neurons also responded to Oxt. In NG neurons isolated from *db/db* mice, by contrast, leptin failed to increase $[Ca^{2+}]_i$ (Fig. 5*B*). Notably, Oxt evoked $[Ca^{2+}]_i$ increases in these NG neurons of *db/db* mice (Fig. 5*B*), and the incidence and amplitude of $[Ca^{2+}]_i$ responses to Oxt were indistinguishable between *db/db* and wild-type mice (Fig. 5, *C* and *D*). Thus Oxt activated leptin-resistant vagal afferent neurons of *db/db* mice.

Single ip injection of 200 μg/kg Oxt into *db/db* mice significantly suppressed food intake for 0.5–3 h after injection to an extent similar to that in normal mice (Fig. 6*A* vs. Fig. 1*A*). We examined whether subchronic administration of Oxt for 2 wk using osmotic minipumps ameliorates hyperphagia in *db/db* mice. The surgery and implantation of osmotic minipumps markedly and transiently decreased daily food intake to a level around 2 g/day in saline and Oxt injection groups (Fig. 6*B*). The daily food intake largely recovered at *day 2* after the surgery/implant and thereafter became stable through *day 12* at the level around 6~8 g/day for saline and 4~6 g/day for Oxt (Fig. 6*B*). Thus subchronic Oxt treatment, compared with saline treatment, significantly reduced daily food intake for the period of *day 2~12* and cumulative food intake for 12 days (Fig. 6*C*). Body weight gain at *day 12* was $-1.8 \pm 0.48$ g in saline-treated group and $-3.2 \pm 0.29$ g in Oxt-treated group, showing a significant reduction of body weight by Oxt (Fig. 6*D*). The minus value of body weight gain at *day 12* is suggested to be due to the transient decrease of daily food intake after surgery/implant. Thus peripheral Oxt injection activated vagal afferents, decreased food intake, and ameliorated obesity in obese *db/db* mice with leptin resistance.

## DISCUSSION

In the present study, ip administration of Oxt (200 μg/kg) suppressed food intake without evoking aversive behavior and induced c-Fos expression in NTS, and these effects were blunted in the mice treated with CAP or that received vagotomy. Oxt evoked membrane depolarization, action potential firings, and $[Ca^{2+}]_i$ increases in the single NG neurons isolated from vagal afferents. The majority of the Oxt-responsive NG neurons also responded to CCK-8 and contained CART, both of which are known to inhibit feeding through vagal afferents. In Type 2 diabetic *db/db* mice, a model of leptin-resistant obesity, Oxt activated NG neurons, while leptin failed to do so. The results indicate that Oxt is able to activate NG neurons under leptin resistance, a condition characteristically associated with obesity. Moreover, peripheral subchronic Oxt infusion ameliorated hyperphagia and obesity in *db/db* mice. These results demonstrate that peripheral administration of Oxt, at least in pharmacological doses, suppresses food intake by activating vagal afferent NG neurons and subsequent signaling to the brain, and suggest that this peripheral Oxt-regulated vagal afferent route provides a novel tool to treat hyperphagia and obesity.

It has previously been shown that a fraction of NG neurons express the Oxt receptor mRNA and protein (52). In the present study, Oxt increased $[Ca^{2+}]_i$ in isolated NG neurons, and this response was inhibited by Oxt receptor antagonist, indicating that Oxt activates vagal afferents via Oxt receptor. Approximately 15% of NG neurons responded to Oxt. This value (15%) of the incidence of Oxt-responsive vagal afferent neurons may be reasonable, due to the following consider-

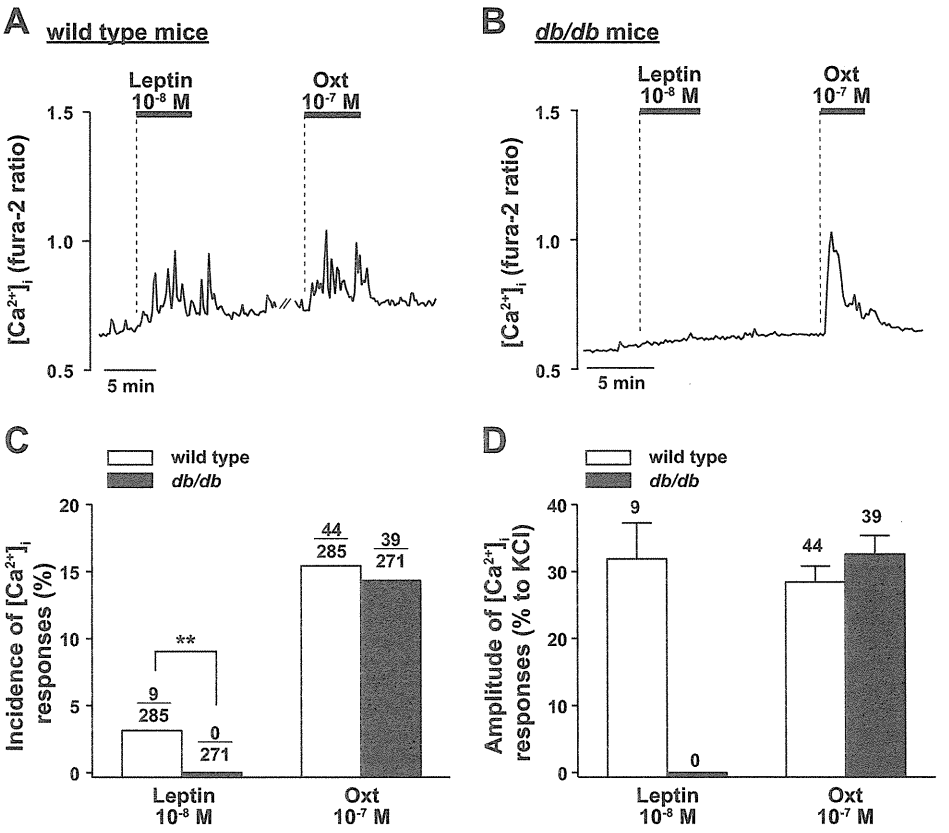


Fig. 5. Blunted leptin action but intact Oxt action to increase $[Ca^{2+}]_i$ in NG neurons of obese diabetic *db/db* mice. *A*: leptin at $10^{-8}$ M and Oxt at $10^{-7}$ M increased $[Ca^{2+}]_i$ in an isolated NG neuron of wild-type BKS mice ($n = 9$). *B*: leptin failed to induce, but Oxt induced, an increase in $[Ca^{2+}]_i$ in an NG neuron of *db/db* mice ($n = 39$). *C* and *D*: incidence (*C*) and amplitude (*D*) of $[Ca^{2+}]_i$ responses to leptin and Oxt in NG neurons of wild-type and *db/db* mice. *C*: numbers above each bar indicate the number of neurons that responded to each peptide over that responding to 55 mM KCl. *D*: numbers on each bar indicate number of neurons that responded to leptin or Oxt. Values are means ± SE. $^{**}P < 0.01$ by $\chi^2$ test.

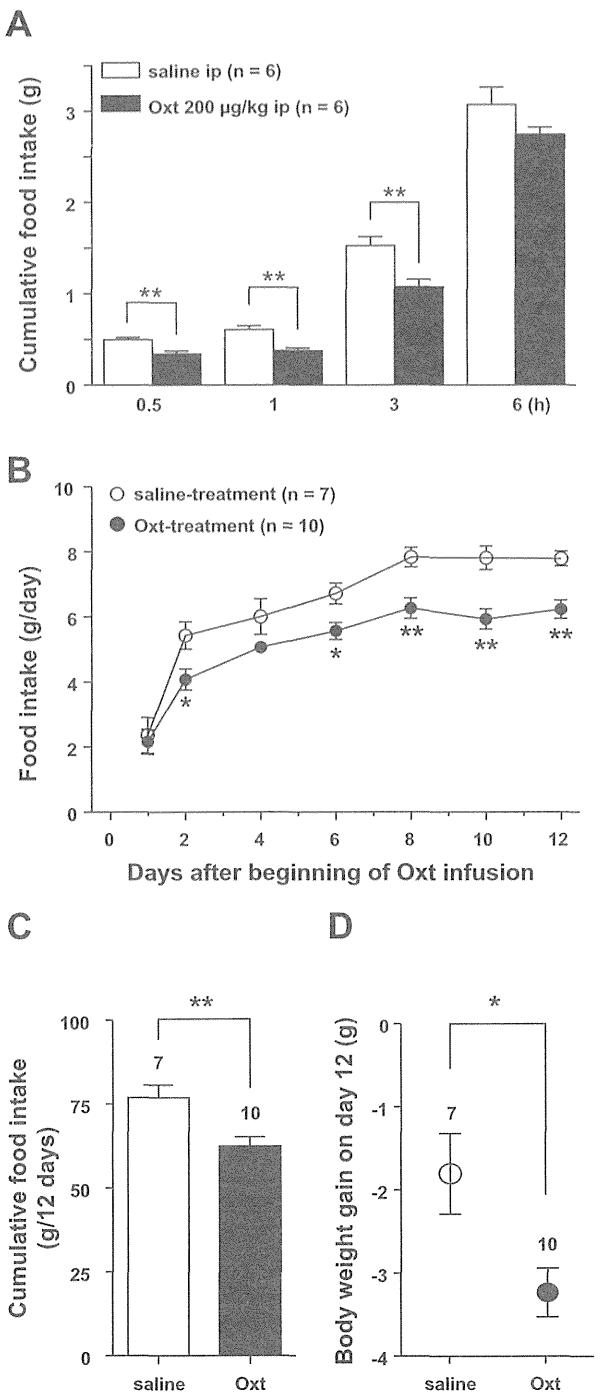


Fig. 6. Single and subchronic Oxt administrations decrease food intake and body weight in obese diabetic *db/db* mice. *A*: cumulative food intake after ip injection of saline (open bar) or Oxt (200 μg/kg, solid bar) in *db/db* mice. *B*: daily food intake during infusion of saline (open circle) or Oxt (1,600 $\mu g \cdot kg^{-1} \cdot day^{-1}$; solid circle) using osmotic minipump. Daily food intake markedly and transiently decreased possibly due to surgery and implant, followed by its substantial recovery on *day 2* and thereafter stable period. *C* and *D*: cumulative food intake for 12 days (*C*) and body weight gain on *day 12* of Oxt infusion (*D*). Values are means ± SE. $^*P < 0.05$, $^{**}P < 0.01$ vs. saline-group by unpaired *t*-test.

ations. First, vagal afferents innervate diverse tissues, forming many distinct subpopulations. A particular subpopulation that innervates a particular tissue could have a distinct ability of sensing specific factors. Second, peptide $YY_{3-36}$, pancreatic polypeptide, and nesfatin-1, the hormones known to decrease food intake via interacting with vagal afferents, increase $[Ca^{2+}]_i$ in ~10% of NG neurons (18, 19). Taken together, it is suggested that, among the highly heterogeneous NG neurons innervating diverse tissues and controlling a variety of functions, its particular subpopulation comprising 10~15% of total NG neurons has the ability to respond to a particular hormone.

The present study showed that Oxt induces action potential firings and increases $[Ca^{2+}]_i$ in a subfraction of NG neurons that respond to CCK-8 and leptin. Previous studies showed that the NG neuron responses to CCK-8 and leptin are implicated in inhibition of feeding. CCK, released from enteroendocrine I cells in response to dietary nutrients (11), directly activates vagal afferent neurons via CCK-1 receptor (28, 45) and thereby rapidly decreases food intake (46). Leptin is produced by the stomach epithelial cells in addition to adipocytes (4). Leptin, released from the stomach in response to food and CCK (4), activates NG neurons of vagal afferents (40) and thereby inhibits food intake for a short time (41). Moreover, leptin receptor is co-expressed with CCK-1 receptor in NG neurons (29), and CCK and leptin synergistically activate NG neurons (40) and decrease food intake (5). These findings by us and others, taken together, suggest that the Oxt activation of CCK- and leptin-responsive NG neurons is linked to inhibition of food intake. Whether Oxt and leptin also cooperatively activate NG neurons and inhibit feeding remains to be studied.

We also found that Oxt activates NG neurons containing CART, a well-known neurotransmitter of vagal afferents (7). CART is co-expressed with CCK-1 and leptin receptors in NG neurons (7, 17), and CCK-8 and leptin stimulate expression and secretion of CART in NG neurons (9, 17). The anorexigenic effect of coadministration of CCK and leptin is blunted in the rats treated with CART small interfering RNA (17), suggesting that CART serves as a neurotransmitter in the NG neurons that respond to CCK and leptin. Accordingly, the Oxt-targeted, CCK- and leptin-responsive CART neurons in vagal afferents may play a role in regulation of feeding.

The anorexigenic effect of ip Oxt at 400 μg/kg in the early phase (0.5~1 h) was blunted by CAP treatment, whereas that in the later phase (3~6 h) significantly remained, indicative of vagal afferent-independent route. It has recently been reported that ip injection of Oxt (450~600 μg/kg) results in increased Oxt levels in the brain, including the hippocampus and amygdala (35), suggesting the transport of Oxt through BBB. Furthermore, the NTS, AP, and hypothalamic arcuate nucleus, the feeding-related areas, are considered to have leaky BBB and express Oxt receptors (54). Therefore, Oxt administered at a high dose (400 μg/kg) might significantly pass through BBB and act on the brain and thereby reduce feeding in the later period. However, further study is definitely needed to verify this possibility.

It remains unclear whether the anorexigenic effect of Oxt via vagal afferent pathway is pharmacological and/or plays a role in the normal regulation of daily food intake. Oxt is synthesized in the neurons in the hypothalamic PVN and SON, and released from the posterior pituitary to circulation. Plasma Oxt concentration is around 0.01~0.1 nM in rodents and humans (23, 38, 56) and is elevated by several times during delivery and lactation (24)

and postprandial periods (51). In the present study using single NG neurons, the effective concentration of Oxt to activate vagal afferents was around 0.1~100 nM (Fig. 1, *A–D*), ranging from the plasma Oxt level to its 1,000-fold higher level. Hence, the plasma Oxt, when elevated during lactation and postprandial periods, may act on a portion of vagal afferent neurons. It has been reported that ip administration of Oxt in a pharmacological dose (12 μg/mouse, around 450~600 μg/kg) results in plasma Oxt concentration around 1.5 nM (35), a dose capable of activating vagal afferent neurons in our study. Therefore, in our study, Oxt ip injection in pharmacological doses (200 and 400 μg/kg) may have reduced food intake, at least in part, via interacting with vagal afferents. More importantly, vagal afferents often terminate at the vicinity of hormone-secreting cells and sense higher concentrations of hormones in a paracrine fashion, as was previously proposed for CCK and insulin (20, 21, 42). This could also be the case for Oxt, in light of previous reports that Oxt is present in the uterus, testis, heart, pancreas, intestinal epithelium, and enteric nervous system (1, 24, 52). Moreover, ip injection of Oxt receptor antagonist was shown to induce hyperphagia during daytime in mice, suggesting that Oxt could regulate diurnal meal patterns (56). These findings by us and others suggest that endogenous Oxt in the periphery possibly interacts with the vagal afferents and regulate feeding. However, further studies are definitely required to verify the potential physiological role of the afferent vagal nerve in sensing Oxt in the circulation and/or peripheral tissues and in regulating feeding.

Obesity induces leptin resistance, a key factor that worsens obesity and leads to metabolic syndrome. Under leptin-resistant conditions, not only leptin itself, but also leptin-dependent agents, lose their ability to induce anorexigenic effect. Recent reports show that diet-induced obesity leads to leptin resistance in vagal afferents (8). Importantly, peripheral Oxt was shown to reduce food intake and body weight in DIO rats and mice (30, 33). The present study used the leptin-resistant obese *db/db* mice and examined the subchronic effect of Oxt released from implanted osmotic minipumps. The surgery and implantation of osmotic minipumps markedly and transiently decreased daily food intake. Daily food intake largely recovered at *day 2* and thereafter became stable. Oxt infusion resulted in significant reductions in the daily food intake during the stable phase and in body weight gain at *day 12*. The present study shows that peripheral subchronic infusion of Oxt decreases food intake and body weight, without aversive behavior. Previous reports show that peripheral Oxt induces no adverse effects on the locomotor activity (30) and blood pressure (30). Our present and previous results indicate that the activation of vagal afferents by peripheral Oxt injection provides a promising tool to treat hyperphagia and obesity. In addition, the vagal afferent-mediated pathway could possibly serve as a signaling route of Oxt for treatment of autism (53), although further study is required to address this issue.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

## AUTHOR CONTRIBUTIONS

Author contributions: T.Y., Y.I., and Y.M. conception and design of research; Y.I., Y.M., S.S., M.Y., T.A., K.K., P.K., and M.K. performed experiments; T.Y., Y.I., Y.M., S.S., and M.K. analyzed data; T.Y., Y.I., Y.M., H.N., and M.K. interpreted results of experiments; T.Y., Y.I., Y.M., and M.K. prepared figures; T.Y., Y.I., and S.S. drafted manuscript; T.Y., Y.I., S.S., and M.K. edited and revised manuscript; T.Y. and Y.I. approved final version of manuscript.

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# Nasal Oxytocin Administration Reduces Food Intake without Affecting Locomotor Activity and Glycemia with c-Fos Induction in Limited Brain Areas

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**Key Words**
Oxytocin · Nasal treatment · Paraventricular nucleus · Dorsal motor nucleus of vagus · Glucose tolerance · Obesity · Insulin release

**Abstract**
Recent studies have considered oxytocin (Oxt) as a possible medicine to treat obesity and hyperphagia. To find the effective and safe route for Oxt treatment, we compared the effects of its nasal and intraperitoneal (IP) administration on food intake, locomotor activity, and glucose tolerance in mice. Nasal Oxt administration decreased food intake without altering locomotor activity and increased the number of c-Fos-immunoreactive (ir) neurons in the paraventricular nucleus (PVN) of the hypothalamus, the area postrema (AP), and the dorsal motor nucleus of vagus (DMNV) of the medulla. IP Oxt administration decreased food intake and locomotor activity and increased the number of c-Fos-ir neurons not only in the PVN, AP, and DMNV but also in the nucleus of solitary tract of the medulla and in the arcuate nucleus of the hypothalamus. In IP glucose tolerance tests, IP Oxt injection attenuated the rise of blood glucose, whereas neither nasal nor intracerebroventricular Oxt affected blood glucose. In isolated islets, Oxt administration potentiated glucose-induced insulin secretion. These results indicate that both nasal and IP Oxt injections reduce food intake to a similar extent and increase the number of c-Fos-ir neurons in common brain regions. IP Oxt administration, in addition, activates broader brain regions, reduces locomotor activity, and affects glucose tolerance possibly by promoting insulin secretion from pancreatic islets. In comparison with IP administration, the nasal route of Oxt administration could exert a similar anorexigenic effect with a lesser effect on peripheral organs.


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## Introduction

Oxytocin (Oxt) is a neurohypophysial hormone that regulates uterine contraction during labor and milk ejection [1]. Oxt is synthesized in the paraventricular (PVN) and supraoptic nucleus of the hypothalamus. It is released peripherally after being shuttled to the pituitary [2], or centrally in the brain to regulate neuronal process [3]. Recent studies have clarified new functions of Oxt in the central nervous system, including increased trust [4], regulation of social recognition [5] and development of mother-infant bonding [6]. In addition, our previous study employed Oxt in the PVN-driven anorexigenic circuit in rats [7].

The anorexigenic effect of Oxt has been reported since the early 1990s. Olson et al. [8] showed that Oxt and its agonist decrease food intake when administered centrally in rats, and Arletti et al. [9] showed that intracerebroventricular (icv) injection of Oxt inhibits food intake together with water intake. More recent studies have shown that mice with deficient Oxt or Oxt receptor (Oxt-R) develop late-onset obesity [10, 11].

Obesity is linked to numerous diseases including type 2 diabetes, cardiovascular events, and certain forms of cancer [12, 13]. Several antiobesity drugs, including diethylpropion, fenproporex, mazindol, fluoxetine, and sibutramine, have been developed [14], but there is little evidence for effective and safe treatments.

We have previously shown that subchronic peripheral Oxt treatment through a subcutaneously implanted osmotic minipump reduces hyperphagia and obesity in high-fat diet-fed obese mice, and that intraperitoneal (IP) Oxt treatment similarly reduces hyperphagia [15]. Zhang et al. [16] have recently reported that the nasal administration of Oxt decreases body weight (BW) in obese subjects. Ott et al. [17] showed that nasal Oxt treatment reduces reward-driven food intake. These reports indicate that subcutaneous, IP, and nasal routes of Oxt treatment have common actions of reducing obesity and hyperphagia. However, a comparison of these actions and underlying mechanisms following different administration routes of Oxt has not been previously undertaken.

We performed a comparative study on the effects of nasal and IP administration of Oxt on feeding, locomotor activity, and glucose tolerance in mice, and found that both nasal and IP Oxt injections reduce food intake to a similar extent with increased c-Fos-immunoreactive (ir) neurons in common brain regions. IP Oxt administration also increased c-Fos-ir neurons in additional brain regions, reduced locomotor activity, and affected glucose tolerance possibly by promoting insulin secretion from pancreatic islets.

## Materials and Methods

*Animals*

Male C57BL/6J mice (aged 6 weeks) were obtained from Japan SLC (Hamamatsu, Japan). Animals were maintained on a 12-hour light/dark cycle. The dark and light phases started at 19:30 h and 7:30 h, respectively. Mice were allowed ad libitum access to water and a standard diet (CE-2; Clea, Osaka, Japan). Experimental procedures and care of animals were carried out according to the Jichi Medical University Institute of Animal Care and Use Committee. The mice used in the experiments were well habituated to minimize stress.

*Measurements of Food Intake and Locomotor Activity after Oxt Administration*

Following deprivation of food for 2 h before the dark phase, the animals received 0.1–10 μg/10 μl nasal Oxt or 40 and 400 μg/kg IP Oxt (Peptide Institute, Osaka, Japan) 30 min before the dark phase. For the nasal administration, 10 μl of Oxt or vehicle was dropped into the nasal cavity. Cumulative food intake was measured for the following 0.5, 1, 2, 6, and 24 h. For the measurements during the dark phase, red light with minimum intensity was used to prevent mice from being stimulated by bright light. Locomotor activity was measured by an activity monitoring system (ACTIMO-100; Shinfactory, Fukuoka, Japan). The doses of nasal Oxt were 0.1, 1 and 10 μg, based on the minimum effective dose of Oxt (1 μg) on food intake determined by pilot experiments. The dose of IP Oxt was based on a previous report [15] showing that 400 μg/kg Oxt markedly and long-lastingly suppressed food intake.

*Immunostaining of c-Fos after Oxt Administration*

Food was deprived for 2 h before the dark phase until perfusion. The animals received nasal (1 μg/10 μl) or IP Oxt (400 μg/kg) administration 30 min before the dark phase. After 90 min, the mice were transcardially perfused as described previously [7]. Coronal sections of 40-μm thickness were cut with a freezing microtome. Sections at 120-μm intervals between –0.58 and –8.0 mm from the bregma were used for c-Fos immunohistochemistry. Sections were rinsed in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), incubated in PBS containing 2% normal goat serum and 2% bovine serum albumin (BSA), then incubated with rabbit anti-c-Fos antiserum (sc-52, 1:5,000; Santa Cruz, Calif., USA). Subsequently, the sections were incubated with biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories Inc., Calif., USA), and an avidin-biotin complex (ABC kit; Vector Laboratories Inc.). Immunoreactions were visualized by incubating in diaminobenzidine (DAB) solution containing nickel ammonium.

Bilateral sections of the hypothalamus and brainstem between –0.58 and –8.0 mm from the bregma were prepared, with c-Fos-ir cells per section counted manually under the microscope. The cell count per section was averaged for all sections in each investigated nucleus of each animal.

*Glucose Tolerance Test and Measurement of Plasma Insulin*

Mice aged 10 weeks (or with a BW of 22–25 g) were cannulated in the lateral ventricle (0.5 mm caudal to bregma, 1.0 mm lateral from the midline, and 2.2 mm below the skull surface; ICM-23G09 Inter Medical, Osaka, Japan) and housed individually. BW and food intake were not significantly different before the operation and for 10 days thereafter. On the day of the experiment, food was deprived at 09:00 h and an IP glucose tolerance test (IPGTT; 2 g/kg) was started at 13:00 h. IPGTT was performed 20 min after nasal (0.1, 1, 10 μg), icv (0.4, 4 μg) or IP (40, 400 μg/kg) Oxt injection. Oxt doses for icv injection were determined based on our previous report [7]. Blood was sampled by cutting the surface of the tail skin under local anesthesia with EMLA cream (AstraZeneca K.K., London, UK) without restraint. Blood glucose levels were measured using Glucocard (Arkray, Kyoto, Japan).

For the measurement of plasma insulin, mice aged 10 weeks (22–26 g BW) were used. IP Oxt injection (400 μg/kg) and IPGTT procedures followed the same protocol and time course. The mice were decapitated, and their blood was collected 10 min after IPGTT. After centrifugation, plasma insulin concentration was measured by ultrasensitive mouse insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan).

*Isolation of Islets and Measurement of Insulin Secretion*

Mice with similar age and BW to those in the IPGTT were used. Islets were isolated as previously described [18] and cultured overnight in DMEM solution (Sigma, St. Louis, Mo., USA). After 30 min of preincubation in 2 mM glucose solution and 30 min under static incubation conditions in Krebs-Ringer Buffer (mM): 120 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $KH_2PO_4$, 1.2 $MgSO_4$, 10 HEPES, 25 $NaHCO_3$, pH 7.4 (with NaOH), plus 0.1% BSA, experimental glucose (2, 5, 20 mM) with or without Oxt (10 nM), insulin secretion was measured. Islets were then incubated overnight at –20°C with acidified ethanol solution (95% ethanol, 5% acetic acid) to extract all insulin. Insulin was measured using a Morinaga mouse ELISA kit (Morinaga Institute of Biological Science, Inc.).

*Statistical Analysis*

All data are presented as means ± SEM. The statistical analysis of experimental data of c-Fos-ir neurons, insulin secretion, and plasma insulin were carried out using Student's t test. Data for food intake, locomotor activity, and glucose tolerance test were analyzed by repeated measures of two-way ANOVA with treatment (saline vs. Oxt including dose responses) and time as factors. Post hoc multiple comparisons were made using Tukey's test. Significance was set at $p < 0.05$ for all analyses.

## Results

*Effect of Oxt Administration on Food Intake and Locomotor Activity*

In order to compare the effects of nasal and IP Oxt administration, we administered an approximately equally effective amount of Oxt for nasal and IP administration (nasal: 0.1–10 μg/25 g BW, i.e. approx. 4–400 μg/kg; IP: 40–400 μg/kg). Nasal administration of Oxt at doses of 0.1–10 μg/10 μl was performed in mice weighing 20–25 g. These doses correspond to approximately 4–400 μg/kg.

Nasal administration of 0.1 μg Oxt did not affect the food intake significantly ($F_{1,60} = 2.53$, $p > 0.05$; fig. 1a). Administration of 1 μg Oxt significantly decreased the food intake ($F_{1,56} = 27.4$, $p < 0.01$; fig. 1b), and 10 μg Oxt ($F_{1,92} = 27.16$, $p < 0.01$; fig. 1c). Tukey's test indicated that nasal administration of 1 μg Oxt reduced the cumulative food intake 6 and 24 h after administration (fig. 1b), and 10 μg Oxt reduced the food intake 0.5, 1, 2, 6, and 24 h after administration (fig. 1c) without changing locomotor activity ($F_{1,299} = 0.10$, $p > 0.05$; fig. 2a, c).

IP administration of 40 μg/kg Oxt failed to significantly affect the food intake ($F_{1,24} = 2.27$, $p > 0.05$; fig. 1d) and 400 μg/kg Oxt markedly decreased the food intake at 0.5, 1, 2, and 6 h ($F_{1,24} = 38.9$, $p < 0.01$; fig. 1e) but not at 24 h. IP injection of 400 μg/kg Oxt showed a tendency to decrease locomotor activity ($F_{1,138} = 2.6$, $p > 0.05$) for the first 7 h and significantly decreased cumulative locomotor activity during the dark phase (fig. 2b, d).

The tendency and time course of reduction in the food intake were similar between nasal and IP administration (fig. 1b, d). However, at a higher dose (approx. 400 μg/kg), nasal administration of Oxt (10 μg/μl; fig. 1c) reduced the cumulative food intake for up to 24 h, but the extent of food intake reduction during 0.5–6 h was smaller compared to IP administration (fig. 1e).

*Effect of Oxt Administration on the Number of c-Fos-ir Neurons*

After nasal administration of 1 μg Oxt, the number of c-Fos-ir neurons was significantly increased in the PVN of the hypothalamus. The number was 137.1 ± 25.6/section in controls versus 284.6 ± 37.4/section in Oxt-injected groups ($p < 0.05$; fig. 3a, b, e). Nasal Oxt administration also increased the number of c-Fos-ir neurons in the medulla, the area postrema (AP; 10.6 ± 3.5/section in controls vs. 27.3 ± 4.7/section in Oxt-injected groups), and the dorsal motor nucleus of vagus (DMNV; 32.3 ± 6.9/section in controls vs. 69.2 ± 4.7/section in Oxt-injected groups; fig. 3c–e). IP administration of Oxt also increased the number of c-Fos-ir neurons in the PVN (71.7 ± 18.1/section in controls vs. 188.0 ± 35.2/section in Oxt-injected groups), DMNV (14.7 ± 3.7/section in controls vs. 43.4 ± 4.8/section in Oxt-injected groups; fig. 3f, g, j), nucleus of the solitary tract (NTS; 39.5 ± 9.8/section in controls vs. 153.3 ± 6.0/section in Oxt-injected groups), and the arcuate nucleus (ARC; 68.9 ± 12.2/section in control vs. 128.6 ± 7.0 in Oxt groups), consistent with our previous

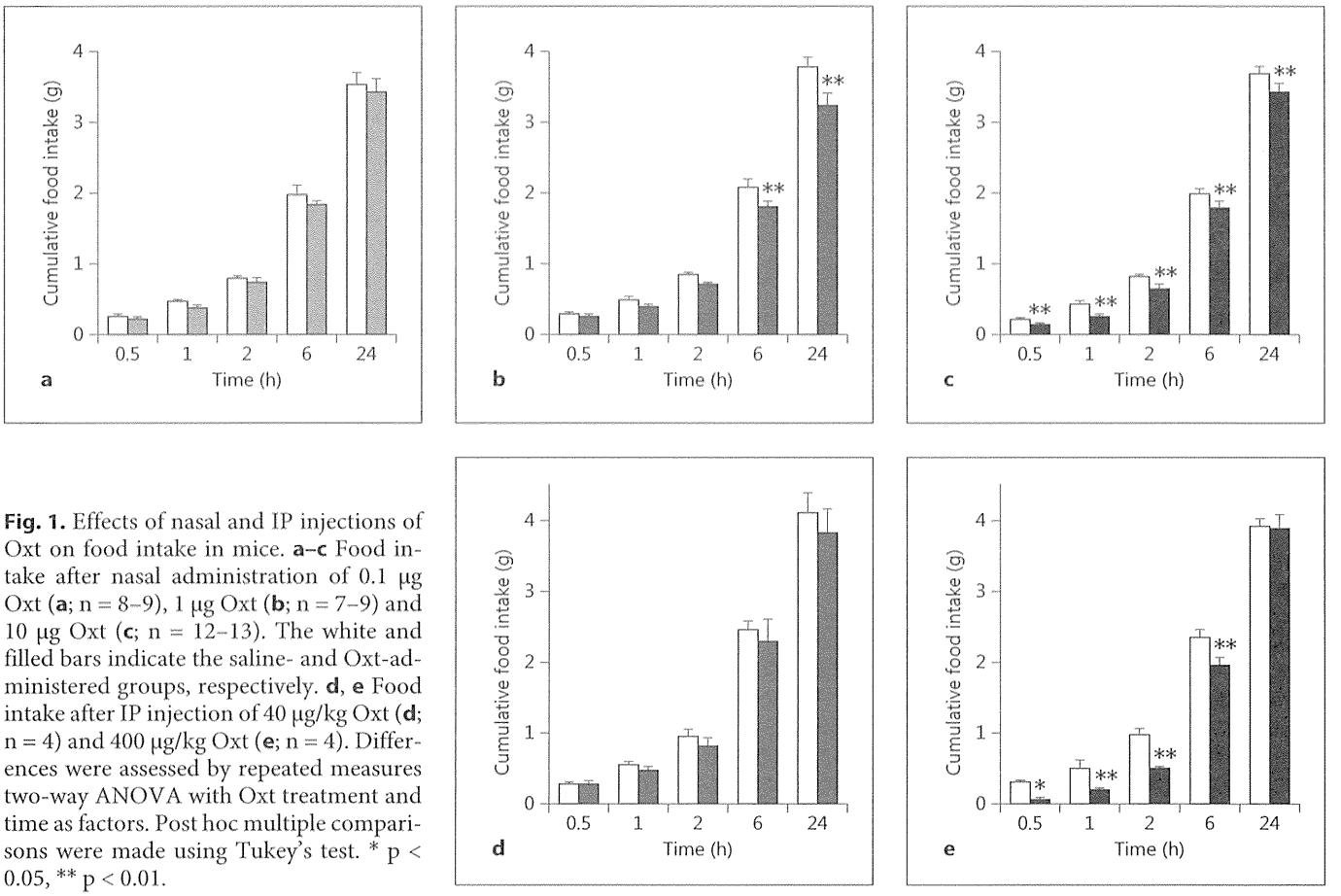



**Fig. 1.** Effects of nasal and IP injections of Oxt on food intake in mice. **a–c** Food intake after nasal administration of 0.1 μg Oxt (**a**; n = 8–9), 1 μg Oxt (**b**; n = 7–9) and 10 μg Oxt (**c**; n = 12–13). The white and filled bars indicate the saline- and Oxt-administered groups, respectively. **d**, **e** Food intake after IP injection of 40 μg/kg Oxt (**d**; n = 4) and 400 μg/kg Oxt (**e**; n = 4). Differences were assessed by repeated measures two-way ANOVA with Oxt treatment and time as factors. Post hoc multiple comparisons were made using Tukey's test. * $p < 0.05$, ** $p < 0.01$.

report [15]. Thus, IP but not nasal administration of Oxt increased c-Fos-ir neurons in the NTS and ARC.

*Effect of Oxt Administration on Glucose Metabolism*

The effect of Oxt on the elevated levels of blood glucose during IPGTT was examined. Nasal and icv Oxt administration had no effect on blood glucose levels in IPGTT ($F_{3,84} = 0.28$, $p > 0.05$, and $F_{2,54} = 1.02$, $p > 0.05$, respectively; fig. 4a, c). In contrast, IP administration of 40 and 400/μg/kg Oxt ($F_{2,36} = 13.10$, $p < 0.01$) dose-dependently attenuated the elevation of blood glucose at 30 and 60 min of IPGTT (fig. 4b). The alteration of glycemia prompted us to examine whether Oxt affects insulin release from pancreatic islets. Ten minutes after IPGTT, plasma insulin concentration was significantly increased (2.19 ± 0.26 ng/ml), compared with the saline-injected group (0.93 ± 0.12 ng/ml; fig. 4d). Incubation of isolated islets with 10 nM Oxt-enhanced insulin secretion in the stimulatory (20 mM), but not in the low (2 mM) and basal (5 mM) glucose conditions (fig. 4e).

## Discussion

It has recently been reported that nasal administration of Oxt reduces BW in obese humans [17]. To the best of our knowledge, the present study is the first to show the effect of nasal Oxt in reducing food intake in rodents without affecting locomotor activity or blood glucose level. This may suggest a selective anorexigenic action of nasal treatment of Oxt.

Neumann et al. [19] reported the increase of brain and plasma Oxt concentrations after nasal administration in rats and mice. The dose (12 μg/10 μl) and application method used in their study are almost identical to those used here (10 μg/10 μl). Hence, we speculate that Oxt levels in the brain and plasma may have been increased after nasal administration of Oxt in the present study. Nasal administration of Oxt may lead to its inhalation into the lungs [20], thereby increasing the plasma Oxt level. If so, pharmacokinetics of Oxt may be similar between nasal and IP applications. Hence, the similar anorexigenic effects of

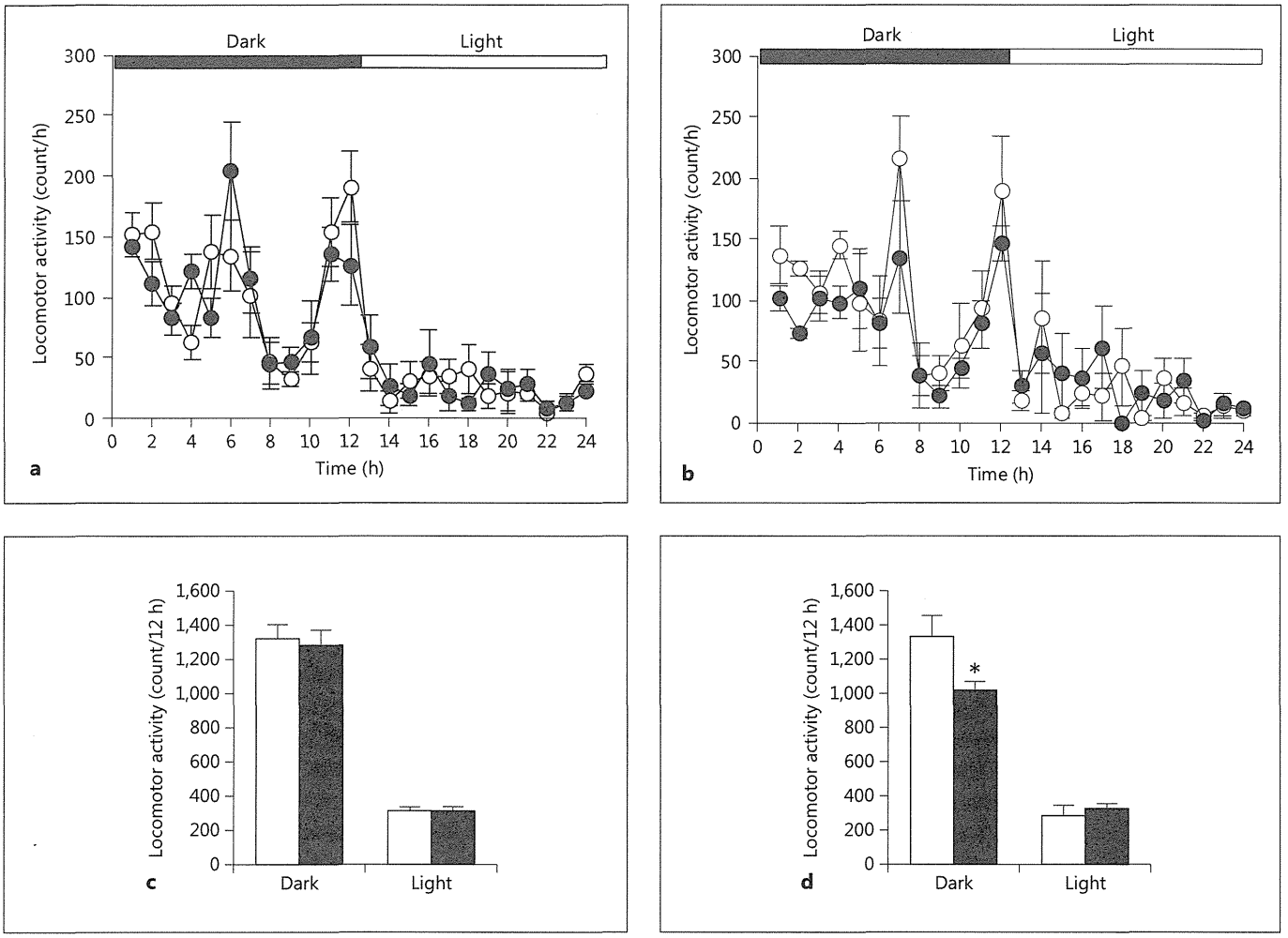



**Fig. 2.** Effects of nasal and IP injections of Oxt on locomotor activity in mice. **a**, **b** Locomotor activity for every 1 h during the 24-hour period after nasal administration of 10 μg Oxt (n = 7–8; **a**) and IP administration of 400 μg/kg Oxt (n = 4; **b**). Nasal and IP administration of Oxt was performed just before the dark phase. Empty and full circles indicate the control and Oxt-injected groups, respectively. Differences were assessed by repeated measures of two-way ANOVA with Oxt treatment and time as factors. Post hoc multiple comparisons were made using Tukey's test. * $p < 0.05$. **c**, **d** Cumulative locomotor activity after nasal (**c**) and IP administration of Oxt (**d**). Open and solid bars indicate the control and Oxt-injected groups. Differences were assessed by Student's t test.

nasal and IP Oxt treatments shown in the present study may have been mediated largely by the increase of peripheral Oxt concentration. In addition, Oxt is reported to cross the blood-brain barrier (BBB) from the blood to the brain by 0.05% [21]. The permeation of Oxt through the BBB depends on the concentration of Oxt in vessels. Since our study used a high dose of Oxt via nasal and IP routes, a significant amount of the peptide may have reached the central nervous system by crossing the BBB [3, 22].

On the other hand, this study has shown that both nasal and IP administration of Oxt induces c-Fos in PVN, AP, and DMNV. In addition, only IP administration of Oxt induces c-Fos in ARC and NTS.

In apparent discrepancy with our results, nasal administration of Oxt (1 μg) has been reported to have no effect on c-Fos-ir neurons in rats [23]. However, this discrepancy may be explained by the dose of Oxt. In the study by Ludwig et al. [23], the dose of Oxt used was 1 μg for rats (BW: 250–300 g). In the present study, it was 1 μg for mice (BW: 25–30 g), a factor 10 times higher when corrected for BW. Oxt doses that induced c-Fos in the present study correspond to 1–100 times of the total Oxt content of ap-

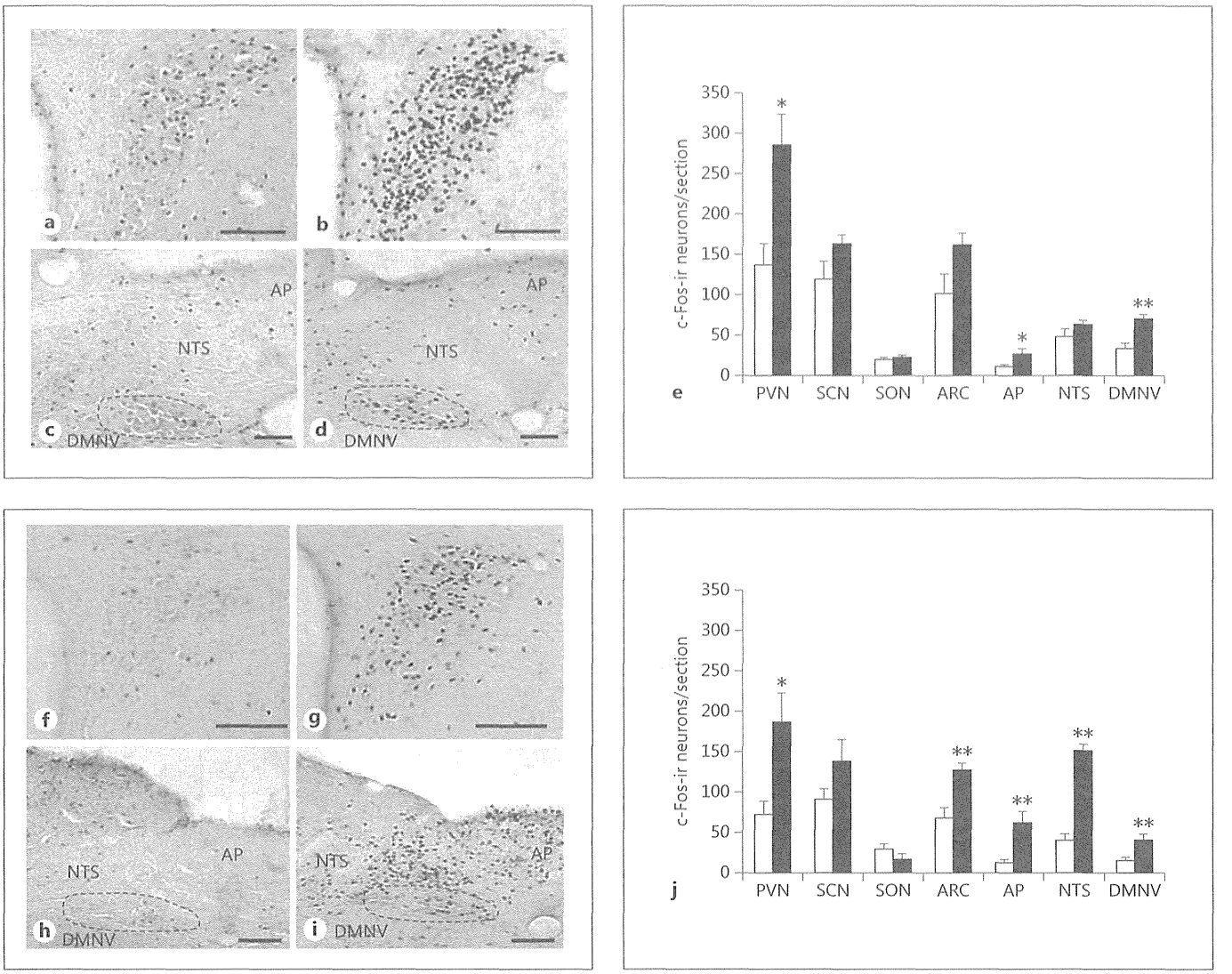
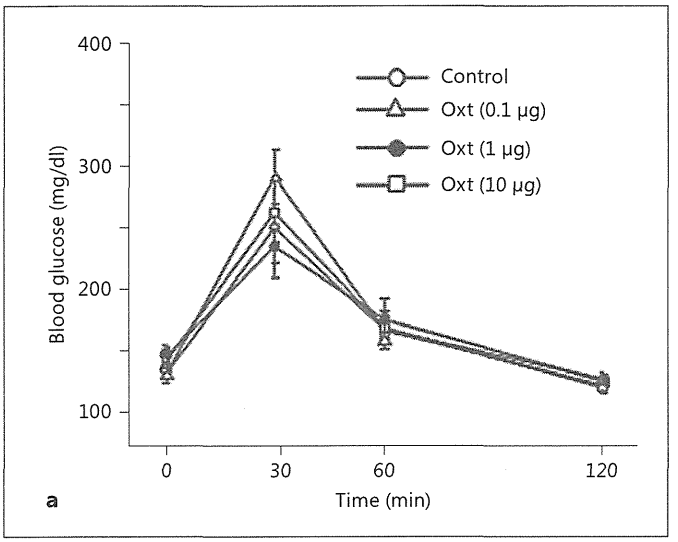
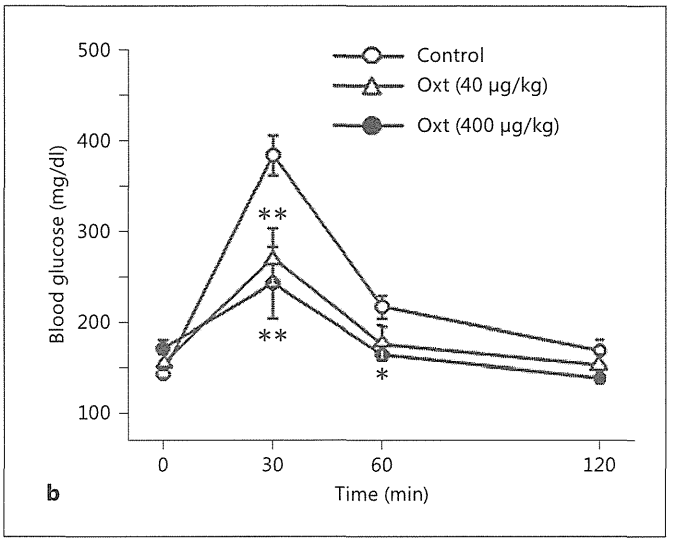


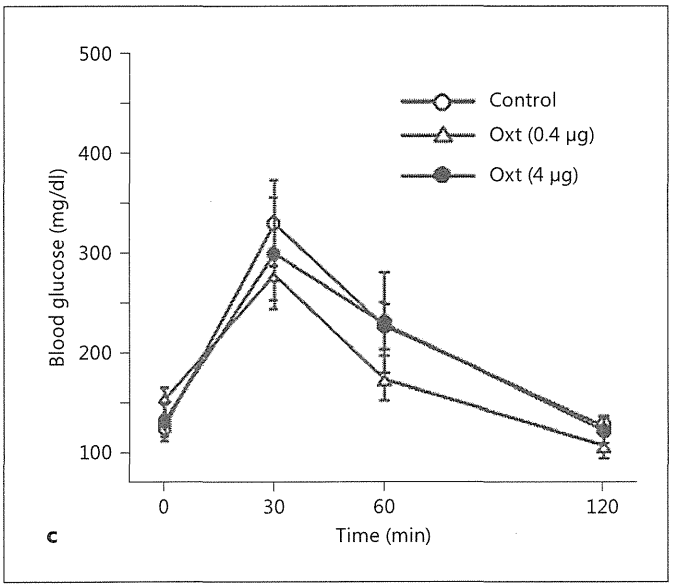

**Fig. 3.** c-Fos-ir neurons after nasal and IP Oxt injection. **a–d** Microphotographs of c-Fos-ir neurons in the PVN (**a**, **b**) and dorsal vagal complex (**c**, **d**) after nasal administration of saline (**a**, **c**) or 1 μg Oxt (**b**, **d**). **e** Number of c-Fos-ir neurons after nasal administration of Oxt in feeding-related areas in the hypothalamus and brain stem (n = 4). **f–i** Microphotographs of c-Fos-ir neurons in PVN (**f**, **g**) and dorsal vagal complex (**h**, **i**) after IP injection of saline (**f**, **h**) or 400 μg/kg Oxt (**g**, **i**). **j** Number of c-Fos-ir neurons after IP injection of Oxt in feeding-related areas (n = 4). Scale bars = 100 μm. Differences were assessed for significance by Student's t test. * $p < 0.05$, ** $p < 0.01$. SCN = Suprachiasmatic nucleus; SON = supraoptic nucleus.

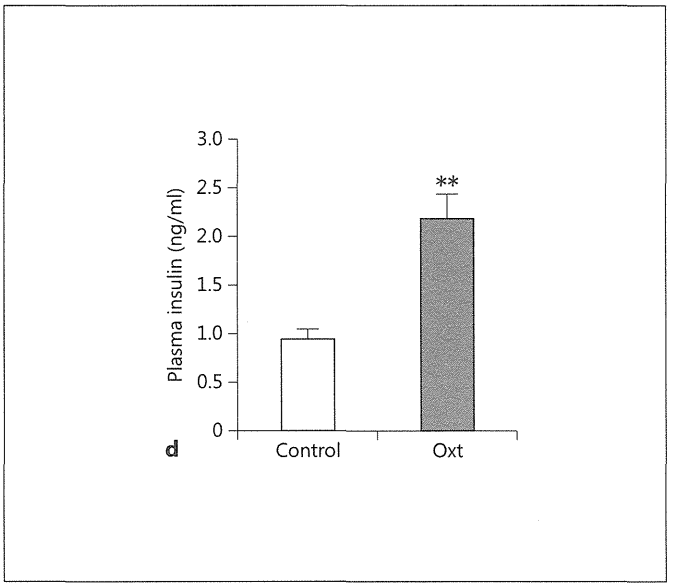
proximately 150 ng in the mouse pituitary [24]. Hence, it is suggested that a relatively high dose of nasal Oxt is required to induce c-Fos expression. In addition, the present study used a higher dose of Oxt (approx. 10 μg nasal, approx. 400 μg/kg IP) than that used in a human study (approx. 48 μg nasal) [17]. The different dose requirement between humans and rodents is possibly due to different anatomical structures and/or to the different approach for nasal Oxt administration between humans (vaporizer or nasal spray) and mice (dropping the Oxt-containing solution on the nose by a pipette).

In this study, Oxt administration via the nasal versus the IP route increased the number of c-Fos-ir neurons in both common and distinct brain areas. Both nasal and IP administration of Oxt increased c-Fos-ir neurons in the PVN, AP, and DMNV. The increase in the PVN may underlie the common action of nasal and IP Oxt administration to suppress food intake, since PVN is recognized as



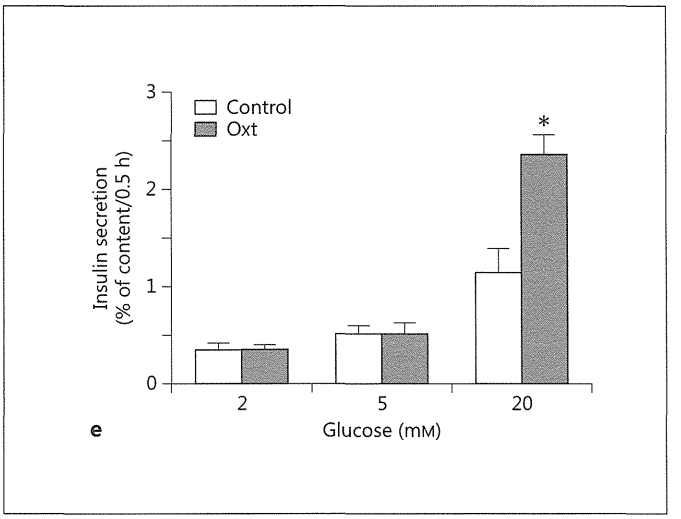




**Fig. 4.** Effect of Oxt on blood glucose levels in glucose tolerance test and insulin levels. **a–c** Blood glucose profiles in IPGTT after Oxt administration via nasal (0.1, 1, and 10 µg; n = 6–11; **a**), IP (40 and 400 µg/kg; n = 5; **b**) and icv administration (0.4 and 4 µg; n = 5–8; **c**). Differences were assessed by repeated measures two-way ANOVA with Oxt treatment and time as factors. For each time point, post hoc multiple comparisons were made using Tukey's test. * $p < 0.05$, ** $p < 0.01$ versus control. **d** Plasma insulin levels after IP administration of Oxt. ** $p < 0.01$. **e** Insulin secretion from isolated pancreatic islets under static incubation with low (2 mM), basal (5 mM), and stimulatory (20 mM) glucose concentrations. Open bars indicate the control group (n = 11) and solid bars indicate the Oxt-treated group (n = 11). Differences were assessed for significance by Student's t test. * $p < 0.05$.

an integrative center for feeding and energy metabolism [25, 26]. The neurons in the ARC of the hypothalamus sense various nutrients and hormones and convey neural information to the neurons in PVN, where peripheral and central information is integrated [27]. In contrast, only IP Oxt increased c-Fos-ir neurons in the ARC and NTS. Several pathways may be considered for the common and distinct brain signalling by nasal and IP Oxt. First, given that the cribriform plate is the only structure separating the nasal cavity from the olfactory bulb in rodents, nasal Oxt administration may activate the Oxt-R present in the olfactory bulb [28, 29]. Second is the route mediated by the AP. c-Fos-ir neurons were increased in the AP after both IP and nasal application of Oxt. The AP is known to have a leaky BBB, and the neurons in the AP project to NTS neurons, which in turn project to the ARC and PVN [30]. Hence, it is possible that nasal and IP injections of Oxt exert their effects on feeding via activating neurons in AP. However, in this study Oxt increased c-Fos-ir neurons in the NTS only after IP injection and not after nasal administration. This may indicate that neural signal transduction through the AP to NTS projection after nasal application of Oxt was not intensive enough to induce c-Fos-ir neurons in the NTS. Third is the route via vagal afferents. In this study, only IP administration of Oxt increased the number of c-Fos-ir neurons in the NTS, where the vagal afferent neurons terminate [31]. Oxt-R is widely expressed in the peripheral tissues including the nodose ganglion [32]. Hence, it is possible that IP-administered Oxt interacts with Oxt-R on the vagal afferent nodose ganglion neurons to signal to the NTS.

Regarding the reduced locomotor activity found only with IP administration of Oxt, the underlying mechanisms could be due to the passage of Oxt from the blood to the brain, thereby activating responsible brain regions, or to the consequences of the peripheral actions of very high levels of Oxt following IP administration. It is important to note that a high peripheral Oxt concentration can also activate vasopressin receptors that have been reported to regulate locomotor activity [33]. Further studies are required to elucidate the mechanism underlying the effect of IP Oxt administration on locomotor activities.

In the present study, nasal and IP administration of Oxt increased the number of c-Fos-ir neurons in the PVN of male mice, suggesting a possible peripheral-central Oxt axis. The result is in agreement with a recent report that the IP administration of Oxt activates PVN Oxt neurons in male rats [34]. Negative feedback effects of Oxt on Oxt neurons were reported under particular conditions in virgin and pregnant female rats, in contrast to positive feedback effects in male and lactating female rats [35–37]. These reports suggest that Oxt activates Oxt neurons in male rodents. Hence, our results could reflect the direct effects of exogenously administered Oxt on PVN Oxt neurons or indirect effects mediated by the nasal/IP Oxt-activated neurons that project to the PVN Oxt neurons.

We have previously reported that chronic Oxt treatment ameliorates the impaired glucose tolerance in high-fat diet-induced obese mice [15]. Since chronic Oxt treatment also ameliorated obesity, the improvement of glucose tolerance by Oxt could be due to its direct effect on glucose metabolism or secondary to the amelioration of obesity. In the present study, IP administration of 40 μg/kg Oxt induced a small effect on food intake but markedly decreased blood glucose levels at 30 min of IPGTT. Furthermore, Oxt elevated plasma insulin level in vivo and enhanced glucose-induced insulin secretion in isolated islets in vitro. These results taken together suggest that IP-administered Oxt substantially reaches the pancreas and directly interacts with islet β-cells to enhance glucose-induced insulin release, thereby improving glucose tolerance. Insulin, released postprandially [38], might enter the brain and inhibit food intake [27]. It was also reported that insulin activates vagal afferent neurons, which could be linked to the regulation of food intake [39]. Therefore, it is possible that insulin secretion induced by IP Oxt may partly contribute to the anorexigenic effect of IP Oxt.

In our study, nasal Oxt administration failed to affect glucose tolerance in mice. In dogs, in contrast, nasal Oxt at a similar dose (3–4 μg/kg) was reported to increase plasma insulin and glucagon levels [40]. Also, in humans, nasal administration of Oxt (24 IU) attenuated the elevated blood glucose following the test buffet [17]. The apparent discrepancy in the effect of nasal Oxt on glucose homeostasis could be due to species differences or methodology of treatment as stated above. The nasally administered Oxt might be more easily transferred to the periphery in dogs and humans than in mice.

In order to assess the direct cerebral effect of Oxt on glucose metabolism, we performed the IPGTT after icv injection of Oxt. icv injection of Oxt (0.4, 4 μg/mice) had no effect on glucose metabolism. Although regulation of glucose metabolism through the brain is established, whether this process may be modulated by Oxt is not clear [41, 42]. Our results suggest that Oxt in the brain has little effect on glucose metabolism under the experimental conditions studied here.

When considering the physiological aspect of Oxt on females, it shows different effects on food intake under different conditions. In the preparturition period, Oxt secretion is very low in order to reduce the risk of preterm delivery and to prevent the loss of the accumulating neurophysial Oxt store [43], which is accompanied by an increase in food intake in both mice and rats [42, 44]. During the parturition and lactation periods, in spite of the extensive activation of Oxt neurons, food intake is dramatically elevated [44]. It is suggested that the involvement of Oxt in feeding behavior is unique in these periods when the energy intake and expenditure are in different states from those in male or virgin animals.

Recent reports have shown that Oxt treatment reduces reward-driven food intake [17]. It is well known that the reward process, including feeding, sexual [45] and social [46] rewards, is related to the mesocorticolimbic dopamine system in the brain [47, 48]. Oxt appears to impact this system [47], in which dopamine is one of the neurotransmitters playing a major role in addiction. It has recently been reported that Oxt modulates the negative aspect of addiction [49] and that Oxt treatment can attenuate the addictive cocaine-seeking behavior in humans [50]. Hyperphagia is considered an addictive behavior for food. The common mechanisms for hyperphagia and addiction have been reported [51]. Therefore, Oxt as a neuropeptide may serve to keep the reward system at a moderate level.

In conclusion, nasal and IP administration of Oxt have similar anorexigenic effects. However, nasal administration of Oxt may have a lesser effect on the peripheral tissue, including pancreatic β-cells, compared to IP administration. Hence, nasal administration may have the advantage of activating specific brain regions without affecting glucose tolerance and locomotion.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## Disclosure Statement

The authors have no conflicts of interest to disclose.

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Original article

# Microarray analysis of 50 patients reveals the critical chromosomal regions responsible for 1p36 deletion syndrome-related complications

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**Abstract**

*Objective:* Monosomy 1p36 syndrome is the most commonly observed subtelomeric deletion syndrome. Patients with this syndrome typically have common clinical features, such as intellectual disability, epilepsy, and characteristic craniofacial features.

*Method:* In cooperation with academic societies, we analyzed the genomic copy number aberrations using chromosomal microarray testing. Finally, the genotype–phenotype correlation among them was examined.

*Results:* We obtained clinical information of 86 patients who had been diagnosed with chromosomal deletions in the 1p36 region. Among them, blood samples were obtained from 50 patients (15 males and 35 females). The precise deletion regions were successfully genotyped. There were variable deletion patterns: pure terminal deletions in 38 patients (76%), including three cases of mosaicism; unbalanced translocations in seven (14%); and interstitial deletions in five (10%). Craniofacial/skeletal features, neurodevelopmental impairments, and cardiac anomalies were commonly observed in patients, with correlation to deletion sizes.

*Conclusion:* The genotype–phenotype correlation analysis narrowed the region responsible for distinctive craniofacial features and intellectual disability into 1.8–2.1 and 1.8–2.2 Mb region, respectively. Patients with deletions larger than 6.2 Mb showed no ambulation, indicating that severe neurodevelopmental prognosis may be modified by haploinsufficiencies of *KCNAB2* and *CHD5*, located at 6.2 Mb away from the telomere. Although the genotype–phenotype correlation for the cardiac abnormalities is unclear, *PRDM16*, *PRKCZ*, and *RERE* may be related to this complication. Our study also revealed that female patients who acquired ambulatory ability were likely to be at risk for obesity.


*Keywords:* 1p36 deletion syndrome; Chromosomal deletion; Genotype–phenotype correlation; Intellectual disability; Ambulation; Epilepsy; Distinctive features

## 1. Introduction

Monosomy 1p36 syndrome is a congenital malformation syndrome caused by the subtelomeric deletion of the short arm of chromosome 1 [1–3]. This syndrome is the most commonly observed subtelomere deletion syndrome, with an estimated incidence of 1:5000–1:10,000 [4,5]. Patients with this syndrome exhibit common clinical features, including intellectual disability (ID) and characteristic craniofacial features; such as straight eyebrows, deep-set eyes, epicanthus, and a pointed chin [6–9]. Although the levels of ID vary among patients, craniofacial features are commonly seen [10]. The patients with the 1p36 deletion syndrome also show many other complications, including hypotonia, seizures, hearing loss, structural heart defects, cardiomyopathy, ophthalmological abnormalities, and behavior abnormalities [7]. Recent advances in microarray-based chromosomal testing have helped us to identify small chromosomal rearrangements that are invisible by conventional G-banded chromosomal tests/karyotyping [11,12]. Using this method, the precise locations of the aberrations can be revealed at the molecular level. These advances have also allowed the study of more in-depth genotype–phenotype correlations for this syndrome, as well as the identification of some of the regions responsible for individual complications [12,13].

In this study, we performed a nation-wide survey for the 1p36 deletion syndrome in Japan. The aim of this study was to identify the chromosomal regions responsible for individual complications in patients with 1p36 deletions. We analyzed the affected genomic regions in 50 patients with 1p36 deletions, and performed correlational analyses of the genotype data with the clinical information.

## 2. Materials and methods

### 2.1. Patients and samples

We performed a nation-wide survey for the 1p36 deletion syndrome with the cooperation of two academic societies; the Japanese Society of Child Neurology and the Japan Society of Pediatric Genetics. The study subjects were Japanese patients who had already been diagnosed using various diagnostic methods, including conventional karyotyping, subtelomere fluorescence *in situ* hybridization (FISH) analysis, multiplex ligation-dependent probe amplification (MLPA), and chromosomal microarray testing. Five patients

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(patient [Pt] 8, 9, 21, 28, and 43), whose clinical features have been previously reported [14–17], were also included in this study. With the questionnaire survey for attending physicians, we accumulated the patients' clinical information, including craniofacial/skeletal features, neurodevelopmental features, brain structural abnormalities, cardiac abnormalities, sensory-organs abnormalities, urogenital abnormalities, endocrinological and nutritional findings among others. This study was approved by the ethics committee in Tokyo Women's Medical University.

On receipt of written informed consents from the families of the patients, we obtained the patients' blood samples to determine genomic copy number losses in the patients. Genomic DNA was extracted from the blood samples using the QIA quick DNA Extraction Kit (QIAGEN, Hamburg, Germany). Metaphase spreads were also prepared from blood samples and used for FISH analyses. In cases, if we could obtain written informed consent, parental samples were also analyzed.

### 2.2. Molecular and cytogenetic analyses

Chromosomal microarray testing was performed using any of the Agilent Oligo Microarray Kits 44, 60, 105, 180, and 244 K (Agilent Technologies, Santa Clara, CA), as described previously [18,19]. Genomic copy number aberrations were visualized using Agilent Genomic Workbench version 6.5 (Agilent Technologies). For cases in which variations of unknown significance were identified or suspected, parental samples were also analyzed. In cases of complex chromosomal rearrangements or mosaicism, metaphase spreads prepared using the patients' samples were used for FISH analyses for confirmation. The bacterial artificial clones were selected from the UCSC genome browser (http://genome.ucsc.edu/) for use as probes. For the target probes, RP11-425E15 (1p36.33: 949,400–1,132,489), RP11-82D16 (1p36.33: 2,046,751–2,208,312), RP11-70N12 (1p36.32: 2,740,703–2,922,551), CTD-3209F18 (1p36.32: 3,530,092–3,769,006), and RP11-933B18 (1p36.31: 5,988,719–6,177,261) were selected, while CTB-167K11 (1q44: 249,250,621–249,250,621) was used as a marker of chromosome 1. All of the genomic regions are described according to the February 2009 human reference sequence (GRCh37/hg19) in this study.

## 3. Results

### 3.1. Molecular-cytogenetic findings

We obtained clinical information from 86 patients with chromosomal deletions involving 1p36 regions. Among them, 50 patients (15 males and 35 females) were successfully genotyped. All of the genotypes were summarized in Tables 1 and 2, and 1p36 deletions identified in the patients were depicted in the genome map (Fig. 2). The minimum and maximum deletion sizes was 0.9 and 12.9 Mb, respectively. Pure terminal deletions were identified in 38 patients (76%). Among them, three patients (Pt 8, 19, and 21) exhibited mosaicism. Pt 8 was first diagnosed with mosaic 1p36 deletion by chromosomal microarray testing, and Pt 21 had been diagnosed with 1p36 deletion using subtelomere FISH analysis; however, mosaicism was not reported at that time [17]. Although the mosaic deletion of 1p36 in Pt 19 had been firstly confirmed by FISH, we could not detect the breakpoint by chromosomal microarray testing due to low frequency (28% mosaic ratio). As the breakpoint was determined to be between two FISH probes (CTD-3209F18 and RP11-933B18), the proximal end of CTD-3209F18 was used as the minimum deletion region in this patient.

Additional aberrations with the sizes over 0.5 Mb were identified in eight patients (Pt 2, 10, 11, 15, 20, 28, 34, and 43) involving chromosomes 4, 7, 8, 13, and Y (Table 2), including a possible benign copy number aberration in Pt 15, which was also observed in the healthy mother. The other seven patients were confirmed to have unbalanced translocations by cytogenetic evaluation (14%), using either G-banding or FISH analysis. Two translocations were diagnosed as de novo, and the others were designated as unknown because of the lack of availability of parental information.

Five patients (Pt 1, 14, 47, 48, and 50) had interstitial deletions (10%) with a deletion size between 0.9 and 10.3 Mb.

### 3.2. Clinical findings

Clinical information of the 50 patients successfully genotyped is summarized in Table 3. Estimated frequencies of each complication are also included in Table 3. Pt 26 and 49 suddenly died at 24 and 10 months old of age, respectively. Pt 49 probably died due to heart failure but Pt 26 died of an unknown cause (detailed information unavailable).

#### 3.2.1. Craniofacial features

Most of the patients showed craniofacial features, including straight eyebrows (84%), deep-set eyes (93%), broad nasal bridge (97%), low set ears (88%), and a pointed chin (89%). Constellations of these findings make distinctive facial impressions for 1p36 deletion syndrome, observed in Pt 3, 6, and 14 (Fig. 1b–d). This observation suggests that hypotelorism is rather characteristic among these patients. On the other hand, Pt 1 did not show deep-set eyes (Fig. 1a). The craniofacial features of three patients (Pt 47, 48, and 50) did not exhibit hypotelorism (Fig. 2e–g). From the genotypic point of view, these three patients (Pt 47, 48, and 50) would be diagnosed as having the proximal 1p36 deletion syndrome [20,21].

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Table 1
The ranges of 1p36 deletions analyzed by chromosomal microarray testing.

| Patient number | Age (year) | Gender | Platform (k) | Start[a] | End[a] | Additional aberration | FISH probe | Mosaic ratio[b] (%) | References |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 14 | F | 180 | 834,101 | 1,770,669 | Interstitial | RP11–425E15 | | |
| 2 | 9 | M | 44 | 1 | 1,820,584 | der(1)t(Y;1), idic(Y) | | | |
| 3 | 6 | F | 180 | 1 | 2,186,829 | | | | |
| 4 | 1 | F | 60 | 1 | 2,239,497 | | | | |
| 5 | 3 | F | 44 | 1 | 2,281,699 | | | | |
| 6 | 5 | F | 60 | 1 | 2,553,982 | | | | |
| 7 | 2 | M | 60 | 1 | 2,553,982 | | | | |
| 8 | 5 | F | 44 | 1 | 3,044,953 | Mosaicism | RP11–82D16 | 70 | Shimada et al. [17] |
| 9 | 13 | F | 44 | 1 | 3,102,718 | | | | Okamoto et al. [14] |
| 10 | 18 | F | 44 | 1 | 3,102,718 | der(1)t(1;7) | | | |
| 11 | 17 | F | 60 | 1 | 3,138,565 | der(1)t(1;8) | | | |
| 12 | 8 | F | 60 | 1 | 3,265,702 | | | | |
| 13 | 11 | F | 244 | 1 | 3,408,152 | | | | |
| 14 | 5 | M | 60 | 1,786,789 | 3,472,907 | Interstitial | | | |
| 15 | 2 | F | 180 | 1 | 3,564,328 | | | | |
| 16 | 13 | M | 60 | 1 | 3,582,084 | | | | |
| 17 | 4 | F | 44 | 1 | 3,607,275 | | | | |
| 18 | 2 | F | 60 | 1 | 3,660,110 | | | | |
| 19 | 3 | F | 60 | 1 | 3,769,006 | Mosaicism | CTD-3209F18 | 28 | |
| 20 | 3 | M | 44 | 1 | 4,070,842 | der(1)t(1;13) | | | |
| 21 | 17 | F | 44 | 1 | 4,481,324 | Mosaicism | RP11–82D16 | 77 | Shimada et al. [17] |
| 22 | 2 | F | 180 | 1 | 4,703,581 | | | | |
| 23 | 6 | M | 60 | 1 | 4,779,157 | | | | |
| 24 | 3 | F | 60 | 1 | 4,843,370 | | | | |
| 25 | 6 | F | 44 | 1 | 4,843,718 | | | | |
| 26 | 2 | M | 60 | 1 | 5,252,985 | | | | |
| 27 | 0 | F | 44 | 1 | 5,252,985 | | | | |
| 28 | 25 | F | 44 | 1 | 5,411,803 | der(1)t(Y;1) | | | Hiraki et al. [15] |
| 29 | 3 | F | 44 | 1 | 6,128,223 | | | | |
| 30 | 3 | F | 60 | 1 | 6,282,562 | | | | |
| 31 | 1 | F | 60 | 1 | 6,282,562 | | | | |
| 32 | 3 | M | 60 | 1 | 6,882,431 | | | | |
| 33 | 7 | M | 60 | 1 | 7,035,075 | | | | |
| 34 | 1 | F | 60 | 1 | 7,187,535 | der(1)t(1;4) | | | |
| 35 | 10 | M | 60 | 1 | 7,392,688 | | | | |
| 36 | 8 | M | 60 | 1 | 7,581,058 | | | | |
| 37 | 3 | F | 44 | 1 | 8,077,959 | | | | |
| 38 | 2 | F | 60 | 1 | 8,104,671 | | | | |
| 39 | 3 | M | 44 | 1 | 8,104,671 | | | | |
| 40 | 4 | M | 44 | 1 | 8,181,042 | | | | |
| 41 | 5 | F | 44 | 1 | 8,181,042 | | | | |
| 42 | 1 | F | 60 | 1 | 8,427,633 | | | | |
| 43 | 3 | M | 60 | 1 | 9,180,975 | der(1)(1;4) | | | Saito et al. [16] |
| 44 | 5 | F | 60 | 1 | 9,251,936 | | | | |
| 45 | 4 | M | 60 | 1 | 9,953,030 | | | | |

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| | | | | | | |
|---|---|---|---|---|---|---|
| 46 | 2 | F | 44 | 1 | 10,001,011 | |
| 47 | 4 | F | 44 | 2,080,309 | 10,869,155 | Interstitial |
| 48 | 8 | F | 60 | 2,785,042 | 12,743,178 | Interstitial |
| 49 | 0 | F | 44 | 1 | 12,917,483 | |
| 50 | 22 | F | 180 | 6,614,950 | 16,890,814 | Interstitial |

[a] The genomic position reffering build19.
[b] The mosaic ratio was confirmed by FISH; F, female; M, male.

### 3.2.2. Neurological features

Almost all patients showed ID (98%) but a patient (Pt 2) having a deletion in the far distal region of 1p36 showed borderline ID, with an intelligence quotient (IQ) of 80. Therefore, this region could be eliminated from the responsible region for ID (Fig. 2). The smallest deletion, an interstitial deletion between genomic positions 0.8 and 1.8 Mb, was identified in Pt 1 (Fig. 2). In spite of having this smallest deletion, Pt 1 had severe ID, i.e., she was locomotive but aphasic and required support for all activities in her daily life. This was probably a consequence of intractable epilepsy associated with tonic seizures, caused by factors other than the interstitial deletion of this region. The proximal and distal ends of the breakpoints in Pt 3 and 14 defined the shortest region of overlap for ID, spanning the 1.8–2.2 Mb region (Fig. 2; region B). Axial hypotonia (92%) and poor sucking (70%) were also commonly observed. Epilepsy, one of the major complications in 1p36 deletion syndrome, was observed in 70% of the patients. Infantile spasms were observed in 16% of the patients.

In this study, many types of structural brain abnormalities were identified; not only in the cerebral cortex but also in the white matter (Table 3), indicating that there is no major pattern. The most frequently observed abnormality was a nonspecific finding with enlargement of lateral ventricles.

### 3.2.3. Cardiac abnormality

Cardiac abnormality is one of the most frequently observed complications in patients with 1p36 deletions. In this study, congenital heart defects and functional abnormalities were observed in 69% (34/49) and 22% (11/49) of the patients, respectively. The most frequently observed patterns were patent ductus arteriosus (PDA; 37% [18/49]) and ventricular septal defects (VSD; 37% [18/49]).

### 3.2.4. Other complications

Many kinds of complications were observed in many organs. Cryptorchidism was the most frequently observed complication in male patients (64% [9/14]). As Pt 14, with a small interstitial deletion spanning from 1.8 to 3.5 Mb, had cryptorchidism, the deleted region was likely involved in abnormalities of the external genitalia (Fig. 2; region H). Hearing problems (39% [19/49]) and strabismus (33% [15/46]) were relatively common among the patients. Obesity was observed in 5 patients (11% [5/46]).

Renal abnormalities were rare and identified only in three patients. Among them, Pt 26, who had a 5.3 Mb deletion, was diagnosed with the autosomal recessive cystic kidney disease of nephronophthisis (this patient died at 2 years of age) [22]. One of the genes responsible for this condition, the nephronophthisis 4 gene

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Table 2
Additional aberrations identified in the patients.

| Patient number | Chr | Start[a] | End[a] | Remark | Attribute | Origin |
|---|---|---|---|---|---|---|
| 2 | Y | 1 | 59,373,566 | der(1)t(Y;1)(p36.3;q12), idic(Y)(q12) | dup | NA |
| 10 | 7 | 1 | 6,870,943 | der(1)t(1;7)(p36.32;p22.1) | dup | NA |
| 11 | 8 | 1 | 3,909,039 | der(1)t(1;8)(p36.22;p23.2) | dup | NA |
| 15 | 1 | 146,324,068 | 149,192,104 | del(1)(q21.1;q21.2) | del | Common with mother |
| 20 | 13 | 100,462,233 | 115,169,878 | der(1)t(1;13)(p26.32;q32.3) | dup | De novo |
| 28 | Y | 26,435,039 | 59,373,566 | der(1)t(Y;1)(q12;p36.32)[#] | dup | NA |
| 34 | 4 | 1 | 13,396,747 | der(1)t(1;4)(p36.31;p15.33) | dup | De novo |
| 43 | 4 | 189,012,426 | 191,154,276 | der(1)t(1;4)(p36.31;q35.2) | dup | NA |

[a] The genomic position reffering build19; dup, duplication; del, deletion; NA, not available.
[#] This case was previously reported by Hiraki et al. [15].

(*NPHP4*), is located on 1p36 (chr1: 5,946,555–5,965,543) [23], proximal to the deletion region of three patients with renal abnormalities (Pt 26, 33, and 35). It is unclear whether there is a correlation between *NPHP4* and the renal abnormalities observed in this study.

## 4. Discussion

### 4.1. Previous genetic studies on the 1p36 deletion syndrome

Many cohort studies have been performed to delineate the phenotypic features of patients with 1p36 deletion syndrome and to evaluate the frequency of complications [1,6,7]. It has been reported that there is no correlation between the deletion size and the number of observed clinical features [24], while the critical region responsible for core phenotypic features, including clefting, hypothyroidism, cardiomyopathy, hearing loss, large fontanel, and hypotonia, has been narrowed down to a region 2.2 Mb from the telomere [3]. Compared to such core phenotypic features, other complications tend to vary with the size of the deletion, and study subjects with larger deletions tend to have more phenotypic features [25], suggesting that the various phenotypic features are dependent on genes involved in the deletion regions. Thus, precise knowledge of the genotype–phenotype correlations could potentially lead to more personalized treatments for individuals with 1p36 deletions and might identify mutations for single gene disorders [3]. The potassium voltage-gated channel, shaker-related subfamily, beta member 2 gene (*KCNAB2*) and the v-ski sarcoma viral oncogene homolog gene (*SKI*) were identified as candidate genes for the epilepsy phenotype and clefting abnormalities, respectively [26,27]. More recently, the PR domain containing 16 gene (*PRDM16*) was identified as a possible candidate gene for cardiomyopathy, as *PRDM16* was included in a minimal deletion among patients with 1p36 deletions associated with cardiomyopathy, while in patients with pure cardiomyopathy, single nucleotide variants of *PRDM16* were identified as the cause of cardiomyopathy [28]. This was one of the most successful studies of genotype–phenotype correlation in patients with 1p36 deletions [28].

### 4.2. Craniofacial features

As mentioned above, a region 2.2 Mb from the telomere has been reported to be responsible for core phenotypic features of 1p36 deletion syndrome [3]. Compared to this, we observed atypical facial features in four patients (Pt 1, 47, 48, and 50) whose deletions did not include the 1.8–2.1 Mb region, in this study. Thus, the region responsible for typical facial features is narrowed into this region (Fig. 2; region A). Because hypotelorism has never been listed in the clinical delineations of 1p36 deletion syndrome reported from Western countries, we did not include this finding in the questionnaire survey and the frequency of this finding in Japanese patients could not be calculated. However, it is commonly observed in Japanese patients with typical 1p36 deletion syndrome. Therefore, hypotelorism may be a characteristic finding among Asian patients.

### 4.3. Neurological features

Although more severe ID was reported to be associated with larger 1p36 deletions [10], the genomic region responsible for severe ID has never been identified. In this study, a patient (Pt 28) having a 5.4 Mb deletion acquired independent gait, while patients with >6.1 Mb deletions had not yet acquired independent gait, and exhibited severe ID. Thus, the region between 5.4 and 6.1 Mb would appear to be the borderline for independent gait (Fig. 2; region C), and the modifier genes for prognosis of development may be located in the region proximal to this borderline. *KCNAB2*, mentioned above, may be one of the modifier genes responsible for severe ID. Chromodomain helicase DNA-binding protein 5 (*CHD5*; chr1: 6,161,847–6,240,194), which encodes a neuron-specific protein, is

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