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

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Abstract

Djungarian hamsters are able to reduce their body weight by more than 30% in anticipation of the winter season. This particular adaptation to extreme environmental conditions is primarily driven by a natural reduction in day length and conserved under laboratory conditions. We used this animal model to investigate hypothalamic gene expression linked to body weight regulation behind this physiological phenomenon. After an initial collective short photoperiod (SP) adaptation for 14 weeks from a preceding long photoperiod (LP), hamsters were re-exposed to LP for either six or 14 weeks, followed by a second re-exposure to SP for eight weeks. Our data showed that re-exposure to LP led to an increase in body weight. In the hypothalamus *Dio2*, *Vimentin*, *Crbp1* and *Grp50* expression increased, but expression of *Dio3, Mct8* and *Srif* decreased. The changes in body weight and gene expression were reversible in most hamsters after a further re-exposure to SP following six or 14 weeks in LP. Interestingly, body weight loss was pronounced in six hamsters re-exposed to SP after 14 weeks in LP, while five hamsters did not respond to SP. In non-responding hamsters, a different gene expression pattern was manifested, with the exception of *Dio2* which was reduced not only in SP re-exposed hamsters but also hamsters maintained in LP. Together these data suggest that body weight regulation seems to be tightly linked to a co-ordinated regulation of several genes in the hypothalamus including those involved in thyroid hormone metabolism.

Introduction

Seasonal mammals show a wide range of adaptations to short photoperiod in anticipation of cold winter seasons and reduced food availability. Together with quiescence of reproduction, improved winter fur and optimized thermoregulation, body weight regulation is a very important component to survive winter (1, 2). Djungarian hamsters (*Phodopus sungorus*), also known as Siberian hamsters, have been used intensively as an animal model for long-term changes in energy balance, because of their particular annual body weight cycle. They reduce body weight well in advance of the winter season and increase body weight ahead of the next reproductive period in spring (3, 4). These changes are primarily driven by seasonal changes in photoperiod and can be easily induced under laboratory conditions by transferring hamsters between summer-like long photoperiod (LP) or winter-like short photoperiod (SP) (5, 6). Internal information about day length is provided by the duration of nocturnal pineal melatonin secretion (7). Over the last decade several genes have been discovered in the hypothalamus that are potentially involved in seasonal adaptations, but their

specific roles are not well understood. Much of our knowledge of genes involved in photoperiod adaptation has come from transfer of animals between static photoperiods representing LP or SP. Commonly this has been confined to a LP to a SP transition and occasionally a further transition from SP to LP. Hamsters continuously kept in SP for more than 20 weeks become insensitive to the SP signal and gradually return to their fully functional LP phenotype including body weight gain and reproductive recrudescence (8-11). This innate long-term-timing process is referred to as the photorefractory response which in nature ensures a timely start of the next reproductive season in spring. This phenomenon has also been used to investigate hypothalamic gene expression in relation to physiological adaptation (8, 12, 13).

The hypothalamus is the centre for energy homeostasis and neuroendocrine regulator of many physiological processes and is thought to be the region of the brain which integrates altered thyroid hormone availability in response to change in day length. TSH-receptor (TSH-r) expressing tanycytes, a glial cell type in the ependymal layer of the third ventricle, is a key component in the mechanism underpinning responsiveness to day length. Tanycytes operate at the interface between the cerebrospinal fluid (CSF) and neurons of the hypothalamus (14, 15) and change in structure (vimentin) as well as function in response to seasonal change in photoperiod (16, 17).

The contemporary view for the involvement of tanycytes in the mechanism of physiological adaptation centres around responding to thyrotropin (TSH) produced by melatonin responsive cells in the *pars tuberalis* (PT) (18). In LP production of TSH in the PT is high, which leads to an increased deiodinase type 2 (DIO2) expression in tanycytes (18, 19). DIO2 is an enzyme involved in thyroid hormone metabolism and there is a large body of evidence to show that thyroid hormone availability specifically in the hypothalamus underpins the mechanism driving seasonal adaptations (8, 18-21). Thyroid hormone availability in the hypothalamus depends on thyroid hormone transport by specific transporters e.g. monocarboxylate transporter 8 (MCT8), as well as the activity of deiodinase enzymes. Whereas DIO2 converts thyroxine by phenolic ring deiodination (T_4) to biologically active 3,3',5-triiodothyronine (T_3), type 3 deiodinase (DIO3) acts as counterpart and inactivates T3 by tyrosyl ring deiodination to 3,3'-diiodothyronine (T_2) (22-24). Both, transporter and enzymes are expressed in tanycytes in Djungarian hamsters and are regulated in a photoperiod dependent manner, *Dio2* being high in LP and low in SP and *Dio3* absent in LP, but transiently expressed on exposure to SP. Several studies have suggested that a low T_3 concentration in the hypothalamus, generated by low *Dio2* expression and high *Dio3* expression, is required for body mass reduction (8, 20, 21, 25).

In addition to genes involved in the thyroid hormone signalling pathway, several other hypothalamic genes are regulated by photoperiod and have been linked to seasonal adaptations (26-28). Cellular retinol-binding protein 1 (CRBP1), that is expressed in tanycytes, is a transport protein for retinol (vitamin A) and involved in the pathway for synthesizing retinoic acid (29). Another protein expressed in the ependymal layer is GPR50, an orphan G-protein-coupled receptor, which despite its homology to melatonin receptors, does not bind melatonin and has been suggested to play a role in sensing components of metabolic homeostasis in the CSF (30, 31). Somatostatin (SRIF) is produced in the arcuate nucleus (ARC) and periventricular nucleus of the hypothalamus and plays a role in the regulation of the growth axis, for which there is evidence that this axis contributes to the seasonal body weight cycle of Djungarian hamsters (8, 32-34).

The aim of this study was to develop a better understanding of photo-responsive genes by investigating their transcriptional plasticity to multiple directional changes between photoperiods. Hamsters adapted to a static SP for more than ten weeks, but then re-exposed to LP have shown that physiological adaptations revert to LP phenotype within a period of six weeks (33, 35). Prior to the onset of changes in physiology the expression of most genes revert back to LP expression levels, with *Dio2* expressed to a greater level than in hamsters continuously kept in LP (8, 12).

From a life history perspective, at least in the laboratory Djungarian hamsters may survive two winter seasons, particularly those born after the summer solstice which adapt to SP physiology soon after birth (9, 34, 36). Therefore the potential for survival for more than one winter season, whether in a laboratory situation or their natural environment, needs to be reflected in an ability to respond to seasonal photoperiod cues to maximize the chances of survival and provide for continuation of the species. Although hypothalamic gene expression studies have been performed on LP to SP transition and a subsequent SP to LP transition, there is no further information on the plasticity of *Dio2* and other critical components of the hypothalamic thyroid hormone pathway in response to a further directional change back to SP following LP exposure. Previously it has been found that responsiveness to melatonin or SP in photorefractory hamsters is only re-established after a return to LP for a minimum of ten weeks in LP (37-39). Based on this response, we hypothesised that a multiple transition model may provide additional insight into the plasticity of hypothalamic gene expression and physiological adaptions. In particular, hamsters transferred from LP to SP for a second time may not be responsive to SP after a short adaptation to LP. Investigating the responsiveness to multiple photoperiod transitions may help in understanding the plasticity of the

hypothalamic thyroid hormone signalling and the importance of neuroendocrine pathways following photorefractory physiological recrudescence.

Material and Methods

Animals and experimental procedure

All experiments and procedures were approved by the local animal welfare authorities (Hamburg, Germany). Fifty-two Djungarian hamsters (*Phodopus sungorus*) of both sexes were bred and raised under artificial long photoperiod (LP; 16 h light : 8 h dark). After weaning, hamsters were singly housed in Makrolon type III cages with food and water *ad libitum* at an ambient temperature (T_a) of 21±1 °C. The experimental protocol is outlined in Figure 1. All animals were culled between Zeitgeber time (ZT) 4-5 in their respective photoperiods. At an age of 3-4 month (=week 0) a cohort of six hamsters (LP_0) was euthanized by CO_2 inhalation and decapitation under LP conditions. The remaining 46 adult hamsters were transferred to an artificial short photoperiod (SP, 8 h light : 16 h dark). Hamsters were weighed once a week. After 14 weeks in SP a group of six hamsters (SP_{14}) was sacrificed and 40 hamsters were transferred back to LP. After six weeks in LP a further cohort of six hamsters ($SP_{14}LP_6$) was killed. At this time point a group of six hamsters was switched back to SP for a second time and culled after eight weeks under SP conditions ($SP_{14}LP_6SP_8$). Six hamsters kept under LP for 14 weeks ($SP_{14}LP_{14}$) after the first switch from SP were culled together with the $SP_{14}LP_6SP_8$ group. The remaining 22 hamsters were split into two groups. Eleven hamsters stayed in LP for another eight weeks ($SP_{14}LP_{22}$) and eleven hamsters were switched back to SP for also eight weeks ($SP_{14}LP_{14}SP_8$). Those hamsters were sacrificed at the end of the experiment. Blood of all hamsters was collected, serum extracted and total T_4 (tT_4) and total T_3 (tT_3) serum concentrations were analysed by radio-immuno assays (RIA) as described before (40). The brains were removed, immediately frozen on dry ice and stored at -80°C until required for *in situ* hybridization.

Radioactive in situ hybridisation

In situ hybridization was used to quantify expression of *Dio2, Dio3, Mct8, Tsh-r, Crbp1, Gpr50* and *Vimentin* mRNA along the third ventricle of the hypothalamus and quantification of *Srif* was restricted to the ARC region within the hypothalamus. The hypothalamic region of interest was between bregma -1.70 and -2.54 mm according to the Mouse Brain Atlas of Franklin & Paxinos (3rd ed., 2008). Frozen brains were cut with a cryostat into 16µm coronal sections, mounted onto polysine-coated slides and stored at -80°C until needed.

For *in situ* hybridization, brain sections were fixed in 4% paraformaldehyde, washed with 0.1 M PBS, incubated in 0.1M triethanolamine (pH 8) and acetylated with 0.25% acetic anhydride. Slides were washed again with 0.1 M PBS and subsequently dehydrated using an ascending ethanol series followed by vacuum drying. Riboprobes were synthesized as previously described from DNA fragments for *Dio2, Dio3, Mct8, Tsh-r, Crbp1, Gpr50*, *Vimentin* and *Srif*, using ^{35}S-UTP with SP6 or T7 polymerases as appropriate (8, 21, 25, 26, 28, 41). 70µl hybridization mixture (formamide, 0.3 M NaCl, 10 mM Tris-HCL (pH 8), 1 mM EDTA, 0.05% transfer RNA, 10 mM dithiothreitol,0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, and 10% dextran sulphate) containing the appropriate radioactive probes (ca. 10^6 cpm) was applied per glass slide and sealed with DPX Mountant. Hybridization was carried out over night at 58°C. After hybridization, slides were washed in 4x SSC (Saline-Sodium Citrate), incubated with RNase A solution at 37°C for 30 minutes and washed in SSC solutions with decreasing concentrations (2x to 0.1x). Finally slides were dehydrated using an ascending ethanol series and air dried before exposed to Kodak BioMax MR Films (Sigma-Aldrich Company Ltd., Poole, Dorset, UK). Autoradiographic films were developed after 18-20h (*Srif, Vimentin*), 5 days (*Mct8*), 6 days (*Gpr50*), 7 days (*Crbp1, Tsh-r*) or 14 days (*Dio2, Dio3*). Autoradiographic films were scanned at 300 dpi and analysed using ImageJ 1.47v software. Integrated optical density (IOD) was obtained in two to three consecutive sections per animal by reference to a standard curve [y=a+b*ln(x-c)] generated from a ^{14}C microscale, was measured. Values were averaged for each animal. Relative gene expression was calculated by defining SP_{14} as 100% for *Dio2, Dio3, Mct8* and *Srif* and LP_0 as 100% for *Crbp1, Tsh-r, Gpr50* and *Vimentin.*

Statistical analysis

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

Results

Body weight

Hamsters started with an initial average body weight of 34.3 ± 4.8 g. During 14 weeks in SP the six groups of hamsters which were transferred to SP showed a body weight reduction of -8.8 ± 4.7 g that reached significance from week 6 (Figure 2A, B, ANOVA p<0.05). The five groups switched back

to LP increased their body weight by 7.1 ± 3.3g after six weeks. The increase reached significance four weeks after the switch to LP (ANOVA $p<0.05$). The groups which experienced extended LP reached a body weight plateau after 12 weeks in LP and had increased their body weight after 14 weeks by 18.1 ± 6.1 g ($SP_{14}LP_{14}$) and by 18.9 ± 5.9 g after 22 weeks ($SP_{14}LP_{22}$).

Hamsters switched back to SP for a second time after six weeks in LP ($SP_{14}LP_6SP_8$) stopped increasing body weight (-2.8 ± 2.07, ANOVA $p=0.11$) and had a significantly lower body weight compared to the corresponding age matched $SP_{14}LP_{14}$ group (ANOVA $p<0.05$). However the range of responsiveness to the second SP varied from no weight loss to 21.6% in one hamster, which is in contrast to weight loss after the first exposure to SP where all $SP_{14}LP_6SP_8$ animals lost 12% or more during eight weeks of SP exposure (Figure 2A, Supplementary Figure 1B).

Of the hamsters re-exposed to SP after 14 weeks in LP ($SP_{14}LP_{14}SP_8$), there was an apparent division in the body weight responsiveness of some hamsters compared to others. We therefore applied a criteria of SP responsiveness to this group which was a body weight loss of 10% or greater from peak value by seven weeks into SP (Figure 4A). This criterion would exclude the classification of hamsters as responsive due to natural variability in body hamster weight which would include variability in food stored in cheek pouches. After the initial exposure to SP from LP, all hamsters in this $SP_{14}LP_{14}SP_8$ group had a body weight loss of 14% or more within seven weeks and continued to lose body weight with an average weight loss of 26.2% ± 6.6% after 14 weeks SP exposure and were therefore clearly all photoresponsive hamsters in the first transition from LP to SP. However in a second re-exposure to SP, six hamsters reduced their body weight by 6.5 ± 4.4 g (15.1 % ± 3.1%, ANOVA $p<0.05$), whereas five hamsters on average did not lose body weight within eight weeks of SP exposure (Figure 4A, -0.7 ±1.3 g, -2.7% ± 2.5%,) with no significant difference between the non-responding hamsters and the corresponding age matched $SP_{14}LP_{22}$ group.

Serum thyroid hormone concentrations

No significant differences in serum thyroid hormone levels were found between the groups (Table 1). Hamsters kept at LP for six weeks after switched back from SP ($SP_{14}LP_6$) showed a trend towards an increase in tT_4 compared to hamsters kept in SP for 14 weeks ($SP_{14}LP_{14}$ vs $SP_{14}LP_6$, t-test $p=0.057$). Serum thyroid hormone concentrations within the group $SP_{14}LP_{14}SP_8$ revealed no differences between hamsters which lost body weight and stable body weight. The $SP_{14}LP_{14}SP_8$ group showed a statistical trend towards higher tT_4 concentrations compared to the corresponding $SP_{14}LP_{22}$ group (t-test $p= 0.077$).

Hypothalamic gene expression

Dio2 expression was lower in hamsters that had experienced only LP as compared to SP_{14} (LP_0 vs SP_{14}, 61 ± 11 %, t-test p=0.03, Figure 3A). After re-exposure from SP to LP *Dio2* expression was increased by more than 6-fold after six weeks ($SP_{14}LP_6$ vs SP_{14}, 630 ± 81%, U-test p=0.002), before it declined in the following eight weeks, but with still elevated mRNA concentrations after 14 weeks in LP ($SP_{14}LP_{14}$ vs SP_{14}, 208 ± 17%, t-test p<0.001). *Dio2* expression was back to original LP_0 mRNA levels and below SP_{14} values after 22 weeks in LP ($SP_{14}LP_{22}$ vs SP_{14}, 44 ± 3%, t-test p<0.001). Hamsters transferred to SP for a second time for eight weeks after six weeks in LP ($SP_{14}LP_6SP_8$) had lower *Dio2* levels compared to SP_{14} ($SP_{14}LP_6SP_8$ vs SP_{14}, 56 ± 5%, t-test p=0.002). Lower *Dio2* expression than the SP_{14} cohort was also observed in hamsters re-exposed to SP for eight weeks after 14 weeks in LP ($SP_{14}LP_{14}SP_8$), but only in hamsters that had reduced their body weight ($SP_{14}LP_{14}SP_8$ body weight loss vs SP_{14}, 50 ± 8%, t-test p=0.003). Hamsters of $SP_{14}LP_{14}SP_8$ that did not reduce body weight on SP re-exposure, showed *Dio2* expression similar to SP_{14} ($SP_{14}LP_{14}SP_8$ constant body weight vs SP_{14}, 73 ± 13%, t-test p=0.17, Figure 3A, 4B). Although marginally higher, *Dio2* expression in hamsters that did not lose body weight was not statistically different from body weight responders ($SP_{14}LP_{14}SP_8$ body weight loss vs $SP_{14}LP_{14}SP_8$ no body weight loss, 68 ± 11%, t-test p=0.2, Figure 3A, 4B).

Deiodinase 3 (Dio3)

Dio3 was not expressed in LP, but at different levels in all hamsters culled during SP (Figure 3B). The $SP_{14}LP_6SP_8$ group was not significantly different from SP_{14} ($SP_{14}LP_6SP_8$ vs SP_{14}, 54 ± 28%, t-test p=0.184). This is due to the large variability in this group. Four hamsters showed no *Dio3* expression comparable to LP animals, whereas two hamsters showed increased gene expression comparable to SP_{14} values. In the $SP_{14}LP_{14}SP_8$ group all hamsters with reduced body weight had increased *Dio3* expression ($SP_{14}LP_{14}SP_8$ body weight loss vs SP_{14}, 184 ± 30%, t-test p=0.04, Figure 3B, 4B), and *Dio3* was only partially induced in hamsters with constant body weight ($SP_{14}LP_{14}SP_8$ constant body weight vs SP_{14}, 46 ± 44%, U-test p=0.13, Figure 3B, 4B). Only one hamster showed increased *Dio3* expression, whereas the other four animals had very low *Dio3* mRNA concentrations (Figure 4B). Within the $SP_{14}LP_{14}SP_8$ group *Dio3* expression was significantly different between hamsters losing body weight and those maintaining constant body weight ($SP_{14}LP_{14}SP_8$ constant body weight vs $SP_{14}LP_{14}SP_8$ body weight loss, 25 ± 21%, U-test p=0.03).

Monocarboxylate transporter 8 (Mct8)

Mct8 expression (Figure 5A) did not differ after 14 weeks in SP (SP_{14}) from LP_0 (LP_0 vs SP_{14}, 87 ± 8%, t-test p=0.22). After re-exposure from SP to LP, *Mct8* expression was reduced in all LP groups. Six weeks after re-exposure ($SP_{14}LP_6$ vs SP_{14}, 43 ± 5%, t-test p<0.001), 14 weeks after re-exposure ($SP_{14}LP_{14}$ vs SP_{14}, 67 ± 9%, t-test p=0.009) and 22 weeks after re-exposure ($SP_{14}LP_{22}$ vs SP_{14}, 51 ± 12%, t-test p<0.001) *Mct8* expression was significantly lower. Hamsters of $SP_{14}LP_6SP_8$ had higher *Mct8* expression as compared to the parallel $SP_{14}LP_{14}$ group ($SP_{14}LP_{14}$ vs $SP_{14}LP_6SP_8$, 177 ± 15%, t-test p=0.003) which was similar to the SP_{14} group ($SP_{14}LP_6SP_8$ vs SP_{14}, 119 ± 10%, t-test p=0.19). The $SP_{14}LP_{14}SP_8$ group showed an interesting pattern. In hamsters with body weight loss, gene expression of *Mct8* was higher than in SP_{14} animals ($SP_{14}LP_{14}SP_8$ body weight loss vs SP_{14}, 129 ± 10%, t-test p=0.03) and their parallel $SP_{14}LP_{22}$ group ($SP_{14}LP_{14}SP_8$ body weight loss vs $SP_{14}LP_{22}$, 251 ± 20% t-test p<0.001). However, hamsters of $SP_{14}LP_{14}SP_8$ maintaining constant body weight had unchanged *Mct8* expression compared to SP_{14} ($SP_{14}LP_{14}SP_8$ constant body weight vs SP_{14}, 86 ± 19%, U-test p=0.6), but expression was lower compared to the hamsters with lost body weight within the $SP_{14}LP_{14}SP_8$ group ($SP_{14}LP_{14}SP_8$ constant body weight vs $SP_{14}LP_{14}SP_8$ body weight loss, 66 ± 12 %, t-test p=0.04).

Thyrotropin receptor (Tsh-r)

In situ hybridization revealed no generalised response caused by directional changes in photoperiod of *Tsh-r* expression (data not shown). The only significant difference (t-test p=0.01) appeared between $SP_{14}LP_{14}$ (116 ± 7%) and $SP_{14}LP_6SP_8$ (85 ± 7%).

Vimentin

Vimentin mRNA expression (Figure 5B) was decreased after 14 weeks in SP (SP_{14} vs LP_0, 37 ± 2%, t-test p<0.001). Six weeks after re-exposure from SP_{14} to LP ($SP_{14}SP_6$) *Vimentin* expression was increased ($SP_{14}LP_6$ vs SP_{14}, 203 ± 13%, U-test p=0.002) and continued to increase reaching 100 ± 6 % of original LP values after eight more weeks in LP ($SP_{14}LP_{14}$ vs SP_{14}, 269 ± 15%, U-test p=0.002). Hamsters from group $SP_{14}LP_6SP_8$ ($SP_{14}LP_6SP_8$ vs $SP_{14}LP_{14}$, 50 ± 7%, t-test p=0.002) and $SP_{14}LP_{14}SP_8$ hamsters with reduced body weight ($SP_{14}LP_{14}SP_8$ body weight loss vs $SP_{14}LP_{22}$, 60 ± 14%, t-test p=0.03) had lower *Vimentin* expression as compared to their parallel control LP groups. $SP_{14}LP_{14}SP_8$ hamsters maintaining constant body weight, had levels comparable to their LP_{22} control animals ($SP_{14}LP_{14}SP_8$ constant body weight vs $SP_{14}LP_{22}$, 105 ± 15%, t-test p=0.8). *Vimentin* expression was significantly lower in hamsters losing body weight than in hamsters maintaining constant body weight ($SP_{14}LP_{14}SP_8$ body weight loss vs $SP_{14}LP_{14}SP_8$ constant body weight, 58 ± 7%, t-test p=0.003)

G protein-coupled receptor 50 (Gpr50)

Abundance of *Gpr50* mRNA (Figure 5C) showed clear differences between hamsters kept at LP or SP. After 14 weeks in SP (SP_{14}) *Gpr50* expression was significantly reduced (SP_{14} vs LP_0, 22 ± 1%, U-test p=0.002). Expression was partially restored six weeks after re-exposure to LP ($SP_{14}LP_6$ vs LP_0, 47 ± 6%, t-test p=0.001) and regained original LP_0 expression after 14 weeks in LP ($SP_{14}LP_{14}$ vs LP_0, 103 ± 9%). Hamsters re-exposed to SP for eight weeks after six weeks in LP ($SP_{14}LP_6SP_8$ vs $SP_{14}LP_{14}$, 33 ± 2%, U-test p=0.002) or 14 weeks in LP ($SP_{14}LP_{14}SP_8$ body weight loss vs $SP_{14}LP_{22}$, 32 ± 3%, U-test p=0.001) had decreased *Gpr50* expression relative to their LP controls, except hamsters, that did not reduce body weight ($SP_{14}LP_{14}SP_8$ constant body weight vs $SP_{14}LP_{22}$, 74 ± 11%, t-test p=0.066). In the $SP_{14}LP_{14}SP_8$ group *Gpr50* expression was lower in hamsters showing a body weight response than in those showing no response to SP exposure. ($SP_{14}LP_{14}SP_8$ body weight loss vs $SP_{14}LP_{14}SP_8$ constant body weight, 44 ± 5%, U-test p=0.004).

Cellular Retinol-Binding Protein 1 (*Crbp1*)

Crbp1 expression was clearly reduced after 14 weeks in SP (SP_{14}) relative to LP_0 (SP_{14} vs LP_0, 11 ± 3%, U-test p=0.002) and increased again after re-exposure to LP (Figure 5D). After six weeks in LP ($SP_{14}LP_6$) the mRNA concentration increased to 64 ± 14% and was not significantly different from LP_0 ($SP_{14}LP_6$ vs LP_0, t-test p=0.15). In the $SP_{14}LP_6SP_8$ group, gene expression returned to SP_{14} levels and was significantly lower than its matched LP group ($SP_{14}LP_6SP_8$ vs $SP_{14}LP_{14}$, 20 ± 4%, t-test p=0.002). Both $SP_{14}LP_{14}SP_8$ groups had higher *Crbp1* expression than the SP_{14} group ($SP_{14}LP_{14}SP_8$ body weight loss vs SP_{14}, 260 ± 65%, t-test p=0.049; $SP_{14}LP_{14}SP_8$ no body weight loss vs SP_{14}, 436 ± 83%, t-test p=0.004). $SP_{14}LP_{14}SP_8$ hamsters had significantly lower mRNA levels than the $SP_{14}LP_{22}$ animals, if they had lost body weight ($SP_{14}LP_{14}SP_8$ body weight loss vs $SP_{14}LP_{22}$, 38 ± 9%, t-test p=0.002), but *Crbp1* expression was not different if body weight remained constant ($SP_{14}LP_{14}SP_8$ constant body weight vs $SP_{14}LP_{22}$, 63 ± 12%, t-test p=0.08).

Somatostatin/somatotropin release inhibiting factor (Srif)

Somatostatin was weakly expressed in all LP groups (Figure 5E). After 14 weeks in SP_{14} somatostatin expression was approximately 7-fold increased relative to LP_0 (SP_{14} vs LP_0, 683 ± 78%, U-test p=0.002). The re-exposure from SP to LP, caused a decrease in *Srif* expression to 7 ± 1% within six weeks ($SP_{14}LP_6$ vs SP_{14}, U-test p=0.002). Hamsters re-exposed to SP for a further eight weeks, after six weeks in LP ($SP_{14}LP_6SP_8$), showed a partial reverse of *Srif* expression to 36 ± 3% ($SP_{14}LP_6SP_8$ vs SP_{14}, U-test p=0.002), in contrast to a low expression in hamsters maintained in continuing LP ($SP_{14}LP_{14}$ vs SP_{14}, 13% ± 2%, t-test p=0.002). After 14 weeks in LP and another eight weeks in SP

($SP_{14}LP_{14}SP_8$) *Srif* only showed increased expression in hamsters that reduced body weight compared to the matching LP group ($SP_{14}LP_{14}SP_8$ body weight loss vs $SP_{14}LP_{22}$, 378 ± 36%, U-test p<0.001). This partial increase was significantly lower as compared to SP_{14} ($SP_{14}LP_{14}SP_8$ body weight loss vs SP_{14}, 60 ± 6%, U-test p=0.01). Hamsters that did not respond to SP re-exposure maintained low *Srif* expression which was comparable to hamsters maintained in LP for 22 weeks ($SP_{14}LP_{14}SP_8$ constant body weight vs SP_{14}, 28 ± 7%, t-test p<0.001). *Srif* expression was significantly higher in hamsters of $SP_{14}LP_{14}SP_8$ that lost body weight compared to those maintaining body weight ($SP_{14}LP_{14}SP_8$ body weight loss vs $SP_{14}LP_{14}SP_8$ constant body weight, 217 ± 20%, t-test p = 0.004)

Discussion

Body weight

Hamsters reduced their body weight during an exposure to SP for 14 weeks. As expected body weight increased after re-exposure to LP, with most hamsters reaching or exceeding their original LP body weight after only six weeks. The body weight increase reached a plateau phase after approximately 12 weeks and ten more weeks in LP had no further significant effect.

Previously it has been described that photorefractory hamsters that had completely regained their LP phenotype in continuous SP exposure for >20 weeks need at least ten weeks in LP to regain SP sensitivity (37-39). We initially hypothesized that only hamsters re-exposed after 14 weeks in LP ($SP_{14}LP_{14}SP_8$), but not after six weeks in LP ($SP_{14}LP_6SP_8$), would be able to respond to a second SP re-exposure. Surprisingly, hamsters re-exposed to SP after only six weeks in LP ($SP_{14}LP_6SP_8$) responded to the photoperiodic signal as they all stopped gaining body weight. Therefore these data do not support our hypothesis for the requirement of an extended LP exposure but however, it is noticeable that weight loss was muted and only some animals started to reduce body weight once more (Supplementary Figure 1B). One explanation for this response may be that during the duration of 14 weeks of SP exposure (the trough of SP adaptations in body weight and reproduction), hamsters had not yet become photorefractory and some SP sensitivity remained or could be partially restored by only six weeks in LP.

In hamsters re-exposed to LP for 14 weeks before a further SP exposure ($SP_{14}LP_{14}SP_8$), a divergent body weight response seemed to be evident. As body weight can fluctuate on a daily basis with gains or losses in some photoperiod non-responsive hamsters, we applied criteria of SP responsiveness as a loss of body weight of 10% or greater from peak value after 7 weeks exposure to SP and to below the weight at the time transfer from LP to SP. Using these criteria the response of hamsters switched back to SP after 14 weeks in LP ($SP_{14}LP_{14}SP_8$) was divided. Half of the hamsters responded with a more pronounced weight loss than hamsters re-exposed to SP after six weeks LP exposure

($SP_{14}LP_6SP_8$). The other half of the $SP_{14}LP_{14}SP_8$ group did not respond to SP re-exposure. These hamsters were not non-responders *per se*, because they had shown a normal SP adaptation after the initial switch from LP to SP for 14 weeks (26.2 % ± 6.6 % body weight loss and a weight loss of 14% or greater within eight weeks of exposure to SP). Furthermore, this differential physiological response was also partially reflected in hypothalamic gene expression. However, hamsters can vary considerably in the rapidity of response to a change in photoperiod direction and this may be evident in our analysis where one non-responder may have been reclassified as responder given a longer duration in SP.

Serum concentrations

Total serum concentrations of T_4 and T_3 showed no significant differences in response to alternating photoperiods. Changes in thyroid hormone concentrations were highly variable between animals, which might have overridden potential differences caused by photoperiodic changes. Other studies were able to measure seasonal fluctuations in serum concentrations, but experimental setup and timing of sampling was different (25, 42). The diversity of different studies and high inter-individual variability in the current data suggest that serum thyroid hormone concentrations, at least in our stable laboratory conditions, are not directly involved in photoperiodic changes of physiology. Rather intracellular T_3 levels, controlled by deiodinases, in specific tissues such as the hypothalamus seem to be regulating metabolism and body weight in a photoperiodic setting.

Gene expression

Most photoperiodic gene transcription changes were located in tanycytes of the ependymal layer of the third ventricle adjacent to the hypothalamus which are critically involved in seasonal adaptation of energy balance, metabolism and growth (14, 43). Djungarian hamsters exposed to SP showed lower expression of *Vimentin* in tanycytes, which has been demonstrated previously and is associated with structural changes of these cells (8, 16, 17).

Thyroid hormone availability in the hypothalamus is regulated by deiodinases, which play a central role in seasonal adaptation (18, 19, 21). The current view is that low *Dio2* and high *Dio3* expression during SP exposure lead to low T_3 concentrations in the hypothalamus, which is considered to be a pre-requisite for many of the adaptive responses to SP. On re-exposure to static LP or naturally lengthening days after the winter solstice, absent *Dio3* expression and an induction of *Dio2* expression contributes to re-establishing LP physiology in the hamster (8, 20, 21, 44).

In our study, ependymal *Dio2* expression did not show a reduction after the initial 14 weeks in SP (SP_{14}) when body weight had maximally decreased. Furthermore, after a substantial increase in

expression following six weeks of re-exposure to LP, by 14 weeks *Dio2* started to decline and by 22 weeks had returned to values found in hamsters at LP_0. A decline of *Dio2* over a static constant LP has previously been noted (12), which together with a study analysing gene expression in natural photoperiod suggests, that *Dio2* expression can become refractory to LP (44). Therefore, *Dio2* at sample points LP_0 and SP_{14} in our study likely reflects the nadir of a refractory *Dio2* expression at the time the hamsters were sampled in each photoperiod.

The SP non-responsive hamsters following a 14 week re-exposure to LP ($SP_{14}LP_{14}SP_8$) also had low *Dio2* expression with the exception of one hamster whose body weight increased during eight weeks in SP (Figure 4A, B). These data suggest that although low tanycyte *Dio2* expression is likely to be a pre-requisite for a sufficient reduction in hypothalamic T_3 availability, continual high levels of hypothalamic T_3 are not required to maintain LP body weight. This is consistent with our previous data obtained over the course of one year in natural photoperiod which showed that body weight continues to increase for four weeks or more after the summer solstice, even though *Dio2* expression is reduced to near minimal expression by mid-July or earlier (44).

Importantly, *Dio2* expression must always be interpreted in combination with *Dio3* expression. In static photoperiods Djungarian hamsters show complete absence of *Dio3* expression in LP and a transient expression in SP with highest levels of expression within about eight to ten weeks of SP exposure, which then becomes refractory and declines to low or absent levels in a continuing SP environment (12, 21, 25, 34). As a result, DIO3 in SP is likely to convert T_4 into rT_3, before it can be converted by DIO2 to T_3, or DIO3 inactivates available T_3 by conversion to T_2. Given similar *Dio2* expression levels in LP_0 and SP_{14}, the data suggest that the key event in physiological SP responsiveness is expression of *Dio3*. Consistent with this notion, *Dio3* only responded weakly overall after exposure to SP following a six week re-exposure to LP ($SP_{14}LP_6SP_8$), when body weight loss was low. After a 14 week re-exposure to LP, a subsequent re-exposure to SP *Dio3* strongly increased in responsive hamsters, and with one exception, was low in non-responders ($SP_{14}LP_{14}SP_{8,}$ Figure 4B). The hamster with an exception in *Dio3* expression levels in the group of non-responders may however, be a late responsive hamster. Body weight of this hamster continued to increase for six weeks after re-exposure to SP and then decreased in the next two weeks before the hamster was culled, but had not reached a weight loss relative to the time of transition from LP to SP (Figure 4A). Had this hamster spent further time in SP, it may have become evident that this hamster was a responder. Taken together, the photoperiodic history i.e. the length of exposure to LP after the first re-exposure from SP may have an impact on the intensity of *Dio3* expression response which is also reflected in the body weight change and has been previously discussed (45).

However, *Mct8* expression also needs to be considered in the context of thyroid hormone availability. The function of *Mct8* in the photoperiod response is not known, but as a bidirectional T4/T3 transporter (46), one possibility is that an increase in *Mct8* in SP could function to facilitate removal of T_4 and T_3 from the hypothalamus to aid reduction in T_3 availability. Similar to *Dio3,* in the context of responsiveness to SP, those hamsters which responded to SP showed higher expression levels of *Mct8*, whereas in SP non-responsive hamsters *Mct8* expression was not significantly increased relative to those in LP at 22 weeks. There is the possibility that other thyroid hormone transporters may have a role to play, but to date OATP1c has not been found in the ependymal layer of the Djungaian hamster and MCT10 has to our knowledge not been examined (47). Nevertheless, our data indicate that an appropriate co-ordinated response of all components of the thyroid hormone metabolism/transport system is likely to be required for successful initiation of SP physiology.

Photoperiod regulated *Tsh-r* expression in the ependymal layer is a possible factor in the transmission of the day length signal provided by a duration dependent melatonin-signal transduction pathway, to a genomic and physiological action. In contrast to a previous study, which showed a lower expression during SP, we found no differences in *Tsh-r* mRNA expression between LP and SP (8). Our results suggest that altered *Tsh-r* expression does not play a critical role in mediating seasonal physiology.

Somatostatin expression in the ARC has been shown to be substantially increased upon exposure to SP and has been proposed to be involved in suppressing the growth hormone axis, hence may be a key player in seasonal body weight regulation (33, 34). Furthermore, evidence obtained from *in vivo* TSH administration studies suggests *Srif* expression is downstream of thyroid signalling in the hypothalamus (48). In accordance with other studies, *Srif* was highly expressed in SP and reduced to minimal expression when hamsters were switched from SP to LP ($SP_{14}LP_6$). After re-exposure to SP from LP, *Srif* expression only achieved 36% ($SP_{14}LP_6SP_8$) and 60% ($SP_{14}LP_{14}SP_8$ responsive hamsters), respectively compared to SP_{14}. This is consistent with the hypothesis for an involvement of ARC *Srif* in the growth hormone axis and the lower expression achieved in the $SP_{14}LP_6SP_8$ group may at least in part account for the smaller body weight loss achieved by this group. Also consistent is that hamsters which were SP non-responsive after 14 weeks of LP re-exposure ($SP_{14}LP_{14}SP_8$ constant body weight) showed a low level of expression that was comparable to their LP counterparts ($SP_{14}LP_{22}$).

In accordance with previous studies *Gpr50* was down regulated during SP in Djungarian hamsters (SP_{14}) (8, 26). Gene expression of *Gpr50* in the ependymal layer was reversed after re-exposure to LP from SP ($SP_{14}LP_6$). However, the increase was retarded and restoration of LP_0 mRNA concentrations was not reached after six weeks, but only after 14 weeks ($SP_{14}LP_{14}$) re-exposure to LP. The function

of GPR50 in seasonal adaptations is not understood, but a recent study in mice has shown that knock-out of GPR50 results in a higher level of expression of *Dio2* in tanycytes (49). In the context of the Djungarian hamster a similar mechanism offers an explanation for a reduction in *Dio2* upon re-exposure to LP for 14 weeks or more, where there is an inverse relationship between *Dio2* and *Gpr50* expression. Furthermore in natural photoperiod *Gpr50* expression only starts to increase at around the time *Dio2* has almost reached a nadir of expression and peaks around the autumnal equinox (44). However, contradicting this explanation is the observed reduction in *Dio2*, but little change of *Gpr50* in non-responsive hamsters of the $SP_{14}LP_{14}SP_8$ group.

Expression of *Crbp1* in the ependymal layer was regulated by photoperiod in a similar manner to the expression of *Gpr50.* CRBP1 is responsible for the intracellular transport of retinol, the substrate for the synthesis of retinoic acid (50) and was, together with other retinoid-signalling genes, discovered as photoperiod-responsive (28). Re-exposure of hamsters from SP to LP (groups $SP_{14}LP_6$ and$SP_{14}LP_{14}$) induced a slow reversion of *Crbp1* gene expression, which has been observed in a previous study (28). *Crbp1* responded again to the second switch from LP to SP after six ($SP_{14}LP_6SP_8$) and 14 weeks ($SP_{14}LP_{14}SP_8$) in LP, but only in the responsive hamsters of this latter group. Ross and colleagues (28) have shown, that in photorefractory hamsters body weight increased, but *Crbp1* expression remained low. Together with our results, this suggests that *Crbp1* was regulated by the photoperiodic signal, but only played a minor role in seasonal body weight regulation. Nonetheless the retinoid-signalling pathway is under photoperiod control, but the function of *Crbp1* gene expression remains unclear.

Taken together all results from non-responding hamsters demonstrate that these animals were insensitive to the SP signal which was largely reflected in gene expression profiles. Why some animals responded to the second photoperiodic switch after 14 weeks, and others did not, remains to be investigated. However, the gene expression data of the non-responding hamsters would suggest a continuation of a LP output of the PT in these SP non-responsive hamsters.

In summary, our data provide additional support for an involvement of a co-ordinated response of the thyroid hormone metabolism/transport system, and other photoperiodically regulated genes in seasonal physiological adaptations. Additionally, *Dio2* expression in response to LP re-exposure supports a previous suggestion that this gene may become refractory to LP; a response which has only be described in the long lived seasonal sheep.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1: Experimental schedule. Hamsters were kept under long photoperiod LP or short photoperiod (SP) and euthanized at different time points.

Figure 2: Body weight changes of all animals (A) and absolute body weights of the distinct groups (B) are expressed as means (±SEM). Animals were kept under short photoperiod (SP, white circle) or long photoperiod (LP, black circle). One cohort was sacrificed at time point A (SP_{14}), one cohort at B ($SP_{14}LP_6$), two cohorts at C ($SP_{14}LP_{14}$, $SP_{14}LP_6SP_8$) and two cohorts at D ($SP_{14}LP_{22}$, $SP_{14}LP_{14}SP_8$). The $SP_{14}LP_{14}SP_8$ (D) group was divided into hamsters which lost weight (white circle) and hamsters with constant body weight (grey circle).

Figure 2: *Dio2* (A) and *Dio3* (B) gene expression in ventricular ependymal cells of all groups. Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to SP_{14}. Black bars represent hamsters culled in long photoperiod (LP), white bars illustrate hamsters from short photoperiod (SP) and the grey bar shows non-responsive animals from SP. White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. * significant difference to SP_{14}; # significant difference between other groups. Autoradiographs show representative brain sections with gene expression close to mean values.

Figure 4: Individual body weight changes (C) and individual *Dio2* and *Dio3* expression values (D) of $SP_{14}LD_{14}SP_8$ hamsters. Data are presented relative to the peak body weight of each individual. White symbols represent SP responsive hamsters losing body weight, grey symbols represent SP non-responsive hamsters maintaining stable body weight.

Figure 5: Gene expression *Mct8* (A), *Vimentin* (B), *Gpr50* (C), *Crbp1* (D) in ventricular ependymal cells and *Srif* (E) in the arcuate nucleus. Data are expressed as means + SEM. Integrated optical intensity (IOD) was normalized to SP_{14} for genes downregulated in LP (A, E) or LP_0 for genes upregulated in LP (B-D). Black bars represent hamsters culled in long photoperiod (LP), white bars

illustrate hamsters from short photoperiod (SP) and the grey bar shows non-responsive animals from SP. White bar at $SP_{14}LP_{14}$ represents group $SP_{14}LP_6SP_8$ and at $SP_{14}LP_{22}$ it represents group $SP_{14}LP_{14}SP_8$. The grey bar represents hamsters from the $SP_{14}LP_{14}SP_8$ group that did not reduce body weight. * significant difference to SP_{14}; # significant difference between other groups. Autoradiographs show representative brain sections with gene expression close to mean values.

Tables

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

Supplementary Figure 1: Individual body weight changes of hamsters from the SP_{14} group exposed to short photoperiod (SP) for the first time (A) and from the $SP_{14}LP_6SP_8$ group exposed to SP the second time after six weeks re-exposure to long photoperiod (LP) (B). Data are presented relative to the peak body weight of each individual.

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