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Photoperiodic Regulation of the Orexigenic Effects of Ghrelin in Siberian Hamsters

Sean P. Bradley¹, Lucia M. Pattullo¹, Priyesh N. Patel¹, and Brian J. Prendergast^{1,2}

¹Department of Psychology, University of Chicago, Chicago, IL, 60637

²Committee on Neurobiology, University of Chicago, Chicago, IL, 60637

Abstract

Animals living in temperate climates with predictable seasonal changes in food availability may use seasonal information to engage different metabolic strategies. Siberian hamsters decrease costs of thermoregulation during winter by reducing food intake and body mass in response to decreasing or short day lengths (SD). These experiments examined whether SD reductions in food intake in hamsters is driven, at least in part, by altered behavioral responses to ghrelin, a gut-derived orexigenic peptide which induces food intake via NPY-dependent mechanisms. Relative to hamsters housed in long day (LD) photoperiods, SD hamsters consumed less food in response to i.p. treatment with ghrelin across a range of doses from 0.03 to 3 mg/kg. To determine whether changes in photoperiod alter behavioral responses ghrelin-induced activation of NPY neurons, c-Fos and NPY expression were quantified in the arcuate nucleus (ARC) via double-label fluorescent immunocytochemistry following i.p. treatment with 0.3 mg/kg ghrelin or saline. Ghrelin induced c-Fos immunoreactivity (-ir) in a greater proportion of NPY-ir neurons of LD relative to SD hamsters. In addition, following ghrelin treatment, a greater proportion of ARC c-Fos-ir neurons were identifiable as NPY-ir in LD relative to SD hamsters. Changes in day length markedly alter the behavioral response to ghrelin. The data also identify photoperiod-induced changes in the ability of ghrelin to activate ARC NPY neurons as a possible mechanism by which changes in day length alter food intake.

Keywords

food intake; arcuate nucleus; seasonality; neuropeptide Y; c-fos

Introduction

One challenge facing all heterotrophic organisms is the need to continuously balance food intake with energy expenditure. Even when food is abundant, an animal must choose when, what, and how much to consume (Schneider, 1992). In non-tropical regions, the winter months are characterized by reduced food availability and increased costs of thermoregulation. Siberian hamsters (*Phodopus sungorus*) contend with the energetic challenges of winter by exhibiting a constellation of physiological adaptations which reduce

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Correspondence to: Sean P. Bradley Department of Psychology 940 E. 57th St. University of Chicago Chicago, IL 60637 Phone: 773-834-3317 Fax: 773-702-6898 spbradley@uchicago.edu.

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basal metabolic rate, including gonadal involution, decreases in body mass, and reductions in food intake (Prendergast et al., 2002). Changes in day length (photoperiod) are sufficient to initiate seasonal adjustments in metabolism, behavior and morphology in this species: short days (13 h light/day) inhibit reproduction, body mass, and food intake in body mass within 8 weeks of onset (Steinlechner & Heldmaier, 1982).

Peripherally produced hormones act in the brain to regulate food intake. The arcuate nucleus of the hypothalamus (ARC) is a major site for integration of peripheral orexigenic and anorexigenic signals. Within the ARC there exist two major populations of neurons that participate in the control of food intake: a population that releases neuropeptide Y (NPY) and agouti-related peptide (AgRP), and a population that releases pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (reviewed in Dhillo, 2007). Central NPY treatments stimulate food intake in a number of species (rat: Stanley and Leibowitz, 1985; chick: Kuenzel et al., 1987; golden hamster Kulkosky et al., 1988; sheep: Miner et al., 1989, Siberian hamster: Boss-Williams and Bartness, 1996). NPY-releasing neurons project in abundance to the lateral hypothalamic area and paraventricular nucleus of the hypothalamus (PVN), areas critical to the regulation of reward and feeding behavior. (Dhillo, 2007).

The stomach-derived peptide ghrelin has been identified as a potent peripheral orexigenic hormone; intraperitoneal injections of ghrelin cause rapid and marked increases in food intake in previously satiated rats (Wren et al., 2000). In addition to increasing food intake, exogenous injections of ghrelin promote non-consummatory appetitive behaviors, such as foraging and food hoarding in Siberian hamsters (Keen-Rhinehart and Bartness, 2005). Ghrelin is produced in the fundus of the stomach, and plasma ghrelin concentrations increase during fasting; peak concentrations occur immediately before an anticipated meal and decline sharply postprandially (reviewed in Geary, 2004). The orexigenic action of ghrelin is mediated by growth hormone secretagogue receptors (GHS-R) which are expressed by the majority of cells in the ARC (Willensen et al., 1999). Agonists of this receptor activate approximately 50% of ARC neurons, inducing c-Fos expression, a marker of neuronal excitation (Dickson and Luckman, 1997). Ghrelin treatments increase expression of NPY mRNA in ARC neurons (Shintani et al., 2001). ARC exposure to ghrelin mimics the effect of NPY on targets of hypothalamic NPY projections (Cowley et al., 2004), and NPY antagonists attenuate ghrelin-induced feeding in mice (Nakazato et al., 2001), indicating that the primary mechanism by which ghrelin stimulates food intake is through the stimulation of NPY production and release. In Siberian hamsters, antagonizing the NPY Y1 receptor abolishes ghrelin-induced foraging and food intake and reduces ghrelin-induced food hoarding (Keen-Rhinehart and Bartness, 2007).

Changes in day length are largely without effect on ingestive responses to NPY (Boss-Williams and Bartness, 1996), but the role of ghrelin in the seasonal regulation of energy balance has yet to be fully characterized. Day length does not alter the production of ghrelin following an extended period of food deprivation: following 48 h of fasting, plasma ghrelin concentrations were comparable between LD- and SD-acclimated Siberian hamsters (Tups et al., 2004). These data suggest that a comparable ghrelin signal is generated in both photoperiods under these conditions of food deprivation. However, upon refeeding following a 48 h fast, LD hamsters tended to engage in greater compensatory food intake relative to SD hamsters (Bartness et al., 1985). Whether, and how, seasonal changes in day length are sufficient to alter behavioral responsiveness to ghrelin is not known.

The present experiment tested the hypothesis that behavioral responsiveness to ghrelin is attenuated in Siberian hamsters following acclimation to short day lengths. Furthermore, in light of the well-established effects of ghrelin on the activity of NPY neurons in the ARC,

we also used double-label fluorescent immunocytochemistry to identify cells expressing NPY and c-Fos, and evaluated whether photoperiodic changes in the orexigenic potency of ghrelin were associated with differential activation of NPY neurons.

Materials and Methods

Animals

All animal treatments described in this experiment conformed to the USDA Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Chicago Institutional Animal Care and Use Committee. Adult male Siberian hamsters were randomly selected from a long-day (15L:9D; LD) breeding colony. All animals in this colony were weaned between 18-21 days after birth and housed 1-4 animals/cage in 28 × 17 × 12 cm polypropylene cages containing bedding (Harlan Sani-Chips, Harlan Inc., Indianapolis, IN). The colony room was maintained at 20±2 °C with relative humidity 50±5%, and all animals were provided with food (Teklad Rodent Diet 8604, Harlan Inc., Indianapolis, IN) and filtered tap water *ad libitum*.

Photoperiod manipulations

For each experiment, adult (3-4 month old) male hamsters were removed from the breeding colony and singly housed. Half of these animals were transferred to a short-day photoperiod (9L:15D; SD), and half remained in their natal long-day photoperiod. The time of the onset of darkness remained the same for all photoperiod conditions.

Experiment 1

A total of 26 hamsters were used in this experiment. On week 0, hamsters were transferred either to SD (n=13) or remained in LD (n=13). During weeks 12-16, each animal received i.p. injections of 0, 0.03, 0.3, or 3 mg/kg ghrelin (Tocris Pharmaceuticals) in 1 ml 0.9% saline coincident with the onset of the dark phase (18:00 CST for both photoperiods) in a pseudorandom, blocked design with all animals receiving each dose once. Data from a pilot study (not shown) specified that orexigenic effects of 3 mg/kg ghrelin, a dose demonstrated to be sufficient to increase food intake in this species (Keen-Rhinehart and Bartness 2003; Keen-Rhinehart and Bartness 2007), endured for a maximum of 2 days, thus successive injections were separated by 1 week to minimize carryover effects. Food intake was measured by weighing the contents of each animal's food hopper immediately before the administration of each injection and 1, 2, 4, 6, 9, 15, 21, and 24 h post injection.

Experiment 2

A separate group of hamsters (n=24) was maintained in LD (n=10) or transferred to SD (n=14). After 12 weeks of photoperiod acclimation, each animal received 0.1 ml injections of 0.3 mg/kg ghrelin (LD: n=6; SD: n=9) or 0.9% saline (LD: n=4; SD: n=5) coincident with the onset of the dark phase as described in Experiment 1. One hour after injection, each animal was anaesthetized with sodium pentobarbital (100 mg/kg; Nembutal, Ovation Pharmaceuticals) and transcardially perfused with 30 ml cold PBS followed by 30 ml 4% paraformaldehyde. Following dissection, brains were postfixed for 24 h in 4% paraformaldehyde, immersed in a 30% sucrose/0.1 M PBS solution for ~48 h, and stored at -80 °C until sectioned for immunocytochemistry.

Immunocytochemistry

Coronal sections (30 µm) were cut on a freezing microtome from the optic chiasm to the appearance of the substantia nigra. Free-floating brain sections were pre-treated in a 1% sodium borohydride solution for 15 m. Afterward, sections were rinsed 6 times in tris-

buffered saline (TBS) before being immersed in 5% normal donkey serum (Millipore, Billerica, MA) and 0.3% Triton-X/TBS solution for 60m to reduce unspecific staining. Following this, brain sections were rinsed 6 times in TBS before being incubated in a dilution of both primary antibodies for 24h at 20 °C [sheep anti-NPY (1:2000, Millipore) and mouse anti-c-Fos (1:4000, Millipore)]. The sections were washed 6 times in TBS and then were incubated in a dilution of both secondary antibodies (donkey-anti-sheep Alexa Fluor 488 [10 mg/ml; Invitrogen, Carlsbad, CA] and donkey-anti-mouse Alexa Fluor 594 [10 mg/ml; Invitrogen]) for 1 h at room temperature. Finally, the sections went through a final 6 washes in TBS, were dehydrated in graded ethanol (50%, 70%, 80%, 90%, and 100%), then were fixed on microscope slides with Permount and stored in a dark environment until image acquisition. Primary antibody specificity was confirmed by conducting negative controls trials omitting each primary antibody, in which no significant fluorescence was observed.

Image Acquisition and Analysis

Confocal microscopy would be required to definitively identify these cells as double-labeled. However, such instrumentation was unavailable to us at the time these studies were performed. Given this constraint, we follow the convention of Grieves et al. (2007) in using cell bodies that appeared to be immunopositive for both substances as an acceptable method of determining colocalization.

Each section was visualized using a CCD camera (Micropublisher 3.3 RTV; Q-Imaging, Surrey, BC, Canada) attached to a Nikon 50i microscope D-FL epi-fluorescence attachment and illuminated by a 100 W mercury lamp and photographed through both 488 and 594 nm fluorescent filters in succession. The ARC was identified morphologically using a mouse stereotaxic atlas (Slotnick and Leonard, 1975) and confirmed by the appearance of dense NPY immunoreactivity (-ir). Photomicrographs permitting colocalization were obtained from each ARC section by obtaining photomicrographs of the same region using both fluorescent filters (QCapture Pro, QImaging) and saved in 8-bit grayscale TIFF format. Image files were exported to Adobe Photoshop CS3 (Adobe Systems, San Jose, CA), and a composite RGB image was created for each set of ARC images such that the red channel contained the c-Fos-ir image (captured through the 594 nm filter), the green channel contained the NPY-ir image (captured through the 488 nm filter), and the blue channel contained a black (0 brightness) mask. In the resulting composite image, c-Fos-ir appeared red, NPY-ir appeared green, and areas both c-Fos-ir and NPY-ir appeared yellow (Paddock et al., 1997).

To facilitate the manual quantification of cell bodies, a threshold filter was applied to each image using ImageJ (NIH, Bethesda, MD) such that only pixels of brightness greater than or equal to 3 standard deviations above background were displayed. In addition to reducing background, this filter eliminated fluorescent glare observed in conventional fluorescent microscopy, providing a conservative visualization of cell bodies. The number of NPY-ir cells, c-Fos-ir cells, and cells immunoreactive for both NPY and c-Fos were recorded bilaterally from 1-2 sections of each ARC.

Statistics

In Experiment 1, the effects of hormone treatment and photoperiod condition on hourly food intake were assessed using one-way ANOVAs at each time point. Pairwise comparisons were conducted using Fisher's PLSD if an omnibus ANOVA was significant. Data in Experiment 2 were analyzed using one-way ANOVAs assessing the effect of hormone treatment and photoperiod condition on observed cell counts or proportions. Pairwise comparisons were conducted using Fisher's PLSD after a significant omnibus ANOVA

result. Observed differences were considered significant if $p \leq 0.05$. All statistical tests were performed using Statview 5.0 (SAS Institute, Cary, NC).

Results

Experiment 1 – Behavioral Responsiveness to Peripheral Ghrelin Treatment

Within the ARC, for both LD and SD groups, at least one dose of ghrelin was effective in increasing food intake in each of the first 2 hours following hormone treatment (LD: $F=8.54$, $p<0.05$; SD: $F=0.59$, $p<0.05$; Fig. 1). No ghrelin-induced increases in food intake were observed during the 2-4 hour post-treatment time interval for either group (LD: $F=0.62$, $p>0.05$; SD: $F=0.38$, $p>0.05$), or during any subsequent interval up to 24 h post treatment (not shown). Pursuant to this observation, ghrelin-induced food intake was compared to food intake in response to saline treatment for each of the first two hours in each photoperiod group.

In the first hour after treatment, all three doses of ghrelin increased food intake in LD animals (.03 mg/kg: $t=-3.98$, $p<0.05$; 0.3 mg/kg: $t=-5.29$, $p<0.05$; 3 mg/kg: $t=-7.14$, $p<0.05$). In contrast, the 0.03 mg/kg dose did not stimulate food intake in SD hamsters ($t=-0.516$, $p>0.05$); rather, ghrelin stimulated food intake in SD animals only at the 0.3 mg/kg dose ($t=-4.01$, $p<0.05$) and 3 mg/kg dose ($t=-4.22$, $p<0.05$).

In the second hour after treatment, ghrelin increased food intake relative to saline in both LD and SD hamsters at the highest dose (3 mg/kg; LD: $t=-2.10$, $p<0.05$, SD: $t=-1.99$, $p<0.05$). SD hamsters given the 0.3 mg/kg dose consumed less food than SD hamsters injected with saline ($t=-2.13$, $p<0.05$).

Experiment 2 – NPY-ir & c-Fos-ir following ghrelin treatment

Within the ARC, c-Fos-ir was evident as punctate fluorescence (Figure 2A); NPY-ir was more diffuse in appearance, indicative of cytoplasmic detection (Figure 2B). Overlay of digital photomicrographs permitted identification of NPY-expressing cells putatively coexpressing c-Fos (Figure 2C).

The number of ARC NPY-ir cells did not differ as a function of photoperiod ($F=0.86$, $df=1$, $p<0.05$) or hormone treatment ($F=.12$, $df=1$, $p>0.05$; Figure 3A). Similarly, the number of ARC c-Fos-ir cells in the ARC was comparable across all treatment groups regardless of photoperiod ($F=3.1$, $df=1$, $p>0.05$) or hormone treatment ($F=.82$, $df=1$, $p>0.05$; Fig. 3B). No significant interactions were observed between day length and hormone treatment condition in either number of c-Fos-ir cells ($F=0.29$, $df=1$, $p>0.05$) or the number of NPY-ir cells ($F=1.59$, $df=1$, $p>0.05$).

The proportion of NPY-ir cells that were also c-Fos-ir was significantly higher in LD relative to SD ($F=4.36$, $df=1$, $p<0.05$), but was not affected by ghrelin injections ($F=0.87$, $df=1$, $p>0.05$; Figure 3C). A greater proportion of NPY-ir cells were also c-Fos-ir in LD animals administered ghrelin compared with SD animals administered either saline or ghrelin ($p<0.05$, both comparisons).

The proportion of c-Fos-ir cells that were also NPY-ir did not differ as a function of photoperiod ($F=2.18$, $df=1$, $p>0.05$) or hormone treatment ($F=2.53$, $df=1$, $p>0.05$). However, a significant interaction between photoperiod and hormone treatment was observed ($F=4.75$, $df=1$, $p<0.05$): a significantly greater proportion of the total population of c-Fos-ir cells were also NPY-ir in ghrelin treated LD hamsters compared to ghrelin treated SD hamsters ($p<0.05$).

Discussion

In LD-acclimated hamsters, ghrelin stimulated food intake at a dosage an order of magnitude lower than the minimally-effective dose observed in SD-acclimated hamsters. These data indicate that photoperiod can alter the threshold of behavioral responsiveness to this orexigenic hormone and implicate ghrelin as a potential component of the mechanism regulating seasonal changes in food intake. Because circulating ghrelin increases preprandially and decreases postprandially (Cummings et al., 2001), a lowered threshold for behavioral responsiveness may lead to more frequent meals in LD hamsters. Similarly, because circulating ghrelin decreases rapidly during a meal, meal size may be correspondingly increased in LD acclimated hamsters. Data regarding the effects of meal size and the temporal aspects of feeding bouts in this species are limited; however, a recent report suggests that these features of feeding can be affected by photoperiod independently of one another: TLQP-2 reduces meal size without affecting meal frequency and is expressed at higher levels in the ARC of SD- relative to LD-housed Siberian hamsters (Jethwa et al., 2007). Adopting a relatively higher threshold for the orexigenic potency of ghrelin may contribute to the suite of photoperiod-induced neuroendocrine changes that culminate in reduced food intake and body mass following exposure to SD in this species.

In addition to their magnitude, the duration of the behavioral changes induced by ghrelin treatments differed between LD- and SD-acclimated hamsters. In LD hamsters, 3 mg/kg ghrelin treatment was effective in stimulating food intake, and this effect endured for 2 hours. In contrast, SD animals, no dose of ghrelin used in the present study was effective in stimulating food intake beyond the first hour post-treatment. A prolonged efficacy of ghrelin in LD relative to SD is also consistent with a lowered threshold of behavioral responsiveness in LD.

In both photoperiods, the lowest effective ghrelin dose (0.03 mg/kg in LD animals, 0.3 mg/kg in SD animals) increased food intake during the first hour after treatment, but decreased in food intake during the second hour; i.e. the bolus of food ingested during the first hour appears to be followed by reduced feeding during the second hour. At doses higher than the minimally effective dose, however, this compensatory reduction in food intake was not evident during the second hour. At these larger doses, the absence of compensatory inhibition of food intake after relatively large, ghrelin-induced bouts of food intake represents an enduring effect of the hormone.

Mechanistically, the present data identify differential responses of the ARC NPY system to ghrelin in LD and SD hamsters. Ghrelin induces both c-Fos (Dickson and Luckman, 1997; Wang, 2002) and NPY mRNA expression in NPY cells in the ARC (Shintani et al., 2001). Thus, we infer that ghrelin-induced c-Fos expression in NPY-ir ARC neurons serves as a reasonable proxy for eventual NPY signaling. c-Fos expression in ARC NPY-ir neurons was significantly higher in ghrelin-treated hamsters housed in LD compared to those housed in SD one hour after injection. Because ghrelin-induced increases in food intake are NPY-dependent (Nakazato et al., 2001; Keen-Rhinehart and Bartness, 2007), and photoperiod does not affect plasma ghrelin concentrations (Tups et al., 2004) this outcome is consistent with an enhanced orexigenic potency of ghrelin in LD relative to SD and suggests that ghrelin elicits greater activation of NPY neurons in LD. Given that the orexigenic effects of ghrelin in this species are dependent on the NPY Y1 receptor (Keen-Rhinehart and Bartness, 2007), yet behavioral responses to NPY are unaffected by photoperiod (Boss-Williams and Bartness, 1996), it seems likely that responses of the ARC to ghrelin may be one important site of photoperiodic regulation. A limitation to this method is that c-Fos is not expressed in neurons that have been inhibited. Therefore, the present analysis cannot identify inhibitory effects of ghrelin on NPY neurons or other types of neurons. Increased ghrelin-induced

activation of ARC NPY neurons would be a mechanism capable of mediating photoperiodic changes in behavioral responsiveness (e.g. food intake) to this orexigenic hormone.

In LD ghrelin-treated hamsters, a greater proportion of c-Fos-ir cells were colocalized with NPY-ir. In other words, in LD hamsters, NPY-expressing neurons were more likely to be activated by ghrelin than other active ARC cell types, perhaps due to an enhanced sensitivity to the hormone. CART/POMC-expressing neurons are more numerous in SD relative to LD hamsters (Khoroshii et al., 2008). Although the present study did not identify the neuropeptide phenotypes of non NPY-ir neurons that were c-Fos-ir, an increase in activation of the CART/POMC population by ghrelin in SD would be expected to inhibit food intake

Further investigation is required to identify the mechanisms by which ghrelin is more effective in activating ARC NPY neurons. Increased ghrelin signaling might explain both the lowered threshold for behavioral responsiveness to ghrelin and the potentiation of ghrelin-induced activation of NPY-ir cells in LD animals. No effort was made in the present study to identify ARC subnuclei. Thus, it is not clear where, within the ARC, these differential effects of ghrelin are manifest. The dmpARC is robustly responsive to DL and plays role in seasonal changes in energy balance. Further specification of the regions within the ARC responsible for the change in response to ghrelin in LD compared with SD may identify whether the dmpARC or other subregions contribute to this response.

In contrast with ghrelin, most gut-derived hormones have anorexigenic effects. The putative satiety peptides bombesin and cholecystokinin (CCK) are released by the gut in response to nutrient-rich chyme, slowing gastric emptying to aid digestion and providing a meal cessation signal (reviewed by Dhillon, 2007). Exogenous injections of bombesin do not inhibit food intake in LD, but do so in SD, and CCK-induced inhibition of food intake is greater in SD relative to LD hamsters (Bartness et al., 1986). The attenuation of responsiveness to ghrelin and the relative enhancement of responsiveness to satiety peptides in SD may work in concert to reduce food intake in SD.

The present study contributes to a growing body of evidence implicating the ARC as an important site for the photoperiodic regulation of energy balance in Siberian hamsters. Photoperiod-induced changes in the expression of POMC (Adam et al., 2000; Mercer et al., 2000; Reddy et al., 2000), AgRP (Mercer et al., 2000), leptin receptor (Adam et al. 2000; Mercer et al., 2000), histamine H3 receptor, VGF (Barrett et al. 2005), and CART (Khoroshii et al., 2008) have been documented in this species. Data on photoperiodic control of NPY mRNA are less consistent, with reports indicating decreases and no changes in NPY mRNA following exposure to SD (Mercer et al., 2000; Reddy et al., 1999). In contrast with photoperiodic changes in the long-term maintenance of metabolism, NPY may function as a more short-term inducer of food intake, particularly after a period of fasting. The present study extends the literature on photoperiod-dependent changes in ARC neuropeptide responses to include another peripheral signal of fasting, ghrelin.

In the ARC, most NPY-expressing neurons coexpress the orexigenic neuropeptide AgRP (Hahn et al., 1998). It is likely, but perhaps not certain, that most of the NPY-ir neurons identified in this study also express AgRP. Thus, the results of the present experiment would also be consistent with photoperiod-induced changes in the ghrelin-induced expression of AgRP. In common with NPY, antagonizing the receptor by which AgRP exerts its orexigenic effect, the melanocortin 3 or 4 receptor (MC-3/4R) inhibits ghrelin-induced food intake (Keen-Rhinehart and Bartness, 2007b), suggesting that both NPY and AgRP are necessary for ghrelin to increase food intake in this species. ARC AgRP expression has been reported to be greater (Mercer et al., 2000), and the expression of the melanocortin 3R lower (Mercer et al., 2001), in SD relative to LD hamsters. Increased ghrelin-induced activation of

NPY/AgRP neurons together with increased expression of MC-3R receptors in LD relative to SD may explain effects of day length on food intake in this species (Mercer et al., 2000, 2001; present data).

In summary, adaptation to SD photoperiods attenuated behavioral responsiveness to the orexigenic hormone ghrelin and decreased ghrelin-induced c-Fos-ir in NPY-ir neurons in the ARC. Photoperiodic changes in responsiveness to ghrelin may contribute to seasonal changes in ingestive behavior in Siberian hamsters. Decreased responsiveness of SD animals to ghrelin may work in concert with increased sensitivity to anorectic peptides and photoperiod-driven changes in ARC gene expression to regulate food intake and appetitive behaviors in a seasonally-appropriate manner. In winter conditions with low food availability and higher energetic costs for foraging, reducing behavioral responsiveness to prolonged periods of fasting may provide adaptive benefits.

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Figure 1.

Mean ($\pm$ SEM) hourly food intake of male Siberian hamsters housed in long -day (LD; 15 h light/day) or short-day (SD; 9 h light/day) photoperiods prior to i.p. injection with either 0.03, 0.3, or 3.0 mg/kg ghrelin or 0.1 ml of 0.9% saline. * $p < 0.05$ difference in food intake compared with saline condition within photoperiod condition and point of observation.

Figure 2. Representative photomicrographs of (A) arcuate nucleus (ARC) immunoreactivity for (B) NPY (green fluorescence), (C) c-Fos (red fluorescence), and (D) a composite image constructed to allow co-localization (yellow).

Figure 3.

Mean ($\pm$ SEM) (A) number of NPY-ir cells, (B) number of c-Fos-ir cells, (C) proportion of NPY-ir cells also c-Fos-ir, and (D) proportion of c-Fos-ir cells also NPY-ir as measured in coronal sections of the ARC of adult male Siberian hamsters housed in long-day (LD; 15 h light/day) or short-day (SD; 9 h light/day) photoperiods one hour after i.p. injection with 0.3 mg/kg ghrelin or 0.1 ml 0.9% saline. * $p < 0.05$, ** $p < 0.05$ vs. all other groups.