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ORIGINAL ARTICLE

Somatostatin is involved in anorexia in mice fed a valine-deficient diet

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Abstract The ingestion of a valine (Val)-deficient diet results in a significant reduction of food intake and body weight within 24 h, and this phenomenon continues throughout the period over which such a diet is supplied. Both microarray and real-time PCR analyses revealed that the expression of somatostatin mRNA was increased in the hypothalamus in anorectic mice that received a Val-deficient diet. On the other hand, when somatostatin was administered intracerebroventricularly to intact animals that were fed a control diet, their 24-h food intake decreased significantly. In addition, Val-deficient but not pair-fed mice or those fasted for 24 h showed a less than 0.5-fold decrease in the hypothalamic mRNA expression levels of Crym, Foxg1, Itpka and two unknown EST clone genes and a more than twofold increase in those of Slc6a3, Bdh1, Ptgr2 and one unknown EST clone gene. These results suggest that hypothalamic somatostatin and genes responsive to Val deficiency may be involved in the central mechanism of anorexia induced by a Val-deficient diet.

Keywords Amino acid deficiency · Valine · Anorexia nervosa · Somatostatin

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Introduction

As a consequence of evolution, animals have lost the ability to synthesize certain proteinogenic amino acids by themselves. Therefore, the acquisition of these nutrients in foods is essential, and consequently, animals have developed a system for rejecting diets in which essential amino acids are imbalanced or deficient (Rogers and Leung 1973; Gietzen and Rogers 2006; Gietzen et al. 2007). Following the rejection of such diets, animals begin foraging for a diet that is balanced in essential amino acids, and the animals develop a conditioned aversion to cues that are associated with the deficient diet (Feurte et al. 2000; Gietzen et al. 2007). The central mechanism responsible for the rejection of diets that are imbalanced or deficient in essential amino acids has not yet been fully explained. However, it was reported recently that a chemosensor in the anterior piriform cortex of the brain that surveys the balance of dietary amino acids; an amino acid-deficient or imbalanced diet causes a specific increase in the uncharged tRNA of the relevant amino acid, which activates general control nonderepressible-2 (GCN2) in the anterior piriform cortex (Hao et al. 2005). The GCN2 pathway is conserved from yeast to mammals, and it senses cellular amino acid deficiencies and/or imbalances (Wilson and Roach 2002). The activated GCN2 triggers the glutamatergic output from neural cells in the anterior piriform cortex (Gietzen et al. 2007). However, the location of this output projection is not precisely known, and the causal link between amino acid deficiency and anorexia remains elusive.

The hypothalamus is widely recognized to be an important center of feeding regulation. The hypothalamic regulation of food intake has been studied intensively (Minokoshi et al. 2004; Morton et al. 2006). We speculated that an increase of an anorexigenic substance rather than a decrease

of an orexigenic substance might be induced by an amino acid-deficient diet (Goto et al. 2010). This hypothesis is supported by our recent observation that anorexia induced by a valine (Val)-deficient diet is not completely ameliorated by the intracerebroventricular (icv) or intraperitoneal (ip) administration of the orexigenic peptides ghrelin, neuropeptide Y (NPY), agouti-related peptide (AGRP) or orexin (also known as hypocretin) (Goto et al. 2010). On the other hand, many anorexigenic substances, including hormones, cytokines, and nutrients, have been reported, including cholecystokinin (CCK) (Gibbs et al. 1973), insulin (Panksepp 1974), corticotropin-releasing hormone (CRH) (Glowa et al. 1992), α-melanocyte-stimulating hormone (α-MSH) (Qu et al. 1996), oxytocin (Fleming 1976), cocaine and amphetamine-regulated transcript (CART) (Kristensen et al. 1998), thyrotropin releasing hormone (TRH) (Vijayan and McCann 1977), somatostatin (Lotter et al. 1981), neurotensin (NT) (Stanley et al. 1983) and neuromedin U (NMU) (Kojima et al. 2000).

In the present study, we analyzed the expression of hypothalamic genes by microarray followed by real-time PCR analyses to search for the genes in the hypothalamus that participate in Val-deficient diet induced anorexia. First, we compared feeding-related gene expression in the hypothalamus between mice fed a Val-deficient diet and a control diet using a microarray membrane containing 109 genes that are known to be directly related to obesity and food regulation. Second, we searched for Val-deficiency-responsive genes in the hypothalamus by administering an anorexia-inducing Val-deficient diet and analyzing the results of a microarray containing 39,000 unique transcripts. In the latter experiment, controls for the Val-deficient group were used to exclude genes that showed changes in expression in response to energy deprivation or hunger. Therefore, in addition to the mice that were fed a control diet ad libitum (control mice), we also evaluated mice with restricted access to a control diet (pair-fed mice) that demonstrated the same food intake as the mice that received a Val-deficient diet (Val-deficient mice) and the mice that were starved for 24 h (24-h starved mice).

Materials and methods

Animals and diets

Male C57BL/6 J mice were housed under controlled temperature (23 ± 1°C) and 12:12-h light:dark conditions (lights on at 07:00 h) with free access to food and water. All of the procedures were performed in accordance with the guidelines for animal care stipulated by the Japanese Physiological Society and approved by the ethics committees of Miyazaki University (permit number 2010-007).

The control and Val-deficient diets were based on a modified AIN93G standard diet in which the casein portion of the diet was replaced with a mixture of equivalent free amino acids that supplied all of the dietary nitrogen. The Val-deficient diet was formulated by omitting valine from the free amino acid mixture and by making an isonitrogenous adjustment with glutamine (Goto et al. 2010).

Before the microarray analysis, we confirmed the influence of the Val-deficient diet on the food intake and body weight of the mice and then confirmed that recovery occurred in response to valine supplementation. 7-week-old mice were individually housed in plastic cages (237L × 120W × 127H) and fed a powdered CRF-1 diet (Oriental Yeast Co., Ltd, Tokyo, Japan) for 4 days to acclimate them to a powder diet. The diet was then replaced with a control diet, and the mice were divided into two groups and fed either the control or the Val-deficient diet for an additional 7 days ($n = 6$/group). Subsequently, all of the mice were returned to the control diet. Changes in body weight and food intake were recorded every day. Next, to examine whether anorexia induced by the Val-deficient diet was normalized by valine supplementation, the mice were fed the Val-deficient diet for 7 days and then switched to a Val-deficient diet that was supplemented with 10, 20, 30 and 40% valine (relative to the percentage of valine in the control diet) for 14 days ($n = 6$/group). Changes in body weight and food intake were recorded every day.

Microarray analysis

A preliminary hypothalamus microarray study was performed at 24, 48 and 72 h after switching from the control to the Val-deficient diet, and this revealed a significant change in gene expression in the hypothalamus at 48 and 72 h, but not at 24 h, after switching from the control to the Val-deficient diet. Therefore, we decided to examine the responses at 2 and 3 days after switching in this experiment.

In the first experiment, mice were killed by decapitation 3 days after switching from the control to the Val-deficient diet. The control mice were fed the control diet continuously. The hypothalamus ($n = 4$/group) was collected by dissection using a mouse coronal brain slicer (RB-SS0.5-C; Muromachi Kikai Co., Ltd, Tokyo, Japan). The whole brain was placed in a brain slicer and then coronal slices 2,000 μm thick were made by cutting along the slicer flute with two razor blades. The frontal position was adjusted to the middle of the optic chiasm. Each brain slice was placed on wet filter paper, and the hypothalamus was obtained by bilateral sagittal cutting at a distance of 1,500 μm from the midpoint (third ventricle), and transverse cutting at a distance of 2,000 μm from the bottom. The hypothalamus sample obtained included the paraventricular nucleus

(PVN), ventromedial hypothalamus, lateral hypothalamus and arcuate nucleus (Arc), but not the anterior piriform cortex. Each individual hypothalamic sample included sufficient mRNA to perform microarray and real-time PCR analyses. Total RNA was extracted from each tissue using an RNeasy Micro Kit (Qiagen, Tokyo, Japan), purified with a RNeasy MinElute Cleanup Kit (Qiagen, Tokyo, Japan), and then synthesized into first-strand cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA). Based on the synthesized cDNA, cRNA was then synthesized using biotin-labeled and unlabeled nucleotides. According to the SuperArray guidelines, after purification of the cRNA using an Array Grade cRNA Cleanup Kit (SuperArray Bioscience Inc., MD, USA), the cRNA was hybridized to a microarray membrane containing 109 genes that are known to be related to obesity and food regulation and 6 housekeeping genes (Oligo GEArray: SuperArray Bioscience Inc.). The expression density of the spots was scanned using a Lumino image analyzer (LAS-4000, Fuji Film, Tokyo Japan). GEArray Analyzer software was used to normalize the data. Gene expression was determined relative to that of the housekeeping gene β-2 microglobulin. The same experiment was repeated three times using different animals ($n = 4 \times 3$ times).

In the second experiment, four groups were prepared: (1) a standard diet group (control diet group), in which the animals were allowed free access to the control diet until sacrifice; (2) a Val-deficient group, in which the animals received the Val-deficient diet for 2 days before sacrifice; (3) a pair-fed group, in which the animals were allowed free access to the control diet for 2 days, but their feeding amount per day was restricted to the same amount as that utilized daily in the Val-deficient group; (4) a 24-h starvation group, in which the animals were fed the control diet and then fasted for 24 h prior to sacrifice. The hypothalamus samples for the microarray analysis were prepared using the same method described for the first experiment. The Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA) was used, which comprises 39,000 probe sets that are designed to detect unique transcripts, including 34,000 well-characterized mouse genes. The hybridization assay procedures, including the solution preparation, were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. The distribution of fluorescent material on the array was determined using the GeneArray Scanner 3000 (Affymetrix). Normalization and analysis of the microarray data were performed using the method described by Shankar et al. (2010) using GeneSpring version 7.3X software (Agilent Technologies, Santa Clara, CA, USA). We selected specific genes that were involved in Val-induced anorexia in the hypothalamus by assuming a significant difference between only the Val-deficient diet group and the control group. In each comparison, genes were filtered based on a minimum change of $\pm$2.0-fold (vs. the control), $-$2.0-fold being designated as 0.5-fold, in the present study. If a significant difference in gene expression was observed between either the pair-fed versus control or the 24-h starvation versus control groups, those genes were excluded as candidates for specific genes that were responsive to Val deficiency.

Quantification of mRNAs in the hypothalamus

Real-time RT–PCR was used to quantify changes in the expression of the somatostatin genes identified by microarray in the first experiment and in the Crym (μ-crystallin), Foxg1 (forkhead box G1), Itpka (inositol 1,4,5-trisphosphate 3-kinase A), Slc6a3 (solute carrier family 6, member 3), Bdh1 (3-hydroxybutyrate dehydrogenase, type 1) and Ptgr2 (prostaglandin reductase 2) genes identified by microarray in the second experiment. The samples used for real-time PCR were identical to those included in the microarray analysis. However, to confirm and characterize the relationship between somatostatin mRNA expression and Val deficiency, the mice that were newly fed the Val-deficient diet were ip-administered 0.2-mg valine/0.5-ml saline twice a day (07:00 h, 19:00 h) for 2 days prior to sacrifice, and the hypothalamus was isolated.

Total RNA was extracted from each tissue using the RNeasy Micro Kit (Qiagen, Tokyo, Japan) and used to synthesize first-strand cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). A single tissue sample was sufficient for measuring mRNA levels. An aliquot of first-strand cDNA (40–100 ng tissue equivalent) was quantified using a 7300 Real-Time PCR System (Applied Biosystems) with TaqMan Gene Expression Master Mix (Applied Biosystems) and primers to amplify β-2 microglobulin and the specific target genes. The probe and primer kits were purchased from Applied Biosystems (TaqMan Gene Expression Assay ID: Mm00437762_ml, GenBank NM_009735 for β-2 microglobulin, and Assay ID:Mm00436671_ml, GenBank NM_009215 for somatostatin, Assay ID: Mm00516679_m1, GenBank NM_016669 for Crym, Assay ID: Mm02059886_s1, GenBank NM_008241 for Foxg1, Assay ID: Mm00525139_m1, GenBank NM_146125 for Itpka, Assay ID: Mm00438388_m1, GenBank NM_010020 for Slc6a3, Assay ID: Mm00558330_m1, GenBank NM_175177 for Bdh1, Assay ID: Mm00518647_m1, GenBank NM_029880 for Ptgr2).

Intraventricular (icv) injection of somatostatin

For icv injection, 24 adult male mice were anesthetized with an ip injection of sodium pentobarbital (Abbott Laboratories, North Chicago, USA; 0.75 mg/10 g body

weight) and then mounted in a Narishige mouse brain stereotaxic instrument (Narishige Group, Tokyo, Japan). A stainless steel cannula (guide cannula, length 7 mm, internal diameter 0.4 mm, external diameter 0.7 mm) was then implanted into the lateral left ventricle, the tip being placed at the following stereotaxic coordinates: 0.22 mm posterior to the bregma; 1.0 mm lateral to the midline; 2.0 mm below the dura. The guide cannula was anchored to the skull with machine screws and dental acrylic. After surgery, the animals were housed individually and allowed to recover for 4 days before icv injection, which was performed with a 30-gauge injection cannula that extended into the lateral ventricle 0.5 mm beyond the guide cannula. The 30-gauge injection cannula was connected to a 10-µl Hamilton syringe via a 10-cm polyethylene tube, and CSF solution with our without somatostatin (Peptide Inc., Osaka, Japan) at 0.1, 0.5, or 1.0 nmol/2 µl ($n = 6 \times 4$) was injected into each free-moving mouse at 18:30 h, and then the 24-h food intake was measured. We checked whether the cannulation had been successful 3 days after the somatostatin experiment by confirming an increase of food intake after icv injection of 0.5 nmol mouse ghrelin. Two mice showing no increase of food intake after ghrelin administration were excluded from the analysis.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM). Comparisons between the control and treated groups were performed by ANOVA followed by Fisher's post hoc test. The data obtained for weight recovery due to Val supplementation were evaluated using Two-way repeated measures ANOVA. $P < 0.05$ was considered statistically significant.

Results

The mice that were fed a Val-deficient diet showed significant reductions of food intake and body weight (Fig. 1a). The reduction of food intake was very rapid and was followed by a gradual decrease in body weight, as expected. After the Val-deficient diet was replaced with the control diet, food intake increased immediately to the normal level (Fig. 1a). The decline of body weight due to the Val-deficient diet was restored in a dose-dependent manner with Val supplementation ($P < 0.05$, %Val-dose × day) (Fig. 1b).

The microarray analysis revealed that somatostatin mRNA increased significantly in the hypothalamus of the mice that received the Val-deficient diet (Fig. 2a). On the other hand, the expression of mRNAs for typical anorexigenic peptides, such as CCK, CRH, NT, POMC, TRH,

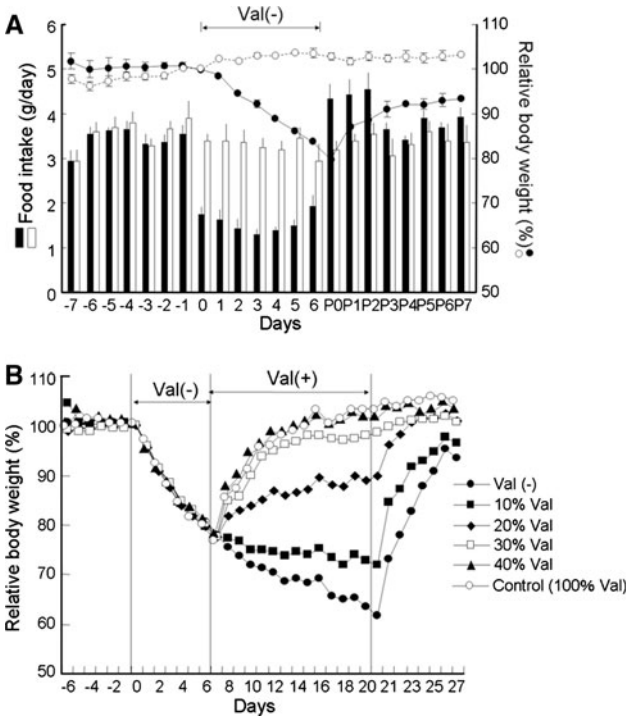

Fig. 1 **a** Average daily food intake and body weights in mice fed a Val-deficient diet (*black bar* and *black circles*) and a control diet (*white bar* and *white circles*). The Val-deficient diet was supplied for 0–6 days. **b** Recovery of body weight in response to valine supplementation in mice that were fed the Val-deficient diet. The diet supplemented with various concentrations of Val was fed to mice beginning at 6 days after initiation of the Val-deficient diet. Data represent the mean ± standard error of the mean (SEM, $n = 6$)

NMU and CART, and for orexigenic peptides, such as orexin (hypocretin; HPC) and NPY, did not change significantly between the Val-deficient and the control diet groups. The quantitative mRNA analysis by real-time PCR also revealed a significant increase in somatostatin mRNA in the mice that received the Val-deficient diet (Fig. 2c). On the other hand, concomitant with the partial inhibition of body weight loss, treatment with Val twice a day for two days reversed the increased expression of somatostatin mRNA caused by the Val-deficient diet (Fig. 2b, c).

In Supplementary Fig. s1, we show the time-course of somatostatin mRNA expression in the hypothalamus after switching from the control diet to the Val-deficient diet and then switching back from the Val-deficient to the control diet. A significant change in the level of somatostatin mRNA in the hypothalamus was observed at 48 and 72 h, but not at 6, 12 and 24 h, after switching to the Val-deficient diet. In addition, the increased level of somatostatin mRNA returned to the basal level 5 days after switching back from the Val-deficient to the control diet.

Next, we examined the possibility that the increased levels of somatostatin in the hypothalamus induced by the Val-deficient diet suppressed food intake. An icv injection

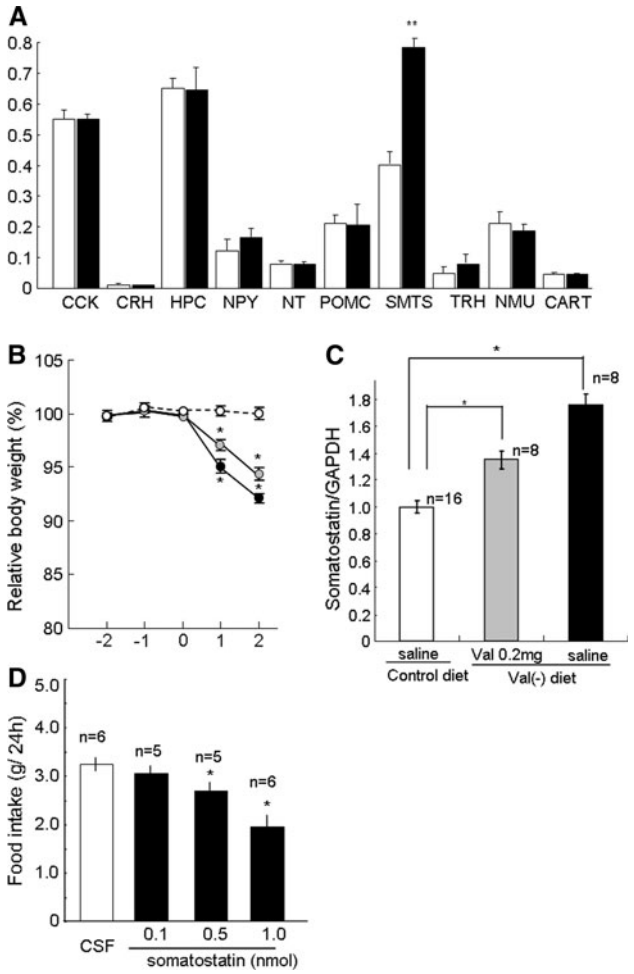

Fig. 2 **a** Relative expression of mRNAs for various hypothalamic neuropeptides in rats fed the Val-deficient (*black bar*) and control (*white bar*) diets. Samples were collected 3 days after feeding the animals the Val-deficient diet. *CCK* cholecystokinin, *CRH* corticotropin-releasing hormone, *HPC* hypocretin, *NPY* neuropeptide Y, *NT* neurotensin, *POMC* proopiomelanocortin, *SMTS* somatostatin, *TRH* thyrotropin hormone-releasing hormone. **b** Relative changes in body weight (compared to day 0) in mice that received the control diet (*white circles*) and the Val-deficient diet (*black circles*) and in mice administered 0.2 mg of Val twice a day after switching to the Val-deficient diet (*gray circles*). **c** Quantitative analysis of the hypothalamic expression levels of somatostatin mRNA in the three groups of mice shown in **b**. **d** Effect of icv injection of somatostatin on food intake in mice. Somatostatin (at a dose of 0.1, 0.5, or 1.0 nmol/2 μl) was injected at 18:30 h, and then the 24-h food intake was measured. All data represent the mean ± SEM. *Asterisks* indicate significant differences versus the control group ($*P < 0.05$)

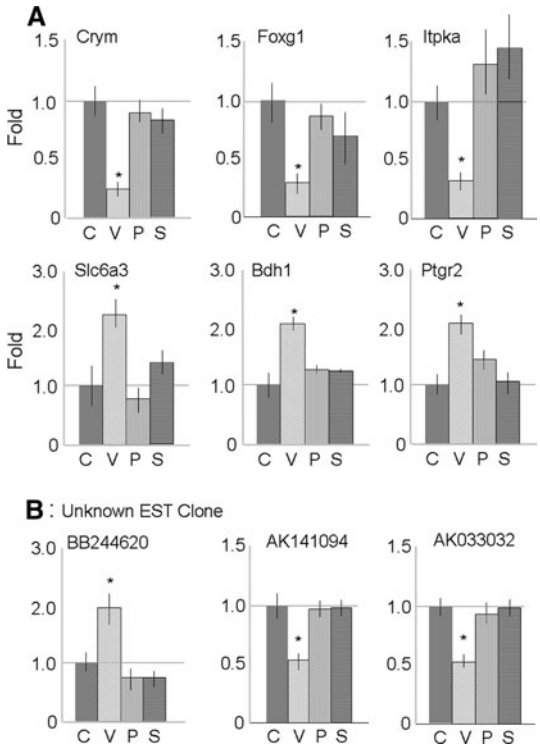

Fig. 3 Real-time PCR to confirm the microarray data for genes that are responsive to the Val-deficient diet. Values are represented relative to the control diet group. Data represent the mean ± SEM ($n = 8$). Asterisks indicate significant differences vs. the control group ($*P < 0.05$). *C* control, *V* Val-deficient, *P* pair-fed for Val deficiency, *S* 24-h starvation

of somatostatin into both mice and rats that had received the control diet resulted in a dose-dependent decrease of food intake (Fig. 2d; Supplementary Fig. s2).

A microarray analysis of 39,000 unique transcripts followed by real-time PCR revealed that the expression of several genes changed significantly in the Val-deficient group relative to the other three groups. The mRNA expression levels of Crym, Foxg1, Itpka and two unknown genes changed less than 0.5-fold in the Val-deficient relative to the control group. On the other hand, the mRNA expression levels of Slc6a3, Bdh1 and Ptgr2 increased more than twofold in the Val-deficient group relative to the other three groups. For these six mRNAs excluding the unknown EST clones, no significant differences were observed between either the pair-fed or the 24-h fasted group and the control group. However, a significant difference was detected between both the pair-fed and the 24-h fasted groups and the Val-deficient group (Fig. 3a).

In addition, the microarray analysis also revealed that some unknown EST clone genes (gene name not yet assigned) were expressed more than twofold or less than 0.5-fold in the Val-deficient group but not in the pair-fed and 24-h fasted groups in comparison to the control. An increased mRNA level of access number BB244620 and decreased mRNA levels of AK141094 and AK033032 mRNA were detected in the only Val-deficient group in comparison with the control (Fig. 3b).

Although we were unable to confirm the following gene expressions by real-time PCR, a microarray analysis showed that the expression levels of the Sgk1, Pdk4, Arl4d, S3-12, Cdkn1a, Sulta1, Ddit4, Arrdc2, 6030422H21Rik,

1810011O10Rik and Nfkbia genes were more than twofold higher and those of the Gm1337, Arpp21, Pitpnm3 and Atp8b1 genes showed a difference of less than 0.5-fold in the Val-deficient, pair-fed and 24-h fasted groups in comparison to the control group (Supplementary Fig. s3). Excluding Arpp21 and Pitpnm3, the mRNA levels showed significant changes in the Val-deficient, pair-fed and 24-h fasted groups in comparison with the control group. These changes in gene expression may be attributable to hunger, stress or energy metabolism in the Val-deficient, pair-fed and 24-h fasted groups.

Discussion

The food intake of the mice that were fed the Val-deficient diet declined to 1/3 of the control level until day 6 and was associated with a decrease in body weight. These results are consistent with previously reported data obtained for rats fed a Val-deficient diet (Goto et al. 2010). This reduction in body weight is considered to result from not only a decline in calorie intake but also an enhancement of endogenous protein catabolism induced by an amino acid imbalance. The rats that were pair-fed the control diet showed a less marked decrease in body weight in comparison to those fed the Val-deficient diet (Nagao et al. 2009, 2010). The results of the present study also demonstrated that the decline in body weight and food intake by the Val-deficient diet was restored in a dose-dependent manner by Val supplementation. These findings suggested that the changes in body weight and food intake were solely attributable to the Val deficiency.

The anorexia induced by the Val-deficient diet is not detrimental, such as that resulting from the ratio of preference relative to the control diet, but rather, it serves as a form of protection against protein catabolism in the body. Although the severity of the anorexia caused by a dietary deficiency of each essential amino acid may vary, this condition has been reported to be triggered by a detection system in the anterior piriform cortex and thereby leads to a further conditioned aversion to the deficient diet (Gietzen et al. 2007; Gietzen and Rogers 2006). However, the mechanism by which an anorexigenic neuropeptide or neurotransmitter is involved in the anorexia induced by a diet that is deficient in an essential amino acid remains unclear.

Only somatostatin mRNA levels demonstrated a significant increase in the hypothalamus of mice that received a Val-deficient diet. In addition, the treatment with Val twice a day for 2 days diminished the increased expression of somatostatin mRNA caused by the Val-deficient diet. These results suggest that hypothalamic somatostatin may be involved in some central regulatory mechanism that is mediated by the Val imbalance. The increase in somatostatin mRNA induced by the Val-deficient diet was suppressed by the administration of Val in accordance with a reduction of the loss in body weight. This result in particular suggests that the increased somatostatin levels may be involved in the anorexia induced by a Val-deficient diet. This possibility was supported by the dose-dependent decrease in food intake detected following the icv injection of somatostatin in both mice and rats fed that received the control diet.

The effect of somatostatin on feeding may be somewhat complex. Administration of somatostatin in the light phase increases food intake in both mice and rats, without any change in 24-h food intake (Stengel et al. 2010). On the other hand, administration of somatostatin during the dark period decreases food intake for 12 h thereafter (Levine and Morley 1982). In addition, a low dose (pmol) and a high dose (nmol) of somatostatin administered icv increase and decrease 1-h feeding in rats, respectively (Feifel and Vaccarino 1990). These results suggest that the effect of somatostatin on feeding may differ according to dose and photoperiod. If the level of somatostatin is increased by a Val-deficient diet, the predominant effect on feeding may be a suppressive one. Although the site of action of the anorexigenic effect of somatostatin remains unclear, it may include the Arc and PVN, which synthesize and release many feeding-regulatory peptides, and contain somatostatin receptors (Fehlmann et al. 2000). In addition, the present study was unable to rule out the possibility that neuropeptide(s) other than somatostatin might be involved in this anorexia, since our mRNA analysis of the whole hypothalamus would not have detected local changes that could very well contribute to the anorexigenic effect of the Val-deficient diet.

It has been shown that central administration of a low dose (pmol) and a high dose (nmol) of somatostatin in the anterior piriform cortex increases and decreases the intake of a threonine-imbalanced diet, respectively. In addition, peripheral administration of somatostatin alters the intake of a threonine-imbalanced diet in rats. These results also suggest that somatostatin may be involved in the response to an amino acid-imbalanced diet.

Although it has been demonstrated that rejection of a deficient diet occurs in less than 6 h (Feurté et al. 2002), no significant change in hypothalamic gene expression was detected at 24 h after switching to a Val-deficient diet in preliminary microarray analysis, and real-time PCR analysis demonstrated no significant change in the level of somatostatin mRNA in the hypothalamus at 6, 12 and 24 h after switching. However, these findings were unable to rule out the possibility that the level of somatostatin may have increased at specific sites in the hypothalamus within 6 h after switching from the control to the Val-deficient

diet, since use of the whole hypothalamus for microarray analysis would not have been able to reveal local changes.

The increased somatostatin mRNA levels in response to the Val-deficient diet were also confirmed using a microarray that contained 39,000 unique transcripts. Although a significant change in the mRNA expression levels of the feeding-related gene AGRP was also observed, this increase was only detected in the 24-h fasted group; it was not observed in the Val-deficient and pair-fed groups (Supplementary Fig. s3).

On the other hand, the potency and time course of the anorectic response to amino acid deficiency depends on the pre-feeding conditions, the type of amino acid deficiency, housing conditions, and so on. Pre-feeding with a high-protein diet would delay the response, because of high levels of circulating amino acids. In addition, housing in a cage equipped with running wheel would also delay and decrease the anorectic response. Therefore, further studies will be required to elucidate the mechanism of the anorectic response to amino acid deficiency under various conditions.

The amino acid imbalance that results from a Val-deficient diet might be detected in the anterior piriform cortex (Hao et al. 2005; Gietzen and Rogers 2006; Gietzen et al. 2007), and this information might be transferred to the hypothalamus to induce the suppression of food intake. On the other hand, the afferent vagal nerve might also transfer information related to the amino acid imbalance following consumption of the Val-deficient diet because a vagotomy is known to inhibit the feeding suppression induced by a diet that is deficient in an essential amino acid (Washburn et al. 1994; Erecius et al. 1996). In addition, we have previously reported that the Val concentration in cerebrospinal fluid (CSF) is depleted significantly in response to a Val-deficient diet (Goto et al. 2010). In addition, the icv injection of Val into Val-deficient rats with a CSF Val concentration that had decreased to approximately half of the normal level caused a distinct increase in the number of c-Fos-positive ependymal cells lining the third ventricle (Goto et al. 2010). Because the same treatment in rats fed the control diet, in which the CSF Val concentrations were expected to be normal, was shown to be completely ineffective, these ependymal cells might be sensitive to any bias in the concentration of essential amino acids in the CSF. The ventral one-third of the third ventricle is known to be composed of specialized ependymal cells known as tanycytes (Berger and Hediger 2001). These cells serve to transport materials from the ventricle to the brain via specialized structures. The distribution of several amino acid transporters has been reported in these cells (Berger and Hediger 2001; Sato et al. 2002) and these transporters might underlie the c-Fos reaction. If these ependymal cells are primarily amino acid-sensing cells, then the resulting signal transduction would be an interesting target for further studies.

Intake of the Val-deficient diet significantly decreased the hypothalamic mRNA expression levels of Crym, Foxg1 and Itpka and increased those of Slc6a3, Bdh1 and Ptgr2. In addition, the microarray analysis demonstrated that the expression levels of some unknown EST clone genes (gene names not yet assigned) were altered by more than twofold or less than 0.5-fold in the Val-deficient but not in the pair-fed or 24-h fasted groups relative to the control. The mRNA levels of accession number BB244620 were higher and those of accession number AK141094 and AK033032 were lower only in the Val-deficient groups relative to control group. Because pair-feeding and 24-h fasting did not affect these mRNA levels of six genes (Crym, Foxg1, Itpka, Slc6a3, Bdh1 and Ptgr2) and three unknown genes (BB244620, AK141094 and AK033032), these genes appear to be involved specifically in the response to dietary Val deficiency and not in responses to hunger or other stresses. In the present study, however, we were unable to clarify the involvement of those genes in Val-induced anorexia. Foxg1, which was previously known as BF-1, has an important role in controlling the process of neurogenesis (Tao and Lai 1992). Iptka is expressed only in neurons and testes, and it may play a role in actin remodeling and cell motility (Windhorst et al. 2008). Slc6a3, which is also known as DAT or DAT1 (dopamine transporter 1), is a crucial regulator of dopamine transmission (Jaber et al. 1997). The enzyme μ-cristallin (Crym) may be involved in amino acid metabolism and have a particularly important role in photoreceptors (Segovia et al. 1997). Ptgr2 was identified recently as a novel prostaglandin reductase that is involved in the regulation of peroxisome proliferator-activated receptor γ activation (PPARγ) (Chou et al. 2007). Bdh1 is a mitochondrial inner membrane enzyme that is associated peripherally with the electron respiratory chain, in which its active site is oriented toward the mitochondrial matrix (Kabine et al. 2003). Further studies are required to elucidate the relationship between these genes and anorexia induced by a Val-deficient diet.

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