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Expression of the genes encoding hypothalamic feeding-related neuropeptides in the streptozotocin-induced diabetic rats with variable hyperglycemia and hyperphagia

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1. Introduction

Feeding behavior is regulated by neural circuits, including hypothalamic neuropeptidergic pathways, and peripheral humoral factors, such as plasma glucose/insulin levels (Schwartz et al., 2000) and leptin/ghrelin signaling (Arora and Anubhuti, 2006; Kageyama et al., 2010). Elevated plasma glucose suppresses feeding behavior, while fasting promotes feeding in the non-diabetic state, via activation of the orexigenic neuropeptidergic pathway, as well as through suppression of the anorexigenic neuropeptidergic pathway in the hypothalamus (Kohno and Yada, 2012; Arora and Anubhuti, 2006). In contrast, patients with diabetes sometimes experience episodes of uncontrollable hyperphagia, despite high plasma glucose levels. In a clinical situation, there are two different types of hyperglycemia in diabetic patients, where plasma glucose level is either elevated in both postprandial and fasted states or only in the postprandial, but not in the fasted state (Ceriello et al., 2014). However, differences in the expression of hypothalamic feeding-related neuropeptide genes between these two types of hyperglycemia have yet to be investigated.

Hyperphagia is caused by hyperglycemia in the diabetic state associated with reduced humoral signaling in the central nervous system, such as that induced via insulin and/or leptin (Leedom and Meehan, 1989; Hernandez and Briese, 1972). Previous studies have revealed that central administration of insulin causes downregulation of neuropeptide Y *(NPY)* and agouti-related protein *(AgRP)* and upregulation of proopiomelanocortin *(POMC)* and cocaine- and amphetamine-regulating transcript *(CART)* genes in the rodent arcuate nucleus (Arc; Schwartz et al., 1992). NPY and AgRP are orexigenic neuropeptides, while POMC and CART are anorexigenic neuropeptides.

Streptozotocin (STZ) is both an anti-microbial agent and a diabetogenic chemical that prevents beta-pancreatic cells from functioning (Lenzen, 2008). STZ-induced diabetic animal models are widely used as the model of type 1 diabetes mellitus; however, they do not always express severe hyperglycemia because of variability of the toxic effects of STZ on beta-pancreatic cells (Deeds et al., 2011; Okabayashi et al., 1985). In addition, peripheral administration of STZ results in animal models with various grades of diabetes, according to STZ dose and route of administration, and animal strain and age (Qinna and Badwan, 2015; Sakata et al., 2012; Damasceno et al., 2013; Sinzato et al., 2009; Kiss et al., 2009; Tsuji et al., 1988; Bonner-Weir et al., 1981).

In the present study, STZ-administered rats were divided into three groups: (1) those that presented hyperglycemia both while feeding ad libitum and during fasting (severe diabetic); (2) those that exhibited hyperglycemia only while feeding ad libitum, but not during fasting (mild diabetic); and (3) rats that were not hyperglycemic while feeding ad libitum or during fasting (non-diabetic).

We evaluated differences in the expression of the hypothalamic feeding-related neuropeptide genes among STZ-administered rats in severe, mild, and non-diabetic states using in situ hybridization histochemistry. To clarify whether the gene expression was influenced by plasma insulin levels, we also re-analyzed and re-evaluated the gene expression levels in all STZ-administered rats divided into two groups according to plasma insulin levels. In the present study, we focused on the expression of *NPY*, *AgRP*, *POMC*, *CART*, corticotropin-releasing hormone *(CRH)*, and thyrotropin-releasing hormone *(TRH)* genes, whose products function as feeding-related and metabolic state-related hypothalamic neuropeptides. In addition, we measured plasma leptin levels and triiodothyronine (T3) levels in all STZ-administered rats.

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2. Materials and methods

2.1. Animals

Adult male 6-week-old aged Wistar rats (140–151 g) were group housed in cages which were maintained under a 12/12-h light/dark cycle (lights on at 07.00 h) at 23–25 °C. They were acclimatized for 1 week before initiation of the experiments and were provided access to food and water ad libitum. Animals used in the present study were maintained in accordance with the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Science" set by the Physiological Society of Japan. The experiments were approved by the Animal Research Committees of University of Occupational and Environmental Health (AE16–009).

2.2. Experimental procedures

STZ (Sigma-Aldrich Japan Co. LLC., Tokyo, Japan) was dissolved in 0.9% saline (Otsuka Pharmaceutical Co. LTD., Tokyo, Japan). The solution was shielded from the light to avoid quenching and freshly prepared before use. STZ (80 mg/kg) was administered intraperitoneally (i.p.) in adult male Wistar rats at day 0. Blood glucose levels were measured every 24 h at 11.00 am from day 3 to day 10 after STZ administration. Body weight and cumulative food intake were measured every 24 h from day 3 to day 10. Fourteen days after STZ administration, after being deprived of food for 12 h prior to the experiment (but not deprived of water), rats were decapitated and blood samples collected. Brains were removed, promptly placed on dry ice, and then stored at −80 °C. Blood glucose levels were measured using a blood glucose meter (Medisafe Mini; Terumo Co., Tokyo, Japan) and plasma insulin concentration was measured using a rat insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan). Plasma leptin levels were measured by a rat leptin ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan). Plasma T3 levels were measured by an ECLIA system (cobas 8000 module, Roche Diagnostics K.K., Tokyo, Japan). STZ-administered rats were divided according to postprandial plasma glucose level on day 3 (Table 1 (1)) into those with plasma glucose levels < 300 mg/dl ($n = 29$) and ≥ 300 mg/dl ($n = 21$). STZ-administered rats were also divided by fasted plasma glucose level on day 14 (Table 1 (2)) into those with plasma glucose levels < 200 mg/dl ($n = 39$) and ≥ 200 mg/dl ($n = 11$). Finally, all rats were divided into PG1 ($n = 29$), PG2 ($n = 10$), and PG3 ($n = 11$), according to plasma glucose profile (Tables 1 and 2) using previously reported criteria (Tan et al., 2015). Rats which exhibited postprandial glucose < 300 mg/dl and fasting glucose < 200 mg/dl were classified as PG1, those with postprandial glucose ≥ 300 mg/dl and fasting glucose < 200 mg/dl as PG2, and those with postprandial glucose ≥ 300 mg/dl and fasting glucose ≥ 200 mg/dl as PG3.

2.3. In situ hybridization histochemistry

Using a cryostat (OTF5000, Bright Instrument Co Ltd., England), brains were sliced into 12 μm thick coronal sections at −20 °C, and thaw mounted on gelatin/chrome alum-coated slides. The paraventricular nucleus (PVN) and Arc regions were determined by referring to the coordinates provided in the rat brain atlas (Paxinos and Watson, 1997).

^{35}S 3′-end-labeled deoxyoligonucleotides complementary to the *NPY*, *AgRP, POMC, CART, CRH,* and *TRH* transcripts were used as probes; their use was confirmed by previous reports (Hashimoto et al., 2007). Sequences of probes used for in situ hybridization histochemistry are shown in Table 3. The detailed protocol and quantitative analysis methods for in situ hybridization histochemistry has been described in previous studies (Ueta et al., 1995; Levy, 1997; Levy and Lightman, 1988a, 1988b, 1988c). Hybridized tissues were exposed to autoradiography films (BioMax XAR film, KODAK, USA) for 1 week. The films were analyzed using an MCID imaging analyzer (Imaging Research, Inc., Ontario, Canada). The mean optical densities (ODs) of the autoradiographs were measured by comparing with simultaneously exposed ^{14}C-labeled microscale samples (Amersham, Bucks, UK). They are represented as arbitrary units calculated using the mean OD values obtained from control rats.

Table 1
Streptozotocin (STZ)-administered rats were divided according to plasma glucose levels under different conditions.

Plasma glucose (mg/dl)	*n*
(1) Plasma glucose in rats fed *ad libitum* after i.p. administration of STZ at day 3	
< 300	29
≥ 300	21
(2) Plasma glucose in rats after fasting for a 12 h dark period after i.p. administration of STZ on day 14	
< 200	39
≥ 200	11

Table 2
Profiles of the three experimental groups (PG1, 2, and 3).

	Plasma glucose (mg/dl)		*n*
	Feeding *ad libitum* after i.p. administration of STZ at day 0 (Table 1 (1))	Fasting for a 12 h dark period after i.p. administration of STZ at day 14 (Table 1 (2))	
PG1	< 300	< 200	29
PG2	≥ 300	< 200	10
PG3	≥ 300	≥ 200	11

2.4. Statistical analysis

The mean ± standard error of the mean (SEM) were calculated for blood glucose, body weight change, and cumulative food intake data. Expression levels of the genes encoding hypothalamic feeding-related neuropeptides from in situ hybridization data are displayed as a percent of those observed in PG1 rats. These data were analyzed by one-way ANOVA followed by a Bonferroni-type adjustment for multiple comparisons (SPSS Statistics version 21 software, IBM Corp., Armonk, NY). The correlation between blood glucose and plasma insulin was analyzed using the Spearman's test. Statistical significance was set at $P < .05$.

3. Results

3.1. Plasma glucose, body weight, and cumulative food intake after STZ treatment

Plasma glucose, body weight, and cumulative food intake were measured from day 3 (0 day in Fig. 1) to day 10 (7 day in Fig. 1) after STZ treatment. Plasma glucose concentration was significantly increased in the PG2 and PG3 groups compared with the PG1 group (Fig. 1a). In addition, body weight was markedly decreased in the PG2 and PG3 groups relative to the PG1 group (Fig. 1b). Moreover, rats in the PG2 and PG3 groups had a significantly higher cumulative food intake compared with those in the PG1 group from day 5 to day 10 after STZ treatment (Fig. 1c).

3.2. Plasma leptin levels in STZ-administered rats

Plasma leptin levels are shown in Table 4. Plasma leptin levels in PG2 and PG3 were significantly lower than those in PG1, while there were no significant differences in plasma leptin levels between PG2 and PG3.

Table 3
Sequences of probes used for *in situ* hybridization histochemistry.

mRNA	Oligonucleotide sequence
NPY	5′-GGA GTA GTA TCT GGC CAT GTC CTC TGC TGG CGC GTC-3′
AgRP	5′-CGA CGC GGA GAA CGA GAC TCG CGG TTC TGT GGA TCT AGC ACC TCT GCC-3′
POMC	5′- CTT CTT GCC CAG CGG CTT GCC CCA GCA GAA GTG CTC CAT GGA CTA GGA-3′
CART	5′-TGG GGA CTT GGC CGT ACT TCT TCT CAT AGA TCG GAA TGC-3′
CRH	5′-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3′
TRH	5′-GTC TTT TTC CTC CTC CTC CCT TTT GCC TGG ATG CTG CGC TTT TGT GAT-3′

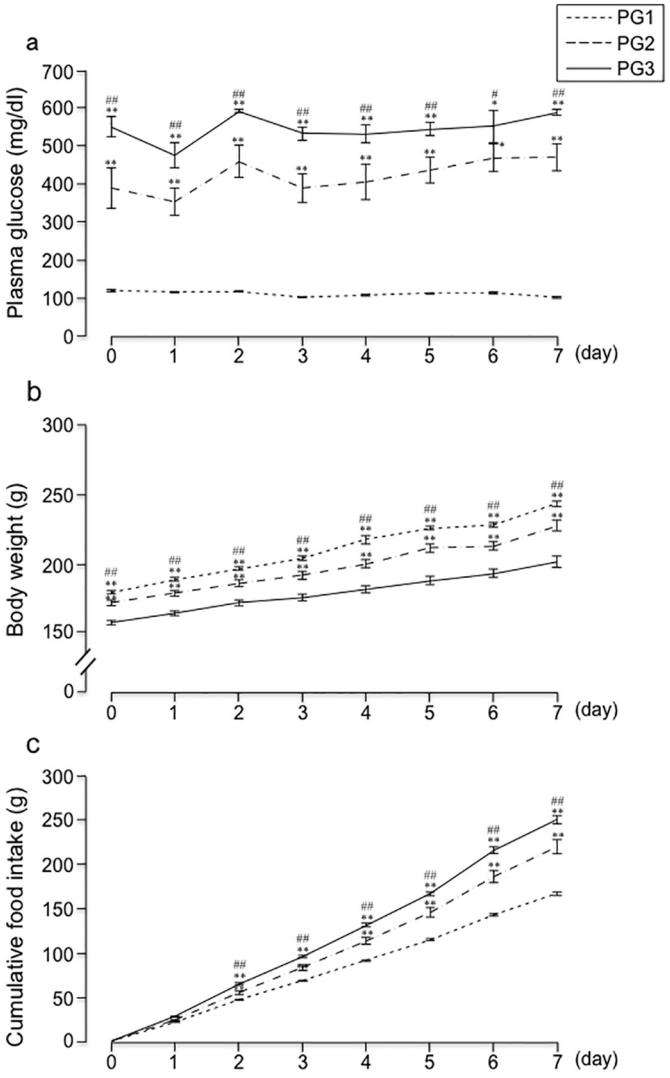

Fig. 1. Plasma glucose, body weight, and cumulative food intake after STZ treatment. Plasma concentration of glucose (a), body weight (b), and cumulative food intake (c) on day 0 after i.p. administration of STZ. Data are presented as means ± standard error of mean (SEM). PG1, $n = 29$; PG2, $n = 10$; PG3, $n = 11$. $^{*}P < .05$ vs. PG1. $^{**}P < .01$ vs. PG1. $^{\#}P < .05$ vs. PG2. $^{\#\#}P < .01$ vs. PG2.

Table 4
Plasma glucose, insulin, leptin levels of the three experimental groups in fasted for a 12 h dark period after i.p. administration of STZ at day 14.

	Plasma glucose (mg/dl)	Plasma insulin (ng/ml)	Plasma leptin (ng/ml)
PG1	89.7 ± 2.2	0.82 ± 0.07	1.56 ± 0.02
PG2	121.9 ± 13.9*	0.88 ± 0.09	1.15 ± 0.03*
PG3	339.4 ± 17.0**	0.36 ± 0.05**	1.14 ± 0.02*

Correlation between hyperglycemia and the expression of the genes encoding hypothalamic feeding-related neuropeptides in STZ-induced diabetic rats.

Digital images of autoradiographs of representative sections stained for *NPY* and *AgRP* (Fig. 2a-1–a-3 and b-1–b-3), *POMC* and *CART* (Fig. 2c-1–c-3 and d-1–d-3), *CRH* (Fig. 2e-1–e-3), and *TRH* (Fig. 2f-1–f-3) are presented. Expression of *NPY* and *AgRP* in the Arc were significantly higher in PG3, but not PG2 group rats, compared with those in animals in the PG1 group (Fig. 3a, b). In addition, *POMC* and *CART mRNA* levels were significantly lower in the Arc of PG3 and PG2 rats than in that of PG1 rats, 2 weeks after STZ administration (Fig. 3c, d). *CRH* expression in the PVN was comparable among all groups (Fig. 3e). Expression of *TRH* in the PVN was significantly lower in the PG3, but not in the PG2 group rats, compared with that in the PG1 group rats (Fig. 3f). Plasma T3 levels were 1.19 ± 0.03 ng/ml in the PG1, 0.86 ± 0.04 ng/ml in the PG2, and 0.70 ± 0.02 ng/ml in the PG3 group rats. Plasma T3 levels in the PG2 and PG3 group rats were significantly lower than those in the PG1 group rats. Plasma T3 levels in the PG3 group rats were also markedly lower than those in the PG2 group rats (data not shown).

3.3. Correlation between plasma glucose and insulin

Preliminary analysis showed that there was an unclear relationship between plasma glucose, gene expression of feeding-related neuropeptides, and insulin in each. Thus, PG1, PG2, and PG3 were analyzed, as described above.

Scatter plot of plasma insulin versus plasma glucose and a regression line determined by simple linear regression analysis of these data are presented in Fig. 4. Plasma insulin levels were significantly correlated with those of plasma glucose ($r = -0.294$, $P = .045$). Simple linear regression analysis indicated that a plasma glucose level of 300 mg/dl was equivalent to a plasma insulin value of 0.5 ng/ml, according to the generated regression equation.

Relationship between expression of the genes encoding hypothalamic feeding-related neuropeptides and plasma insulin in hyperglycemic rats.

Rats in the PG2 and PG3 groups were divided in two subgroups: those with plasma insulin ≥ 0.5 ng/ml (PI1, $n = 12$) and < 0.5 ng/ml (PI2, $n = 7$). Expression of *NPY* (Fig. 5a) and *AgRP* in the Arc (Fig. 5b) did not differ significantly, and nor did that of *CRH* in the PVN (Fig. 5e). However, expression of *POMC* and *CART* in the Arc (Fig. 5c, d) and *TRH* in the PVN (Fig. 5f) was significantly lower in PI2 than in PI1 rats.

4. Discussion

Here, we demonstrate, for the first time, that the expression levels of the genes encoding hypothalamic feeding-related neuropeptides in the hypothalamus exhibit dynamic changes depending on hyperglycemia severity. Levels of *NPY* and *CART* in rats with hyperglycemia induced by STZ administration have been investigated in previous studies (Marks et al., 1993). This may promote feeding behavior in response to intracellular starvation. Starvation induces both activation of NPY/AgRP neurons and inhibition of POMC/CART neurons to maintain energy homeostasis (Schwartz et al., 2000). The data generated in the

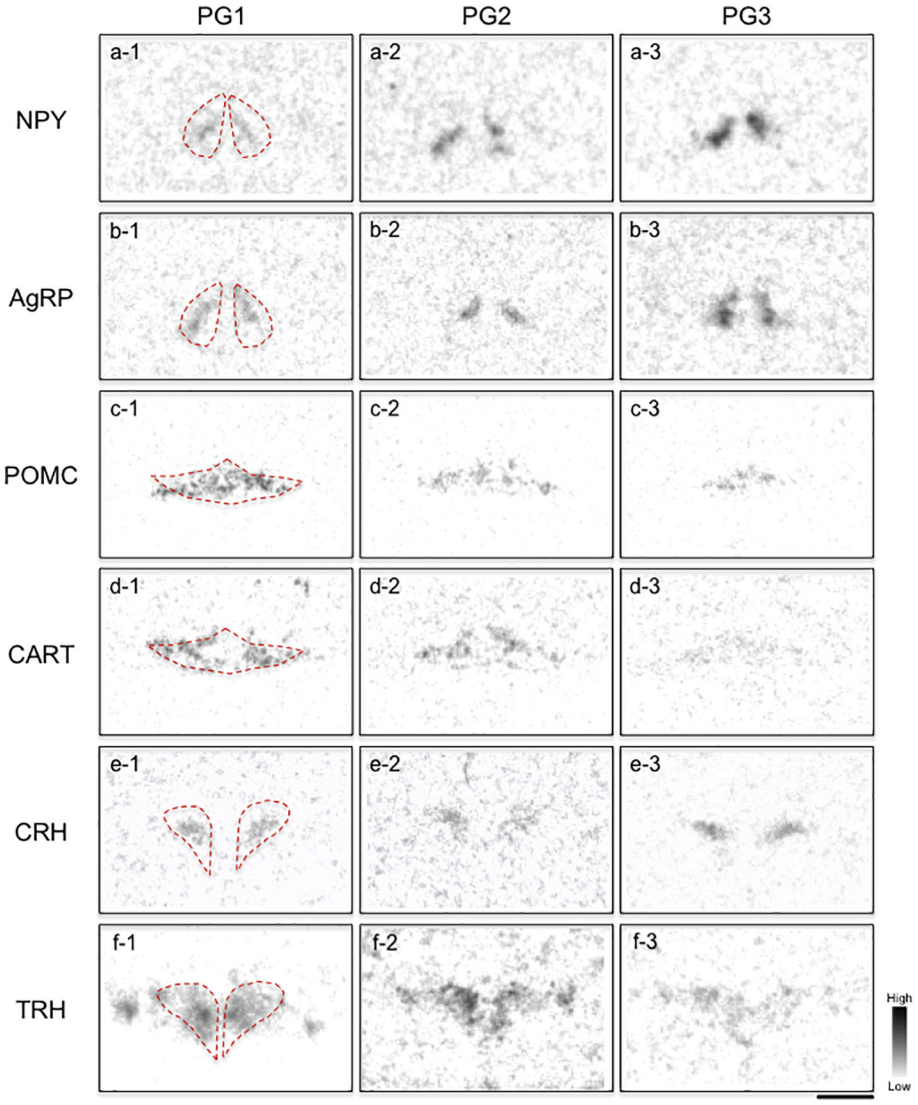

Fig. 2. Digital image of Representative digital images obtained from autoradiographs of brain sections hybridized with 35S-labeled oligonucleotide probes complementary to *NPY* (a), *AgRP* (b), *POMC* (c), and *CART* (d) in the Arc, and *CRH* (e), and *TRH* (f) in the PVN, 2 weeks after i.p. administration of STZ. Analyzed areas are surrounded by red dotted lines (a-1, b-1, c-1, d-1, e-1, f-1). Scale bars indicate 1 mm. Signal intensity ranges from high (black) to low (white). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

present study indicate that altered *NPY*, *AgRP*, *POMC*, and *CART* gene expression levels reflect intracellular starvation caused by hyperglycemia, consistent with previous reports (Sucajtys-Szulc et al., 2010; Cahill et al., 1966; Schwartz et al., 1995; Palou et al., 2009).

Several studies have focused on changes in hypothalamic neuropeptides under hyperglycemic conditions, with hyperglycemia defined as a plasma glucose level of 300–500 mg/dl with ad libitum feeding (Havel et al., 2000). However, it is unclear whether the expression of the genes encoding hypothalamic neuropeptides differed between animals in a persistent hyperglycemic state and those in a postprandial hyperglycemic state, which develop hyperglycemia only after eating. Our data reveal that expression of the genes encoding anorexigenic neuropeptides, such as *POMC* and *CART*, in the Arc was markedly decreased in persistent hyperglycemic rats relative to postprandial hyperglycemic rats, while the expression of the *NPY* and *AgRP* remained unchanged. We consider that these differences likely reflect variation between POMC/CART neurons and NPY/AgRP neurons in their sensitivity and adaptation to environmental changes, such as hyperglycemia and hypoinsulinemia.

The PVN is one of the most important nuclei as it is involved in integration of the neuroendocrine and autonomic nervous systems (Ferguson et al., 2008) and also contributes to the regulation of feeding and metabolic balance (Waterson and Horvath, 2015). We focused on expression of the *CRH* and *TRH* genes, which regulate adrenocorticotropic hormone (ACTH) and thyroid stimulating hormone (TSH), respectively, in the PVN, as they encode neuropeptides important for maintenance of plasma glucose levels and metabolic balance in the whole body via regulation of glucocorticoid and thyroid hormones. Glucose-sensing neurons are located in the lateral, arcuate, and ventromedial hypothalamic regions (Burdakov et al., 2005), whereas, the PVN does not contain glucose-sensing neurons. Thus, our findings that *CRH* remains unchanged, even in the hyperglycemic state are logical. Level of the *TRH* mRNA was dramatically lower in the severe hyperglycemic state than in a non-hyperglycemic state. This difference may be attributable to low levels of plasma triiodothyronine (T3), resulting from impaired activity of 5′-deiodinase, an enzyme that converts thyroxine (T4) into T3 in peripheral tissues. The low T3 state is considered an adaptive response to severely impaired metabolic conditions (Chopra, 1997), which could lead to central hypothyroidism through alteration of the set point of the HPT axis, eventually resulting in decreased *TRH* gene expression in the PVN. O'Mara et al. showed that type I 5′-deiodinase activity was markedly reduced in diabetic rats (O'Mara

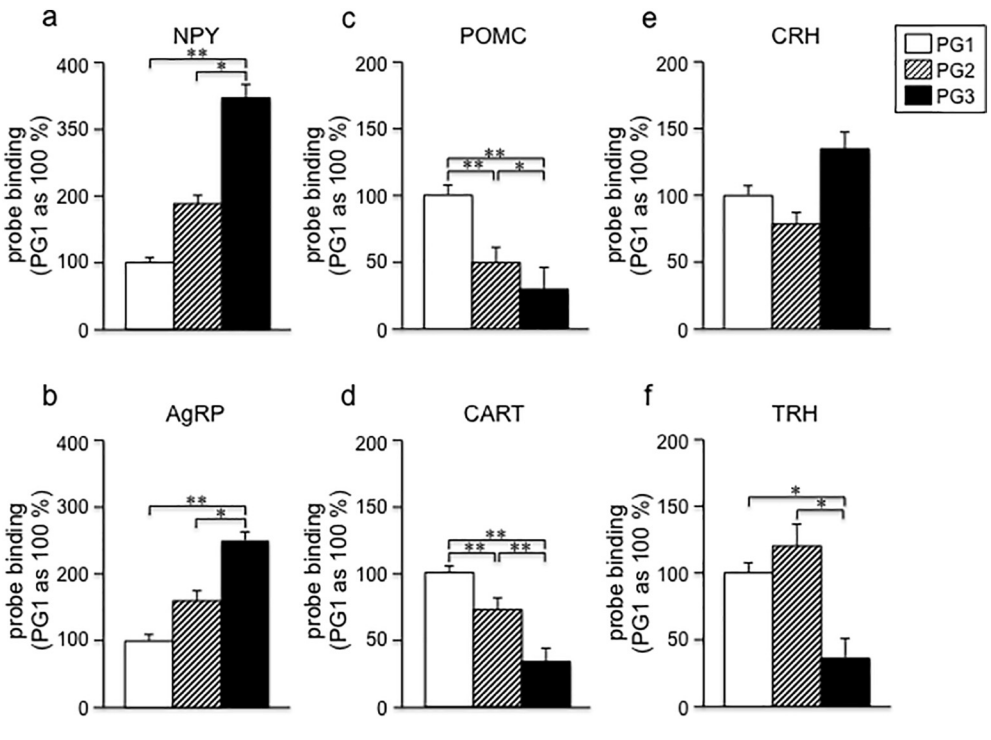

Fig. 3. Expression of the *hypothalamic feeding-related neuropeptide* genes 2 weeks after i.p. administration of STZ. *NPY* (a), *AgRP* (b), *POMC* (c), and *CART* (d) in the Arc, and *CRH* (e), and *TRH* (f) in the PVN. Signals for PG1 were set at 100%. Data are presented as the mean ± SEM. PG1, $n = 29$; PG2, $n = 10$; PG3, $n = 11$. $^{*}P < .05$ and $^{**}P < .01$ vs. PG1.

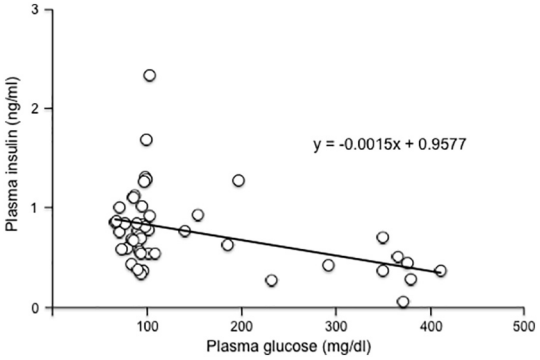

Fig. 4. Scatter plot analysis of plasma insulin against plasma glucose (PG). Each dot represents an individual data point and a regression line generated by simple linear regression analysis is shown. Calculation of Spearman's correlation coefficient indicated that plasma insulin was significantly correlated with plasma glucose ($r = -0.294$, $P = .045$).

et al., 1993). Hypothalamic TRH content in diabetic rats is also reduced by almost 50% compared with control rats (GonzÁlez et al., 1980). Hence, it is possible that persistent hyperglycemia caused low T3, resulting in central hypothyroidism, or that hypothalamic TRH content was significantly decreased in hyperglycemic rats in our study. In addition, both NPY and AgRP inhibit *TRH* gene expression (Warner and Beckett, 2010). Up-regulation of *NPY* and *AgRP* in severely diabetic rats may affect the synthesis of *TRH* via this pathway. It is difficult to explain why decreased plasma insulin led to inhibition of expression of the *TRH* gene and this finding warrants further investigation.

Insulin receptors are particularly highly expressed in the Arc (Van Houten et al., 1979) and inhibition of insulin signaling in the brain has orexigenic effects (Bruning et al., 2000). In several reports using STZ-induced diabetic models, it was shown that the expression of orexigenic neuropeptide genes, such as *NPY* and *AgRP*, was stimulated and that of anorectic neuropeptide genes, such as *POMC*, was inhibited. In addition, the endogenous insulin level in rats with STZ-induced diabetes was lower than that in control rats. In these reports, the expression of *NPY* and *AgRP* in the Arc was decreased and that of *POMC* was increased after insulin treatment using subcutaneous injection, intracerebroventricular administration, and an implant impregnated with insulin (Havel et al., 2000; Sindelar et al., 2002; Filippi et al., 2013; Schwartz et al., 1992; Kim et al., 1999; Sipols et al., 1995). Thus, previous studies have revealed that exogenous insulin alters the levels of hypothalamic neuropeptides; however, few studies have focused on the direct relationship between endogenous insulin and the expression of genes encoding hypothalamic neuropeptides. Reduced endogenous insulin secretion may affect the expression of these hypothalamic neuropeptide genes; however, our results do not provide a rationale for the observed changes. We speculate that POMC/CART neurons may be more sensitive to endogenous insulin than NPY/AgRP neurons. Another possibility is that NPY/AgRP neurons may adapt more rapidly to altered plasma insulin than POMC/CART neurons, so that only changes in *POMC* and *CART* gene expression were observed.

The expression of hypothalamic feeding-related neuropeptides was regulated by peripheral humoral factors such as leptin and ghrelin. Reduced leptin signaling lowered *POMC* levels (Schwartz et al., 1997), while leptin suppressed *AgRP* levels (Wilson et al., 1999). As a result, leptin reduced food intake in normal animals. In previous reports, plasma leptin levels decreased in STZ-induced diabetic rats and leptin levels contributed to diabetic hyperphagia (Sindelar et al., 2000; Sivitz et al., 1998). In the present study, leptin levels in the PG2 and PG3 group rats were significantly lower than in the PG1 group, while there were no significant differences in leptin levels between the PG2 and PG3 groups (Table 4). Decrease in *POMC* and increase in *AgRP* in PG3 rats compared with PG1 rats may be caused by the reduction in plasma leptin levels. However, the decrease in leptin did not affect the significantly lower levels of *POMC* and *AgRP* in PG3 rats than in PG2 rats, because there was no significant difference in leptin levels between the PG2 and PG3 groups. In addition, previous studies have reported that plasma ghrelin levels in STZ-induced diabetic rats and mice were significantly higher than in control rats (Ishii et al., 2002; Masaoka et al., 2003; Tsubone, 2005). Ghrelin is the orexigenic hormone produced in the peripheral tissue, which affects feeding behavior (Kageyama et al., 2010). Ghrelin enhanced NPY expression in STZ-induces diabetic mice (Dong et al., 2006). The present study indicates that ghrelin levels might be involved in the change in hypothalamic feeding-related

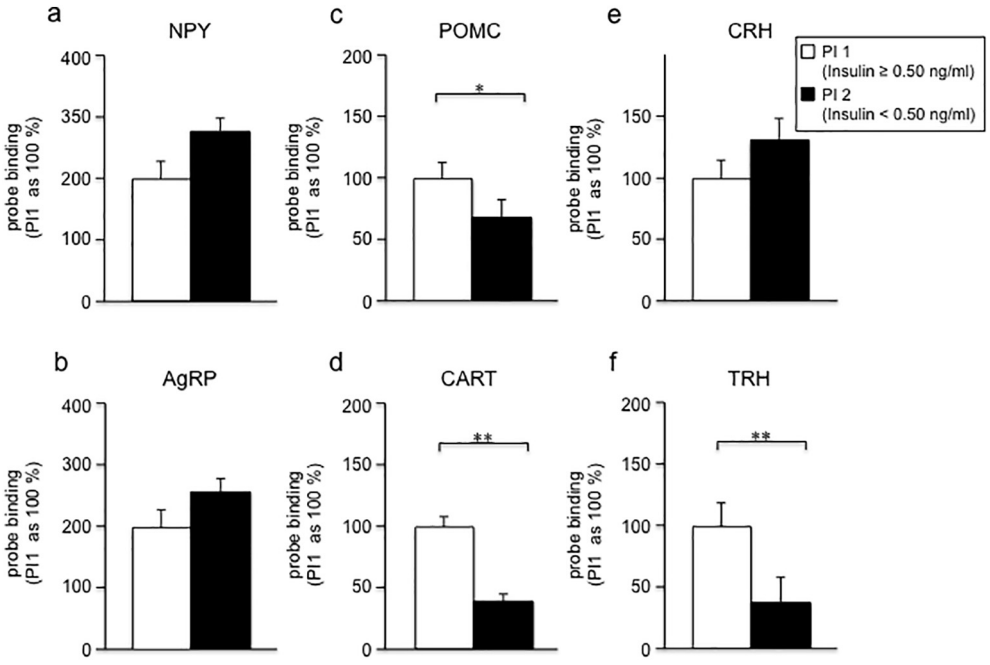

Fig. 5. Expression of the genes encoding *hypothalamic feeding-related neuropeptides* 2 weeks after i.p. administration of STZ in PI1 (insulin ≥ 0.5 ng/ml) and PI2 (insulin < 0.5 ng/ml) rats. *NPY* (a) and *AgRP* (b), *POMC* (c), and *CART* (d) in the Arc, and *CRH* (e) and *TRH* (f) in the PVN. Signals for PI1 were set at 100%. Data are presented as the mean ± SEM. PI1, $n = 12$; PI2, $n = 7$. $^{*}P < .05$ and $^{**}P < .01$ vs. PI1.

neuropeptides and hyperphagia in diabetic rats; however, we did not measure ghrelin levels in the present study. Further studies would clarify any relationship between ghrelin signaling and feeding behavior in diabetic animals.

In the STZ-induced diabetic model, inflammatory processes may be involved in the diabetic state. Although we tried to measure TNF (one of the proinflammatory cytokines) in STZ-administered rats, TNF levels were under the detectable concentration in all cases. Further studies should be performed to evaluate the involvement of inflammatory processes in STZ-induced diabetic models.

In conclusion, the expression of the hypothalamic neuropeptide genes differed depending on the degree of hyperglycemia and the level of residual insulin secretion in STZ-induced diabetic rats. Plasma glucose and insulin levels may be one of important factors that regulate the hypothalamic feeding-related neuropeptides dynamically, causing hyperphagia in STZ-induced diabetic rats. Our data may provide explanations and therapeutic suggestions for human hyperphagia induced by hyperglycemia or hypoinsulinemia.

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Conflict of interest

The authors declare that they have no conflict of interest

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Author contributions

This study was designed by S.S., M.Y., Y.O., Y.T., and Y.U. The experiments were performed by S.S., M.Y., H.N., K.N., K.T., H.U., Y.M., R.S., T.M., and H.H. Data were analyzed by S.S. The manuscript and figures were prepared by S.S. and Y.U. Final approval was by Y.U. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work regarding questions related to the accuracy. All authors listed qualify for authorship, and all those who qualify for authorship are listed.

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