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Involvement of brain ketone bodies and the noradrenergic pathway in diabetic hyperphagia in rats

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Abstract Uncontrolled type 1 diabetes leads to hyperphagia and severe ketosis. This study was conducted to test the hypothesis that ketone bodies act on the hindbrain as a starvation signal to induce diabetic hyperphagia. Injection of an inhibitor of monocarboxylate transporter 1, a ketone body transporter, into the fourth ventricle normalized the increase in food intake in streptozotocin (STZ)-induced diabetic rats. Blockade of catecholamine synthesis in the hypothalamic paraventricular nucleus (PVN) also restored food intake to normal levels in diabetic animals. On the other hand, hindbrain injection of the ketone body induced feeding, hyperglycemia, and fatty acid mobilization via increased sympathetic activity and also norepinephrine release in the PVN. This result provides evidence that hyperphagia in STZ-induced type 1 diabetes is signaled by a ketone body sensed in the hindbrain, and mediated by noradrenergic inputs to the PVN.

Keywords Feeding regulation · Ketosis · Monocarboxylate transporter 1 · Energy sensing · Paraventricular nucleus

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Introduction

Diabetic hyperphagia is one of the classical symptoms of type 1 diabetes, and the pathogenesis has been intensively studied for many years [1]. Food intake is regulated by central and peripheral signals, including neurotransmitters, hormones, and energy substrates [2, 3]. It has been suggested insulin and leptin are important peripheral signals controlling food intake, and plasma concentrations of both hormones are low in type 1 diabetes. Receptors for both insulin and leptin exist in the hypothalamus, and are associated with regulation of food intake [4, 5], and injection of either insulin or leptin into the brain attenuated diabetic hyperphagia [6, 7]. Therefore, insulin and leptin have been regarded as peripheral signals related to diabetic hyperphagia.

Energy substrates, in addition to providing an energy source, also act as signals regulating food intake. A decrease in blood glucose levels stimulates food intake in rats and mice [8, 9]. Glucose sensing has also been reported to be related to diabetic hyperphagia [10, 11]. For instance, 48-h fasting increased mRNA levels of hypothalamic orexigenic peptides, for example agouti-related protein (Agrp) and neuropeptide Y (NPY) in streptozotocin (STZ)-induced diabetic rats with insulin and leptin deficiency [11]. Blood glucose levels are believed to be sensed by brain energy sensors that may be located within the hypothalamus and brainstem [8, 9, 12, 13]. These brain energy sensors might be specialized neurons that respond to changes in local extracellular glucose levels to control food intake [14–16]. Ependymocytes in the hypothalamus and brainstem also have the capacity to sense blood glucose levels [17–21].

The ketone body is an energy substrate that is overproduced by fatty acid oxidation to serve as an alternative

energy source of glucose during malnutrition. Ketone bodies are thought to suppress hunger, because previous studies have demonstrated that peripheral injection of 3-hydroxybutyrate (3HB) reduced food intake is by improving energy metabolism [22–24]. On the other hand, the notion that ketone bodies provide a satiety signal might be inconsistent with some pathophysiologic conditions, because 72-h fasting increases plasma ketone body levels and mRNA expression of orexigenic peptide genes in the hypothalamus [25, 26], and reduces mRNA expression of gut cholecystokinin, an anorexigenic peptide [27].

Uncontrolled type 1 diabetes causes severe ketosis and hyperphagia [28–30]. Overproduced ketone bodies, therefore, may contribute to a pathogenesis of diabetic hyperphagia. Hypothalamic neuronal cell line shows a dose-dependent increase in in-vitro expression of Agrp, an orexigenic peptide, when the cells are exposed to 3HB [31]. In this study, we examined the potential function of ketone bodies as a hunger signal to stimulate food intake under severe diabetic conditions.

Materials and methods

Animals

Male 7-week-old Wistar–Imamichi strain rats were obtained from the Institute for Animal Reproduction (Ibaraki, Japan) and were housed in a controlled (14 h light/ 10 h darkness, 5:00 h lights on) environment with free access to food and water. All surgical procedures were performed under isoflurane anesthesia and aseptic conditions. Blood collection, drug injection through brain cannula, and microdialysis were performed on freely moving conscious animals. The study was approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Brain surgery

A guide cannula for fourth ventricle (4V) injection (23G; Plastic One, Roanoke, VA, USA) or for paraventricular nucleus (PVN) microdialysis (0.5 mm outer diameter, AG-12; Eicom, Kyoto, Japan) was stereotaxically implanted 1 week before each experiment. Stereotaxic coordinates for the PVN and 4V cannulas were determined according to a rat brain atlas [32]. The coordinates were 1.9 mm posterior and 7.6 mm ventral to the bregma and 0.5 mm lateral to midline for the PVN and 12.5 mm posterior and 8.0 mm ventral to the bregma at midline for the 4V.

At the end of the experiment, the same amount of 3% brilliant blue as in the experiments was used to infuse the PVN and 4V to verify cannula placement in the animals.

The location of the 4V cannula was verified by visual inspection. The animals with the PVN cannula were perfused with saline, followed by 10% formalin under deep anesthesia. Coronal sections of the brain (50 μm) were stained with thionin, and PVN cannula placement was verified under a microscope.

Effect of a monocarboxylate transporter 1 inhibitor on STZ-induced diabetic hyperphagia

Diabetic rats were produced by a single intravenous (i.v.) injection of STZ (Sigma, St Louis, MO, USA) at 65 mg/kg body weight in 0.01 M citrate buffer (pH 4.5) through an indwelling atrial cannula (silicone rubber tubing: i.d., 0.5 mm; o.d., 1.0 mm; Shin-Etsu Polymer, Tokyo, Japan) that had been inserted a day before STZ treatment through the right jugular vein. Non-diabetic control animals received only the citrate buffer. Blood samples (150 μl) were collected daily at 10:00 h for 6 days through an indwelling atrial cannula to determine plasma concentrations of glucose and ketone body. STZ was administered 1 week after implanting the brain cannula into the 4V. p-Chloromercuribenzene sulfonic acid (pCMBS; Toronto Research Chemicals, ON, Canada), an inhibitor of monocarboxylate transporter 1 (MCT1), a ketone body transporter [33, 34], was injected into the 4V of STZ-induced diabetic rats (5 or 10 nmol/2 μl in ultrapure water at a flow rate of 0.5 μl/min for 4 min) at 10:30 h 8 days after the STZ injection to determine whether diabetic hyperphagia is caused by MCT1-mediated uptake of overproduced ketone bodies. Food intake was measured, and blood samples were obtained after drug treatment for 24 h. Vehicle-treated controls received a 4V injection with ultrapure water.

No apparent behavioral disorder was found after 4V pCMBS injection in diabetic and non-diabetic rats, suggesting that the drug did not have a non-specific effect on normal neuronal functions.

MCT1 detection by immunohistochemistry and RT-PCR

Intact male rats were deeply anesthetized with sodium pentobarbital and perfused with 4% paraformaldehyde. Coronal brain sections (50 μm) were incubated with a mixture of the chicken polyclonal antibody to MCT1 (AB1286; Chemicon, Temecula, CA, USA) and mouse monoclonal antibody to vimentin (MAB1633; Chemicon) for 4 days at 4°C, followed by FITC-conjugated rabbit anti-chicken IgY (303-095-003; Jackson, West Grove, PA, USA) and Alexa Fluor 594-conjugated donkey anti-mouse IgG (A21203; Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. Cell nuclei were visualized using 4,6-diamidino-2-phenylindole (Sigma). Fluorescence images

were obtained by use of an Apotome microscope (Carl Zeiss, Oberkochen, Germany).

Total RNAs were extracted from the tissue containing the wall of the 4V in intact male rats ($n = 3$). cDNAs synthesized with the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) were amplified by PCR. All primer sequences are listed in Table 1. MCT1 is a protein encoded by the solute carrier family 16, member 1 (*Slc16a1*) gene. RT-PCR for the mRNA was performed under the following conditions: 95°C for 5 min, 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, using Amplitaq Gold polymerase (Applied Biosystems).

Correlation of energy substrate concentrations between cerebrospinal fluid and blood

Cerebrospinal fluid (CSF) was collected from diabetic animals and non-diabetic controls by suboccipital puncture in the cisterna magna under anesthesia to determine the correlation of energy substrate levels between CSF and blood. CSF (20–30 μl) and blood (150 μl) were collected 7 or 8 days after STZ treatment.

CSF levels of glucose (Glucose C-Test; Wako, Osaka, Japan), 3HB (Sanwa, Nagoya, Japan), and non-esterified fatty acids (NEFA, NEFA C-test; Wako) were determined with commercial kits as described previously [35, 36].

Involvement of catecholaminergic inputs to the PVN in mediating diabetic hyperphagia

Some diabetic rats were implanted with paraffin pellets (0.66 mm in diameter; 3 mm in length) containing alpha-methyl-*p*-tyrosine (AMPT, a catecholamine synthesis inhibitor; 1.22 ± 0.06 mg/pellet; Sigma) into the dorsal edge of the bilateral PVN 8 days after STZ treatment, because catecholaminergic inputs to the nucleus have been reported to be involved in the induction of food intake [37, 38]. Controls were implanted with paraffin pellets without AMPT. Daily food intake was measured with an automatic feeding monitor (BioDAQ; Research Diets, NJ, USA). Body weight was measured daily at 10:00 h. Blood samples (150 μl) were collected through an indwelling atrial cannula at 1, 3, and 6 days after AMPT implantation at 10:00 h to determine plasma concentrations of glucose, 3HB, and NEFA. Plasma energy substrates were determined as described in detail above.

Effect of MCT1 inhibitor administration into the 4V on orexigenic peptide mRNA expression in the hypothalamic arcuate nucleus

pCMBS (10 nmol/2 μl in ultrapure water at a flow rate of 0.5 μl/min for 4 min), an MCT1 inhibitor, was injected into the 4V, 8 days after STZ or vehicle (citrate buffer) treatment, at 10:30 h, to determine mRNA expression of arcuate nucleus (ARC) orexigenic peptides after injection of the MCT1 inhibitor. Hypothalamic ARC-median eminence (ME) tissues were dissected from the brain, by use of a microknife, 3 h after pCMBS injection into the 4V. Total RNA was extracted from the brain tissues containing the ARC and ME. Vehicle-treated controls were injected with ultrapure water.

Total RNA was extracted from the brain tissues. cDNAs were synthesized as described above. RNase-treated cDNAs were processed by real-time PCR using TaqMan universal PCR Master Mix (Applied Biosystems) in an ABI Prism 7500 Real Time PCR System. We used *Actb* as the invariant control. *Agrp* encodes Agrp. *Npy* encodes NPY. All primer sequences are described in Table 1.

Table 1 Primers used in this study

Peptide	Gene	Oligonucleotide primer sequences (5′ → 3′)	
		Forward	Reverse
RT-PCR			
MCT1	*Slc 16a1*	TCTGGCTGTGGCTTGATTGCAGCTT	GACTGACAGCTTTTCTCCTTTGGGA
Actb	*Actb*	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA
Real-time PCR			
Npy	*Npy*	CCATGTGGTGATGGGAAATG	CAACGACAACAAGGGCCCTGG
Agrp	*Agrp*	TTGGCAGAGGTGCTAGATCCA	AGGACTCGTGCAGCCTTACAC
Actb	*Actb*	ATGTGCTGCCTGACGGTCAG	GGAAGGCTGGAAGAGAGCCT
	Probe (5′ → 3′)		
Npy	FAM-GTGCCACCACCAGGCTGGATTCC-TAMRA		
Agrp	FAM-CGAGTCTCGTTCTCCGCGTCGC-TAMRA		
Actb	HEX-CATCACTATCGGCAATGAGCGGTTCC-TAMRA		

Effects of 3HB injection into the 4V on food intake

3HB (DL-β-hydroxybutyric acid sodium salt) was injected (8 or 16 μmol/2 μl in artificial CSF at 1 μl/min for 2 min; Sigma) into the 4V at 13:00 h in intact male rats. Food intake was measured 3 h after 3HB injection. For vehicle-treated controls artificial CSF was injected into the 4V. pCMBS (0.01, 0.1, or 1 μmol/2 μl in ultrapure water at a flow rate of 1 μl/min) was injected into the 4V just before 3HB injection.

Effects of 3HB injection into the 4V on norepinephrine release in the PVN, with microdialysis, and orexigenic peptide expression in the ARC

Norepinephrine (NE) release in the PVN induced by injecting ketone bodies into the 4V was determined by microdialysis and high-performance liquid chromatography with electrochemical detection (HPLC–ECD) in intact male rats. A microdialysis probe (50 kDa cutoff, 2 mm in length, 0.22 mm outer diameter; A-I-12-02; Eicom) was inserted into the PVN through the guide cannula 2 h before microdialysate collection. The PVN was perfused continuously with Ringer's solution (147 mM NaCl, 4 mM KCl, and 2.3 mM CaCl$_2$) using a microinfusion pump (ESP-64; Eicom) at 1 μl/min. Microdialysate was collected into tubes, on ice, containing 5 μl 0.02 M HCl every 20 min for 5 h starting at 11:00 h. 3HB (16 μmol/2 μl in artificial CSF) was injected into the 4V at 1 μl/min for 2 min, starting at 13:00 h.

The HPLC system consisted of a reverse-phase column (Eicom Eicompack CA-5ODS; outer diameter 2.1 mm, length 150 mm) and an ECD (Eicom) used at an oxidation potential of +450 mV. The mobile phase for microdialysis samples was 0.1 M phosphate buffer (pH 6.0) containing EDTA-2Na (50 mg/l), sodium 1-octanesulfonate (400 mg/l), and 5% methanol. The least detectable NE level in the microdialysate was 1.25 pg, and the intra and inter-assay coefficients of variation (CVs) were 5.6 and 7.4%, respectively.

Hypothalamic ARC tissues were collected 3 h after 3HB injection at 13:00 h into the 4V. Tissues were collected and processed for real-time PCR for Agrp and Npy as described above.

Energy substrate mobilization by 4V or i.v. 3HB injection

3HB (16 μmol/2 μl in artificial CSF) was injected at 1 μl/min for 2 min into the 4V of intact male rats. Blood samples were collected at 12 and 30-min intervals during the first and last 2 h of the sampling period, respectively, through an indwelling atrial cannula, to determine changes in plasma levels of glucose, 3HB, and NEFA. For measurement of plasma NE and epinephrine levels, blood samples were obtained every 12 min after 3HB injection into the 4V. NE and epinephrine levels were determined as described in detail above. Catecholamines in plasma samples (100 μl) were absorbed on to 30 mg activated alumina, and eluted with 70 μl 4% acetic acid containing 0.1 mM EDTA-2Na. The least detectable level of plasma NE and epinephrine (E) was 12.5 pg, intra-assay CVs were 7.0% (NE) and 9.0% (E), and inter-assay CVs were 27.3% (NE) and 10.0% (E), respectively.

To elucidate whether or not the 3HB injected into the 4V stimulates feeding by a central action or a peripheral action, because of leakage into the circulation, 3HB was administered intravenously at the highest dose which was injected into the 4V (16 μmol in artificial CSF) at 13:00 h. Vehicle-treated controls were injected with artificial CSF. Energy substrates were determined as described above in detail.

Statistical analysis

All data are reported as means ± SEM. Statistical differences in food intake and plasma glucose among groups (Fig. 1) were analyzed by two-way ANOVA (STZ and pCMBS treatments) followed by the Bonferroni test. Statistical differences in food intake, body weight, plasma glucose, 3HB, and NEFA (Fig. 4a–e) among groups were determined by two-way ANOVA (treatment and time), followed by the Bonferroni test. Statistical differences in mRNA levels of Agrp and Npy (Fig. 4g) were analyzed by two-way ANOVA (STZ and pCMBS treatments) followed by the Bonferroni test. Statistical differences in food intake (Fig. 5) were determined by Fischer's PLSD test after one-way ANOVA. Statistical differences in PVN NE release (Fig. 6a) and plasma glucose, 3HB, NEFA, NE, and epinephrine (Fig. 7) were analyzed by two-way ANOVA (treatment and time), followed by the Bonferroni test. Statistical differences in mRNA levels of Agrp and Npy (Fig. 6c) between groups were determined by use of the unpaired Student t test.

Results

Diabetic hyperphagia was normalized by inhibiting ketone body transport in rat hindbrain

STZ-induced diabetic rats exhibited hyperphagia (Fig. 1a). Injection of pCMBS, a ketone transporter inhibitor, into the 4V reduced the increased food intake in diabetic rats to non-diabetic levels (Fig. 1a). pCMBS had no effect on food intake in non-diabetic control rats. On the other hand, plasma glucose levels remained at a high level even after pCMBS administration (Fig. 1b).
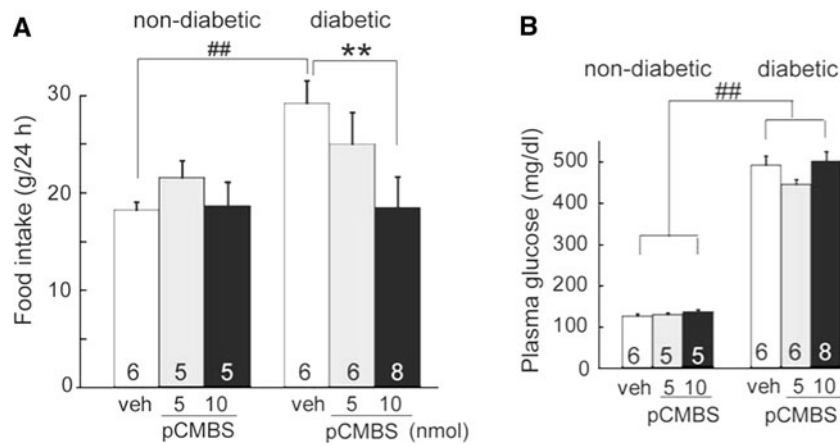
Fig. 1 Role of ketone bodies in diabetic hyperphagia. Effect of fourth-ventricle (4V) injection of *p*-chloromercuribenzene sulfonic acid (pCMBS), a monocarboxylate transporter 1 (MCT1) inhibitor, on food intake (**a**) and plasma glucose levels (**b**) in streptozotocin (STZ)-induced diabetic and in non-diabetic rats. pCMBS was injected at 10:30 h 8 days after STZ treatment. Food intake and plasma glucose levels were determined 24 h after 4V pCMBS injection. Statistical differences were determined by two-way ANOVA (group and treatment) followed by the Bonferroni test. $**P < 0.01$, versus ultrapure water-treated controls. $^{##}P < 0.01$, versus non-diabetic animals. Statistical differences in plasma glucose levels between groups are not shown. *Numbers* in each column represent the number of animals used. Values are means ± SEM
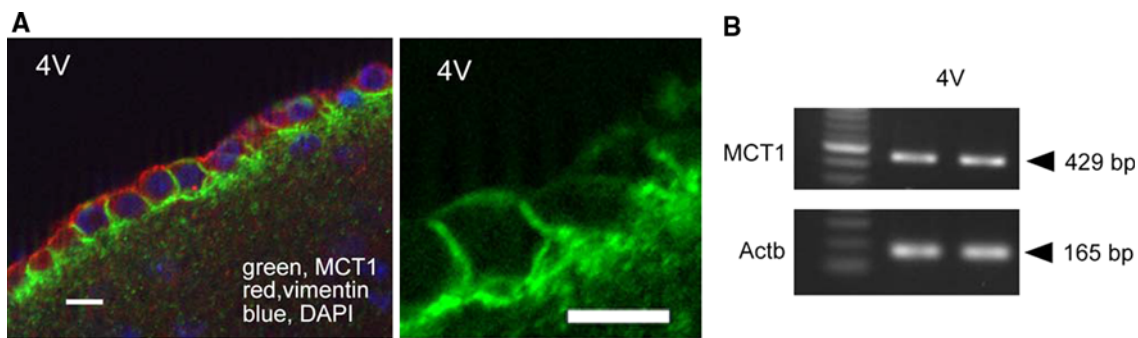

Fig. 2 Expression of MCT1 around the 4V. Immunofluorescence localization of MCT1 (a ketone body transporter, *green*), vimentin (an ependymocyte marker, *red*), and 4,6-diamidino-2-phenylindole (DAPI, cell nuclei, *blue*) around the 4V indicating the location of MCT1 in the ependymocytes in normal male rats (**a**). *Scale bars* 20 μm. Mct1 (official symbol, Slc16a1) mRNA expression in the tissue around the 4V in normal male rats (**b**). The product size for the Mct1 primer was assumed to be 429 bp. Actb (165 bp) was used as an internal standard (color figure online)

Localization of MCT1 in ependymocytes

MCT1 immunoreactivity was found on cells immunopositive to vimentin, a marker of ependymocytes, around the 4V (Fig. 2a). MCT1 (official symbol, Slc16a1) mRNA was detected by RT-PCR in tissues containing ependymocytes around the 4V (Fig. 2b).

Correlation between CSF and plasma concentrations of energy substrates in diabetic and non-diabetic rats

The STZ-induced diabetic rat model exhibited ketosis and hyperphagia (Fig. 3a). In this model, food intake positively correlated with plasma levels of 3HB, a ketone body (Fig. 3a). CSF 3HB levels were significantly elevated in diabetic rats (0.27 ± 0.06) compared with non-diabetic controls (0.08 ± 0.01 mmol/l, $P = 0.03$), and positively correlated with plasma 3HB levels (Fig. 3b). CSF glucose levels were significantly elevated in diabetic rats (149.3 ± 7.5) compared with non-diabetic rats (74.3 ± 3.5 mg/dl, $P = 0.000017$), and positively correlated with plasma glucose levels at a ratio of 0.3 ± 0.02 (Fig. 3c), as previously reported [39]. CSF NEFA levels were undetectable in both diabetic and non-diabetic animals, despite the higher plasma NEFA levels in diabetic rats (1.17 ± 0.2) compared with non-diabetic rats (0.39 ± 0.1 mEq/l, $P = 0.018$) (Fig. 3d).

Involvement of paraventricular catecholaminergic inputs in diabetic hyperphagia

When catecholamine release was blocked by microimplantation of the catecholamine synthesis inhibitor AMPT
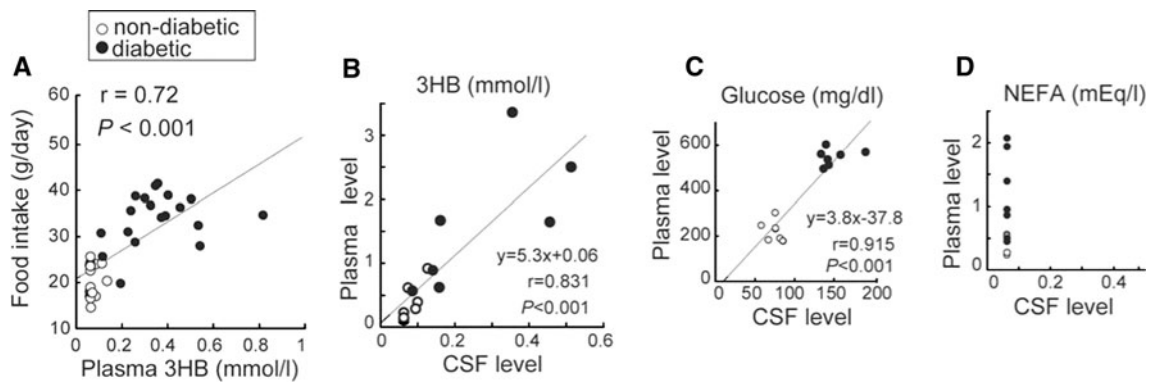
Fig. 3 Relationship between food intake and ketone body. Correlation between food intake and plasma 3HB levels in non-diabetic ($n = 16$) and diabetic ($n = 20$) rats (**a**). 24-hour food intake was measured, cerebrospinal fluid (CSF) or blood samples were obtained 7 or 8 days after STZ or vehicle treatment. Pearson's correlation coefficient is shown. Correlation between CSF and plasma levels of 3-hydroxybutyrate (3HB) (**b**), glucose (**c**), or non-esterified fatty acids (NEFA) (**d**) in non-diabetic and diabetic rats ($n = 7$ for each group). Pearson's correlation coefficient is shown

into the bilateral PVN of diabetic rats, food intake was restored to normal levels by 6 days after AMPT treatment (Fig. 4a). Plasma 3HB was reduced by inhibiting PVN catecholamine synthesis (Fig. 4c). Body weight and plasma levels of glucose and NEFA were not significantly different in AMPT-implanted animals compared with vehicle-treated diabetic rats, and levels remained higher than those in non-diabetic controls (Fig. 4b, d, e).

Diabetic hyperphagia was cured by injection of MCT1 inhibitor (Fig. 1a), but hypothalamic mRNA levels of *Agrp* and *Npy* remained high even after pCMBS injection into the 4V (Fig. 4g).

Increase in food intake and PVN NE release induced by 4V 3HB injection

Injection of 3HB at 8 and 16 μmol into the 4V increased food intake in non-diabetic normal male rats (Fig. 5). In normal rats the 3HB-induced increase in food intake was blocked by inhibition of ketone body transport in the hindbrain by pCMBS (Fig. 5).

NE release in the PVN significantly increased 40 min after 4V 3HB injection in non-diabetic normal rats (Fig. 6a).

Agrp mRNA in the hypothalamic ARC was significantly increased by 3HB injection into the 4V. Npy mRNA levels in the ARC were not affected by the injection (Fig. 6c).

Energy substrate mobilization by 4V 3HB injection

4V 3HB injection increased plasma levels of glucose, 3HB, and NEFA (Fig. 7a–c). Plasma NE and epinephrine levels increased after the injection of 3HB into the 4V (Fig. 7d, e). Peripheral (i.v.) 3HB injection had no significant effect on plasma glucose, 3HB, and NEFA levels except for

transient 3HB increase just after the exogenous 3HB injection (Fig. 7f–h).

Discussion

This study revealed that ketone bodies act as a hunger signal to induce hyperphagia in a diabetic condition, because blockade of MCT1 ameliorated diabetic hyperphagia. Some metabolic products are believed to function as energy signals that are detected by brain energy sensors to control food intake [2]. Our results suggest that overproduced ketone bodies are sensed by the brain through MCT1, and thus would cause hyperphagia under a diabetic condition. Indeed, injection of 3HB into the hindbrain (4V) induced food intake, and shifted energy metabolism from glycolysis to fatty acid oxidation.

STZ-induced type 1 diabetes induced severe ketosis and hyperphagia. We showed that food intake positively correlated with plasma 3HB levels, which in turn positively correlated with CSF 3HB levels. This study showed that ependymocytes lining a wall of the 4V express MCT1. Collectively, the ketone body level in the CSF, which reflects plasma levels, seems to be sensed by hindbrain ependymocytes and to induce diabetic hyperphagia. It has been suggested a glucose sensor is located in hindbrain ependymocytes [17–21]. We speculate that ependymocytes also sense ketone body and then integrate energy information to control food intake. It is likely that pCMBS blocked MCT1 activity on the cell membrane of the ependymocytes, not on the mitochondria membrane, because pCMBS is a hydrophilic compound and may not pass the cell membrane [33]. In addition, pCMBS is believed to be a specific MCT1 inhibitor [33] and may not inhibit MCT2 activity, which is the predominant neuronal
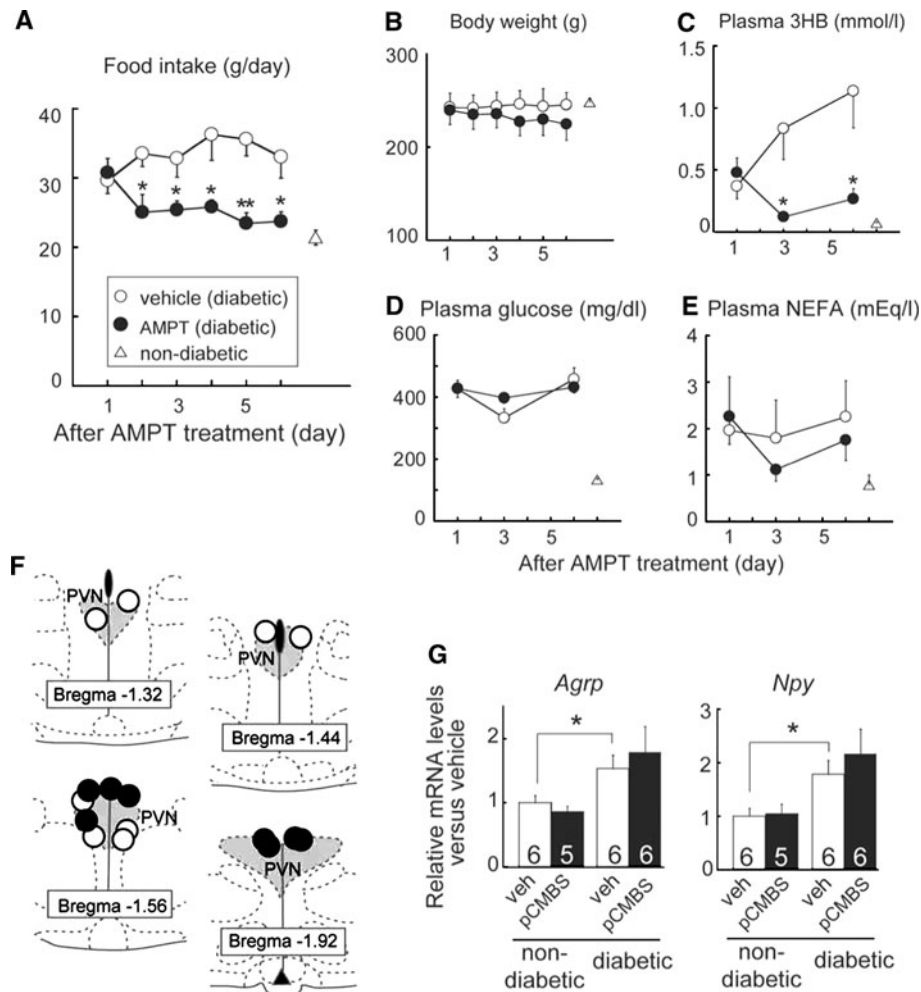
Fig. 4 Involvement of noradrenergic neurons in diabetic hyperphagia. Effect of bilateral alpha-methyl-*p*-tyrosine (AMPT), a catecholamine synthesis inhibitor, microimplants in the hypothalamic paraventricular nucleus (PVN) of STZ-induced diabetic rats on food intake (**a**), body weight (**b**), and plasma levels of 3HB (**c**), glucose (**d**), and NEFA (**e**) ($n = 4$ for each group). The *open triangles* show normal levels of 24-h food intake, body weight, and plasma metabolites for reference. Statistical differences were determined by two-way ANOVA (treatment and day) followed by the Bonferroni test. *$P < 0.05$, **$P < 0.01$, versus vehicle-treated controls. Schematic drawings of the placement of the microimplants (**f**). *Open* and *closed circles* show vehicle-treated controls and AMPT-treated rats, respectively. mRNA levels of agouti-related protein (Agrp) and neuropeptide Y (Npy) in the arcuate nucleus (ARC) (**g**). ARC tissue samples were collected 3 h after injection of pCMBS or vehicle into the 4V. Statistical differences were determined by two-way ANOVA (group and treatment) followed by Student's *t* test. *$P < 0.05$, versus non-diabetic controls. Values are means ± SEM

MCT [40]. Therefore, pCMBS might not inhibit neuronal uptake of ketone bodies but indeed inhibits uptake by ependymocytes.

There are still other possibilities for interpretation of the effect of pCMBS injection into the 4V on food intake in this study. The study does not exclude the possibility that the ependymocytes around the lateral ventricle (LV) or third ventricle (3 V) or some neurons are also a ketone body sensor in the brain. It has been reported that MCT1 is expressed in endothelial cells in the choroid plexus and ependymocytes around the LV and 3 V [41]. MCT1 expression increases in endothelial cells forming the brood–brain barrier under starvation [41]. Second, it is also possible that pCMBS might have blocked lactate uptake,

because MCT1 has also been reported to be involved in lactate transport [40]. There points remain to be clarified in the future studies.

This study suggests that ketone body-induced diabetic hyperphagia is mainly mediated by catecholaminergic inputs to the PVN, because the diabetic hyperphagia was attenuated to normal levels by PVN implants of AMPT, a catecholamine synthesis inhibitor. To our knowledge, this is the first evidence of a positive correlation between PVN catecholaminergic activation and diabetic hyperphagia. In fact, this study showed that injection of 3HB into the 4V increased NE release in the PVN. The involvement of PVN catecholaminergic inputs in feeding has been well documented. Injection of NE into the
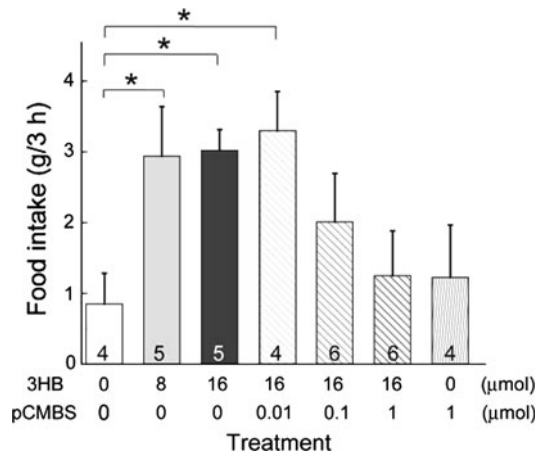
Fig. 5 Effects on food intake of 3HB injection into the 4V. Food intake for 3 h after 3HB injection with or without pCMBS, an MCT1 inhibitor, into the 4V in normal male rats. pCMBS was injected into the 4V immediately before the 4V 3HB administration at 13:00 h. Statistical difference determined by Fischer's PLSD test following one-way ANOVA. *$P < 0.05$, versus vehicle-treated controls. *Numbers* in each column represent the number of animals used. Values are means ± SEM
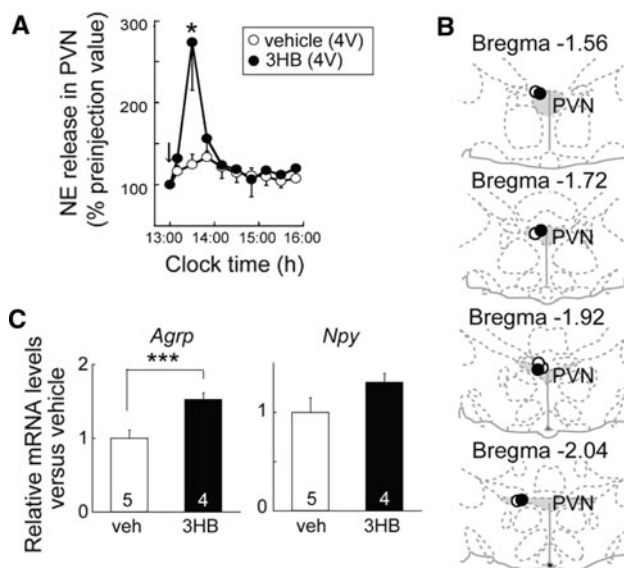

Fig. 6 Effect of 3HB in the 4V on norepinephrine (NE) release in the PVN and on hypothalamic orexigenic peptides. NE release in the hypothalamic PVN was determined by microdialysis and HPLC–ECD after injection of 3HB into the 4V of normal male rats (**a**) ($n = 4–5$ per group). Schematic drawings of the placement of the tip end of the microdialysis probes (**b**). *Open* and *closed circles* show vehicle-treated controls and 3HB-treated rats, respectively. Statistical differences were determined by two-way ANOVA (treatment and clock time) followed by the Bonferroni test. *$P < 0.05$, versus vehicle-treated controls. Values are means ± SEM. mRNA expression of Agrp and Npy in the hypothalamic ARC in male rats injected into the 4V with 3HB (16 μmol) (**c**). ARC tissue samples were collected 3 h after 3HB injection into the 4V. Statistical differences were determined by use of the unpaired Student's *t* test. ***$P < 0.001$, versus vehicle-treated controls. *Numbers* in each column represent the number of animals used. Values are means ± SEM

hypothalamic PVN potently elicits food intake in rats, and the PVN NE synthesis rate increases in 24-h fasting mice [37, 38]. NE stimulates feeding via the α2-adrenergic receptor in the PVN in rats [42]. PVN NE-induced feeding is dependent on circulating corticosterone, and the PVN α2-adrenergic receptor is up-regulated by corticosterone in rats [43, 44]. Because type 1 diabetes shows hypercorticism [45], the feeding response to NE via the PVN α2 receptor may increase under diabetic conditions. The increase in PVN responsiveness to NE may be implicated in the pathogenesis of diabetic hyperphagia. We, however, do not actually know the area of diffusion of AMPT in this study and cannot exclude the possibility that areas other than the PVN are involved in the AMPT-induced decrease in food intake. It should be noted that the PVN AMPT implantation reduced plasma 3HB levels in diabetic rats. There is another possibility that normalized diabetic hyperphagia by AMPT implantation may be associated with the decrease in plasma 3HB levels. Future studies will be needed to address these points.

The ARC mRNA expression of orexigenic peptides, for example Agrp and Npy, was not altered by injection of an MCT1 inhibitor in diabetic rats, whereas injection of 3HB into the 4V increased Agrp expression in the ARC. The reason for the discrepancy is not clear, but ketone body-induced hyperphagia seems to be mediated mainly by noradrenergic inputs into the PVN, because AMPT implantation into the PVN normalized diabetic hyperphagia. NPY and Agrp are co-expressed in the ARC [46], and their expression is directly regulated by insulin and leptin [47, 48]. NPY and Agrp expression increased in STZ-induced diabetic rats, in which insulin deficiency and low leptin secretion are observed [29, 30]. These results suggest that ketone body injection alters NPY and Agrp expression by changing insulin and leptin tone in intact animals and that the diabetic ketosis may induce hyperphagia mainly by the noradrenergic pathway. The involvement of insulin/leptin-NPY/Agrp signaling in the hyperphagia remains to be determined.

Noradrenergic neurons projecting into the PVN mainly arise from the A1 and A2 regions of the solitary tract nucleus (NTS) and the A6 region of the locus coeruleus [49]. 2-Deoxy-ᴅ-glucose-induced glucoprivation has been reported to induce c-Fos expression in the NTS and A6 of rats. [13, 50, 51]. Catecholaminergic cell bodies in the A1 and C1 coexpressed NPY [49], and have been shown to be related to glucoprivic feeding [52]. Hence, signals from a ketone body sensor may be integrated in noradrenergic cell bodies in the A2 and/or A1/C1 and/or A6 regions to stimulate PVN NE release and feeding, although the neural pathway from the sensor to noradrenergic neurons is still unclear.

Fig. 7 Effects of 4V or i.v. 3HB injection on plasma metabolites and catecholamines. Changes in plasma levels of glucose (**a**), 3HB (**b**), NEFA (**c**), NE (**d**), and epinephrine (**e**) after 3HB injection into the 4V ($n = 4$–6 per group) at 13:00 h in normal male rats. Changes in plasma levels of glucose (**f**), 3HB (**g**), and NEFA (**h**) after intravenous 3HB (16 μmol) injection ($n = 4$–6 per group) in normal male rats. Vehicle-treated controls were injected with artificial CSF. Statistical differences were determined by two-way ANOVA (treatment and time) followed by the Bonferroni test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, versus vehicle-treated controls. Values are means ± SEM
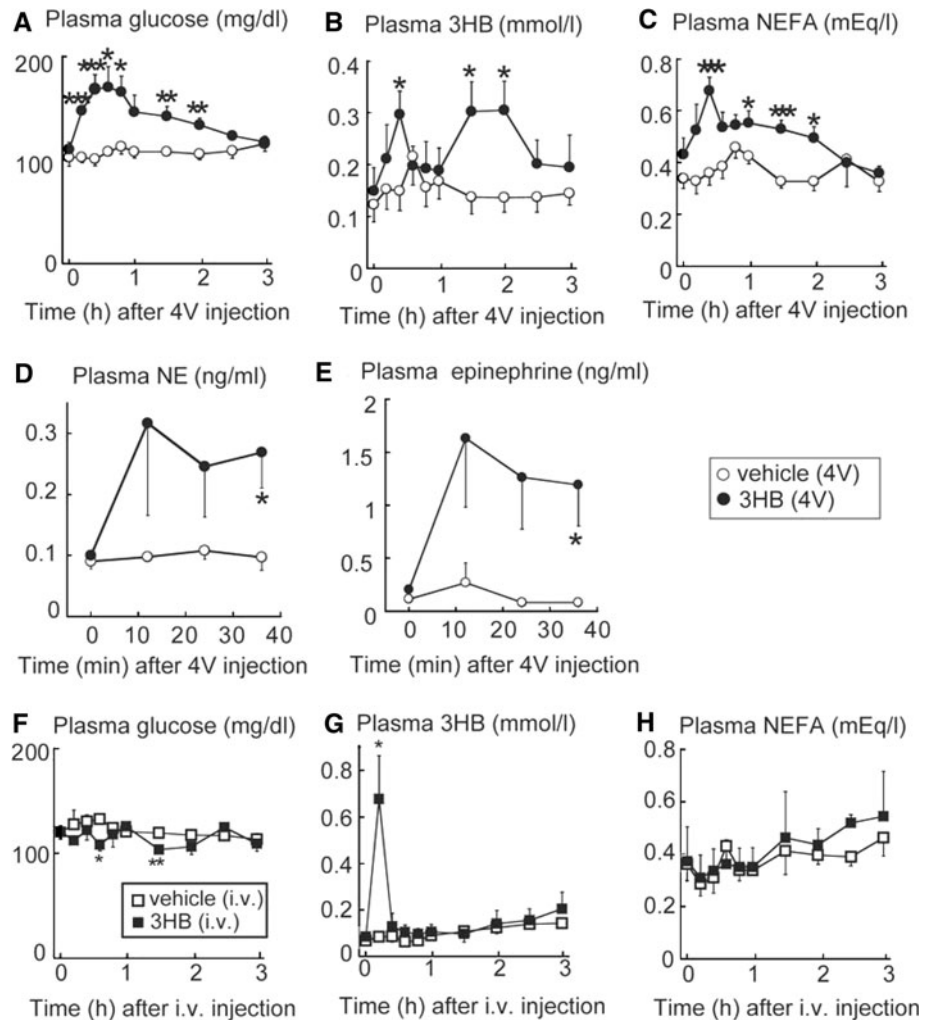
Ketone bodies have long been regarded as an anorectic signal, because a single peripheral 3HB injection reduces food intake in normal rats [22–24]. In contrast, this study showed that the ketone body is sensed by the brain to induce food intake, because diabetic hyperphagia was alleviated by inhibition of uptake of ketone bodies, and 3HB injection into the 4V induced food intake. The subcellular mechanism of ketone body sensing in brain energy sensors remains unknown. Ketone bodies have been known to inhibit glucose oxidation by inhibition of pyruvate dehydrogenase, which catalyzes oxidative decarboxylation of pyruvate in the mitochondria [53]. Pyruvate dehydrogenase activity is reduced in various tissues under fasting or diabetes, showing ketosis as the main symptom [54, 55]. Thus, inhibition of glucose oxidation by ketone body signaling in specific brain sensors may be a part of the mechanism for sensing ketone bodies and for integrating competing energy-related information.

This result is inconsistent with several previous reports on 3HB administration. Fisler et al. [23] showed that subcutaneous 3HB injection reduced food intake in dietary-fat-resistant S 5B/P1 rats, but not in dietary-fat-sensitive Osborne–Mendel rats, through hepatic vagus nerve-independent pathways. On the other hand, Langhans et al. [56] showed that ketone body induces hypophagia via the vagal afferent system. Infusion of 3HB into the 3 V reduced feeding in Osborne–Mendel rats, but not S 5B/P1 rats [24]. Davis et al. [57] reported that infusion of 3HB into the 3 V did not reduce food intake. In some studies, 3HB was chronically infused into the cerebroventricle at a smaller dose, and others used acute peripheral injection [23]. In our study, the dose of 3HB acutely injected into the 4V in normal rats was based on plasma levels of ketone body in alloxan-induced diabetic rats [28]. The doses cannot simply be compared but our 3HB doses were rather higher than in the previous study with 3 V infusion [24]. Thus, the inconsistency of the effect of 3HB could be because of the different animal models, doses, or administration route and duration. Further studies will be required to address these points.

We propose here the hypothesis that increased ketone bodies in the brain contributed to diabetic hyperphagia. An increase in CSF ketone bodies activates a specific neural

pathway, noradrenergic inputs to the PVN, resulting in diabetic hyperphagia. Hindbrain energy sensors may integrate the information signals from CSF energy substrates, for example ketone bodies and glucose, and transmit the information via the noradrenergic pathway to control food intake. Our findings might be of therapeutic importance for eating disorders under a negative energy balance accompanied by ketosis.

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