Somatostatin agonist pasireotide inhibits exercise stimulated growth in the male Siberian hamster (*Phodopus sungorus*)

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Abstract

The Siberian hamster (*Phodopus sungorus*) is a seasonal mammal, exhibiting a suite of physiologically and behaviourally distinct traits dependent on the time of year and governed by changes in perceived day length (photoperiod). These attributes include significant weight loss, reduced food intake, gonadal atrophy, and pelage change with short day photoperiod as in winter. The central mechanisms driving seasonal phenotype change during winter are mediated by a reduced availability of hypothalamic tri-iodothyronine (T3), but downstream mechanisms responsible for physiological and behavioural changes are yet to be fully elucidated. With access to a running wheel (RW) in short photoperiod, Siberian hamsters which have undergone photoperiod mediated weight loss override photoperiod-drive for reduced body weight and regain weight similar to a hamster held in long days. These changes occur despite retaining the majority of hypothalamic gene expression profiles appropriate for short day hamsters. Utilising the somatostatin agonist pasireotide, we have recently provided evidence for an involvement of the growth hormone axis (GH axis) in the seasonal regulation of bodyweight. In the present study we employed pasireotide to test for the possible involvement of the GH axis in running wheel induced body weight regulation. Pasireotide successfully inhibited exercise stimulated growth in short day hamsters and this was accompanied by altered hypothalamic gene expression of key GH axis components. Our data provides support for an involvement of the GH axis in the RW response in Siberian hamsters.

Introduction

Many species that have evolved for life in a seasonally variable environment have developed physiological strategies to optimise survival. The Siberian hamster is well described for its ability to reduce energy expenditure in winter or short day photoperiod (SD), with a suite of physiological adaptations that include reduced body mass and food intake, cessation of reproduction, altered pelage and the ability to employ a controlled hypometabolic state in the form of daily torpor (1). These responses which occur naturally in the wild, can be induced in the laboratory by providing a shortened photoperiod length (2), allowing for the convenient investigation of underlying mechanisms driving these altered physiological processes. This seasonal phenotypic plasticity is underpinned by an alteration of hypothalamic gene expression primarily of deiodinase enzymes which regulate local thyroid hormone (T3) availability (3). In the Siberian hamster, approximately 50% of SD weight loss comes from fat mass; the remaining is composed of lean, or fat-free mass (4, 5), and in male hamsters fat is mainly lost from abdominal depots (6). This physiological response occurs in a variety of small mammals and was first observed in the seasonal common shrew by Dehnel and later termed Dehnel's phenomenon (7, 8).

In the Siberian hamster, Dehnel's phenomenon is reversed by wheel running activity (4, 9-11), a phenomenon that has also been observed in the Syrian hamster (*Mesocricetus auratus*) (12-15) and gerbils (*Meriones unguiculatus*) (16). With access to a running wheel (RW) the Siberian hamster will run for long durations during the normal active phase, and in response will increase food intake and gain weight (4, 10). This response occurs to a lesser extent in hamsters adapted to long day photoperiod (LD; 16:8 h light:dark), but is particularly apparent in hamsters adapted to SD (8:16 h light:dark) (10, 17). SD hamsters undertaking RW exercise maintain photoperiod appropriate pelage while torpor is prevented and testicular atrophy is partially reversed, and at the same time will gain body mass so that over a period of weeks they increase to a size more appropriate for an LD hamster (4, 9, 11). This increase in body mass is composed of both lean and fat tissue (4, 11), accompanied by increased overall body length, bone length and mineral content (10, 11) and is thus indicative of somatic growth. Despite attaining LD metabolic physiology, SD exercised hamsters largely retain hypothalamic gene expression profiles of SD sedentary hamsters, with only evidence of a partial reversal of the photoperiod mediated reduction in *Pomc* gene expression and a slower temporal increase in arcuate nucleus (ARC), somatostatin (*Srif*) mRNA (8).

Somatostatin is an inhibitory hormone that is expressed widely in the central nervous system and is an integral component of the growth hormone (GH) axis. The majority of SRIF projections from the hypothalamus to the median eminence are from neurons in the periventricular nucleus (PeVN),

which play the main role in the regulation of pituitary GH secretion (18, 19). To positively regulate GH secretion, growth hormone-releasing hormone (GHRH) is produced in the ventromedial hypothalamus and ARC, with the latter being those neurons primarily projecting to the median eminence in order to reach the somatotrophs of the anterior pituitary via the hypophyseal portal system (20).

Pasireotide (also called SOM230) is a somatostatin analogue that was developed to treat GH secreting pituitary tumours in acromegaly (21). Pasireotide acts with high affinity at 4 of the 5 known somatostatin G-protein coupled receptor subtypes ($SSTR_{1,2,3,5}$), with particularly high affinity for $SSTR_5$ (22, 23). Pasireotide mimics the action of somatostatin, inhibiting the secretion of pituitary GH in human and rodent tissue *in vitro* (24), and GH and IGF-1 secretion *in vivo*, which is in turn accompanied by impaired body mass gain in rodents (25, 26). We have previously shown that following 7 weeks of administration to LD hamsters, pasireotide led to significant weight loss, accompanied by a decrease in circulating IGF-1. However, SD hamsters administered with pasireotide did not lose further weight, but when subsequently switched back to LD, pasireotide significantly inhibited weight re-gain stimulated by LD photoperiod. In addition, pasireotide altered hypothalamic expression of GH axis genes including *Srif* in the PeVN, *Ghrh* in the ARC and supressed a rise in circulating IGF-1, suggesting a key role for the GH axis in the regulation of seasonal body mass (27). In the Syrian hamster, reversal of photoperiod induced physiology by exercise is accompanied by growth and an increase in pulsatile secretion of several pituitary hormones including GH (28). Together with the aforementioned study, we hypothesised that the GH axis may also be involved in the RW stimulated weight gain demonstrated in the Siberian hamster. Therefore, we established an experiment to mirror our previous study, with pasireotide administered in a long acting release form (LAR – 28 day) to SD acclimated hamsters, given access to a RW instead of manipulating the photoperiod.

Methods

Animals and tissue collection

All animal experiments and general husbandry were in accordance with the German Animal Welfare Act, and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (license no. 12/1023). All experiments were performed using adult male Siberian hamsters from a colony maintained at the University of Veterinary Medicine, Hannover. Hamsters were bred under natural photoperiod at a latitude of 52°N under ambient temperatures, before transferral

to artificial LD (16:8 h light:dark) after weaning. Food (hamster breeding diet, Altromin 7014, Lage, Germany) and water were available *ad libitum* throughout, supplemented by a weekly slice of apple, and during experiments the hamsters were singly housed. 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). For hamsters provided with a running wheel (RW; 14.5cm inner diameter), wheel revolutions were registered and stored at 6 min intervals. For comparison, mean daily distance run per hamster, calculated over the 49 day experiment was compared. In the pasireotide experiment, further sub-analyses were carried out, with daily running distance compared over the course of the experiment, and to assess if the behavioural pattern differed over the course of the dark (active) phase, wheel revolutions in 2 h bins were compared, taking the mean wheel revolutions during each bin for each hamster over the 49 day experiment. At the end of the experiments, in order to determine any acute effects of exercise, non-fasted hamsters were sacrificed by CO_2 overdose followed by cervical dislocation in the dark (active) phase between zeitgeber time (ZT) 12 and 13, where ZT 0 and ZT8 corresponds to time of lights on and off respectively. This timing was chosen so that we could take into account potential changes in circulating hormone levels due to RW activity. The brain, and trunk blood (from which serum was prepared), were collected and stored at -70˚C before use. Testes and liver were dissected and immediately weighed. In the pasireotide experiment these were returned to carcasses, which were stored at -70˚C and later thawed for body composition analysis.

Pasireotide RW experiment

Thirty adult male Siberian hamsters were initially acclimated to SD (8:16 h light:dark) for 10 weeks (69-72 days) before they received a subcutaneous dose of pasireotide LAR (Novartis, Basel Switzerland;160mg/kg based on weight on day of administration) or vehicle only. On the day of administration, hamsters were further split into running wheel and sedentary groups, of which one vehicle and one pasireotide group received a RW (RW-Vehicle, RW-Pasireotide both n=7) and the remaining two groups did not (Sedentary-Vehicle, Sedentary-Pasireotide, both n=8). Pasireotide and vehicle treatment was repeated 28 days following the first administration. Body mass was measured from day 2 onwards following pasireotide or vehicle treatment at 4-day intervals, and the change in body mass from the start of pasireotide or vehicle administration was calculated and compared. The initial ambient temperature of 22 ± 1°C was lowered to 20 ± 1°C on day 20 to better facilitate the expression of torpor, which has been reported elsewhere (29).

Hamsters were sacrificed as described above, after 49 days (7 weeks) of treatment and/or running wheel access.

Body composition of dissected hamsters (with brain removed) was determined by nuclear magnetic resonance imaging (MRI; Echo MRI™ Whole Body Composition Analyser, Echo Medical Systems, Houston, Texas). Previously frozen decapitated bodies were doubly sealed in plastic bags, sealed bags then disinfected and warmed to 37°C in a water bath before measurement. Three measurements per carcass were taken, and the mean of these measurements accepted. Data are compared directly and as a ratio of lean:fat mass. Serum glucose was determined by ACCU-CHEK AVIVA glucose monitor and test strips. ELISAs were performed on thawed serum according to manufacturers' instructions. Serum insulin concentration was measured using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden; cat. no. 10-1250-01), with intra assay CVs of 6.93% and 6.35%, and an inter assay CV of 6.65%. One RW-Vehicle sample was excluded due to a very high %CV between technical replicates, reducing this group to an n=6.

12 week RW experiment

Food intake and hypothalamic *Ghrh* mRNA expression was investigated in a 12-week RW experiment (4). Briefly, 48 adult male Siberian hamsters were acclimated to either LD or SD, as described above, for 2 weeks before given access to a RW (LD-RW, SD-RW; both n=10) or not (LD-C, n=10; SD-C, n=8). The hamsters were sacrificed as described above, after 84 days (12 weeks) following RW introduction as described above. Body mass and RW revolutions were measured during the course of the experiment and terminal organ mass (liver and testes) were recorded. Due to the loss of one LD-RW slide during preparation, the *Ghrh* in situ experiment was reduced to n=9 for this group. Because of excessive crumbling of the food, food intake data was excluded for several hamsters, reducing sample sizes; SD-RW: n=4, SD-Sedentary: n=5, LD-RW: n=10, LD-Sedentary, n=9.

Open flow Respirometry Experiments

Sedentary hamsters were monitored for a period of 2 or 3 days between days 13-20 of the experiment, by open flow respirometry, carried out in their home cages with dimensions 24.5cm x 15cm x 15cm and a volume approximately 5.5L. VO_2 and VCO_2 were measured with a FOXBOX field gas analyser (Sable systems, NV, USA) at a flow rate of 35-40 L/hour. Measurements were taken every 1 in 6 minutes, for 5 hamsters per session, and were adjusted

according to an air reference channel. The body mass specific metabolic rate and respiratory quotient (RQ) were calculated, taking the bodyweight as the mean from the two closest weigh dates (4 days apart).

Riboprobe synthesis

Riboprobes complementary to DNA sequence fragments were generated from Siberian hamster, mouse or rat brain cDNA by RT-PCR as previously described (3, 27, 30-32). Templates were generated by PCR amplification of the insert from plasmid DNA with M13 forward and reverse primers located 5' upstream to polymerase transcription sites in host vectors. Approximately 100ng of PCR product were used in an *in vitro* transcription reaction with T7, T3 or SP6 polymerases as appropriate in the presence of ^{35}S-uridine 5-triphosphate (Perkin-Elmer, Bucks, UK) for radioactive in situ hybridisation.

In situ hybridisation

Coronal hypothalamic sections were cryosectioned at 14µm and mounted on poly-L-lysine coated slides (ThermoScientific, Rockford, IL, USA). Radiolabelled in situ hybridisation was carried out as previously described (33). Briefly, slides were fixed in 4% PFA-0.1 M PB, acetylated in 0.25 % acetic anhydride-0.1 M triethanolamine, pH 8. Radioactive probes (approx. 106 counts per minute per slide) were applied to the slides in 70 µl hybridisation buffer containing 0.3 M NaCl, 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.05% tRNA, 10 mM DTT, 0.02 % Ficoll, 0.02 % polyvinylpyrrolidone, 0.02 % BSA and 10 % dextran sulphate. Hybridisation was performed overnight (approx. 16 h) at 58˚C. The following day, slides were washed in 4 x SSC (1 x SSC is 0.15 M NaCl, 15 mM sodium citrate), treated with Ribonuclease A (20 µg/µl) at 37°C and washed in SSC to a stringency of 0.1 x at 60˚C. Dehydrated slides were exposed to Biomax MR film (Kodak, Rochester, NY, USA) for 16 h – 14 days as appropriate.

Image analysis

Autoradiographic films were scanned at 600 d.p.i. to a computer running Image Pro Plus v. 6.8 or v. 7.0 (Media Cybernetics, Marlow, UK). Integrated optical density of mRNA expression was obtained in reference to a ^{14}C microscale and measured in 2-5 sections per slide for each probe as appropriate, and the accumulated count (arbitrary units) was compared. For presentation purposes,

integrated optical density is expressed relative to the sedentary-vehicle or LD-C group, whose value is defined as 1.

Statistical Analysis

Data are expressed as mean ± SEM and analysis was carried out using Minitab v. 15.0 (Minitab, PA, USA) or GraphPad Prism v. 7.0 (Graphpad, CA, USA). Statistical tests used were 2-way ANOVA (general linear model) with Tukey post hoc tests, or two sample t-tests unless stated. P-values less than 0.05 were considered statistically significant. Where data did not conform to assumptions of an ANOVA, it was transformed by log10 or square root, and statistics were performed on transformed data (log_{10}: serum glucose and insulin data; square root: *Ghrh* in situ data). When data could not be transformed to fit assumptions of the parametric test, Kruskall-Wallis (KW) and/or Mann-Whitney (MW) tests were performed, these instances are indicated in the text as appropriate. RW time course data were compared by 2-way RM-ANOVA followed by Sidak's multiple comparison tests for differences between pasireotide and vehicle data. Correlations between distance travelled and change in body mass were investigated by linear regression. For the change in body mass data, 2-way ANOVAs were carried out at each time point, with pasireotide and RW access as factors.

Results

Effect of pasireotide on RW activity stimulated weight gain, body composition and organ mass

Representative actograms are shown in figure 1A, here the RW activity is double plotted by aligning two consecutive days horizontally and each 24h is plotted twice (34), with the full 49 days shown. Pasireotide treatment did not significantly alter the daily distance run by RW hamsters, (Vehicle: 24,458 ± 3945 revolutions, 11.14 ± 1.80 km; pasireotide: 32,947 ± 4520 revolutions, 15.01 ± 1.93 km. $p=0.185$, figure 1B). RW hamsters can be expected to decrease daily wheel running activity over time (10), and despite a brief drop in activity following the 2nd injection for vehicle hamsters, pasireotide did not alter daily wheel running activity over the course of the experiment, with only an overall effect of time ($F(48, 624)=2.038$, $p<0.001$); however there were no significant effects in post hoc analyses (figure 1C). In order to detect if pasireotide altered the pattern of wheel running behaviour, mean revolutions per 2 h of the dark phase from the 49 experimental days were compared. There was a significant time effect ($F(7, 91)=30.31$, $p<0.001$), with the greater amount of wheel running occurring in the first 10 h of the dark phase, but no overall significant pasireotide effect was detected. And despite a significant interaction ($F(7, 91)=2.722$, $p=0.013$) at no individual 2 h time-point did vehicle and pasireotide RW activity

significantly differ (figure 1D). Very little wheel running activity occurred during the light phase, and this did not differ with pasireotide (RW-Vehicle: 1.93±0.56 revolutions, RW-Pasireotide: 1.56±0.65 revolutions).

As expected, RW activity caused weight gain (figure 2A; day 49: $F(1,26)=72.38$, $p<0.001$), and the increase in body mass for RW-Vehicle hamsters reached significance compared to all others after only 10 days. Pasireotide attenuated RW induced weight gain (day 49: $F(1,26)=13.58$, $p=0.001$), with RW-pasireotide hamsters reaching significant weight gain compared to sedentary counterparts by day 34, and differing from all other groups by day 46. The RW stimulated weight gain was attenuated by pasireotide, demonstrated by a significant interaction between these factors (day 49: $F(1,26)=6.05$, $p=0.021$). No correlation was found between the cumulative distance run over the course of the experiment and change in body mass for either pasireotide or vehicle treated hamsters (figure 2B $r^2=0.004$, $p=0.894$; $r^2=0.182$, $p=0.292$ respectively). Furthermore, we determined whether pasireotide might directly alter energy expenditure using open flow respirometry. In sedentary animals, metabolic rate and respiratory quotient did not differ in pasireotide compared with vehicle treated animals, measured overall, or in either the light or dark phase (supplementary table 1 and supplementary figure 1).

Overall, lean mass of dissected carcasses was significantly increased by RW activity ($F(1,26)=35.78$, $p<0.001$), and this was suppressed by pasireotide treatment ($F(1,26)=13.94$, $p=0.001$). There was a significant interaction between factors ($F(1,26)=15.43$, $p=0.001$); with the RW-vehicle hamsters having a greater lean mass than all other treatment combinations ($p<0.001$ all comparisons; figure 2C). Fat mass was only raised by RW activity ($F(1,26)=7.25$, $p=0.012$; figure 2D). Since body composition is a relative measure, and that the final body mass overall differed dramatically between groups, the ratio of lean-to-fat mass in dissected carcasses was compared, and RW hamsters were found to have a small but significantly greater proportion of fat mass overall (sedentary-vehicle: 0.905:0.095 ± 0.017, sedentary-pasireotide: 0.872:0.128 ± 0.020, RW-vehicle: 0.861:0.139 ± 0.024, RW-pasireotide: 0.836:0.164 ± 0.012 lean:fat mass; $F(1,26)=4.37$, $p=0.047$; figure 2E).

Paired testes mass was significantly increased by RW activity (KW; $p<0.001$) but not altered by pasireotide (KW; $p=0.852$) and within RW and sedentary groups there were no significant effects of pasireotide (figure 2F). Liver mass was used as an indicator of internal organ mass and was significantly increased by RW activity (figure 2G; $F(1,26)=23.74$, $p<0.001$), and the effect of pasireotide and interaction between treatments both approaching significance ($F(1,26)=2.94$, $p=0.098$ and $F(1,26)=3.32$, $p=0.080$ respectively; figure 2G). In post hoc analyses, RW-Vehicle

hamsters had significantly greater liver mass than sedentary counterparts ($p<0.001$) but compared with RW-Pasireotide, this difference did not reach significance ($p=0.098$). Interestingly, when liver mass was compared as a proportion of body mass, the trend for a pasireotide effect was abolished and only the RW effect remained ($F(1,26)=7.18$, $p=0.013$; Sedentary-Vehicle: 48.13±1.64mg/g; Sedentary-Pasireotide: 47.38±1.23mg/g; RW-Vehicle: 53.78±2.40mg/g; RW-Pasireotide: 51.67±1.62mg/g).

Effects of RW activity on serum glucose and insulin concentrations

Terminal (non-fasted) glucose concentrations did not differ between any of the treatment groups (Figure 3A), however serum insulin was significantly raised in RW hamsters ($F(1,25)=4.42$, $p=0.046$) and this was independent of pasireotide treatment (figure 3B).

Effect of RW activity and pasireotide hypothalamic mRNA expression

Two key genes in the regulation of photoperiod regulated seasonal phenotype change are those encoding for deiodinase enzymes types II and III (*Dio2* and *Dio3*) which regulate central availability of active thyroid hormone. In accordance with previous work (4, 27), when measured by *in situ* hybridization, expression of neither *Dio2* nor *Dio3* differed with RW activity or pasireotide treatment (figures 4A and 4B respectively). Since RW stimulated weight gain in the Siberian hamster is accompanied by increased food intake (4), expression of two appetite regulating peptides *Pomc* and *Npy* were measured in the ARC. *Pomc* expression was raised by RW activity independent of pasireotide ($F(1,26)=13.05$, $p=0.001$, figure 4C) and this was also the case for *Npy* expression ($F(1,26)=5.40$, $p=0.029$; figure 4D).

In order to determine whether the observed growth effects may be mediated by an alteration of the central growth hormone axis or by systemic feedback to this axis, hypothalamic expression of *Srif* in the ARC and PeVN as well as *Ghrh* and *Gh-r* in the ARC were measured. Arcuate nucleus *Srif* expression was not altered by RW activity or pasireotide, although a decrease with RW activity approached significance ($F(1,26)=3.54$, $p=0.071$; figure 4E). *Srif* expression in the PeVN was significantly increased with RW activity ($F(1,26)=14.93$, $p=0.001$) and decreased by pasireotide ($F(1,26)=8.63$ $p=0.007$; figure 4F). Expression of *Ghrh* in the ARC was increased only by RW activity ($F(1,26)=5.44$ $p=0.028$, figure 4G). Similarly, *Gh-r* expression in the ARC was unaltered by pasireotide and significantly increased overall in RW hamsters ($F(1,26)=9.33$, $p=0.005$; figure 4H).

Effect of RW on Ghrh expression and food intake in LD and SD hamsters

In a second RW experiment, hamsters exposed to LD or SD for a period of two weeks before 12 weeks of RW access, body weight was significantly decreased for the SD-Sedentary hamsters compared to all others by day 35 ($p \leq 0.05$). At the end of the experiment there were significant effects on body mass, with both photoperiod and RW activity, with interaction (Photoperiod: $F(1,34)=16.46$, $p<0.001$; RW Activity: $F(1,34)=33.23$, $p<0.001$; Interaction: $F(1,34)=5.263$, $p=0.028$ figure 5A). For the RW hamsters, photoperiod did not affect the mean distance run (figures 5B, C). As previously described (4), food intake increased for RW hamsters and with LD photoperiod, (Photoperiod: $F(1,24)=5.15$, $p=0.033$; RW Activity: $F(1,24)=50.71$, $p<0.001$; Interaction: $F(1,24)=0.09$, $p=0.764$, supplementary figure 2). As expected, paired testes mass was significantly reduced in SD hamsters with no overall effect of RW activity (KW; Photoperiod: $H=27.08$, $p<0.001$; RW Activity: $p=0.279$). However, in pairwise comparisons both SD-Sedentary and SD-RW hamsters had significantly different paired testes mass compared to all other groups (MW; $p<0.001$, all comparisons), with the SD-RW hamsters having a mid-range mass compared to the SD-Sedentary and LD hamsters (figure 5D). RW access significantly increased liver mass, with interaction between RW access and photoperiod (Photoperiod: $F(1,34)=2.19$, $p=0.148$; RW Activity: $F(1,34)=22.82$, $p<0.001$; Interaction: $F(1,34)=6.10$, $p=0.019$), with SD-C having a lower liver mass than all other groups (vs. LD-C $p=0.049$; vs. SD-RW, $p<0.001$; vs. LD-RW $p<0.001$; figure E). Liver mass as a function of body mass did not significantly differ between groups (LD-RW: 46.29 ± 1.50mg/g; LD-C 45.74 ± 1.65mg/g; SD-RW: 49.83 ± 3.01mg/g; SD-C: 44.53 ± 0.63mg/g).

Expression of ARC *Ghrh* was significantly raised by RW overall and with a trend for an effect of photoperiod (RW Activity: $F(1,33)=8.18$, $p=0.007$; Photoperiod: $F(1,33)=3.32$, $p=0.078$; Interaction: $F(1,33)=0.85$, $P=0.362$, figure 5F).

Discussion

The present study aimed to provide evidence to support the hypothesis that the GH axis is involved in the exercise induced growth response of the Siberian hamster by disrupting the hypothesised stimulation of this axis with the somatostatin analogue, pasireotide and by measuring the expression of key GH axis components in the hypothalamus.

Pasireotide treatment significantly retarded the body weight increase caused by access to a RW. Further, access to RW increased expression of *Srif* in the PeVN indicative of increased feedback to the inhibitory arm of the GH axis, and increased *Ghrh* expression in the ARC, indicative of increased stimulatory drive to the GH axis. Furthermore, pasireotide reduced the RW-induced *Srif* expression in the PeVN indicative of a reduction in GH feedback to the hypothalamus. Since pasireotide is not expected to cross the blood brain barrier, and reduces circulating IGF-1 (25-27), the most likely explanations for pasireotide retardation of RW induced growth are inhibition of GH secretion from the pituitary or inhibition of IGF-1 secretion from the liver, although as discussed below, other mechanisms may be involved.

The RW response phenomenon may have evolved in this species in order to take advantage of favourable conditions such as a mild winter or an early spring to reproduce early and maximise offspring number and survival. Wheel running is not a natural behaviour but can represent a natural drive to be active. Indeed, running wheels placed in the wild are taken advantage of by a surprising variety of species (35) and it may be a self-rewarding behaviour. The hamsters of the present study ran for comparable distances to that previously reported (11) and any trend for reduced mean daily distance run in vehicle hamsters did not reach significance. A temporary and non-significant decline in daily distance run by vehicle hamsters following the second administration may account for this apparent trend. As expected for animals housed in SD (36), further sub-analysis of daily RW activity over the dark period showed the peak of activity in the first of half of the night for all RW hamsters, independent of pasireotide. Therefore, we conclude that RW behaviour was largely unaltered by administration of the drug.

In exercising SD hamsters, weight gain was retarded by pasireotide, with overall lean mass being similar to sedentary counterparts in contrast to the vehicle treated RW hamsters. Weight gain was however, not completely inhibited by pasireotide. A contribution to weight gain comes from fat mass which increased with RW activity and did not differ between vehicle and pasireotide treatments. Although we found no direct effect of pasireotide on metabolic energy expenditure in sedentary hamsters, it might be argued that a trend for increase in RW activity lead to increased energy expenditure and therefore the diversion of energy from growth. However, this explanation is unlikely since change in body mass did not correlate with the total distance run. Furthermore, fat mass would be the first source of additional energy mobilised for an increased energy expenditure (37), but this was similar in all RW hamsters.

In the absence of altered energy expenditure, the retarded growth of RW-Pasireotide hamsters might be explained by a reduction in energy intake. One key component of the weight gain experienced by exercising hamsters is increased food intake (4, 10, 11). We did not measure food

intake in the present pasireotide experiment, but clearly caloric intake was sufficient to sustain a similar increase in fat mass in vehicle and pasireotide treated hamsters. A lower food intake in pasireotide treated hamsters would likely occur because of a lower basal metabolic rate due to less lean tissue and less energy required to sustain RW induced muscle accretion and organ growth (38). This would also be consistent with a reduced orexigenic drive from the GH axis in the brain where GH has been shown to stimulate NPY neurons, and increase both *Npy* and *Agrp* expression (39-41).

Food intake can be stimulated and suppressed by appetite regulating neuropeptides expressed in the hypothalamus, and POMC and NPY are both implicated in appetite control (42). In our previous study RW activity was accompanied by an increase in *Pomc* but no alteration of *Npy* expression (4). In the present study, in addition to an increase in *Pomc*, an increase in *Npy* expression was also observed. Increased *Pomc* expression may not necessarily equate to an increased anorexigenic drive as this may depend on the impact of RW activity on downstream processing enzymes which are photoperiodically regulated in a manner to increase anorexic α-MSH production from POMC precursor peptide in SD (43). However, the discrepancy between these two findings for *Npy* may lie in the time of sampling, since hamsters in the present study were sacrificed during the dark RW active phase when an energy deficit due to activity will be greater in comparison with our previous study when hamsters were killed during the early light phase when hamsters were not exercising (4).

Testicular atrophy was partially retarded in RW hamsters, with no significant effect of pasireotide treatment, suggesting RW activity had a broad stimulation of neuroendocrine axes. This is similar to a stimulatory effect of RW activity in Syrian hamsters where exercise has been shown to inhibit or reverse photoperiod induced reduction in prolactin, follicle-stimulating hormone, lueteinzing hormone and testosterone, and reverse reproductive quiescence (44, 45). Notably, RW stimulated growth was first observed in the Siberian hamster in castrated male animals (9), and so growth is unlikely to be driven by testosterone produced by the partially recrudesced testes of RW hamsters. Additionally, exercise induced growth has been demonstrated in both male and female Syrian hamsters (45) and Siberian hamsters (11).

In line with previous work (4), insulin concentration was increased in serum of RW hamsters, although serum glucose levels did not differ. Any subtle differences in serum glucose might have been masked by the non-fasted state of the hamsters at sacrifice. Although the photoperiodic difference in circulating insulin in male Siberian hamsters is well established (4, 32), intraperitoneal glucose tolerance tests have revealed no photoperiod differences in glucose clearance for Siberian hamsters (46). A chronic difference in circulating insulin can be considered

indicative of a more obese and generally insulin resistant state, as has been described for LD hamsters with regard to central insulin signalling (47). Insulin has a lipogenic activity in Siberian hamster adipocytes (48); therefore a greater concentration of insulin in the serum of RW hamsters may provide a mechanism for the increase and maintenance of fat deposition similar to a LD hamster, in addition to any insulin driven increase in lean mass (49). The lack of pasireotide effect on circulating insulin concentrations supports this interpretation, since fat mass was also unaffected.

Alterations in the balance of the central thyroid hormone system are essential for driving seasonal phenotype change in the Siberian hamster (3, 50). However, as previously reported for RW (4) and pasireotide treated hamsters (27), there were no measurable effects on *Dio2* and *Dio3* expression, indicating action downstream of the integration of the photoperiodic cue.

Gh-r expression remained unaltered in the ARC by pasireotide, however there was a stimulatory effect of wheel running on expression of this receptor mRNA. This may have allowed for increased sensitivity to circulating GH, and suggests that tachyphylaxis of this receptor was not a problem with the hypothesised increase in circulating GH. Unfortunately, there was not enough remaining tissue to compare photoperiodic expression of GH-R and so it remains to be seen whether there is photoperiodic regulation of this receptor.

In the present study, the effect of wheel running to stimulate increased ARC *Ghrh* expression was evident in both experiments, and in both LD and SD hamsters. Whereas we previously found that pasireotide significantly increased *Ghrh* expression in sedentary hamsters independent of photoperiod, the effect of pasireotide on *Ghrh* expression did not appear to be additive to the RW effect. Wheel running activity has been shown to suppress the SD stimulated increase in ARC *Srif* expression, an effect that appears to depend on the length of time that the hamsters had access to a RW and/or the length of time in SD photoperiod (4). Although not quite achieving significance, the present data demonstrated a trend to suppression by RW activity consistent with the previous study, but pasireotide did not alter ARC *Srif* expression. This indicates that pasireotide does not affect the photoperiodic drive on ARC *Srif* expression.

As previously described (27) expression of *Srif* in the PeVN was suppressed by pasireotide. Together with increased *Ghrh* expression in the ARC by RW activity, the findings are consistent with altered feedback of the GH axis to these neurons. Although we cannot definitively conclude the primary driver of growth caused by RW activity is GH, the following support the notion GH underpins this mechanism; 1) robust increase in pulsatile circulating GH in Syrian hamsters caused by RW activity (28, 51); 2) the persistence of RW induced growth in castrated hamsters (9), 3) the known stimulatory effect of GH and suppressive effect of pasireotide on circulating IGF-1 levels

in the Siberian hamster (27) and 4) suppressive effect of pasireotide on RW and LD photoperiod induced growth. However, due to insufficient serum we were unable to provide the definitive confirmation from measurement of circulating IGF-1 measurements. Thus any additional mechanism of inducing somatic growth may still be possible such as a contribution from increased circulating insulin (49), although evidence from Syrian hamsters suggests insulin is less likely to contribute to RW induced somatic growth (52).

The seasonally adaptive physiology of the Siberian hamster has traditionally been studied as a tool to understand mechanisms for the regulation of appetite, body mass and metabolism. The exercise response that occurs in this species may be counterintuitive when considering that in general, exercise is known to have health promoting effects in human physiology across several different parameters. However even in human physiology, on an individual level there can be substantial variability in beneficial effects, or even detrimental effects of exercise on specific health parameters (53-56) that may be tied to compensatory eating (57). Considering the current level of reported obesity and overweight worldwide, with the main treatments being to reduce caloric intake and increase energy expenditure, it is important to understand how in certain individuals, specific exercise regimes or treatments might be beneficial to counteract poor exercise response. We propose that the Siberian hamster exercise response can be considered a model for poor exercise response in humans, typified by the increased serum insulin concentration, weight gain and no reduction in fat mass. Therefore, understanding what drives these physiological changes in the hamster could inform human medicine.

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Conflicts of interest

Dr Herbert Schmid is an employee of Norvatis AG. Other authors have no conflict of interest.

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Figures

Figure 1: Pasireotide does not alter distance run in SD acclimated Siberian hamsters with RW access. Representative double plotted actograms for a RW-vehicle and a RW-pasireotide hamster (A). Mean distance run / day / hamster over 49 d for hamsters treated with vehicle or pasireotide and access to a RW (B). Daily RW distance for pasireotide and vehicle hamsters (C), and mean wheel revolutions in 2 h bins during the dark phase over the course of the experiment (D). n.s.: no significant differences. Both groups n = 7. Data are expressed as mean ± SEM.

Figure 2: Pasireotide inhibits RW stimulated weight gain in SD acclimated Siberian hamsters. Body mass change (A) of hamsters acclimated to SD for 69-72 d before given access to a RW or not, and treated with pasireotide or vehicle for 49 d. (B) No correlation was found between distance travelled in 49 days and change in body mass in RW hamsters. Carcass lean (C) and fat (D) tissue mass, measured by MRI, and relative lean and fat mass (E). Paired testes mass (F), and liver mass (G) at time of sacrifice are also shown. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$, vs all other groups or as indicated; #: $p<0.05$ vs sedentary-pasireotide. †: $p<0.001$ vs RW-vehicle RW groups both n = 7, sedentary groups both n = 8. Data are expressed as mean ± SEM.

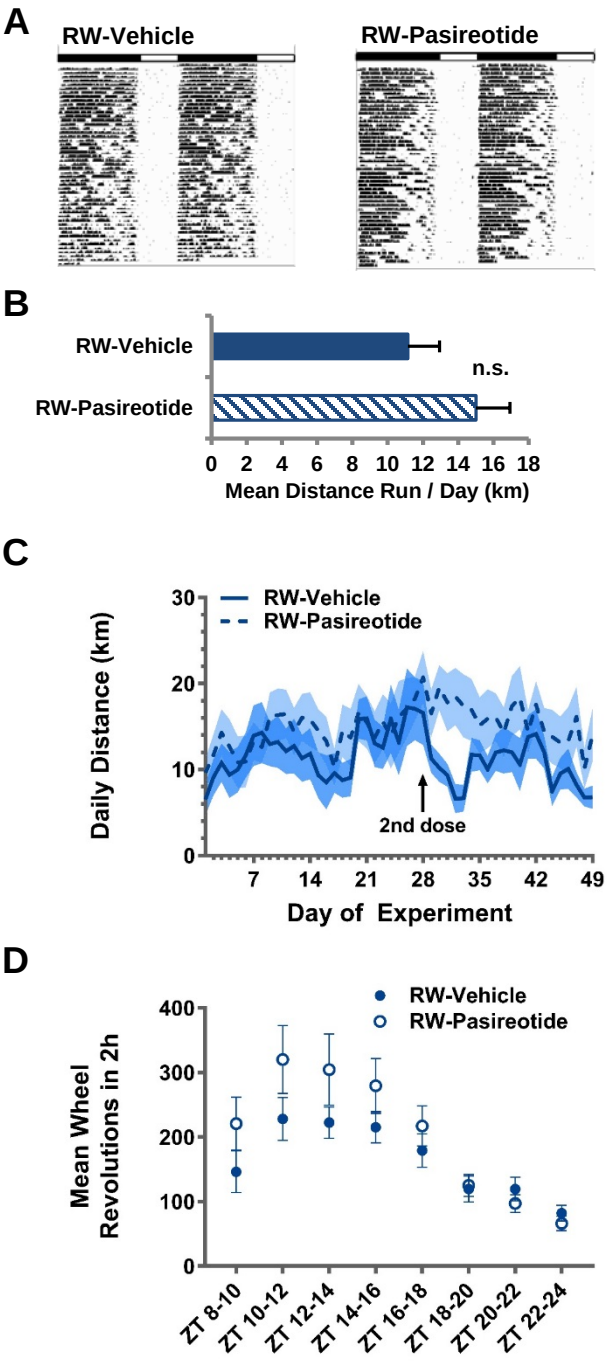
Figure 3: Pasireotide did not alter glucose homeostasis in SD acclimated Siberian hamsters. Terminal serum glucose (A) and insulin (B). *$p < 0.05$. RW-vehicle, n = 6; RW-pasireotide, n = 7; sedentary groups, both n = 8. Data are expressed as mean ± SEM.

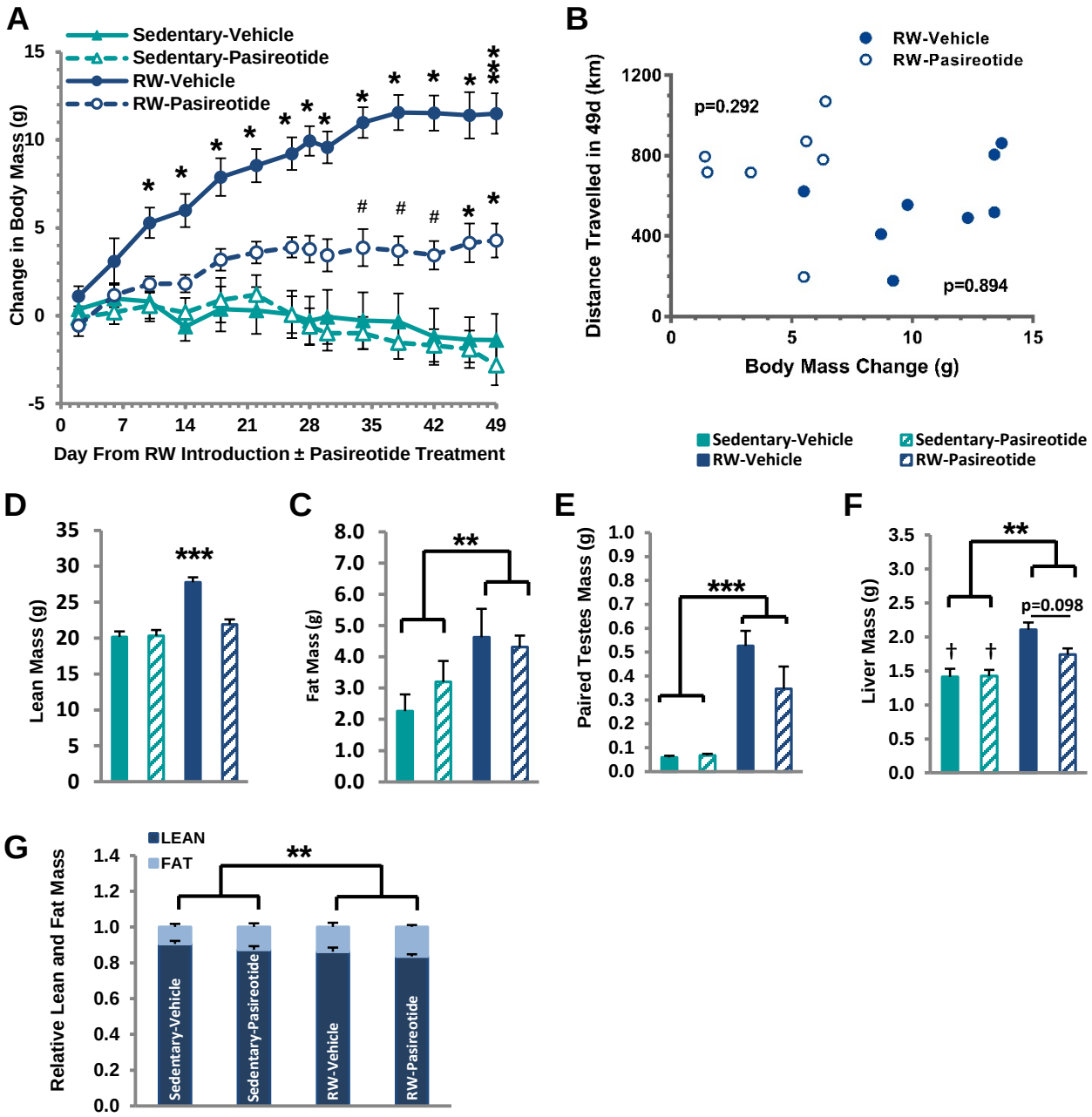
Figure 4: RW stimulated increased gene expression of appetite regulating peptides and GH axis components, while pasireotide only altered expression of PeVN *Srif*. Relative mRNA expression of *Dio2* (A), *Dio3* (B), *Pomc* (C), *Npy* (D) and *Srif* (E) in the arcuate nucleus (ARC), *Srif* in the periventricular nucleus (PeVN; F), and *Ghrh* (G) and *Gh-r* (H) in the ARC. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$, n.s.: no significant differences. RW groups both n = 7, sedentary groups both n = 8. Data are expressed as mean ± SEM.

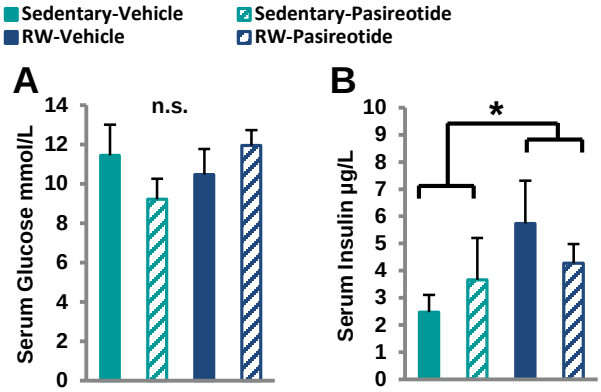
Figure 5: RW access stimulates hypothalamic *Ghrh* expression in LD and SD hamsters. Siberian hamsters were acclimated to SD for 14 d or remained in LD before given access to a RW or not for a further 84 d. RW access caused a positive change in body mass (A), representative actograms for LD-RW and SD-RW hamsters for the full course of the experiment (B), and distance run / hamster / day (C). Terminal organ mass of paired testes (D) and liver (E), and relative *Ghrh* mRNA expression in the arcuate nucleus (F). *$p < 0.05$, ***$p<0.001$, vs all other groups, **$p < 0.01$ as indicated; #: $p < 0.05$ LD vs SD; †: $p < 0.05$ RW vs Sedentary. A-C, E, F: SD-sedentary, n = 8; all other groups n = 10. D: SD-RW: n=4, SD-Sedentary: n=5, LD-RW: n=10, LD-Sedentary, n=9. G:

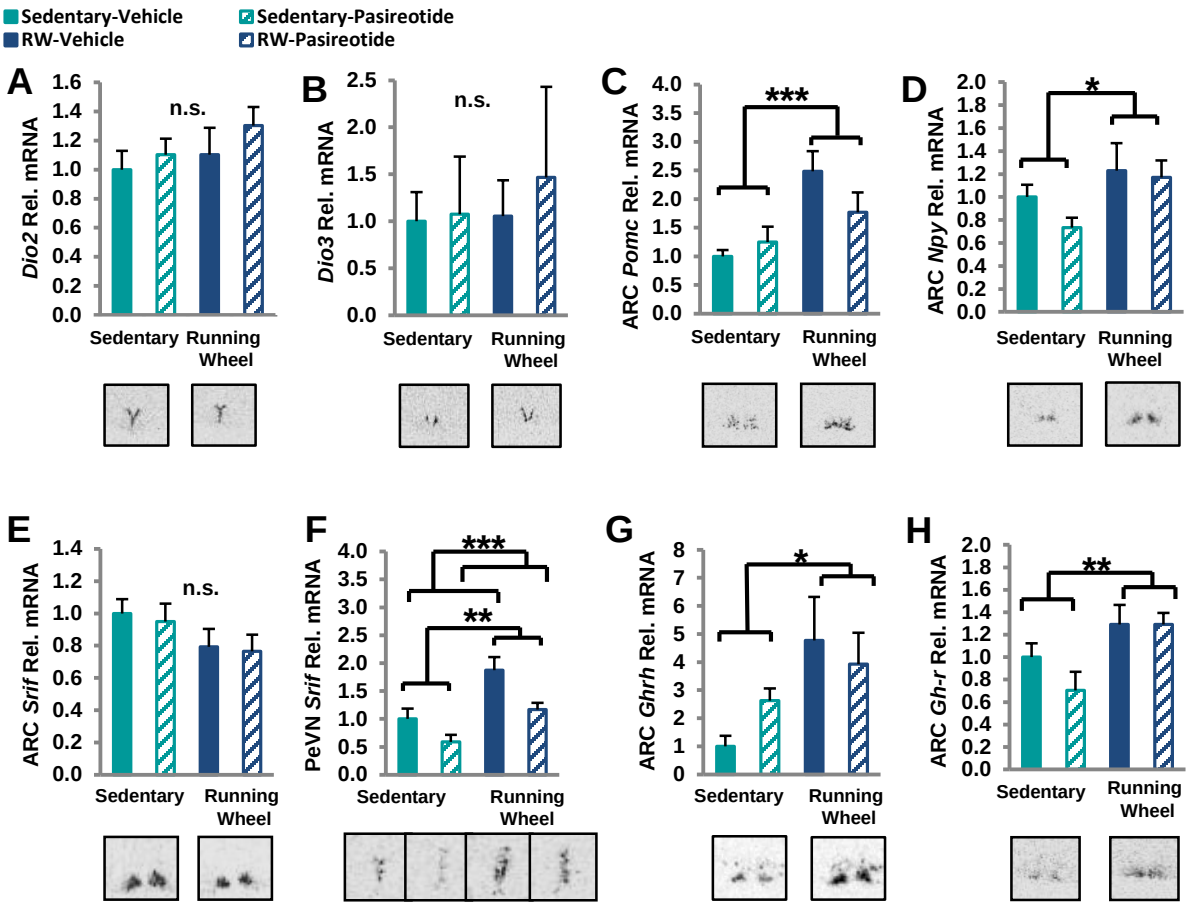
677 SD-sedentary n = 8, LD-RW n = 9, SD-RW and LD-sedentary both n = 10. Data are expressed as
678 mean ± SEM.

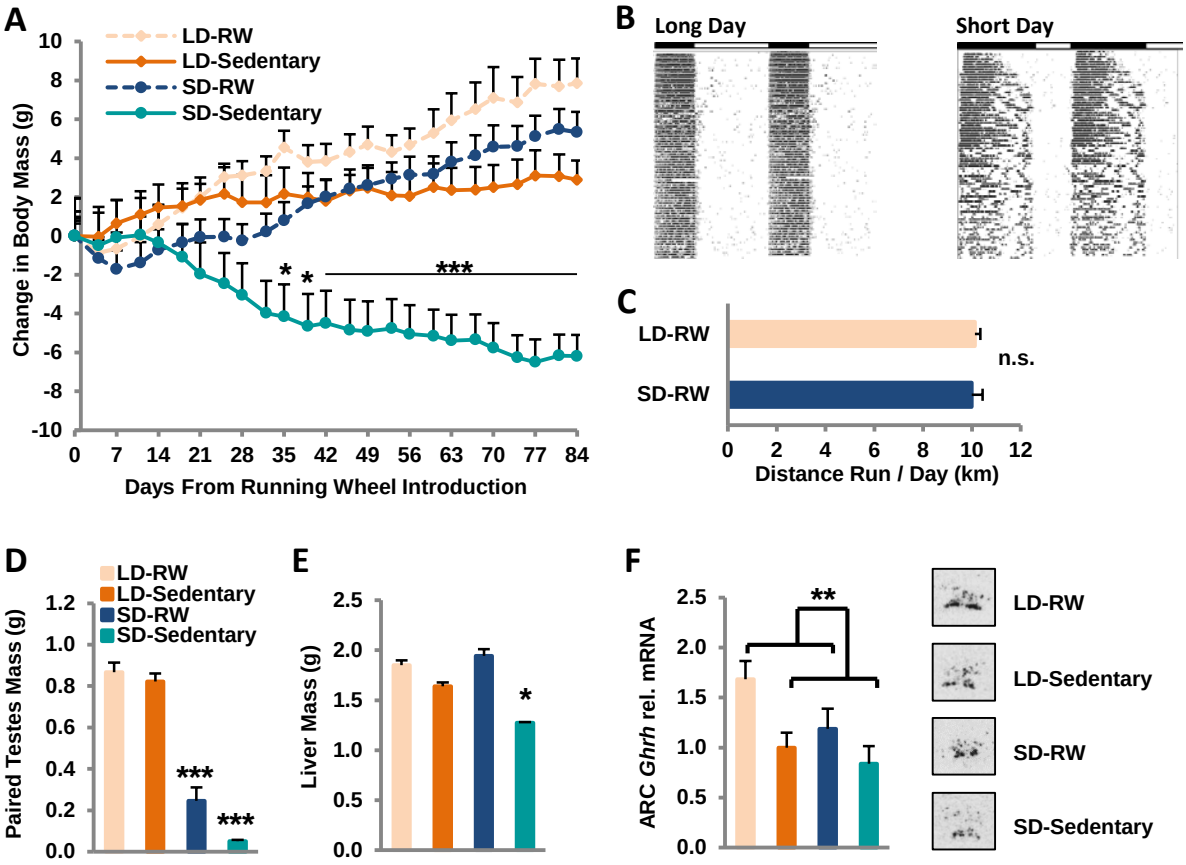
679


A
RW-Vehicle
RW-Pasireotide
B
RW-Vehicle
RW-Pasireotide
n.s.
0 2 4 6 8 10 12 14 16 18
Mean Distance Run / Day (km)
C
RW-Vehicle
RW-Pasireotide
Daily Distance (km)
30
20
10
0
2nd dose
7 14 21 28 35 42 49
Day of Experiment
D
RW-Vehicle
RW-Pasireotide
Mean Wheel Revolutions in 2h
400
300
200
100
0
ZT 8-10
ZT 10-12
ZT 12-14
ZT 14-16
ZT 16-18
ZT 18-20
ZT 20-22
ZT 22-24


A
Sedentary-Vehicle
Sedentary-Pasireotide
RW-Vehicle
RW-Pasireotide
Change in Body Mass (g)
Day From RW Introduction ± Pasireotide Treatment
B
RW-Vehicle
RW-Pasireotide
p=0.292
p=0.894
Distance Travelled in 49d (km)
Body Mass Change (g)
Sedentary-Vehicle
Sedentary-Pasireotide
RW-Vehicle
RW-Pasireotide
D
Lean Mass (g)
C
Fat Mass (g)
E
Paired Testes Mass (g)
F
Liver Mass (g)
p=0.098
G
LEAN
FAT
Relative Lean and Fat Mass
Sedentary-Vehicle
Sedentary-Pasireotide
RW-Vehicle
RW-Pasireotide


Sedentary-Vehicle
Sedentary-Pasireotide
RW-Vehicle
RW-Pasireotide
A
n.s.
Serum Glucose mmol/L
14
12
10
8
6
4
2
0
B
*
Serum Insulin µg/L
10
9
8
7
6
5
4
3
2
1
0


Sedentary-Vehicle
Sedentary-Pasireotide
RW-Vehicle
RW-Pasireotide
A
Dio2 Rel. mRNA
n.s.
Sedentary
Running Wheel
B
Dio3 Rel. mRNA
n.s.
Sedentary
Running Wheel
C
ARC Pomc Rel. mRNA
***
Sedentary
Running Wheel
D
ARC Npy Rel. mRNA
*
Sedentary
Running Wheel
E
ARC Srif Rel. mRNA
n.s.
Sedentary
Running Wheel
F
PeVN Srif Rel. mRNA
***
**
Sedentary
Running Wheel
G
ARC Ghrh Rel. mRNA
*
Sedentary
Running Wheel
H
ARC Gh-r Rel. mRNA
**
Sedentary
Running Wheel


A
LD-RW
LD-Sedentary
SD-RW
SD-Sedentary
Change in Body Mass (g)
Days From Running Wheel Introduction
*
*
***
B
Long Day
Short Day
C
LD-RW
SD-RW
n.s.
Distance Run / Day (km)
D
Paired Testes Mass (g)
***
***
E
Liver Mass (g)
*
F
ARC Ghrh rel. mRNA
**
LD-RW
LD-Sedentary
SD-RW
SD-Sedentary

Supplementary Material

Somatostatin agonist pasireotide inhibits exercise stimulated growth in the male Siberian hamster (*Phodopus sungorus*)

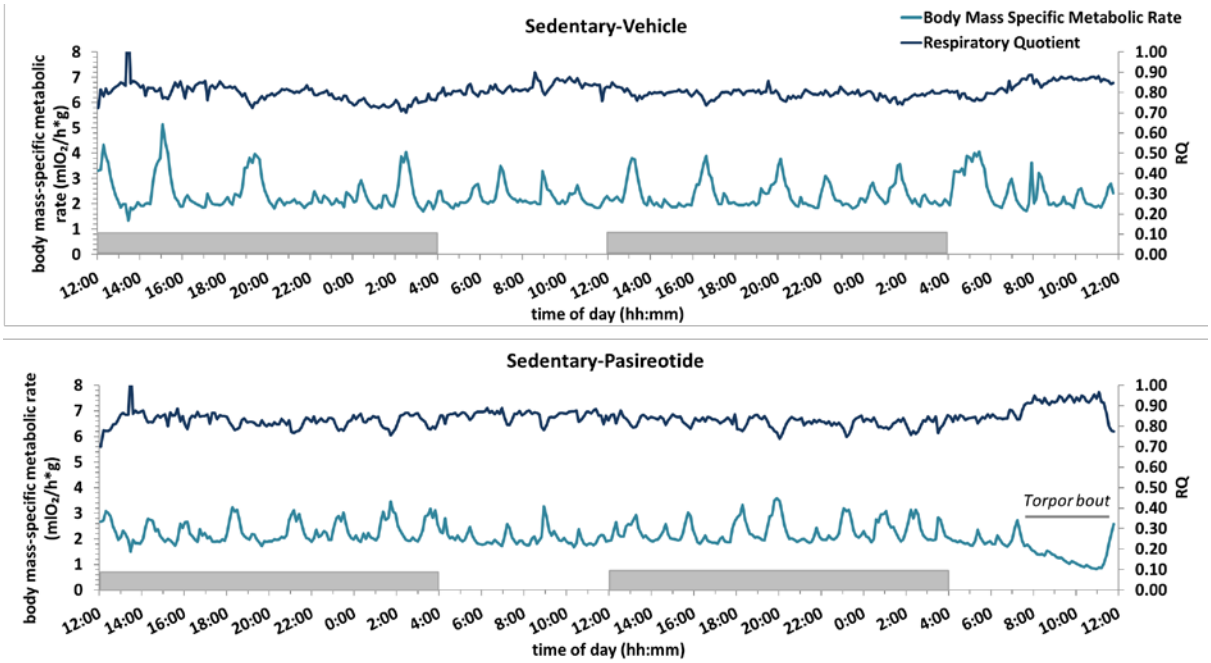
Open flow Respirometry Experiments

Sedentary hamsters were monitored for a period of 2 or 3 days between days 13-20 of the experiment, by open flow respirometry, carried out in their home cages with dimensions 24.5cm x 15cm x 15cm and a volume approximately 5.5L. VO_2 and VCO_2 were measured with a FOXBOX field gas analyser (Sable systems, NV, USA) at a flow rate of 35-40 L/hour. Measurements were taken every 1 in 6 minutes, for 5 hamsters per session, and were adjusted according to an air reference channel. The body mass specific metabolic rate and respiratory quotient (RQ) were calculated, taking the bodyweight as the mean from the two closest weigh dates (4 days apart). Supplementary table 1 illustrate mean RQ over the measurement period, and supplementary figure 1 demonstrates an example for vehicle and pasireotide treated sedentary hamsters over the course of a 2-day measurement period.

Supplemental table 1: Respiratory quotient (RQ) is unchanged by pasireotide in sedentary hamsters.

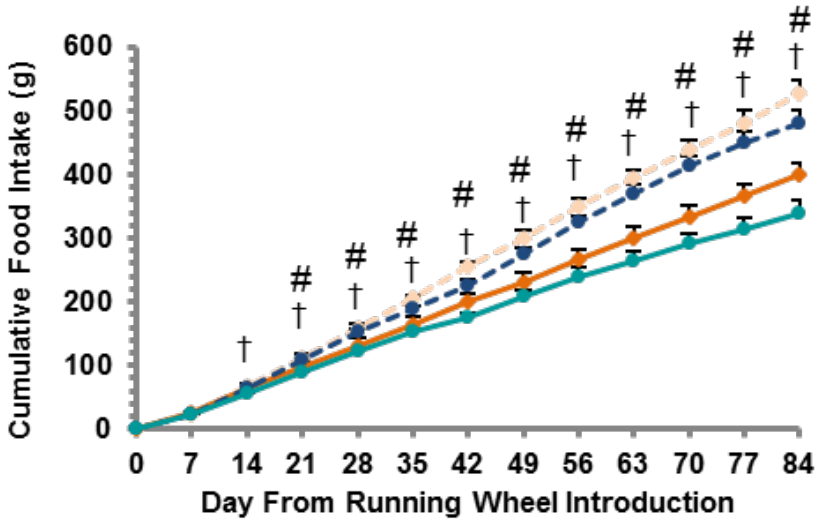
	Light Phase RQ	Dark Phase RQ	Overall RQ
Pasireotide	0.846 ± 0.015	0.824 ± 0.012	0.831 ± 0.013
Vehicle	0.821 ± 0.017	0.810 ± 0.012	0.813 ± 0.013

Supplementary figure 1.



Representative example metabolic rate and respiratory quotient (RQ) traces for a sedentary-vehicle and a sedentary-pasireotide hamster over 48h in the 3rd week of experiment. Grey bars indicate the dark phase, and a torpor bout is indicated for the sedentary-pasireotide hamster during the light phase on the 2nd measurement day. The effects of pasireotide on torpor in these hamsters has previously been discussed (1).

Supplementary Figure 2: LD/SD experiment food intake data



Food intake was measured on a weekly basis by weighing the difference in food weight in the cage hoppers, and plotted as cumulative food intake. Because of excessive crumbling of the food, food intake data was excluded for several hamsters, reducing sample sizes; SD-RW: n=4, SD-Sedentary: n=5, LD-RW: n=10, LD-Sedentary, n=9. As previously described (2), food intake increased for RW hamsters and with LD photoperiod, (Photoperiod: $F(1,24)=5.15$, $p=0.033$; RW Activity: $F(1,24)=50.71$, $p<0.001$; Interaction: $F(1,24)=0.09$, $p=0.764$, figure). #: $p < 0.05$ LD vs SD; †: $p < 0.05$ RW vs Sedentary.

References

1. Scherbarth F, Diedrich V, Dumbell RA, Schmid HA, Steinlechner S, Barrett P. Somatostatin receptor activation is involved in the control of daily torpor in a seasonal mammal. *American journal of physiology Regulatory, integrative and comparative physiology*. 2015; **309**(6): R668-74.

2. Petri I, Dumbell R, Scherbarth F, Steinlechner S, Barrett P. Effect of Exercise on Photoperiod-Regulated Hypothalamic Gene Expression and Peripheral Hormones in the Seasonal Dwarf Hamster Phodopus sungorus. *PLoS ONE*. 2014; **9**(3): e90253.