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ORIGINAL PAPER

Involvement of orexin-A neurons but not melanin-concentrating hormone neurons in the short-term regulation of food intake in rats

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Abstract In order to elucidate the involvement of melanin-concentrating hormone (MCH) and orexin-A (ORX-A) neurons of the perifornical/lateral hypothalamic areas (PF/LH) in the regulation of food intake induced by acutely reduced glucose availability, we examined the food intake response and c-Fos expression in the MCH and ORX-A neurons in the PF/LH during 2-deoxy-D-glucose (2DG)-induced glucoprivation (400 mg/kg; i.v.) and systemic insulin-induced hypoglycemia (5 U/kg; s.c.) in male Wistar rats. The administration of both 2DG and insulin stimulated food intake and induced c-Fos expression in the ORX-A neurons corresponding to food intake, but not in the MCH neurons. These data indicate that ORX-A neurons, but not MCH neurons, play a role in the short-term regulation of food intake, and that the input signals for the neurons containing MCH and ORX-A are different, and these neurons play different roles in the regulation of feeding behavior.

Keywords Melanin-concentrating hormone · Orexin · Feeding behavior · Glucosensitive neurons · Glucoprivation · Hypoglycemia

Introduction

The short-term regulation of ingestive behavior is mediated mainly by reduced glucose availability, or plasma glucose concentration, and glucosensitive neurons are involved in the short-term regulation of food intake [1–4]. It is well-established that glucosensitive neurons are located in the lateral hypothalamic area [2, 5]. It is also known that the neurons expressing melanin-concentrating hormone (MCH) and orexin-A (ORX-A) are distributed at the perifornical and lateral hypothalamic areas (PF/LH) of the hypothalamus [4–6], and it is known that both of these peptides are orexigenic. Furthermore, some studies have shown that the ORX-A neurons interact morphologically with the MCH neurons [7, 8].

ORX-A neurons are possibly glucosensitive and believed to be involved in the short-term regulation of food intake; several studies, but not all [9], have demonstrated that reduced glucose availability induced by systemic and intracranial administration of 2-deoxy-D-glucose (2DG), or systemic insulin-induced hypoglycemia, stimulated c-Fos expression in the ORX-A neurons or orexin mRNA expression [10–13]. These results suggest that the ORX-A neurons are almost certainly responsive to acutely reduced glucose availability. However, the involvement of ORX-A neurons in the short-term regulation of food intake has not been fully established, because most of the previous studies did not examine the time course of the plasma glucose concentration, the metabolic status, and the corresponding feeding behavior in addition to examining the activation of ORX-A neurons during glucoprivation or hypoglycemia.

In contrast to ORX-A neurons, the knowledge available concerning the involvement of MCH neurons in the short-term regulation of food intake is limited and controversial. The results of several studies showed that MCH neurons are not responsive to reduced glucose availability [12, 14, 15]. In contrast, it has been also reported that MCH neurons are possibly involved in the glucose sensing system, because hypothalamic MCH mRNA levels are increased by hypoglycemic stimulations such as fasting [16–18] or

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treatment with insulin [19] or 2DG [9]. Another study demonstrated that glucose infusion after fasting reduced the expression of the phosphorylated cAMP response element-binding protein, suggesting that MCH neurons might be involved in food intake behavior in response to fasting, and these neurons are responsive to the plasma glucose level [16]. However, this study did not directly demonstrate the involvement of MCH neurons in the regulation of food intake induced by reduced glucose availability. Thus, it is still uncertain whether or not the MCH neurons are responsive to acutely reduced glucose availability and responsible for the short-term regulation of food intake.

In the present study, in order to elucidate the involvement of ORX-A and MCH neurons in the short-term regulation of food intake, we examined the food intake response and the c-Fos expression in MCH and ORX neurons in the PF/LH. We also examined the c-Fos expression in the arcuate nucleus (Arc), because neuropeptide Y (NPY) neurons reportedly have projections to ORX-A and MCH neurons and are glucosensitive [3, 5]. The present study included a behavioral experiment and a separate immunohistochemical experiment in the same rats. In the behavioral experiment, we examined the plasma glucose level and body temperature as indices of metabolic status, and also examined food intake response. First, we determined the time course of the feeding behavior during the 2DG-induced glucoprivation and insulin-induced hypoglycemia. Then in the immunohistochemical experiment, we examined the c-Fos expression in the ORX-A and MCH neurons in the PF/LH and c-Fos expression in the Arc at a specific timing, corresponding to 2 h after the rats started eating in the behavioral study, because the peak c-Fos expression reportedly occurs 1.5–2 h after stimulation [20, 21]. In the immunohistochemical experiment, the rats were not provided access to food in order to eliminate the effect of feeding on the activity of ORX-A and MCH neurons. We believe that the results of this study provide useful insights into the mechanisms responsible for the short-term regulation of food intake.

Materials and methods

Nine-week-old male Wistar rats weighing 200–220 g (Jcr: Wistar; CLEA Japan, Japan) were used in the present study. All experiments were conducted in accordance with the guidelines for animal care and use of Nara Women's University and the guiding principles for the care and use of animals in the field of physiological sciences of the Physiological Society of Japan. All of the experimental procedures employed were approved by the ethical committee for animal care and use of Nara Women's University.

The animals were housed in a 12 h/12 h light/dark cycle environment with lights on at 07:00, and given ad libitum access to food (standard rodent chow; CE-2, CLEA, Japan) and tap water before the experiments. Temperature and humidity were controlled at 23 °C and 40 % relative humidity.

Surgical procedures

After the rats were anesthetized with pentobarbital sodium (50 mg/kg), an intravenous catheter (3 Fr vinyl tubing, ATOM, Japan) was inserted into the right jugular vein, with the tip of the catheter placed at the inferior vena cava/right atrium while the free end of the catheter was passed subcutaneously and exteriorized dorsally behind the neck through the midscapular incision. A miniature temperature data logger was implanted in the abdominal cavity to measure the body core temperature (intraabdominal temperature; T_{abdo}). The first experiment (behavioral experiment) was conducted at least 4 days after the surgical operation to allow for recovery from surgical stress. The patency of the venous catheter was maintained by flushing every day with heparinized (100 U/ml) isotonic saline. During the recovery period, the rats were kept in individual cages. Food and water intake and body weight were measured daily after the surgery, and rats showing abnormal water or food intake and/or abnormal weight gain were excluded from the experiments.

Behavioral experiment

In this experiment, we examined the effects of systemic 2DG-induced glucoprivation and systemic insulin-induced hypoglycemia on food intake behavior, plasma glucose concentration, and T_{abdo}. The experiments were started at 10:00. One hour before the experiment, access to food was removed. In the glucoprivation study, a 0.2-ml blood sample was drawn, and then either 2DG (Sigma-Aldrich; 400 mg/kg body weight in 0.2 ml/kg body weight distilled water; $n = 8$) or the same volume of saline (control; $n = 5$) was infused through the venous catheter. In the study of insulin-induced hypoglycemia, rats were injected subcutaneously with insulin (Sigma-Aldrich; 5 U/kg body weight in 0.1 ml/kg body weight saline; $n = 9$) or the same amount of saline (control; $n = 5$). Access to food was provided immediately after the administration of 2DG, while access to food was provided 30 min after the injection of insulin; until then, the plasma glucose concentration decreased to about half of the baseline level in the preliminary experiment. A blood sample was taken and food intake was measured at every 60 min after the onset of access to food.

Plasma glucose concentration was measured with a mutarotase-glucose oxidase method (Glucose CII-test Wako; Wako Pure Chemicals, Osaka). However, this measurement method did not allow for a separate determination of the endogenous plasma glucose from the exogenous 2DG. T_{abdo} was measured every 5 min with a miniature data logger placed in the abdominal cavity (SubCue; Canada). Food intake was measured by manually weighing chow hoppers on a digital scale and corrected by subtracting the weight of food spillage.

Immunohistochemical experiments

Three days after the behavioral study, we performed immunohistochemical experiments to examine the c-Fos expression in the ORX-A and the MCH neurons in the PF/LH, and also the c-Fos expression in the Arc.

One hour after the removal of access to food, rats were injected with either 2DG (i.v.), insulin (s.c.), or saline (i.v. or s.c.) and then deeply anesthetized with pentobarbital 3 h after the 2DG administration, and 2.5 h after the insulin administration. In the immunohistochemical experiment, access to water, but not food, was provided after the injection. From the behavioral experimental data in which we determined the timing for the onset of feeding after the injection, we decided that the timing for the examination of the expression of c-Fos could be set at 2 h after the rats started feeding. Immediately after the rats were deeply anesthetized, the rats were transcardially perfused with ice-cold phosphate-buffered saline (PBS), followed by 4 % paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) for fixation. The brain was removed and immersed in the fixative for more than 2 days at 4 °C. The fixed brain was immersed in 15 % sucrose in PBS for 1 day and 25 % sucrose in PBS for 2 days at 4 °C for cryoprotection. Frozen sections were cut coronally at a thickness of 30 μm with a cryostat microtome (Leica CM3050S, Germany). Free-floating sections were pretreated with 0.1 % H_2O_2 in PBS for 20 min and then preincubated with 5 % normal horse serum in PBS containing 0.3 % Triton X-100 (TPBS) for 2 h at room temperature. Then, the sections were incubated with polyclonal anti-c-Fos antibody (Sc-52, Santa Cruz Biotechnology, CA) diluted at 1:4,000 in TPBS containing 1 % normal horse serum for 16 h at 4 °C. Then, the sections were incubated with biotinylated horse anti-rabbit IgG (dilution 1:400; BA-1100, Vector, CA) for 2 h, followed by the ABC Elite kit solution (dilution 1:400; Vector, CA) for 2 h. Visualization of the antibodies was performed with 0.02 % 3,3-diaminobenzidine (DAB) and 0.01 % H_2O_2 in a 50 mM Tris HCl buffer (pH 7.4). After the DAB reaction, the sections were preincubated with 5 % normal donkey serum in TPBS for 1 h, and incubated overnight at 4 °C with anti-ORX-A (dilution 1:200; Sc-8070; Santa Cruz Biotechnology, CA) and anti-MCH (dilution 1:2,000; Phoenix Pharmaceutical, CA) antibodies in TPBS containing 1 % normal donkey serum. The sections were then incubated with donkey anti-goat IgG conjugated with Texas Red (1:200; Sc-3856, Santa Cruz Biotechnology, CA) and donkey anti-rabbit IgG conjugated with FITC (1:200; Sc-0.2090, Santa Cruz Biotechnology, CA) in TPBS for 2 h. The sections were rinsed with 50 mM Tris HCl buffer, mounted on gelatin-coated glass slides, and coverslipped with the mounting media for fluorescence (VECTASHIELD HardSet; Vector).

Regions including the PF/LH and Arc were identified using the rat brain stereotaxic atlas [22]. The sections were carefully matched across all animals in all experimental groups. Four sections of every 5th section containing the LH/PFA and Arc (in between −2.56 and −3.60 mm from the Bregma) [22] were observed using a fluorescent microscope (Olympus BX-51, Tokyo, Japan), and bright field images for c-Fos and fluorescence images for ORX-A and MCH neurons were obtained using a cooled-CCD camera (Retiga 4000R; QImaging, Canada). The brightfield c-Fos images were converted into 8-bit images and inverted, and these three images were merged (ImageJ, NIH, USA). The total number of neurons that expressed ORX-A and MCH, and the number of ORX-A and MCH neurons that expressed c-Fos were counted bilaterally in the images obtained from each side (1,516 × 1,516 μm). The number of the c-Fos immunoreactive nuclei in the Arc was counted unilaterally. The percentage of the ORX-A and MCH neurons that expressed c-Fos was calculated and compared among the rats administered 2DG, insulin, and saline control. Because the number of ORX-A and MCH neurons and the number of c-Fos expressions in the ORX-A and MCH neurons did not show any differences between the i.v. saline infusion (control for 2DG experiment) and the s.c. saline injection (control for insulin experiment), we pooled the data as saline control.

Data were shown as means with their standard errors. A two-way analysis of variance (ANOVA) with repeated measures (one within and one between factors) was performed to determine the effects of treatment (between factors) and time (within factors) on food intake, plasma glucose concentration, and T_{abdo}. A one-way ANOVA was performed to determine the effects of the treatments on the number of c-Fos-ir nuclei in the ORX-A and MCH neurons at the PF/LH. Differences between data of specific comparisons were determined using the Tukey–Kramer post hoc test. Values of $p < 0.05$ were considered statistically significant.

Results

Systemic 2DG administration stimulated food intake (Fig. 1, top), and rats started eating 1 h after the access to food was provided (1 h after the 2DG administration). Plasma glucose (including 2DG) concentration increased following the i.v. 2DG injections, with a peak (376.9 ± 18.7 mg/dl) at 60 min after the injections. The concentration decreased towards the baseline level, but remained higher at 4 h after the injections (Fig. 1 middle). Plasma glucose (including 2DG) was significantly higher with the 2DG administration compared with the saline during the time period of 60–240 min after the injection. 2DG administration decreased T_{abdo}, with a nadir at ~75 min after the injections, and T_{abdo} was significantly lower with the 2DG administration compared with the saline during the time period of 60–135 min after the injection.

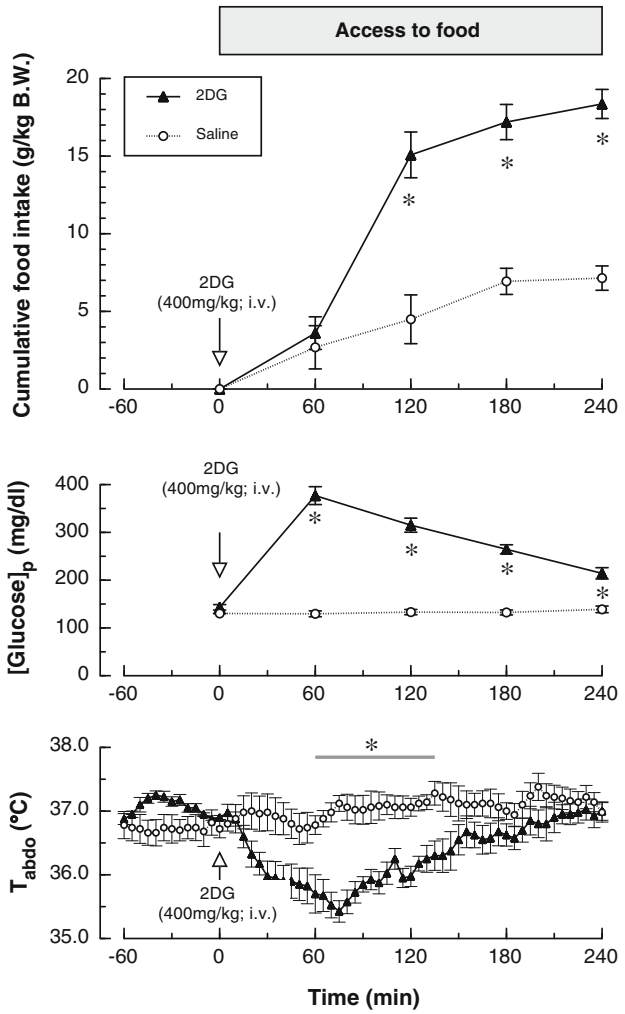

Fig. 1 Food intake (*top*), plasma glucose concentration ([Glucose]$_p$) (*middle*), and body core temperature (intra-abdominal temperature; T_{abdo}) *(bottom)* in response to intravenous injections of 2-deoxy-D-glucose (2DG; 400 mg/kg b.w.; $n = 8$) and saline ($n = 5$). The data are shown as mean ± SE for each group. *Stars* indicate significant differences between the 2DG-injected group and saline control

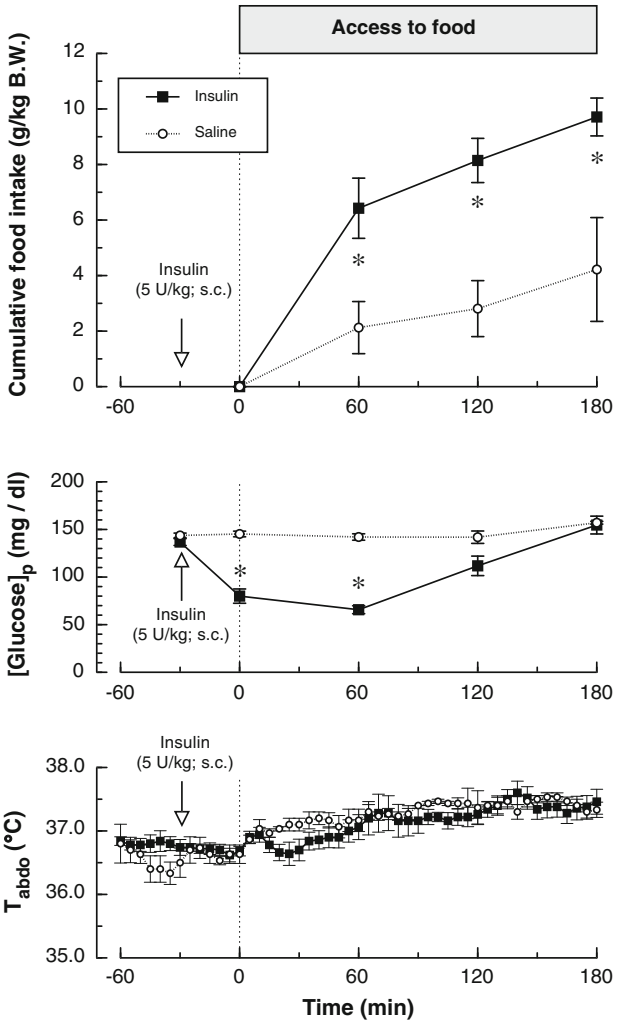

Fig. 2 Food intake (*top*), plasma glucose concentration([Glucose]$_p$) (*middle*), and body core temperature (intra-abdominal temperature; T_{abdo}) (*bottom*) in response to subcutaneous injections of insulin (5 U/kg b.w.; $n = 9$) and saline ($n = 5$). The data are shown as mean ± SE for each group. *Stars* indicate significant differences between the insulin-injected group and saline control

Systemic insulin administration stimulated food intake, and the rats started eating immediately after access to food was provided (30 min after the insulin administration) (Fig. 2 top). The plasma glucose concentration decreased from 136.8 ± 3.6 to 80.0 ± 7.5 mg/dl at 30 min after the injection when access to food was provided, and to 65.7 ± 4.1 mg/dl at 90 min after the s.c. insulin injections, and returned to the baseline level at 2.5 h after the injections (Fig. 2 middle).

After the insulin administration, the T_{abdo} values did not show any differences from the T_{abdo} values after the saline injections (Fig. 2 bottom).

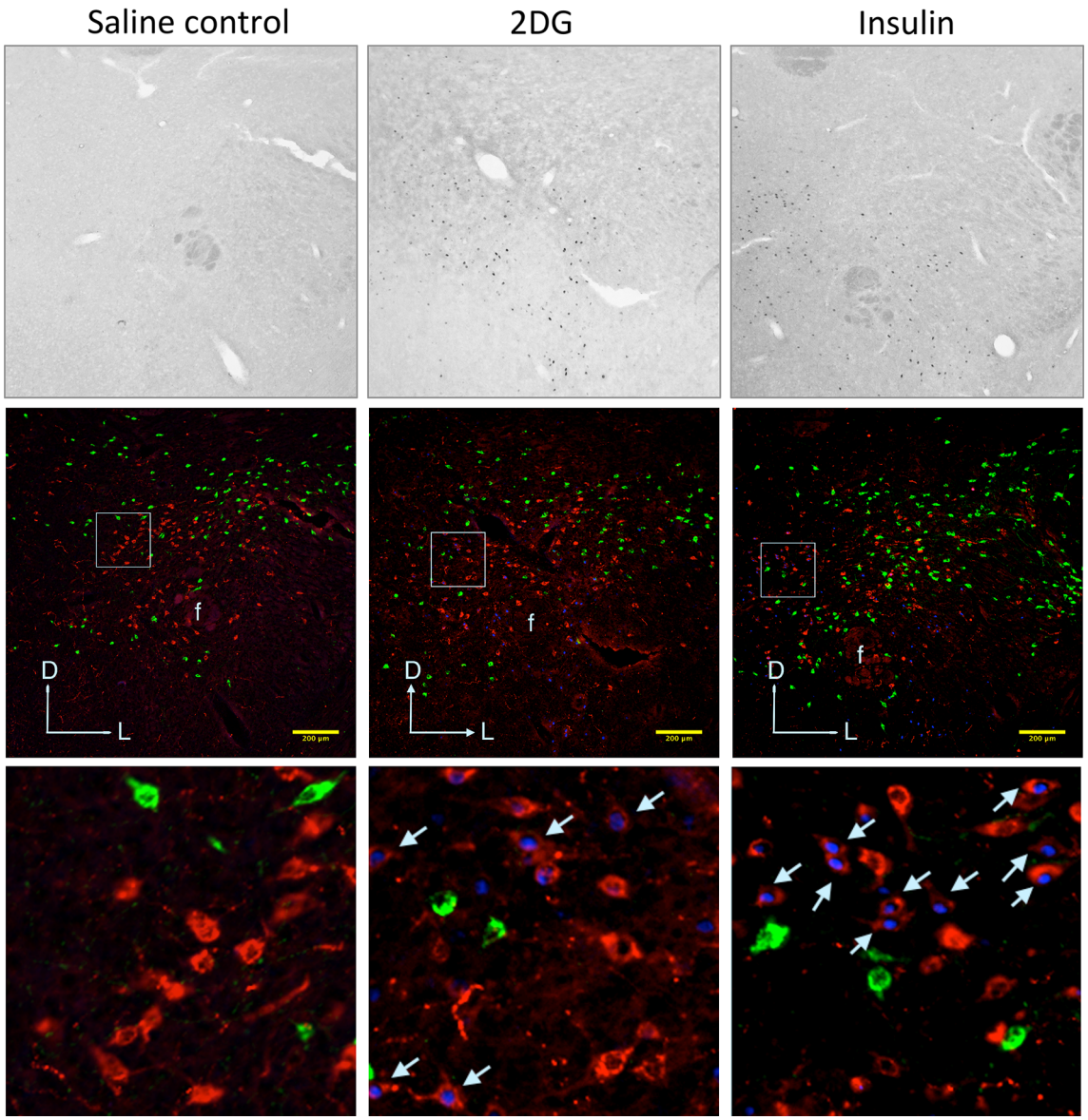

Fig. 3 The representative microscope images of c-Fos-immunoreactive nuclei (*top*) and the merged microscope images of ORX-A-immunoreactive neurons (*red*), MCH-immunoreactive neurons (*green*), and c-Fos immunoreactive nuclei (*blue*) after saline (*left*), 2DG (*middle*), or insulin (*right*) injections (*middle*). The bottom images are higher-power magnification of the boxed area in the middle images showing the representative region of c-Fos-ir and ORX-A-ir coexpression. *Scale bars* = 200 μm. *f*, fornix; *D*, dorsal and *L*, lateral direction. *Arrows* indicate the colocalization of c-Fos-ir and ORX-A-ir

The ORX-A and MCH neurons were distinctly located in separate regions, and there were almost no ORX-A neurons colocalized with MCH neurons in the PF/LH (Fig. 3). The ORX-A neurons were mainly located in the perifornical region, and the MCH neurons were in a more lateral region (Fig. 3). Both the 2DG-induced glucoprivation and insulin-induced hypoglycemia increased the c-Fos expression in the ORX neurons (Fig. 3) and the percentage of ORX-A neurons showing c-Fos expression in the PF/LH (Fig. 4). In contrast, the acute hypoglycemic stress did not increase c-Fos expression in the MCH neurons (Figs. 3, 4). The number of c-Fos immunoreactive nuclei in the Arc was also increased by systemic 2DG and insulin administration (Fig. 5).

Discussion

The primary finding of the present study was that an acute reduction of glucose availability, induced by either systemic 2DG or insulin administration, stimulated food intake and c-Fos expression in the ORX-A neurons in the PF/LH. The reduced glucose availability also induced

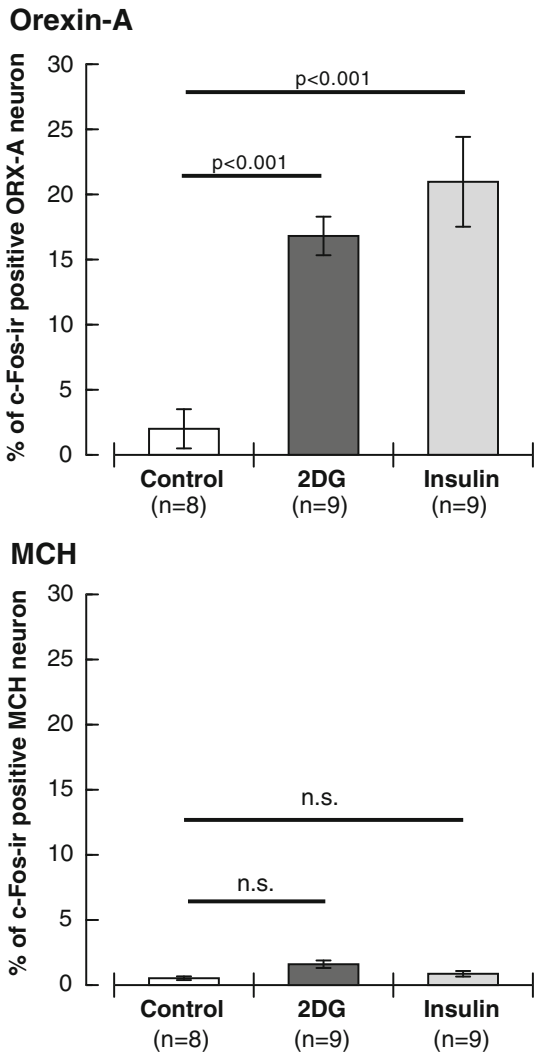

Fig. 4 The effect of 2DG and insulin administration on the percentages (%) of c-Fos-ir positive ORX-A neurons (*top*) and c-Fos-ir positive MCH neurons (*bottom*). The data are shown as mean ± SE. The number of rats examined and statistical differences are shown in the figure

c-Fos expression in the Arc, especially in the medial region. In contrast, these hypoglycemic stimuli did not activate MCH neurons; almost none of the MCH neurons expressed c-Fos after these hypoglycemic stimulations. Thus, our results indicated that ORX-A neurons in the PF/LH are possibly involved in the short-term regulation of food intake induced by acutely reduced glucose availability, while MCH neurons are not involved. Our results also suggested that the input stimuli activating ORX-A and MCH neurons are different [23], and that these neurons differentially play a role in the regulation of ingestive behavior, although both of these peptides are orexigenic.

It has been reported that insulin treatment stimulated MCH mRNA expression, determined by in situ hybridization in rats [19]. However, the dose of insulin in that study was relatively too high, and plasma glucose concentration decreased to 0.2–1.5 and 0–0.2 mM at 3 and 5 h after administration, respectively, clearly indicating the plasma glucose level was far outside the physiological range for the short-term regulation of feeding behavior. In addition, that study did not examine the food intake response, but it did show that MCH mRNA expression was increased 5 h after the injection. The enhanced expression of MCH mRNA 5 h after insulin administration is not likely to indicate that MCH neurons are responsible for short-term regulation of food intake. Thus, it remained unclear whether or not MCH neurons are involved in the short-term regulation of food intake. Sergeyev et al. [9] reported that the relatively high dose of 2DG administration (600 mg/kg) stimulated food intake and MCH mRNA expression, but not ORX mRNA at 2 h after the injection, using an in situ hybridization technique. However, access to food was provided before examining the mRNA level, suggesting that eating possibly affected the response of the MCH and the ORX-A neurons. Further, in contrast to these studies, several studies have demonstrated that the acute reduction of glucose availability does not activate MCH neurons [12, 14, 15]. In the present study, we also found that the MCH neurons were not activated, either by the 2DG-induced glucoprivation or the insulin-induced hypoglycemia, at the time point corresponding to the onset of feeding behavior. Thus, it is unlikely that MCH neurons are responsive to acutely reduced glucose availability, or that they are involved in the short-term regulation of feeding behavior.

It has also been reported that both ORX-A and MCH neurons are activated by fasting [2], and that the activation was reversed by glucose infusion after fasting, indicating that an increased plasma glucose level plays a role in the deactivation of both MCH and ORX-A neurons [16, 24]. These results suggest that both ORX-A and MCH neurons are possibly responsive to changes in plasma glucose level. The results of our present study demonstrated that the ORX-A neurons are activated either by 2DG-induced glucoprivation or insulin-induced hypoglycemia, but that MCH neurons are not. These results suggested that fasting-induced activation of MCH neurons is not mediated by reduced glucose availability, but by other factors related to fasting [9, 17, 18], and that an increased glucose level can attenuate the activity of both ORX-A and MCH neurons as a satiety factor [16, 24].

We performed the behavioral and immunohistochemical experiments separately, employing the same rats. In the behavioral experiment, we examined the effect of an acute reduction in glucose availability on food intake response. We found that reduced plasma glucose concentration induced by systemic insulin administration immediately stimulated ingestive behavior. However, systemic 2DG

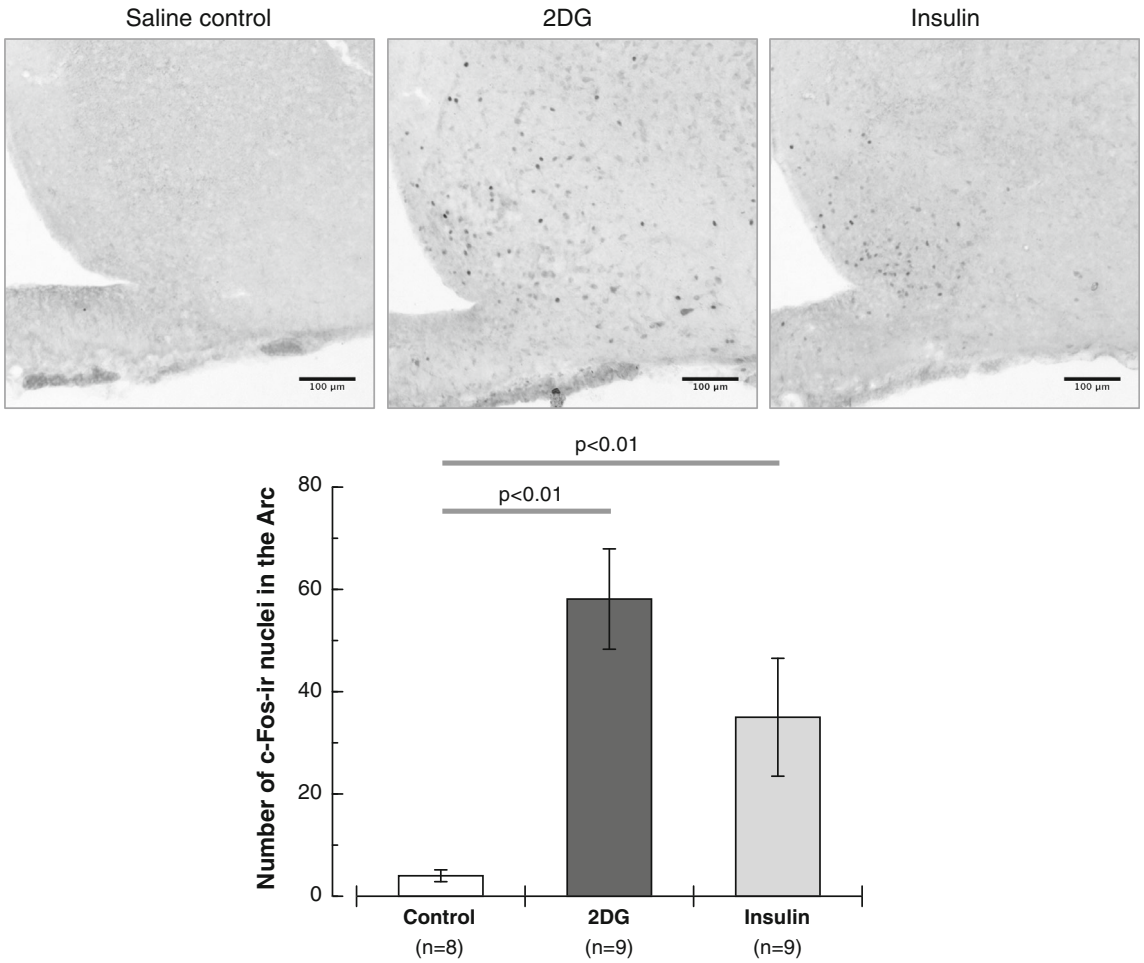

Fig. 5 The representative image of c-Fos immunoreactive nuclei in the arcuate nucleus (*top*) and the effect of 2DG and insulin administrations on the number of c-Fos-ir positive nuclei in the arcuate nucleus (*bottom*). The data are shown as mean ± SE. The number of rats examined and statistical differences are shown in the figure. *Scale bars* = 100 μm

administration (400 mg/kg b.w.) did not stimulate food intake immediately, but induced the onset of food intake at 1 h after the 2DG administration, when body core temperature was reduced significantly. In the behavioral experiment, we determined the timing for the onset of feeding after the injections, which was about 1 h after the 2DG injections and 30 min after the insulin injection (immediately after access to food was provided). Because the maximal c-Fos expression reportedly occurs about 1.5–2 h after stimulation [20, 21], we decided that the timing for examining the expression of c-Fos could be set at 2 h after the rats started to feed. In the histochemical experiment, we did not provide food during the glucoprivation or hypoglycemia state in order to eliminate the effect of feeding behavior on the ORX-A and MCH neurons. Thus, we believe that the present study provided the appropriate conditions to elucidate the role of ORX-A and MCH neurons in the short-term regulation of feeding.

In the present study, we measured T_{abdo} as an index of the central glucopenia in the 2DG administration experiment. It has been reported that systemic injection with a relatively high dose of 2DG decreases the metabolic rate, which could contribute to the reduction in body core temperature [25]. However, central injections of 2DG also reportedly decrease body temperature and induce systemic hyperglycemia via a central mechanism, suggesting that body temperature could be used as an index of central glucopenia [25, 26]. The data in the present study indicated that 2DG may act both peripherally and centrally, and that central glucopenia activated ORX-A neurons and neurons in the Arc.

In the present study, in addition to the activation of ORX-A neurons, the neurons in the Arc were also activated by the acute reduction in glucose availability. Although the cell bodies of the NPY neurons could not be identified immunohistochemically, we assumed that the c-Fos expression occurred mainly in the NPY neurons, rather than in the proopiomelanocortin neurons, because c-Fos expression in the Arc was more prominent in the medial region where the NPY neurons are mainly located [27], and the reduced

glucose availability stimulated food intake. The NPY neurons are known to connect reciprocally with both ORX-A and MCH neurons, and they are glucosensitive [3, 5, 8]. Our data indicated that the activation of ORX-A neurons and the neurons in the Arc, probably NPY neurons, is stimulated by acutely reduced glucose availability, and that these neurons are possibly involved in the short-term regulation of feeding behavior [28, 29]. In contrast, the MCH neurons are not responsible for the short-term regulation of food intake [29]. However, a recent study demonstrated that NPY/AgRP neurons are not necessary for generating or mediating the orexigenic response to glucose deficiency [30]. It is expected that additional studies will further elucidate the neuronal interactions involved in short-term regulation of feeding behavior.

The c-Fos expression in the ORX-A neurons tended to be higher after the insulin treatment compared with the 2DG treatment, while the c-Fos expression in the Arc tended to be higher after the 2DG treatment compared with the insulin treatment; however, there were no statistical differences in c-Fos expression in the arcuate nucleus or the ORX-A neurons between these treatments. These results prompted us to speculate that the actions of insulin and 2DG vary, although both induce reduced glucose availability. Insulin receptors are reportedly located in the arcuate nucleus [4, 31] and are thought to attenuate the activity of NPY neurons [4]. Therefore, insulin induces food intake response and activation of ORX-A neurons via hypoglycemia, and at the same time, it might directly inhibit NPY neurons via insulin receptors in the arcuate nucleus. This mechanism might contribute to the relatively lower c-Fos expression in the arcuate nucleus after insulin administration compared with after 2DG administration, with similar c-Fos expression in the ORX-A neurons.

In summary, both 2DG-induced glucoprivation and insulin-induced hypoglycemia stimulated food intake, and induced c-Fos expression in ORX-A neurons and neurons in the Arc, but not in MCH neurons. These results indicated that while ORX-A neurons and neurons in the Arc, probably NPY neurons, play a role in the short-term regulation of food intake, MCH neurons do not. Our results also suggested that the input signals stimulating the neurons containing MCH and ORX-A are different, and that these neurons play different roles in the regulation of feeding behavior.

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Conflict of interest None of the authors had a personal or financial conflict of interest.

Refereces

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