6000
5000
4000
3000
2000
1000
0
LD
SD

Fig. 5

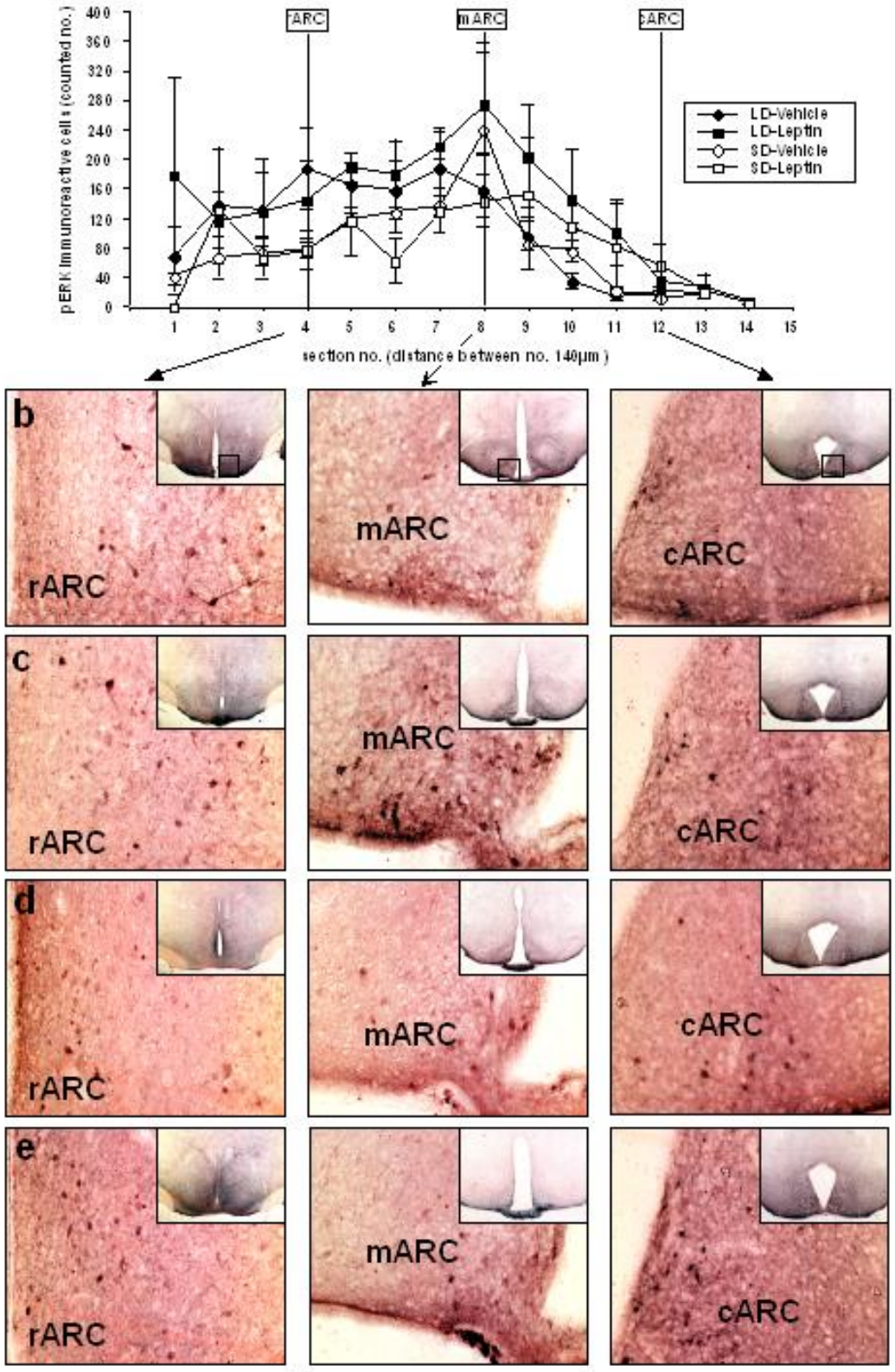
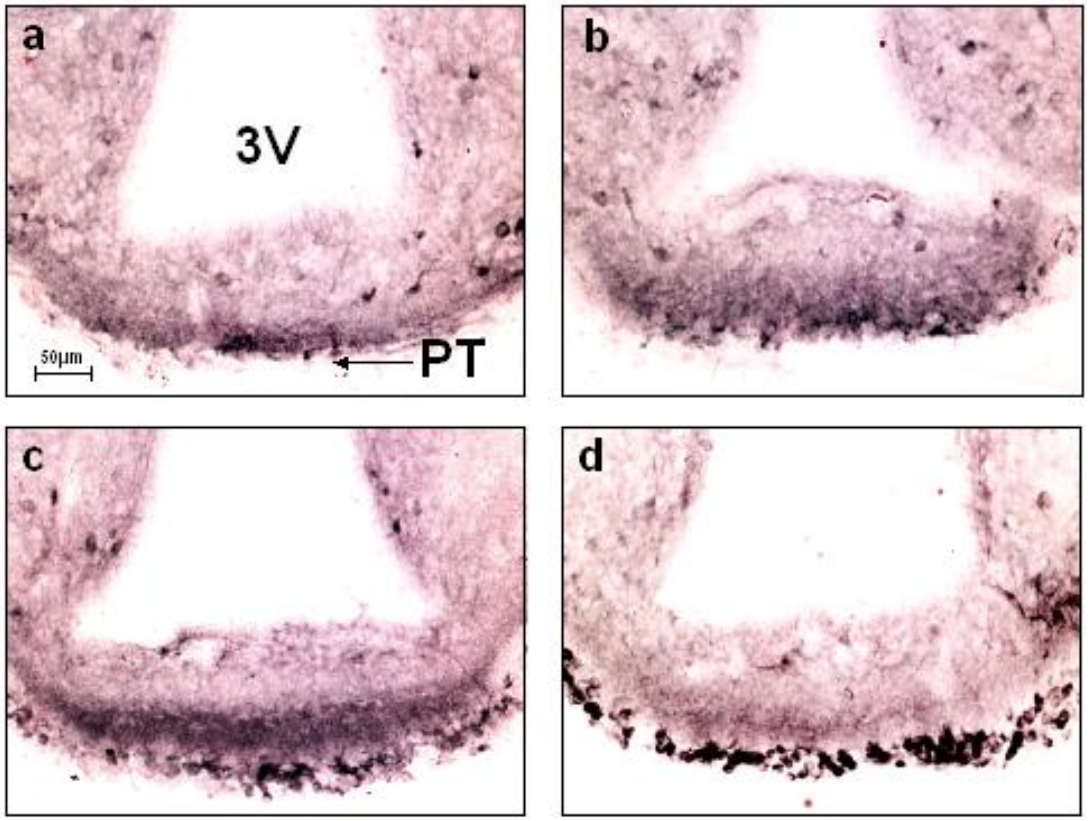

pERK Immunoreactive cells (counted no.)
400
360
320
280
240
200
160
120
80
40
0
rARC
mARC
cARC
LD-Vehicle
LD-Leptin
SD-Vehicle
SD-Leptin
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
section no. (distance between no. 140μm)
b
rARC
mARC
cARC
c
rARC
mARC
cARC
d
rARC
mARC
cARC
e
rARC
mARC
cARC

Fig. 6


a
3V
50µm
PT
b
c
d

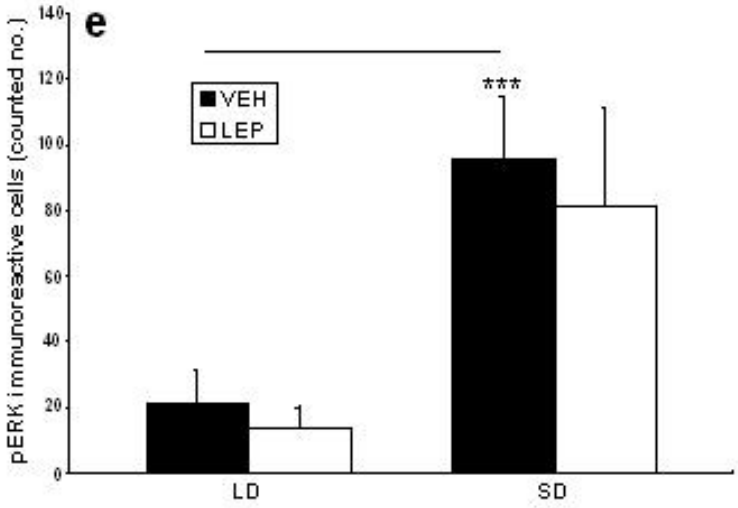

e
pERK immunoreactive cells (counted no.)
140
120
100
80
60
40
20
0
VEH
LEP
***
LD
SD

Fig. 7

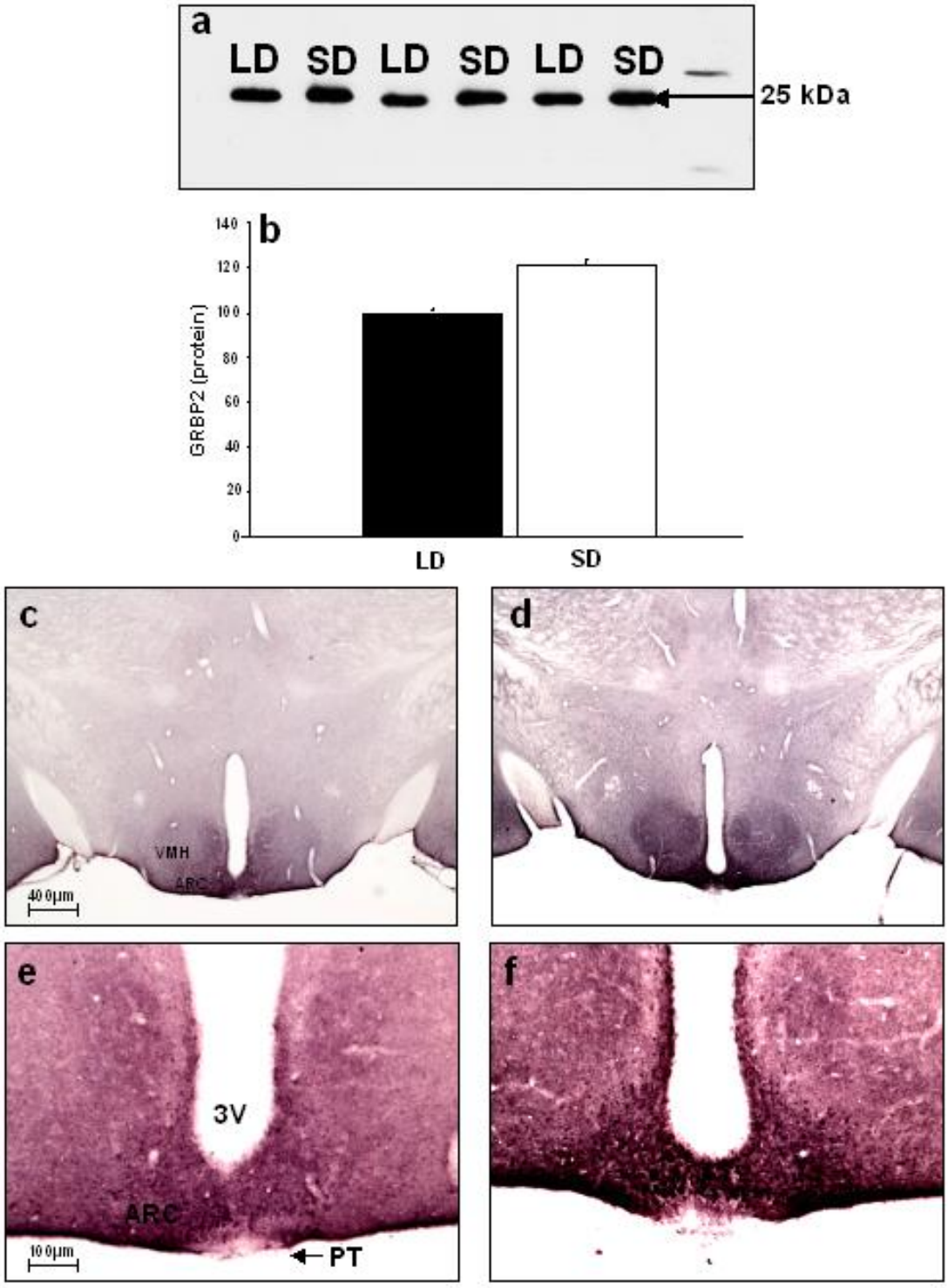

a
LD
SD
LD
SD
LD
SD
25 kDa
b
GRBP2 (protein)
140
120
100
80
60
40
20
0
LD
SD
c
VMH
ARC
400µm
d
e
3V
ARC
100µm
PT
f

Fig. 8

Photoperiodic regulation of insulin receptor mRNA and intracellular insulin signalling in the arcuate nucleus of the Siberian hamster, *Phodopus sungorus*

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Abstract

During the last five years it has been well established that photoperiod induced changes in body weight in the seasonal hamster, *Phodopus sungorus,* are accompanied by a marked seasonal cycle in leptin sensitivity. In the current study we investigated the possible involvement of insulin signalling in seasonal body weight regulation. We analysed the expression pattern and relative intensity of insulin receptor (IR), Pi3-Kinase (Pi3K) and protein tyrosine phosphatase 1B (PTP1B) mRNAs by *in situ* hybridisation in the brains of juvenile female hamsters acclimated to either long (LD) or short photoperiods (SD) for eight weeks, with or without superimposed food deprivation for 48h. Furthermore, the hypothalamic concentration and distribution of *phospho*-AKT, a marker of Pi3K activity was determined by immunoblotting and immunohistochemistry. A specific and localised signal for the IR transcript and all investigated downstream signalling components was detected within the arcuate nucleus of the hypothalamus. Eight weeks acclimation to SD led to a substantial downregulation of IR, PTP1B gene expression and *phospho*-AKT concentration in this brain region, whereas Pi3K mRNA was unchanged. Food deprivation induced a decrease in PTP1B and a trend towards lowered IR gene expression in LD but not in SD. Additionally, a striking increase in PTP1B gene expression in the thalamus was observed after food deprivation in both photoperiods. The counterintuitive data question a central role of hypothalamic insulin signalling in maintaining energy homeostasis. SD-induced reduction in insulin signalling may be due to the decline in body fat stores mediated by enhanced leptin signalling. The increased anorexigenic tone of leptin may overwrite central insulin signalling to prevent catabolic overdrive.

Introduction

Seasonal mammals like the Siberian hamster, *Phodopus sungorus* (also known as the Djungarian hamster), undergo an anticipatory seasonal cycle in energy balance in response to the annual change in photoperiod. Acclimation to short photoperiod induces alterations in physiology such as a reduction of food intake, energy expenditure and body weight which are reversible after transfer back to LD. During the complete cycle, precise adjustment of seasonally-appropriate parameters regulating energy balance requires „cross-talk“ between peripheral “adiposity signals” and the brain (1,2). To date only two molecules have been identified - leptin and insulin - that meet the criteria proposed for “adiposity signals” (3). These hormones circulate in proportion to body fat mass and are transported into the brain from the blood stream where they bind to their receptors, and in particular those in the arcuate nucleus, a key neuronal centre for control of energy homeostasis. Activation of signal transduction pathways distal to their receptors integrates these peripheral signals into a neuronal response. Leptin and insulin exhibit similarities in their central effects - both display catabolic actions - and accumulating evidence suggests that „cross-talk“ between these hormones, particularly at the level of their central intracellular signal transduction, leads to synergistic action in the regulation of energy balance (3-5).

The insulin receptor is a tetrameric complex composed of two α- and β-subunits. Although not related to the leptin receptor, the insulin receptor shares the feature of possessesing intrinsic tyrosine-protein-kinase activity leading to activation of downstream signalling pathways (6). Cellular interaction of leptin and insulin signalling is most likely to occur via a pathway involving Pi3K (5,7,8). Upon insulin binding, Pi3K is activated by insulin-receptor-substrate (IRS) proteins which have been phosphorylated by the IR (9,10). Pi3K catalyses the phosphorylation of phosphatidylinositol (4,5) bisphosphate (PIP2) to phosphatidylinositol (3,4,5) trisphosphate (PIP3), which in turn activates downstream targets

like AKT (also known as protein kinase B), a pivotal molecule for most of insulin's effects (3,11).

Inhibitory molecules such as the suppressor of cytokine signalling 3 (SOCS3) and several protein tyrosine phosphatases [(PTPs) PTPα, LAR, CD45, PTPε and PTP1B] are thought to deactivate insulin signalling whereas some of them (SOCS3 and PTP1B) even exhibit synergistic effects in terms of inhibition of both leptin and IR signalling (12,13). Among these molecules, substantial evidence supports PTP1B as being the central player in controlling insulin action. PTP1B knockout in mice and knockdown by antisense oligonucleotides in diabetic rodents leads to enhanced insulin sensitivity (14-17). These animals maintain euglycemia (in the fed state) with one-half the level of insulin observed in wild-type littermates, and surprisingly are resistant to diet-induced obesity. These attributes make this phosphatase a very attractive candidate for obesity and type 2 diabetes research.

Our knowledge of insulin signalling pathways, and the molecules involved, is derived from studies investigating insulin's action in the periphery. However, although recent studies reported blockade of ICV insulin actions on both food intake and hepatic glucose production by ICV pre-treatment with Pi3K inhibitors (7,8), the details of central insulin signal transduction remain limited.

Recent studies have unmasked a seasonal cycle in sensitivity to the adipocyte derived hormone, leptin, in *Phodopus sungorus* (18-20). We proposed that SOCS3 plays an important role in mediating seasonal modifications in leptin sensitivity, suggesting that the underlying molecular mechanism is centred on intracellular signal transduction of leptin receptors in the arcuate nucleus, the brain-region with strongest density of SOCS3 gene expression (21).

The close association of leptin- and insulin signalling raises the question of whether modifications in insulin signal transduction within the arcuate nucleus are implicated in

seasonal body weight regulation. In the present study, we investigated this hypothesis by analysing (*in situ* hybridisation) hypothalamic IR, PTP1B and Pi3K gene expression in juvenile female hamsters that had been acclimated to either SD or LD for a period of eight weeks. Furthermore, we investigated phosphorylation of AKT in the hypothalamus, detected with phospho-specific antibodies by immunoblotting and immunohistochemistry, and determined the effect of photoperiod on the hypothalamic content of this pivotal insulin signalling molecule.

Materials and Methods

All procedures involving animals were licensed under the Animals (Scientific Procedures) Act of 1986 and received approval from the Ethical Review Committee at the Rowett Research Institute. Siberian hamsters were drawn from the Rowett breeding colony (22-24), and were gestated and suckled in long day (LD) photoperiod (16:8-h light:dark cycle). All Siberian hamsters were weaned at three weeks of age and were individually housed at weaning. Where specified, hamsters were transferred into a short day (SD) photoperiod (8:16-h light:dark cycle), but with all other environmental conditions unaltered; rooms were maintained at 22°C. Food (Labsure pelleted diet; Special Diet Services, Witham, Essex, UK) and water were available *ad libitum*, or, where specified, hamsters were food deprived for 48h. All animals were killed in the middle of the light phase by either cervical dislocation (for *in situ* hybridisation and immunoblotting) or by transcardial perfusion with 4% paraformaldehyde under deep sodium pentabarbitone anaesthesia (for immunohistochemistry). Brains were either rapidly removed and frozen on dry ice for *in situ* hybridisation, for immunoblotting fresh hypothalami were excised with anatomical precision, or, as it was the case for immunohistochemsitry, brains were frozen in isopentane cooled on dry ice, respectively.

Experimental procedure

For all experiments, juvenile female hamsters were employed, which were either maintained in LD for eight weeks (n=19) or transferred to SD for the same period (n=19) post weaning. For analysis of IR, PTP1B and Pi3K gene expression by *in situ* hybridisation in either photoperiod, six food deprived (48h) and six *ad libitum*-fed control animals were killed. For detection of *phospho*-AKT by immunoblotting or immunohistochemistry, respectively, three or four brains from *ad libitum*-fed hamsters from each photoperiod were used. PTP1B mRNA

in *ad libitum*-fed animals was analysed in archived brain sections from an earlier experiment performed under identical conditions (21).

Hypothalamic gene expression

Messenger RNA levels were quantified by *in situ* hybridisation in 20 µm coronal hypothalamic sections, using techniques described in detail elsewhere (23). Riboprobes complementary to IR, Pi3K and PTP1B were generated from cloned cDNA from the hypothalamus of the Siberian hamster. cDNA synthesis was performed using a cDNA synthesis Kit (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. Primers for amplification of the three fragments were designed using Primer Select (Table 1, Lasergene, DNA-Star Software). The IR amplicons were generated by PCR with 35 cycles of 94°C for 1 min, 55°C for 1 min 40 s, and 72°C for 2 min and a final extension at 72°C for 10 min. For the amplification of Pi3K and PTP1B, the annealing temperature was adjusted to 50°C for Pi3K and 59°C for PTP1B. DNA fragments were ligated into pGEM-T-Easy [(IR and Pi3K), Promega, Madison, USA] or pPCR-Script Amp SK(+) [(PTP1B) Stratagene, La Jolla, USA] transformed into E. coli DH5α and sequenced. For cRNA synthesis of antisense riboprobes by *in vitro* transcription SP6-Polymerase [(Invitrogen, Carlsbad, USA) IR and Pi3K] or T3-Polymerase [(Invitrogen) PTP1B] were used. To generate the sense control for all three riboprobes, cRNA synthesis was performed by T7-Polymerase (Invitrogen).

As previously described (23), forebrain sections, 20µm were collected throughout the extent of the ARC onto a set of eight slides with six or seven sections mounted on each slide. Accordingly, slides spanned the hypothalamic region approximating from -2.7mm to -1.46mm relative to Bregma according to the atlas of the mouse brain (25). One slide from each animal was hybridised. Briefly, slides were fixed, acetylated, and hybridised overnight at 58°C using [^{35}S]-labelled cRNA probes (1-2 x 10^7 cpm/ml). Slides were treated with RNase A, desalted, with a final high stringency wash (30 min) in 0.1 x SSC at 60°C, dried

and apposed to Kodak Biomax MR Film (Kodak, Rochester, New York, USA). Autoradiographic images were quantified using the Image-Pro Plus system. Corresponding sections of individual animals were matched according to the atlas of the mouse brain. Three-four sections from the ARC of each animal spanning from –2.54mm to –1.94mm relative to Bregma were analysed. Data were manipulated using a standard curve generated from ^{14}C autoradiographic micro-scales (Amersham Pharmacia Biotech), and the integrated intensities of the hybridisation signals were computed.

Immunohistochemistry

For immunohistochemistry, animals were anesthetised with Euthatal (Rhone Merieux, Harlow, UK), transcardially perfused with 0.9% saline containing heparin (1000 U/litre) followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed and postfixed in the same fixative overnight at 4°C. On the next morning brains were transferred to 30% sucrose in 0.1M phosphate buffer for dehydration and cryoprotection. When brains had sunk they were frozen in isopentane cooled on dry ice for 1 min and sectioned coronally at 35 µm throughout the extent of the hypothalamus (additionally the nucleus tractus solitarius region of the hindbrain was cut) on a freezing microtome, collected in four series and stored in cryoprotectant at 4°C. Free-floating sections were incubated in 1% H_2O_2, 10% Methanol diluted in H_2O for 15 min to quench endogenous peroxide followed by incubation in blocking solution [5% bovine serum albumin (BSA), 0.5% Triton X-100 in phosphate buffer] for 45 min. Sections were incubated over night at 4°C with anti-phospho-AKT primary antibody (Ser473, IHC-specific, Cell Signaling Technology, USA # 9277) diluted in blocking solution (1:100). On the next day, sections were incubated with a biotinylated secondary goat anti-rabbit antibody for 1 h (1:1000, in blocking solution containing 3% BSA), and then treated with ABC (Vector Laboratories, Inc., Burlingame, CA) solution for 2 h. Between steps sections were washed in phosphate buffer containing 0.25%

Triton X-100. Finally, the signal was developed by Nickel-DAB solution (Vector Laboratories, Inc., Burlingame, CA), giving a gray/black precipitate. Section images were captured using a Polaroid DMCe digital camera mounted on a Zeiss Axioskop (Jena, Germany).

Immunoblotting

For immunoblotting hypothalami were immediately excised with anatomical precision from freshly prepared brains, weighed, and rapidly frozen in liquid nitrogen. Using a glass homogenizer tissues were homogenised in buffer containing phosphatase- and protease-inhibitors (10mM Hepes pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5 mM PMSF, 20mM NaF and 1mM Na_3V0_4) and incubated on ice for 10 min. For detection of *phospho*-AKT, the cytoplasmic fraction was separated from the nuclear part by centrifugation for 15 min at 3300g. The protein content of the supernatant containing the cytoplasmic fraction was determined by the Bradford assay, and equal amounts of protein were loaded into each lane. Immunoblotting-analysis was performed by standard method (26). Samples were separated on a 12.5% SDS-PAGE and, after transfer to nitrocellulose membrane, stained with red Ponceau dye to ensure accurate protein loading and transfer. *Phospho*-AKT was detected with a polyclonal rabbit antibody specific for immnunoblotting [(Ser473, Cell Signaling Technology, USA # 9271 1:1000]. Goat anti-rabbit-IgG-HRPO conjugate (Dako-Cytomation, Glostrup, Denmark) was used as the secondary antibody (1:10000). Immunodetection was performed by enhanced chemiluminescence using ECL-reagent. Autoradiographs were quantified densitometrically using Scion-Image (Scion-Corporation, Maryland, USA) software. For technical validation the immunoblot was repeated twice.

Statistical analysis

Data were analysed by One or Two Way Analysis of Variance followed by Student-Newman-Keuls multiple comparison test, as appropriate, using SigmaStat statistical software

(Jandel Corp., Erkrath, Germany). Where data failed equal variance or normality tests they were analysed by One Way Analysis of Variance on Ranks followed by Dunn's multiple comparison test. Results are presented as means ± SEM, and differences considered significant if $P<0.05$.

Results:

Distribution of insulin signalling components in the hamster brain

IR, Pi3K and PTP1B gene expression:

The species-specific probes to IR, Pi3K and PTP1B mRNA had an identity of 95%, 91% and 90% in nucleic acid sequence to *Rattus norvegicus* or *Mus musculus*, respectively (for accession numbers see Table 1). Within the investigated brain region, neuroanatomical structures which hybridised the three riboprobes are listed in Table 2, along with their estimated relative intensities (see also Fig.1). Of particular interest were the localised and intense hybridisation signals of IR, Pi3K and PTP1B in the arcuate nucleus. For all three candidate genes, sense probes synthesised from the cloned cDNA generated a low intensity non-specific signal (Fig.1d).

Phospho-AKT:

Using immunohistochemistry *phospho*-AKT positive cells were detected in the arcuate nucleus. The very specific signal was conspicuously confined to this brain region (Fig.3c). Beyond the arcuate nucleus, *phospho*-AKT immunoreactive cells were only present in the nucleus tractus solitarius of the hindbrain (data not shown).

Effect of photoperiod and/or food deprivation on insulin signalling components

Effect of photoperiod and food deprivation on insulin receptor gene expression

Body weight change at 8 wks post-weaning was similar to an identical experiment reported earlier (21). Over the eight-week period, SD hamsters gained 10.2 ± 1.3 g, while hamsters in LD gained 14.2 ± 1.2. In juvenile female hamsters acclimated to SD photoperiod for 8 weeks, IR gene expression was decreased significantly compared to hamsters maintained in LD for the same period (*Two way ANOVA; F=5.909; P< 0.05;* Fig.1a). Although there was a trend to decreased IR gene expression in response to food deprivation in LD this difference was not significant and there was no significant interaction between photoperiod and food deprivation. IR gene expression in structures analysed outwith the arcuate nucleus was unaffected by either photoperiod or feeding status.

SD induced down-regulation of IR to about 40% of the expression level in LD hamsters was confirmed by repeating the experiment with 11-12 *ad libitum* fed female hamsters in each photoperiod [eight weeks post weaning, (*One way ANOVA on Ranks; H=8.37; P< 0.001;* data not shown)].

Effect of photoperiod and food deprivation on Pi3K gene expression

There was no effect of photoperiod, feeding status or interaction of both parameters on Pi3K mRNA in any of the brain regions examined (Fig.1b) although the latter almost achieved significance with p=0.061.

Effect of photoperiod and food deprivation on PTP1b gene expression

A highly significant reduction of PTP1B mRNA levels in the arcuate nucleus was observed (Fig.1c) in response to SD acclimation (Two way ANOVA; F=52.24; P < 0.001), to food deprivation (Two way ANOVA, F=7.74; P < 0.05). Additionally, there was a significant interaction between photoperiod and feeding status (Two way ANOVA, F=7.14; P < 0.05).

Multiple group-wise comparisons revealed a significant reduction of arcuate nucleus PTP1B gene expression induced by food deprivation in LD but not in SD (Fig.1c). There was also a highly significant increase in PTP1B gene expression in the thalamus in response to food deprivation (Two way ANOVA, $F=39.73$; $P < 0.0001$) but not in response to photoperiod (Fig.1c and Fig.2). PTP1B gene expression was unaffected by photoperiod or feeding status in other analysed structures.

Effect of photoperiod on the phosphorylation of AKT

As a marker for Pi3K activity we determined the hypothalamic content of phosphorylated AKT. As shown in Fig.3a, a single conspicuous and specific band of the expected size (60 kDa) for *phospho*-AKT could be determined in hypothalamic lysates of the Siberian hamster. Exposure of juvenile female hamsters to SD for eight weeks led to a striking reduction of *phospho*-AKT in the hypothalamus compared to LD hamsters as revealed by quantification of the immunoblot (Fig. 3b).

These differences were investigated at a neuroanatomical level by immunohistochemistry. As mentioned above, *phospho*-AKT immunoreactivity was restricted to the arcuate nucleus and photoperiod induced differences were confined to this region (Fig.3c). Thus, the changes observed by immunoblotting of total lysates were due to localised changes in the arcuate nucleus. Consistently, in all four analysed animals from each photoperiod, *phospho*-AKT immunoreactivity was substantially lower in SD compared to LD.

Discussion

Over the last five years accumulating evidence has revealed a seasonal switch in leptin sensitivity in *Phodopus sungorus* reflected by increased leptin sensitivity in SD and the establishment of central leptin resistance in LD (18-21). Leptin exhibits conspicuous similarities in its central effects with the second hormone beside leptin that meets the criteria

for an “adiposity signal”, insulin, and growing literature suggests convergence of central signalling at the level downstream of their respective receptors (3,5). The present study was designed to investigate seasonal alterations in central signal transduction of insulin. Here we report seasonal regulation of central IR gene expression and alterations in the hypothalamic *phospho*-AKT content in a seasonal species. These changes are associated with differential regulation of PTP1B gene expression, the main inhibitor of insulin signalling.

The neuroanatomical gene expression pattern of IR and Pi3K is consistent with those reported in rats and mice, respectively (27,28). Expression was mainly confined to the arcuate nucleus, a key neuronal centre for the integrated regulation of body weight. This together with the fact that seasonal differential gene expression was restricted to this brain area indicates that insulin signalling may be involved in the seasonal body weight cycle exhibited by *Phodopus sungorus*.

Seasonally induced alterations in circulating insulin levels were reported in the related species *Phodopus campbelli* (29). Here, in adult male hamsters after a 20 week acclimation, SD plasma insulin levels fell to about 20 % of the level in LD controls (29). Additionally, in the Siberian hamster reduced circulating insulin in response to SD was described (Timothy Bartness, personal communication).

Presumed elevation of insulin levels in LD coincident with increased IR gene expression in the arcuate nucleus suggests a causal link. Entry of insulin into specific brain areas across the blood-brain barrier is well established (6,30). Although regional uptake of insulin into the brain does not directly correlate with the localization of the IR [the pons-medulla was the region with the greatest insulin passage K_i:0.764 µl/g min, but it only contains relatively few insulin IR`s, (30)], intriguingly, the second largest flux of circulating insulin into the brain was reported within the hypothalamus (K_i:0.731 µl/g min), substantiating the importance of regional agglomeration of insulin receptor molecules in this area. Although it is well

documented that brain insulin is probably largely of peripheral origin, the possibility of local insulin synthesis cannot be excluded, and remains controversial. It has been reported that the concentrations of insulin in the cerebrospinal fluid and insulin entry in the hypothalamus positively correlate with feeding status, which may provide a link between the seasonal alterations in IR gene expression and the marked cycle in seasonal body weight which is reflected by alterations in food intake (31-33). An important association of brain IR and feeding status is also substantiated by the trend to decreased IR gene expression in the arcuate nucleus in LD hamsters in response to food deprivation (48h). This effect could not be observed in SD suggesting that these animals exhibit a basal IR gene expression level.

However, considering the potent anorexigenic action of insulin, SD induced downregulation of circulating insulin concentration appears to be paradoxical. Recent studies demonstrated that third cerebral ventricle (ICV) administration of insulin decreases food intake and body weight (5,34-36). Furthermore, diminished insulin signalling, as it was achieved by neuronal knockout, knockdown of the IR (by administration of antisense oligonucleotides against the IR) or neuronal specific reduction of insulin signalling distal of its receptor (by ICV treatment with Pi3K inhibitors) led to hyperphagia and an increase in body weight (8,37,38). However, in our study we revealed diminished hypothalamic signal transduction of this hormone (as exemplified by reduction of IR mRNA and *phospho*-AKT protein) in SD, times when body weight is low and food intake comparatively reduced. Regarding the fundamental catabolic action of central insulin (i.e. reducing food intake and body weight) the finding that both IR gene expression and insulin signalling via *phospho*-AKT are downregulated in SD hamsters appears to be counterintuitive. Conceivably, reduced insulin signalling in SD may be the **result** rather than the trigger of an increased catabolic tone in SD. Possibly, the high leptin sensitivity associated with an enhanced catabolic effect in SD overwrites the central insulin signal and prevents a catabolic overdrive which would have been resulted from additive actions of both hormones. This hypothetical scenario may be

critical for the survival of Siberian hamsters in harsh winter conditions, times when food is limited. However, our study supports central insulin signalling mediated via Pi3K since both IR and *phospho*-AKT were consistently coregulated in response to photoperiod within the arcuate nucleus.

Beside the Pi3K pathway, other pathways [JAK-STAT and ERK; (39,40)] have been implicated in peripheral insulin signalling. Convergence of both insulin and leptin signalling upon the level of these other signalling pathways beyond signalling through Pi3K is plausible.

Intriguing though is the role of PTP1B as a central player in modulating insulin signal transduction. This attribute may be challenged by the fact that gene expression of this inhibitory molecule is reduced in SD when also insulin signalling is minimal. Assuming that the reduction of PTP1B mRNA is reflected at the protein level, lowered content of this inhibitor in the arcuate nucleus would be expected to result in increased insulin signalling. Nevertheless, the hypothalamic *phospho*-AKT content was substantially reduced in this photoperiod. However, we did not investigate functional interactions of the insulin signalling components. Although the expression levels in response to SD and food deprivation for 48h of both IR and PTP1B are almost identical indicating strong interaction, it is possible that changes in brain PTP1B gene expression may not be exclusively associated with insulin signalling. The intriguing finding that PTP1B gene expression is massively induced by food deprivation in the thalamus, a region which in Siberian hamsters does not express either IR or the long form of the leptin receptor [with full signal transduction capacity (LRb)], indicates that PTP1B may be involved in signalling of other feeding status regulatory hormones, whose identity is presently unknown.

PTP1B has also been associated with intracellular signalling of the second “adiposity signal”, leptin. This factor exhibits its inhibitory effects on leptin signalling most likely via deactivation of the Januskinase 2, a with the leptin receptor associated enzyme (41). Seasonal

modulation of leptin signalling by PTP1B may be plausible. Supporting evidence for this hypothesis is contributed by the identical neuroanatomical expression patterns of both PTP1B and leptin receptor mRNA within the arcuate nucleus of the Siberian hamster (42). Moreover, the mRNA of SOCS3, a possible key modulator in the mediation of the seasonal cycle in leptin sensitivity, reveals a very similar hypothalamic neuroanatomical expression pattern like PTP1B mRNA and in response to SD arcuate nucleus SOCS3 and PTP1B gene expression are diminished to a strikingly similar extent (21). PTP1B and SOCS3 may exhibit potent synergistic actions in terms of inhibition of insulin and leptin signalling. However, the complementary deactivatory attributes of both molecules are unlikely to be primarily displayed on the level of the Pi3K (due to the lack of increased Pi3K activity in SD), it is rather plausible that intracellular signalling is modified by additional recruitment of the JAK-STAT or ERK signalling pathways. Diminished inhibition of leptin signalling in SD on the level of the JAK-STAT pathway is also supported by the very recent finding of a substantial increase of *phospho*-STAT3 positive neurons within the hypothalamus in response to a single intraperitoneal leptin injection (Tups A., unpublished results). These data substantiate the close association of leptin and insulin signalling within the hypothalamus. The drop of both circulating insulin levels and insulin signalling in SD may be primarily the response to the reduced fat mass (insulin meets the criteria of an adiposity signal thus insulin levels are proportional to body fat mass) and the metabolic modifications in SD. The apparent paradoxically reduced anorexigenic drive of insulin implicated by marked diminished insulin signalling may be antagonized by increased activation of the JAK-STAT pathway induced by increased leptin sensitivity in SD. Thus convergence of both insulin and leptin signalling within the hypothalamus and partial compensation of different parts of their shared signal transduction cascades may be a fascinating powerful mechanism for the dynamic regulation of energy homeostasis.

In summary, we provide evidence that seasonal body weight regulation is be associated with modulations in arcuate nucleus insulin signal transduction. However, the direction of change in neuronal insulin signalling is counterintuitive related to the central catabolic action of this pathway. Reduced insulin signalling in SD may result from enhanced leptin signalling in SD as a consequence of reduced body fat stores. Insulin- and leptin receptors are both expressed in the arcuate nucleus of Siberian hamsters but intracellular signalling of both hormones is inverse making it unlikely that both hormones exhibit synergistic body weight regulatory effects in the hypothalamus. However, we cannot rule out possible inverse "cross-talk" of both hormones. Beyond Pi3K, insulin and leptin share further signal transduction pathways. This "cross-talk" of insulin and leptin within the hypothalamus, distal of their respective receptors, remains an enigma whose resolution will certainly enable us to better understand the complex mechanisms maintaining energy homeostasis and that may be perturbed in obesity.

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Fig1: Gene expression of IR (a), Pi3K (b) and PTP1B (c) in juvenile female hamsters fed *ad libitum* or food deprived for 48h in long- (LD) or short daylength [8 weeks acclimation (SD)] detected by *in situ* hybridisation to antisense ^{35}S-labelled riboprobes or the respective sense controls (d). Left panel: autoradiographs of representative coronal brain-sections of animals either *ad libitum* fed or food deprived for 48h (food dep.) in either photoperiod. Right panel: Bar chart of quantified gene expression in the arcuate nucleus from 3-4 sections of each animal (n=6 animals/group). Values are expressed as percentages of values in LD hamsters fed *ad libitum*. Means ± SEM, * P< 0.05, *** P< 0.001; ARC: arcuate nucleus; CA 1-3: CA 1-3 region; cc: cerebral cortex; cp: choroid plexus; dg: dentate gyrus; hb: habenular nucleus; pc: piriform cortex; tha: thalamus

Fig2: PTP1B gene expression in the thalamus of juvenile female Siberian hamsters. Hamsters were either *ad libitum* fed or food deprived for 48h (food dep.) (n= 6) in long (LD) or short day-length (SD). Values are expressed as percentages of values in LD hamsters fed *ad libitum*. Means ± SEM, *** P< 0.001

Fig3: Short daylength (SD) induced down-regulation of *phospho*-AKT in the hypothalamus of juvenile female hamsters acclimated to long- (LD) or SD for eight weeks. (a) Depicting a confined specific band for *phospho*-AKT by immunoblotting in the hypothalamus of 3 animals from each photoperiod. (b) Densitometric analysis of the immunoblot shown in (a), Means ± SEM (c) *Phospho*-AKT in the arcuate nucleus detected by immunohistochemistry. Shown are photomicrographs of coronal sections of one (from a group of four animals in each photoperiod) representative animal in each photoperiod. ARC: arcuate nucleus; 3V: third ventricle

Table1: Oligonucleotides used for cloning of the respective candidate genes for *in situ* hybridisation

Probes	Primers	Oligonucleotide sequence	Fragment size (bp)	Access. No. (rat/mouse)
Insulin receptor	forward reverse	5'-CTGCGGCCCGATGCTGAGA-3' 5'-CCCTTGCCCCCTTTCCGATAGTAA-3'	229	8393620
Pi3K	forward reverse	5'-AACGCGAAGGCAACGAGAAGGAA-3' 5'-AACGCGAAGGCAACGAGAAGGAA-3'	219	6981357
PTP1B	forward reverse	5'-TGGCCACAGCAAGAAGAAAAGGAG-3' 5'-TGAGCCCCATGCGGAACC-3'	409	292901

Table2: Distribution of mRNA in the investigated brain region

Structure	Probe (rel. density in LD ad lib.)		
	IR	**Pi3K**	**PTP1B**
CA1-3	+++	+++	+++
dentategyrus	+++	+++	+++
habenular nucleus	+++	-	+++
choroid plexus	++	+++	+
cerebral cortex	-	+++	+
piriform cortex	++	+	++
amygdala	+	+	+
optic tract	++	+	+
arcuate nucleus	++	++	++
thalamus	-	-	++

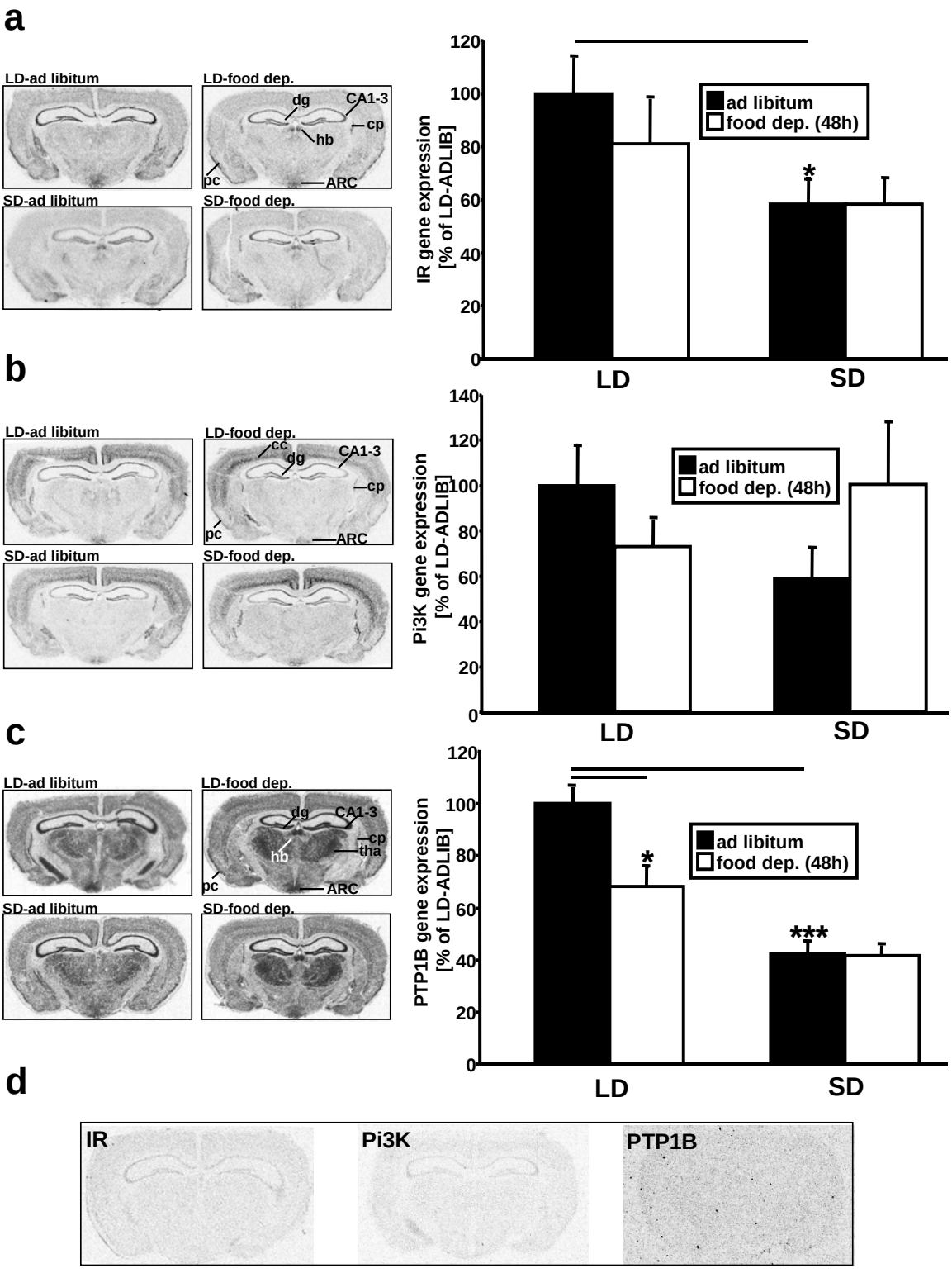
a
LD-ad libitum
LD-food dep.
dg
CA1-3
cp
hb
pc
ARC
SD-ad libitum
SD-food dep.
IR gene expression [% of LD-ADLIB]
ad libitum
food dep. (48h)
*
LD
SD
b
cc
Pi3K gene expression [% of LD-ADLIB]
c
tha
PTP1B gene expression [% of LD-ADLIB]
***
d
IR
Pi3K
PTP1B

Fig. 1

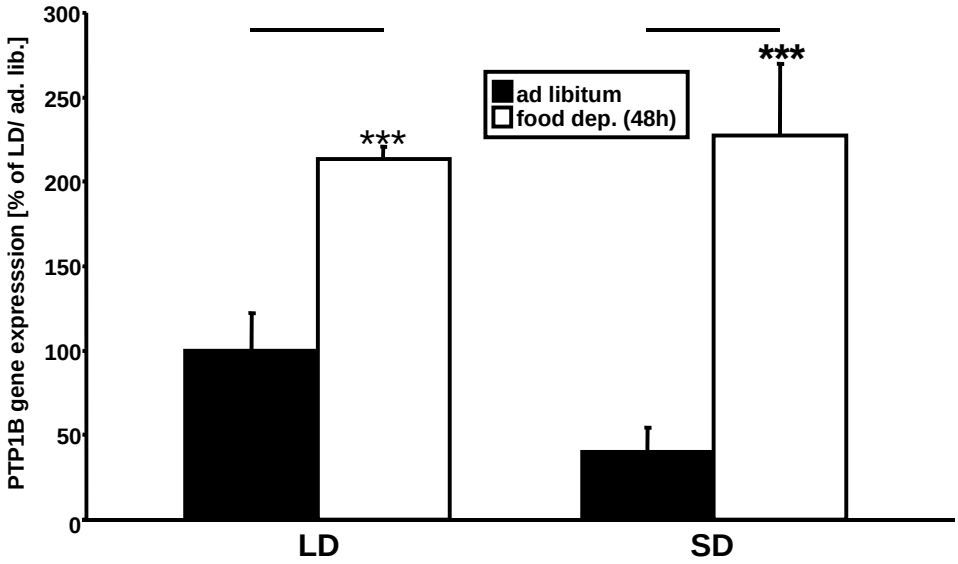
PTP1B gene expresssion [% of LD/ ad. lib.]
300
250
200
150
100
50
0
***
***
ad libitum
food dep. (48h)
LD
SD

Fig. 2

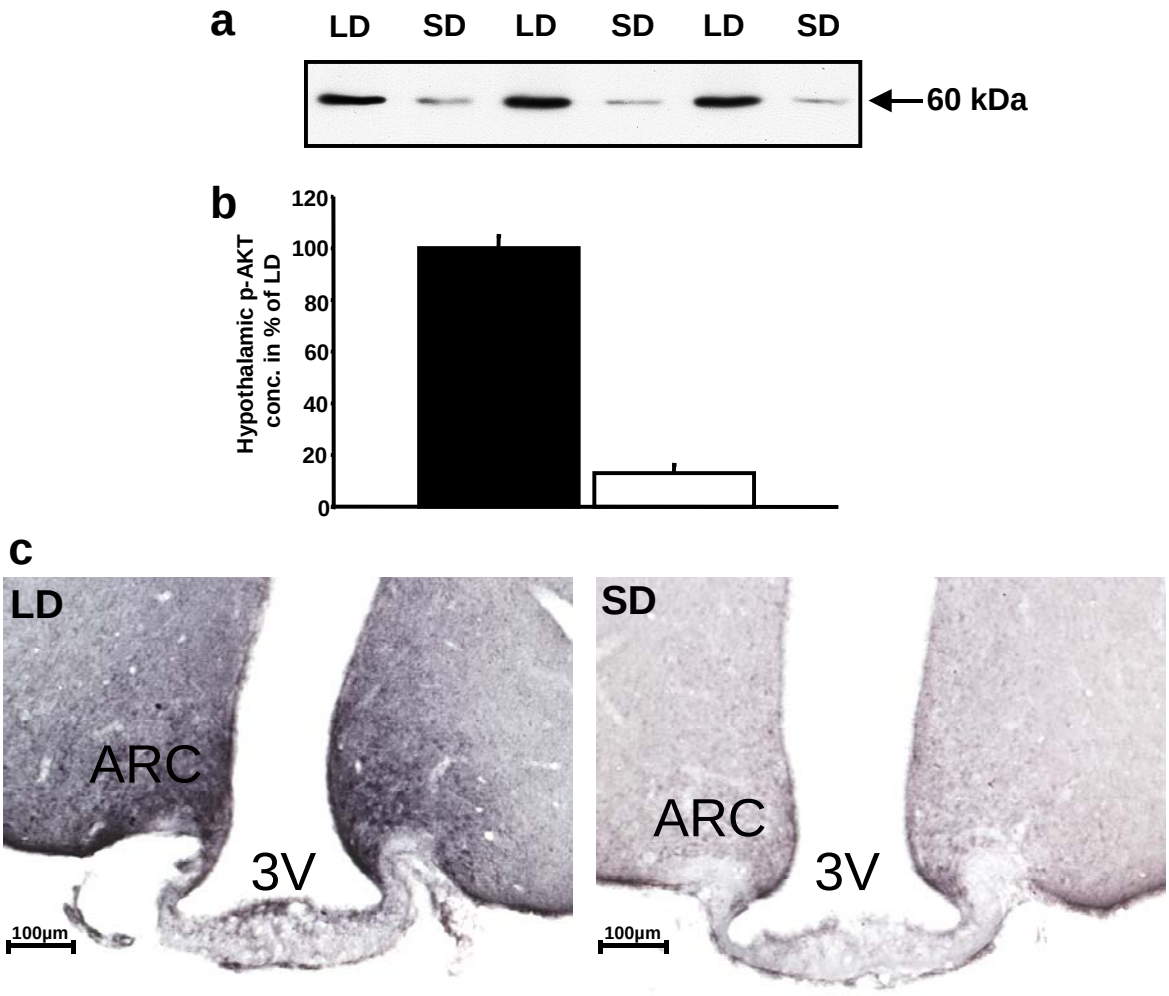
a
LD
SD
LD
SD
LD
SD
60 kDa
b
Hypothalamic p-AKT conc. in % of LD
120
100
80
60
40
20
0
c
LD
ARC
3V
100µm
SD
ARC
3V
100µm

Fig.3

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Circulating Ghrelin Levels and Central Ghrelin Receptor Expression are Elevated in Response to Food Deprivation in a Seasonal Mammal *(Phodopus sungorus)*

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Key words: energy balance, photoperiod, seasonal body weight regulation, leptin.

Abstract

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor (GHSR). However, the functional interaction of ligand and receptor is not very well understood. We demonstrate that GHSR mRNA is up-regulated after food deprivation (48 h) in the hypothalamic arcuate nucleus and ventromedial nucleus of the seasonal Siberian hamster, *Phodopus sungorus*. This increase is accompanied by a two-fold elevation of circulating ghrelin concentration. Chronic changes in feeding state imposed by food restriction over a period of 12 weeks during long day-length induced increased GHSR gene expression, whereas food restriction for 6 weeks had no effect. *Phodopus sungorus* reveals remarkable seasonal changes in body weight, fat mass and circulating leptin levels. Ghrelin is generally regarded as having opposing effects on appetite and body weight with respect to those exhibited by leptin. However, our study revealed that seasonal adaptations were not accompanied by changes in either GHSR gene expression or circulating ghrelin concentration. Therefore, we suggest that ghrelin only plays a minor role in modulating long-term seasonal body weight cycles. Our findings imply that ghrelin predominantly acts as a short-term regulator of feeding.

Ghrelin, a 28-amino-acid gut peptide, has been identified as an endogenous ligand of the growth hormone secretagogue receptor (GHSR) and shown to stimulate growth hormone (GH) secretion (1, 2). However, accumulating evidence suggests that its major physiological role may be related to the regulation of energy homeostasis (3–5). Ghrelin is produced by the stomach and circulating plasma ghrelin concentrations are dynamically related to feeding state (6, 7). Thus, in man, it has been demonstrated that circulating ghrelin levels are decreased in chronic (obesity) and acute (feeding) states of positive energy balance. By contrast, plasma ghrelin levels are increased by fasting and in patients with anorexia nervosa (7–10). Furthermore, peripheral and central (intracerebroventricular) ghrelin administration in mice and rats caused weight gain by either reducing fat utilization or by a dose-dependent increase in food intake (5). Ghrelin modifies energy homeostasis independent of its GH-releasing activity, as demonstrated by studies performed in GH-deficient rats (4). Ghrelin undergoes post-translational processing where the hydroxyl group of one of its serine residues is acylated by *n*-octanoic acid (1, 11). Acylation of this peptide is regarded to be essential for its endocrine actions because it facilitates transport across the blood–brain barrier and is essential for binding to GHSR (12–14).

GHSR was originally cloned in 1996 from the pituitary of several species, including humans and the rat (15, 16). The name GHSR derived from a class of synthetic molecules, the growth hormone secretagogues (GHSs) which represent the first identified ligands of this receptor. GHSR is a G-protein-coupled receptor (15) and is encoded by a single gene across different species (17). In the rat, central GHSR mRNA expression is confined to the hypothalamus and the pituitary gland (18).

Recently, a link between feeding status and GHSR mRNA expression was demonstrated. Total hypothalamic mRNA of GHSR was increased after food deprivation (48 h) in the rat (19). However, whether the anatomical localization of mRNA changes according to feeding status within the hypothalamus is unknown. It may be important to establish whether feeding-induced mRNA changes occur in distinct hypothalamic nuclei that are important centres in the modulation of body weight.

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In the present study, we localized GHSR mRNA within the hypothalamus via *in situ* hybridization and investigated changes in the expression profile of GHSR mRNA within distinct hypothalamic nuclei. Moreover, we determined serum levels of total circulating ghrelin. We performed our studies in the seasonal Siberian hamster (*Phodopus sungorus*, also known as Djungarian hamster), which represents a unique model for the investigation of energy homeostasis. *Phodopus sungorus* reveals a remarkable natural body weight cycle determined by the prevailing photoperiod and reflected in changes of circulating leptin levels (20–22). Short day exposure (SD), either as a gradual change (natural conditions) or as an abrupt change (laboratory conditions), leads to a progressive reduction in body weight. This animal model allowed us to investigate the functional role of circulating ghrelin and its centrally expressed receptor in relation to chronically changed body weight induced by photoperiod. Effects of food deprivation on the interplay of this feeding related gut hormone and its receptor were analysed and, beyond that, the impact of food restriction and of the anorexigenic cytokine leptin on GHSR gene expression was studied, both in animals with a naturally high body weight and in animals that are reaching their body weight nadir induced by SD exposure.

Materials and methods

Animals

Procedures involving animals were licensed under the Animals (Scientific Procedures) Act of 1986 and received approval from the Ethical Review Committee at the Rowett Research Institute. All experimental animals were drawn from the Rowett breeding colony of Siberian hamsters (23–25), and were gestated and suckled in long day (LD) 16 : 8 h light/dark cycle. All hamsters were weaned at 3 weeks of age, and were individually housed either at weaning or, in the case of adult animals, at least 2 weeks before food deprivation. Where specified, hamsters were maintained from weaning in a short day (SD) 8 : 16 h light/dark cycle, but with all other environmental conditions unaltered. Food (Labsure pelleted diet; Special Diet Services, Witham, Essex, UK) and water were available *ad libitum* unless specified, and rooms were maintained at 22 °C. All animals were killed by cervical dislocation in the middle of the light phase, trunk blood was collected and brains were rapidly removed and frozen on dry ice.

Experimental procedure

To investigate acute changes in GHSR mRNA expression induced by food deprivation (48 h), archived brain sections were used from juvenile female LD hamsters weaned in LD and then held in LD (n = 12) or SD (n = 12) photoperiods. Eight weeks after weaning, half of the animals (n = 6) in each photoperiod were deprived of food while the remainder continued to feed *ad libitum* (26).

Chronic changes in GHSR gene expression following food restriction for 6 and 12 weeks, respectively, were examined by analysing archived brain sections of three groups of juvenile female hamsters. One group was maintained in LD (LD-ADLIB), the second was transferred to SD (SD-ADLIB) and the third group was also maintained in LD (LD-REST) but was food restricted so that the body weight trajectory was matched with that of the SD group (26).

In another experiment, the effect of leptin on GHSR gene expression was investigated. Archived brain sections (26) of juvenile female hamsters which had received a single intraperitoneal leptin injection 15, 30, 60 or 120 min before cervical dislocation were analysed. Control groups were injected with vehicle (26).

Serum ghrelin concentration was investigated in a second food deprivation experiment, which was carried out exactly as above with a new group of hamsters (n = 24). This repeated study was performed due to insufficient blood sampled from the first set of animals. To substantiate the results obtained from juvenile female hamsters, we also determined serum ghrelin concentration in adult male hamsters. Twenty adult male LD hamsters, aged 5–6 months, were divided into two groups, one of which was deprived of food for 48 h whereas the other group continued to feed *ad libitum*.

Radioimmunoassay

Serum concentrations of total immunoreactive ghrelin were measured using the commercially available radioimmunoassay kit from Phoenix Pharmaceuticals, Inc. (catalogue no. RK 031-31, Belmont, CA, USA).

Hypothalamic gene expression

Messenger RNA levels were quantified by *in situ* hybridization in 20-μm coronal hypothalamic sections, using techniques previously described in detail (24). A riboprobe complementary to GHSR (type 1a and b) was generated from cloned cDNA from the hypothalamus of rat. cDNA synthesis was performed by reverse transcription (cDNA synthesis Kit, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The 449-bp (Genebank NM032075) fragment of rat GHSR was amplified by polymerase chain reaction (PCR) with 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 68 °C for 1 min and finally one cycle at 72 °C for 10 min. For the amplification, the primers 5′-GCGCTCTTCGTGGTGGGCATCT-3′ and 5′-GTGGCGCGGCATTCGTTGGT-3′ were used. The DNA fragments were ligated into PCR-script Amp cloning vector (Stratagene, Basingstoke, UK) and transformed into JM 109 cells (Promega, Southampton, UK). Automated sequencing was performed to verify the sequence of interest.

As previously described (24), 20-μm forebrain sections were collected throughout the extent of the arcuate nucleus and the caudal part of the ventromedial nucleus (VMH), to which GHSR gene expression is confined, onto a set of eight slides with six or seven sections mounted on each slide. Accordingly, slides spanned the hypothalamic region approximating from −2.7 mm to −1.25 mm relative to Bregma according to the atlas of the mouse brain (27). One slide from each animal was hybridized. Briefly, slides were fixed, acetylated, and hybridized overnight at 58 °C using [^{35}S]-labelled cRNA probes ($1–2 \times 10^7$ c.p.m./ml). Slides were treated with RNase A, desalted, with a final high stringency wash (30 min) in 0.1 × SSC at 60 °C, dried and apposed to Kodak Biomax MR Film (Kodak, Rochester, NY, USA). Autoradiographic images were quantified using the Image-Pro Plus system. Equivalent sections of individual animals were matched according to the atlas of the mouse brain. Four sections from the arcuate nucleus and 3 sections from VMH of each animal spanning from −2.54 mm to 0.94 mm relative to Bregma were analysed. Integrated optical densities were calculated using a standard curve generated from ^{14}C autoradiographic microscales (Amersham Pharmacia Biotech, UK Ltd, Bucks, UK).

Statistical analysis

Data were analysed by one- or two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison test, as appropriate, using SigmaStat statistical software (Jandel Corp., Erkrath, Germany). Where data failed normality tests, they were analysed by one-way ANOVA on ranks followed by Dunn's multiple comparison test. Data are presented as mean ± SEM. $P < 0.05$ was considered to be statistically significant.

Results

Localization of GHSR mRNA and protein in the hamster hypothalamus

The riboprobe complementary to rat GHSR mRNA hybridized within the hypothalamus of the Siberian hamster to the arcuate nucleus and the VMH (Fig. 1), as well as to the paraventricular (PVN) and suprachiasmatic nucleus (SCN) (data not shown). Differential gene expression was not observed in the PVN or SCN in any of the experiments reported below. A sense probe synthesized from the cloned

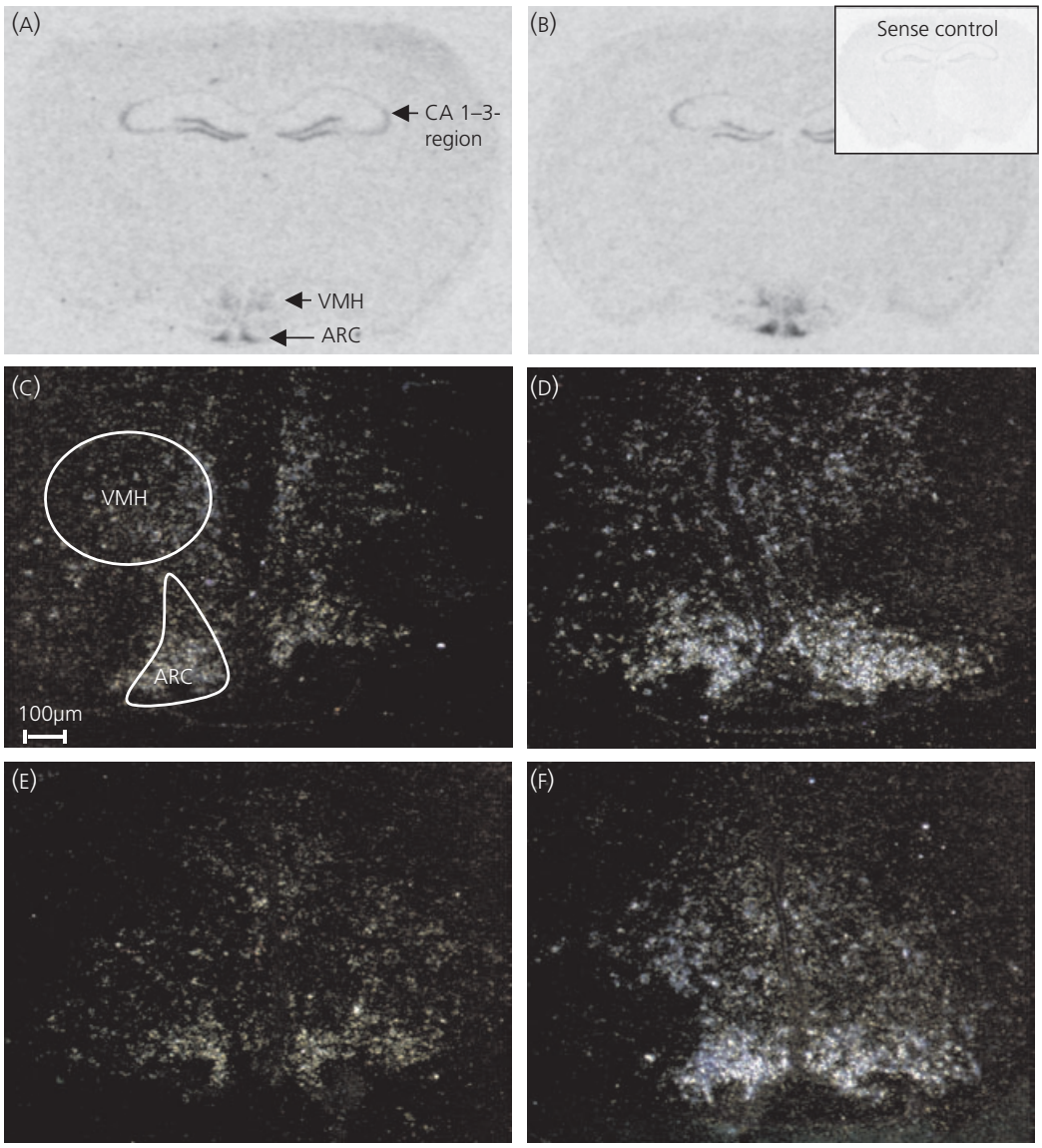

FIG. 1. Autoradiographs of LD female Siberian hamster brain sections (20 µm coronal sections; 8 weeks post weaning) either *ad libitum* fed (A) or 48-h food deprived (B) following *in situ* hybridization to an antisense ^{35}S-labelled riboprobe to growth hormone secretagogue receptor mRNA (inset depicting sense control). Also shown are representative sections of animals from each photoperiod. (C) to (F) Dark field photomicrographs showing high resolution images of the respective hypothalamic regions in LD (C,D) and SD (E,F) depicting induction of GHSR gene expression after food deprivation (C,E: *ad libitum*; D,F: food deprived). ARC, Arcuate nucleus; CA 1–3, CA 1–3 region; VMH, ventromedial hypothalamus.

rat cDNA generated a low intensity nonspecific signal (data not shown).

Effect of food deprivation(48 h) on GHSR gene expression in LD and SD hamsters

As described previously (26), SD hamsters gained 10.9 ± 1.0 g body weight, while hamsters in LD gained 16.8 ± 0.8 g during the 8 weeks following weaning. Food deprivation for 48 h led to a loss in body weight of 13.4 ± 2.3% in LD hamsters and 17.9 ± 2.3% in SD hamsters.

We found no difference in hypothalamic arcuate nucleus and VMH GHSR mRNA expression between LD and SD *ad libitum* fed hamsters, although there was a trend to increased gene expression in the arcuate nucleus and VMH in SD, which came close to, but did not achieve, statistical significance. However, food deprivation for 48 h led to a marked increase in GHSR gene expression in the arcuate nucleus (two-way ANOVA; $F = 18.17$; $P < 0.001$; Figs 1 and 2A) and VMH (two-way ANOVA; $F = 4.99$; $P < 0.05$; Figs 1 and 2B) irrespective of photoperiod.

Effect of chronic food restriction on GHSR gene expression in LD and SD hamsters

This experiment investigated changes in GHSR gene expression related to chronic manipulation of feeding state. As described previously (26), the body weight trajectory of LD-REST hamsters was matched to the body weight traject-

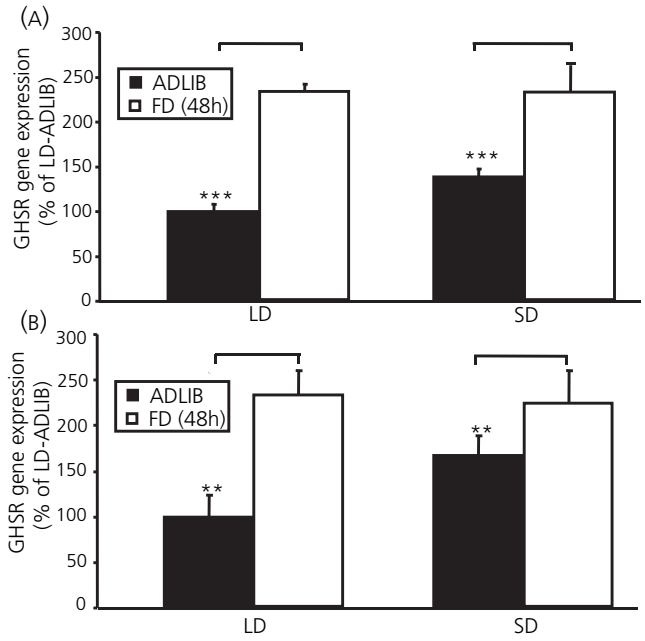

FIG. 2. Growth hormone secretagogue receptor (GHSR) gene expression in the hypothalamic arcuate nucleus (A) and in the ventromedial hypothalamus (B) of juvenile female Siberian hamsters. Hamsters were either *ad libitum* fed (ADLIB) or food deprived for 48 h (FD) (n = 6) in long (LD) or short day-length (SD). Values are expressed as percentages of values in LD hamsters fed *ad libitum*. Data are mean ± SEM. **P < 0.01, ***P < 0.001.

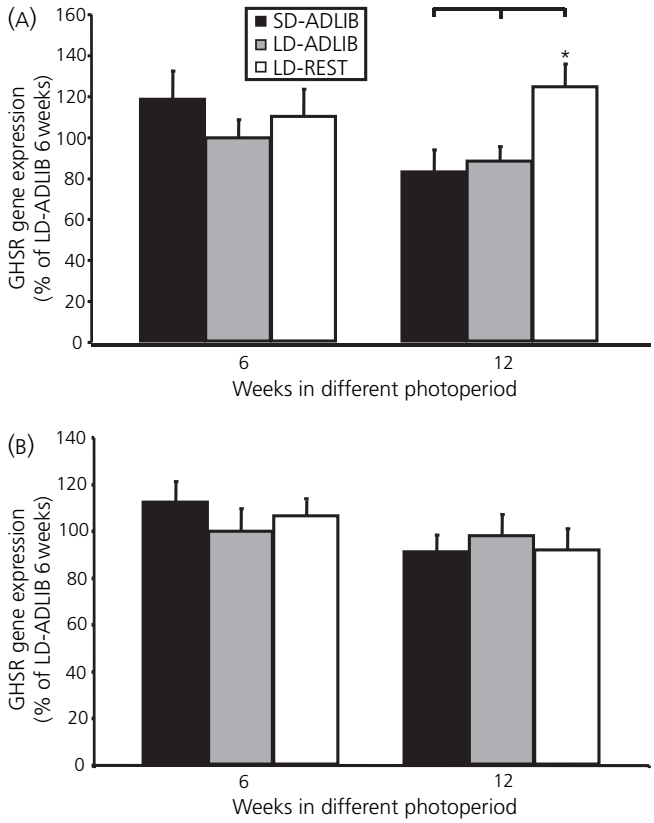

FIG. 3. Growth hormone secretagogue receptor (GHSR) gene expression in the hypothalamic arcuate nucleus (A) and in the ventromedial hypothalamus (B) of juvenile female Siberian hamsters (n = 9–12 in each group), fed *ad libitum* in long (LD-ADLIB) or short day-length (SD-ADLIB) for either 6 or 12 weeks, or held in long day-length with restricted food from day 0 post weaning onwards to mimic short-day-length body weight trajectory (LD-REST). Values are expressed as percentages of values in LD hamsters fed *ad libitum* (6 weeks). Data are mean ± SEM, *P < 0.05

ory of SD hamsters, whereas LD hamsters which continued to feed *ad libitum* gained significantly more weight than the remaining two groups (approximately 30% after 12 weeks).

Neither SD acclimation nor food restriction for 6 weeks had significant effects on arcuate nucleus (Fig. 3A) GHSR gene expression. By contrast, after 12 weeks, the LD-REST group showed significantly elevated arcuate nucleus GHSR gene expression compared to the LD-ADLIB and SD-ADLIB animals (one-way ANOVA on ranks; $H = 5.39$; $P < 0.01$). GHSR gene expression in the VMH (Fig. 3B) after either food restriction period was not different from the respective *ad libitum* fed groups.

Effect of leptin injection on GHSR gene expression

There was no effect of leptin injection on GHSR gene expression in the arcuate nucleus in either LD or SD hamsters over the 15–120 min time course postinjection compared to the vehicle-injected controls. Furthermore, GHSR gene expression of LD and SD vehicle injected hamsters was not different (Fig. 4).

Serum ghrelin concentration

In this repetition of the first experiment, over the 8-week postweaning period, SD hamsters gained 10.7 ± 1.0 g, while hamsters in LD gained 15.4 ± 1.1 g. Following food deprivation, LD hamsters lost 18.4 ± 2.4%, and SD hamsters 20.4 ± 3.2%, of their initial body weight before food deprivation. These values are similar to those observed previously (26).

Serum ghrelin levels recorded in the Siberian hamster are within the range measured in different mammalian species (28–30). No significant effect of photoperiod on serum ghrelin concentration was observed, although there was a trend for higher levels in SD. In food-deprived juvenile female hamsters, serum ghrelin concentration was significantly elevated in comparison to *ad libitum* fed hamsters (Fig. 5). This increase was observed in both LD and SD hamsters with a slightly greater elevation in SD (Fig. 5). The overall effect of feeding status was highly significant (two-way ANOVA; $F = 9.20$; $P < 0.001$). A similar increase in serum ghrelin concentration was also apparent in adult male LD hamsters after 48 h of food deprivation (LD-ADLIB: 795.2 ± 83.5 pg/ml, LD-FD: 1510.6 ± 189.0 pg/ml; n = 10 in each group, *t*-test; −3.31; $P < 0.001$).

Discussion

In this report, we demonstrate, for the first time, a marked elevation of GHSR gene expression in the arcuate nucleus and VMH in response to food deprivation for 48 h. By contrast chronic food restriction imposed to match SD body weight trajectory in LD hamsters (LD-REST) for 6 weeks

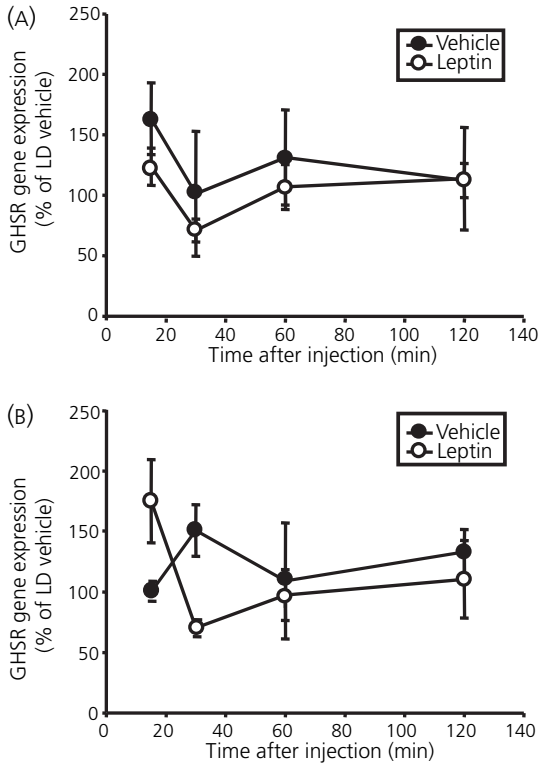

FIG. 4. Time-dependent effect of leptin injection on Growth hormone secretagogue receptor (GHSR) gene expression in the hypothalamic arcuate nucleus of juvenile female hamsters held in short (A) or long (B) day-length for 8 weeks (n = 3). Leptin was injected intraperitoneally at different time-points (15, 30, 60 and 120 min) before preparation of the brains; values are expressed as percentages of values in LD 15 min vehicle. Data are mean ± SEM.

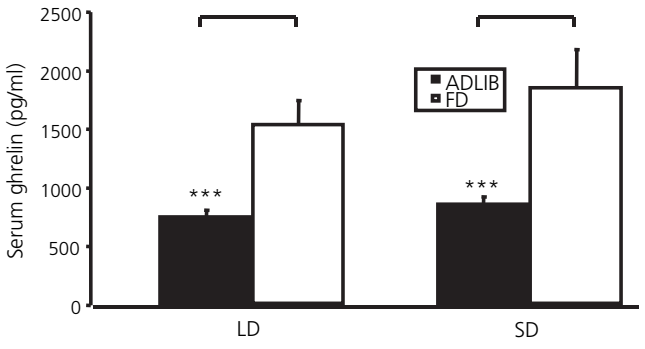

FIG. 5. Serum ghrelin levels in juvenile female Siberian hamsters 8 weeks post weaning. Hamsters were either *ad libitum* fed (ADLIB) or food deprived for 48 h (FD) (n = 4–6) in long (LD) or short day-length (SD). Data are mean ± SEM. ***P < 0.001.

had no effect and after 12 weeks led to only a slight increase in GHSR gene expression. Remarkably, the distinct photoperiod induced changes in body weight of *P. sungorus* did not affect GHSR gene expression within the examined hypothalamic regions. The arcuate nucleus and the VMH are both regarded to be key centres for the central integration of peripheral signals that convey energy homeostasis (31). Across different mammalian species, including the seasonal hamster *P. sungorus*, it is well established that body weight regulatory hormones such as leptin are processed in these hypothalamic nuclei to transduce their bioenergetic information into a central response. Recently, GHSR was demonstrated to be the primary ghrelin receptor that is able to modulate appetite in mice (32). We demonstrate that an increase of GHSR gene expression following food deprivation (48 h) is associated with a two-fold elevation of serum ghrelin levels. In addition to the fact that hypothalamic differential GHSR gene expression is confined to the arcuate nucleus and VMH, this implies that the orexigenic function of ghrelin would appear to depend on signal processing in these hypothalamic nuclei.

Ad libitum fed LD acclimated hamsters adjust their body weight corresponding to a postulated 'set-point' encoded by unknown neuronal mechanisms. In the present study, LD-REST hamsters were manipulated to a much lower body weight than imposed by this 'set point'. By contrast, SD hamsters defend a body weight that is appropriate to the lowered 'set-point' induced by SD acclimation. In terms of appetite, SD-ADLIB and LD-REST hamsters, despite having the same body weight, are in different satiety states due to their photoperiodic history. Therefore, LD-REST hamsters are expected to exhibit a permanently increased appetite, reflecting the drive to regain their individual body weight to the desired LD 'set point'. Indeed after 12 weeks of food restriction, GHSR gene expression in the arcuate nucleus of LD-REST hamsters was significantly elevated by approximately 50% compared to LD-ADLIB hamsters, but food restriction for 6 weeks had no effect. This discrepancy may be explained by the respective body weight differentials established between LD-ADLIB and LD-REST hamsters in these studies. In juvenile female hamsters, 6 weeks of food restriction led to a body weight differential of 6.3 g between LD-ADLIB (27.7 ± 2.6 g) and LD-REST (21.4 ± 1.4 g), whereas 12 weeks of food restriction caused a body weight differential of 8.6 g between LD-ADLIB (30.6 ± 2.6 g) and LD-REST (22.0 ± 1.0 g) (26). The larger body weight differential established after 12 weeks of food restriction may be indicative for stronger appetite in these hamsters. Interestingly, imposed food restriction in LD-REST hamsters led to a more dramatic decrease of circulating leptin levels after 12 weeks compared to 6 weeks [6 weeks: LD-ADLIB: 26.3 ± 0.8, LD-REST: 15.4 ± 3.2 ng/ml, SD-ADLIB: 10.8 ± 1.5; 12 weeks: LD-ADLIB: 26.7 ± 8.1; LD-REST: 3.8 ± 0.6 and SD-ADLIB: 9.1 ± 2.4 ng/ml (26)]. Only after 12 weeks were serum leptin levels in LD-REST hamsters clearly decreased even below the level measured in SD-ADLIB hamsters. Thus, leptin may exert an inhibitory effect on GHSR gene expression that is released only in catabolic states associated with extremely low serum leptin levels. The inhibitory potential of leptin on ghrelin sensitivity may be fully exploited in *ad libitum* fed hamsters. This may be one reason why leptin injections had no effect on GHSR gene expression in *ad libitum* fed hamsters with normal leptin levels. Hewson *et al.* (33) demonstrated that leptin alters ghrelin sensitivity only in food deprived rats, in which ghrelin and ghrelin mimetics were able to increase the number of cells expressing Fos protein in the arcuate nucleus. Our observed increase in GHSR gene expression and the accompanied elevation in circulating ghrelin concentration,

after 48 h of food deprivation, although being in a different species, may be a general mechanism through which ghrelin sensitivity could be altered in this catabolic state. This may be mediated by the abrupt decline of circulating leptin levels induced by food deprivation (26). Clearly, the increase in gene expression after 12 weeks of food restriction was less profound than that exhibited following food deprivation for 48 h. Together with the finding that GHSR gene expression in the VMH was not at all affected by chronic food restriction but clearly induced by acute food deprivation, our data support the hypothesis that ghrelin is primarily involved in the short-term regulation of appetite and body weight.

Across different species, including man, a negative correlation between circulating ghrelin and leptin has been described (10, 28, 34); obesity is associated with high leptin and low ghrelin levels. However, in *P. sungorus*, ghrelin levels were not negatively correlated with body weight. Although LD and SD hamsters exhibited a body weight differential of 15% after 8 weeks acclimation to the opposite photoperiod, no changes in serum ghrelin concentration could be detected. As published previously (26), serum leptin levels in LD hamsters are elevated by two- to three-fold in LD compared to SD. Thus, the lack of SD photoperiod-induced changes of both GHSR gene expression as well as circulating ghrelin levels implies that in seasonal body weight regulation, leptin may not counteract ghrelin. Barazzoni *et al*. (34) demonstrated that, in lean rats, subcutaneous leptin infusion prevented the rise in serum ghrelin levels in response to moderate caloric restriction. However, our results in *P. sungorus* suggest that leptin has no effect on serum ghrelin levels in the ad libitum fed state.

Long-term changes in serum leptin concentrations induced by photoperiod in the female juvenile hamster do not affect central expression of GHSR or ghrelin serum concentration, suggesting that ghrelin does not play a major role in seasonal body weight regulation. To generalize this conclusion, further photoperiod experiments in adult male and female hamsters are required. As such, ghrelin does not appear to be a functional antagonist to leptin (at least for the regulation of long-term body weight changes) but perhaps a signal regulating responses to short-term changes in energy homeostasis, such as food deprivation. The data obtained from the seasonal species *P. sungorus* contribute additional information to the poorly understood interaction of leptin and ghrelin. This discrepancy implies that the interaction of the important feeding related hormones leptin and ghrelin, an interesting enigma in body weight regulation, certainly requires further investigation.

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PC1/3 and PC2 gene expression and post-translational endoproteolytic POMC processing is regulated by photoperiod in the seasonal Siberian hamster (*Phodopus sungorus*).

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Short title: Photoperiod regulates POMC processing in the Siberian hamster.

Keywords: Seasonal body weight regulation, proteolytic processing, photoperiod, prohormone convertases, POMC.

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Abstract:

A remarkable feature of the seasonal adaptation displayed by the Siberian hamster (*Phodopus sungorus*) is the ability to decrease food intake and body weight (by up to 40%) in response to shortening photoperiod. The regulating neuroendocrine systems involved in this adaptation and their neuroanatomical and molecular bases are poorly understood. We investigated the effect of photoperiod on the expression of prohormone convertases 1 (PC1/3) and 2 (PC2) and the endoproteolytic processing of the neuropeptide precursor pro-opiomelanocortin (POMC) within key energy balance regulating centres of the hypothalamus. We compared mRNA levels and protein distribution of PC1/3, PC2, POMC, ACTH, α-MSH, β-endorphin and orexin A in selected hypothalamic areas of long day (LD, 16h light: 8h dark), short day (SD, 8h light: 16h dark) and natural-day (ND, photoperiod depending on time of the year) acclimated Siberian hamsters. The gene expression of PC2 was significantly higher within the arcuate nucleus (ARC, $P<0.01$) in SD and in ND (vs. LD), and is reflected in the daylength profile between October and April in the latter. PC1/3 gene expression in the ARC and lateral hypothalamus was higher in ND but not in SD compared with respective LD controls. The immunoreactivity of PC1 cleaved neuropeptides such as ACTH in the ARC, and orexin A in the LH, were not affected by photoperiod changes. However, increased levels of PC2 mRNA and protein were associated with higher abundance of the mature neuropeptides α-MSH and β-endorphin ($P<0.01$) in SD. This study provides a possible explanation for previous paradoxical findings showing lower food intake in SD associated with decreased POMC mRNA levels. Our results suggest that a major part of neuroendocrine body weight control in seasonal adaptation may be effected by post-translational processing mediated by the prohormone convertases PC1/3 and PC2, in addition to regulation of gene expression of neuropeptide precursors.

Introduction:

Seasonal animals such as the Siberian hamster (*Phodopus sungorus*) exhibit remarkable physiological and metabolic adaptations in response to seasonal changing environment. These adaptations include changes in pelage insulation and colour, reproductive activity, food intake and body weight (1). The drive to reduce food intake in shortening winter photoperiod persists even if food is provided *ad libitum* demonstrating the importance of this regulatory energy balance mechanism. A key neuronal centre that regulates these physiological responses is the hypothalamus, an area of the central nervous system (CNS) that integrates photoperiodic and peripheral inputs in a complex network of interacting orexigenic and anorexigenic neuropeptides (2, 3). The Siberian hamster processes information on changing photoperiod through the pineal hormone, melatonin, and about internal energy stores via peripherally released hormones such as leptin and ghrelin, to generate appropriate responses in terms of energy balance regulation (4, 5, 6). The voluntary decrease in food intake and body weight in SD presumably reflects the increased activity of anorexigenic components of this neuroendocrine system. One of the neuropeptides that would meet this criterion is alpha-melanocyte-stimulating hormone (α-MSH; 7, 8), a product of the 30-32 kDa molecule pro-opiomelanocortin (POMC), which exerts an inhibitory control on food intake and energy storage through its action in the CNS at the melanocortin 3 and 4 receptors (9). Unexpectedly, previous studies demonstrated decreased gene expression of the precursor POMC in SD which could in principal result in lower concentrations of α-MSH during winter (10). However, most neuropeptide precursors like POMC have to undergo post-translational processing by proteolytic cleavage before their products acquire biological activity. The post-translational process is accomplished by highly specific cleavage enzymes (prohormone convertases) and is therefore an essential step not only as a part of the protein biosynthetic process but also as a regulatory step in neuropeptide synthesis. In mammals, prohormone

convertases 1/3 (PC1/3) and 2 (PC2), that are members of the subtilisin-like proprotein convertases, have been identified to be responsible for the proteolytic processing of neuropeptides and peptide hormones in neuronal endocrine tissue (11).

Both PC1/3 and PC2 are expressed in neuroendocrine tissues such as hypothalamic neurons and cleave prohormones at paired basic residues. The cleavage-specificity of PC1/3 and PC2 in POMC processing was reported by cell transfection experiments. It has been demonstrated that PC1/3 cleaves POMC into large intermediate molecules such as adrenocorticotropic hormone (ACTH) and β-lipotropin, whereas PC2 subsequently cleaves ACTH and β-lipotropin into α-MSH and β-endorphin, respectively (Fig. 1; 12, 13). Thus, coordinated cleavage activity of both prohormone convertases is necessary to process neuropeptide precursors such as POMC into specific neuropeptides. As PC1/3 and PC2 are essential for the post-translational processing of various neuropeptide precursors it is likely that changes in gene expression and biosynthesis have fundamental effects on the maturation of neuropeptides and hence energy homeostasis.

We hypothesised that POMC processing is photoperiodically regulated by differential expression of PC1/3 and PC2. It is likely that decreasing body weight in SD acclimated hamsters is associated with higher levels of anorexigenic neuropeptides such as α-MSH despite down-regulated gene expression of POMC. We suggest that differential endoproteolytic activity of prohormone convertases in SD and LD is responsible for photoperiod-regulated biosynthesis of smaller POMC-derived neuropeptides. To test this, gene expression of PC1/3 and PC2 was investigated in hamsters exposed to ambient photoperiod in winter (October – April) to profile long term effects. In addition, we measured mRNA expression levels of PC1/3 and PC2 following transfer of Siberian hamsters back into LD, after 14 weeks in artificial SD photoperiod. This experimental setup provided a better assessment of the temporal responsiveness of photoperiod-induced regulation of gene expression. Neuroanatomical protein distribution and differential expression of PC1/3, PC2,

POMC, ACTH, α-MSH, and β-endorphin in SD and LD acclimated hamsters were investigated by immunohistochemistry. In a second approach, we used dual fluorescence immunohistochemistry to colocalise the prohormone convertases with POMC and the derived neuropeptides to evaluate the ratio of proteolytic activity of PC1/3 and PC2 in SD and LD, respectively. In addition, the post-translational fate of prepro-orexin as a candidate of orexigenic neuropeptide precursors was analysed.

Materials & Methods:

Animals and experimental procedures: All described procedures were in accordance with German animal welfare regulation, or were licensed under the UK Home Office Animals (Scientific Procedures) Act, 1986, and had local ethical approval.

Siberian hamsters (*Phodopus sungorus*) were drawn from breeding colonies established in the Biology Faculty in Marburg (Germany) and at the Rowett Research Institute in Aberdeen (Scotland). All animals were housed individually and had *ad libitum* access to food (Marburg: Standard breeding chow diet, 7014, Altromin, Lage, Germany; Aberdeen: Labsure pelleted diet, Special Diet Services, Witham, Essex, UK) and water. Body weights were assessed weekly. Photoperiods referred to in this article are defined as LD (long day, 16h light, 8h dark), SD (8h light, 16h dark) and ND (natural day, with day length depending on time of the year).

Experiment 1: Siberian hamsters (n=72, Marburg colony) were born and reared in ND at 23° C. At the age of 4-6 months they were divided into two groups. One group (n=36, matched for sexes) was transferred to LD whereas the other (n=36, matched for sexes) was maintained in ND and exposed to the progressive change in natural day length from October until April. At intervals of 40 days (Oct, Nov, Jan, Feb, Mar, Apr) hamsters from the LD and ND group (3 males, 3 females/group) were killed with CO_2 and decapitated. Brains were immediately dissected, frozen on dry ice and stored at -80° C until required. The day length in ND photoperiod was calculated using the Sunrise/Sunset Calculator software (National Oceanic and Atmospheric Administration, Washington D.C; USA) based on the geographical location of the breeding facility in Marburg (8°46'17,7"/50°48'17,5").

Experiment 2: Male Siberian hamsters (n = 32, Aberdeen colony) were housed individually at 22° C. Hamsters used in this experiment were born and reared in LD. When 4-6 months old, half the animals (n = 16) were transferred to SD. After 14 weeks (wk 0) a group of LD and SD hamsters (n = 4/group) were killed by cervical dislocation. All remaining SD hamsters were transferred back to LD photoperiod. LD controls and hamsters transferred back from SD to LD (n = 4/group) were then killed at intervals of two weeks (wk 2, 4 and 6; 14). Brains were immediately dissected, frozen on dry ice and stored at –80° C until required.

In situ hybridisation: Messenger RNA levels for PC1/3 and PC2 were quantified by *in situ* hybridisation in 15 µm coronal sections, corresponding to –1.5 to –3.7mm relative to Bregma (15), using techniques described in detail elsewhere (16). Riboprobes complementary to partial fragments of PC1/3 and PC2 gene were generated from cloned Siberian hamster brain cDNA. The amplification of the PC1/3 (248 bp, GenBank AY625692) and PC2 (232 bp GenBank AY625693) fragments was performed by PCR using the following primers: 5'-ATGGGGGTCGTCAAGGAGATAACT-3' and 5'-GATGCCAGCAGCAGCCAGAGGTG-3' (rat PC1/3, GenBank M76705) and 5'-GCGGCCGGGCTTCTCTTCT-3' and 5'-GCTGCCGCTTGTGATGTAGG-3' (rat PC2, GenBank M76706), respectively. Both DNA fragments were ligated into pGEM-T-easy (Promega, Madison, WI, USA), transformed into *Escherichia coli* DH5α and sequenced. Sequence alignment of the species-specific fragments cloned from *P. sungorus* revealed a 96.4% (PC1/3) and 97% (PC2) identity to rat prohormone convertases at the nucleotide level. Hybridised slides were apposed with Kodak BioMax MR film and, where appropriate, were coated with LM-1 film emulsion (Amersham Bioscience UK Limited, Buckinghamshire, UK). The levels of hypothalamic mRNAs were analysed and quantified by computerized densitometry (Image Pro-Plus software, Version 5.5.1; Media Cybernetics, Wokingham, Berkshire, UK) of *in situ* hybridisation autoradiograms. Micropictures of emulsion autoradiography sections were taken by bright field microscopy

using an Olympus BX-50 microscope (Olympus Microscopes Ltd., Middlesex, UK) with attached digital camera system (Hitachi HV-C20, Hitachi Europe Ltd., Maidenhead, Berkshire, UK).

Dual immunostaining: Male Siberian Hamsters (n = 24, Aberdeen colony) were kept under conditions described above (Exp. 2). Half of them (n = 12) were transferred to SD. After 14 weeks in LD or SD, hamsters were anaesthetized with sodium pentobarbital and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains were dissected and transferred into a 4% PFA-PBS solution (8h, 4° C), followed by cryoprotection in 30% sucrose - 0.1 M PBS (48h, 4°C), and were deep frozen in isopentane over dry ice (1 min). Coronal sections (35 µm) of the brain, corresponding to –1.5 to –3.7mm relative to Bregma (15), were processed on a cryostat. Free-floating sections were treated with blocking solution (BS) containing 3% bovine serum albumin (BSA) in 0.5 % Triton X-100 - 0.1 M PBS (0.5% PBS-T) for 1 h to block non-specific reactions. Then, sections of LD (n = 3) and SD (n = 3) hamster brains were incubated with polyclonal rabbit anti-orexin A (1:200, H-003-30, Phoenix Pharmaceuticals Inc; Belmont, USA), anti-POMC (1:100, H-029-30, Phoenix), or anti-*β*-endorphin (1:100, H-022-33, Phoenix) in BS overnight (4° C). Following washes in 0.25% PBS-T, sections were incubated for 2h with unconjugated goat anti-rabbit Fab-fragment antibody (111-007-003, Jackson ImmunoResearch, West Grove, USA) diluted 1:60 in BS at room temperature (RT). Sections were rinsed briefly in 0.25% PBS-T and incubated with Cy3 (Ex_{max} 554 nm, Em_{max} 566 nm) conjugated donkey anti-goat secondary antibody in BS (1:250, 705-165-147, Jackson) for 2h at RT, rinsed again in 0.25% PBS-T and incubated with the second polyclonal rabbit anti-PC1/3 primary antibody (1:400, AB1260, Chemicon Inc; Temecula, USA) or anti-PC2 (1:400, AB1262, Chemicon), in BS overnight at 4°C. Sections were incubated with Alexa 488 dye (Ex_{max} 492 nm, Em_{max} 520 nm) conjugated goat anti-rabbit secondary antibody (1:250, Molecular Probes, Eugene, USA) in BS for 2h at RT.

Colocalisation for α-MSH was performed with polyclonal sheep anti-α-MSH antibody (1:15.000, Chemicon) in BS overnight at 4° C. In this case different host species in which the applied primary antibodies were raised made an intermediate step of Fab-fragment incubation obsolete. α-MSH was visualized by incubation with Fluorescein (Ex_{max} 494 nm, Em_{max} 520 nm) conjugated donkey anti-sheep secondary antibody (1:100, AP184F, Chemicon) in BS for 2 h at RT. Incubation with the second primary antibodies and secondary antibody matched the steps described above. Sections were then rinsed in PBS, mounted on gelatin-coated slides, air-dried, dehydrated in graded alcohol, cleared in xylene and coverslipped with Enthelan (Merck Biosciences, Darmstadt, Germany). Sections were examined under a conventional Leica DMR epifluorescent microscope (Leica Microsystems, Wetzlar, Germany). Cell bodies in comparable extensions of 105 µm (3 sections) within the ARC were counted blindly. Images were taken by a Hamamatsu C5810 digital camera system mounted on the microscope. Merger of images was performed by colour channel overlay using image processing software (Adobe Photoshop version 7.0). The anatomical localization of neuropeptides within the brain of Siberian hamsters was annotated according to the atlas of the golden hamster brain (15).

Controls: For controls, each of the primary antibodies was pre-incubated with its complementary peptide (α-MSH, 043-01, Phoenix; β-endorphin, 022-33, Phoenix; orexin-A, 003-30, Phoenix; POMC, 029-30, Phoenix; PC 1/3, AB5011, Abcam; PC2, AB5012, Abcam), prior to application. Incubation with preadsorbed primary antibodies resulted in no staining. Additional negative controls were performed by incubation of sections lacking primary antisera. Labeling of the primary antibodies by incubation with interchanged secondary antibodies showed an identical staining pattern.

Single immunostaining: Female Siberian hamsters (n = 20, Marburg colony) at 7 months of age were divided into two groups of 10. One group was kept in LD, whereas the other was transferred to SD. After 14 weeks, hamsters were killed in a CO_2 atmosphere and decapitated. Brains were excised, fixed in 4% PFA (48 h, 4°C), and cryoprotected in 20% sucrose in 0.1 M PBS for 24 hr at 4° C. Brains were deep frozen in isopentane over dry ice (1 min) and stored in –80° C until required. Coronal sections were cut on a cryostat at 30 µm. Endogenous peroxidase activity was inhibited in sections using 80% PBS, 10% methanol and 10% H_2O_2 for 15 min at RT. Free-floating sections were rinsed in PBS and 0.5% PBS-T. Following pre-incubation in a blocking solution containing 0.5% PBS-T and 3% BSA, sections were incubated with primary polyclonal rabbit anti-ACTH (Phoenix; H-001-21) antibody diluted 1:350 in BS overnight at 4°C. Following washing in 0.5% PBS-T, sections were then incubated with peroxidase-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, 111-035-144) diluted 1:500 in BS for 1 hr at RT. Using Vector SG substrate kit for peroxidase (SK-4700, Vector Labs, Burlingame, USA), the colour reaction resulted in dark-gray/blue immunostaining. Sections were then rinsed in PBS, mounted on gelatin-coated slides, air-dried, dehydrated in graded alcohol, cleared in xylene and coverslipped with Enthelan (Merck). Immunoreactive cell bodies were counted blinded, using a Zeiss Axioskop (Carl Zeiss, Jena, Germany) microscope (objective, 20X). Images were taken by a mounted Polaroid DMCe digital camera.

Controls: The specificity of primary antibody was tested by adding an excess of ACTH-(Phoenix; 001-21) peptide to the primary antibody for 3 hrs at RT before application to sections, or by omission of the primary antibody. Brain sections incubated either with preadsorbed primary antiserum or in the absence of primary antibodies did not exhibit any ACTH-ir (data not shown).

Statistical analysis: Data were analysed by two-way ANOVA followed by the Student-Newman-Keuls multiple comparison test, where appropriate (for *in situ* experiments), and one-way ANOVA (for immunohistochemistry data) using a statistical software package (SigmaStat, Jandel Corp.). Data from *in situ* hybridisation experiments are presented as means ± SEM; Data for immunohistochemistry experiments are presented as percentage values of LD control ± SEM. A probability value of $p < 0.05$ was considered as statistically significant.

Results:

Effect of seasonal changing photoperiod on body weight

The body weight trajectory of ND animals was inversely related to the seasonal change in ambient photoperiod (Fig. 2). Beginning with an average body weight of 32.3 g (± 3.4 g) in October, ND body weight decreased by 17.3% to a minimum of 26.7 g (± 2.1 g) in January, followed by a weight gain of 53.9% to a body weight of 41.1 g (± 3.9 g) in April. Control group animals kept in constant LD photoperiod (16h light : 8h dark) maintained an average body weight of 38.3 g (± 3.6 g) throughout the six months of the experiment. As a result, mean body weights of ND and LD animals differed by 12 g in January ($P < 0.01$), and were also significantly different in November ($P < 0.05$).

Effect of natural photoperiod on gene expression of PC1/3 and PC2

PC1/3 and PC2 mRNA were detected in various areas of the hamster hypothalamus with region-specific intensity differences. Gene expression of both PC1/3 and PC2 was observed in arcuate nucleus (ARC), paraventricular nucleus (PVN) and ventromedial nucleus (VMH), although gene expression in the PVN and VMH was close to the limit of detection and consequently was not quantified. In addition, PC1/3 mRNA was observed in the lateral hypothalamus (LH). Autoradiographs of PC1/3 and PC2 in the ARC revealed similar expression patterns to previously observed POMC mRNA distribution in this area (10). In addition, high concentrations of PC2 mRNA were observed in a small group of neurons within the dorsal medial posterior part of the arcuate nucleus (dmpARC).

Gene expression of PC1/3 within the ARC (Fig. 3A) and LH (Fig. 3B) revealed a significant overall effect of photoperiod ($P < 0.05$ for both regions) with higher levels of mRNA in ND (vs. LD controls). However, PC1/3 mRNA levels in the ARC and LH were not correlated with the profile of changing photoperiod in ND; there were no effects of time

(month) and no time * photoperiod interaction. In the LH (Fig. 3B), the apparent reflection of ND photoperiod in trends to increased levels of PC1/3 mRNA from October until January followed by a decline to February were not statistically significant.

Gene expression of PC2 in the ARC (Fig. 3C) and the dmpARC (Fig. 3D) also revealed strong effects of photoperiod with higher levels of mRNA in ND ($P < 0.001$ for both regions). In addition, a seasonal pattern of PC2 gene expression in the ARC (Fig. 3C, quantified area excluding the dmpARC) was observed in ND hamsters (two-way ANOVA: $P < 0.01$ for effect of time; $P < 0.05$ for time * photoperiod interaction) with maximal mRNA levels observed in January (multiple comparison: $P < 0.05$). Although a similar temporal gene expression profile was apparent for gene expression of PC2 in the dmpARC (and for PC1/3 in LH) this could not be statistically consolidated (Fig. 3D).

Effect of transfer of hamsters from SD to LD on body weight

Body weights of the Siberian hamsters used in these experiments have been documented previously (14). 14 weeks in SD resulted in a 27% reduction in body weight, compared to LD controls. Transfer back to LD had little effect on body weight for the first two weeks, but thereafter body weight increased significantly ($P < 0.001$) and achieved a level similar to that of LD controls by 6 weeks.

Effect of transfer of hamsters from SD to LD on gene expression of PC1/3 and PC2

Gene expression of PC1/3 in the ARC and LH did not change significantly after 14 weeks in SD (Fig. 3E, F, wk0). PC1/3 mRNA levels in the ARC and LH were unaffected by transfer from SD back to LD photoperiod (wk2, wk4, wk6) and were similar to those of LD controls. There were no effects of photoperiod or time, and no interaction. Neuroanatomical distribution patterns of PC1/3 mRNA analysed by emulsion autoradiography in the ARC (Fig. 3I, a-b) and LH (Fig. 3I,c-d) of SD and LD (wk0) also showed no apparent differences.

Photoperiod had no overall effect on PC2 gene expression in the ARC (Fig. 3G) or dmpARC (Fig. 3H) but PC2 gene expression revealed a significant effect of time ($P < 0.001$ for both regions), and a time * photoperiod interaction ($P < 0.001$ for both regions). Significantly higher levels of PC2 mRNA were found in the ARC ($P < 0.05$) and dmpARC ($P < 0.05$) of SD animals compared to LD controls after 14 weeks in SD photoperiod (wk0). This observation was corroborated by emulsion autoradiographs showing a higher content of silver grains with PC2 probes within the ARC (Fig. 3I, e-f) and dmpARC (Fig. 3I, g-h) of SD hamsters at time point wk0. Following the transfer from SD back to LD photoperiod, gene expression of PC2 in the ARC decreased to a nadir at wk4. A significant down regulation of gene expression was observed after transfer from SD back to LD at all three time points (wk2, wk4, wk6; $P < 0.05$, vs. wk0 SD, respectively). Between 4 and 6 weeks after transfer back to LD, mRNA levels of PC2 increased significantly ($P < 0.05$). In the dmpARC, gene expression of PC2 was decreased after 2 weeks and remained significantly lower until 6 weeks (wk2, wk4, wk6; $P < 0.05$, vs. wk0 SD, respectively) after transfer back to LD.

Effect of photoperiod on protein expression of PC1/3, PC2, POMC, ACTH, α-MSH, β-endorphin and orexin A

Immunoreactive cells and fibres for PC1/3, PC2, POMC, ACTH, α-MSH, β-endorphin and orexin A were observed in different hypothalamic areas of the Siberian hamster brain. Immunolocalised distribution patterns of PC1/3 and PC2 protein matched the mRNA pattern, except for a lack of PC2-ir in the dmpARC. Unlike the strong signal detected for PC2 mRNA in this region, little immunoreactivity for its protein could be observed (data not shown).

In the ARC, there was no effect of photoperiod on the number of counted PC1/3-ir cells (Fig. 4A; B, a-b); hamsters kept in LD had 167 ±15 ir-cells and those in SD 153 ±11 ir-cells within the investigated region of the ARC. However immunohistochemical staining of PC2 in the ARC showed 125% more ir-cells in SD (88 ±19 ir-cells), leading to a significant

difference ($P < 0.01$) compared to those counted in LD controls (39 ±6 ir-cells) (Fig. 4A; B, c-d). POMC-ir in LD (178 ±22 ir-cells) and SD (156 ±10 ir-cells) revealed no significant difference in ir-cell number (Fig. 4A; B, e-f). ACTH-ir (LD, 105 ±14; SD, 81 ±9 ir-cells) as well as α-MSH-ir (LD, 47 ±11; SD, 54 ±13 ir-cells) levels in the ARC were also unaffected by photoperiod (Fig. 4A; B, g-h, k-l). In contrast, the density of α-MSH-ir fibres appeared greater in SD, but was not quantifiable (Fig. 4A; B, k-l). The neuroanatomical distribution pattern of β-endorphin-ir was similar to that of α-MSH-ir, but was mainly concentrated in cell bodies. Counting of β-endorphin-ir cells revealed 76% ($P < 0.01$) more neurons in SD (74 ±18 ir-cells) compared to LD (42 ±9 ir-cells) (Fig. 4A; B, i-j). Quantification of PC1/3-ir (LD, 119 ±16; SD, 104 ±13 ir-cells) and orexin A-ir (LD, 105 ±14; SD, 97 ±11 ir-cells) within the LH did not reveal differences between SD and LD controls (Fig. 4A; B, m-p).

Effect of photoperiod on colocalisation of PC1/3- and PC2- with POMC-, α-MSH-, β-endorphin- and orexin A-immunoreactivity

Dual fluorescence immunohistochemistry in the ARC showed no significant differences between the proportion of POMC-ir cells colocalised with PC1/3-ir cells in SD and LD. In both LD (87.5% ±21) and SD (94.1% ±14.7), nearly all POMC-ir cells were also PC1/3-ir positive (Fig. 5A,F). In contrast, the overall level of PC2-ir colocalisation with POMC-ir was lower and revealed significantly ($P < 0.01$) more POMC-ir cells which also contained PC2-ir in SD (59.14% ±12.9) than in LD (22.9% ±8; Fig. 5B, F). Colocalisation of PC2-ir cells with α-MSH and β-endorphin immunoreactivity (Fig. 5C, D) revealed a nearly complete match of these POMC derived neuropeptides and the cleavage mediating prohormone convertase in SD and LD (Fig. 5 F; α-MSH, LD, 89.1% ±12.6; SD, 93.2% ±21.5; β-endorphin, LD, 90.9% ±13.5; SD, 86.4% ±16.3;). In the LH, there was no effect of photoperiod on the colocalisation of orexin A-ir and PC1/3-ir (Fig. 5 E, F); the neuropeptide product of prepro-orexin was

localised with the majority of PC1/3-ir cells in this area in both LD (92% ±18.4) and SD (83.2% ±18.7).

Discussion:

Seasonal body weight in mammals is regulated by a complex interaction of neuropeptides in a hypothalamic network of neurons that integrates environmental photoperiod inputs. Most of these energy balance-regulating neuropeptides derive from larger biologically inactive precursors and have to undergo post-translational processing by endoproteolytic cleavage. This study presents evidence substantiating the hypothesis that an important part of the photoperiod-driven regulation of POMC product biosynthesis is mediated by post-translational processing through PC1/3 and PC2, and thus provides valuable information over and above the control of precursor gene expression at a transcriptional level.

Neuroanatomical distribution patterns of PC1/3 and PC2 transcripts in the hamster hypothalamus match with previously described localisations in other rodent species such as rats and mice (17, 18). Hamsters kept in ND and SD displayed typical physiological adaptations to reducing or reduced photoperiod including change of pelage colour, reduction of reproductive tissue, reduced food intake and body weight loss. These photoperiod-induced physiological changes were not accompanied by temporal change in PC1/3 mRNA levels. Gene expression of PC1/3 in ARC and LH was higher overall in ND (vs. LD) but this effect was not observed after 14 weeks in SD (vs. LD) artificial photoperiod, suggesting some impact of prior photoperiodic history. At present there is no clear explanation for this unexpected difference between ND and SD as gene expression of PC1/3 in summer ND (May – September) was not measured. Furthermore, short term change of photoperiod induced by transfer from SD to LD was also without discernible effect on gene expression of PC1/3 in ARC and LH, suggesting that on a transcriptional level PC1/3 is not directly regulated by photoperiodic inputs. However, previous observations have demonstrated a regulatory effect of the adipose tissue hormone, leptin, on gene expression of PC1/3 in LH and ARC since

reduced PC1/3 mRNA levels observed in obese *ob/ob* mice were upregulated in response to leptin injection (19).

In contrast to PC1/3, gene expression of PC2 in the ARC and dmpARC broadly paralleled the profile of changing ambient ND photoperiod in winter resulting in elevated mRNA when photoperiod was shortest. This photoperiod dependency was substantiated by upregulated PC2 gene expression in hamsters kept in SD for 14 weeks. After transfer from SD to LD, mRNA levels of PC2 decreased rapidly, and within 2 weeks levels were similar to those in LD. This acute regulatory change in PC2 gene expression preceded body weight loss and is therefore unlikely to be a secondary effect of metabolic and physiological changes. The photoperiod-driven gene expression profile suggests that PC2 may be an important part of a molecular neuroendocrine mechanism that is closely related to the integration of photoperiod information and the mediation of seasonal responses. Previous studies demonstrated a photoperiod-dependent differential gene expression of the neuropeptide precursor POMC in the ARC with lower mRNA levels in SD (20, 21). At first this observation appears paradoxical since down-regulation of POMC would most likely result in lower levels of its derived neuropeptide, α-MSH, whereas photoperiod-induced changes in metabolism and physiology such as reduced food intake and body weight loss would appear to require a higher concentration of the anorexic peptide, α-MSH. Artificial square-wave photoperiod transformation did not affect gene expression of PC1/3 and consequently cleavage activity of PC1/3 most likely results in unaltered levels of larger POMC derivates such as ACTH and β-lipotropin, which are cleaved by PC1/3. In contrast, increased gene expression of PC2 in SD is likely to increase proteolytic activity of PC2 at specific cleavage sites resulting in higher levels of smaller peptides such as α-MSH, β-MSH, γ-MSH, β-endorphin, CLIP and methionine-enkephalin.

Despite reported decreased gene expression of POMC in SD, protein distribution in neurons of the ARC remained unaltered by photoperiod suggesting that gene expression may

not be the primary regulator of POMC product biosynthesis. Gene expression of PC1/3 in SD (vs. LD) animals was also unaffected by photoperiod and was reflected in similar levels of PC1/3 protein in SD and LD acclimated hamsters. The transcriptional regulation of PC2 by photoperiod in the ARC was also reflected at a translational level as there was more PC2 protein detected in SD than LD animals. Similar levels of PC1/3 and POMC protein in SD and LD are reflected in unaltered ACTH-ir, with ACTH peptide known to be a direct result of POMC cleavage by PC1/3 (22). Although inhibitory properties of ACTH on food intake (23) would presume an accumulation of this peptide in SD animals, our results imply a minor role in regulation of seasonal body weight. In contrast, increased PC2 protein in SD animals was accompanied by higher levels of α-MSH-ir fibres and β-endorphin-ir cells, in line with previous studies demonstrating that α-MSH production varies directly in accordance with the expression of PC2 (24). Similar observations were reported for the maturation of β-endorphin (25). Our results corroborate these findings as PC2 up-regulation in SD results in higher concentrations of β-endorphin. Current opinions on the physiological function of β-endorphin are conflicting; pharmacological studies generally indicate a short term stimulatory effect of opioids on food intake (26, 27), but longer term regulation of energy balance has not been reported (28, 29), although β-END$^{-/-}$ mice (30) are characterised by an obese phenotype. These latter findings may support our observations of increased β-endorphin protein levels in states of reduced feeding behaviour and negative energy balance in SD, and the time scale over which these changes are manifest. These characteristics are consistent with our results since seasonal regulation of energy balance is a long-term process rather than an acute induced inhibition of food intake. In contrast to immunoreactivity of β-endorphin, which is more confined to the cell body, α-MSH-ir was widely distributed throughout fibres and boutons of neurons in the ARC. Thus, the biological relevance of quantification of relative α-MSH protein content by counting of ir-neurons is questionable. Appraisal of SD and LD α-MSH-ir distribution patterns revealed more intense staining of α-MSH-ir fibres in SD and

hence higher levels of protein in SD. This observation is supported by the fact that the concentrations of α-MSH and β-endorphin are closely correlated in agreement with their production in equimolar amounts as products of the same precursor (31).

Therefore it is unlikely that less α-MSH than β-endorphin is processed and the immunoreactivity patterns observed in the current study could reflect different rates of transport and routes of intracellular trafficking within the neuronal network. Visually apparent increased levels of α-MSH could be appropriate to the state of negative energy balance in SD (32, 7). Combined with increased expression of β-endorphin in SD, these findings suggest a complementary interaction between the melanocortin, α-MSH, and the opioid, β-endorphin, on seasonal regulation of energy homeostasis, rather than opposing effects.

Colocalisation of PC1/3-ir with POMC-ir in ARC showed almost complete coexpression. This observation implies that cleavage of POMC by PC1/3 is a fundamental process that provides the same relative amounts of PC1/3-cleaved POMC-derived peptides independent of changing photoperiod in SD. We substantiated this hypothesis by demonstrating levels of ACTH-ir that were nearly equal in SD and LD. Even though we did not scrutinize the proteolytic processing fate of β-lipotropin, the intermediate precursor of β-endorphin, similar results would be expected since β-lipotropin is cleaved by PC1/3 in a similar manner to ACTH. α-MSH and β-endorphin were almost completely colocalised with PC2 in SD and LD reflecting their derivation from larger intermediate POMC fragments by proteolytic PC2 processing. As a result of higher protein concentrations of PC2 in SD (vs. LD), more α-MSH-ir and β-endorphin-ir was processed and could be observed in animals that were exposed to short photoperiod.

Colocalisations of PC1/3 and orexin A in LH demonstrated no regulatory effect of photoperiod. The orexigenic neuropeptides, orexin A and orexin B, are highly specifically localized in the LH and are generated by proteolytic processing of the precursor peptide

prepro-orexin (33), whose gene expression in the LH was previously colocalised with mRNA encoding for PC1/3 (19). Immunohistochemical colocalisation of PC1/3-ir and orexin-A-ir in this study confirmed the close relationship between these two neuroendocrine components at a protein level. It has been previously demonstrated that gene expression of prepro-orexin in LH is, like gene expression of PC1/3 in LH, unaffected by photoperiod (10, 21). Our observations of equivalent levels of PC1/3-ir and orexin-A-ir are consistent with these published studies in SD and LD. In addition, we recently demonstrated that photoperiod had no effect on the second prepro-orexin derived neuropeptide, orexin-B, as no differences of orexin-B-ir were found in LH of SD and LD acclimated hamsters (34). Combined, these observations suggest that PC1/3 does not play a major role in the seasonal regulation of post-translational neuropeptide maturation processes in the LH and hence in seasonal energy balance.

Interestingly, despite distinct gene expression of PC2 in dmpARC, immunoreactive protein was not observed and consequently the function of PC2 mRNA within these neurons remains unclear. This phenomenon could reflect rapid protein denaturation in this nucleus or the ability of neuronal cells to transport mRNA and perform protein biosynthesis in remote locations away from cell bodies (35, 36). Thus, PC2 mRNA could be transported to, and protein synthesis located in, regions and areas within the hypothalamus other than its origin in the dmpARC. Post-translational modifications of proPC2 to PC2 may be also a reason for the failure to detect PC2-ir (37). Neuronal projections from the dmpARC to other nuclei and the integration of dmpARC neurons in the hypothalamic neuroendocrine network remain to be established. There is growing evidence that the dmpARC is a functionally important component of the neuroendocrine network that regulates energy balance during seasonal adaptation; previous studies have identified a number of photoperiod regulated genes in this area (38, 39, 14). One of the identified neuropeptides within this subdivision of the ARC is

the precursor proVGF, which is cleaved by PC1/3 and PC2 into biological active energy balance regulating peptides (40, 41). In contrast to VGF, where gene expression is increased in the dmpARC in SD but decreased in the ARC, gene expression of PC2 was up regulated in both hypothalamic areas in SD. Interestingly, VGF-ir in the ARC is, like PC2-ir, also characterised by the apparent absence of protein in the dmpARC despite abundant mRNA expressed in this sub-nucleus (P. Barrett, unpublished data). Therefore PC2 might be transported away from its site of synthesis in the dmpARC very quickly to perform subsequent post-translational processing of ProVGF at a different location of the CNS. ProVGF mRNA in the dmpARC suggests a possible target of post-translational PC2 activity as mRNA levels of proVGF are also significantly increased in SD hamsters and respond promptly, like gene expression of PC2, following photoperiod manipulation.

Thus, our results demonstrate that decreasing photoperiod up-regulates PC2 gene expression and PC2 protein, whereas gene expression and protein of PC1/3 are unaffected by SD. We hypothesise that this intensifies proteolytic processing of POMC intermediate derivatives (ACTH and β-lipotropin), and is the reason for higher concentrations of POMC-derived α-MSH and β-endorphin in short photoperiod despite apparently paradoxical findings showing down-regulated POMC gene expression in SD. In contrast, unaltered PC1/3 gene expression and protein in LH and ARC suggests that regulation by photoperiod is not accomplished via proteolytic processing at PC1/3 specific sites. Thus, regulation of proteolytic processing activity by photoperiod via coordinated expression of PC1/3 and PC2 at transcriptional and translational levels is critical for the maturation of neuropeptide precursors. This photoperiod-driven regulatory mechanism could therefore be an additional universal control point for other energy balance related neuropeptide precursors such as proNPY, proTRH and CART. In addition, we provide further evidence for the dmpARC as an area with distinct photoperiod

influenced neuroendocrine activity, suggesting that this subdivision of hypothalamic arcuate neurons is an important integral part of the seasonal energy balance regulation network.

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Figure legends:

Fig.1: Diagrammatic representation of post-translational endoproteolytic POMC processing by prohormone convertases 1/3 (PC1/3) and 2 (PC2). Cleavage sites are marked by paired basic amino acids R (Lysine) and K (Arginine). Sites believed but not confirmed as being processed by PC2 are indicated by hatched triangles. (ACTH, adrenocorticotropic hormone; CLIP, corticotropin- like intermediate peptide; MET ENK, methionine enkephalin).

Fig.2: Body weight of Siberian hamsters kept in constant LD (16h light : 8h dark; dashed line) or dynamic ND photoperiod (day length depending on ambient light during winter; dotted line) over 6 months from October until April (means ± SEM, n = 6/time point). Photoperiod ranged from a minimum of 8:07h light in January to a maximum of 14:19h light in April. **, $P < 0.01$; *, $P < 0.05$, ND vs. LD.

Fig.3: Gene expression of PC1/3 and PC2 in selected areas of the Siberian hamster hypothalamus. A-D, Effect of changing photoperiod during winter on expression of PC1/3 (A, ARC; B, LH) and PC2 (C, ARC; D, dmpARC) genes. mRNA levels are expressed as mean percentages of LD controls in October (± SEM, n = 6/time point). E-H, PC1/3 (E, ARC; F, LH) and PC2 (G, ARC; H, dmpARC) gene expression after switch from SD (wk0) back to LD (wk2, wk4, wk6) photoperiod. mRNA levels are expressed as mean percentages of LD controls at wk0 (± SEM, n = 4/group). LD is indicated by dashed lines, whereas dotted curves mark ND (A-D) or SD (E-H). I, Representative autoradiographs showing gene expression of PC1/3 and PC2 in quantified areas of the hypothalamus in LD and SD animals. Significances ($P < 0.05$) are marked by asterisks (*) for same time points but different photoperiods and

crosses (+) for same photoperiod (ND or SD, respectively) but different time points. Scale bars, 100 µm (a-d) and 80 µm (e-h).

Fig.4: A, Quantitative analysis of immunoreactive (-ir) cells in selected areas of the Siberian hamster hypothalamus. Data are number of ir-cells expressed as % of LD controls (**, $P < 0.01$, n = 3/group). B, Representative photomicrographs showing neuroanatomical distribution of immunoreactive cells in comparable hypothalamic areas of LD and SD animals. Colour inverted images of immunofluorescence stained sections (a-f, i-p) or peroxidase / substrate stained sections (g-h). 3V, third ventricle; ARC, arcuate nucleus; LH, lateral hypothalamus. Scale bars, 100 µm (a-l) and 180 µm (m-p).

Fig.5: A-D**,** Photomicrographs showing immunofluorescence double staining of PC1/3-ir or PC2-ir with POMC-ir and its derived neuropeptides in the arcuate nucleus and E, PC1/3-ir and orexin A-ir double staining in the lateral hypothalamus. Images derive from SD hamsters. For each panel the upper row shows low magnification images and the lower row high magnification images of selected (boxed) areas. Merged images (centre) demonstrate colocalisation of immunoreactive products. Colocalisation is indicated by solid arrows, single cell-ir by dashed arrows. F, Quantitative colocalisation analysis of prohormone convertase-ir (PC1/3 or PC2) and neuropeptide-ir. Values are expressed as mean percentages (± SEM) of counted POMC-ir, α-MSH-ir, β-endorphin-ir and orexin A-ir cells in LD and SD (n = 3/group). 3V, third ventricle; ARC, arcuate nucleus; LH, lateral hypothalamus; opt, optical tract. Scale bars, low magnification images, 100 µm; high magnification images, 40 µm.

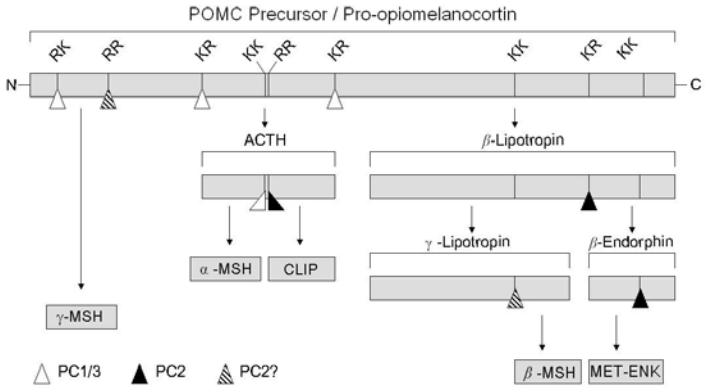
POMC Precursor / Pro-opiomelanocortin
RK
RR
KR
KK
RR
KR
KK
KR
KK
N
C
ACTH
β-Lipotropin
α -MSH
CLIP
γ -Lipotropin
β-Endorphin
γ-MSH
β -MSH
MET-ENK
PC1/3
PC2
PC2?

FIG.1

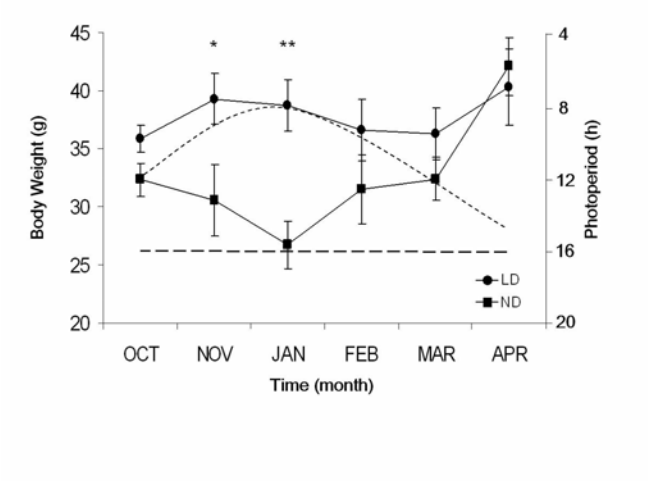
45
40
35
30
25
20
4
8
12
16
20
*
**
Body Weight (g)
Photoperiod (h)
LD
ND
OCT
NOV
JAN
FEB
MAR
APR
Time (month)

FIG.2

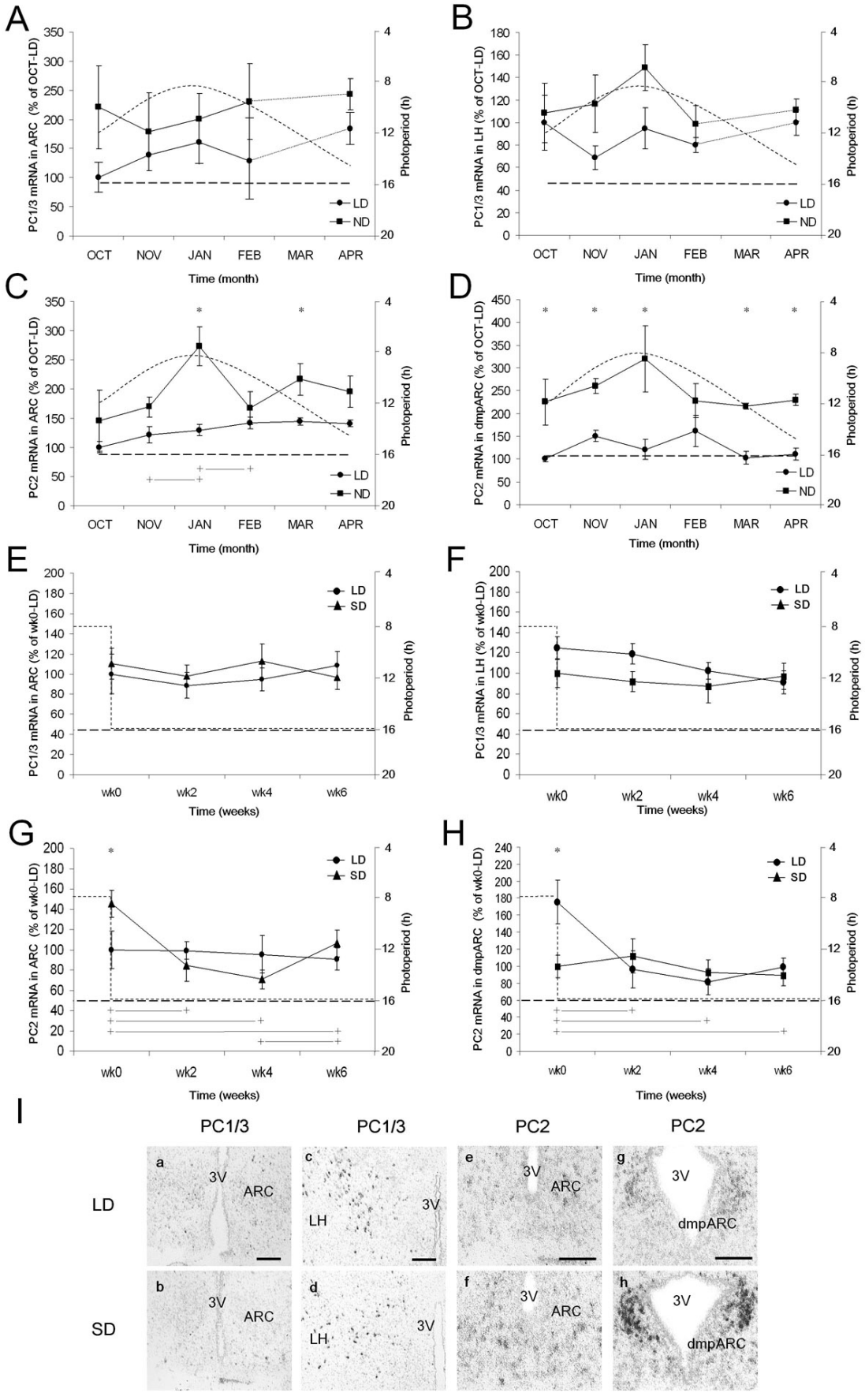
A
PC1/3 mRNA in ARC (% of OCT-LD)
Photoperiod (h)
LD
ND
OCT NOV JAN FEB MAR APR
Time (month)
B
PC1/3 mRNA in LH (% of OCT-LD)
C
PC2 mRNA in ARC (% of OCT-LD)
D
PC2 mRNA in dmpARC (% of OCT-LD)
E
PC1/3 mRNA in ARC (% of wk0-LD)
SD
wk0 wk2 wk4 wk6
Time (weeks)
F
PC1/3 mRNA in LH (% of wk0-LD)
G
PC2 mRNA in ARC (% of wk0-LD)
H
PC2 mRNA in dmpARC (% of wk0-LD)
I
PC1/3
PC1/3
PC2
PC2
LD
SD
a
b
c
d
e
f
g
h
3V
ARC
LH
dmpARC

FIG.3

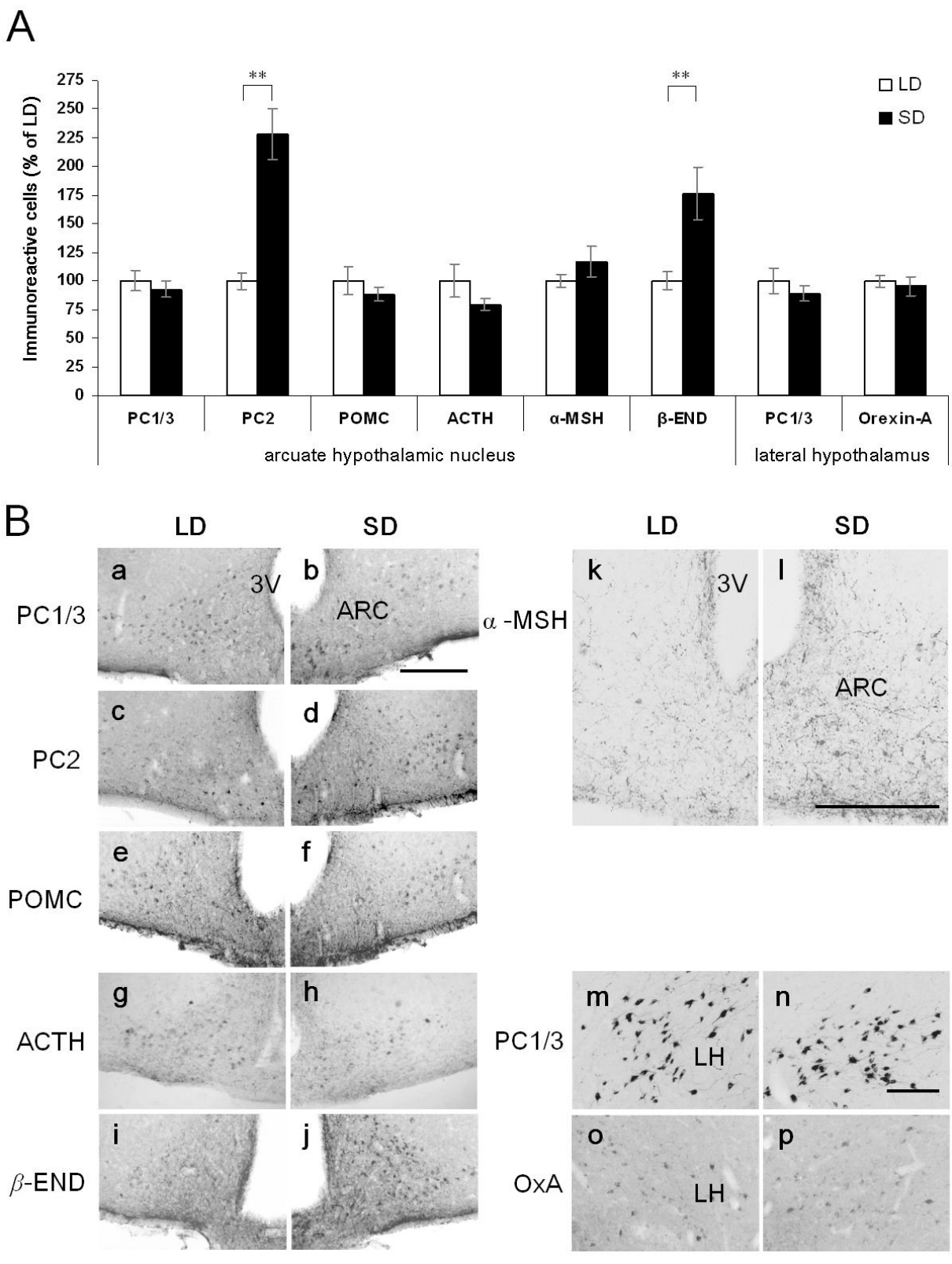
A
Immunoreactive cells (% of LD)
275
250
225
200
175
150
125
100
75
50
25
0
**
**
LD
SD
PC1/3
PC2
POMC
ACTH
α-MSH
β-END
PC1/3
Orexin-A
arcuate hypothalamic nucleus
lateral hypothalamus
B
LD
SD
LD
SD
a
3V
b
ARC
PC1/3
α -MSH
k
3V
l
ARC
c
d
PC2
e
f
POMC
g
h
ACTH
PC1/3
m
LH
n
i
j
β-END
OxA
o
LH
p

FIG.4

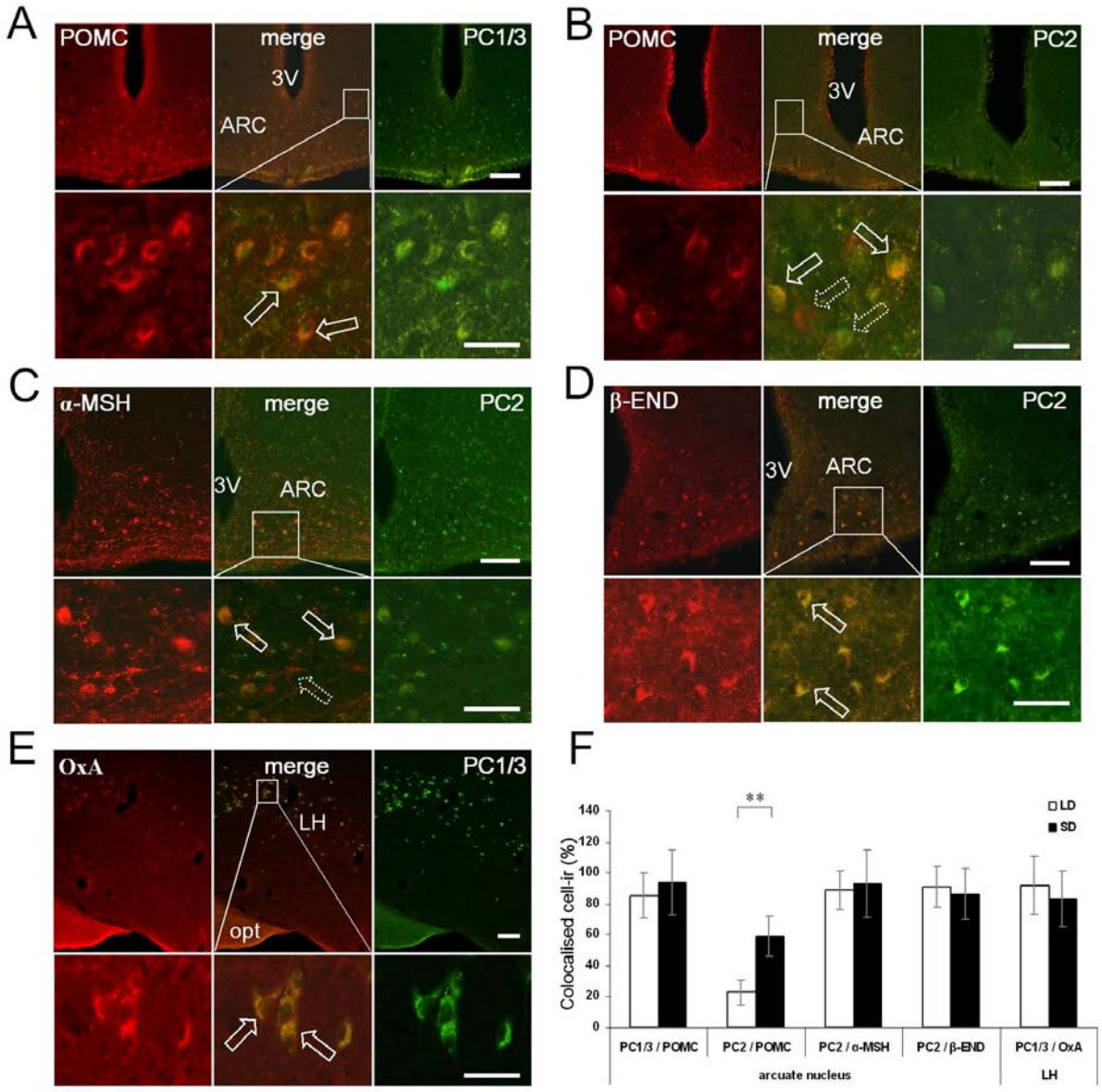
A
POMC
merge
PC1/3
3V
ARC
B
POMC
merge
PC2
3V
ARC
C
α-MSH
merge
PC2
3V
ARC
D
β-END
merge
PC2
3V
ARC
E
OxA
merge
PC1/3
LH
opt
F
**
LD
SD
Colocalised cell-ir (%)
140
120
100
80
60
40
20
0
PC1/3 / POMC
PC2 / POMC
PC2 / α-MSH
PC2 / β-END
PC1/3 / OxA
arcuate nucleus
LH

FIG.5

Zusammenfassung

Die vorliegende Dissertation befasst sich mit der Charakterisierung neuroendokriner Signalwege der saisonalen Körpergewichtsregulation des Dsungarischen Zwerghamsters (*Phodopus sungorus*). Sie untersucht eingehend die zentrale Verarbeitung der „Adipositassignale“ Leptin und Insulin (beide inhibieren die Nahrungsaufnahme) und des die Nahrungsaufnahme stimulierenden Hormons Ghrelin im Hypothalamus, der als neuronales Zentrum der Gewichtsregulation fungiert. Ein weiteres Ziel dieser Arbeit war die Identifizierung der molekularen Grundlage des Phänomens der Leptinresistenz, einem Schlüssereignis bei der Ausbildung von Adipositas, sowie die Charakterisierung einer möglichen Konvergenz zentraler Leptin- und Insulinsignalwege.

Die hypothalamische Signalverarbeitung dieser beiden Hormone war deutlich saisonal reguliert, welches eine zentrale Bedeutung dieser humoralen Signale für die jahreszeitlichen Veränderungen des Körpergewichts impliziert. Die molekulare Grundlage saisonal induzierter Leptinresistenz konnte weitgehend aufgeklärt werden: Sie wird durch eine Modulation der Signaltransduktionskaskade distal des Leptinrezeptors hervorgerufen. Darüber hinaus widersprechen die Ergebnisse der vorliegenden Arbeit der gängigen Meinung möglicher synergistischer Effekte der anorexigenen Hormone Leptin und Insulin in Bezug auf ihre hypothalamische Signalverarbeitung.

Des weiteren konnte gezeigt werden, dass Ghrelin und seine zentrale Verarbeitung über den Ghrelinrezeptor höchstwahrscheinlich eine zentrale Rolle bei der akuten Regulation der Nahrungsaufnahme spielt während es chronische Veränderungen der Energiehomöostase (saisonale Gewichtszyklen) nicht beeinflusst.

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April 2002	Diploma of Biology: equivalent to Master of Science	

07/2002 – 12/2002 Scholar of the Marie Curie PhD training site (funded by the European Union) at the Rowett Research Institute, Scotland, Division for Energy Balance and Obesity

since 01/2003 Scholar of the Boehringer Ingelheim Fonds funding the PhD at the Philipps University of Marburg, Department of Animal Physiology under supervision of Dr. Martin Klingenspor and at the Rowett Research Institute, Scotland, Division for Energy Balance and Obesity under supervision of Dr. Julian Mercer.

Relevant Work Experience

07/99 - 09/1999 Bayer AG Leverkusen, Germany
Student trainee in the department of animal healthcare:
Establishment of a new molecular method to detect infections of cattle with nematodes.

02/00 - 03/2000 Bayer AG Leverkusen, Germany
07/00 - 09/2000
Students job in the department of metabolism research:
Survey about metabolism of new substances in plant-care research

Topic of Diploma Thesis

"Regulation of the expression of factors involved in the intracellular signalling cascade distal of the leptin receptor in *Phodopus sungorus*"

Original Publications

- "Circulating ghrelin levels and central ghrelin receptor expression are elevated in response *to* food deprivation in the seasonal hamster (*Phodopus sungorus*)" **Tups A**., Helwig M., Khorooshi MH., Archer ZA., Klingenspor M. and Mercer G; J Neuroendocrinol. 2004 Nov;16(11):922-8. (b)

- "Photoperiodic regulation of leptin sensitivity in the Siberian hamster, *Phodopus sungorus*, is reflected in arcuate nucleus SOCS-3 (suppressor of cytokine signaling) gene expression." **Tups A**, Ellis C, Moar KM, Logie TJ, Adam CL, Mercer JG, Klingenspor M; Endocrinology. 2004 Mar;145(3):1185-93. Epub 2003 Nov 26. (a)

Reviews

- "Neuropeptides and anticipatory changes in behaviour and physiology: seasonal body weight regulation in the Siberian hamster." Mercer JG, **Tups A**; Eur J Pharmacol. 2003 Nov 7;480(1-3):43-50.

- "The leptin resistance paradox: molecular dissection of leptin signalling pathways in the central nervous system." **Tups A**, B.I.F. Futura Vol. 18 (2003) No. 2

Manuscripts in Preparation

- "The suppressor of cytokine signalling, SOCS3, plays an essential role in the regulation of seasonal body weight in the Siberian hamster, *Phodopus sungorus"* **Tups A,** Barrett P, Ross AW, Morgan PJ, Klingenspor M, Mercer JG

- "Photoperiodic regulation of insulin receptor mRNA and intracellular insulin signalling in the arcuate nucleus of the Siberian hamster, *Phodopus s. sungorus"* **Tups A**, Helwig M, Stöhr S, Mercer JG and Klingenspor M

- "The JAK-STAT and ERK signalling pathways and their implication in seasonal body weight regulation in the Siberian hamster, *Phodopus sungorus"* **Tups A**, Stöhr S, Krol E, Barrett P, Mercer JG, Klingenspor M

- "PC1/3 and PC2 gene expression and post translational endoproteolytic POMC processing is regulated by photoperiod in the seasonal Siberian hamster (*Phodopus sungorus*)."
  Helwig M, Khorooshi RMH., **Tups** A, Barrett P, Braulke LJ, Mercer JG, Klingenspor M

Meetings (Attendance and/or active participation)

- 9th Conference of the European Pineal and Biological Rhythms Society, Aberdeen United Kingdom, 19.07-22.07.02

- The 5th International Congress of Neuroendocrinology, Bristol United Kingdom, 31.08.- 04.09.02.

- Disorders of Body Weight Regulation – Clinical Aspects and Identification of Novel Drug Targets Partnering Day, 29.01.-31.01.2003, Marburg, Germany.

- High Level Scientific Conference "Molecular Mechanisms in Metabolic Diseases: Obesity, Diabetes Type 2, Lipid disorders and Atherosclerosis – Basic Science towards Clinical Application", 10. –12.07.03 at Reisensburg Castle, Germany.
  Poster and Oral presentation: "SOCS3 as a potential modulator of leptin sensitivity: lessons from a seasonal mammal." **Tups A**

- 7th Annual Meeting of the Neuroendocrinolgy Section of the German Society of Endocrinology (DGE), 17.10.-18.10.03, Lübeck Germany.

Poster: "SOCS3 as a potential modulator of leptin sensitivity: lessons from as seasonal mammal" **Tups A**, Klingenspor M, Mercer JG

- Meeting of the British Society for Neuroendocrinology, Sep. 2003, Manchester United Kingdom.
  Poster and Oral presentation: "SOCS3 as a potential modulator of leptin sensitivity: lessons from a seasonal mammal." **Tups A**, Klingenspor M, Mercer JG (Poster Prize, see awards)

- 12th International Congress of Endocrinology, Lisbon Portugal, 31.08-04.09.04
  Poster: "Circulating ghrelin levels and central ghrelin receptor expression is elevated in response to food deprivation in the seasonal hamster *(Phodopus sungorus)"* **Tups A**, Helwig M, Khorooshi M, Archer Z, Klingenspor M, Mercer JG

- Neuroscience 2004 Annual Meeting, San Diego United States, 23.10-28.10.04
  Poster: "The suppressor of cytokine signalling, SOCS3, plays an essential role in the regulation of seasonal body weight in the Siberian hamster, *Phodopus sungorus"* **Tups A,** Barrett P, Ross AW, Morgan PJ, Mercer JG and Klingenspor M

- **Annual oral presentations** on the symposium of the Aberdeen Centre for Energy Regulation and Obesity (ACERO) in Nov. 2002 and 2003, Aberdeen United Kingdom

International research visits

May 2005: Oral presentations at neuroendocrine seminars at the University of Otago, Dunedin, New Zealand and at the Vollum Institute, Portland, Oregon.

Awards

Awarded for a promotion program for talented young scientists including several training courses at the Bayer AG (October 1999).

- Awarded for a Marie Curie PhD training site scholarship of the European Union (July 2002).

- Awarded for a scholarship of the Boehringer Ingelheim Fonds (January 2003).

- Poster Prize for the best student presentation at the British Society for Neuroendocrinology Meeting in November 2003

Society Membership

Member of the International Society for Neuroscience, Washington DC United States

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Erklärung

Ich versichere, dass ich meine Dissertation

Molecular and Neuroendocrine Determinants of Seasonal Body Weight Regulation

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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(Ort/Datum) (Unterschrift mit Vor- und Zuname)