change despite food deprivation after PCPA pretreatment, it can be referred that the neuropeptides may have possible interactions with 5-HT.

Several studies have demonstrated the correlations between 5-HT and hypothalamic feeding-regulating neuropeptides [8, 9, 16–18], although which kind of feeding-regulating peptides are affected by the serotonergic system is unclear. Here, we comprehensively examined the interactions between the serotonergic system and feeding-regulating neuropeptides in the hypothalamus after 48 h food deprivation with or without PCPA pretreatment, using in situ hybridization histochemistry (IHC) in rats.

Materials and methods

Animals

Adult male Wistar rats (180–190 g body weight) were individually housed and maintained in temperature controlled (23–25 °C) conditions under a 12.12 h light/dark cycle (lights on at 0700 hours). All experiments were performed in strict accordance with the guidelines on the use and care of laboratory animals issued by the Physiological Society of Japan, and were approved by the Ethics Committee of Animal Care and Experimentation of University of Occupational and Environmental Health.

Test substance

PCPA (Sigma-Aldrich Japan, Tokyo, Japan) was dissolved in 0.9 % sterile physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) (0.6 mg/1 mL).

Determination of hypothalamic monoamine concentrations

Hypothalamic concentrations of noradrenaline (NA), dopamine (DA), and 5-HT were measured by high-performance liquid chromatography with electrochemical detection (HPLC-ECD). PCPA or saline were administered by intraperitoneally (i.p.) injection daily for 2 days. The rats were decapitated 48 h after second saline ($n = 8$) or PCPA ($n = 8$) treatment followed by removal of the hypothalamus. Briefly, samples were frozen onto dry-ice and stored at −80 °C before the measurements. Samples were homogenized in 0.2 M perchloric acid and centrifuged (8,000g) at 4 °C for 30 min. The supernatant was collected and analyzed with HPLC-ECD system (Hitachi, Japan). HPLC-ECD conditions were a modified method of Wetherell et al. [19]. Briefly, separations were performed using a 4.6 × 150 mm ODS C_{18} column. The mobile

phase consisted of 0.1 M Na_2PO_4, 0.8 mM OSA, 0.5 M EDTA, and 10 % methanol, and was adjusted to pH 3.63 with phosphoric acid. Column temperature was 40 °C, flow rate 1.0 mL/min, and the detector was set at a potential of +0.75 V relative to an Ag/AgCl reference electrode. The working standard solution was prepared in 0.2 M perchloric acid containing 0.5 mM EDTA and 0.05 mg/mL DHBA was stored at −80 °C.

Experimental procedure

All the rats had ad libitum access to water throughout the experiments. The rats were divided into four groups: saline + ad libitum access to food (SAF, $n = 13$), saline + food deprivation for 48 h (SFD, $n = 13$), PCPA + ad libitum access to food (PAF, $n = 14$), and PCPA + food deprivation for 48 h (PFD, $n = 14$). Saline (10 mL/kg body weight as a single daily dose) or PCPA (200 mg/10 mL/kg body weight as a single daily dose) was administered i.p. at day 0 and day 1 (0900–1000 hours). After the administration of saline or PCPA at day 1, food was deprived in SFD and PFD. Body weights and food intake in all the experimental groups were measured every 24 h from day 1 to day 6.

On day 3, some of those rats (SAF, $n = 7$, SFD, $n = 7$, PAF, $n = 8$, PFD, $n = 8$) were decapitated immediately after the treatment without being anesthetized, followed by prompt removal of the brain onto dry ice, then storing at −80 °C. Trunk blood samples were taken during decapitation, and were collected into chilled reaction tubes (Greiner Bio-One) containing an aprotinin/EDTA mixture. The blood samples were Immediately,centrifuged for 10 min at 4 °C, 1,000g, after which, a 15-μL sample of plasma was taken for measuring plasma osmolality (P-Osm) using a ONE-TEN osmometer (FISKE, Norwood, MA, USA), 500 μL for plasma 5-HT (SRL, Tokyo, Japan), 500 μL for plasma leptin (SRL), 1,000 μL for plasma active and desacyl ghrelin (SRL), and 10 μL for measuring plasma glucose (PG) using a Medisafe Reader GR-101 (Terumo, Tokyo, Japan). Finally, 100 μL of 1 M HCl was added to each tube for measuring active and desacyl ghrelin in order to protect against decomposition.

In situ hybridization histochemistry

The removed brains were cut into 12-μm slices and thaw-mounted on gelatin/chrome alum-coated slides. The locations of the hypothalamic areas, including the supraoptic nucleus (SON), paraventricular nucleus (PVN), arcuate nucleus (ARC), and lateral hypothalamic area (LHA), were determined according to coordinates of the rat brain atlas.

[35]S 3'- end-labeled deoxyoligonucleotide complementary to transcripts encoding *oxytocin*, *CRH*, *TRH*, *pro-opiomelanocortin* (*POMC*), *cocaine and amphetamine-regulated transcript* (*CART*), *NPY*, *agouti-related protein* (*AgRP*), *MCH*, and *orexin* were used (*oxytocin* probe sequence, 5'-CTC GGA GAA GGC AGA CTC AGG GTC GCA GGC-3'; *CRH* probe sequence, 5'-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3'; *TRH* probe sequence, 5'-GTC TTT TTC CTC CTC CTC CCT TTT GCC TGG ATG CTG CGC TTT TGT GAT-3'; *POMC* probe sequence, 5'-TGG CTG CTC TCC AGG CAC CAG CTC CAC ACA TCT ATG GAG G-3'; *CART* probe sequence, 5'-TCC TTC TCG TGG GAC GCA TCA TCC ACG GCA GAG TAG ATG TCC AGG-3'; *NPY* probe sequence, 5'-CAA ATG GAT GAT TGG TCA TTT CAA CAT AGA GTT GGG GGC TTG CT-3'; *AgRP* probe sequence, 5'-CGA CGC GGA GAA CGA GAC TCG CGG TTC TGT GGA TCT AGC ACC TCT GCC-3'; *MCH* probe sequence, 5'-CCA ACA GGG TCG GTA GAC TCG TCC CAG CAT-3'; and *orexin* probe sequence, 5'-TCC TCA TAG TCT GGA GGC AGG TGG AAG GGT TCC CCA CTG CTA GTG-3') The specificity of these probes were confirmed by previous studies [20, 21].

The probe was 3'-end-labeled using terminal deoxynucleotidyl transferase and [35S] dATP. The in situ hybridization protocol has been previously described in detail [22]. Briefly, sections were fixed in 4 % (w/v) formaldehyde for 5 min and incubated in saline containing 0.25 % (v/v) acetic anhydride and 0.1 M triethanolamine for 10 min and then dehydrated, delipidated in chloroform, and partially rehydrated. Hybridization was carried out overnight at 37 °C in 45 μL of hybridization buffer under a Nescofilm (Bando Kagaku, Osaka, Japan) cover slip. A total count of 1×10^5 c.p.m. for the *oxytocin* transcripts and 1×10^6 c.p.m. for the *CRH*, *TRH*, *POMC*, *CART*, *NPY*, *AgRP*, *MCH* and *orexin* transcripts were used per slide. After hybridization, the sections were washed 4 times with SSC (150 mM NaCl and 15 mM sodium citrate) for 1 h at 55 °C and for an additional hour with two changes of SSC at room temperature. The hybridized sections containing hypothalamus were exposed for autoradiography (Hyperfilm; Amersham, Bucks, UK) for 6 h for the *oxytocin* probe, 5 days for the *MCH* and *orexin* probe, and 1 week for the *CRH*, *TRH*, *POMC*, *CART*, *NPY*, and *AgRP* probe. The resulting images were analyzed by computerized densitometry using a MCID imaging analyzer (Imaging Research, Ontario, Canada). The mean optical densities (OD) of the autoradiographs were measured by comparison with simultaneously exposed ^{14}C-labeled microscale samples (Amersham) and represented in arbitrary units setting the mean OD obtained from control rats.

Statistical analysis

The mean ± SEM was calculated from the results of the change in body weight, cumulative food intake, plasma measurements, and ISH studies. In the results of ISH, the expression levels of the genes were expressed as a percentage of PAF. All data were analyzed by one-way ANOVA followed by a Bonferroni-type adjustment for multiple comparisons (Origin Pro v.8.5 J; Lightstone, Tokyo, Japan). Statistical significance was set at $P < 0.05$.

Results

Hypothalamic monoamine concentrations after PCPA treatment

Hypothalamic NA, DA, and 5-HT were measured using HPLC (Fig. 1). No statistically differences were observed in hypothalamic NA and DA; however, hypothalamic 5-HT levels nearly depleted in PCPA-treated group compared to saline-treated group (Fig. 1).

Changes in body weight

The body weight of each group was measured from day 1 to day 6 (Fig. 2). The body weight gradually increased during the experiments in SAF (Fig. 2). The body weight in PAF gradually increased after day 1, but was statistically different compared to SAF after day 1. The body weight decreased after PCPA administration (day 1) in PFD and PAF compared to SAF (Fig. 2). A decrease in body weight was observed in SFD and PFD after starting food
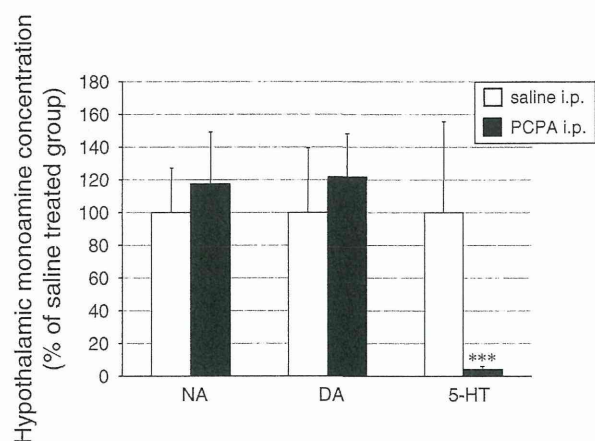

Fig. 1 Hypothalamic monoamine concentration after i.p. administration of saline or PCPA. These data were measured by HPLC. The amount of each monoamine level represents % from saline group. Data are presented as mean ± SEM. ***$P < 0.001$ vs. saline group. *NA* noradrenaline, *DA* dopamine, *5-HT* serotonin
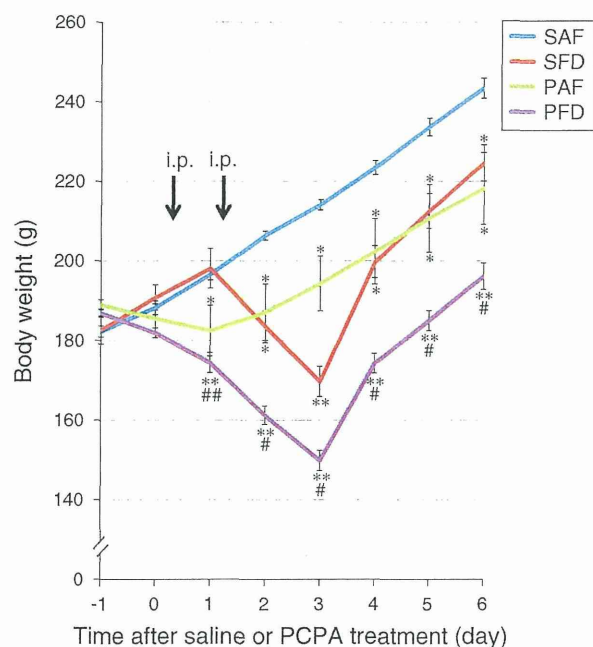
Fig. 2 Changes in body weight changes in body weight from day 1 to day 6. *Arrows* indicate saline or PCPA i.p. administration. Data are presented as mean ± SEM. *$P < 0.05$ vs. SAF, **$P < 0.01$ vs. SAF and PAF, #$P < 0.05$ vs. SFD, ##$P < 0.01$ vs. SFD. *SAF* saline + ad libitum access to food, *SFD* saline + food deprivation for 48 h, *PAF* PCPA + ad libitum access to food, *PFD* PCPA + food deprivation for 48 h

deprivation (Fig. 2). There were no statistical differences between the body weight of SFD and PAF after day 4.

Food intake

Cumulative food intake during the experiment was measured from day 1 to day,6 (Fig. 3). Cumulative food intake was comparable in all among the groups at day 0 and day 1. Cumulative food intake in SFD and PFD was null after starting food deprivation (Fig. 3). Cumulative food intake in PFD decreased at days 4, 5, and 6 compared to SFD (Fig. 3).

Plasma measurement

PG, P-Osm, 5-HT, leptin, and active/desacyl ghrelin concentration at day 3 were measured. PG in SFD (62 ± 2.4 mg/dL) and PFD (75 ± 4.1 mg/dL) decreased significantly compared to that in SAF (108 ± 1.8 mg/dL) and PAF (105 ± 3.9 mg/dL). No statistical differences were seen between SAF and PAF or between SFD and PFD. There were no significant differences in P-Osm among all the experimental groups (SAF: 311 ± 1.3 mOsm/kg; SFD: 310 ± 2.78 mOsm/kg; PAF: 307 ± 2.2 mOsm/kg; PFD: 310 ± 1.7 mOsm/kg). Plasma 5-HT level significantly
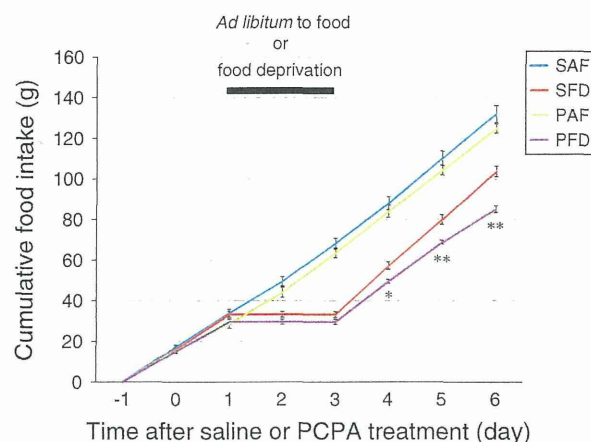

Fig. 3 Changes in food intake Cumulative food intake (g) from day 1 to day 6. Data are presented as mean ± SEM. *$P < 0.05$ vs. SFD, **$P < 0.01$ vs. PFD. Saline or PCPA was i.p. administered at day 0 and day 1

decreased in SFD (384 ± 19.4 ng/mL) compared to SAF (532 ± 42.0 ng/mL) (Fig. 4a). That in PAF (75 ± 14.3 ng/mL) and PFD (35 ± 10.3 ng/mL) was almost depleted compared to SAF and SFD (Fig. 4a). It also significantly decreased in PFD compared to PAF (Fig. 4a). Plasma leptin level in SFD (1.5 ± 0.1 ng/mL) and PFD (1.7 ± 0.1 ng/mL) significantly decreased compared to SAF (3.9 ± 0.3 ng/mL) and PAF (3.4 ± 0.5 ng/mL) (Fig. 4b). No statistical differences were observed between SAF and PAF or SFD and PFD. Plasma desacyl ghrelin in SFD (793.3 ± 83.0 fmol/mL) and PFD (751.6 ± 72.0 fmol/mL) significantly increased compared to those of SAF (213.3 ± 32.9 fmol/mL) and PAF (344 ± 48.6 fmol/mL) (Fig. 4c). There were statistical differences between SAF and PAF (Fig. 4c). Plasma active ghrelin increased in SFD (20.2 ± 6.2 fmol/mL) compared to SAF (5.3 ± 0.6 fmol/mL) (Fig. 4d). Those in PAF (53.0 ± 11.6 fmol/mL) and PFD (65.8 ± 5.8 fmol/mL) significantly increased compared to SAF and SFD (Fig. 4d). The ratio of plasma active/desacyl ghrelin $\times 10^2$ in PAF (15.3 ± 1.9) and PFD (9.1 ± 0.9) significantly increased compared to those in SAF (2.6 ± 0.3) and SFD (2.5 ± 0.6) (Fig. 4e). There were statistical differences between PAF and PFD (Fig. 4e).

Feeding-regulating peptides in the SON and the PVN

The feeding-regulating peptides in the SON and the PVN were measured by ISH followed by quantification using MCID. The gene expression of the *oxytocin* in the SON and the PVN was comparable among all the experimental groups (Fig. 5a A-a–A-d, B-a–B-d, b). The gene expression of the *CRH* and the *TRH* in the PVN in SFD (Fig. 5a C-b, D-b, b) and PFD (Fig. 5a D-b, D-d, b) decreased significantly compared to those in SAF (Fig. 5a C-a, D-a,
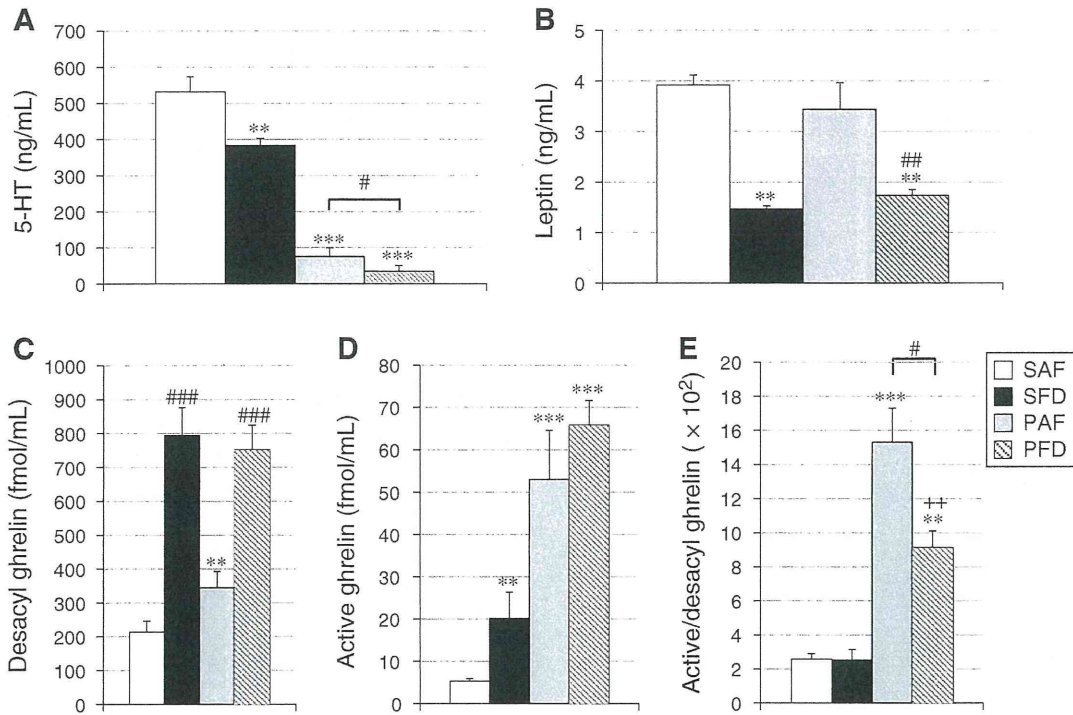
Fig. 4 Plasma concentrations of 5-HT, leptin, and active/desacyl ghrelin ratio Plasma levels of 5-HT (**a**), leptin (**b**), desacyl ghrelin (**c**), active ghrelin (**d**), and the ration of active/desacyl ghrelin (**e**) are shown. Data are presented as mean ± SEM. **$P < 0.01$ vs. SAF ***$P < 0.001$ vs. SAF and SFD, #$P < 0.05$, ##$P < 0.01$ vs. PAF, ###$P < 0.001$ vs. SAF and SFD, ++$P < 0.01$ vs. SFD

b) and PAF (Fig. 5a C–c, D-c, b). There were no statistical differences between SAF and PAF, or between SFD and PFD in the gene expression of the *oxytocin*, *CRH*, and *TRH*.

Feeding-regulating peptides in the ARC and the LHA

The feeding-regulating peptides in the ARC and the LHA were measured by ISH followed by quantification using MCID. The gene expression of the *POMC* and the *CART* in the ARC in SFD (Fig. 6a A-b, B-b, and b) decreased significantly compared to SAF (Fig. 6a A-a, B-a, b), whereas those in PFD (Fig. 6a A-d, B-d, b) were comparable to PAF (Fig. 6a A-c, B-c, b). The gene expression of the *NPY* and *AgRP* in the ARC in SFD (Fig. 6a C-b, D-b, b) and PFD (Fig. 6a C-d, D-d, b) increased significantly compared to SAF (Fig. 6a C-a, D-a, b) and PAF (Fig. 6a C–c, D-c, b). With regard to the gene expression of the *AgRP*, there were no significant differences between SFD (Fig. 6a D-b, b) and PFD (Fig. 6a D-d, b). However, the gene expression of the *NPY* in SFD (Fig. 6a C-b, b) and PFD (Fig. 6a C-d, b) differed significantly.

The gene expression of the *MCH* in the LHA in SFD (Fig. 6a E-b, b) and PFD (Fig. 6a E-d, b) increased significantly compared to those in SAF (Fig. 6a E-a, b) and

PAF (Fig. 6a E-c, b). The gene expression of the *MCH* did not differ between SFD and PFD.

The gene expression of the *orexin* was comparable among all the experimental groups (Fig. 6a F-a–F-d, b). No significant differences were observed between SAF and PAF in the gene expression of the *POMC*, *CART*, *NPY*, *AgRP*, *MCH*, and *orexin*.

Discussion

The present study comprehensively showed the relationships between the serotonergic system and feeding-regulating peptides in the hypothalamus. These results imply that the gene expression of the *POMC*, *CART* and *NPY* in the ARC may be involved in the serotonergic system in the hypothalamus.

After the PCPA treatment, hypothalamic contents of NA, DA, and 5-HT were measured by HPLC to confirm the depletion of 5-HT after the treatment. As shown in Fig. 1, although NA and DA did not change with or without PCPA, 5-HT level nearly depleted after PCPA treatment, which was consistent with a previous report [10]. We made sure that the 5-HT level in the hypothalamus definitely depleted after the PCPA treatment.
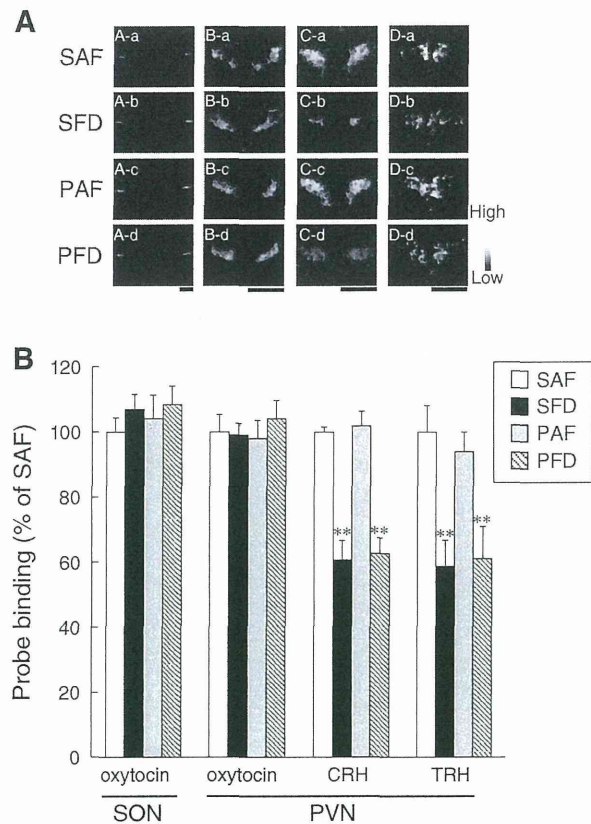
Fig. 5 Feeding-regulating peptides in the SON and the PVN a Digital images of ISH of *oxytocin* in the SON (*A-a–A-d*) and the PVN (*B-a–B-d*), *CRH* in the PVN (*C-a–C-d*), *TRH* in the PVN (*D-a–D-d*). *Scale bar* 1 mm. **b** Quantification of mRNA signals using MCID. PAF data are presented as 100 %. Data are presented as mean ± SEM. **P < 0.01 vs. SAF and PAF

Fig. 6 Feeding-regulating peptides in the ARC and the LHA a Digital images of ISH of *POMC* in the ARC (*A-a–A-d*), *CART* in the ARC (*B-a–B-d*), *NPY* in the ARC (*C-a–C-d*), *AgRP* in the ARC (*D-a–D-d*), *MCH* in the LHA (*E-a–E-d*), and *orexin* in the LHA (*F-a–F-d*). *Scale bar* 1 mm. **b** Quantification of mRNA signals using MCID. PAF data are presented as 100 %. Data are presented as mean ± SEM. #P < 0.05 vs. SFD, **P < 0.01 vs. SAF and PAF

After PCPA systemic treatment, the body weight decreased in PFD and PAF (Fig. 2) compared to SAF. Although the body weight in PAF after day 3 decreased significantly compared to SAF, food intakes after day 3 were comparable to SAF; thus, it was expected that there would be no significant effects on the gene expression of the feeding-regulating neuropeptides in the hypothalamus in our experimental situation after day 3.

Cumulative food intake in PFD decreased at days 4, 5, and 6 compared to those in SFD (Fig. 3). After food deprivation in the PCPA-treated group, the gene expression of the *POMC*, *CART*, and *NPY* seemed likely to be involved in 5-HT system in the hypothalamus according to our results of ISH which will be described later. Although this cumulative food intake data appear to suggest that serotonin facilitates food intake which possibly contradicts the previous concept that serotonin inhibits food intake, the body weights themselves also decreased after serotonin depletion. The decrease of body weights may be caused by complex factors, not just by decreased food intake. Thus,
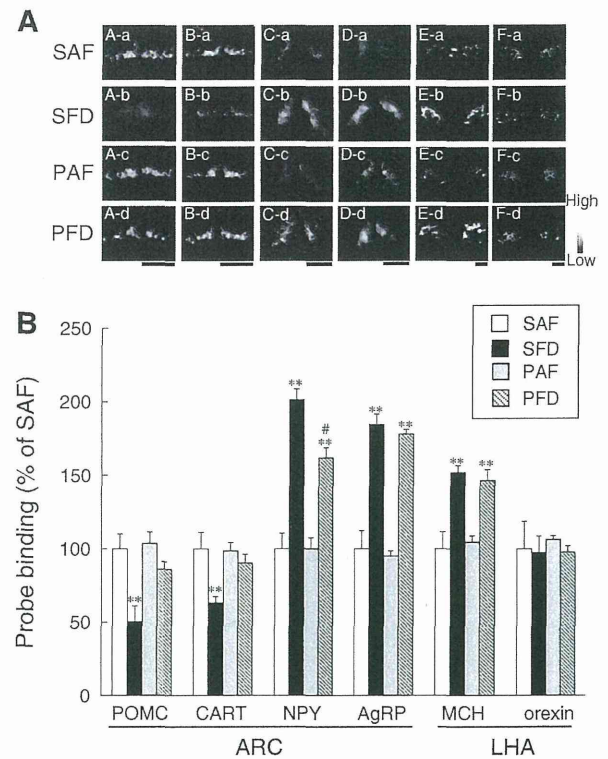
we decided to evaluate accumulating food intake but not food intake per body weight. This analysis appears on the surface to produce opposite results when the food intake is expressed as per body weight, but in fact complex factors may be involved in decreased food intake in each animal after 5-HT-depletion. This point should be examined by further study. We have not examined the gene expression of the feeding-regulating peptides in the hypothalamus after re-feeding; however, it is speculated that some of the feeding-regulating peptides in the hypothalamus may not change despite re-feeding with PCPA treatment.

The significant decrease in PG in SFD and PFD compared to that in SAF and PAF might have been due to the effect of food deprivation. No statistical differences were seen between SAF and PAF or between SFD and PFD, and P-Osm did not differ among all the experimental groups, suggesting that we can compare the feeding-regulating neuropeptides in the hypothalamus in this experiment. Plasma 5-HT dramatically decreased after PCPA treatment (Fig. 4a), but not as completely as that of the hypothalamus. And it tended to decrease with food deprivation.

These result suggests that peripheral 5-HT was also degraded by PCPA as was hypothalamic 5-HT. As previously described [23, 24], food restriction decreased 5-HT levels, which is consistent with our results. Because leptin is a satiety molecule [25, 26], it significantly decreased in food deprivation. But there were no statistical changes between SAF and PAF or SFD and PFD, suggesting that PCPA treatment did not affect plasma leptin levels.

Food deprivation for 48 h significantly increased plasma desacyl ghrelin concentration, indicating the adaptive response to starvation [27]. This was the case in plasma-active ghrelin in SFD; however, it significantly increased in PAF and PFD. These results may indicate that PCPA systemic treatment itself decreased food intake, which resulted in increasing active ghrelin secretion as a secondary response. One possible explanation is that a large amount of active ghrelin had already been secreted by the PCPA treatment in PAF and additional food deprivation produced no further changes in PFD. However, it is difficult to evaluate whether 5-HT may be involved in the regulation of circulating active ghrelin from the data obtained from the present study. Further study should be carried out to confirm this and to elucidate its mechanism.

Among all the feeding-regulating neuropeptides we examined, there was no remarkable difference between SAF and PAF, which indicates that PCPA itself did not affect the peptides under the free feeding condition. Therefore, if the feeding-regulating peptide was markedly different between SFD and PFD, it may be said that the peptide is regulated via the serotonergic system in the hypothalamus.

The gene expression of the *oxytocin*, which is recognized as an anorexic peptide [28], in the SON and the PVN, did not differ despite food deprivation (Fig. 5a, b). It is considered that no significant difference can be detected as a state of *oxytocin* mRNA. Further study, such as a comparison with heteronuclear RNA expression [29], is needed.

The gene expression of the *CRH* and the *TRH* in the PVN decreased significantly after 48 h food deprivation (Fig. 5a, b), as previously described [9, 11–13]. They also decreased after food deprivation under the PCPA pretreatment, which suggests that 5-HT in the brain does not affect the gene expression of *CRH* and *TRH*. Although it has been described that 5-HT 2C receptors exists in the CRH neuron and that there is a possible interaction between 5-HT and CRH [30], our experiment was performed under a condition of almost completely depleted 5-HT, and we examined only one point, so a simple comparison cannot be made. The time course of the gene expression of the *CRH* may have gradually changed after the start of food deprivation.

As expected, the gene expression of the *POMC* and *CART* decreased significantly after food deprivation (Fig. 6a, b). Strikingly, however, they remained unchanged by PCPA pretreatment despite food deprivation. Thus, the effects of PCPA are not statistically confirmed. CART in the ARC is co-stored with POMC [31] and 5-HT receptors exist in the POMC neuron in the ARC [17, 32]. Our results indicate that the serotonergic system in the hypothalamus may project to the POMC and CART neurons in the ARC, which is consistent with previous reports [17, 33].

The gene expression of the *NPY* in the ARC increased significantly after food deprivation (Fig. 6a, b). While the gene expression of the *NPY* also increased significantly after food deprivation with PCPA pretreatment, the extent of the rate of increase was not uniform. The gene expression of the *NPY* in PFD did not increase adequately compared to SFD. As the NPY neurons in the ARC are also expressed in 5-HT receptors [34], there may have been a possible interaction with 5-HT. Taken together with our results, 5-HT may be partially related to NPY regulation.

As previously reported [15], the gene expression of the *MCH* increased significantly after food deprivation (Fig. 6a, b). The variation was to the same degree as with PCPA pretreatment. Our results indicate that 5-HT had no effect to the gene expression of the *MCH*. As with the result of *oxytocin*, the gene expression of the *orexin* did not differ despite food deprivation, which indicates that 5-HT in the hypothalamus may not affect the gene expression of the *MCH*.

In conclusion, we have shown that there may have been possible interaction between the serotonergic system in the hypothalamus and the gene expression of the *POMC*, *CART*, and *NPY* in the ARC. These findings provide evidence of the mechanism of the neural circuit that mediates feeding-regulating neuropeptide reaction in response to 5-HT in the hypothalamus.

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ORIGINAL ARTICLE

An analysis of genotype–phenotype correlations and survival outcomes in patients with primary hyperparathyroidism caused by multiple endocrine neoplasia type 1: the experience at a single institution

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Abstract

Purpose To examine the clinical characteristics and survival outcomes of patients with primary hyperparathyroidism (PHPT) in multiple endocrine neoplasia type 1 (MEN1) in relation to the *MEN1* gene mutation.

Methods The study population included the patients, positive for the *MEN1* gene mutation, who underwent parathyroidectomy between 1983 and 2009 at a single tertiary referral center. Manifestations of the syndrome, other tumors and causes of death were retrospectively correlated with the specific types and locations of *MEN1* gene mutations.

Results Thirty-two patients from 19 families were diagnosed as having MEN1 on genetic examinations. Mutations were most common in exons 2, 7 and 10. A phenotypic analysis of the main MEN1 tumor types among the 32 patients revealed that PHPT was the most common (100 %), followed in order by pancreatic neuroendocrine tumors (PNETs) (53 %) and pituitary tumors (38 %). Death due to MEN1-related disease occurred in five patients (16 %), including malignant PNET in three cases (exons 2, 3), pituitary crisis in one case (exon 2) and thymic cancer in one case (large deletion).

Conclusions Premature deaths related to MEN1 are due to the development of malignant PNET, pituitary crisis or thymic tumors associated with mutations in exons 2, 3 and a large deletion.

Keywords Multiple endocrine neoplasia type 1 ·
Primary hyperparathyroidism ·
Genotype–phenotype analysis

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal-dominant disease characterized by tumors of the parathyroid, pancreas and pituitary. Its prevalence has been estimated to be 1–10 per 100,000 individuals [1]. The clinical manifestations of MEN1 include hyperparathyroidism (over 90 % of cases), pancreatic islet cell tumors (30–80 % of cases) and pituitary tumors (15–50 % of cases) [1, 2]. Other complications such as facial angiofibromas, lipomas, carcinoids and adrenal tumors have also been identified [1, 2].

The locus of the gene responsible for MEN1 was assigned to the long arm of chromosome 11 in 198 [3, 8] and the gene was isolated by positional cloning in 1997 [4]. *MEN1* is a tumor-suppressor gene spanning 9 kb and containing 10 exons. It encodes the 610-amino-acid protein known as menin [5].

Investigators have attempted to correlate various mutations of *MEN1* with clinical manifestations or patient survival; however, no significant correlations have emerged. We herein present an analysis of the genotype–phenotype expression and genotype–prognosis correlations based on long-term observation of MEN1 patients who underwent

parathyroid surgery for primary hyperparathyroidism (PHPT) at our institution.

Methods

Patients

Between 1983 and 2009, 68 patients from 46 kindreds with PHPT or multiglandular disease underwent parathyroidectomy at our department. To be deemed as having MEN1, they had to meet the following criteria: (1) multiglandular disease with a histopathological diagnosis of parathyroid hyperplasia and (2) satisfying the practical criteria for MEN1 reported by Brandi et al. [2].

Clinical manifestations

All patients had PHPT with serum calcium levels exceeding 10.0 mg/dl (normal 8.5–9.9 mg/dl) and serum intact-PTH levels exceeding 66 pg/ml (normal 10–65 pg/ml). The presence or absence of MEN1-associated diseases was determined by measuring the serum levels of gastrin, insulin, glucagon, prolactin, GH and IGF-1 and by performing imaging studies (MRI, CT) of the pituitary and pancreas.

MEN1 gene

All 68 patients had undergone a *MEN1* gene analysis after providing their informed consent. From each patient, a blood sample was collected and genomic DNA was extracted using a QIA Amp blood kit (Qiagen, Hilden, Germany). All protein-coding regions of exons 2 through 10 of the *MEN1* gene were amplified using polymerase chain reaction (PCR). A PCR assay and sequence analyses were performed, as previously described elsewhere [6]. All identified mutations were confirmed by cloning the PCR products into the pCR vector (Invitrogen, Carlsbad, CA, USA) and nucleotide sequencing. For one case involving a large deletion, the mutation was detected as reported previously [7].

Statistical analysis

The overall survival after initial parathyroidectomy was estimated with the Kaplan–Meier method using the JMP (version 8, SAS Institute Japan Ltd., Tokyo, Japan) statistical software package.

We analyzed genotype–phenotype correlations and the survival outcomes of the MEN1 patients.

Results

Among the 68 patients, 25 did not undergo genetic examinations, while one was negative and 42 were positive for the *MEN1* gene mutation. Ten of the latter 42 patients were excluded from the study due to incomplete clinical data. The remaining 32 patients from 19 kindreds comprised the study population. Five patients were male and 27 were female. The average age and standard deviation at initial parathyroidectomy was 45.6 ± 15 years (range 20–73 years), and the average follow-up period and standard deviation was 153 ± 97 months (range 1–396 months).

A phenotypic analysis of the main MEN1 manifestations among the 32 patients revealed that PHPT was the most common (32 patients, 100 %), followed in order by pancreatic neuroendocrine tumors (PNETs) (16 patients, 50 %) and pituitary tumors (15 patients, 47 %). In the 26 patients without *MEN1* gene confirmation, PHPT was the most common manifestation (26 patients, 100 %), followed in order by pancreatic tumors (10 patients, 38.5 %), pituitary tumors (nine patients, 24.6 %), adrenal tumors (three patients, 11.5 %), thymic tumors (three patients, 11.5 %) and malignant tumors (two patients, 7.7 %) (Table 1).

Genotype–phenotype analysis

The locations and types of *MEN1* mutations are summarized in Table 2. Mutations were most common in exons 2 (10 patients from four kindreds) and 10 (five patients from four kindreds). In exons 3 and 7, five patients (three kindreds) and four patients (three kindreds) had mutations, respectively. The genotype–phenotype associations of tumors of the pancreas and pituitary are shown in Table 3. Sixteen patients developed tumors of the pancreas. Seven

Table 1 Characteristics of the patients without *MEN1 gene* confirmation

Total	26
PHPT	26 (100 %)
Pituitary tumor	9 (34.6 %)
PRL	5
Microadenoma	4
Pancreatic tumor	10 (38.5 %)
Non-functioning	7
Gastrinoma	2
Gastrinoma	1
Adrenal tumor	3 (11.5 %)
Thymic tumor	3 (11.5 %)
Others	2 (7.7 %)
Abdominal desmoids	1
Laryngeal Cancer	1

Table 2 The site and type of the *MEN 1* gene

Location	Type	No. of kindreds	No. of patients
Exon 2			
249_252delGTCT	Frameshift	2	5
358_360delAAG	In-frame deletion	1	4
133G > T	Nonsense	1	1
Exon 3			
455T > A	Nonsense	1	2
512_520delGGGATGTCC	In-frame deletion	2	3
Intron 4			
784 − 9G > A	Splicing site mutation	1	1
824 + 1G > A	Splicing site mutation	1	1
Exon 6			
878delC	Frameshift	1	1
Exon 7			
959C > T	Missense	1	1
955_956insT	Frameshift	1	1
914G > A	Missense	1	2
Exon9			
1324C > T	Nonsense	1	2
Exon 10			
1546_1547insC	Frameshift	3	4
1387G > T	Nonsense	1	1
Large deletion		1	3

patients had PNETs, including four patients with gastrinomas, one patient with an insulinoma, one patient with a glucagonoma and one patient with a malignant tumor. Non-functional pancreatic tumors were observed in nine patients. Mutations in the *MEN1* gene were found at exons 2, 3, 6 and 10, and a large deletion was present in one case. Among 11 patients with functional pituitary adenomas, nine had prolactinomas, one had an ACTH-producing adenoma and one had an adenoma that produced both prolactin and growth hormone. Non-functional pituitary tumors were found in two patients. No particular trends were observed between the sites of mutation and the clinical manifestations in the pancreas or pituitary.

Table 3 also shows the genotype–phenotype correlations of thymic tumors and other malignant diseases. Three patients had lesions in the thymus. One patient with a large deletion mutation had a carcinoma of the thymus, and her son was also found to have a thymic carcinoid. The clinical details of another patient with an exon 3 mutation were not available. Other malignant tumors were found in three patients: one patient with a mutation in exon 2, who also had malignant PNET, died

of meningioma; another patient with a mutation in exon 2 developed lung cancer; one patient who had a mutation in exon 10 developed both papillary carcinoma of the thyroid and osteomyelodysplasia.

Survival

Five (16 %) patients died of MEN1-related disease during the follow-up period. The overall survival rates of the patients at 5, 10 and 20 years were estimated to be 99.6 % (95 % CI 88.4–99.9), 95 % (95 % CI 80.8–98.9) and 74.5 % (95 % CI 51.1–89.1), respectively (Fig. 1). The mutations in the patients who died involved exons 2 and 3, and a large deletion. Two patients died of liver metastases from gastrinomas, one patient died of malignant PNET and meningioma and one patient died due to pituitary crisis. The patient with the large deletion died of thymic cancer.

Discussion

The first description of a patient with both parathyroid and pituitary tumors was published in 1903 [8]. Half a century later, in 1952, based on a case series of patients with tumors of the parathyroid, pituitary and pancreatic islets, Underdahl et al. [9] recognized the combination to be a new syndrome. Subsequently, Wermer [6] hypothesized that the syndrome was caused by a mutation in an autosomal gene with a high penetrance.

In our present series, *MEN1* gene mutations were observed in exons 2, 3, 4, 6, 7, 9 and 10, and a large deletion was also recognized. The most frequently observed mutation site was exon 2. The distribution of the mutation sites in our patients was similar to that in a previous report of 24 other Japanese cases [10], although it was different from the results reported by Turner [11]. In our series, frame-shift mutations were the most common form, in agreement with the results of previous reports [12, 13].

Although most manifestations of MEN 1 are thought to be benign, the life expectancy of affected patients has become an important issue. Dean et al. [7] reported that MEN1 patients have a lower 20-year survival rate than normal age- and sex-matched US citizens (64 vs. 81 %). Wilkinson et al. [14] reported the results of a retrospective survey on causes of death in a MEN1 family in Tasmania dating from 1861. Of the 46 family members who had a high probability of having MEN1, 20 (43 %) died due to MEN1-associated conditions, comprising 12 cases of neoplasia and eight cases of hypercalcemia [14]. Wilson et al. [15] observed a dramatic change in the causes of death in their population. Gastrointestinal (GI) hemorrhage was the most common cause of death in the young generation