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Caloric restriction and physical activity in zebrafish (*Danio rerio*)

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Abstract

Understanding the mechanism of energy flux may be critical for explaining how obesity has emerged as a public health epidemic. It is known that changes in caloric intake predictably alter physical activity levels (PA) in mammals. Here, our goal was to test the hypothesis that fasting induces a biphasic pattern of change in PA by measuring PA before and after long-term food deprivation in zebrafish. Compared to control-fed fish, food-deprived fish showed a significant increase in PA levels during the first 2 days of food deprivation. Subsequently, however, fasted fish showed a significant chronic decrease in PA compared to fish fed at weight-maintenance levels. These data are comparable to those seen with mammals, which also show a biphasic response of PA to caloric restriction. In a separate group of fish, long-term food deprivation, associated with decreases in PA, induced a significant increase in brain preproorexin mRNA levels compared to fed controls. No change in orexin mRNA was seen after 2 days of food deprivation. The finding that orexin mRNA expression is altered only after long-term starvation suggests that orexin may be coupled with the changes in PA seen at this time. Thus, the association between negative energy balance and reductions in PA occurs across genera in biology and is associated with predictable neurological changes in brain gene expression.

Keywords: Locomotor activity; Food restriction; Fasting; Orexin; Preproorexin mRNA

Caloric restriction (CR) is associated with prolonged longevity and a myriad of biological changes that impact systems including metabolic, immune, musculoskeletal, and neurological systems [14,20]. We are particularly interested in the interaction between CR and physical activity (PA) for several reasons. First, there is an overt effect of changes in energy balance on PA. Short-term positive energy balance or caloric excess is associated with increased PA in humans [22]. Moreover, there is a negative relationship between PA and adiposity in humans [23]. In addition to positive energy balance affecting PA, negative energy balance or CR is also associated with altered PA. In the short-term, CR is paradoxically associated with increased PA (possibly due to a foraging response), whereas in the longer term, PA declines [26,29,31,37]. The second reason is that life prolongation is only known to occur with one nutritional manipulation, namely CR [11]. One potential mechanism of this effect could be through the decline in PA and thence decreased oxidative damage [14,20]. Conversely, higher PA levels are associated with decreased mortality and morbidity in humans. This paradox may be crucial for understanding the process of aging and how PA impacts it. Since PA occurs in most living species, from unicellular phylogenies to more complex ones, a fundamental mechanism may exist that defines the level of PA within a species and regulates the amount of PA according to fluxes in nutritional availability. Moreover, uncovering the neural and hormonal mechanisms, which underlie changes in PA levels, may also help us to understand the link between PA andobesity.

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We argue that if the relationship between fasting and PA is fundamental in biology, then this relationship should be reproducible across genera. If such a relationship were readily definable in a lower phylogeny species, it would not only reaffirm the generalized nature of the phenomenon in biology but also provide a model that would allow the genetic mechanism of the relationship between fasting and PA to be studied. Part of the problem in examining the interplay between CR and PA is that experimentation, particularly with longitudinal studies, can be logistically prohibitive. To examine whether fasting is associated with short-term increase and longer-term suppression of PA in zebrafish, we devised and validated technologies to address two hypotheses. Our hypothesis was that fasting in zebrafish would be similar to mammals in that first, fasting would be immediately followed by increased PA and, second, PA would decline after prolonged fasting.

One likely neural mechanism for food intake to alter PA levels is through the orexin/hypocretin system [16]. In rodent models, central treatment with orexin-A induces sharp increases in PA levels [16]. In addition, since orexins can also increase food intake [33], it is likely that the orexin system is sensitive to alterations in energy balance. We hypothesize that fasting alters PA through its effects on orexin levels. We further predict that orexin production will be altered as PA changes over the course of caloric restriction.

Adult zebrafish (aged 6–8 months; $N = 58$) were obtained from Scientific Hatcheries (Huntington Beach, CA). Fish were housed at $73 \pm 0.5\,^{\circ}$F in an acrylic cylinder (30 cm diameter, 20 cm high, 15 L volume) filled to 2.5 cm depth with sterile deionized water (pH 7.50) treated with StressCoat and StressZyme (10 mL/L; Aquarium Pharmaceuticals, Chalfont, PA). Fish were kept in a 14:10 h light:dark cycle.

Physical activity was measured ($N = 18$) using arrays of infrared beams and sensors (Opto-M Varimex Minor, Columbus Instruments, Columbus, OH) that passed through each tank in two axes at 17 beams per inch. Beam breaks were counted continuously and binned every minute. The PA measurement system was housed in a light- and sound-proof container that was thermostatically regulated to within $0.2\,^{\circ}$C. The precision of the system was verified in separate experiments using two approaches. One was by using a dead zebrafish through which a wire was inserted. The wired fish was then moved through different distances in the horizontal axis within the tank. Using the second approach, the beam breaks were compared with the horizontal distance traveled, which was calculated by observing videotapes of fish whose tank was placed on top of a 1 cm square grid.

For at least 4 days prior to the experiment, the fish were housed alone in a cylindrical tank and allowed to acclimate; the environment was identical to the experimental conditions. Fish were fed twice daily (08:00 and 15:00) at weight-maintenance food intake of 10% body weight.

The first experiment lasted 20 days throughout which each fish remained individually housed in a cylindrical tank as described above. For the first 3 days of the experiment (Days 1–3), each fish was fed at weight-maintenance food intake. Starting on Day 4, each fish was randomly assigned to receive either continued weight maintenance feeding or food restriction for 14 days (Days 4–17; $N = 9$ per group). Food restricted fish were completely deprived of food for the duration of the treatment. Using several sensor arrays that operated concomitantly, the random assignation was such that PA measurements began on a weight maintenance-fed (control) fish at the same time as a fasted fish, in separate tanks. From Days 18–20, all fish were fed at weight maintenance needs.

Beam breaks/minute were averaged to calculate mean PA/day for each fish. The PA data were analyzed using a two-way repeated-measures ANOVA to compare PA in fish from the two groups (fasted versus control fish) over the 21 days of the experiment; baseline PA was defined as the mean PA for Days 1–3; short-term PA measured for 48 h starting on Day 4; long-term PA was the mean PA for Days 14–17. To test for differences between groups at specific time points, unpaired *t*-tests were used. Statistical significance was defined as $P < 0.05$.

Body weight was measured on Day 3 and Day 17. Fish were removed from their tank using a net, momentarily blot-dried on gauze, and placed in a container containing tank water, positioned on a torn digital balance. Fish weight was determined as the weight of the container plus the fish minus the weight of the container before the fish was added. The mean of three measurements was recorded.

We wanted to determine if changes in brain orexin expression were associated with the short-term increase or the long-term decrease in physical activity in zebrafish. For these studies, separate groups of fish were used ($N = 10$ in each of four groups, CR and control-fed fish at each Days 5 and 17 of the study); the RNA from individual fish was used for these analyses (no pooling of tissue was done). The protocol as delineated above was repeated except that the experiment ended on Day 5 or Day 17 (7 h after lights-on) such that fish had either been fed at weight maintenance needs or fasted for 2 days or 14 days. At the end of the Day 5 or Day 17, fish were decapitated and their heads removed over dry ice. Pestle and mortar homogenization was then performed in a bath of liquid nitrogen and total RNA was isolated from zebrafish brain according to the TRI Reagent protocol as previously described [12,19] with minor modifications [6]. Briefly, tissue was homogenized with Trizol reagent (Gibco BRL, Paisley, UK) and chloroform. After phase separation, the aqueous phase was removed and total RNA was precipitated with isopropanol. The subsequent RNA pellet was washed with 75% ethanol and then stored at $-80\,^{\circ}$C in 100% ethanol until quantification. Aliquots of RNA were reconstituted in DNAase/RNAse-free water and analyzed spectrophotometrically (DU-640, Beckman Coulter, Fullerton, CA) at 230, 260, 270, 280, and 320 nm. A denaturing gel was run to confirm RNA integrity.

Splicing patterns and cDNA sequence for the zebrafish preproorexin gene are currently unpublished. The cDNA sequence used for the preproorexin primer design was derived within the product of a previously described primer set, which

Table 1
Primer sequences for prepro-orexin and β-actin

Orexin-A (GenBank Accession, BX005093)	
Forward 5′-TCTACGAGATGCTGTGCCGAG-3′	
Reverse 5′-CGTTTGCCAAGAGTGAGAATC-3′	
β-Actin (GenBank Accession, AF025305)	
Forward 5′-TGTTCCAGCCATCCTTCTTGG-3′	
Reverse 5′-CCAGACAGAGTATTTACGCTCCG-3′	

also encoded the orexin-A protein [15]. The reverse complement of the zebrafish contiguous DNA sequence (BX005093) spanning 131848–132121, was used to create the orexin-A primer, which was specific for orexin-A and verified by reverse translation. The primers for orexin-A and β-actin (using published splice variants and sequence data from NCBI) were created using MacVector 7.2 (Accerlys, San Diego, CA) (Table 1).

One-step real-time RT-PCR was performed using 100 ng of total RNA, the reagents provided in the Roche RNA Amplification Kit SYBR Green I, and a Roche LightCycler (Roche Applied Science, Indianapolis, IN). RT-PCR was performed as follows: reverse transcription (30 min, 42 °C), denaturation (30 s, 95 °C), followed by 35 cycles of cDNA amplification consisting of a 15 s denaturation at 95 °C, primer annealing for 20 s at 59 °C (orexin-A), and 58 °C (β-actin), and product elongation for 15 s at 72 °C. Data acquisition was taken at the end of each amplification cycle at a temperature slightly lower than the temperature required to melt the PCR product, 84 °C (orexin-A) and 83 °C (β-actin). Amplification products from PCR were determined by electrophoresis in a 3% Nuseive gel. PCR products were purified (QIAquick PCR Purification Kit, Valencia, CA) and then verified by capillary electrophoresis.

The $2^{-\Delta\Delta CT}$ method was used to calculate relative orexin-A and β-actin mRNA and fold changes in mRNA levels [24]. Fold change in orexin-A mRNA compared to β-actin mRNA was expressed as the ratio of the mean relative orexin-A mRNA and mean relative β-actin mRNA. Data were analyzed using StatView 5.0 (Cary, NC) and are expressed as mean ± S.E.M. Means were compared by unpaired (between group) *t*-tests and an alpha level of 0.05 was used for the statistical tests.

All the fish survived the experiment without evidence of physical harm. In the fish randomized to weight maintenance, body weight did not change significantly (data are stated as mean ± S.E.M.), 0.388 ± 0.036 g on Day 3 to 0.435 ± 0.038 g on Day 18. In the fasted fish, weight decreased from 0.392 ± 0.023 g to 0.336 ± 0.020 g ($P < 0.001$).

The PA validation experiments demonstrated that all movements of >1 cm were captured regardless of where the fish was within the cylindrical tank. By using light scattered off ceiling-mounted polystyrene ballasts, we eliminated the effect of light shimmering against each fish's body. Counts versus distance traveled showed an intraclass correlation coefficient of $r > 0.98$, indicating that we accurately capture PA data in zebrafish.

There was a significant interaction between group and day ($F_{(20,300)} = 4.521$, $P < 0.001$). To address our first hypothesis, that short-term fasting is associated with increased PA, we compared the PA for the fish on Day 4 with baseline (Days 1–3). On average, PA increased with short-term CR from 12.8 ± 2.5 to 37.2 ± 13.4 beam breaks. In the CR group, one fish was particularly active on Day 3, with a PA an average of 133.78 beam breaks/min, though the CR fish showed significantly more PA compared to control fish after the outlier fish was removed from the analysis ($P < 0.0005$). In Fig. 1, the grey data points represent mean PA of the CR fish physical activity after the outlier data were removed on Days 4 and 5. In the weight maintained (control) group, PA remained exactly the same over this time period (mean beam breaks/min: 15.3 ± 1.9 on Days 1–3; 15.3 ± 1.9 on Day 4).

To address our second hypothesis, that longer term fasting is associated with decreased PA, we compared the PA for the fish at Day 17 with baseline PA (Days 1–3). The number of beam breaks/min declined substantially with CR from 12.8 (±2.5) to 1.7 (±0.4). In the control group, PA did not change significantly over the same time period, with 15.3 (±1.9) beam breaks on Days 1–3 and 14.7 (±1.3) on Day 18. Overall, the pattern for the changes in PA were highly characteristic and occurred in all fish studied (Fig. 1).

Both orexin-A and β-actin primer sets yielded amplification products that were detected as single products at 109 and 240 nucleotides, respectively (portion shown in Fig. 2A). The nucleotide sequences of the PCR products determined by sequencing were verified to the reverse complement of the zebrafish contiguous DNA sequence spanning 131848–132121 and the zebrafish β-actin mRNA sequence (GenBank Accession, BX005093 and AF025305, respectively; Fig. 2B). Fish experiencing 2 days of CR showed no significant change in orexin-A mRNA, from 0.797 (±0.089)

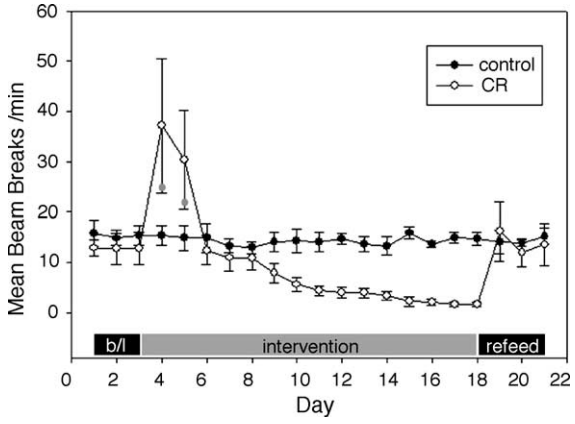

Fig. 1. Physical activity (PA) levels in zebrafish before, during, and after fasting. Significant increases in PA were seen in fish during the first 2 days of fasting (i.e., Days 4 and 5 of "intervention") compared to controls (baseline, "b/l"). After long-term fasting, significant decreases in PA were seen in fasted fish compared to controls. All fish were fed ("refeed") at weight-maintenance levels starting on Day 18. Grey points represent the mean PA/day for the calorically restricted (CR) fish after the outlier point was removed on days 4 (S.E.M. = 4.4) and 5 (S.E.M. = 3.6). $N = 9$/group (including outlier).

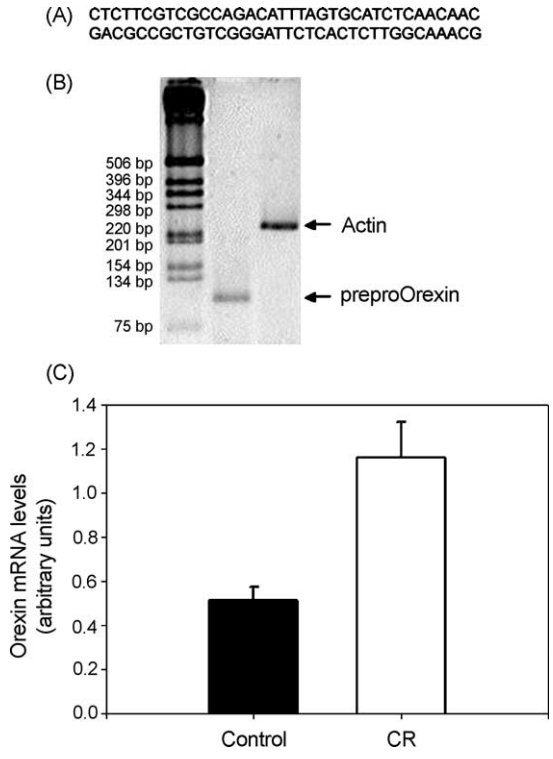

Fig. 2. (A) Portion of the sequence of orexin-A PCR product used for verification. (B) Gel indicating that orexin-A and β-actin primer sets yielded PCR amplification products that were detected as single products at 109 and 240 nucleotides, respectively. (C) Significant effect of fasting on relative orexin-A gene expression in whole zebrafish brains compared to fed controls ($P = 0.0015$). $N = 10$/group.

in the control-fed fish to 1.086 (±0.203) in the CR fish (data not shown). After 14 days of CR, relative orexin-A mRNA expression level in the fasted animals displayed a two-fold increase in comparison to the fed controls. As shown in Fig. 2C, significantly higher orexin-A mRNA expression levels were detected in the fasted animals (1.236 ± 0.211) compared to the fed controls (0.511 ± 0.063; $P < 0.01$) after long-term CR. One outlier (>2 S.D. from the mean) was removed from the CR group for analysis; the groups still showed a significant difference after the outlier was removed ($P < 0.01$).

These data are the first to demonstrate that zebrafish show alterations in PA after fasting. Fasted fish showed an increase in PA after 2 days, followed by a chronic decrease in PA. This biphasic pattern has also been seen in fasted mammals [26,29,31,37]. The similarity in the changes in PA seen during fasting across species implies that the effect of fasting on PA is a general phenomenon. A fundamental mechanism may therefore exist to regulate PA levels according to changes in nutritional intake. This information could be critical in understanding how PA interacts with energy provision, or the amount of energy available to the individual, in obesity [23].

Orexin-A and orexin-B (also known as hypocretin 1 and 2) are produced from a common peptide precursor (preproorexin) to form 33 and 28 amino acid residues, respectively [9,30]. In mammals, orexin producing neurons reside in the caudal aspect of the lateral, perifornical, posterior and dorsomedial hypothalamus [9,30]. The orexin neurons project diffusely throughout the central nervous system in a pattern that matches the wide distribution of orexin receptors, OXR1 and OXR2, which are metabotropic G-protein coupled receptors [1,7–9,28,30,35]. Because of the ability of orexins to induce intense increases in PA in mammals as well as goldfish [36], and the association of increased brain orexin levels with increased PA [16,18], we hypothesized that sustained fasting may induce changes in PA through altering brain orexin. Although short-term CR had no significant effect on orexin-A mRNA, long-term fasting was associated with specific and substantial increases in preproorexin mRNA levels in the zebrafish brain (Fig. 2C). These results are consistent with data from mammals demonstrating that long-term, but not short-term (48 h or less), fasting or food restriction increases orexin gene expression, receptor expression, or CSF orexin levels [5,17,25,27,32,39,40] (however see [2] for contrasting results) as well as Fos expression in orexin neurons [3,4,10,21].

Orexin in fish appears to have a similar effect on feeding as in mammals [36]. The current data imply that the increased orexin mRNA levels seen in CR zebrafish are linked to orexin's role in appetite regulation; the possible role of orexin in the decreased PA after starvation, however, cannot be discounted. Our results showing increased orexin expression in zebrafish subjected to long-term CR suggest that interoceptive cues mediating hunger signaling are elevated, which is consistent with the drive to feed during extreme caloric deprivation. It is possible that any effect of reduced locomotor activity on orexin gene expression is masked by the elevated orexin expression simulated by the strong appetitive drive during fasting. Moreover, measuring preproorexin gene expression in whole brain does not allow for the detection of differences in preproorexin gene expression between subpopulations of orexin neurons, or between activity of the cleaved products, orexin-A and orexin-B, which may serve different functions within the brain [34,38]. Alternatively, it is interesting that the decrease in PA seen after long-term CR was associated with changes in orexin gene expression whereas the increase in PA seen after 2 days of CR was not. This suggests that the profound decline in PA seen after prolonged CR may be mechanistically linked to the increase in zebrafish orexin gene expression.

There is a strong link between energy provision and PA, and this link may be critical to the understanding of obesity [22,23]. Both obesity and CR strongly affect energy provision, and both affect PA as well. Investigating the link between CR and PA may provide important insights into the mechanisms that link PA and decreased energy provision. These mechanisms may then generalize to the coupling between increased energy intake and PA in as well, allowing us to further elucidate the physiological changes underlying obesity.

In summary, the data described here demonstrate that the effects of fasting on both PA and brain orexin are consistent with previous reports in mammals. This strengthens the hypothesis that fasting has consistent and predictable consequences with respect to physical activity. These effects may represent a biological phenomenon affecting many classes of vertebrates, not only mammals. Moreover, the zebrafish represents a valuable model to examine the genetic and molecular underpinnings of changes in PA after fasting and other manipulations, as these changes in PA can be rapidly and accurately assessed. The large amount of information on neural and molecular mechanisms [13], as well as the wealth of molecular genetic tools available to zebrafish studies [13], makes the zebrafish a powerful model to investigate the molecular and genetic underpinnings of changes in PA with changes in energy intake.

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References

[1] M. Backberg, G. Hervieu, S. Wilson, B. Meister, Orexin receptor-1 (OX-R1) immunoreactivity in chemically identified neurons of the hypothalamus: focus on orexin targets involved in control of food and water intake, Eur. J. Neurosci. 15 (2002) 315–328.
[2] F. Bertile, H. Oudart, F. Criscuolo, Y.L. Maho, T. Raclot, Hypothalamic gene expression in long-term fasted rats: relationship with body fat, Biochem. Biophys. Res. Commun. 303 (2003) 1106–1113.
[3] K.P. Briski, P.W. Sylvester, Hypothalamic orexin-A-immunpositive neurons express Fos in response to central glucopenia, Neuroreport 12 (2001) 531–534.
[4] X.J. Cai, M.L. Evans, C.A. Lister, R.A. Leslie, J.R. Arch, S. Wilson, G. Williams, Hypoglycemia activates orexin neurons and selectively increases hypothalamic orexin-B levels: responses inhibited by feeding and possibly mediated by the nucleus of the solitary tract, Diabetes 50 (2001) 105–112.
[5] X.J. Cai, P.S. Widdowson, J. Harrold, S. Wilson, R.E. Buckingham, J.R. Arch, M. Tadayyon, J.C. Clapham, J. Wilding, G. Williams, Hypothalamic orexin expression: modulation by blood glucose and feeding, Diabetes 48 (1999) 2132–2137.
[6] P. Chomczynski, A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples, Biotechniques 15 (1993) 532–534, 536–537.
[7] J.E. Cluderay, D.C. Harrison, G.J. Hervieu, Protein distribution of the orexin-2 receptor in the rat central nervous system, Regul. Pept. 104 (2002) 131–144.
[8] Y. Date, Y. Ueta, H. Yamashita, H. Yamaguchi, S. Matsukura, K. Kangawa, T. Sakurai, M. Yanagisawa, M. Nakazato, Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 748–753.
[9] L. de Lecea, T.S. Kilduff, C. Peyron, X. Gao, P.E. Foye, P.E. Danielson, C. Fukuhara, E.L. Battenberg, V.T. Gautvik, F.S. Bartlett II, W.N. Frankel, A.N. van den Pol, F.E. Bloom, K.M. Gautvik, J.G. Sutcliffe, The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 322–327.
[10] S. Diano, B. Horvath, H.F. Urbanski, P. Sotonyi, T.L. Horvath, Fasting activates the nonhuman primate hypocretin (orexin) system and its postsynaptic targets, Endocrinology 144 (2003) 3774–3778.
[11] G. Fernandes, E.J. Yunis, R.A. Good, Influence of diet on survival of mice, Proc. Natl. Acad. Sci. U.S.A. 73 (1976) 1279–1283.
[12] M.J. Glass, J.E. Briggs, C.J. Billington, C.M. Kotz, A.S. Levine, Opioid receptor blockade in rat nucleus tractus solitarius alters amygdala dynorphin gene expression, Am. J. Physiol. Regul. Integr. Comp. Physiol. 283 (2002) R161–R167.
[13] S. Guo, Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? Genes Brain Behav. 3 (2004) 63–74.
[14] L.K. Heilbronn, E. Ravussin, Calorie restriction and aging: review of the literature and implications for studies in humans, Am. J. Clin. Nutr. 78 (2003) 361–369.
[15] J. Kaslin, J.M. Nystedt, M. Ostergard, N. Peitsaro, P. Panula, The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems, J. Neurosci. 24 (2004) 2678–2689.
[16] K. Kiwaki, C.M. Kotz, C. Wang, L. Lanningham-Foster, J.A. Levine, Orexin A (hypocretin 1) injected into hypothalamic paraventricular nucleus and spontaneous physical activity in rats, Am. J. Physiol. Endocrinol. Metab. 286 (2004) E551–E559.
[17] G. Komaki, Y. Matsumoto, H. Nishikata, K. Kawai, T. Nozaki, M. Takii, H. Sogawa, C. Kubo, Orexin-A and leptin change inversely in fasting non-obese subjects, Eur. J. Endocrinol. 144 (2001) 645–651.
[18] C.M. Kotz, J.A. Teske, J.A. Levine, C. Wang, Feeding and activity induced by orexin A in the lateral hypothalamus in rats, Regul. Pept. 104 (2002) 27–32.
[19] C.M. Kotz, D. Weldon, C.J. Billington, A.S. Levine, Age-related changes in brain proDynorphin gene expression in the rat, Neurobiol. Aging 25 (2004) 1343–1347.
[20] J. Koubova, L. Guarente, How does calorie restriction work? Genes Dev. 17 (2003) 313–321.
[21] T. Kurose, Y. Ueta, Y. Yamamoto, R. Serino, Y. Ozaki, J. Saito, S. Nagata, H. Yamashita, Effects of restricted feeding on the activity of hypothalamic Orexin (OX)-A containing neurons and OX2 receptor mRNA level in the paraventricular nucleus of rats, Regul. Pept. 104 (2002) 145–151.
[22] J.A. Levine, N.L. Eberhardt, M.D. Jensen, Role of nonexercise activity thermogenesis in resistance to fat gain in humans, Science 283 (1999) 212–214.
[23] J.A. Levine, L.M. Lanningham-Foster, S.K. McCrady, A.C. Krizan, L.R. Olson, P.H. Kane, M.D. Jensen, M.M. Clark, Interindividual variation in posture allocation: possible role in human obesity, Science 307 (2005) 584–586.
[24] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[25] X.Y. Lu, D. Bagnol, S. Burke, H. Akil, S.J. Watson, Differential distribution and regulation of OX1 and OX2 orexin/hypocretin receptor messenger RNA in the brain upon fasting, Horm. Behav. 37 (2000) 335–344.
[26] S.E. Lynn, C.W. Breuner, J.C. Wingfield, Short-term fasting affects locomotor activity, corticosterone, and corticosterone binding globulin in a migratory songbird, Horm. Behav. 43 (2003) 150–157.
[27] M.S. Mondal, M. Nakazato, Y. Date, N. Murakami, M. Yanagisawa, S. Matsukura, Widespread distribution of orexin in rat brain and its regulation upon fasting, Biochem. Biophys. Res. Commun. 256 (1999) 495–499.
[28] C. Peyron, D.K. Tighe, A.N. van den Pol, L. de Lecea, H.C. Heller, J.G. Sutcliffe, T.S. Kilduff, Neurons containing hypocretin (orexin) project to multiple neuronal systems, J. Neurosci. 18 (1998) 9996–10015.

[29] J.C. Russell, W.F. Epling, D. Pierce, R.M. Amy, D.P. Boer, Induction of voluntary prolonged running by rats, J. Appl. Physiol. 63 (1987) 2549–2553.
[30] T. Sakurai, A. Amemiya, M. Ishii, I. Matsuzaki, R.M. Chemelli, H. Tanaka, S.C. Williams, J.A. Richardson, G.P. Kozlowski, S. Wilson, J.R. Arch, R.E. Buckingham, A.C. Haynes, S.A. Carr, R.S. Annan, D.E. McNulty, W.S. Liu, J.A. Terrett, N.A. Elshourbagy, D.J. Bergsma, M. Yanagisawa, Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior, Cell 92 (1998) 573–585.
[31] T. Severinsen, I.C. Munch, Body core temperature during food restriction in rats, Acta Physiol. Scand. 165 (1999) 299–305.
[32] I. Swart, J.M. Overton, T.A. Houpt, The effect of food deprivation and experimental diabetes on orexin and NPY mRNA levels, Peptides 22 (2001) 2175–2179.
[33] D.C. Sweet, A.S. Levine, C.J. Billington, C.M. Kotz, Feeding response to central orexins, Brain Res. 821 (1999) 535–538.
[34] M. Szekely, E. Petervari, M. Balasko, I. Hernadi, B. Uzsoki, Effects of orexins on energy balance and thermoregulation, Regul. Pept. 104 (2002) 47–53.
[35] S. Taheri, D. Sunter, C. Dakin, S. Moyes, L. Seal, J. Gardiner, M. Rossi, M. Ghatei, S. Bloom, Diurnal variation in orexin A immunoreactivity and prepro-orexin mRNA in the rat central nervous system, Neurosci. Lett. 279 (2000) 109–112.
[36] H. Volkoff, R.E. Peter, Effects of CART peptides on food consumption, feeding and associated behaviors in the goldfish, *Carassius auratus*: actions on neuropeptide Y- and orexin A-induced feeding, Brain Res. 887 (2000) 125–133.
[37] J.L. Weed, M.A. Lane, G.S. Roth, D.L. Speer, D.K. Ingram, Activity measures in rhesus monkeys on long-term calorie restriction, Physiol. Behav. 62 (1997) 97–103.
[38] J.T. Willie, R.M. Chemelli, C.M. Sinton, M. Yanagisawa, To eat or to sleep? Orexin in the regulation of feeding and wakefulness, Annu. Rev. Neurosci. 24 (2001) 429–458.
[39] M.F. Wu, J. John, N. Maidment, H.A. Lam, J.M. Siegel, Hypocretin release in normal and narcoleptic dogs after food and sleep deprivation, eating, and movement, Am. J. Physiol. Regul. Integr. Comp. Physiol. 283 (2002) R1079–R1086.
[40] Y. Yamamoto, Y. Ueta, R. Serino, M. Nomura, I. Shibuya, H. Yamashita, Effects of food restriction on the hypothalamic prepro-orexin gene expression in genetically obese mice, Brain Res. Bull. 51 (2000) 515–521.