Somatostatin agonist pasireotide promotes a physiological state resembling short-day acclimation in the photoperiodic male Siberian hamster (*Phodopus sungorus*)

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Abstract

The timing of growth in seasonal mammals is inextricably linked to food availability. This is exemplified in the Siberian hamster (*Phodopus sungorus*) which uses the annual cycle of photoperiod to optimally programme energy expenditure in anticipation of seasonal fluctuations in food resources. During the autumn, energy expenditure is progressively minimized by physiological adaptations including a 30% reduction in body mass, comprising of a reduction in both fat and lean tissues. However, the mechanistic basis of this adaptation is still unexplained. We hypothesised that growth hormone (GH) was a likely candidate to underpin these reversible changes in body mass.

Administration of pasireotide, a long acting somatostatin receptor agonist developed for the treatment of acromegaly, to male hamsters in long-day photoperiod (LD) produced a body weight loss. This comprised of a reduction in lean and fat mass, including kidneys, testes, and brown adipose tissue typically found in short-day (SD) housed hamsters. Furthermore, when administered to hamsters switched from SD to LD pasireotide retarded the body weight increase compared to vehicle treated hamsters. Pasireotide did not alter photoperiod-mediated changes in hypothalamic energy balance gene expression, but altered expression of *Srif* mRNA expression in the periventricular nucleus and *Ghrh* mRNA expression in the arcuate nucleus consistent with a reduction in GH feedback and concurrent with reduced serum IGF-1. Conversely, GH treatment of SD hamsters increased body mass which included increased mass of liver and kidneys. Together these data point to a role for the GH axis in the determination of seasonal body mass of the Siberian hamster.

Introduction

Maintenance of endothermy during winter months is a physiological challenge for seasonal mammals as food resources are diminished when energy requirements are increased by seasonal environmental changes. Consequently many seasonal mammals have adapted physiology and behaviours to minimize energy expenditure including a cessation of reproduction and slowing of growth, but some small mammals can actively reduce body mass, a change comprised of a reduction in lean, fat, organ and skeletal masses (Dehnel's phenomenon) (1, 2). In temperate climates photoperiod is the predominant seasonal cue that anticipates the forthcoming food and climatic environment. The Siberian hamster is an exemplar of a photoperiodic mammal using seasonal changes in photoperiod to anticipate food availability (3-5). As autumn approaches, the Siberian hamster reduces body mass which includes a reduction in the mass of internal organs such as the kidney, liver, adrenal gland, white adipose tissue, brown adipose tissue, an involution of the reproductive organs and a reduction in skeletal mass may also occur (6, 7). The winter reduction in body mass of the Siberian hamster is initiated by a shortening of day length following the summer solstice and can be simulated in the laboratory by switching hamsters from long day length (LD) to short day length (SD). Conversely during spring the body mass of the Siberian hamster increases (8), reversing the reductions of fat mass and lean mass and can be enabled in the laboratory by switching hamsters from SD to LD (9, 10).

The seasonal physiological adaptations are largely determined by a central mechanism governed by thyroid hormone (T3) availability to the hypothalamus (11), which in turn regulates a cohort of genes which contribute to physiological adaptions appropriate for the season (9, 12-15). Conceptually, the change in body mass during the transition from winter to summer (SD to LD) could be considered as a growth response. This is supported by evidence from studies on growth in red deer showing a correlation between growth hormone (GH),

IGF-1 and seasonal growth (16, 17). Furthermore, mean levels of circulating growth hormone over a 24h period correlate with photoperiod appropriate body weight in the seasonal rodent, the golden hamster (*Mesocricetus auratus*, 18), which might point to a general role for the GH axis in the regulation of seasonal body size. We therefore hypothesised that hypothalamic neuroendocrine regulation of GH would be involved in the seasonal body mass cycle of the Siberian hamster. GH is produced in the pituitary with secretion promoted by growth hormone releasing hormone (GHRH) from neurons in the arcuate nucleus (ARC) and inhibited by somatostatin (referred to as somatotropin release-inhibiting factor - SRIF) produced by neurons of the periventricular nucleus (PeVN). Circulating GH concentration is regulated by a negative feedback pathway to the hypothalamus, acting to suppress GHRH stimulated synthesis and secretion. As GH falls, GHRH secretion increases and SRIF decreases. Through this feedback mechanism, GH is released in a pulsatile manner (19-21). Interestingly, in the Siberian hamster we and others have shown that *Srif* expressed in the ARC is strongly influenced by photoperiod and downstream of central thyroid hormone balance (10, 14, 15), and so may play a role in the seasonal regulation of the GH axis and thus seasonal body mass.

Pasireotide is a somatostatin analogue developed for the treatment of acromegaly, which target somatostatin receptors (SSTRs) at the pituitary to reduce GH secretion (22, 23, 24). Pasireotide has a high affinity for four of the five somatostatin receptor subtypes with highest affinity for $SSTR_5$ ($SSTR_{5>2>3>1,}$ 39-fold greater than octreotide, 23). We have utilized pasireotide to suppress pituitary secretion of GH and assessed the effect on seasonal body mass and photoperiod regulated hypothalamic gene expression that underpins the physiological responses of the Siberian hamster. Our data show that GH is a likely proponent of seasonal body mass regulation, but also provide evidence that somatostatin may play a broad role in the seasonal regulation of the hypothalamic neuroendocrine axis.

Materials and Methods

Animals

Animal husbandry and all experiments were in accordance with the German Animal Welfare Act and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (ref: 13/1246, 14/1453). Adult male Siberian hamsters were taken from a colony maintained at the University of Veterinary Medicine, Hannover, having been bred under natural photoperiod (at 52°N latitude) and ambient temperatures before the summer solstice. Hamsters were then transferred to artificial long days (LD: 16:8h light:dark; lights on: 04:00-20:00 CET; 20-22°C) after weaning and were a minimum of 3 months old at the start of experiments. Food (hamster breeding diet, Altromin 7014, Lage, Germany) and water were available ad libitum, supplemented weekly by a slice of apple. Overhead lighting was provided by fluorescent tubes (Lumilux LF11, Osram, Germany) resulting in a light intensity of ca. 200–350 Lux at cage level. During the dark phase, illumination was limited to dim red light of <5 Lux (Osram, Darkroom red, 15 W). Experimental hamsters were singly housed indoors throughout all experiments. Hamsters were sacrificed by overdose of CO_2 followed by cervical dislocation. The brain and trunk blood, from which serum was isolated, were collected and stored at -70°C before use. Organs, epididymal white adipose tissue (EWAT) and, where applicable, interscapular brown adipose tissue (IBAT) were dissected out and immediately weighed; testes, kidneys and EWAT were returned to carcasses which were stored at -70°C and later thawed for body composition analysis by MRI (EchoMRI Whole Body Composition Analyser, Echo Medical Systems, Houston, Texas).

LD Experiment

Sixteen age and weight matched hamsters (3-4 months of age) were maintained in LD, 8 hamsters received subcutaneously a dose of long acting release (LAR) pasireotide (Novartis, Basel Switzerland; 160mg/kg based on weight on day of administration) and 8 hamsters received vehicle (25) only (8 hamsters per day; 4 from each group per day). Twenty-eight days following the first injection, pasireotide was re-administered and hamsters were sacrificed at 49 days following first administration.

Switchback Experiment

Thirty-two Siberian hamsters were acclimated to short day photoperiod (SD; 8:16h light:dark; lights on: 09:00-17:00 CET; 20 ± 1°C) for a period of 10 weeks (69-72 days). One hamster did not respond to SD and was excluded from the experiment. The hamsters were divided into four weight matched groups (three groups of 8 and one group of 7). Two groups (n=8 per group) received a subcutaneous dose of LAR pasireotide (160mg/kg based on weight on day of administration) or received vehicle only (1 group n=8 and 1 group n= 7). Injections were carried out over the course of 4 days (8 hamsters per day; 2 from each group per day) to accommodate consistent killing and retrieval of tissues between 4-5h after lights off over a 4 day period. One group of hamsters which had received vehicle (SD-vehicle n=7) and one group which had received pasireotide (SD-pasireotide, n=8) remained in SD. The remaining vehicle treated (SWB-vehicle, n=8) and pasireotide treated (SWB-pasireotide, n=8) groups were returned to LD (16:8h light dark). Twenty-eight days following the first injection, hamsters were re-administered pasireotide or vehicle as appropriate. One SWB-pasireotide hamster had one testis and epididymal fat pad removed due to exposure outside the body cavity during radiotelemetry implant surgery (data to be reported separately), thus reducing the sample size to n=7 for EWAT and paired testes mass analyses. Hamsters were sacrificed at 49 days following first administration.

GH Experiment

Siberian hamsters that had been held in SD for 12 weeks were either switched back to LD (n=6) or administered bovine GH ip. (1mg/kg bovine growth hormone – NIDDK, Bethesda, MD, USA, catalogue no: AFP-1032SC; in phosphate buffered saline (PBS) or PBS alone in 200µl doses in the final hour of the light phase, daily for 28d (n=12 per group). GH was administered at the end of the light phase to extend a possible circadian elevation in GH over the dark phase, in order to increase the duration of proposed elevated GH which has been shown to exist in other rodents (18) and be important to interpretation of the GH in LD Siberian hamsters. A stock preparation of GH was made at 1 mg/ml in PBS under aseptic conditions, and stored at -20°C in 250µl aliquots. The stock solution was diluted daily at 1 µl/g hamster body mass made up to 200µl with PBS just prior to use.

Serum glucose and hormone determination

Serum glucose was measured with a glucose monitor and test strips (Accu-Chek, Roche) in duplicate. Serum IGF-1 (established as a biomarker reflecting circulating GH levels, 26) and insulin were determined by ELISA (Mercodia, Uppsala, Sweden and R&D Systems, MN, USA, respectively), following kit instructions for mouse samples. For the insulin ELISA, samples were diluted 2-fold in sample buffer.

Riboprobe synthesis

Riboprobes complementary to gene fragments were generated as previously described (11, 27-29). To ascertain possible effects on the sensitivity of the GH-axis by pasireotide

treatment, additional riboprobes were synthesised for growth hormone receptor (*Gh-r*) and growth hormone releasing hormone (*Ghrh*); For *Gh-r;* primers were F: 5'-AAGTGCGKGTGAGATCCAGACAAC-3' and R: 5'-GGTACGYCCAGAATCRTCATCCT-3', based on a consensus between mouse and rat sequences (mouse Genbank NM_010284 bases 1066-1089 forward primer and bases 1534-1556 reverse primer). For *Ghrh,* primers were based on the partial sequence of Mesocricetus auratus (golden hamster, Genbank NM_001281590) 1. F: 5'-GATGCCACTCTGGGTGTTCTTTG-3' (bases 26-48), R: 5'-ATCAGGATGGGGGTTTTATTGTAT-3' (bases 464-441). These primers amplified several products and therefore a secondary amplification was performed with the original forward primer and a reverse primer located 3 nucleotides within the target sequence R: 5'-AGGATGGGGGTTTTATTGTATTTA-3' (bases 461-438). This resulted in the amplification of a single product of the correct sequence. Templates for riboprobe synthesis were generated as described previously (10)

In situ hybridisation

Frozen coronal hypothalamic sections were cut at 14µm and mounted on poly-L-lysine coated slides (ThermoScientific, Rockford, IL, USA). Radiolabelled *in situ* hybridisation was carried out as previously described (10). Due to poor tissue preservation ARC region SWB-vehicle sample size was reduced to n=7 and in the *pomc* experiment SWB-Pasireotide sample size was reduced to n=7.

Autoradiographic image analysis

Exposed autoradiographic films were scanned at 600 dpi on an Epson scanner linked to a computer running Image-Pro PLUS analysis software (v4.1.0.0, Media Cybernetics, Wokingham, USA). Integrated optical density was obtained by reference to a ^{14}C microscale

and was determined for three to five sections per slide for each probe, depending on the gene considered, and the accumulated count taken. Relative integrated optical density (IOD), an indirect measure of mRNA expression, was expressed relative to the control group (typically LD-Veh or SD-Veh unless otherwise stated) whose value was defined as 1, and statistical analysis was carried out on raw IOD values.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses were carried out using Minitab v.15.0 (Minitab, PA, USA) unless stated otherwise. Data were compared by t-test, assuming equal variances, one-way or two-way ANOVA as appropriate, with Tukey *post hoc* comparisons. Where data did not conform to the assumptions of the test, it was transformed by $\log_{10}$ or square root, and statistics performed on transformed data (LD experiment: $\log_{10}$: serum insulin; SWB experiment: $\log_{10}$: serum IGF-1, kidney mass, serum glucose, ARC *Gh-r,* ARC *Srif*; square root: ARC *Ghrh*, ARC *Npy*; GH experiment: $\log_{10}$: IBAT of 28 day GH treated hamsters). When data could not be transformed to fit assumptions of the parametric test, Kruskal-Wallis (KW) and Mann-Whitney (MW) tests were performed as appropriate (SWB experiment: paired testes mass, *Dio3* and ARC *Gh-r* mRNA expression; GH experiment: paired testes mass). P-values less than 0.05 were considered statistically significant. When insulin was measured in the SWB experiment, some samples exceeded the limit of the assay and so the data were bound from above, with only the upper limit of the assay being observed rather than the actual value. Therefore a Cox regression was carried out with Bonferroni correction for multiple testing using the statistical software R and $p<0.05$ after this correction was considered statistically significant.

Results

Short-day-like weight loss in long-day hamsters is induced by somatostatin agonists

To test our hypothesis that the adaptation of SD body mass is a growth related phenomenon, pasireotide a long-acting somatostatin analogue was administered subcutaneously to hamsters held in long-day photoperiod (LD) in a 28d slow release particle formula releasing over a period of 7 weeks. In hamsters treated with pasireotide there was a progressive loss of body mass (15.0±3.2%, $P<0.001$ vs vehicle; Fig 1A). The weight loss trajectory in these LD hamsters was similar to the weight loss trajectory experienced by hamsters transferred from LD to SD (9,11). Weight loss induced by pasireotide was accompanied by a reduction in lean mass ($P=0.020$) and a trend for fat mass reduction ($P=0.091$) (Fig 1B,C). Analysis of serum parameters showed pasireotide decreased circulating insulin-like growth factor-1 (IGF-1; $P=0.022$, Fig 1D), increased blood glucose concentration ($p=0.009$, Fig 1F), but had no effect on serum insulin concentration (Fig 1E). In comparison to vehicle treated hamsters, weight loss by pasireotide was manifest in tissue mass reductions which included a decrease in kidney mass ($P=0.004$), epididymal white adipose tissue mass (EWAT, $P=0.010$), interscapular brown adipose tissue mass (IBAT, $P=0.006$) and paired testes mass (74.7±5.6%, $P<0.001$), but liver mass was unchanged (table 1).

Hypothalamic gene expression

At the level of the hypothalamus, there was no effect of pasireotide on mRNA expression of *Dio2 Srif*, *Pomc*, and *Npy* expression the ARC or GH-r in either the ARC or PVN (Fig 2A,B; Supplementary Fig 1A-D). However, *Ghrh* expression in the ARC was increased by 2.10±0.22 fold by pasireotide treatment ($p<0.001$) while *Srif* expression the PeVN was

decreased to approximately one-fifth of vehicle expression (0.22±0.07 fold, p<0.001, Figure 2C, D).

Pasireotide impairs photoperiod induced weight gain

Hamsters previously acclimated to SD for 10 weeks with a loss of 21% body mass, were administered pasireotide or vehicle and then switched back (SWB) to LD for 7 weeks or maintained in SD. Analysis of change in body mass shows there was an effect of photoperiod and pasireotide with interaction between photoperiod and treatment (Photoperiod: $F(1,27)=83.45$ $p<0.001$; Pasireotide: $F(1,27)=21.85$, $p<0.001$; Interaction: $F(1,27)=4.75$, $p=0.038$, Fig 3A), so that after 7 weeks administration, SWB-vehicle hamsters had increased body mass more than all other groups. More specifically, SWB-vehicle administered hamsters had regained an average 11.5±1.0g body mass while hamsters administered pasireotide, growth was restricted to 4.1±1.5g. Overall SWB hamsters gained lean mass, but this was significantly less in pasireotide hamsters and there was no interaction between photoperiod and treatment (Photoperiod: $F(1,27)=23.82$ $p<0.001$; Pasireotide: $F(1,27)=7.01$, $p=0.013$; Interaction: $F(1,27)=1.18$, $p=0.287$, Fig 3B). Similarly, fat mass was increased in SWB hamsters and suppressed by pasireotide treatment with no interaction (Photoperiod: $F(1,27)=28.06$ $p<0.001$; Pasireotide: $F(1,27)=4.83$, $p=0.037$; Interaction: $F(1,27)=2.26$, $p=0.144$, Fig 3C). Serum IGF-1 concentration was increased in SWB hamsters and suppressed by pasireotide, with SD-pasireotide having significantly lower and SWB-vehicle having significantly higher concentration compared to all other treatment groups ($P<0.05$ all comparisons after Bonferroni correction; Fig 3D). As noted in LD hamsters, there was an effect of pasireotide to significantly increase serum glucose concentration, independent of photoperiod (Photoperiod: $F(1,27)=1.18$ $p=0.286$; Pasireotide: $F(1,27)=4.84$, $p=0.036$; Interaction: $F(1,27)=0.32$, $p=0.578$, Fig 3F). Serum insulin concentration was increased significantly by SWB to LD (comparisons where $p<0.05$ after Bonferroni correction: SD-

Vehicle vs. SWB-Vehicle; SD-Pasireotide vs. SWB-Pasireotide; SD-Pasireotide vs. SWB-Vehicle, Fig 3E).

Liver mass was significantly increased in SWB hamsters, but there was no effect of pasireotide or interaction between photoperiod and treatment (Photoperiod: $F(1,27)=29.62$ $p<0.001$; Pasireotide: $F(1,27)=2.01$, $p=0.168$; Interaction: $F(1,27)=0.80$, $p=0.378$, table 2). Kidney mass was significantly increased in SWB hamsters, and suppressed by pasireotide treatment, but there was no interaction between photoperiod and treatment (Photoperiod: $F(1,27)=27.88$ $p<0.001$; Pasireotide: $F(1,27)=16.94$, $p<0.001$; Interaction: $F(1,27)=0.82$, $p=0.374$, table 2). EWAT was significantly increased in SWB hamsters with no effect of pasireotide and no interaction (Photoperiod: $F(1,27)=32.45$ $p<0.001$; Pasireotide: $F(1,27)=1.03$, $p=0.319$; Interaction: $F(1,27)=1.00$, $p=0.327$, table 2). Similarly, IBAT was significantly increased in SWB hamsters, with a tendency of pasireotide to decrease IBAT mass approaching significance, but no interaction between photoperiod and treatment (Photoperiod: $F(1,27)=20.27$ $p<0.001$; Pasireotide: $F(1,27)=4.10$, $p=0.053$; Interaction: $F(1,27)=1.81$, $p=0.189$, table 2). Paired testes mass was significantly increased in SWB hamsters, but there was no overall effect of pasireotide (KW; Photoperiod: $p<0.001$; Pasireotide: $p=0.115$). Within SD there was no effect of pasireotide (MW; $p=0.643$), but within SWB hamsters, pasireotide significantly suppressed testes mass (MW: $p=0.003$ table 2).

Hypothalamic gene expression

Expression of *Dio2* was significantly increased by SWB from SD to LD but remained unaffected by pasireotide treatment with no interaction (Photoperiod: $F(1,26)=46.74$, $p<0.001$; Pasireotide: $F(1,26)=0.86$ $p=0.362$; Interaction: $F(1,26)=0.51$, $p=0.482$, Fig 4A). Expression of *Dio3* was very low in in the ventricular ependymal layer of SD hamsters and

was undetectable in any of the SWB hamsters, with no effect of pasireotide (KW; Photoperiod: p=0.001; Pasireotide: p=0.915. MW within SD p=1.000, Fig 4B)

Expression of *Srif* in the ARC was significantly reduced in SWB hamsters with no effect of pasireotide or interaction between photoperiod and treatment (Photoperiod: F(1,26)=17.30, p<0001; Pasireotide: F(1,26)=0.35 p=0.558; Interaction: F(1,26)=0.16, p=0.694, Fig 4C), but expression of S*rif* in the PeVN was significantly reduced by pasireotide treatment, with a trend towards an effect of short day photoperiod to reduce expression, but no interaction between photoperiod and treatment (Photoperiod: F(1,27)=2.93, p=0.098; Pasireotide: F(1,27)=8.37 p=0.007; Interaction: F(1,27)=0.42, p=0.524. Fig 4D).

Pasireotide significantly increased ARC *Ghrh* independently of photoperiod (SD-Pasireotide 1.95±0.31 fold change; SWB-Vehicle: 1.12±0.31 fold change; SWB-Pasireotide: 2.98±0.34 fold change. Photoperiod: F(1,26)=2.75, p=0.109; Pasireotide: F(1,26)=19.62 p<0.001; Interaction: F(1,26)=0.76, p=0.391; Fig 4E).

Expression of *Pomc* was significantly raised in SWB hamsters but was unchanged by pasireotide treatment (Photoperiod: F(1,25)=9.87, p=0.004; Pasireotide: F(1,25)=1.45 p=0.239; Interaction: F(1,26)=1.32, p=0.261, Supplemental Fig 2A). *Npy* expression was not significantly altered by photoperiod or pasireotide treatment (Photoperiod: F(1,26)=0.01, p=0.905; Pasireotide: F(1,26)=0.04 p=0.851; Interaction: F(1,26)=0.28, p=0.603, Supplemental Fig 2B).

Expression of *Gh-r* in the ARC or PVN was not significantly altered by photoperiod or pasireotide (ARC: Photoperiod: F(1,26)=2.83, p=0.104; Pasireotide: F(1,26)=0.03 p=0.860;

306 Interaction: F(1,26)=1.14, p=0.296, Supplemental Fig 2C; PVN: KW; Photoperiod: p=0.665;
307 Pasireotide: p=0.604, Supplemental Fig 2D).

308

309 ***Growth hormone induces body weight increase in SD hamsters***

310 Daily intraperitoneal injection of GH for 28 days in hamsters previously acclimated to SD for
311 12 weeks with an average 25% (10g) loss of body mass showed a mean increase of
312 4.15±0.15g. This increase paralleled that observed in a separate cohort of hamsters switched
313 from SD to LD (SWB, 4.76±0.76g) over the same period of time (SWB and GH $P<0.001$ vs
314 vehicle; Fig 5A). Lean mass was significantly increased for GH treated hamsters only
315 ($P=0.023$, Fig 5B), whereas fat mass increased only in SWB hamsters ($P=0.002$, Fig 5C).
316 Serum IGF-1 concentrations were compared by Cox regression. This analysis showed SWB
317 hamsters had greater concentrations of circulating IGF-1 compared to both vehicle and GH
318 treated hamsters (Fig 5D, $P<0.05$ after Bonferroni correction). The small increase in IGF-1 in
319 GH treated hamsters was not significant. Serum insulin was significantly raised by GH
320 treatment ($P=0.011$) and this was also the case in SWB compared to vehicle hamsters
321 ($P=0.008$, Fig 5E).

322 In GH treated hamsters, growth was accompanied by increased liver mass ($P=0.021$) and
323 kidney mass ($P=0.026$). There was a significant increase in EWAT in SWB hamsters
324 compared to vehicle and GH ($P<0.001$ and $P=0.014$ respectively, table 3), but GH did not
325 increase EWAT mass relative to vehicle hamsters. IBAT after 4 weeks in LD or following
326 GH treatment was not significantly different from vehicle hamsters (table 3). As expected,
327 testes mass was significantly increased in SWB ($P<0.001$), but in GH treated hamsters the
328 small increase in testis mass did not reach statistical significance (table 3).

329

Discussion

The Siberian hamster is one of a number of small seasonal mammals that reduce body mass during winter. Even though this weight loss has been extensively described, the mechanisms are not understood. Here we provide evidence to support the view that the GH axis is a potential proponent of the seasonal neuroendocrine axis underpinning this body mass change.

The seasonal adaptation of reproductive organ regression and recrudescence is well established in the Siberian hamster, but less well known are the photoperiod-mediated changes in the mass of internal tissues. Seasonal changes in organ mass were first described in the common shrew (*Sorex araneus)* by Dehnel (referred to as Dehnel's phenomenon; 1,2). Although several previous studies on body mass regulation of the Siberian hamster have demonstrated reduction in muscle and organ mass in short photoperiod (6, 30, 31), with the exception of adipose tissue and testes mass (32), this is the first report showing that increase in body mass during increased day length involves increase in mass of those organs which were reduced during SD exposure. We would therefore consider the Siberian hamster as a member of a group of mammals that exhibit Dehnel's phenomenon.

We initiated these studies with the hypothesis that GH underpins the seasonal body weight changes in the Siberian hamster. Although photoperiod-regulated effects on frequency and amplitude of pulsatile GH secretion have been previously demonstrated in the golden hamster (18), due to the pulsatile nature of GH secretion and small size of the Siberian hamster, direct measurement of GH was not feasible. In the absence of a GH receptor antagonist for use in rodents, we approached our hypothesis by utilizing pasireotide, a somatostatin receptor agonist that was developed for the treatment of excess GH production by pituitary tumours/adenomas (acromegaly) and inhibition of tumour growth (25, 33, 34). This long

353 acting somatostatin receptor agonist was delivered in an encapsulated form which steadily
354 releases the agonist over a period of 28 days.

355 Pasireotide, when administered to male hamsters in LD, caused a gradual body mass loss
356 similar to that which can be observed in hamsters transferred from LD to SD photoperiod.
357 MRI analysis of body composition showed a loss of lean mass which was reflected in a loss
358 of kidney mass and testes mass both of which occur in response to SD (6, 30, 31). However, a
359 reduction in liver mass observed in SD hamsters was not obtained.

360 In this LD paradigm, fat mass determined by MRI showed a trend toward a reduction by
361 pasireotide. This trend is supported by significantly lower EWAT and IBAT measurement in
362 pasireotide treated hamsters and is characteristic of hamsters held in SD (7, 35). This may
363 reflect an involvement of GH or another neuroendocrine pathway in EWAT regulation, but
364 interestingly pasireotide reveals a neuroendocrine basis for regulating IBAT mass, an
365 important fat depot in thermogenesis and energy expenditure (36).

366 As expected, pasireotide treatment resulted in a reduction of circulating IGF-1, which as a
367 biomarker of endogenous GH is indicative of a suppression of GH secretion (26), a
368 conclusion which is supported by increased *Ghrh* expression in the ARC and reduced *Srif*
369 expression in the PeVN as a result of reduced GH feedback to the hypothalamus (19-21).
370 Furthermore in a preliminary experiment with the somatostatin agonist octreotide, a similar
371 trajectory of body mass loss occurred with terminal liver, kidney and EWAT masses similar
372 to pasireotide treated hamsters, but only a small effect on paired testis weight (data not
373 shown).

374 Pasireotide has highest affinity for $SSTR_5$ (22, 23), thus the effect of pasireotide to reduce
375 growth would be consistent with data for $SSTR_5$ subtype distribution in the rat pituitary
376 where $SSTR_5$ is present on 72% of somatotropic cells. A reduction in GH may contribute to

loss of testicular mass through an action on testicular Leydig cells (37), but the effect of the somatostatin analogues could also be due in part to antagonism of FSH at testicular Sertoli cells (38). However, this seems unlikely as the effect on sertoli cells appears to be mediated by $SSTR_2$ (39). A further possibility is a direct effect of pasireotide on hypothalamic *Kiss-1* or *Rfrp3* expression, but inaccessibility of somatostatin analogues to the brain renders this unlikely (see below). A more likely explanation is the inhibition of pituitary LH and FSH secretion by pasireotide (38), where in the rat 21% of gonadtropes express $SSTR_5$ (40). With pasireotide treatment, we did not systematically observe for an effect on pelage, but no striking change in pelage was noted by the end of 7 weeks of treatment. Although in the rat 36% of lactotropes express $SSTR_5$, no effect of pasireotide on prolactin secretion from rat pituicyte primary cell cultures has been found (41), suggesting prolactin secretion may not be subject to regulation by somatostatin. However, further analysis of $SSTR_5$ distribution and other somatostatin receptor subtypes in the hamster pituitary is required to understand the mechanistic basis for the action of somatostatin and related analogues.

Pasireotide also impaired body mass accretion in hamsters switched from SD (at or close to the body weight nadir) to LD photoperiod (SWB hamsters). This was reflected in an overall reduction in lean mass and fat mass in pasireotide treated hamsters. Although measurement of tissue mass showed no significant effect of pasireotide to suppress tissue regrowth in SWB hamsters, the 7.4g difference between vehicle and pasireotide treated hamsters is likely to be due to the sum of differences of tissues which individually have not reached statistical significance. The exception to this was testes mass. Paired testes mass was increased in SWB hamsters, but this increase was significantly albeit not completely retarded by pasireotide.

Circulating IGF-1 was increased in SWB-vehicle hamsters but was decreased in both SD and SWB hamsters treated with pasireotide compared to vehicle treated hamsters indicative of suppression of pituitary GH secretion. A reduction in GH secretion and reduced GH feedback

to the hypothalamus by pasireotide is also supported by evidence of increased *Ghrh* mRNA expression in the ARC and reduced *Srif* expression in the PeVN (19,20). The inability of pasireotide to completely suppress LD physiology in SWB hamsters is likely to be due to restoration of stimulatory hormones from the hypothalamus, driven by the substantial increase in *Dio2* expression in the hypothalamus (10) competing with the inhibitory action of pasireotide via SSTR signalling on appropriate neuroendocrine cells of the pituitary.

Pasireotide in both experiments resulted in a small hyperglycaemic response as observed in humans and rodents (42,43). In hamsters switched from SD to LD, increased insulin was consistent with previous observation of elevated levels in LD (27,29), but no significant suppression of serum insulin was found in either experiment after pasireotide treatment, as reported in human and rodent studies (42,43). However, a suppressive effect on insulin may have been masked as the hamsters were not fasted prior to taking the terminal blood samples.

In this study, we found no evidence to suggest pasireotide has an effect on photoperiod gene expression or central homeostatic mechanisms of energy balance to account for the observed effect on body and organ masses (31, 44-46). Therefore the most parsimonious explanation for the observed effect of pasireotide is an inhibition of GH secretion from the pituitary gland. This is supported by an observed increase following pasireotide treatment in *Ghrh* expression in the ARC and decrease in *Srif* expression in the PeVN which is consistent with an inhibition of GH feedback to the homeostatic control of GH output from the pituitary gland. Additionally, somatostatin and somatostatin analogues are known to have little or poor accessibility to the brain and may actively be transported from CNS to blood (47-49). Therefore a direct effect of the analogues in the brain is unlikely and the observed effects are

more likely to be due to the presence of SSTRs on relevant hormone secreting cells of the pituitary.

We and Klosen et al (10, 14, 15, 31) have recently demonstrated a robust induction of *Srif* expression in the ARC of SD hamsters and we have hypothesised that these neurons may be important to seasonal growth (10, 14, 31). Klosen et al also showed that in SD hamsters, TSH reduces *Srif* expression to the LD state suggesting expression is downstream of photoperiod mediated change in hypothalamic T3 concentration (15). Unlike *Srif* expression in the PeVN, ARC expression was not affected by pasireotide. Consequently, if SRIF neurons in the ARC are important for seasonal physiological responses including growth, these neurons are resistant to GH feedback, which might be anticipated for a regulatory system that must reflect the prevailing photoperiod.

In the context of a broad role for somatostatin in suppressing the seasonal neuroendocrine axis, it is interesting to note that *Srif* expression is one of only a small number of genes showing increased expression in the ARC in SD photoperiod (10). We would hypothesize that ARC expressing *Srif* neurons project to the pituitary enabling direct regulation and possibly setting the tone of GH secretion upon which circadian regulation mediated by SRIF neurons of the PeVN is imposed. Some support for this hypothesis may be evident in the reported SD increase in somatostatin content of median eminence of the golden hamster and the observation of limited projection of SRIF containing ARC neurons to the median eminence in the dog (50, 51).

Daily GH injections resulted in increased growth of SD adapted Siberian hamsters, reaching significance compared to SD vehicle control hamsters within 14 days. This growth was in advance of growth in hamsters switched to LD, the latter not attaining significant difference until 21 days after SWB. However, both groups finished with the same body mass increase 28 days following application of GH treatment or exposure to LD. Analysis of serum IGF-1

showed a significant increase in SWB hamsters with several samples exceeding the top standard in the ELISA assay. This limited the statistical analysis to a Cox regression and consequently the small increase in IGF-1 as a result of GH injection was not detected as significant. The response of IGF-1 to GH injection was poor, but it has previously been noted that IGF-1 may not be a reliable pharmacodynamic marker of exogenous GH treatment in rodents (52). The increase in body mass was contributed by an increase in both liver and kidney mass for GH treated hamsters. Changes in these tissues approached significance in SWB hamsters, and had the hamsters remained in LD for a further 2-4 weeks to recover to their starting body weight, changes in this group would likely have achieved significance. The SWB hamsters, however, had significant increases in EWAT, IBAT and testes masses, none of which were increased by GH treatment suggesting that GH by itself is not involved in the seasonal regulation of these tissues.

Interestingly, liver mass was unresponsive to pasireotide in either the LD or SWB hamsters, but did respond to GH treatment in SD hamsters. This may indicate that while a pharmacological dose of GH is capable of inducing liver growth, regulation of liver mass in the Siberian hamster is not dependent on endogenous GH or IGF-1. A possible mediator of liver mass could be seasonal changes in prolactin (31, 53), the latter being regulated by tuberalin released by the pars tuberalis during summer and suppressed by melatonin during winter (54, 55) and known to promote normal liver growth, survival and regeneration in rodents (56).

Summary

Taken together, these data support a view of an involvement of the GH axis in photoperiod mediated body weight regulation of the Siberian hamster. A previous study in the rat had shown pasireotide reduced circulating IGF-1 and completely suppressed growth (32).

Although a direct action of pasireotide on peripheral tissues cannot be ruled out, we would reason that a reduction in GH underpins the response observed; firstly GH increases lean mass of SD hamsters, secondly somatostatin would have to act at several different tissues to reduce or suppress their mass simultaneously in a manner which mimicked the gradual decline seen SD or increase in LD respectively. A seasonal regulation of the GH axis is supported by studies in red deer showing a correlation between GH, IGF-1 and seasonal growth (16, 17) and photoperiod dependent GH secretion in the golden hamster (18). Therefore our data would extend the concept of a seasonal regulation of the GH axis to both large and small seasonal mammals. Intriguingly, the effect of pasireotide on multiple physiological parameters indicates somatostatin may have a broad role in suppressing multiple neuroendocrine pathways and provides evidence for an involvement of the neuroendocrine axes in regulating seasonal physiological adaptations that have not hitherto been considered to be under neuroendocrine regulation (white and brown adipose tissue). Although further work is required to demonstrate an involvement of GH in other seasonal mammals that exhibit Dehnel's phenomenon, (1, 2) our data may explain in part, the biological basis of this phenomenon.

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Conflict of interest

Herbert Schmid is an employee of Norvatis AG

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660 Table 1

Tissue (g)	Vehicle	Pasireotide
Liver	1.32 ± 0.07	1.25 ± 0.02
Kidneys	0.41 ± 0.02	0.34 ± 0.01***
EWAT	0.69 ± 0.07	0.45 ± 0.04*
IBAT	0.20 ± 0.01	0.15 ± 0.01**
Paired Testes	0.76 ± 0.04	0.19 ± 0.04***

661

662

663 Table 2

Tissue (g)	SD		SWB		Result of statistical comparison	
	Vehicle	Pasireotide	Vehicle	Pasireotide		
Liver	1.23 ± 0.05	1.19 ± 0.05	1.77 ± 1.11	1.57 ± 0.08	Photoperiod	P < 0.001
					Drug	NS
					Interaction	NS
Kidneys	0.38 ± 0.02	0.32 ± 0.01	0.48 ± 0.02	0.40 ± 0.01	Photoperiod	P < 0.001
					Drug	P < 0.001
					Interaction	NS
EWAT	0.37 ± 0.10	0.21 ± 0.03	0.75 ± 0.08	0.75 ± 0.10	Photoperiod	P < 0.001
					Drug	NS
					Interaction	NS
IBAT	0.11 ± 0.02	0.10 ± 0.01	0.24 ± 0.04	0.15 ± 0.01	Photoperiod	P < 0.001
					Drug	NS
					Interaction	NS
Paired Testes	0.13 ± 0.04	0.08 ± 0.02	0.88 ± 0.06	0.51 ± 0.07**	Photoperiod (KW)	P < 0.001
					Drug (KW)	NS

664

665

666 Table 3

667

Tissue (g)	Vehicle	GH	SWB
Liver	1.11 ± 0.04	1.36 ± 0.07*	1.37 ± 0.11
Kidneys	0.32 ± 0.01	0.38 ± 0.01*	0.37 ± 0.02
EWAT	0.18 ± 0.02	0.26 ± 0.03†	0.41 ± 0.05***
IBAT	0.11 ± 0.01	0.12 ± 0.01	0.19 ± 0.04
Paired Testes	0.06 ± 0.01	0.10 ± 0.02†††	0.51 ± 0.01***

668

669

Table legends

Table 1: Terminal tissue masses of LD-Siberian hamsters treated with vehicle or pasireotide for 49 d. Data are expressed as mean ± SEM. Significant differences signify the effect of pasireotide on organ mass, compared by paired t-test. *P < 0.05, ** P<0.01, ***P <0.001. IBAT, interscapular brown adipose tissue; EWAT, epididymal white adipose tissue. All groups n = 8.

Table 2: Terminal tissue masses of Siberian hamsters acclimated to SD before switch back to LD (SWB) or remaining in SD and treatment with pasireotide or vehicle for 49 d. Data are expressed as mean ± SEM. Statistical comparisons are by 2-way ANOVA or Kruskall-Wallis tests (KW) as indicated. ** P<0.01 effect of pasireotide within SWB, Mann-Whitney test. IBAT, interscapular brown adipose tissue; EWAT, epididymal white adipose tissue. SD-Vehicle n = 7, all other groups n = 8.

Table 3: Terminal tissue mass of SD Siberian hamsters treated with vehicle, pasireotide or switched back to LD (SWB) for 28 d. Data are expressed as mean ± SEM. Statistical comparisons are by 1 – way ANOVA with the exception of paired testes mass, compared by Mann-Whitney tests. † P < 0.05, ††† P < 0.001 compared to SWB. * P < 0.05, *** P < 0.001 compared to Vehicle. IBAT, interscapular brown adipose tissue; EWAT, epididymal white adipose tissue; SD, short day photoperiod (8 : 16 h light : dark). Vehicle and GH both n = 12, SWB n = 6.

692 **Figure legends**

693 Figure 1. Pasireotide reduces body mass in LD-acclimated male Siberian hamsters. Change in
694 body mass (A) of hamsters housed in LD treated with vehicle or pasireotide for 49 days,
695 statistical differences indicated are between vehicle and pasireotide groups. Overall fat mass
696 (B) and lean mass (C) of vehicle and pasireotide treated hamsters post dissection, measured
697 by MRI. Terminal serum IGF-1 (D) and insulin (E) measured by ELISA and serum glucose
698 (F). Statistical comparison carried out by paired t-test, *P<0.05, **P<0.01, ***P<0.001, n.s.:
699 not significant. All groups n = 8. Data are all expressed as mean ± SEM.

700

701 Figure 2. Pasireotide does not directly alter photoperiod programmed hypothalamic gene
702 expression, but does alter mRNA expression stimulated by GH-axis feedback to the
703 hypothalamus in LD acclimated Siberian hamsters. Relative mRNA expression of *Dio2* (A),
704 *Srif* (B), and *Ghrh* (D) in the arcuate nucleus (ARC) and periventricular nucleus (PeVN - D).
705 Statistical comparisons are by paired t-test; ***p<0.001, n.s.: no significant differences;
706 example images showing mRNA expression pattern are shown for vehicle and pasireotide
707 treatments. All groups n = 8. Data are all expressed as mean ± SEM relative to vehicle group
708 data. Shown is a representative section of the named mRNA expression in the measured
709 region of the hypothalamus.

710

711 Figure 3. Pasireotide inhibits LD photoperiod driven body mass accretion. Change in body
712 mass of hamsters treated with vehicle or pasireotide and returned to LD or remaining in SD
713 photoperiod (A) **p<0.01, ***p<0.001 and different to all other groups, a: p<0.05 SWB-
714 Pasireotide vs SD-Pasireotide; b: p<0.05 SWB-Pasireotide vs both SD groups. Lean (B) and

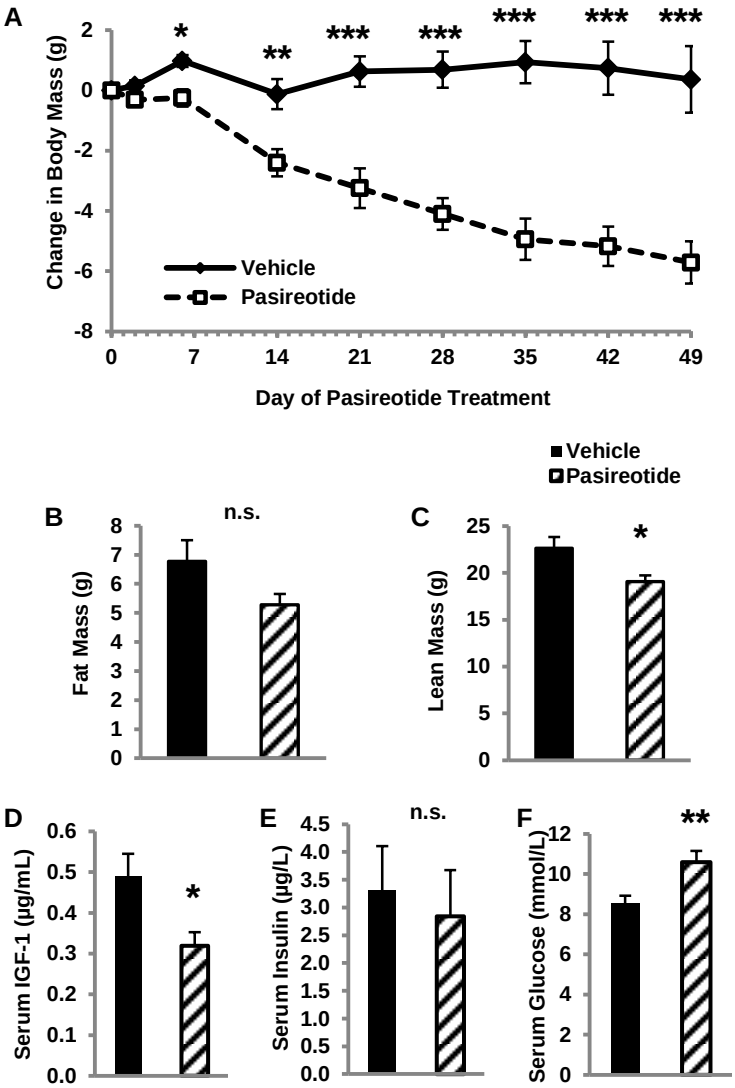
715 fat (C) mass measured by MRI *p<0.05, ***p<0.001. Terminal serum IGF-1(D); * p<0.05
716 compared to all groups. Terminal insulin (E); *p<0.05. Serum glucose (F); *p<0.05.
717 Statistical comparisons are by 2-way ANOVA with the exception of E, compared by Cox
718 regression. SD-Vehicle n = 7, SD-Pasireotide, SWB-Vehicle and SWB-Pasireotide all n = 8;
719 data are all expressed as mean ± SEM.

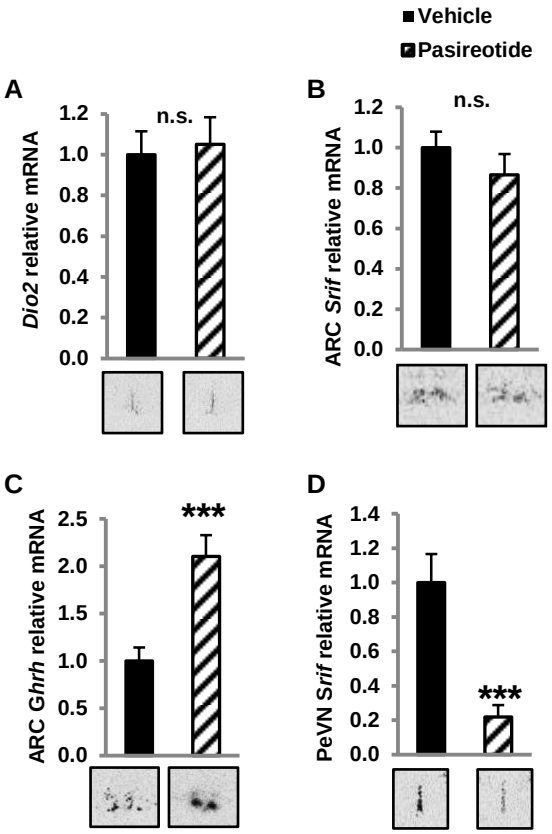
720

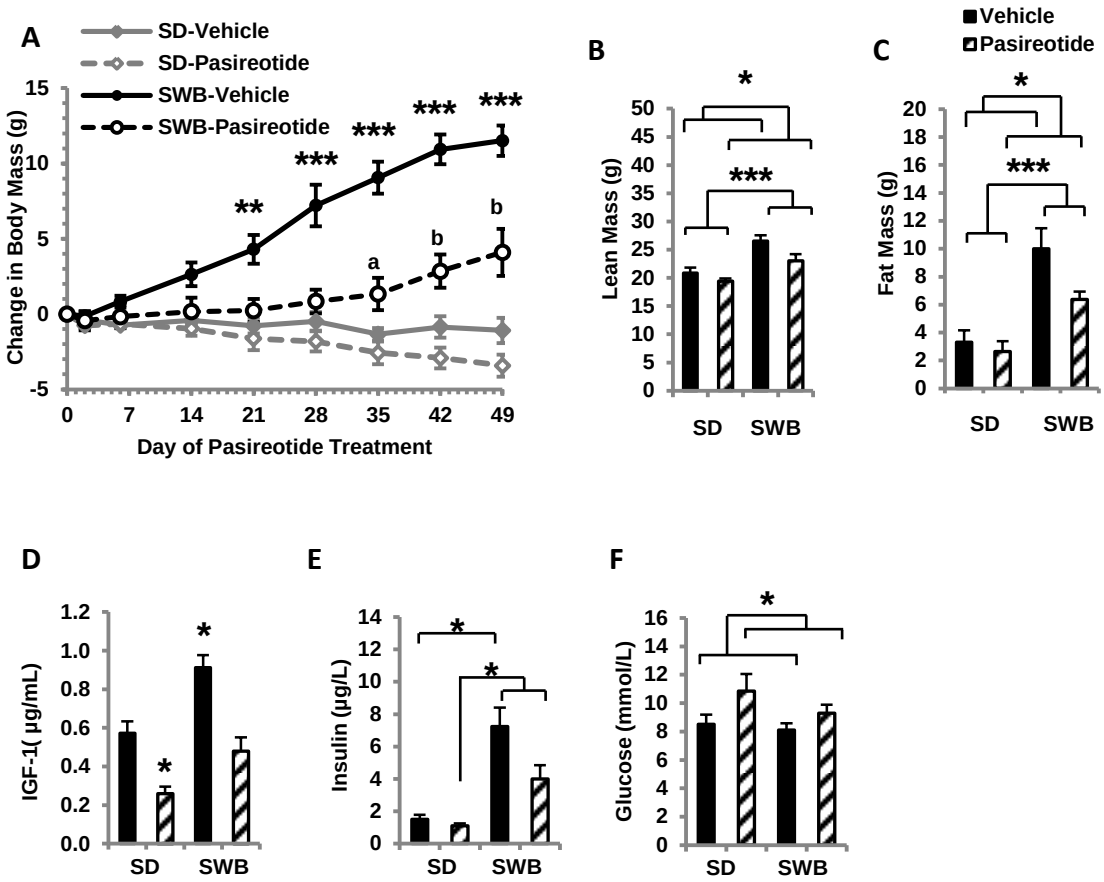
721 Figure 4. Pasireotide does not directly alter photoperiod programmed hypothalamic gene
722 expression in SD and SWB hamsters, but does alter mRNA expression stimulated by GH-axis
723 feedback to the hypothalamus. Relative mRNA expression of *Dio 2* (A), *Dio 3* (B), *Srif* (C) in
724 the arcuate nucleus (ARC), *Srif* (D) in the Periventricular nucleus (PeVN) and of *Ghrh* (E)
725 in the ARC. Statistical comparison is by 2-way ANOVA or Kruskal-Wallis and Mann
726 Whitney tests (B), **p<0.01, ***p<0.001, n.s.: no significant differences; example images
727 showing mRNA expression pattern are shown for SD and SWB treatments (A-C) or Vehicle
728 and Pasireotide treatments (D,E). All groups n=8 except for panels A and B: SD-Vehicle n =
729 7; Panels C, D and E: ARC *Srif* and *Ghrh*, SD-Vehicle n = 7, SWB-Vehicle n = 7. Data are
730 expressed as mean ± SEM, relative to SD-Vehicle.

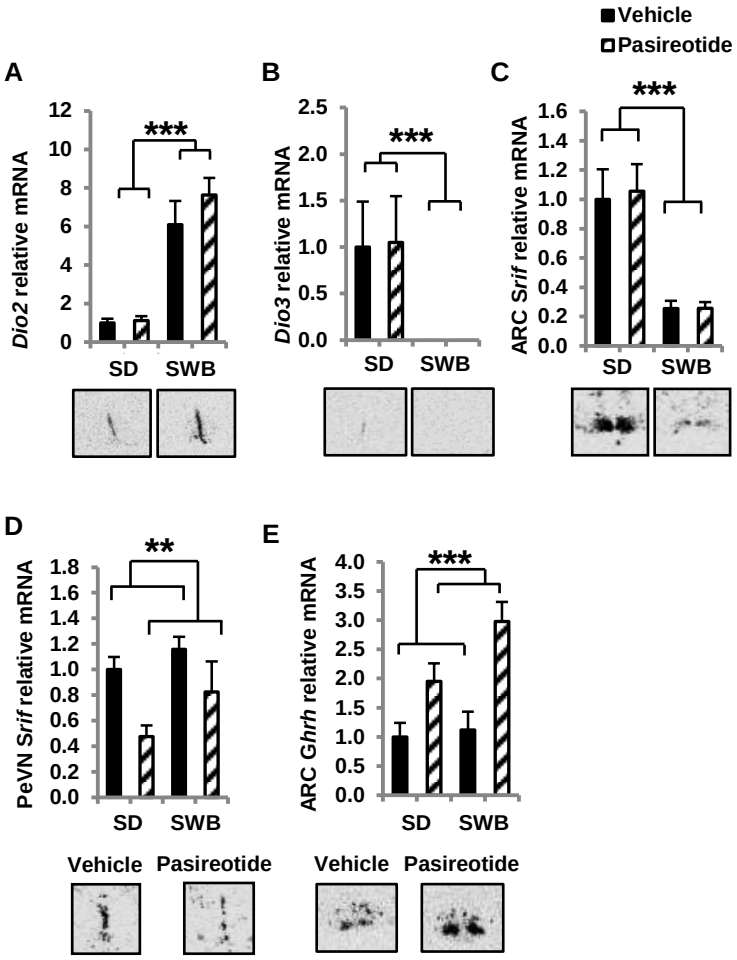
731

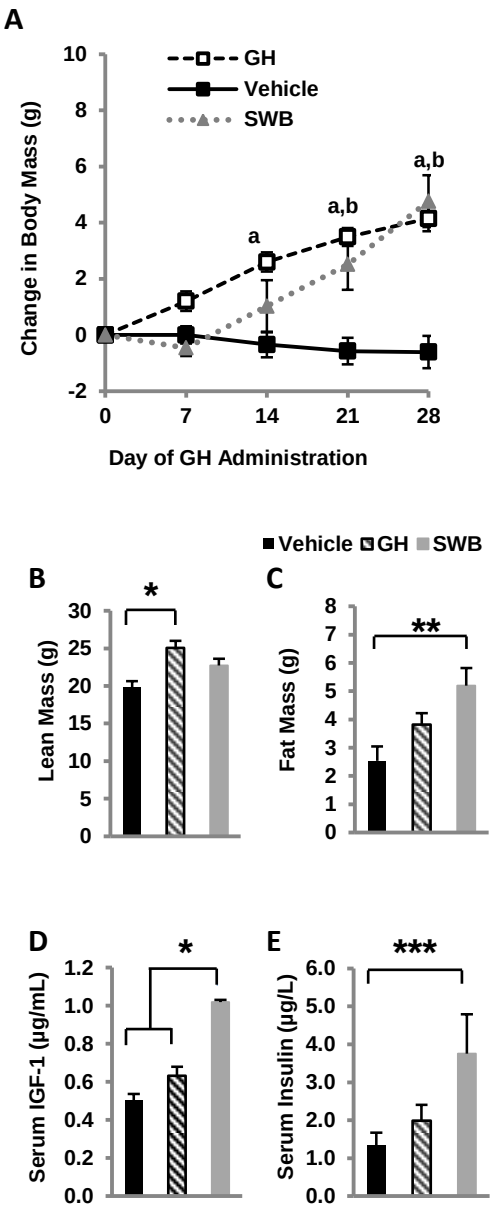
732 Figure 5. Comparison of effect of exogenous bovine GH and LD photoperiod (SWB) on body
733 mass, and circulating IGF-1 and insulin concentrations in SD Siberian hamsters. Change in
734 body mass of hamsters previously acclimated to SD before daily administration of GH or
735 vehicle or switched to LD for 28d; a:P<0.05 Vehicle vs. GH; b:P<0.05 SWB vs. Vehicle (A);
736 effect on lean mass (B); fat mass (C); terminal serum IGF-1(D) and insulin (E) concentration.
737 Comparisons are by 1-way ANOVA; *P<0.05, ***P<0.001; Vehicle and GH both n = 12,
738 SWB n = 6. Data are all expressed as mean ± SEM.


A
Change in Body Mass (g)
2
0
-2
-4
-6
-8
*
**
***
***
***
***
***
Vehicle
Pasireotide
0
7
14
21
28
35
42
49
Day of Pasireotide Treatment
Vehicle
Pasireotide
B
n.s.
Fat Mass (g)
8
7
6
5
4
3
2
1
0
C
Lean Mass (g)
25
20
15
10
5
0
*
D
Serum IGF-1 (µg/mL)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
E
n.s.
Serum Insulin (µg/L)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
F
**
Serum Glucose (mmol/L)
12
10
8
6
4
2
0


Vehicle
Pasireotide
A
Dio2 relative mRNA
n.s.
B
ARC Srif relative mRNA
n.s.
C
ARC Ghrh relative mRNA
***
D
PeVN Srif relative mRNA
***


A
SD-Vehicle
SD-Pasireotide
SWB-Vehicle
SWB-Pasireotide
Change in Body Mass (g)
Day of Pasireotide Treatment
B
Lean Mass (g)
SD
SWB
C
Vehicle
Pasireotide
Fat Mass (g)
SD
SWB
D
IGF-1(µg/mL)
SD
SWB
E
Insulin (µg/L)
SD
SWB
F
Glucose (mmol/L)
SD
SWB


Vehicle
Pasireotide
A
B
C
Dio2 relative mRNA
Dio3 relative mRNA
ARC Srif relative mRNA
***
SD
SWB
D
E
PeVN Srif relative mRNA
ARC Ghrh relative mRNA
**
Vehicle
Pasireotide


A
GH
Vehicle
SWB
Change in Body Mass (g)
10
8
6
4
2
0
-2
0
7
14
21
28
Day of GH Administration
a
a,b
a,b
Vehicle
GH
SWB
B
Lean Mass (g)
30
25
20
15
10
5
0
*
C
Fat Mass (g)
8
7
6
5
4
3
2
1
0
**
D
Serum IGF-1 (µg/mL)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
E
Serum Insulin (µg/L)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
***

Somatostatin agonist pasireotide promotes a physiological state resembling short-day acclimatization in the photoperiodic male Siberian hamster (*Phodopus sungorus*)

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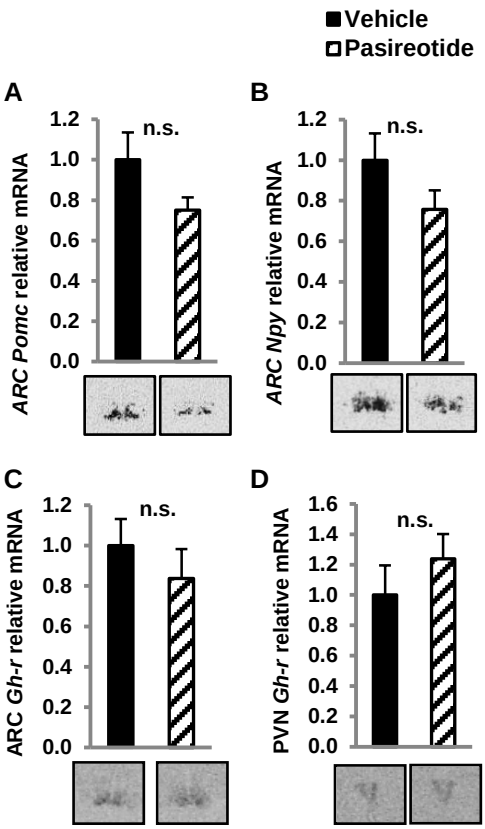
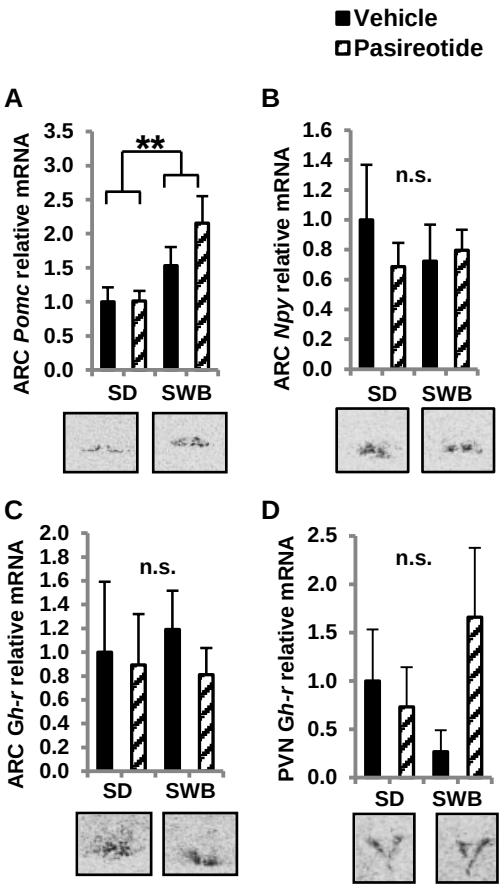
[3]Novartis Pharma AG, WSJ-103.5.10.1, CH-4002 Basel, Switzerland



Supplementary figure 1. Relative mRNA expression in the hypothalamus of LD hamsters vehicle or pasireotide treated of Pomc (A), Npy (B), Gh-r (C) in the arcuate nucleus (ARC) and Gh-r (D) in the periventricular nucleus (PeVN). No significant differences (n.s.) were found, n=8 for both groups.



Supplementary figure 2. Relative mRNA expression in the hypothalamus of SD acclimated hamsters remaining in SD or to LD (SWB) following administration of either vehicle or pasireotide. Pomc (A), Npy (B), Gh-r (C) in the arcuate nucleus (ARC) and Gh-r (D) in the periventricular nucleus. **p<0.01, n.s. no significant differences; n=8 all groups except SD-vehicle, n=7.