



## Intracerebroventricular injection of ghrelin decreases wheel running activity in rats



Yumiko Miyatake<sup>a,1</sup>, Tetsuya Shiuchi<sup>b,c,1</sup>, Kazuaki Mawatari<sup>d</sup>, Satomi Toda<sup>a</sup>, Yasuko Taniguchi<sup>a</sup>, Akari Futami<sup>a</sup>, Fukiko Sato<sup>a</sup>, Masashi Kuroda<sup>a</sup>, Mayu Sebe<sup>a</sup>, Rie Tsutsumi<sup>a</sup>, Nagakatsu Harada<sup>a</sup>, Yasuhiko Minokoshi<sup>e</sup>, Tadahiro Kitamura<sup>f</sup>, Koro Gotoh<sup>g</sup>, Masaki Ueno<sup>h</sup>, Yutaka Nakaya<sup>a</sup>, Hiroshi Sakaue<sup>a,\*</sup>

<sup>a</sup> Department of Nutrition and Metabolism, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Tokushima, 770-8503, Japan

<sup>b</sup> Department of Integrative Physiology, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Tokushima, 770-8503, Japan

<sup>c</sup> PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama, 332-0012, Japan

<sup>d</sup> Department of Preventive Environment and Nutrition, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Tokushima, 770-8503, Japan

<sup>e</sup> Department of Developmental Physiology, National Institute for Physiological Sciences, Okazaki, Aichi, 444-8585, Japan

<sup>f</sup> Metabolic Signal Research Center, Institute for Molecular and Cellular Regulation, Maebashi, Gunma University, 371-8512, Japan

<sup>g</sup> Department of Endocrinology, Metabolism, Rheumatology and Nephrology, Faculty of Medicine, Oita University, Yufu, Oita, 879-5593, Japan

<sup>h</sup> Department of Pathology and Host Defense, Faculty of Medicine, Kagawa University, Miki, Kagawa, 761-0793, Japan

### ARTICLE INFO

#### Article history:

Received 15 April 2016

Received in revised form

28 September 2016

Accepted 1 November 2016

Available online 5 November 2016

#### Keywords:

Ghrelin

Voluntary exercise

Central nervous system

### ABSTRACT

There is an increasing interest in elucidating the molecular mechanisms by which voluntary exercise is regulated. In this study, we examined how the central nervous system regulates exercise. We used SPORTS rats, which were established in our laboratory as a highly voluntary murine exercise model. SPORTS rats showed lower levels of serum ghrelin compared with those of the parental line of Wistar rats. Intracerebroventricular and intraperitoneal injection of ghrelin decreased wheel-running activity in SPORTS rats. In addition, daily injection of the ghrelin inhibitor JMV3002 into the lateral ventricles of Wistar rats increased wheel-running activity. Co-administration of obestatin inhibited ghrelin-induced increases in food intake but did not inhibit ghrelin-induced suppression of voluntary exercise in rats. Growth hormone secretagogue receptor (GHSR) in the hypothalamus and hippocampus of SPORTS rats was not different from that in control rats. We created an arcuate nucleus destruction model by administering monosodium glutamate (MSG) to neonatal SPORTS rats. Injection of ghrelin into MSG-treated rats decreased voluntary exercise but did not increase food intake, suggesting that wheel-running activity is not controlled by the arcuate nucleus neurons that regulate feeding. These results provide new insights into the mechanism by which ghrelin regulates voluntary activity independent of arcuate nucleus neurons.

© 2016 Elsevier Inc. All rights reserved.

### 1. Introduction

Exercise is considered beneficial for the treatment of obesity and life style-related diseases because, it is reported to improve insulin resistance and prevent the progression of obesity and diabetes [1]. Obese animals show low levels of locomotor activity [2,3],

and obese humans generally express low motivation for performing exercise. Forced exercise training causes stress responses in terms of excess production of stress hormones and hypertrophy of the adrenal gland [4,5]. Therefore, voluntary exercise leads to improved therapeutic effects over forced exercise. Elucidating the mechanistic basis of the motivation for voluntary exercise is important for discovering new targets for diabetes treatments, and this topic has become a subject of growing interest.

It is thought that the motivation for movement is controlled by the brain only. Orexin (also known as hypocretin) is a neuronal peptide expressed in the lateral hypothalamic area of the brain that promotes the stimulation of food intake, spontaneous

\* Corresponding author at: Department of Nutrition and Metabolism, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto, Tokushima, 770-8503, Japan.

E-mail address: [hsakaue@tokushima-u.ac.jp](mailto:hsakaue@tokushima-u.ac.jp) (H. Sakaue).

<sup>1</sup> These authors contributed equally to this work.

activity, and energy expenditure, thereby increasing non-exercise-activity thermogenesis (NEAT) [6–9]. Elevation of NEAT in turn leads to inhibited pathogenesis associated with obesity and diabetes. Orexin production in the brain also regulates voluntary exercise, and it is difficult to interfere with and override this stimulation. In contrast, few reports have described the involvement of humoral factors in regulating voluntary exercise. Although there are known systems in the brain for the activation of motivation, such as the ventral tegmental area-dopaminergic system or the opioid system in the nucleus accumbens [10,11], it is important to elucidate novel control mechanisms for voluntary exercise involving humoral factors.

In this study, we used a unique model rat, which has been named the SPORTS (Spontaneously Running Tokushima-Shikoku) rat. SPORTS rats were established in our laboratory from an original line of rats derived from the Wistar strain, and they exhibit the unique characteristic of highly voluntary wheel-running [12]. We hypothesized that the cause of the SPORTS rat's highly voluntary exercise capacity was a change in a gastrointestinal hormone. This knowledge may contribute to the establishment of a novel strategy for the promotion of exercise.

## 2. Materials and methods

### 2.1. Animals

We used male Wistar rats (SLC, Inc. Shizuoka, Japan) and SPORTS rats, which were previously established in our laboratory [12], in this study. Twelve- to sixteen-week-old rats were used for this study because at this age, they exhibit running activity that is almost 6-times higher than that of age-matched Wistar rats [12]. All rats were housed individually at a constant room temperature of  $23 \pm 1^\circ\text{C}$  with a 12-h light-dark cycle (lights on at 10 AM) and were fed a standard non-purified diet (Oriental Yeast, Tokyo, Japan) with food and water available ad libitum. This study conformed to the guidelines for the care and use of laboratory animals of Tokushima University Graduate School Institute of Biomedical Sciences. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

### 2.2. Intraperitoneal injection experiments

Twelve-week-old male SPORTS rats were divided into two groups and received an intraperitoneal (ip) injection of vehicle (0.9% saline) or ghrelin (Asubio Pharma Co., Ltd., Kobe, Japan; 100  $\mu\text{g}/\text{kg}$  body weight) (each  $n=6$ ).

### 2.3. Intracerebroventricular injection experiments

Rats were anesthetized by ip injection of sodium pentobarbital (1 ml/kg body weight), and a stainless-steel guide cannula was inserted from the bregma into the lateral ventricle (AP =  $-1.0$  mm, L =  $+1.5$  mm, H =  $+3.6$  mm). The guide cannula was fixed in the skull with adhesive and dental acrylic cement. A stainless-steel dummy cannula was inserted into the guide and kept there until the start of the experiment. After surgery, rats were allowed to recover for at least 1 week. Thereafter, SPORTS rats were divided into groups based on the substance(s) to be administered, receiving a 5- $\mu\text{l}$  intracerebroventricular (icv) injection of vehicle (0.9% saline), ghrelin (Asubio Pharma Co., Ltd.; 1  $\mu\text{g}/\mu\text{l}$ ), obestatin (Asubio Pharma Co., Ltd.; 1  $\mu\text{g}/\mu\text{l}$ ), or corticotropin-releasing hormone (Sigma-Aldrich, St. Louis, MO, USA; 1  $\mu\text{g}/\mu\text{l}$ ) or a 10- $\mu\text{l}$  icv injection of ghrelin+obestatin (5  $\mu\text{g}/5 \mu\text{l}$  each) at 10 PM. Wistar rats were divided into two groups and received a 5- $\mu\text{l}$  icv acute injection of vehicle (0.9% saline) or JMV3002 (Cayman Chemical Company, Ann Arbor, MI, USA; 0.25  $\mu\text{g}/5 \mu\text{l}$ ) at 10 PM for 10 consecutive days.

JMV3002 concentration was determined based on a previous report [13].

### 2.4. Measurement of wheel-running activity

After ip or icv injection, data were acquired at 2-h intervals for 48 h. Wheel-running activity was measured in a wheel-running cage (Shinano Ltd., Tokyo, Japan).

### 2.5. Measurement of expiratory metabolism

We used Oxymax (Columbus Instruments, Columbus, OH, USA) to measure oxygen consumption ( $\text{VO}_2$ ) and resting energy requirement (RER). Twelve-week-old male SPORTS rats were placed in the Comprehensive Lab Animal Monitoring System (CLAMS) with free access to food and water, allowing them to acclimatize in individual metabolic cages for 48 h before any measurements. The data were acquired at 12-h intervals for 24 h.

### 2.6. Destruction of the arcuate nucleus by monosodium glutamate (MSG) administration

SPORTS rats received a single dose of MSG (4 mg/g body weight) at the age of 3–5 days postpartum. The dose of MSG administered was determined as previously described [14]. The brains of exercising 12- to 16-week-old male SPORTS rats were perfusion-fixed and used for immunostaining of NeuN.

### 2.7. Measurement of plasma levels of ghrelin

Male SPORTS and control rats aged 4 weeks were housed individually in cages without an exercise wheel until 12 weeks of age (SPORTS rat  $n=11$ , Wistar rat  $n=12$ ). We then took blood samples and killed the rats by exsanguination from the abdominal aorta. Blood samples were obtained with EDTA-2Na and 500 kIU/ml aprotinin (Sigma-Aldrich) to prevent degradation of ghrelin and were then centrifuged. Plasma was separated and added to 1 mol/l HCl. Samples were stored at  $-80^\circ\text{C}$  until analysis. Plasma levels of ghrelin were measured using an Active Ghrelin ELISA Kit (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) and a Desacyl-Ghrelin ELISA Kit (Mitsubishi Chemical Medience Corporation), according to the manufacturer's recommended protocols.

### 2.8. Real-time quantitative PCR analysis

Hypothalamus and hippocampus were isolated from 11 to 17-week-old male SPORTS and Wistar rats ( $n=8$  each). The isolated tissues were stored at  $-80^\circ\text{C}$  until further use. RNA was extracted using a phenol/chloroform extraction procedure. Total RNA was isolated using Isoplus (TAKARA BIO, Inc., Shiga, Japan). RNA concentrations and purities were calculated using A.260 UV absorbances and A.260/A.280 ratios. Total RNA was subjected to reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using a 7500 Real-Time PCR instrument (Applied Biosystems) and Power SYBR Green (Applied Biosystems). The sequences of primers used were 5'-GTTAGCCCTGACTATCCTGGAA-3' and 5'-AGGCAGGCAGATCTCTGAGTA-3' for ghrelin, 5'-TGGATTGCTCCAGTCTTC-3' and 5'-AAGGATCCGTGATGCAGAG-3' for ghrelin O-acyltransferase (GOAT), 5'-ACCAGAACCACAAGCAGACA-3' and 5'-CGAAGACTTGAAAAGAGGT-3' for growth hormone secretagogue receptor type 1 (GHSR), and 5'-CCCGCGAGTACAACCTTCT-3' and 5'-CGTCATCCATGGCGAACT-3' for  $\beta$ -actin.  $\beta$ -actin was used as an internal control, and the results

obtained were normalized to  $\beta$ -actin expression to generate relative expression values.

### 2.9. Immunohistochemistry

In the staining of c-fos, 12 rats underwent icv administration of saline or ghrelin. At 1.5 h post-injection, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and transcardially perfused with isotonic phosphate-buffered saline (PBS). This was followed by fixation in 4% paraformaldehyde in 0.1 M phosphate buffer before the brains were excised. The brains were removed and post-fixed for 24 h. Brains were sectioned using a freezing sliding microtome, and 40- $\mu$ m sections were collected and stored in 0.1 M PBS with 0.1% sodium azide (Wako, Osaka, Japan) at 4°C (16–20°C). Brain sections were stained for c-fos expression using an avidin-biotinylated peroxidase complex method, as described previously [15]. The number of c-fos-positive cells was scored by observers blind to the identity of the subjects in five similar coronal sections from a given brain area of interest. Statistical analysis was performed on the average from the bilateral areas of each animal. A negative control for c-fos staining was included in the experiment, in which the antiserum recognizing c-fos was replaced with the normal serum and further incubation with secondary antibody was performed as usual. In the staining of NeuN, control or MSG-treated SPORTS rats were perfused and fixed as described above. The tissue was then embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), and stored at –80°C until analysis. Serial 30- $\mu$ m cryosections were prepared with the use of a cryostat (Leica CM1850, Wetzlar, Germany). Sections received a 10-min quenching treatment with 3% H<sub>2</sub>O<sub>2</sub> in methanol. For DAB staining of NeuN, brain sections were exposed for 2 h at room temperature to 3% normal donkey serum and were then incubated overnight at 4°C with rabbit antibodies to NeuN (1:500 dilution) (Cell Signaling Technology, MA, US). After washing the sections with PBS, immune complexes were detected by VECTASTAIN ABC HRP kit (peroxidase, rabbit IgG) (Vector Laboratories, Burlingame, CA, USA). Sections were colored with a Peroxidase Stain DAB Kit (brown stain) (Nacal Tesque, Kyoto, Japan) and Metal Enhancer for DAB Stain (Nacal Tesque). Sections were finally examined with a microscope (Leica DM4000B). Brain sections were carefully matched on the basis of the shape of brain structures, and three sections corresponding to the arcuate nucleus (between 1.26 and –2.54 mm posterior to the bregma) (Franklin and Paxinos, 1997) were selected per rat. NeuN expression and the number of c-fos-positive cells were determined at the site of the arcuate nucleus.

### 2.10. Sucrose preference test

Twelve-week-old male SPORTS and Wistar rats ( $n = 8$  each) were given a choice between 2% sucrose solution or water for 4 days in their home cages. The amount of each consumed was measured daily, and the two bottles were switched daily to reduce a side bias. Sucrose preference was calculated as a percentage of the volume of sucrose consumed and averaged over the 4 days of testing.

### 2.11. Statistical analysis

Results are expressed as means  $\pm$  SEM. Data from two groups were analyzed using Student's *t*-tests. Data from more than two groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. *P* values < 0.05 were considered statistically significant.

## 3. Results

### 3.1. SPORTS rats exhibit low plasma levels of ghrelin

Plasma desacyl-ghrelin levels in SPORTS rats were significantly lower compared to those observed in Wistar rats (Fig. 1A,  $P < 0.01$ ). In contrast, there was no significant difference between SPORTS and Wistar rats in plasma active ghrelin levels (Fig. 1B). Ghrelin was isolated previously from the stomach as an endogenous ligand that facilitates growth hormone secretion [16], although it is also produced in the hypothalamic arcuate nucleus (ARC) of neurons. Therefore, we measured ghrelin mRNA expression in the stomachs of SPORTS rats. Quantitative real-time PCR results showed that ghrelin mRNA expression in the stomach was not significantly different (Fig. 1C). Moreover, ghrelin is known to be activated by ghrelin *o*-acyltransferase (GOAT) [17]. We tested the possibility that there is a difference in ghrelin-activating enzyme expression between SPORTS and Wistar rats, but GOAT mRNA expression was not significantly different in the stomachs of SPORTS and Wistar rats (Fig. 1D). Together, these data suggest that ghrelin production is normal in the stomach but that desacyl-ghrelin is decreased in the plasma of SPORTS rats. Intraperitoneal injection of ghrelin in SPORTS rats similarly decreased wheel-running activity (Fig. 1E,  $P < 0.05$ ).

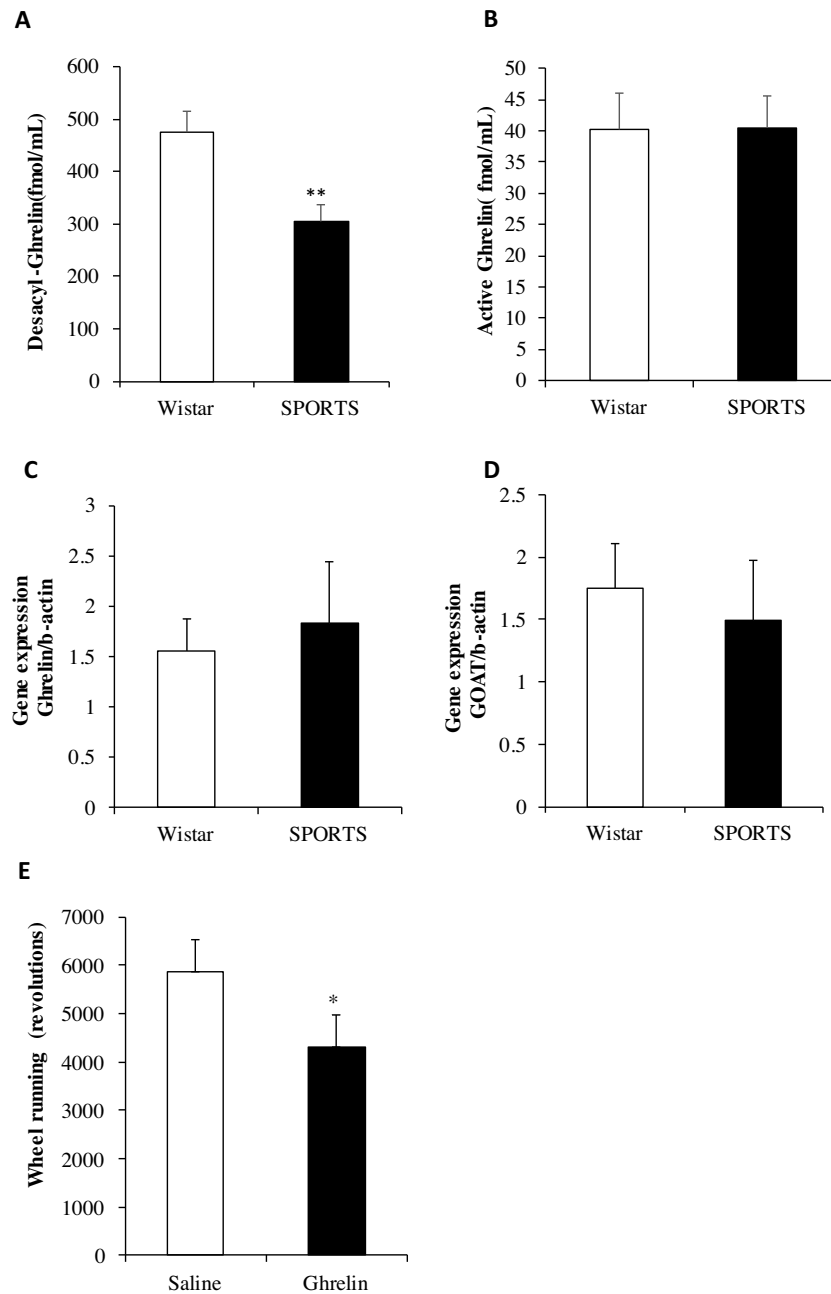
### 3.2. Ghrelin production in the brain influences wheel-running activity

Rats were administered four different concentrations of ghrelin by icv injection, after which wheel-running activities were measured. Ghrelin administration at 0.2  $\mu$ g/rat did not affect voluntary activity, but ghrelin administration of >1  $\mu$ g/rat significantly suppressed wheel-running activity of the rats (Fig. 2A,  $P < 0.05$ ). In contrast, administration of the ghrelin inhibitor JMV3002 for 10 consecutive days increased wheel-running activity in Wistar rats (Fig. 2B,  $P < 0.05$ ). These results demonstrate that ghrelin activity in the central nervous system suppresses wheel-running activity.

### 3.3. Ghrelin regulates exercise independently of food intake

Obestatin was identified from a precursor gene of ghrelin and antagonizes the effect of ghrelin activity on feeding behavior [18]. We examined whether obestatin inhibits the exercise-suppressing effect of ghrelin. Rats were administered saline, ghrelin, or ghrelin plus obestatin through icv injection at 10 PM, which was the beginning of the dark phase. Injection of ghrelin into the lateral ventricles of SPORTS rats decreased wheel-running activities during the dark phase compared with those observed following saline injection, but obestatin did not negate the exercise-suppressing effect of ghrelin (Fig. 3A,  $P < 0.01$ ). Intraperitoneal administration of ghrelin in SPORTS rats increased food intake, and this effect was reversed by obestatin administration (Fig. 3B,  $P < 0.05$ ). Moreover, ip injection of ghrelin in SPORTS rats similarly decreased wheel-running activity (Fig. 1C,  $P < 0.05$ ). In contrast, co-administration of ghrelin and obestatin did not show an antagonistic effect on the decreased wheel-running activity caused by ghrelin. These data indicate that peripheral ghrelin suppresses voluntary exercise to the same degree as feeding behavior; however, this effect is not inhibited by obestatin, despite the fact that ghrelin-induced food intake is reduced by obestatin.

Ghrelin has been reported to increase food intake and suppress energy metabolism [19]. In this study, however, we found that neither the injection of ghrelin nor the co-administration of ghrelin and obestatin in SPORTS rats changed oxygen consumption or RER in SPORTS rats (Fig. 3C, D), suggesting that alteration of the whole-body energy metabolism is not caused by ghrelin-induced increases



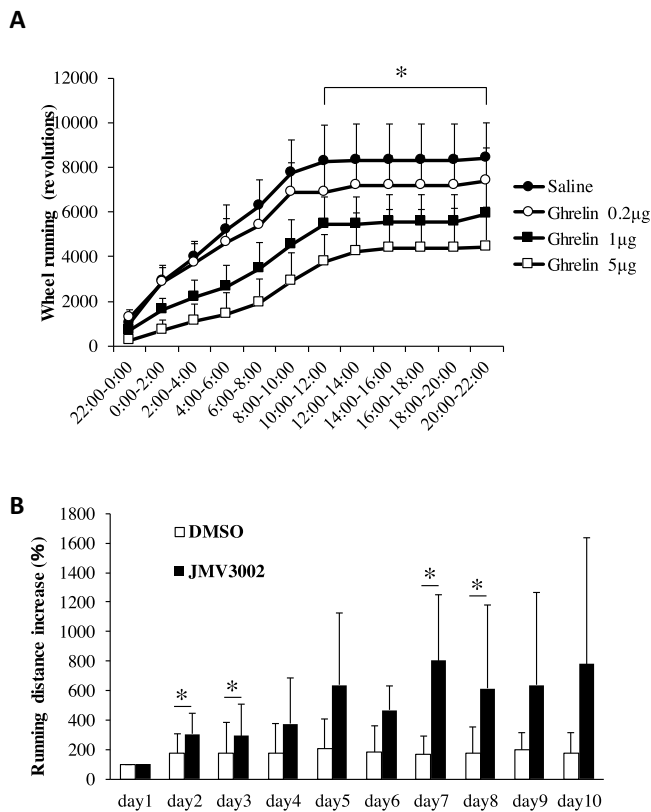
**Fig. 1.** Low levels of plasma ghrelin affect voluntary exercise in SPORTS rats. (A), SPORTS rats ( $n = 11$ ) showed significantly lower plasma desacyl-ghrelin levels than Wistar rats ( $n = 12$ ). (B), SPORTS rats ( $n = 8$ ) and Wistar rats ( $n = 8$ ) did not significantly differ in plasma active ghrelin levels. (C), Stomach ghrelin mRNA levels were not significantly different in Wistar ( $n = 8$ ) and SPORTS ( $n = 8$ ) rats. (D), Stomach GOAT mRNA levels were not significantly different in Wistar ( $n = 8$ ) and SPORTS ( $n = 8$ ) rats. (E), Intraperitoneal injection of ghrelin decreased wheel-running activity in SPORTS rats compared with saline injection (saline,  $n = 6$ ; ghrelin,  $n = 6$ ). The values shown are the means  $\pm$  SE. \* $P < 0.05$  SPORTS vs. Wistar rats.

in voluntary exercise. In addition, motivation for sucrose solution did not differ between Wistar and SPORTS rats (Supplemental Fig. 1). The above results raise the possibility that peripheral ghrelin regulates both feeding behavior and motivation for voluntary exercise independently of the central nervous system without effecting food preferences.

#### 3.4. The paraventricular nucleus is not a target of exercise regulation

Next, we measured *c-fos* expression to identify neuronal activity in the hypothalamus of SPORTS rats. *C-fos* expression is a marker of excitement in nerve cells [20]. We observed higher *c-fos* expres-

sion in the paraventricular nuclei (PVNs) of non-exercised SPORTS rats compared with that in non-exercised Wistar rats. Icv injection of ghrelin abolished *c-fos* expression in the PVNs of SPORTS rats (Supplemental Fig. 2,  $P < 0.05$ ). In contrast, *c-fos* expression in ARC cells was increased by ghrelin treatment, in agreement with a previous report that icv injection of ghrelin increases *c-fos* expression in neuropeptide Y (NPY) neurons [19]. In addition, NPY neurons are reported to suppress corticotropin-releasing hormone (CRH) production and secretion [21]. Therefore, we studied the CRH neurons of the PVN because ghrelin may indirectly inhibit CRH neuronal activity via NPY neurons to enhance voluntary exercise. Rats were injected simultaneously with ghrelin and CRH to determine whether ghrelin suppressed the motivation for exercise through



**Fig. 2.** Effect of intracerebroventricular injection of ghrelin and the ghrelin inhibitor JMV3002. (A), Injection of ghrelin showed a dose-dependent suppressive effect on wheel-running activity in SPORTS rats (saline,  $n = 6$ ;  $0.2 \mu\text{g}$  ghrelin,  $n = 6$ ;  $1 \mu\text{g}$  ghrelin,  $n = 6$ ;  $5 \mu\text{g}$  ghrelin,  $n = 11$ ). The values shown are the means  $\pm$  SE  $*P < 0.05$  saline vs. ghrelin ( $5 \mu\text{g}$ ). (B), Continuous injection of JMV3002 increased wheel-running activity in Wistar rats ( $n = 10$  each). The values shown are the means  $\pm$  SE.  $*P < 0.05$ .

regulating CRH neuronal activity. However, CRH did not antagonize the effect of ghrelin on voluntary exercise (Supplemental Fig. 3). This result suggests that ghrelin regulates wheel-running activity via a pathway that does not involve CRH neurons.

### 3.5. GHSR expression level in the hypothalamus and hippocampus were no difference compared with Wistar and SPORTS rats

GHSR is expressed in various parts of the brain, and it has been hypothesized that ghrelin regulates exercise behavior via the central nervous system, in addition to promoting growth-hormone secretion [22–24]. We examined the expression of GHSR, which is a ghrelin receptor that cannot bind obestatin. GHSR expression in the hypothalamus and hippocampus of SPORTS rats was not different that in Wistar rats (Fig. 4A, B).

### 3.6. MSG treatment does not affect the exercise-suppressing effect of ghrelin in SPORTS rats

Based on the above results, we hypothesized that the ARC is the target of voluntary exercise regulation by ghrelin. Results from previous studies have demonstrated that MSG injection during the neonatal period can damage the mediobasal hypothalamus, especially the ARC neurons, of mice and rats [25–27]. Therefore, we performed ip injection of MSG into SPORTS-rat neonates to examine the effects on ARC neurons. MSG-treated SPORTS rats showed no significant differences in wheel-running behavior during growth (Supplemental Fig. 4A), body weight gain, or food intake (data not shown), compared with those of non-MSG-treated SPORTS rats.

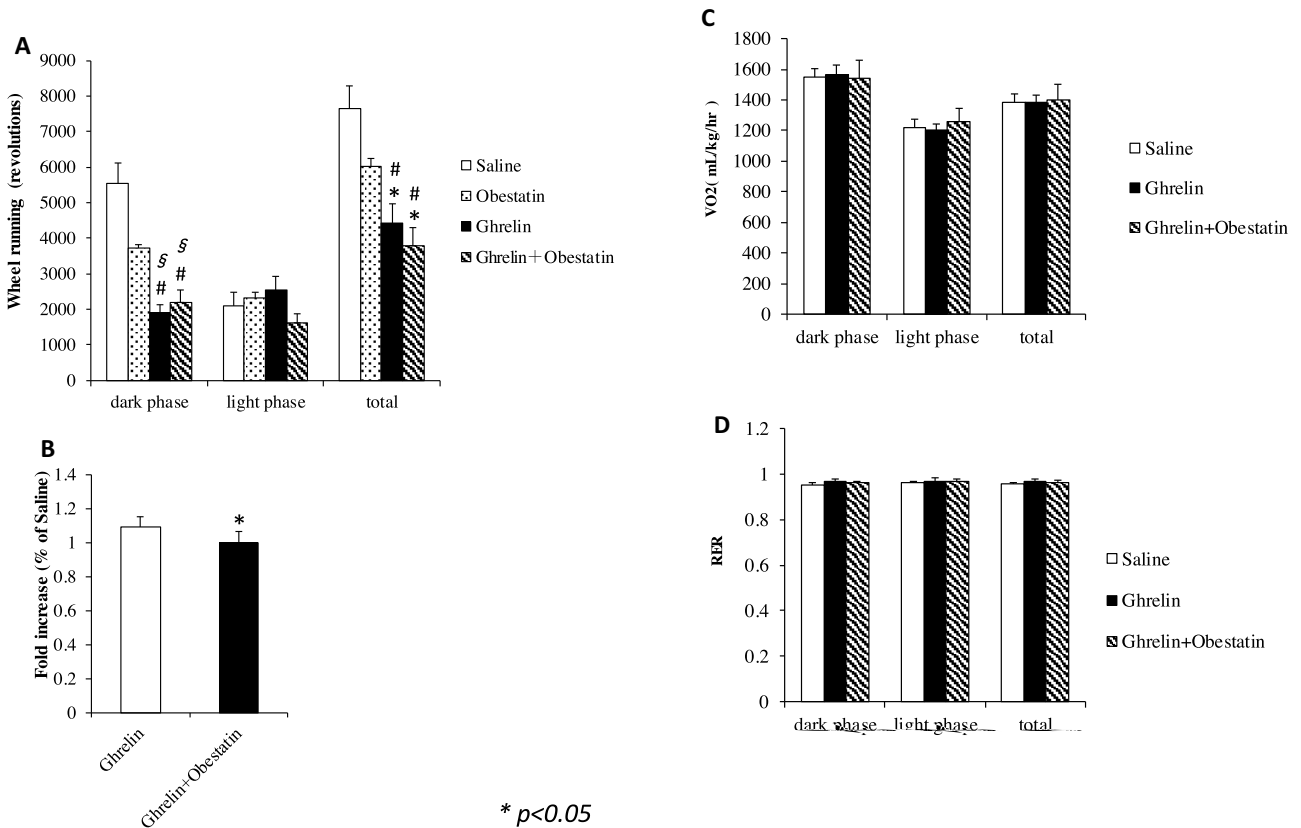
Expression of NeuN, which is a neuron-specific nuclear protein, decreased significantly in the ARCs of MSG-treated SPORTS rats (Supplemental Fig. 4B–D) [28]. When we injected ghrelin into the lateral ventricle, food intake was not increased in MSG-treated SPORTS rats (Fig. 5A). This result is consistent with previous data showing that ghrelin stimulates food intake via the ARC [22]. Interestingly, wheel-running activity was decreased by icv injection of ghrelin even in MSG-treated SPORTS rats (Fig. 5B,  $P < 0.05$ ). These results suggest that the wheel-running activity is not controlled by the ARC neurons that regulate feeding.

## 4. Discussion

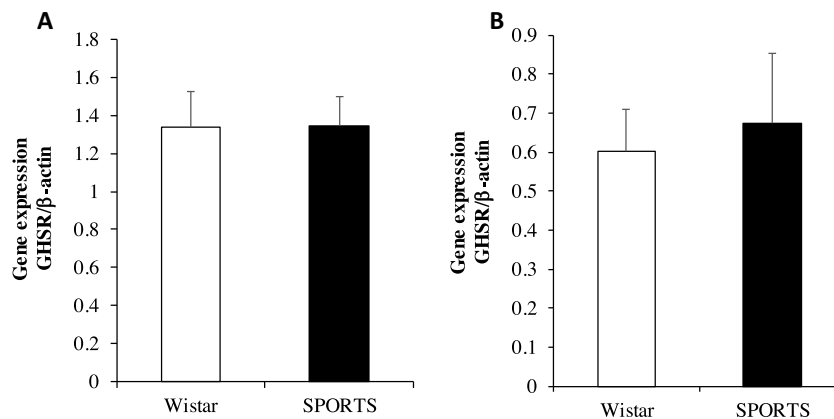
In this study, we investigated a peptide in the blood that may be important in highly voluntary exercise activity in SPORTS rats, observing that plasma levels of ghrelin in SPORTS rats were lower than those of Wistar rats. The ghrelin peptide is predominantly produced in the stomach; however, ghrelin mRNA expression in the stomachs of SPORTS rats was comparable to that observed in Wistar rats, indicating that the low levels of plasma ghrelin observed in SPORTS rats may not be the result of low ghrelin transcription. In addition, we examined GOAT mRNA expression in the stomach of SPORTS rats. GOAT plays an important role in the process of activation of ghrelin [17], but GOAT mRNA expression levels in the stomachs of SPORTS rats were not significantly different from those of Wistar rats. Therefore, GOAT does not appear to be the cause of the low levels of desacyl-ghrelin. The ratio of active ghrelin to desacyl-ghrelin is important in a variety of metabolic disorders [29]. The high locomotor activity of SPORTS rats may therefore be due to a change in this ratio.

Secreted ghrelin passes peripherally through the blood-brain barrier and transmits a signal to the hypothalamus [30]. This fact along with our findings suggests the possibility that peripheral ghrelin regulates voluntary exercise via the brain. Icv injection of ghrelin in SPORTS rats suppressed wheel-running exercise in a dose-dependent manner. We injected ghrelin at 10 PM because exercise levels are highest in rats from 10 P.M. to 2 A.M. Icv injection of ghrelin suppressed only dark-phase exercise, while the amount of exercise during the light phase increased in ghrelin-injected rats. This suggests that the exercise-suppressive effect of ghrelin is short-term and is accompanied by a compensatory increase in exercise during the light phase. Injection of a ghrelin inhibitor into the lateral ventricle increased voluntary activity in Wistar rats. Because ip injection of ghrelin also inhibited voluntary wheel-running in SPORTS rats, these results collectively suggest that peripheral ghrelin inhibits exercise motivation via the central nervous system.

We also investigated the inhibitory effect of the obestatin hormone on ghrelin-induced suppression of voluntary exercise. Obestatin has been identified as a precursor of ghrelin that functions as an antagonist of ghrelin [18,31]. Our findings show that the orexigenic action of ghrelin was antagonized by obestatin but that the suppressive effect of ghrelin on voluntary exercise was not reversed by obestatin treatment. Similarly, icv injection of obestatin did not exert an effect on exercise control. Therefore, the ghrelin/obestatin ratio may not be an important regulator of voluntary exercise. These results raise the possibility that the mechanism of the ghrelin-induced suppressive effect on voluntary exercise differs from that of the neuronal pathway of ghrelin-induced feeding behavior. While the ratio of ghrelin to obestatin is important in a variety of metabolic disorders, as is the ratio of active ghrelin to desacyl-ghrelin [28], we did not assess levels of plasma obestatin in our study. Many questions remain regarding the metabolic control action of obestatin.



**Fig. 3.** Antagonistic effects of intracerebroventricular ghrelin injection on obestatin activity. (A), Co-administration of ghrelin and obestatin did not inhibit wheel-running suppression by ghrelin (saline,  $n = 12$ ; ghrelin,  $n = 12$ ; obestatin,  $n = 6$ ; ghrelin + obestatin,  $n = 13$ ). The values shown are the means  $\pm$  SE. \* $P < 0.05$  vs obestatin, #  $P < 0.01$  vs saline, §  $P < 0.01$  vs obestatin. (B), Obestatin suppressed the ghrelin-dependent effect on food intake in SPORTS rats (ghrelin,  $n = 12$ ; ghrelin + obestatin,  $n = 13$ ). The values shown are the means  $\pm$  SE. \* $P < 0.05$ , ghrelin vs. ghrelin + obestatin. (C – D),  $VO_2$  and RER values did not significantly differ following ghrelin or ghrelin plus obestatin administration (saline,  $n = 5$ ; ghrelin,  $n = 5$ ; ghrelin + obestatin,  $n = 5$ ). The values shown are means  $\pm$  SE.

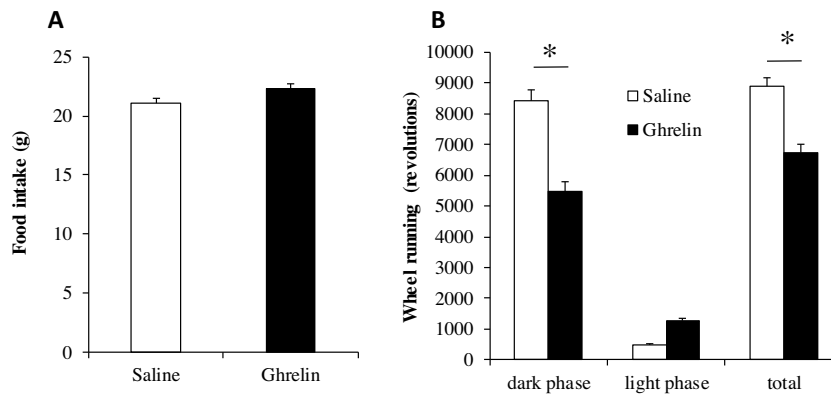


**Fig. 4.** Gene expression of GHSR. (A), Hypothalamus GHSR mRNA levels were not significantly different in Wistar ( $n = 8$ ) and SPORTS ( $n = 8$ ) rats. (B), Hippocampus GHSR mRNA levels were not significantly different in Wistar ( $n = 8$ ) and SPORTS ( $n = 8$ ) rats. The values shown are the means  $\pm$  SE.

To determine the effective site of ghrelin function in the brain, we measured *c-fos* expression, which is an index of neuronal activation [20]. SPORTS rats showed high expression of *c-fos* in the PVN. *C-fos* expression was abolished by icv injection of ghrelin in the PVN but increased in the ARC. It has been generally reported that ghrelin activates ARC neurons, such as NPY/AgRP-related peptide (AgRP) neurons, thereby increasing food intake [22]. NPY/AgRP neurons project into the PVN and inhibit the neuronal activity of CRH, which is a hormone known to suppress feeding behavior [32]. Therefore, we hypothesized that CRH neurons function in a downstream pathway associated with ghrelin-induced exercise

motivation. However, icv injection of CRH did not affect voluntary exercise, and co-administration of CRH and ghrelin did not reverse the observed decrease in wheel-running activity, suggesting that CRH is not involved in ghrelin-induced suppression of voluntary exercise in SPORTS rats.

Moreover, we examined the effect of ghrelin injection on MSG-treated SPORTS rats. MSG has been reported to result in the necrosis of nerve cells in the ARC by causing hyperexcitation in neurons [25–27]. Continuous MSG administration in neonatal mice causes morbid obesity, diabetes, and non-alcoholic fatty liver disease during growth, whereas a single MSG treatment causes



**Fig. 5.** Effect of intracerebroventricular ghrelin injection in MSG-treated SPORTS rats. (A), Ghrelin-dependent promotion of food intake was not observed. (B), Wheel-running suppression in ghrelin-treated rats and non-MSG-treated SPORTS rats. The values shown are the means  $\pm$  SE. \* $P < 0.05$ , saline vs. ghrelin (saline,  $n = 12$ ; ghrelin,  $n = 12$ ).

mild effects in mice [14]. In this study, we used a single MSG injection to exclude the possibility that exercise motivation was reduced due to metabolic disease development following continual MSG exposure. Our data revealed no significant differences between MSG-treated and non-MSG-treated SPORTS rats in daily food intake, body weight gain, or wheel-running activity, indicating that MSG-treated SPORTS rats underwent normal growth, although neuronal cell numbers in the ARC decreased about 40%. After the MSG-treated SPORTS rats reached the age of 12 weeks, we injected ghrelin into the lateral ventricles of the rats. We observed that while the promoting effect of ghrelin on food intake was inhibited, the suppressive effect of ghrelin on exercise motivation remained. These findings confirm that ghrelin regulates wheel-running activity through a mechanism that does not involve the arcuate hypothalamus. In our study, we were not able to present evidence that ghrelin regulates exercise motivation via the reward system, but peripheral ghrelin activates the reward system via dopamine neurons [33]. Morishima et al. examined neurotransmitter concentrations in some regions of the brain responsible for movement in SPORTS rat and found that extracellular norepinephrine levels in the hippocampi of SPORTS rats are higher than those of Wistar rats [34]. The palatability of sucrose did not differ between SPORTS rats and Wistar rats. In contrast, extracellular dopamine levels in the striata of SPORTS rats were not significantly different from those of Wistar rats. Therefore, we speculate that the exercise-suppressive effect of ghrelin may not work via the reward system but instead may act via the norepinephrine system in the hippocampus.

Ghrelin is a gastric hormone that stimulates feeding behavior via the vagal afferent nerve [35] or direct activation of the hypothalamus through the blood-brain barrier [30]. In addition, ghrelin causes an anabolic effect by stimulating the secretion of growth hormone and insulin-like growth factor [16]. A substantial body of research has been conducted to investigate the effect of exercise on circulating ghrelin levels in adult humans [36]. It has been reported that acute exercise transiently decreases blood ghrelin concentrations in humans [37] and rodents [38]. Physical exercise may serve as a bottleneck behavior for anabolic activities. Thus, we propose that ghrelin suppresses motivation for physical activity to enhance whole-body anabolic activity via the brain. Although Swallow et al. selectively bred mice for highly voluntary wheel running to investigate the phenomenon and genomics of high exercise performance as observed in SPORTS rats [40], the involvement of ghrelin in exercise performance was not demonstrated. We suggest that the low levels of ghrelin observed in this study serve a key role in highly voluntary exercise behavior in SPORTS rats. However, we were not able to determine the reason behind the low plasma ghrelin levels

in SPORTS rats. One possibility is that ghrelin levels are reduced via a compensatory mechanism following a period of high activity. Moreover, some reports have showed that plasma ghrelin levels are related to physical activity levels; for example, obese rats exhibit low plasma ghrelin levels, while individuals with anorexia nervosa exhibit high plasma ghrelin levels [29,39]. Although these findings differ from those of our study, a number of conditions differ (e.g., animal species, food, disease model). Various factors may be involved in the relationship between ghrelin and physical activity. Additional research is needed to elucidate the mechanism whereby circulating ghrelin levels are decreased in SPORTS rats.

## 5. Conclusions

SPORTS rats exhibit low levels of plasma ghrelin, suppressing the motivation for voluntary exercise. This finding may contribute to the establishment of novel strategies for the promotion of spontaneous exercise.

## Statement of interest

All authors declare no conflicts of interest.

## Acknowledgements

We thank members of the Support Center for Advanced Medical Sciences, Institute of Biomedical sciences, Tokushima University Graduate School for performing real-time PCR experiments. This work was supported in part by a Grant-in-Aid for Scientific Research on Innovative Areas (Grant No. 25126718 to H.S. and Grant No. 26560398 to T.S.) and a Grant-in-Aid for Scientific Research (C) (Grant No. 24591333 to H.S.) from the Japan Society for the Promotion of Science. This work was also supported by funding to T.S. from the Uehara Memorial Foundation.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.peptides.2016.11.005>.

## References

- [1] N.F. Sheard, Moderate changes in weight and physical activity can prevent or delay the development of type2 diabetes mellitus in susceptible individuals, *Nutr. Rev.* 61 (2003) 76–79.
- [2] N. Harada, E. Takishita, N. Ishimura, A. Minami, S. Sakamoto, Y. Nakaya, Combined effect of ACE inhibitor and exercise training on insulin resistance in type 2 diabetic rats, *Life Sci.* 70 (2002) 1811–1820.

- [3] S. Sakamoto, T. Muto, M. Yokota, N. Ishimura, Y. Niwa, N. Harada, K. Okada, N. Nakaya, Comparison between short-term food restriction and exercise on whole body glucose disposal in high-fat rats, *J. Med. Invest.* 47 (2000) 138–144.
- [4] B.N. Greenwood, T.E. Foley, H.E. Day, J. Campisi, S.H. Hammack, S. Campeau, S.F. Maier, M. Fleshner, Freewheel running prevents learned helplessness/behavioral depression: role of dorsal raphe serotonergic neurons, *J. Neurosci.* 23 (2003) 2889–2898.
- [5] P.R. Burghardt, L.J. Fulk, G.A. Hand, M.A. Wilson, The effects of chronic treadmill and wheel running on behavior in rats, *Brain Res.* 1019 (2004) 84–96.
- [6] 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.
- [7] J.A. Levine, Nonexercise activity thermogenesis – liberating the life-force, *J. Intern. Med.* 262 (2007) 273–287.
- [8] M.M. Taylor, W.K. Samson, The other side of the orexins: endocrine and metabolic actions, *Am. J. Physiol. Endocrinol. Metab.* 284 (2003) E13–E17.
- [9] 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.
- [10] S.E. Hyman, R.C. Malenka, E.J. Nestler, Neural Mechanisms of addiction: the role of reward-related learning and memory, *Annu. Rev. Neurosci.* 29 (2006) 565–598.
- [11] A.E. Kelley, B.A. Baldo, W.E. Pratt, M.J. Will, Corticostriatal-hypothalamic circuitry and food motivation: integration of energy, action and reward, *Physiol. Behav.* 86 (5) (2005) 773–795.
- [12] M. Morishima-Yamato, F. Hisaoka, S. Shinomiya, N. Harada, H. Matoba, A. Takahashi, Y. Nakaya, Cloning and establishment of a line of rats for high levels of voluntary wheel running, *Life Sci.* 77 (2005) 551–561.
- [13] A. Moulin, L. Demange, J. Ryan, D. Mousseaux, P. Sanchez, G. Bergé, D. Gagne, D. Perrissoud, V. Locatelli, A. Torsello, J.C. Galleyrand, J.A. Fehrentz, J. Martinez, New trisubstituted 1,2,4-triazole derivatives as potent ghrelin receptor antagonists. 3. Synthesis and pharmacological in vitro and in vivo evaluations, *J. Med. Chem.* 51 (3) (2008) 689–693.
- [14] Y. Sasaki, W. Suzuki, T. Shimada, S. Iizuka, S. Nakamura, M. Nagata, M. Fujimoto, K. Tsuneyama, R. Hokao, K. Miyamoto, M. Aburada, Dose dependent development of diabetes mellitus and non-alcoholic steatohepatitis in monosodium glutamate-induced obese mice, *Life Sci.* 85 (2009) 490–498.
- [15] K. Fujiwara, K. Gotoh, S. Chiba, T. Masaki, I. Katsuragi, T. Kakuma, H. Yoshimatsu, Intraportal administration of DPP-IV inhibitor regulates insulin secretion and food intake mediated by the hepatic vagal afferent nerve in rats, *J. Neurochem.* 121 (2012) 66–76.
- [16] M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, K. Kangawa, Ghrelin is a growth-hormone-releasing acylated peptide from stomach, *Nature* 402 (1999) 656–660.
- [17] J. Yang, M.S. Brown, G. Liang, N.V. Grishin, J.L. Goldstein, Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone, *Cell* 132 (2008) 387–396.
- [18] J.V. Zhang, P.G. Ren, O. Avsian-Kretschmer, C.W. Luo, R. Rauch, C. Klein, A.J. Hsueh, Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake, *Science* 310 (2005) 996–999.
- [19] M. Nakazato, N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa, S. Matsukura, A role for ghrelin in the central regulation of feeding, *Nature* 409 (2001) 194–198.
- [20] J.I. Morgan, D.R. Cohen, J.L. Hempstead, T. Curran, Mapping patterns of c-fos expression in the central nervous system after seizure, *Science* 237 (1987) 192–197.
- [21] E.L. Dimitrov, M.R. DeJoseph, M.S. Brownfield, J.H. Urban, Involvement of neuropeptide Y Y1 receptors in the regulation of neuroendocrine corticotropin-releasing hormone, *Endocrinology* 148 (2007) 3666–3673.
- [22] X.M. Guan, H. Yu, O.C. Palyha, K.K. McKee, S.D. Feighner, D.J. Sirinathsinghji, R.C. Smith, L.H. Van der Ploeg, A.D. Howard, Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues, *Brain Res. Mol. Brain Res.* 48 (1997) 23–29.
- [23] A.D. Howard, S.D. Feighner, D.F. Cully, J.P. Arena, P.A. Liberatore, C.I. Rosenblum, M. Hamelin, D.L. Hreniuk, O.C. Palyha, J. Anderson, P.S. Paresse, C. Diaz, M. Chou, K.K. Liu, K.K. McKee, S.S. Pong, L.Y. Chaung, A. Elbrecht, M. Dashkevich, R. Heavens, M. Rigby, D.J. Sirinathsinghji, D.C. Dean, D.G. Melillo, A.A. Patchett, R. Nargund, P.R. Griffin, J.A. DeMartino, S.K. Gupta, J.M. Schaeffer, R.G. Smith, L.H. Van der Ploeg, A receptor in pituitary and hypothalamus that functions in growth hormone release, *Science* 273 (1996) 974–977.
- [24] K.K. McKee, O.C. Palyha, S.D. Feighner, D.L. Hreniuk, C.P. Tan, M.S. Phillips, R.G. Smith, L.H. Van der Ploeg, A.D. Howard, Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors, *Mol. Endocrinol.* 11 (1997) 415–423.
- [25] J.S. Kizer, C.B. Nemeroff, W.W. Youngblood, Neurotoxic amino acids and structurally related analogues, *Pharmacol. Rev.* 29 (1977) 301–318.
- [26] J.W. Olney, Brain lesions, obesity and other disturbances in mice treated with monosodium glutamate, *Science* 164 (1969) 719–721.
- [27] T.W. Redding, A.V. Schally, A. Arimura, I. Wakabayashi, Effect of monosodium glutamate on some endocrine functions, *Neuroendocrinology* 8 (1971) 242–255.
- [28] R.J. Mullen, C.R. Buck, A.M. Smith, NeuN, a neuronal specific nuclear protein in vertebrates, *Development* 116 (1) (1992) 201–211.
- [29] R. Hassouna, P. Zizzari, V. Tolle, The ghrelin/obestatin balance in the physiological and pathological control of growth hormone secretion, body composition and food intake, *J. Neuroendocrinol.* 22 (2010) 793–804.
- [30] M. Schaeffer, F. Langlet, C. Lafont, F. Molino, D.J. Hodson, T. Roux, L. Lamarque, P. Verdié, E. Bourrier, B. Dehouck, J.L. Banères, J. Martinez, P.F. Méry, J. Marie, E. Trinquet, J.A. Fehrentz, V. Prévot, P. Mollard, Rapid sensing of circulating ghrelin by hypothalamic appetite-modifying neurons, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 1512–1517.
- [31] S.Q. Tang, Q.Y. Jiang, Y.L. Zhang, X.T. Zhu, G. Shu, P. Gao, D.Y. Feng, X.Q. Wang, X.Y. Dong, Obestatin: its physicochemical characteristics and physiological functions, *Peptides* 29 (2008) 639–645.
- [32] T.L. Bale, W.W. Vale, CRF and CRF receptors: role in stress responsibility and other behaviors, *Annu. Rev. Pharmacol. Toxicol.* 44 (2004) 525–557.
- [33] R.D. Palmiter, Is dopamine a physiologically relevant mediator of feeding behavior? *Trends Neurosci.* 30 (2007) 375–381.
- [34] M. Morishima, N. Harada, S. Hara, A. Sano, H. Seno, A. Takahashi, Y. Morita, Y. Nakaya, Monoamine oxidase A activity and norepinephrine level in hippocampus determine hyperwheel running in SPORTS rats, *Neuropsychopharmacology* 31 (2006) 2627–2638.
- [35] Y. Date, N. Murakami, K. Toshinai, S. Matukura, A. Nijima, H. Matsuo, K. Kangawa, M. Nakazato, The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats, *Gastroenterology* 123 (2002) 1120–1128.
- [36] J.A. King, L.K. Wasse, D.J. Stensel, Acute exercise increases feeding latency in healthy normal weight young males but does not alter energy intake, *Appetite* 61 (2013) 45–51.
- [37] T. Shiiya, H. Ueno, K. Toshinai, T. Kawagoe, S. Naito, T. Tobina, Y. Nishida, M. Shindo, K. Kangawa, H. Tanaka, M. Nakazato, Significant lowering of plasma ghrelin but not des-acyl ghrelin in response to acute exercise in men, *Endocr. J.* 58 (2011) 335–342.
- [38] A. Ghanbari-Niaki, A. Jafari, M. Moradi, R.R. Kraemer, Short-, moderate-, and long-term treadmill training protocols reduce plasma, fundus, but not small intestine ghrelin concentrations in male rats, *J. Endocrinol. Invest.* 34 (2011) 439–443.
- [39] H. Mifune, Y. Tajiri, Y. Nishi, K. Hara, S. Iwata, I. Tokubuchi, R. Mitsuzono, K. Yamada, M. Kojima, Voluntary exercise contributed to an amelioration of abnormal feeding behavior, locomotor activity and ghrelin production concomitantly with a weight reduction in high fat diet-induced obese rats, *Peptides* 71 (2015) 49–55.
- [40] J.G. Swallow, P.A. Carter, T. Garland Jr., Artificial selection for increased wheel-running behavior in house mice, *Behav. Genet.* 28 (1998) 227–237.