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Reduced Carnitine Level Causes Death from Hypoglycemia: Possible Involvement of Suppression of Hypothalamic Orexin Expression During Weaning Period

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Abstract. The mechanism of onset of hypoglycemia in patients with carnitine deficiency has yet to be determined. Using mice with systemic carnitine deficiency (JVS mice), we examined this mechanism, focusing on the weaning period (days 14–28 postpartum). For normal mice, the survival rate was 100%, and no hypoglycemia was observed at all. Gastric lactose began to decrease on day 17, and cellulose increased sharply in amount thereafter. For JVS mice, the survival rate was 77% on day 14 and 28% on day 28. From day 21 on, hypoglycemia was noted. Gastric lactose had disappeared almost completely by day 17, and cellulose was almost undetectable from days 14 to 28. Expression of orexin mRNA in the hypothalamus did not differ between JVS and normal mice on day 14, but was suppressed in JVS mice on days 21 and 28. When JVS mice were fed a carnitine-rich diet, suppression of expression of orexin mRNA in hypothalamus was eliminated, and on day 28 lactose and cellulose were detected in the stomach without hypoglycemia. In conclusion, the suppression of the expression of orexin in the hypothalamus during the weaning period may be involved in the marked anorexia in JVS mice, which eventually leads to death from hypoglycemia.

Key words: Carnitine, Death, Orexin

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CARNITINE is essential for the transport of long-chain acyl coenzyme A into the mitochondrial matrix, and plays an important role in cellular energy metabolism [1]. Although the biochemical roles of carnitine have been well studied *in vitro* [2, 3], the understanding of its *in vivo* roles remains limited [4, 5].

Hypoglycemia is one of the severe complications of carnitine deficiency, and is sometimes fatal [6]. According to one theory proposed for the mechanism of onset of hypoglycemia in patients with carnitine deficiency, inhibition of β-oxidation of mitochondria in organs of patients stimulates the consumption of glucose (glucose-fatty acid cycle theory, 7). However some in-

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vestigators doubt this theory, and have suggested that although the glucose-fatty acid cycle is operative in physiological conditions, it is unclear whether it operates in pathological conditions as well [8]. The onset of hypoglycemia in the presence of carnitine deficiency may thus involve unknown mechanisms. However, the study of this in humans is faced with various obstacles and limitations.

The juvenile visceral steatosis mouse (JVS mouse) develops systemic carnitine deficiency due to the defect of the carnitine carrier (OCTN 2) [9, 10]. It is useful for pathophysiological evaluation and analysis of carnitine deficiency [11]. In 1996, we reported that JVS mice developed hypoglycemia at week 2 after birth [12]. In view of a previous report that decreased glucose utilization increases food intake [13, 14], we placed solid diet on the floor of cages, but JVS mice consumed little of it. Notably, although inhibition of systemic fatty acid oxidation with mercaptoacetate or methyl palmoxirate increased food consumption by mice [15, 16], appetite appeared to be low in the JVS mice with disturbance of systemic fatty acid oxidation. On the other hand, it is reported that elevation of long-chain fatty acid levels in the hypothalamus can induce anorexia [17, 18]. The mechanism of regulation of feeding behavior by the central nervous system, which primarily involves the hypothalamus, has recently been partially clarified [19].

We therefore examined the expression of the genes encoding: 1) orexin and neuropeptide Y (NPY), which are potent stimulators of feeding behavior [20, 21], and 2) proopiomelanocortin (POMC), which produces α-melanocyte stimulating hormone (a potent inhibitor of feeding behavior) [22].

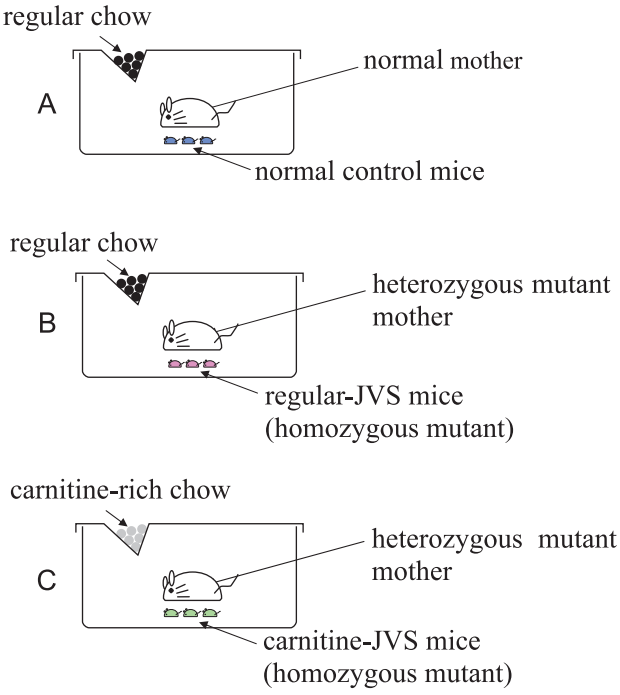


Fig. 1. Experimental design
Three setups were prepared. A: A normal mother was fed regular chow and cared for her normal infants, which were considered normal control mice. B: A heterozygous mutant mother was fed regular chow and cared for her JVS infants (homozygous mutant mice), which were termed regular-JVS mice. C: A heterozygous mutant mother was fed carnitine-rich chow and cared for her JVS infants (homozygous mutant mice), which were termed carnitine-JVS mice. Hematological parameters, gastric content, and expression of peptides in brain were analyzed for the infants.

Methods

Experimental animals

All animals were maintained under specific pathogen-free conditions, and experiments were carried out according to the Guide for Animal Experimentation, School of Medicine, The University of Tokushima. The mice used for this study were JVS mice produced by *in vitro* fertilization according to the method reported by Suenaga *et al.* [23]. C57BL/6J mice (Clea Japan, Inc., Japan) served as normal controls. As regular chow, MF pellet diet (Oriental Yeast Co., Ltd., Tokyo, Japan) was employed. As carnitine-rich chow, MF pellet diet supplemented with L-carnitine (containing 0.28 g L-carnitine per 100 g) was used. Mating and raising of mice were performed in the following fashion. Normal male mice fed regular chow were mated with normal female mice fed regular chow. Each newborn mouse was housed in another cage and fed regular chow (Fig. 1A). Male JVS mice fed carnitine-rich chow were mated with female heterozygous mutants fed carnitine-rich chow. Each newborn mouse was housed in another cage and fed regular chow or carnitine-rich chow. The new infants of JVS mice fed regular chow were termed regular-JVS mice (Fig. 1B), while new infants of JVS mice fed carnitine-rich chow were termed carnitine-JVS mice (Fig. 1C). Experiments were performed only in male infants of the normal control mouse group, the regular-JVS mouse

group, and the carnitine-JVS mouse group.

The ages of individual mice were determined, with the day of birth considered day 0. Mice which appeared to have fatty liver when viewed through the abdominal wall on day 4 were considered JVS mice [9]. On each day from day 4 through day 56 after birth, the number of surviving infants was counted at 0900 h–1200 h. Within each cage, a diet supplier was placed at a height allowing 17-day-old mice from both the normal control mouse group and the JVS mouse group to orally ingest the diet easily by standing on the hindlimbs. The animal quarters were lit between 0730 h and 1930 h each day (light period) and kept dark between 1930 h and 0730 h the following day (dark period).

Blood metabolites and hormones

Blood sampling was usually performed between 0900 h and 1200 h, though for experiments involving analysis of gastric contents was carried out between 2300 h and 2330 h. The animals were fed *ad libitum* in all experiments other than those involving hormone measurement.

After an intraperitoneal dose of sodium pentobarbital (0.05 mg/g body weight), blood was collected under anesthesia from the auricle. Whole blood was neutralized or separated into serum or transferred into a tube containing EDTA-2Na to separate plasma. During the experiments designed to examine the time course of changes in blood glucose level from day 14 to day 35, the tip of the tail was cut with scissors and blood was sampled in a volume of 2 μL, followed by neutralization. Blood sampling was repeated in this fashion from the same mouse.

Blood glucose and blood β-hydroxybutyrate were measured by the enzymatic method [24]. Blood carnitine was quantified by isotopic assay [9]. Concentrations in whole blood were calculated, based on the assumption that whole blood specific gravity was 1.054 and the weight of water was 86.7%. For measurement of ammonia, 8 μL of blood was taken into a tube containing 12 μg EDTA-2Na, and the mixture was tested with an ammonia measuring kit (Pocket Chem BA, ARKRAY, Japan).

For measurement of blood hormone or adiponectin, each 23-day-old mouse was fasted beginning at 1900 h and blood was sampled from the auricle at 0900 h–1200 h the following day. Blood was also sampled from each 24-day-old mouse which had been allowed free access to diet. Commercial kits were used for measurement of blood levels of leptin (Quantikine M mouse leptin, R&D Systems, Wiesbaden, Germany), glucagon (YK090 Glucagon [Rat/Human] ELISA Kit, Yanaihara Institute Inc., Shizuoka, Japan), insulin (Mouse Insulin ELISA [Ultra-sensitive] Kit, AKRIN-031, Shibayagi, Gunma, Japan), ghrelin (Active Ghrelin ELISA Kit, Mitsubishi Kagaku Iatron, Inc.,Tokyo, Japan), and adiponectin (Mouse/Rat Adiponectin ELISA Kit, Otsuka Pharmaceutical, Tokyo Japan).

Analysis of gastric content

To examine the relationship between blood glucose level and amount of milk and solid diet ingested, infant mice were allowed free access to milk and solid diet, and gastric content was collected from each animal at 2300 h–2330 h, at which time the mice were expected to have ingested adequate amounts of milk and solid diet. All gastric content in each animal was taken into a 1.5 mL plastic tube and freeze-dried. Then, 0.1 M HEPES (pH 7.4) was added in a volume of 10 μL to the freeze-dried gastric content (0.37 mg) to obtain a homogenate (4°C), in which cellulose level was measured by enzymatic assay [25] as a marker of solid diet. The remaining homogenate was centrifuged for 20 minutes at 20,000 × g, and the supernatant was combined with the equal amount of 6% perchloric acid. The mixture was neutralized with 1 M K_2CO_3 and centrifuged for 10 minutes at 20,000 × g. The supernatant was harvested to measure lactose level by enzymatic assay [26] as a marker of milk and carnitine level.

Analysis of orexin, NPY, and POMC mRNA expression in the hypothalamus

Under anesthesia with intraperitoneal sodium pentobarbital (0.05 mg/g body weight), the entire brain was removed from each animal, frozen at –20°C, and stored at –80°C. Frozen brain tissue was cut into 12 μm frozen sections with a cryostat. The sections were exposed to a ^{35}S-labeled synthetic oligonucleotide probe complementary to the orexin gene for analysis of orexin expression [27]. Expression of NPY and POMC was determined in the same fashion [28].

Quantitative analysis of behavior

The spontaneous activity of the mice at 20–28 days

was measured for 20 minutes using an animal movement analysis system (SCANET SV-10, MATYS, Japan), which consists of a rectangular enclosure (30 × 48 cm) with a side wall equipped with 144 pairs of photosensors. Each pair of photo sensors scanned the movement of the animal at 0.1-s intervals. The amount of spontaneous activity of each animal was expressed as the frequency of responses per 20 minutes measured with an infrared sensor [29].

Statistical analysis

Survival rate was compared by Kaplan-Meier analysis and the log-rank statistic. Results are presented as means ± SE. *In situ* hybridization histochemistry data were examined using one-way analysis of variance (ANOVA), followed by the Scheffé test for multiple comparisons. Differences between two groups were examined using Student's *t*-test. The level of significance was set at $P<0.05$ for all analyses.

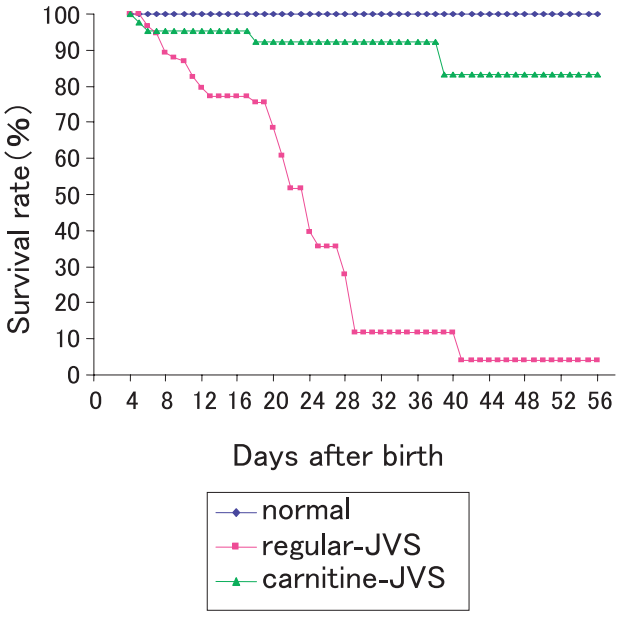


Fig. 2. Survival curves obtained using the Kaplan-Meier method The numbers of normal control mice (◆), regular-JVS mice (■), and carnitine-JVS mice (▲) were counted daily from 4 days after birth to 56 days. On the fourth day after birth, the numbers of normal control mice, regular-JVS mice, and carnitine-JVS mice were 24, 93, and 44, respectively.

Results

Survival rate from day 4 to week 8 after birth

The survival rate for the normal control mice was 100% from day 4 to week 8 after birth. In the regular-JVS mice, the first death was recorded on day 6, and the survival rate was 77% at day 14 and decreased slightly after that. The survival rate in the regular-JVS mice decreased sharply after day 20, and was 61% at day 21 and 28% at day 28. On day 29, the survival rate in this group was 11%, and remained at this level until day 40. By day 41, the rate had decreased to 4% and remained at this level until week 8. Survival rate thus differed significantly between the normal control mice and regular-JVS mice ($P<0.001$). The survival rate for the carnitine-JVS mice was 95% on day 6, 92% on day 28, and 83% on day 39 through day 56. Survival rate thus differed significantly between the carnitine-JVS mice and regular-JVS mice ($P<0.001$) (Fig. 2).

Blood glucose and β-hydroxybutyrate levels from week 1 to week 8 after birth

In the normal control mice, blood glucose level remained above 100 mg/dl from week 1 to week 8. In the regular-JVS group, some animals died during the course of the study, and blood glucose level in the surviving mice of this group was lower than that in the normal control mice at weeks 1, 2, 3, 4, and 5.

The blood glucose level at week 4 was significantly higher in the carnitine-JVS mice than in regular-JVS mice ($P<0.01$) (Fig. 3A).

In the normal control mice, blood β-hydroxybutyrate level was 2084.7 ± 181.2 μM at week 1 and decreased with age thereafter. In the regular-JVS mice, it was 1009.1 ± 107.8 μM at week 1 and lower than that in the normal control mice, but increased thereafter to week 4, and subsequently decreased. In the carnitine-JVS mice, it was 1121.9 ± 196.4 μM at week 1, and increased thereafter to week 3, and subsequently decreased (Fig. 3B).

Blood glucose level from day 14 to day 35

As shown in Figure 2, the survival rate in the regular-JVS group decreased sharply after day 20, and this decrease continued until day 36. This rate appeared to be related to low glucose level, as shown in Figure 3A. Therefore, to follow the time course of change in blood

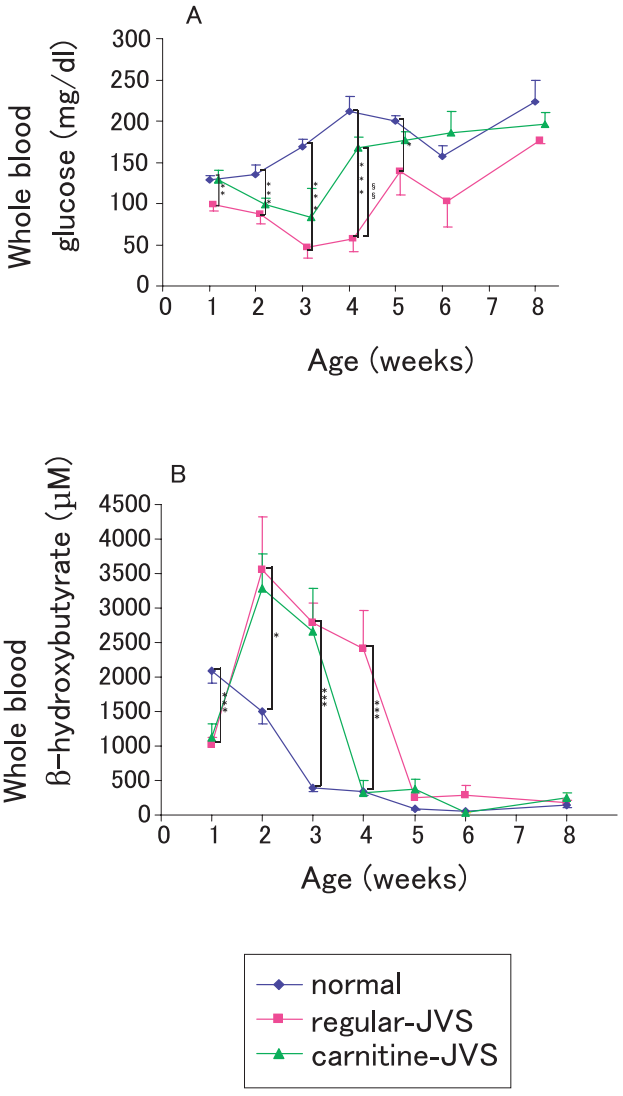


Fig. 3. Changes in whole blood glucose (A) and β-hydroxybutyrate (B) levels
Surviving regular-JVS mice (n = 3–10) and carnitine-JVS mice (n = 3–7) were subjected to examination of blood glucose and β-hydroxybutyrate levels. The normal control mice (n = 3–16) were kept alive until eight weeks after birth. Values are means ± SE. **P*<0.05, ***P*<0.01, ****P*<0.001: between normal control and regular-JVS mice. §§*P*<0.01: between regular-JVS and carnitine-JVS mice.

glucose level between day 14 and day 35, blood was frequently sampled from 35 regular-JVS mice.

In the normal control mice, blood glucose levels were 117.4 ± 2.4 mg/dl on day 14, 126.6 ± 9.8 mg/dl on day 17, 139.5 ± 5.1 mg/dl on day 21, 160.3 ± 15.1 mg/dl on day 24, 151.1 ± 10.7 mg/dl on day 28, and 168.9 ± 15.5 mg/dl on day 35. In the regular-JVS mice, blood glucose level on day 14 was 83.2 ± 3.9 mg/dl, and lower than that in the normal control mice (*P*<0.001). Of the 35 regular-JVS mice, 30 died by day 35 (Fig. 4A). In the mice that had died by day 35, blood glucose level decreased with age. Mean blood glucose level on the day before death was 15.7 ± 3.4 mg/dl. Mean duration of survival was 28.1 days. In the surviving mice, mean blood glucose level began to decrease but was never below 20 mg/dl, and on day 35 was 94.2 ± 29.3 mg/dl (Fig. 4B). Blood glucose level on day 14 did not differ significantly between the carnitine-JVS mice (98.8 ± 21.6 mg/dl) and regular-JVS mice. After day 14, it decreased in the carnitine-JVS mice until day 21 (49.7 ± 21.7 mg/dl), increased to 164.3 ± 19.7 mg/dl on day 24 (*P*<0.01 vs day 21), and remained high on days 28 (170.1 ± 8.4 mg/dl) and day 35 (172.9 ± 15.1 mg/dl) (Fig. 4).

Gastric content, serum carnitine, and serum glucose levels during the dark period

1) Gastric content

Dry weight

Dry weight of gastric content in the normal control mice changed little from day 14 (31.3 ± 7.9 mg) to day 17 (30.1 ± 2.6 mg), but rose sharply on day 19 (146.0 ± 21.5 mg, *P*<0.01 vs day 17). In the regular-JVS mice, dry weight decreased from day 14 (25.2 ± 9.6 mg) to day 17 (6.45 ± 4.07 mg). On day 21 (1.50 ± 0.42 mg) and day 28 (0.16 ± 0.12 mg), it was below 4% of the level in the normal control mice. Dry weight in the carnitine-JVS mice was 23.8 ± 8.2 mg on day 14, 3.00 ± 2.30 mg on day 17, 11.7 ± 7.1 mg on day 19, 37.1 ± 11.0 mg on day 21, and 72.9 ± 20.1 mg on day 28. Dry weight in this group was significantly greater on day 21 (*P*<0.05) and day 28 (*P*<0.01) than on day 17. Compared to the regular-JVS mice, it was significantly higher in the carnitine-JVS mice on day 28 (*P*<0.01) (Fig. 5A, B, C).

Milk-derived lactose

Each mg (dry weight) of regular chow and carnitine-rich chow contained 2.50 ± 0.44 nmol and 2.59 ± 0.17 nmol of lactose, respectively, with no significant difference between the two diets.

In the normal control mice, the total amount of milk-derived lactose in gastric content was 1951.8 ± 255.1 nmol on day 14 and decreased thereafter, reaching 1790.3 ± 393.8 nmol on day 17, 493.5 ± 263.8 nmol

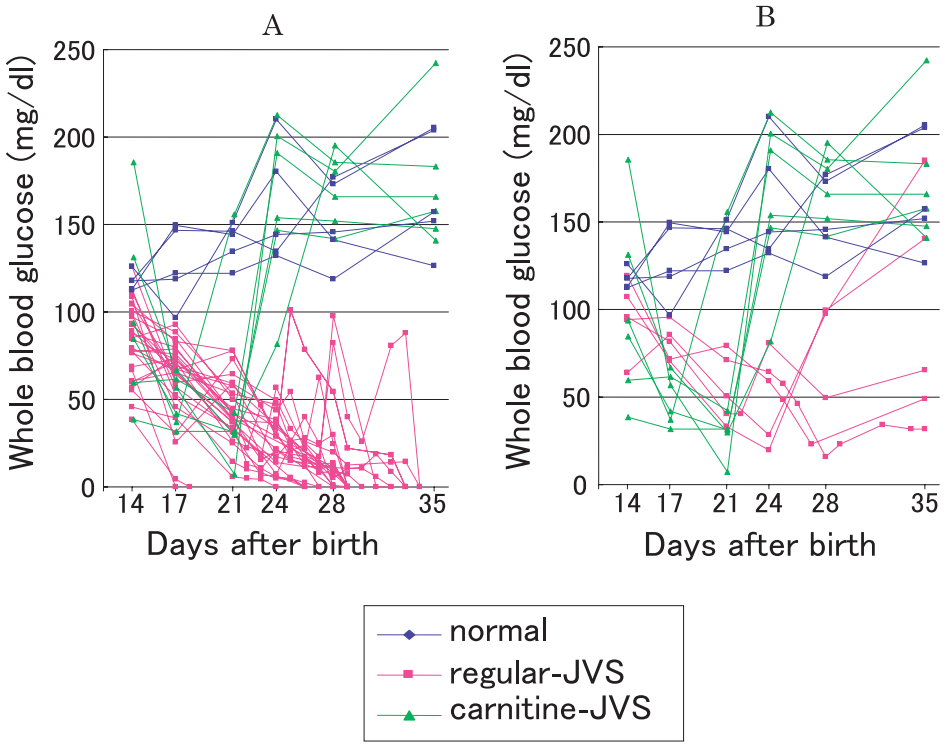


Fig. 4. Changes in glucose level in whole blood from day 14 to day 35
Glucose levels of normal control mice (n = 5), regular-JVS mice (n = 35), and carnitine-JVS mice (n = 6) were measured from day 14 after birth to day 35. All normal control and carnitine-JVS mice survived. A: Regular-JVS mice which died before day 35 (n = 30). B: Regular-JVS mice which survived until day 35 (n = 5).

on day 19, 206.0 ± 36.3 nmol on day 21, and 223.3 ± 154.2 nmol on day 28. The total amount of milk-derived lactose in the regular-JVS mice was 1418.6 ± 613.9 nmol on day 14, and decreased sharply to 278.4 ± 239.9 nmol on day 17 and was significantly lower (P<0.05) than that of the normal control mice on that day. On day 21 (13.1 ± 7.5 nmol), it was below 6% of the level in the normal control mice. Lactose was not detectable in gastric content on day 28 for the regular-JVS mice. In the carnitine-JVS mice, the total amount of milk-derived lactose was 355.5 ± 208.1 nmol on day 14, decreased after day 17, and rose again to 1358.5 ± 581.1 nmol on day 21. Compared to the regular-JVS mice, this level was significantly higher in the carnitine-JVS mice (P<0.01 on day 21, P<0.05 on day 28) (Fig. 5D, E, F).

Cellulose

Each mg (dry weight) of regular chow and carnitine-rich chow contained 2249.1 ± 160.7 nmol and 2269.5 ± 86.9 nmol of cellulose, respectively, with no significant difference between the two diets.

In the normal control mice, the total amount of cellulose in gastric content was 2.59 ± 0.57 µmol on day 14, 21.7 ± 9.7 µmol on day 17, 388.1 ± 162.4 µmol on day 19, 453.7 ± 135.9 µmol on day 21, and 257.3 ± 93.4 µmol on day 28. The total amount of cellulose in gastric content of the regular-JVS mice was 0.02 ± 0.01 µmol on day 14, 0.12 ± 0.08 µmol on day 17, 0.07 ± 0.02 µmol on day 21, and 0.02 ± 0.02 µmol on day 28. It was below 0.8% of the level in the normal control mice on each of the corresponding days. In the carnitine-JVS mice, the total amount of cellulose was 3.16 ± 2.57 µmol on day 14, 0.32 ± 0.31 µmol on day 17, 57.9 ± 37.1 µmol on day 19, 54.2 ± 49.8 µmol on day 21, and 254.8 ± 84.6 µmol on day 28 (P<0.05 vs day 17). Compared to the regular-JVS mice, this level was significantly higher in the carnitine-JVS mice (P<0.01 on day 28) (Fig. 5G,H,I).

Carnitine

Each mg (dry weight) of regular chow and carnitine-rich chow contained 0.18 ± 0.01 nmol and 17.9 ± 0.8 nmol of carnitine, respectively. The carnitine level of

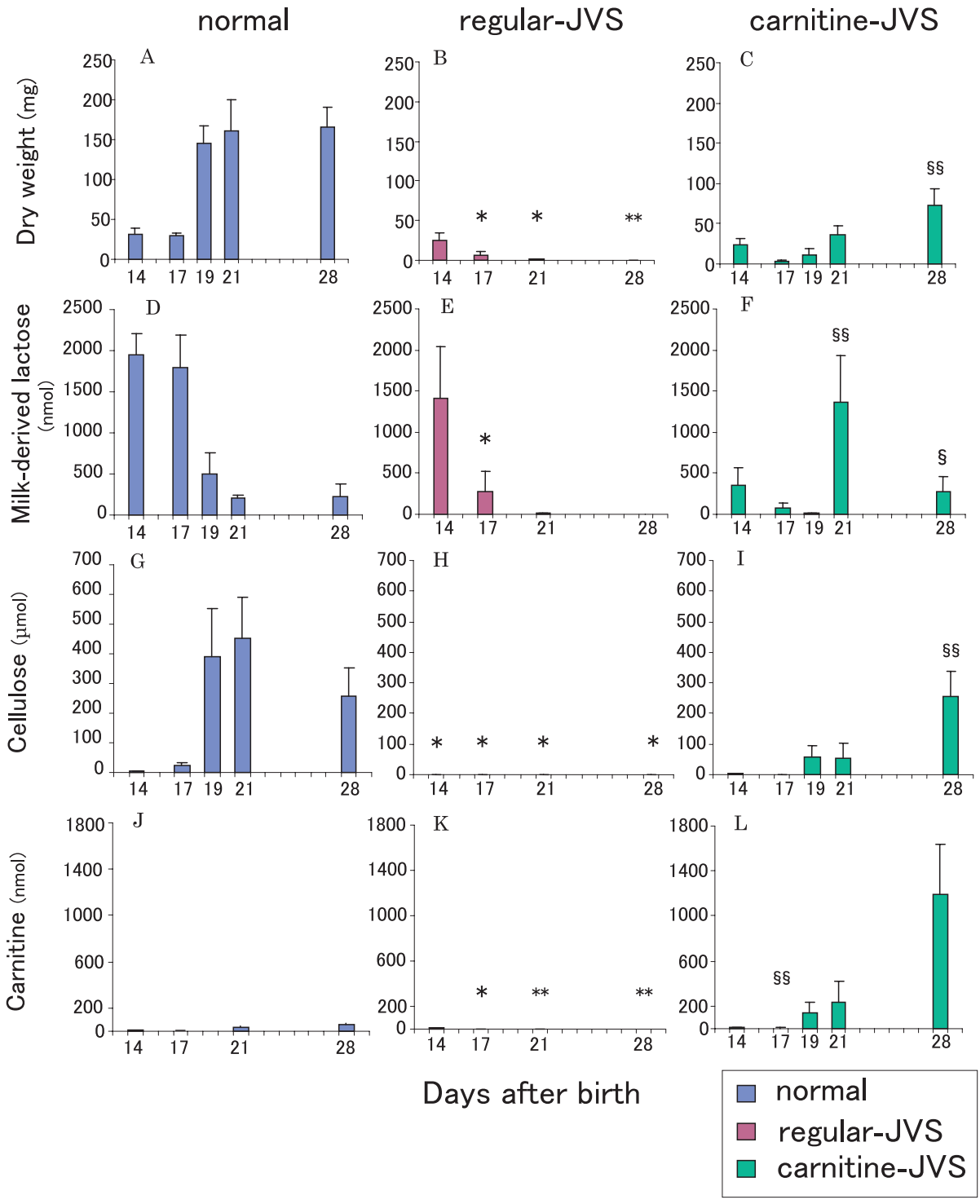
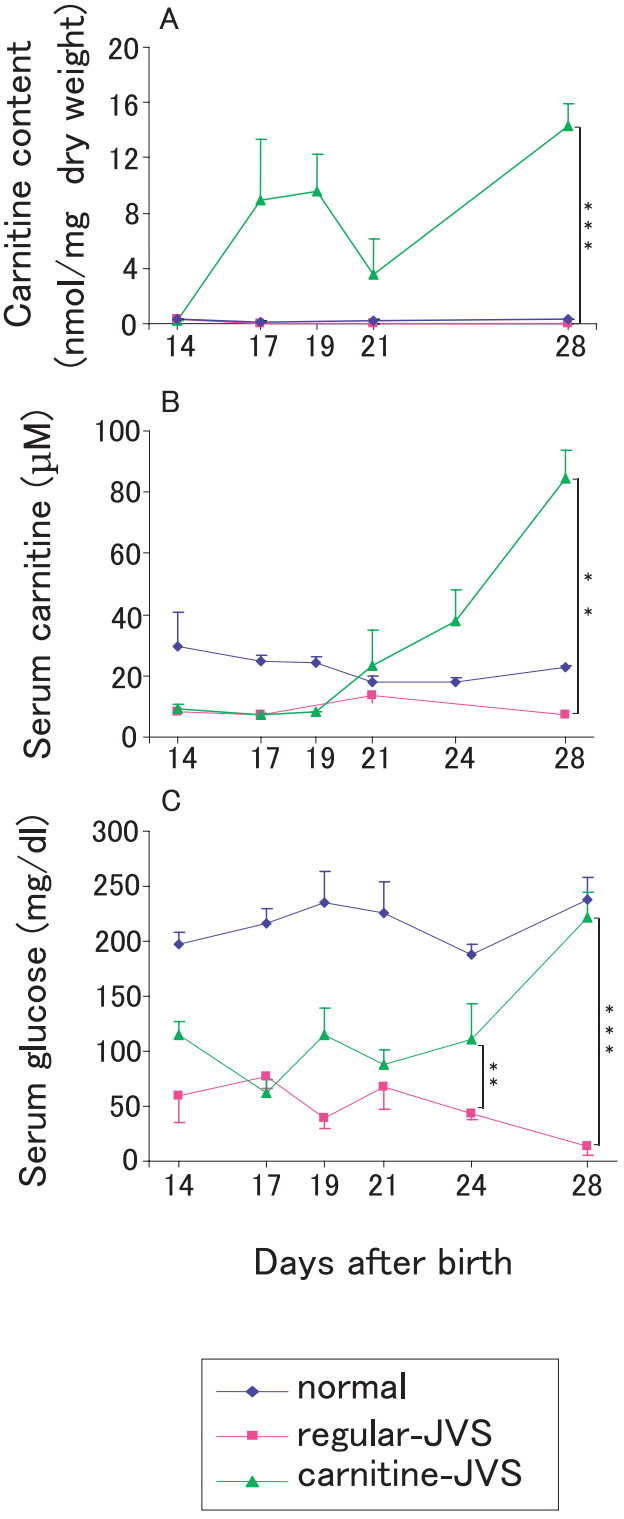


Fig. 5. Total dry weights (A, B, and C), amounts of lactose derived from milk (D, E, and F), cellulose (G, H, and I), and carnitine (J, K, and L) of gastric content during dark period
Sampling of gastric content was performed between 2300 h–2330 h. In normal control mice (n = 3–14), after day 19, the amount of lactose derived from milk decreased, while that of cellulose increased. In regular-JVS mice (n = 3–6), amounts of lactose derived from milk and cellulose were almost undetectable in the stomach after day 21. In carnitine-JVS mice (n = 3–7), lactose derived from milk increased after day 21, and cellulose was increased after day 19. Carnitine content was increased after day 19. Values are the means ± SE. **P*<0.05, ***P*<0.01 : normal control versus regular-JVS mice. §*P*<0.05, §§*P*<0.01: regular-JVS versus carnitine-JVS mice.

carnitine-rich chow was significantly higher than that of regular chow ($P<0.001$).

In the normal control mice, total carnitine level in gastric content was 14.2 ± 2.9 nmol on day 14, 4.88 ± 0.64 nmol on day 17, 39.7 ± 6.3 nmol on day 21, and 60.6 ± 6.9 nmol on day 28. It was significantly higher on day 21 ($P<0.01$) and day 28 ($P<0.001$) than on day 14. In the regular-JVS mice, total carnitine level was 10.5 ± 2.3 nmol on day 14, 0.74 ± 0.73 nmol on day 17, 0.03 ± 0.02 nmol on day 21, and not detectable on day 28. It was lower on days 17, 21, and 28 ($P<0.05$, each case) than on day 14. In the carnitine-JVS mice, total carnitine was 9.27 ± 2.79 nmol on day 14, 5.72 ± 0.26 nmol on day 17, 137.8 ± 95.1 nmol on day 19, 230.7 ± 194.4 nmol on day 21, and 1194.9 ± 446.5 nmol on day 28 (Fig. 5J, K, L).



As shown in Figure 6A, in the normal control mice, the amount of carnitine per mg (dry weight) gastric content was 0.35 ± 0.02 nmol on day 14, 0.16 ± 0.03 nmol on day 17, 0.26 ± 0.03 nmol on day 21 and 0.32 ± 0.02 nmol on day 28, respectively. In the regular-JVS mice, the amount of carnitine per mg (dry weight) of gastric content was 0.30 ± 0.05 nmol on day 14, 0.03 ± 0.03 nmol on day 17, 0.03 ± 0.01 nmol on day 21, and not detectable on day 28. It was significantly lower than that in the normal control mice on day 17 ($P<0.05$), day 21 ($P<0.01$), and day 28 ($P<0.001$). In the carnitine-JVS mice, the amount of carnitine per mg (dry weight) gastric content was 0.25 ± 0.01 nmol on day 14, 8.88 ± 4.44 nmol on day 17, 9.62 ± 2.67 nmol on day 19, 3.55 ± 2.58 nmol on day 21, and 14.3 ± 1.6 nmol on day 28. It was significantly higher on days 19 ($P<0.05$) and 28 ($P<0.001$) than on day 14. Compared to the regular-JVS mice, it was significantly higher in the carnitine-JVS mice on day 28 alone ($P<0.001$). There was no significant difference in the amount of carnitine per mg (dry weight) gastric content and the carnitine-rich chow on day 28.

2) Serum carnitine

In the normal control mice, serum carnitine level

Fig. 6. Carnitine content per dry weight of gastric content (A), serum carnitine (B), and serum glucose (C) levels during dark period
Sampling of residue in the stomach and serum was simultaneously performed between 2300 h–2330 h. In carnitine-JVS mice, carnitine content per dry weight was increased after day 17. Serum carnitine level tended to increase after day 21. Serum glucose levels were significantly higher on day 24 and day 28 than in regular-JVS mice. Values are means ± SE. $^{**}P<0.01$, $^{***}P<0.001$: regular-JVS mice (n = 3–12) versus carnitine-JVS mice (n = 3–14).

during the dark period was 29.7 ± 10.9 μM on day 14, 24.9 ± 1.6 μM on day 17, 24.2 ± 2.0 μM on day 19, 18.1 ± 1.7 μM on day 21, 17.8 ± 1.8 μM on day 24, and 22.8 ± 0.5 μM on day 28. Serum carnitine level in the regular-JVS mice was 8.44 ± 0.33 μM on day 14, 7.08 ± 0.44 μM on day 17 (P<0.001 vs normal control mice), 13.4 ± 2.0 μM on day 21, and 7.29 ± 0.67 μM on day 28 (P<0.001 vs normal control mice). In the carnitine-JVS mice, serum carnitine level was 9.22 ± 1.23 μM on day 14, 7.37 ± 0.38 μM on day 17, 8.22 ± 0.25 μM on day 19, and tended to increase thereafter. It was significantly higher on day 24 (37.9 ± 10.3 μM, P<0.05) and day 28 (84.4 ± 9.2 μM, P<0.01) than on day 19. Compared to the regular-JVS mice, it was significantly higher in the carnitine-JVS mice on day 28 (P<0.01) (Fig. 6B).

3) Serum glucose

In the normal control mice, serum glucose level during the dark period was 196.8 ± 11.2 mg/dl on day 14, 216.5 ± 12.8 mg/dl on day 17, 234.5 ± 28.5 mg/dl on day 19, 225.9 ± 28.5 mg/dl on day 21, 187.8 ± 9.2 mg/dl on day 24, and 238.4 ± 19.3 mg/dl on day 28. In the regular-JVS mice, serum glucose level was 60.1 ± 25.5 mg/dl on day 14, 76.5 ± 9.6 mg/dl on day 17, 38.6 ± 9.5 mg/dl on day 19, 67.9 ± 20.4 mg/dl on day 21, 43.3 ± 5.5 mg/dl on day 24, and 12.8 ± 6.8 mg/dl on day 28. In the carnitine-JVS mice, serum glucose level was 115.3 ± 12.2 mg/dl on day 14, and did not differ significantly from that in the regular-JVS mice. On day 17, it was 61.5 ± 12.5 mg/dl, and significantly lower than that on day 14 (P<0.05). Blood glucose level in this group then tended to rise, reaching 221.7 ± 22.5 mg/dl on day 28 (significantly higher than that on day 17, P<0.001). Blood glucose levels in the carnitine-JVS mice were significantly higher on day 24 (P<0.01) and day 28 (P<0.001) than those in the regular-JVS mice (Fig. 6C).

Analysis of orexin, NPY, and POMC mRNA in the hypothalamus

1) Orexin gene expression

Orexin gene expression was observed in the lateral hypothalamic area, the posterior hypothalamic area, and the perifornical nucleus in mice from weeks 1 to 5 and week 8 after birth. In normal control mice, the expression of the orexin gene was detected at week 1, and gradually increased and was significantly higher at week 2 and 3 than that at week 1. There were no significant differences among weeks 3, 4, 5, and 8. In the regular-JVS mice, there was no significant difference

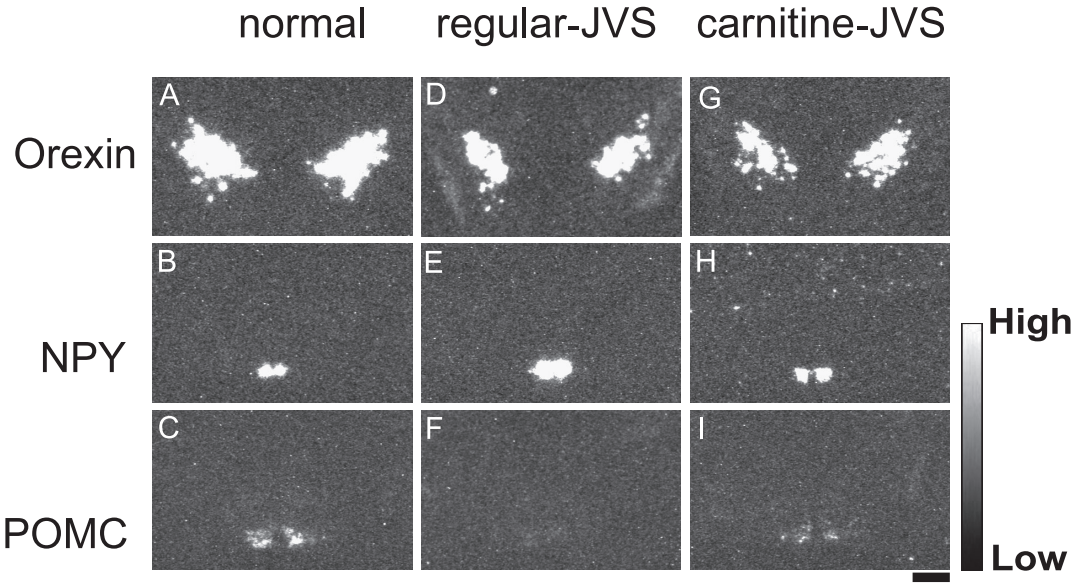


Fig. 7. Representative autoradiographs of corresponding sections hybridized with ^{35}S-labeled oligonucleotide probes for orexin (A, D, and G) in the lateral hypothalamic area, and NPY (B, E, and H) and POMC (C, F, and I) in the arcuate nucleus at week 4 Sections A, B, and C were obtained from normal control mouse. Sections D, E, and F were obtained from regular-JVS mouse. Sections G, H, and I were obtained from carnitine-JVS mouse. 'White' indicates the most intense signal and 'Black' the least intense signal. Scale bar = 1 mm.

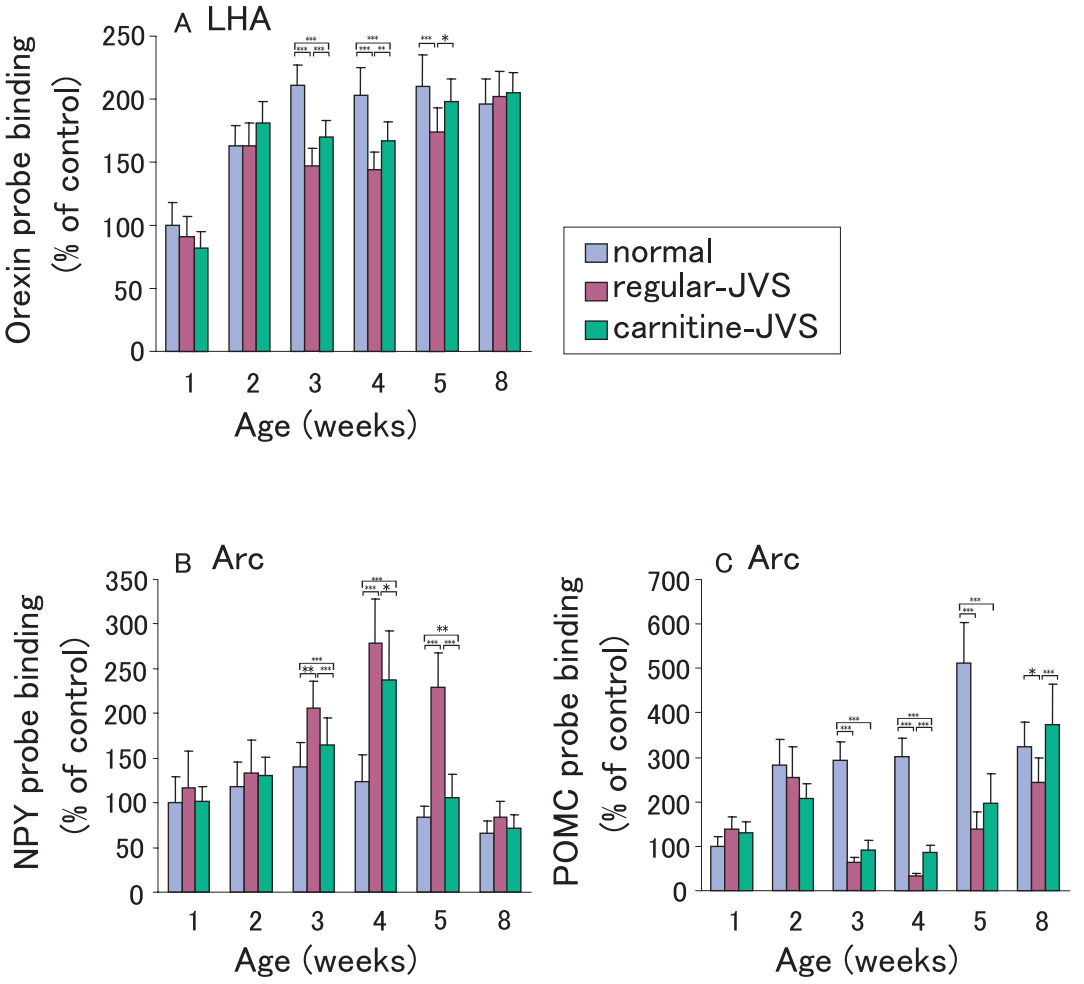


Fig. 8. Effects of carnitine treatment on prevalences of orexin transcript in the lateral hypothalamic area (LHA), NPY transcript in the arcuate nucleus (Arc), and POMC transcript in Arc
Values are percentages of the value obtained at week 1 for the normal control mice. Values are means ± SE (n = 3). **P*<0.05, ***P*<0.01, ****P*<0.001.

in the expression of the orexin gene at week 1 or 2 compared with normal control mice. However, it was significantly lower than in the normal control mice at weeks 3, 4 (Fig. 7A, D), and 5 (Fig. 8A). In the carnitine-JVS mice, orexin gene expression at weeks 3, 4 (Fig. 7D, G), and 5 was significantly higher than in the regular-JVS mice. However, at weeks 3 and 4, it was still lower in the carnitine-JVS mice than in the normal control mice (Fig. 8A).

2) NPY gene expression

NPY gene expression was observed in the arcuate nucleus from weeks 1 to 5 and week 8 after birth. In normal control mice, the expression of the NPY gene was detected at week 1, gradually increased to week 3, and then decreased. In the regular-JVS mice, the expression of the NPY gene was significantly higher than in the normal control mice at weeks 3, 4 (Fig. 7B, E), and 5 (Fig. 8B). NPY gene expression at weeks 3, 4 (Fig. 7E, H), and 5 was significantly lower in the carnitine-JVS mice than in the regular-JVS mice, although the level of the expression in the carnitine-JVS mice at these points of time was still higher than that in the normal control mice (Fig. 8B).

3) POMC gene expression

POMC gene expression was observed in the arcuate nucleus from weeks 1 to 5 and week 8 after birth. In the normal control mice, the expression of the POMC gene was detected at week 1 and was markedly in-

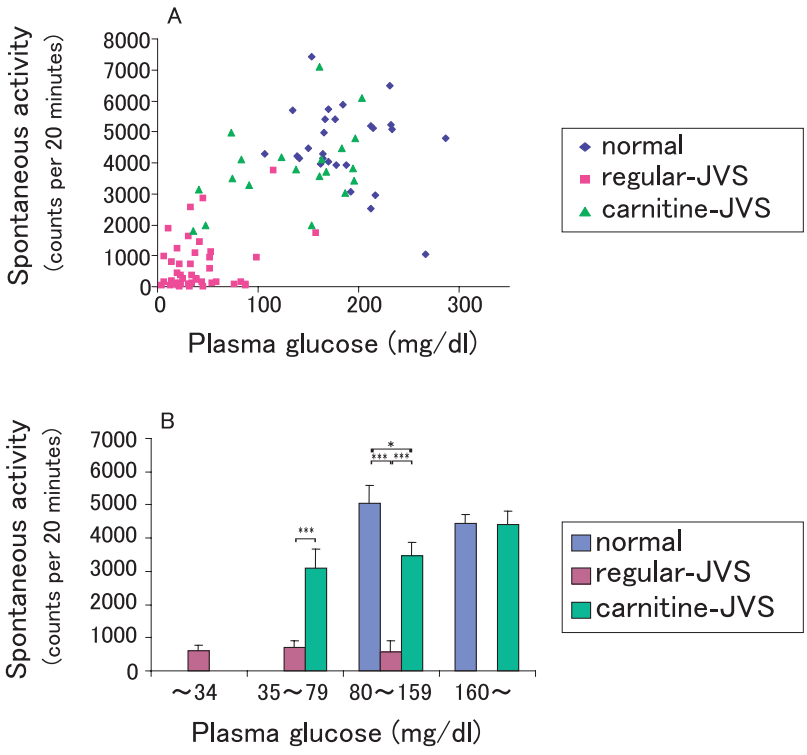


Fig. 9. Relationship between spontaneous activity and plasma glucose level
Spontaneous activity is expressed in count per 20 minutes. See details in text. A: Individual data are plotted along with plasma glucose level. Numbers; normal control mice = 27, regular-JVS mice = 46, carnitine-JVS mice = 20. B: Spontaneous activity for glucose level ranges (–34, 35–79, 80–159, 160 mg/dl–). Numbers; normal control mice = 6–21, regular-JVS mice = 5–28, carnitine-JVS mice = 3–10. Values are means ± SE. **P*<0.05, ****P*<0.001.

creased at weeks 2, 3, 4, 5, and 8. In the regular-JVS mice, expression of the POMC gene was significantly lower than in the normal control mice at weeks 3, 4, 5 (Fig. 7C, F), and 8 (Fig. 8C). In the carnitine-JVS mice, the expression of the POMC gene at week 4 (Fig. 7F, I) and 8 was significantly higher than in the regular-JVS mice. The expression of the POMC gene in carnitine-JVS mice at week 3, 4 and 5 were still lower than that in the normal control mice (Fig. 8C).

Animal behavior

Figure 9A shows a plot of spontaneous activity (Y-axis) against serum glucose level (X-axis). Serum glucose level ranged from 106.9 to 286.7 mg/dl in normal control mice and from 4.4 to 157.9 mg/dl in the regular-JVS mice (Fig. 9A). When the serum glucose level was 35–79 mg/dl, the spontaneous activity for the regular-JVS mice was 690.9 ± 223.5. When serum glucose level was 80–159 mg/dl, the spontaneous activity was 5043.2 ± 529.5 for the normal control mice and 1106.5 ± 591.4 for the regular-JVS mice (P<0.001) (Fig. 9B). In the carnitine-JVS mice, serum glucose level ranged from 35.3 to 203.1 mg/dl (Fig. 9A). When serum glucose level was 35–79 mg/dl, the spontaneous activity for the carnitine-JVS mice was 3084.6 ± 578.4, and 4.5 times that for the regular-JVS mice. When serum glucose level was 80–159 mg/dl, the spontaneous activity for the carnitine-JVS mice was 3470.4 ± 404.3, and 6 times that for the regular-JVS mice but lower than that for the normal control mice (P<0.05). When serum glucose level was above 160 mg/dl, the spontaneous activity did not differ between the carnitine-JVS mice and normal control mice (Fig. 9B). When serum control level was above 106.9 mg/dl, the spontaneous activity for the carnitine-JVS mice was 4164.8 ± 359.8, which did not differ from that for the normal control mice, 4570.3 ± 247.3.

Blood hormone levels on day 24

Blood leptin level during the period with free access

to diet differed significantly between the normal control mice (3.34 ± 0.80 ng/ml, n = 10) and the regular-JVS mice (0.05 ± 0.05 ng/ml, n = 9) (P<0.01). Blood glucagon level was 72.0 ± 8.9 pg/ml in the normal control mice (n = 5) and 218.3 ± 46.0 pg/ml in the regular-JVS mice (n = 4) (P<0.01). Blood insulin level was 136.2 ± 26.9 pg/ml in the normal control mice (n = 4) and 34.2 ± 5.2 pg/ml in the regular-JVS mice (n = 4) (P<0.01). Blood ghrelin level was 23.9 ± 5.0 fmol/ml in the normal control mice (n = 5) and 8.60 ± 4.14 fmol/ml in the regular-JVS mice (n = 4).

After overnight fasting, blood leptin level was 0.43 ± 0.06 ng/ml in the normal control mice (n = 9) and 0.71 ± 0.71 ng/ml in the regular-JVS mice (n = 4). Blood glucagon levels were 173.0 ± 17.0 pg/ml (n = 13) and 353.4 ± 22.0 pg/ml (n = 8) in the same two groups (P<0.001), while blood insulin levels were 79.2 ± 39.1 pg/ml (n = 4) and 35.1 ± 4.0 pg/ml (n = 4) and blood ghrelin levels were 40.6 ± 3.6 fmol/ml (n = 13) and 7.31 ± 0.63 fmol/ml (n = 7) (P<0.001).

Discussion

Of the regular-JVS mice, 90% died from hypoglycemia during the weaning period. The following discussion focused on this period.

First, the causes of hypoglycemia were considered. The energy sources supporting metabolism in the brain are glucose and ketone bodies [3]. We confirmed that simultaneous reduction of both of these was not observed in the regular-JVS mice (Fig. 3). During the weaning period, insulin level exhibited no elevation, and glucagon level rose. The blood level of adiponectin, which decreases blood glucose level [30, 31], was low. These results suggest that hypoglycemia may be due to factors other than these. Because the strength of feeding of the mice appeared to be weak, we checked their gastric contents, and found that they had refused solid diet (Fig. 5H). We therefore concluded that their hypoglycemia was due to anorexia.

Second, the causes of anorexia were considered. Elevated ketone body (Fig. 3) may be one of the factors. However, ketone bodies do not strongly suppress eating [16, 32]. When levels of appetite-related peptides expressions in the hypothalamus were determined, the expression of the gene encoding NPY was found to be high, while that of the gene encoding POMC, which was involved in the formation of α-melanocyte stimulating hormone, was low. These changes may be the result of anorexia-induced starvation [21, 22, 33]. It remains unclear what is responsible for the anorexia caused by carnitine deficiency.

Yamamoto *et al.* found in Sprague-Dawley rats that orexin mRNA level rose dramatically during the weaning period [34], and this change is thought to be associated with feeding behavior during the weaning period [35]. The present study of normal mice also demonstrated this phenomenon. Surprisingly, however, we found that elevation of orexin mRNA was suppressed in regular-JVS mice (Fig. 8). This suppression was partially reversed by replacement of carnitine in JVS mice. Thus, the suppression of orexin mRNA in the hypothalamus during the weaning period may play a role in the induction of anorexia in JVS mice. One possible cause of suppression of orexin expression is reduced blood ghrelin level [36]. However, since all hypoglycemia, hypoleptinemia, elevated NPY expression, and reduced POMC expression can increase the expression of orexin, it seems likely that some unknown mechanism of suppression of orexin expression is operative in regular-JVS mice.

As shown in Figure 9, the regular-JVS mice exhibited reduced activity even in the absence of hypoglycemia. This reduction may also be one of the factors responsible for anorexia, and also appears to be associated with reduced orexin level [37–39].

It has been reported that elevation of long-chain fatty acid levels in the hypothalamus by intracerebroventricular treatment with fatty acid synthase inhibitor or by knocking out of carnitine palmitoyltransferase-1 can induce anorexia [17, 18]. Because brain carnitine level is low in the JVS mice [40], carnitine palmitoyltransferase-1 flux may be reduced in them as well, leading to anorexia through a similar mechanism. On the other hand, some investigators have reported that inhibition of systemic fatty acid degradation with mercaptoacetate or methyl palmoxirate resulted in stimulation rather than suppression of feeding [15, 16]. The details of experiments using such inhibitors in the weaning period are unclear.

Third, the effects of carnitine were considered. In this experiment, carnitine was given in the form of feeding with carnitine-rich chow, rather than by a non-physiological method of administration such as injection. As a result, the carnitine-JVS mice were no longer hypoglycemic and their mortality rate decreased dramatically. One open question is how blood car-

nitine level increased in these mice. As shown in Figures 5L and 6A, gastric carnitine content rose on day 17 after birth, suggesting that the mice had ingested the carnitine-rich chow available at a certain location in the cage or scattered over the floor. On the basis of this finding, we concluded that, feeding with carnitine-rich chow increased their blood carnitine level, resulting in increases in orexin expression, consumption of food, and blood glucose, with avoidance of death. We additionally considered the possibility that the metabolism within the carnitine-JVS mice was modified by the entry of carnitine via the milk of hetero-mothers which had ingested carnitine-rich chow [41]. However, the blood carnitine levels of the carnitine-JVS mice in weeks 1 and 2 after birth did not differ from the regular-JVS mice. Therefore, the likelihood of the above-mentioned mechanism seems quite low, even if it cannot be completely ruled out.

Fourth, whether these findings can be extended to humans is unclear. In the present study, JVS mice, *i.e.*, with carnitine transporter (OCTN2) deficiency, were used. In humans, reduced carnitine level is noted in the presence of OCTN2 deficiency or secondary carnitine deficiency [6, 11]. Although these conditions have diverse symptoms, the findings of the present study are expected to aid the study of eating disorders, behavioral abnormalities and sleep disturbance in humans [42–46].

The findings noted above suggest the following mechanism. For feeding, mice probably have two mechanisms, one for milk feeding and another for solid diet feeding. The milk feeding mechanism begins to operate immediately after birth and ceases about 17 days after birth. It is operative even during carnitine deficiency. The solid diet feeding mechanism then begins to function. This sequence is genetically controlled. It is possible that blood carnitine level needs to be kept normal so that the second mechanism can operate. If blood carnitine level decreases, the second mechanism becomes inoperable.

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