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Dietary GABA and its combination with vigabatrin mimic calorie restriction and induce antiobesity-like effects in lean mice

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ABSTRACT

The anti-obesity and anti-diabetic effects of γ-aminobutyric acid (GABA) are mainly expressed via glucose and insulin regulation in mice. It is unknown whether dietary GABA exerts anti-obesity effects via other pathways. Herein, we report that a high dietary GABA intake (5%) significantly suppressed food intake (−30%), body-weight gain, and fat accumulation, induced ketogenesis, improved glucose metabolism, and elevated the levels of circulating GABA and β-aminoisobutyric acid. These changes suggest that 5% GABA intake possibly induces calorie restriction. Interestingly, a combination of low-dose dietary GABA (0.5% and 2%) and vigabatrin (inhibitor of GABA transaminase (GABA-T)) markedly increased the levels of circulating GABA and strongly exerted antiobesity-like effects. Brain GABA levels increased slightly upon 5% GABA intake, but significantly upon intake of the low-dose GABA–vigabatrin combination. Therefore, the manipulation of peripheral and brain GABA metabolism by targeting GABA-T may lead to the development of novel interventions for overeating and obesity.

1. Introduction

Obesity is a serious global health issue that is linked to metabolic syndromes and various diseases (WHO, 2020). Overeating and imbalanced energy expenditure are major factors that result in overweight, and eventually, obesity (Adan, 2013). Therefore, theoretically, reducing food intake and increasing energy expenditure are the simplest ways to treat obesity. However, obese people tend to fail at controlling food intake and maintaining weight loss. In addition, anti-obesity drugs are not successful treatments, and many have been removed from clinical use owing to their harmful side effects (Adan, 2013). Hence, research on alternative and novel interventions for obesity management is in demand.

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter. Recent research suggests that GABA signaling in the brain plays a crucial role in regulating food intake and energy balance (Delgado, 2013; Tong, Ye, Jones, Elmquist, & Lowell, 2008; Wu, Boyle, & Palmiter, 2009; Yeo & Heisler, 2012). Studies suggest that an imbalance in brain GABA homeostasis is associated with obesity (Basil et al., 2016; Boutin et al., 2003; Broder-Fingert, Brazauskas, Lindgren, Iannuzzi, & Van Cleave, 2014; Daniels, Nick, Liu, Cassedy, & Glauser, 2009; Gaetz et al., 2014; Petroff, Hyder, Rothman, & Mattson, 2001). Changes in the *GAD2* gene, which encodes the glutamic acid decarboxylase enzyme (GAD65) responsible for GABA synthesis, were found to be associated with human obesity (Boutin et al., 2003). Interestingly, overweight/obesity is prevalent in patients with neurological disorders, such as epilepsy (39% vs. 14% of the control subjects), autism (23% vs. 6% of the control subjects), and Down syndrome (48% vs. 12% of the control subjects), whose brain GABA levels are often found to be low (Basil et al., 2016; Broder-Fingert et al., 2014; Daniels et al., 2009; Gaetz et al., 2014; Petroff et al., 2001). Emerging research suggests that GABA is a possible mediator in the gut–brain axis that regulates obesity via both the peripheral and central nervous systems. Transplantation of fecal microbiota from lean donors to obese patients was found to improve insulin sensitivity, in which GABA is the greatest increase and change among all plasma metabolites with an increase in GABA producers, *Lactobacillus* bacteria (Kootte et al., 2017). Oral intake of GABA-producing probiotics such as *Lactobacillus rhamnosus* and *Lactobacillus brevis* elevated GABA levels in

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gut and brain with the improvement of obesity and depressive behavioral abnormalities via the vagus nerve (Bravo et al., 2011; Janik et al., 2016; Patterson et al., 2019). Although these observations suggest a possible link between peripheral and brain GABA homeostases and obesity, the exact mechanisms remain unclear, and more studies are needed for their elucidation.

So far, dietary GABA has been mostly reported to exert anti-obesity effects through its peripheral actions on improving glucose metabolism via the regulation of pancreatic β-cell function and on antioxidant defense and anti-inflammation (Hwang et al., 2019; Tian et al., 2011; Untereiner et al., 2019; Xie, Xia, Qiao, Shi, & Le, 2015; Xie, Xia, & Le, 2014). However, other possible mechanisms underlying its anti-obesity effects have not been investigated. Moreover, there has been no study concerning the effects of GABA administration on feeding behavior (food intake) and brain GABA metabolism.

In our previous study, we surprisingly found that dietary GABA highly suppressed food intake and body weight gain in lean mice (Kumrungsee et al., 2020). Thus, this study aimed to elaborate the previous finding by further elucidating the mechanisms underlying antiobesity-like effects of dietary GABA through assessing plasma lipid and glucose profiles, peripheral and brain GABA levels, and other plasma metabolites. Additionally, to determine if increased endogenous levels of peripheral and brain GABA contribute to antiobesity-like effects, vigabatrin, a GABA degrading enzyme (GABA transaminase (GABA-T)) inhibitor, generally used as a drug for epilepsy treatment via the elevation of brain GABA levels (Blancquaert et al., 2016; Walzer et al., 2011), was applied in this study.

2. Materials and methods

2.1. Animals and GABA diets

It is to be noted that the present study presents an elaboration on results in my previous work (Kumrungsee et al., 2020).

All the mice used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals established by Hiroshima University, and the procedures were approved by the Ethics Committee of the University (Ethical Approval No. 17–19). The mice were housed in metal cages, in groups of two, in a temperature-controlled room (24 ± 1 °C) with a 12 h light/dark cycle (lights on from 08:00–20:00). The mice had free access to food and drinking water, and were acclimatized with a non-purified commercial rodent diet (MF, Oriental Yeast, Tokyo, Japan) for 7 days before being subjected to experiments.

A total of 32 male albino ICR mice (5 weeks old, Charles River Japan, Hino, Japan) were randomly divided into four groups ($n = 8$/group) and received a basal diet mixed with 0 (control), 5 (0.5%), 20 (2%), or 50 (5%) g GABA/kg diet for 6 weeks. The basal diet was composed of the following components (g/kg diet): α-cornstarch, 402; casein, 200; sucrose, 200; corn oil, 100; cellulose, 50; AIN-93G mineral mixture, 35; AIN-93 vitamin mixture, 10; and L-cystine, 3, as described previously (Komatsu et al., 2001; Reeves, Nielsen, & Fahey, 1993). Two mice were house together in one cage. The body weight and food intake of mice were measured every 2 days, in which food intake was measured per cage. At the end of each experiment, all the mice were fasted for 6 h before being sacrificed under isoflurane anesthesia (between 13:00 and 15:00). Blood was collected from their abdominal veins into tubes containing heparin, an anticoagulant, on ice. Then, plasma was obtained by centrifugation at 3,500 rpm for 20 min, and stored at −80 °C. Gastrocnemius muscles, soleus muscles, colon, liver, kidney, heart, spleen, and fat tissues were harvested immediately, weighed, snapped frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.2. Vigabatrin administration

A total of 30 male albino ICR mice (6 weeks of age at the beginning of the treatment) were divided into five groups ($n = 6$/group) and received the basal diet mixed with 0 (control), 5 (0.5%), or 20 (2%) g GABA/kg diet with or without vigabatrin injection for 2 weeks. Two mice were house together in one cage. The body weight and food intake of mice were measured every day, in which food intake was measured per cage. GABA-degrading enzyme inhibitor vigabatrin (Sabril®, Sanof Aventis, Patheon, France) at a dose of 250 mg/kg body weight was administered daily by subcutaneous (SC) injection to mice fed 0.5% or 2% GABA diets. The same volume was used for saline injections to mice fed 0% (control), 0.5%, or 2% GABA diets. Two mice were removed during the experiment due to fighting with their mates (leaving $n = 5$ in some groups). At the end of each experiment, all the mice were fasted for 6 h before being sacrificed under isoflurane anesthesia (between 13:00 and 15:00). The last vigabatrin injection was 3 h before sacrifice. Blood, gastrocnemius muscles, soleus muscles, colon, liver, kidney, heart, spleen, and fat tissues were collected, handled, and stored as described in Section 2.1.

2.3. Analysis of plasma lipid and glucose

Triacylglycerol (TAG), free fatty acid (FFA), ketone body, and glucose levels were determined as described previously (Limpimwong, Kumrungsee, Kato, Yanaka, & Thongngam, 2017). Briefly, plasma samples were analyzed using a Beckman Coulter AU480 analyzer (Beckman Coulter, Krefeld, Germany), an automated chemistry instrument for turbidimetric, spectrophotometric, and ion-selective electrode measurements, according to the manufacturer's protocol.

2.4. Analysis of GABA and β-aminoisobutyric acid (BAIBA) in plasma, liver, and muscle

Four samples from each group were randomly picked and subjected to GABA and BAIBA (Sigma-Aldrich, St. Louis, MO, USA) measurement using an *o*-phthalaldehyde (OPA)-based high-performance liquid chromatography (HPLC) method described previously (Kamisaki et al., 1990; Kumrungsee et al., 2019), with some modification. Briefly, plasma was extracted using acetonitrile at a ratio of 1:3 (plasma:acetonitrile), and liver or muscle tissue was homogenized in 8 volumes of methanol. Then, the extracts were subjected to centrifugation at 15,000*g* for 15 min at 4 °C to collect the supernatants. A Cosmosil 5C18-MS-II column (4.6 × 150 mm; Nacalai Tesque) with isocratic elution of 0.1 mol/L sodium citrate (pH 3.5)–acetonitrile–methanol [60:30:10 (v/v)] and a fluorometric detector set at an excitation wavelength of 350 nm and emission wavelength of 440 nm was used to monitor the compounds. Representative HPLC chromatograms of GABA and BAIBA in standard solutions and sample supernatants are shown in Supplementary Fig. 1. The limits of detection for GABA and BAIBA are 0.36 pmol and 0.33 ρmol, respectively.

2.5. Analysis of GABA in brain

Cortex and hippocampal tissues were homogenized in 8 volumes of methanol containing an internal standard (20 μM methionine sulfone), as previously reported (Kumrungsee et al., 2019; Soga et al., 2003). Then, the supernatants were concentrated via evaporation to dryness and re-suspended in methanol before analysis. GABA was detected using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) (Waters, Milford, MA), as described previously (Waterval, Scheijen, Ortmans-Ploemen, Habets-van der Poel, & Bierau, 2009), with some modifications. Briefly, liquid chromatography was performed at 30 °C using an Acquity UPLC BEH C18 (1.7 μm, 2.1 × 50 mm) column (Waters) and a gradient system with the mobile phase consisting of buffer A (5 mM perfluoroheptanoic acid (PFHpA; Sigma-Aldrich, Louis, MO) in Milli-Q water) and buffer B (5 mM PFHpA in methanol), at a flow rate of 400 μL/min. The gradient program was started with an initial condition of 95% A and 5% B. Then, a linear gradient from 40% to 50% B was applied in 10 min, followed by 50% to

100% B in 0.5 min with a holding period of 1 min. The gradient was finally reinstated to the initial conditions in 0.5 min, with equilibration for 5 min before the next injection. The run-to-run time was 17.5 min. The injected volume was 5 μL. Mass spectrometric analysis was performed via multiple reaction monitoring (MRM) in the ESI-positive mode. The desolvation and source temperatures were 400 and 120 °C, respectively. The capillary voltage, cone voltage, and MRM were set at 3 kV, 10 V, and 103.9 > 86.9 *m/z*, respectively, as described previously (Kumrungsee et al., 2020). Nitrogen gas was used in both the desolvation and cone gas flows. MRM and daughter-ion scans were performed using argon as the collision gas.

2.6. Statistical analysis

The number of animals in each experiment is stated in the respective figure captions. All the values were expressed as means with their standard deviations (SD). Statistical comparison of two groups was performed by the unpaired Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparisons test for more groups. For body weight, two-way ANOVA followed by Tukey's multiple comparisons test and Bonferroni's multiple comparisons test was applied. GraphPad Prism 8 (GraphPad Software, CA, USA) was used for all analyses. For all the tests, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Dietary GABA suppresses food intake and body weight gain

Compared with the control group (0% GABA), the 5% GABA intake group significantly reduced food intake (−30%, $P = 0.0007$) and suppressed body weight gain (−80%, $P < 0.0001$); however, there were no significant differences in these parameters between the control, 0.5% GABA intake, and 2% GABA intake groups (Fig. 1A and B). Notably, 5% GABA intake suppressed body weight gain, but did not induce body weight loss; the final body weight was similar to the initial body weight (35.1 ± 2.5 vs. 32.1 ± 0.5 g, $P = 0.272$, Fig. 1B).

3.2. Dietary GABA suppresses fat accumulation, but conserves skeletal muscle and colon masses

As shown in Fig. 1C, the epididymal white adipose tissue (eWAT) weight per gram body weight of the 5% GABA intake group, but not those of the 0.5% and 2% GABA intake groups, was significantly lower than that of the control group (−68%, $P < 0.0001$). Interestingly, the gastrocnemius muscle and colon weights per gram body weight of the 5% GABA intake group were higher than those of the control group ($P = 0.001$ and $P < 0.0001$, respectively, Fig. 1D and E). Compared to those of the control group, the weights of the eWAT, gastrocnemius muscle, and colon in the 5% GABA intake group were −76%, −7%, and +5%, respectively (Supplementary Table 1). Other tissue weights per gram body weight, including those of the liver, kidney, heart, spleen, and soleus muscle, were not significantly different among the groups (data not shown).

3.3. Dietary GABA affects plasma lipid and glucose profiles

As shown in Table 1, the 5% GABA intake group exhibited a decrease in plasma TAG levels compared with those of the control group ($P = 0.052$), but no significant difference in FFA levels. The levels of ketone bodies were slightly higher in the 5% GABA intake group than in

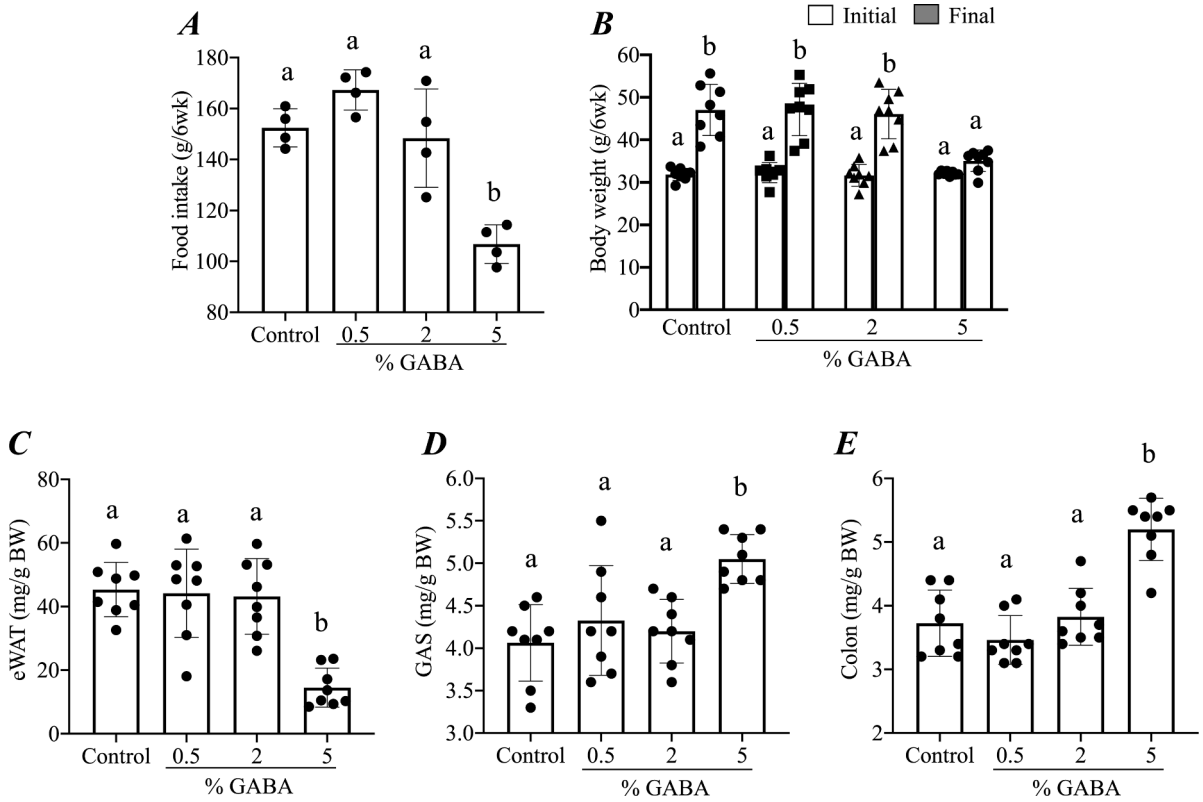

Fig. 1. Effect of GABA intake on food consumption per mouse (A), body weight (B), and tissue weight (C–E). The weights of epididymal white adipose tissue (eWAT), gastrocnemius (GAS) muscle, and colon are reported per gram body weight. Values are expressed, with individual data points (dots), as means ± SD ($n = 4$ and 8). Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test. For body weight, two-way ANOVA followed by Tukey's multiple comparisons test and Bonferroni's multiple comparisons test was applied. The different letters (a and b) above the bars indicate significant differences between groups; *P* values < 0.05 were considered significant. To be noted, food consumption (A) and body weight (B) are represented from the previous study (Kumrungsee et al., 2020).

Table 1
Effect of GABA intake on plasma lipid and glucose parameters.

	Control	5% GABA	*P* value
TAG (mg/dL)	314 ± 223	132 ± 93	0.052
FFA (μmol/L)	466 ± 104	418 ± 66	0.281
Ketone bodies (μmol/L)	116 ± 29	336 ± 341	0.092
Glucose (mg/dL)	140 ± 22	110 ± 18	0.010

All values are expressed as means ± SD ($n = 8$). *P* values vs. control were determined using the unpaired Student's *t*-test. TAG: triacylglycerol; FFA: free fatty acid.

the control group ($P = 0.092$). The concentrations of fasting blood glucose in the 5% GABA-fed mice were significantly lower than those in the controls ($P = 0.010$).

3.4. Dietary GABA increases GABA levels in plasma and brain and BAIBA levels in plasma and liver

Compared with the control group, the 5% GABA intake group had significantly elevated plasma GABA concentrations (0.4 ± 0.5 vs. 5.5 ± 2.4 μM, $P = 0.0003$), whereas the other GABA intake groups showed no significant differences (Fig. 2A). To determine if dietary GABA could elevate GABA levels in the brain, the GABA levels in the cortex and hippocampus were measured. No dietary GABA intake level had any effect on the GABA concentrations in the cortex, but 5% GABA intake slightly increased the GABA concentrations in the hippocampus (3.9 ± 0.5 vs. 5.0 ± 1.0 μmol/g, $P = 0.0617$).

Having the same molecular weight as that of GABA, BAIBA was measured in this study. Since BAIBA has been proposed to be myokine, a compound secreted from skeletal muscle, we measured its concentrations in gastrocnemius muscles and found that BAIBA was undetectable under the measurement conditions used in this study (data not shown). In plasma, the BAIBA levels of the 0%, 0.5%, and 2% GABA intake groups were not significantly different from each other, while these three groups had lower levels than that of the 5% GABA intake group (Fig. 2B). Plasma BAIBA levels exhibited a positive correlation with plasma GABA levels ($r = 0.733$, $P = 0.0012$, Fig. 2C). In the liver, the BAIBA levels in the 5% GABA fed-mouse livers were significantly higher than those in mice from other groups (9.3 ± 2.0 (cont) vs. 29.5 ± 2.9 (5% GABA) nmol/g, $P < 0.0001$, Fig. 2D). The plasma BAIBA concentrations reported herein are in a range similar to those of previous works demonstrating mouse and human plasma compositions (Roberts et al., 2014; Stautemas et al., 2019).

3.5. Vigabatrin administration inhibits GABA-T activity in liver, elevates plasma GABA levels, and decreases food intake and body weight

To confirm that the suppression of food intake observed in the 5% GABA intake group was not due to a distaste for the food, we injected vigabatrin (a GABA-degrading enzyme inhibitor) into mice receiving 0.5% or 2% GABA, which were the GABA intake levels that had no effect on food intake (Fig. 1A). As a result, vigabatrin administration significantly inhibited GABA-T activity in the liver, a primary organ that highly expresses GABA-T (Blancquaert et al., 2016), in both 0.5% and 2% GABA intake groups (Kumrungsee et al., 2020). As shown in Fig. 3A, compared to the control group, 0.5% and 2% GABA intake without vigabatrin injection (Vig (−)) did not increase the plasma GABA levels. However, upon injection, vigabatrin inhibited GABA-T activity in the

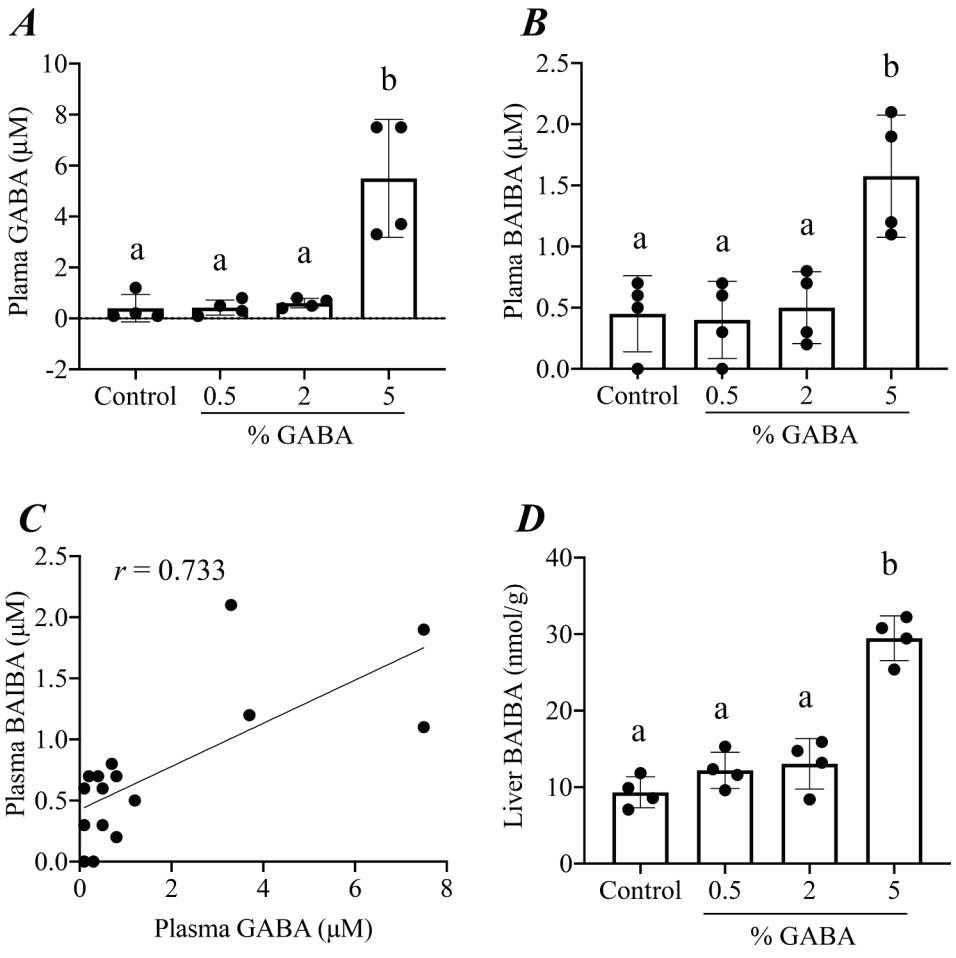

Fig. 2. Effect of GABA intake on GABA and BAIBA levels in plasma and liver. The levels of plasma GABA (A), plasma BAIBA (B), and hepatic BAIBA (D) were analyzed using OPA-HPLC and UPLC-MS/MS, as described in Section 2. Values are expressed, with individual data points (dots), as means ± SD and per wet weight of tissues ($n = 4$). The differences in the numbers of data points ($n = 4$) and animal per group ($n = 8$) are due to random picking of tissue and plasma samples from mice in each group for extraction and analysis, and not due to cutting of data. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test. The different letters (a and b) above the bars indicate significant differences between groups; *P* values < 0.05 were considered significant. Pearson's correlation coefficient analysis was applied to show a correlation between plasma GABA levels and plasma BAIBA levels (C).

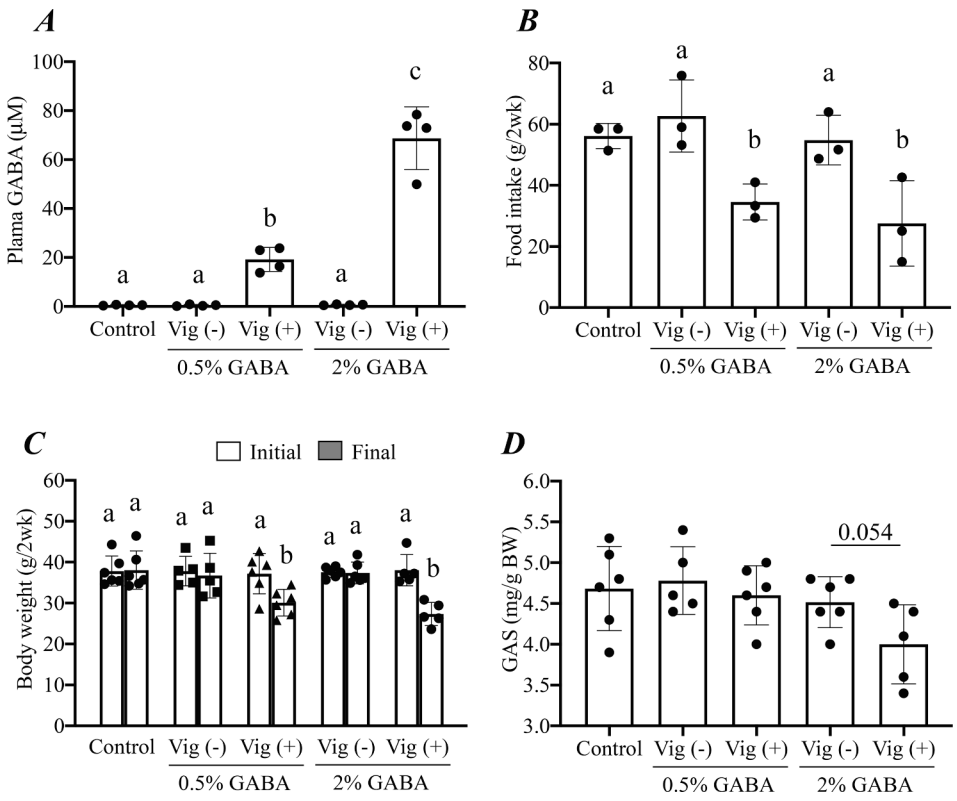

Fig. 3. Effect of vigabatrin administration on GABA levels in plasma (A), food intake (B), body weight (C), and gastrocnemius (GAS) muscle (D). The levels of plasma GABA were analyzed using OPA-HPLC, as described in Section 2. The differences in the numbers of data points in (A) and (B) ($n = 4$) and animal per group ($n = 5$–6) are due to random picking of tissue and plasma samples from mice in each group for extraction and analysis, and not due to cutting of data. The body weight and food intake of mice were measured every day. The weights of GAS muscle are reported per gram body weight. Values are expressed, with individual data points (dots), as means ± SD ($n = 3$–6). Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test. For body weight, two-way ANOVA followed by Tukey's multiple comparisons test and Bonferroni's multiple comparisons test was applied. The different letters (a, b, and c) above the bars indicate significant differences between groups; P values < 0.05 were considered significant.

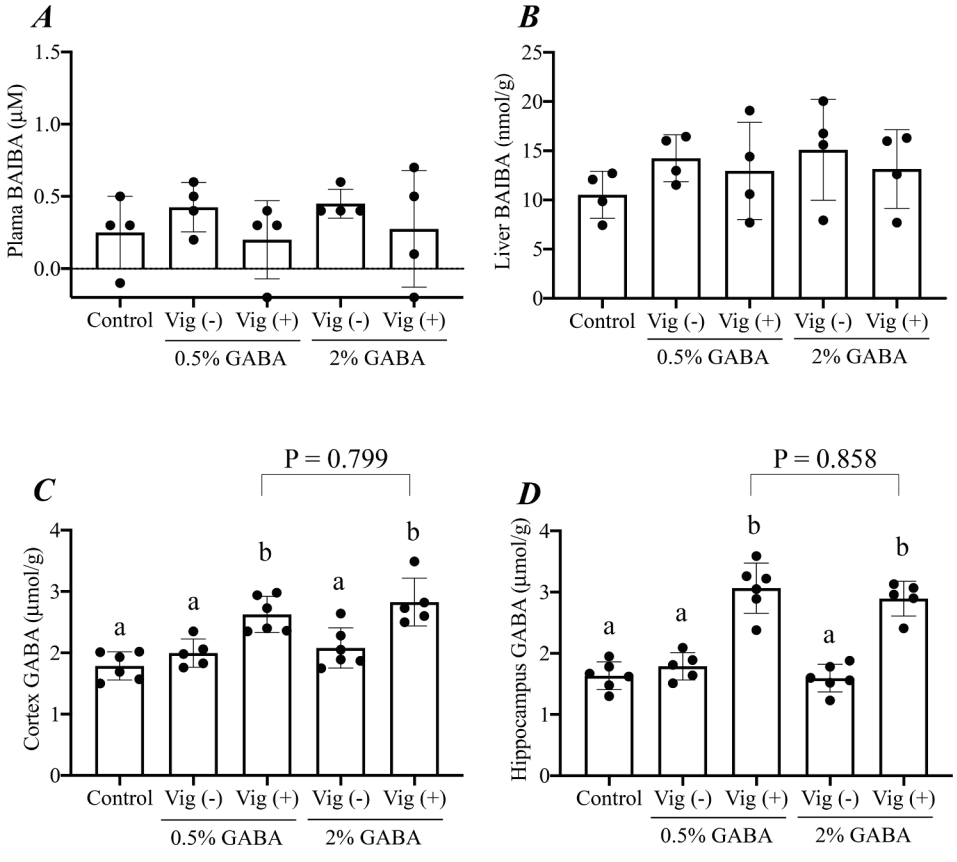

Fig. 4. Effect of vigabatrin administration on BAIBA levels in plasma and liver and GABA levels in cortex and hippocampus. The levels of plasma BAIBA (A), hepatic BAIBA (B), cortex GABA (C), and hippocampal GABA (D) were analyzed using OPA-HPLC and UPLC-MS/MS, as described in Section 2. Values are expressed, with individual data points (dots), as means ± SD and per wet weight of tissues ($n = 3$–6). The differences in the numbers of data points in (A) and (B) ($n = 4$) and animal per group ($n = 5$–6) are due to random picking of tissue and plasma samples from mice in each group for extraction and analysis, and not due to cutting of data. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test. The different letters (a and b) above the bars indicate significant differences between groups; P values < 0.05 were considered significant.

liver and then protected GABA from the degradation pathway, resulting in highly increased GABA concentrations in the plasma, as shown in Fig. 3A (19.2 ± 4.9 vs. 0.5 ± 0.3 μM for 0.5% GABA intake with (Vig (+)) or without (Vig (−)) vigabatrin injection, respectively, $P = 0.0046$; and 68.7 ± 12.8 vs. 0.7 ± 0.2 μM for 2% GABA intake with (Vig (+)) or without (Vig (−)) vigabatrin injection, respectively, $P < 0.0001$).

Although the treatment period was short (2 weeks), vigabatrin administration highly suppressed food intake (−45% in the 0.5% GABA intake group, $P = 0.0297$, and −50% in the 2% GABA intake group, $P = 0.0356$, Fig. 3B). Notably, vigabatrin administration induced body weight loss in both the 0.5% and 2% GABA intake groups, as shown in Fig. 3C, in which the final body weight was lower than the initial body weight (37.2 ± 4.9 (initial) vs. 30.1 ± 3.3 (final) g for 0.5% GABA intake with vigabatrin injection (Vig (+)), $P < 0.0001$; and 38.0 ± 3.8 (initial) vs. 27.3 ± 2.8 (final) g for 2% GABA intake with vigabatrin injection (Vig (+)), $P < 0.0001$).

Vigabatrin administration had no effect on tissue weights (per gram body weight) of the liver, kidney, heart, spleen, and soleus muscle; however, eWATs were eliminated in all the mice treated with vigabatrin (data not show). Vigabatrin had no effect on gastrocnemius muscle weight, but slightly decreased the muscle weight in the 2% GABA intake group ($P = 0.054$, Fig. 3D).

3.6. *Vigabatrin administration has no effect on BAIBA levels in plasma and liver but increases GABA levels in brain*

Since plasma GABA levels had a positive correlation with plasma BAIBA levels (Fig. 2C), we were interested to determine if plasma GABA levels increased by vigabatrin could exhibit the same trend. However, there was no significant difference in plasma and hepatic BAIBA levels among the groups (Fig. 4A and B). Since GABA-T is highly expressed in the brain (Blancquaert et al., 2016) and vigabatrin administration is generally used as a treatment for epilepsy via the elevation of brain GABA levels (Walzer et al., 2011), we hypothesized that vigabatrin administration may exert antiobesity-like effects via central nervous system. We found that vigabatrin administration significantly increased the GABA levels in both the cortex (2.6 ± 0.3 vs. 2.0 ± 0.2 μmol/g for 0.5% GABA intake with (Vig (+)) or without (Vig (−)) vigabatrin injection, respectively, $P = 0.0157$; and 2.8 ± 0.4 vs. 2.1 ± 0.3 μmol/g for 2% GABA intake with (Vig (+)) or without (Vig (−)) vigabatrin injection, respectively, $P = 0.0034$) and hippocampus (3.1 ± 0.4 vs. 1.8 ± 0.2 μmol/g for 0.5% GABA intake with (Vig (+)) or without (Vig (−)) vigabatrin injection, respectively, $P < 0.0001$; and 2.9 ± 0.3 vs. 1.6 ± 0.2 μmol/g for 2% GABA intake with (Vig (+)) or without (Vig (−)) vigabatrin injection, respectively, $P < 0.0001$) (Fig. 4C and D). Despite the plasma GABA levels in the 2% GABA intake group being significantly higher than those in the 0.5% GABA intake group by 2.6-fold owing to vigabatrin administration (Fig. 3A), vigabatrin-induced elevated GABA levels in the cortex and hippocampus were not significantly different between the 0.5% and 2% GABA intake groups ($P = 0.799$ (cortex) and $P = 0.858$ (hippocampus), Fig. 4C and D). This suggests that the increase in brain GABA levels are mostly attributable to the effect of vigabatrin on brain GABA metabolism, and not to that on peripheral GABA metabolism. Although the GABA-T activity in the brain was not measured in this study owing to the limitation in tissue amount, it can be postulated that vigabatrin administration inhibits brain GABA-T activity subsequent to an increase in brain GABA concentrations.

4. Discussion

In this study, we demonstrated that a high dose of GABA (5%) significantly suppressed food intake and body weight gain in a period (6 weeks) shorter than that observed in previous studies (over 20 weeks) (Hwang et al., 2019; Soltani et al., 2011; Tian et al., 2011; Untereiner et al., 2019; Xie et al., 2015; Xie, Xia, & Le, 2014). It seems likely that a decrease in body weight gain is mostly attributable to enhanced body fat utilization, as reflected by a similar decrease in fat tissue (−68% (Fig. 1C) and −76% (Supplementary Table 1)) with body weight gain (−80%, Fig. 1B). Since 5% GABA intake significantly elevated the levels of circulating GABA, which accompanied a decrease in plasma TAG levels and an increase in plasma ketone body levels, we hypothesized that this elevated level of plasma GABA may induce a shift in fuel sources toward lipid and ketone utilization. In addition, 5% GABA intake lowered the levels of fasting blood glucose, which possibly suggests its effects on improving insulin sensitivity via the regulation of β-cell function, as previously reported (Hwang et al., 2019; Purwana et al., 2014; Soltani et al., 2011; Tian et al., 2011; Untereiner et al., 2019). All changes mentioned above with a 30% decrease in food intake indicate that 5% dietary GABA possibly induced the mice to undergo calorie restriction.

To date, GABA has been reported to exert anti-obesity effects via its anti-inflammatory, antioxidant, glucose metabolism–improving, and β-cell–regulating functions (Hwang et al., 2019; Tian et al., 2011; Untereiner et al., 2019; Xie et al., 2015; Xie, Xia, & Le, 2014). For mice fed a high-fat diet, addition of GABA (0.6–2 mg/mL) to drinking water for over 20 weeks suppressed body weight gain and decreased body fat mass (Tian et al., 2011; Xie et al., 2015; Xie, Xia, & Le, 2014). For lean and diabetic mice, addition of GABA (6 mg/mL) to drinking water for over 10–20 weeks improved glucose metabolism with an increase in β-cell mass and function but no change in body weight (Purwana et al., 2014; Soltani et al., 2011; Untereiner et al., 2019). In a human study (healthy subjects), oral administration of GABA (2–6 g/day) led to an increase in the levels of circulating insulin and glucagon, but no significant changes in the insulin-to-glucagon ratio and plasma glucose levels. Based on these studies, the anti-obesity effects of GABA have mostly been investigated through its peripheral effects, especially on pancreatic β-cells. Most studies neglect to report the peripheral circulating levels of GABA and plasma metabolites. Moreover, there has been no study concerning the effects of GABA administration on feeding behavior (food intake) and brain GABA metabolism.

In the present study, we found that 5% GABA intake highly elevated the levels of circulating GABA and BAIBA. Recently, BAIBA was proposed to be a myokine, a compound produced and released by muscle fibers, especially during exercise, and that mediates a cross-talk between skeletal muscle and adipose tissue to exert a fat-burning effect (Roberts et al., 2014; Stautemas et al., 2019). Roberts et al. (2014) demonstrated that muscle cells that overexpressed peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, a transcriptional coactivator that regulates the response of muscles to exercise, secreted high amounts of both BAIBA and GABA. This team also demonstrated that dietary BAIBA increased the levels of circulating BAIBA from approximately 1 to 2 μM, which is in a range similar to that reported herein (Roberts et al., 2014). This increase in the level of circulating BAIBA caused a decrease in body weight gain and body fat, as well as improved glucose tolerance in lean mice (Roberts et al., 2014), which is similar to the phenomenon observed in our study. Interestingly, Steinhauser et al. (2018) reported a coincident increase in plasma GABA, BAIBA, and ketone bodies at an early stage of fasting in healthy human subjects. This finding suggests the relationship of GABA and BAIBA with fatty acid oxidation and energy expenditure, similar to our result showing a positive correlation between plasma GABA and BAIBA levels (Fig. 2C). BAIBA was found to enhance hepatic fat oxidation, causing fat wasting and ketogenesis in lean mice (Maisonneuve et al., 2004). GABA itself has also been demonstrated to exert direct effects on fatty acid oxidation in human endothelial cells (Sen et al., 2016). Moreover, oral administration of BAIBA to mice that were partially deficient in leptin (ob/+) and fed a high-calorie diet was found to restore plasma leptin levels, improve glucose tolerance, limit hypertriglyceridemia, increase fatty acid oxidation and ketogenesis, and reduce body weight gain and fat mass (Begriche et al., 2008). These findings led us to hypothesize that the antiobesity-like effects of dietary GABA observed in our study are possibly attributable, at least partly, to an elevation in the levels of

circulating BAIBA, in addition to the effects of GABA itself. However, further studies to demonstrate the BAIBA levels in fat tissues are needed to support this speculation.

Since we found that BAIBA was undetectable in muscles, it can be speculated that the liver may be a new target organ for BAIBA production, which has been also proposed by the recent study (Stautemas et al., 2019). Generally, BAIBA has two enantiomers of R-BAIBA and S-BAIBA, which are derived from thymine and valine catabolic pathways, respectively (Stautemas et al., 2019). Enzymes in both catabolic pathways are highly present in liver and kidney; especially, GABA-T, an enzyme in the final step of S-BAIBA production from valine catabolism, is highly expressed in the liver, but quite poorly expressed in muscles (Van Kuilenburg, Van Lenthe, & Van Gennip, 2006; Blancquaert et al., 2016). From these notion, it can be hypothesized that suppression of food intake by 5% dietary GABA induces mice to undergo calorie restriction that in turn increases valine catabolism and S-BAIBA production in the liver. This hypothesis can be supported by the previous study reporting a significant increase in plasma branched-chain amino acids and BAIBA in human subjects under fasting condition (Steinhauser et al., 2018). Moreover, an increase in plasma and hepatic BAIBA levels was not observed after vigabatrin treatment (Fig. 4A and 4B); this was possibly because hepatic GABA-T was almost completely suppressed, and hence there was no available active GABA-T to convert the valine catabolite to S-BAIBA. In the present study, total BAIBA (both S-BAIBA and R-BAIBA) was measured. It is of great interest to further examine if 5% dietary GABA contributes to R-BAIBA production, since thymine degradation also takes place in liver (Stautemas et al., 2019). To date, there have been three main approaches to increase endogenous BAIBA levels: exercise, direct BAIBA intake, and starvation. Our study is the first to demonstrate that dietary or nutritional factors can stimulate endogenous BAIBA synthesis. This study may inspire a new idea for developing dietary interventions for obesity that induce endogenous BAIBA synthesis.

To explain the suppressive effects of 5% GABA intake on feeding behavior and body weight gain, we propose three possible mechanisms, namely, distaste for food, peripheral effects, and central nervous effects. Interestingly, studies on the effects of dietary amino acids, including GABA, on feeding behavior also demonstrated that a high dose of dietary GABA (2–5%), similar to the dose used in our study, strongly suppressed the food intake and growth of various animals such as mice, rats, and cats (Tews, Repa, Nguyen, & Harper, 1985; Tews, Repa, & Harper 1984; Tews, 1981; Tews, Riegel, & Harper, 1980). These results suggested that the mechanism underlying the suppression of food intake by GABA does not depend solely on the responses to taste and odor. They demonstrated that intubation or injection of GABA suppressed food intake by rats, indicating that the effects are not directly due to the presence of GABA in the diet (Tews, Repa, & Harper, 1988). The addition of certain flavoring agents, such as condensed milk and saccharin, to the diet, in order to increase the palatability, did not increase the consumption of the GABA-containing diet by rats (Tews, Repa, & Harper, 1988, 1984). Interestingly, the deleterious effects of dietary GABA on food intake were suppressed when the plasma GABA levels were decreased by increasing hepatic GABA-T activity (Tews, Rogers, Morris, & Harper, 1984; Tews, 1981; Tews et al., 1980), which is similar to our finding that a decrease in food intake (Fig. 1A) is associated with an increase in plasma GABA levels (Fig. 2A). With the belief that GABA does not cross the blood–brain barrier (BBB), they concluded that the suppressive effects of dietary GABA on food intake and growth were not due to the central nervous functions, but due to the action of GABA on its receptors in the gut, which made animals fall sick, or due to its peripheral effects via certain metabolic products. In our study, we found that 5% GABA intake slightly increased GABA levels in the hippocampus (+28%, Kumrungsee et al., 2020), suggesting that dietary GABA may be able to cross the BBB, especially in the hippocampus, where the permeability of the BBB is high (Liu, Wang., Zhang, Wei, & Li, 2012; Vorbrodt, Dobrogowska, Ueno, & Tarnawski, 1995). Since the GABA levels reported herein are the 6 h fasting levels, with most of the absorbed GABA probably degraded by GABA-T in the brain (Tews, Rogers, Morris, & Harper, 1984), it is of much interest to test if a greater increase in hippocampal GABA levels will be observed in the morning, when the greatest food intake in the dark has just finished. Taken together with previous findings (Tews, Repa, & Harper, 1988, 1984; Tews et al., 1985, 1984; Tews, 1981; Tews et al.,1980), the results suggest that the suppressive effects of 5% GABA intake on food intake and body weight gain may be attributable to its peripheral effects, i.e., an increase in energy expenditure or body fat utilization, possibly by GABA and BAIBA, and an improvement in glucose metabolism, possibly by β-cell regulation, and to its central nervous effects. Although the effects of taste and odor of GABA are possibly negligible, some minor adverse effects such as the dizziness and sore throat reported in a human study (Li et al., 2015) cannot be ignored, and need to be further examined.

To obtain more evidence to explain the antiobesity-like effects of dietary GABA, we injected vigabatrin, a GABA-degrading enzyme inhibitor, into mice receiving 0.5% or 2% GABA, the low levels of dietary GABA that had no effect on food intake and body weight. Vigabatrin strongly decreased hepatic GABA-T activity, resulting in an increase in the levels of circulating and brain GABA and decreases in food intake and body weight. This confirmed our hypothesis that depressed food intake is not likely due to distaste for the GABA diet, but possibly due to peripheral or central nervous effects. Under vigabatrin treatment, it appears that the level of dietary GABA causes a significant change in the levels of circulating GABA (+3656% and +9575% for 0.5% and 2% GABA, respectively, Fig. 3A), but small changes in food intake suppression (−45% and −50% for 0.5% and 2% GABA, respectively, Fig. 3B). Taken together with the fact that vigabatrin also affects GABA-T in the brain, these results suggest that central nervous system GABA may have strong effects on food intake and body weight gain in addition to its peripheral effects. Indeed, vigabatrin clearly increased the GABA levels in the cortex by +32% and +36%, and in the hippocampus by +71% and +82%, for 0.5% and 2% GABA intake, respectively.

In the brain, the hypothalamus is the control center of feeding behavior. GABA exerts different effects on feeding or appetite in different parts of the hypothalamus (de Vrind, Rozeboom, Wolterink-Donselaar, Luijendijk-Berg, & Adan, 2019; Delgado, 2013; Holmberg et al., 2018; Tong et al., 2008; Vong et al., 2011). In the lateral hypothalamic (LH) area, GABA stimulates satiety, decreasing feeding, whereas in the paraventricular nucleus (PVN), it stimulates appetite, promoting hunger and feeding (Delgado, 2013; Holmberg et al., 2018; Rada, Mendialdua, Hernandez, & Hoebel, 2003). Injection of a GABA agonist into the LH area decreases feeding, whereas injection of a GABA antagonist increases feeding (Kelly, Alheid, Newberg, & Grossman, 1977; Tsujii & Bray, 1991). Rada et al. (2003) showed that at the end of the meal, when satiation is reached, GABA levels in the LH area increase significantly and peak, in order to stimulate satiety and stop feeding. On the other hand, a steroid hormone, allopregnanolone, was found to activate $GABA_A$ receptors in the PVN, resulting in increases in food intake and body weight (Holmberg et al., 2018). In addition to its well-known neuronal actions in the hypothalamus, GABA regulates food intake and body weight by acting on the frontal cortex and hippocampus, in which lower GABA levels are associated with higher body weight gain in rats fed a high-fat diet (Sandoval-Salazar, Ramírez-Emiliano, Trejo-Bahena, Oviedo-Solís, & Solís-Ortiz, 2016). This result is in a good agreement with those of our study demonstrating a negative correlation between the cortex and hippocampal GABA levels and food intake and body weight gain. Low levels of whole brain GABA was found to be associated with obesity (Fisler, Shimizu, & Bray, 1989); however, GABA acts on many parts of the brain, and the complete mechanisms behind its control on feeding are sophisticated and not fully understood (Delgado, 2013). Hence, we still do not know how dietary GABA and vigabatrin suppress food intake and body weight gain at the molecular level. It is of great interest to further investigate how dietary GABA or its combination with vigabatrin affects the GABA levels in other areas of the brain,

such as the LH area and PVN, as well as the whole brain.

It is evident that both dietary GABA and its combination with vigabatrin can exert antiobesity-like effects via an identical mechanism that increases the endogenous GABA levels. Dietary GABA might exert its effects via peripheral actions such as the production of BAIBA, which in turn may induce fat oxidation. Moreover, dietary GABA may also exert antiobesity-like effects via the regulation of glucose metabolism by improving β-cell functions, as previously reported. On the other hand, the dietary GABA–vigabatrin combination tends to suppress food intake and body weight gain by impacting the central nervous system. The dose of vigabatrin (250 mg/kg) used in our study showed severely suppressive effects on food intake and body weight gain within 2 weeks. This is similar to the results of previous works demonstrating that vigabatrin (a range of 50–500 mg/kg) decreases food intake and body weight in a dose dependent manner (Walzer et al., 2011; DeMarco et al., 2008). Based on these findings, and the possibility of inducing a greater energy expenditure using the combination of vigabatrin with 2% GABA, reflected by a decrease in muscle mass in this group (Fig. 3D), we propose that a combination of dietary GABA and vigabatrin may be a new intervention for food consumption and body weight controls. This idea is similar to that behind the use of certain current anti-obesity drugs, such as Qsymia (phentermine and topiramate) and Empatic (bupropion and zonisamide), which are combinations of antiepileptic drugs (topiramate and zonisamide) that regulate GABAergic signaling in the brain (Adan, 2013). Further studies are necessary to determine the optimal doses of dietary GABA and vigabatrin required to increase the levels of both circulating and brain GABA. Lowering the doses of GABA and vigabatrin while increasing the level of endogenous GABA will guarantee safe use with lower side effects. Moreover, food factors or natural compounds having an inhibitory effect on GABA-T activity may constitute a new type of anti-obesity or anti-overeating agent.

In this study, research limitations are using only lean mice to demonstrate the antiobesity-like effects and the safety of high doses of GABA intake or vigabatrin administration is not investigated. Thus, further studies on obese mice and safety are needed to verify these issues.

5. Conclusions

This study demonstrates that dietary GABA (5%) suppresses food intake and mimics calorie restriction, thereby depressing body weight gain, inducing hypolipidemia and ketogenesis, and improving glucose metabolism. We propose that the novel mechanism behind these antiobesity-like effects is possibly partly mediated via peripheral GABA and BAIBA. A slight increase in GABA levels in the hippocampus upon 5% GABA intake may contribute to feeding regulation. The combination of vigabatrin and dietary GABA highly elevates the levels of peripheral circulating and brain GABA and severely suppresses food intake and body weight gain. The possible antiobesity-like effects of the vigabatrin–dietary GABA combination are most likely largely attributable to the control on feeding orchestrated by elevated brain GABA levels, and partly to peripheral GABA effects. Manipulation of peripheral and brain GABA metabolism by targeting GABA-T is possibly a new approach to control feeding behavior and body weight gain.

Ethics Statements

All the mice used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals established by Hiroshima University, and the procedures were approved by the Ethics Committee of the University (Ethical Approval No. 17-19).

CRediT authorship contribution statement

Kanako Sato: Investigation, Formal analysis, Data curation. **Takumi Komaru:** Investigation, Formal analysis, Data curation. **Takeshi Arima:** Visualization. **Chanakarn Jardson:** Visualization. **Noriyuki Yanaka:** Conceptualization, Methodology, Writing - review & editing. **Thanutchaporn Kumrungsee:** Conceptualization, Methodology, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104367.

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