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ORIGINAL INVESTIGATION

Effects of L-theanine on anxiety-like behavior, cerebrospinal fluid amino acid profile, and hippocampal activity in Wistar Kyoto rats

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Abstract
Rationale and objectives The amino acid L-theanine (N-ethyl-L-glutamine) has historically been considered a relaxing agent. In the present study, we examined the effects of repeated L-theanine administration on behavior, levels of amino acids in the cerebrospinal fluid (CSF), and hippocampal activity in Wistar Kyoto (WKY) rats, an animal model of anxiety and depressive disorders.
Methods Behavioral tests were performed after 7–10 days of L-theanine (0.4 mg kg^{-1} day^{-1}) or saline administration, followed by CSF sampling for high-performance liquid chromatography (HPLC) analysis. An independent set of animals was subjected to [^{18}F]fluorodeoxyglucose positron emission tomography (PET) scanning after the same dose of L-theanine or saline administration for 7 days.
Results In the elevated plus maze test, the time spent in the open arms was significantly longer in the L-theanine group than in the saline group ($P = 0.035$). In addition, significantly lower CSF glutamate ($P = 0.039$) and higher methionine ($P = 0.024$) concentrations were observed in the L-theanine group than in the saline group. A significant increase in the standard uptake value ratio was observed in the hippocampus/cerebellum of the L-theanine group ($P < 0.001$).
Conclusions These results suggest that L-theanine enhances hippocampal activity and exerts anxiolytic effects, which may be mediated by changes in glutamate and methionine levels in the brain. Further study is required to more fully elucidate the mechanisms underlying the effects of L-theanine.

Keywords Amino acids · Cerebrospinal fluid · [^{18}F]Fluorodeoxyglucose · L-Theanine (N-ethyl-L-glutamine) · Positron emission tomography · Wistar Kyoto rats

Introduction

The amino acid L-theanine (N-ethyl-L-glutamine) was originally identified in green tea (Sakato 1950) and has historically been recognized as a relaxing agent (Nathan et al. 2006). Previous studies have indicated that L-theanine can cross the blood-brain barrier (Yokogoshi et al. 1998a), and that dietary administration of L-theanine (4000 mg kg^{-1} day^{-1}, 13-week) exerts no adverse effects in rats (Borzelleca et al. 2006).

Accumulating evidence has demonstrated the beneficial effects of L-theanine on emotional behavior and psychiatric symptoms. Several research groups have observed that L-theanine improves anxiety-like, psychosocial (Unno et al. 2013a; Wise et al. 2012), and depression-like (Unno et al. 2011, 2013a; Wakabayashi et al. 2012; Yin et al. 2011) behaviors in animals. Moreover, several studies have documented the effects of L-theanine on stress responses (Kimura et al. 2007; Lu et al. 2004; Nobre et al. 2008; Unno et al. 2013b),

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anxiety (Hidese et al. 2016; Rogers et al. 2008; Unno et al. 2013b; Yoto et al. 2012), depressive symptoms (Cross et al. 2011; Hidese et al. 2016), and schizophrenic symptoms (Kardashev et al. 2015; Ota et al. 2015; Ritsner et al. 2011; White et al. 2016) in humans. Although previous research has indicated that L-theanine modulates the activity of brain neurotransmitters such as serotonin, dopamine, and γ-aminobutyric acid (GABA) (Yamada et al. 2007; Yokogoshi et al. 1998a, b), the molecular basis underlying these effects remains poorly understood.

Several amino acids act as neurotransmitters themselves, or are precursors to neurotransmitters (Kurian et al. 2011). Previous studies, including those by our own research group, have suggested that patients with psychiatric disorders exhibit altered amino acid profiles in the cerebrospinal fluid (CSF; Kaddurah-Daouk et al. 2012; Ogawa et al. 2015). In one post-mortem study, elevated glutamate levels were observed in the frontal cortex of patients with major depression (Hashimoto et al. 2007). Magnetic resonance spectroscopy (MRS) studies have also revealed that patients with major depressive disorder (MDD) exhibit increased levels of glutamate in the hippocampus (Hermens et al. 2015). Since it is an amino acid, L-theanine may alter amino acid profiles via competitive inhibition of transporters, which may in turn influence neurotransmitter function (Zhou and Danbolt 2014). Indeed, Kakuda et al. (2008) reported that L-theanine inhibits glutamate release from cultured neurons, while our previous MRS findings indicate that L-theanine affects glutamate and glutamine levels in humans (Ota et al. 2015). Based on these findings, we aimed to investigate the effects of L-theanine on extracellular amino acid levels in the brain.

Reduced glucose metabolism in brain regions such as the prefrontal cortex (Videbech 2000) and limbic system (Su et al. 2014) has been documented in patients with MDD, and previous research has indicated that antidepressant treatment significantly increases hippocampal glucose metabolism in mice (Jang et al. 2009). Moreover, glutamatergic signaling mediates reductions in glucose metabolism in the prefrontal cortex and hippocampus in a rat model of brain injury (Omata et al. 2003). Thus, we hypothesized that L-theanine administration ameliorates impaired glucose metabolism in the hippocampus. To test this hypothesis, we employed [^{18}F]fluorodeoxyglucose (FDG) micro-positron emission tomography (PET) to determine whether L-theanine elevates glucose in a manner similar to that of antidepressants in the hippocampus.

In the present study, we investigated whether repeated L-theanine administration alters anxiety- and/or depression-like behaviors in Wistar Kyoto (WKY) rats, which have been proposed as an animal model of anxiety vulnerability (McAuley et al. 2009) and refractory depression (Will et al. 2003). We further aimed to determine whether such changes are accompanied by changes in CSF amino acid profiles and hippocampal glucose metabolism.

Materials and methods

Animals

All experimental procedures were approved by the Ethics Review Committee for Animal Experimentation at the National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP, Tokyo, Japan). All efforts were made to minimize the number of animals used and their suffering. To avoid the effect of behavioral tasks on PET measurement, we utilized two separate groups of rats (one for PET assessments and the other for behavioral and CSF assessments). Sixteen and 25 adult (age, 9 weeks) male WKY rats weighing 206–246 and 206–242 g at the start of the PET and behavioral experiments, respectively, were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). All rats were housed in individual cages and given free access to food and water. The housing room was maintained at a constant temperature of 22 ± 1 °C under a 12-h/12-h light/dark cycle. Lights were turned on at 8:00 a.m., which was regarded as zeitgeber time (ZT) 0.

L-Theanine administration and experimental schedule

L-Theanine was kindly provided by Taiyo Kagaku (Yokkaichi, Mie, Japan). The prepared L-theanine solution and saline were aliquoted into microtubes and stored at − 25 °C until use, at which time they were thawed prior to administration. Prior to the experiments, all rats were handled daily for a few minutes for several days. The L-theanine administration and experimental schedules are summarized in Fig. 1. L-Theanine (0.4 mg/kg body weight) or saline was administered via intraperitoneal (i.p.) injection once per day at a volume of 1 mL/kg. The L-theanine doses used in previous animal studies ranged widely between 1.0 and 5000 mg/kg (Schallier et al. 2013; Unno et al. 2011, 2013a; Wise et al. 2012; Yin et al. 2011; Yokogoshi et al. 1998a, b). Such studies have demonstrated that even 1 mg/kg of orally administered L-theanine significantly ameliorates depression-like behaviors (Yin et al. 2011) and stress responses (Unno et al. 2013a). Previously, we observed that subchronic i.p. administration of L-theanine at a daily dose of 0.4 mg/kg produced significant behavioral effects (Wakabayashi et al. 2012), which were comparable to those observed following oral administration at 1 mg/kg. We therefore adopted a dose of 0.4 mg/kg i.p. in the present study.

PET scans were performed after 7 days of L-theanine or saline treatment. Behavioral tests were performed for three consecutive days after 7 days of L-theanine or saline treatment, following which rats were euthanized and CSF was collected. During the 3-day behavioral testing period, daily injections were maintained to prevent

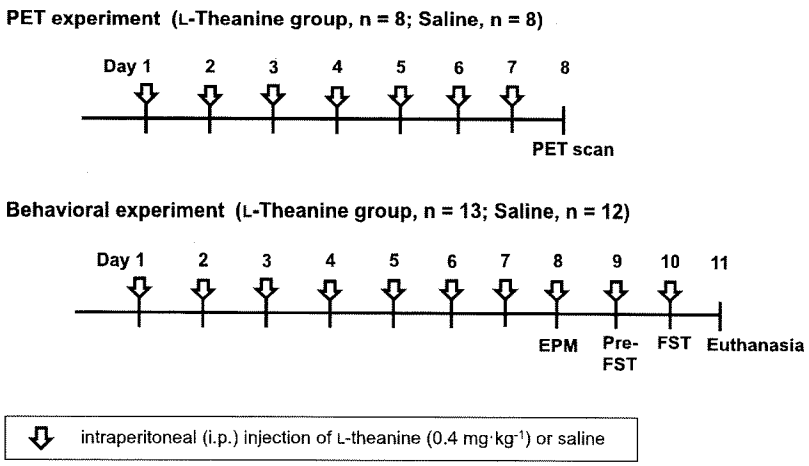

Fig. 1 Experimental schedule. We conducted both behavioral and PET imaging experiments. Intraperitoneal (i.p.) injections were administered once per day between zeitgeber time (ZT) 5–7 and 10–12 in the PET and behavioral groups, respectively (arrows indicate injections). Eight rats from the PET experiment were included in each L-theanine or saline group, while 13 and 12 behavioral experimental rats were included in the L-theanine and saline groups, respectively. CSF samples were collected after behavioral tests. Abbreviations: PET, positron emission tomography; EPM, elevated plus maze; FST, forced swim test; CSF, cerebrospinal fluid

withdrawal effects. Rats of the relevant group were fasted 16 h prior to initiation of the PET experiment.

Behavioral assessments

In the elevated plus maze (EPM) test, the total distance during the test and total time spent in the open arms were automatically measured. In the forced swim test (FST), the total durations of immobility, swimming, and climbing were calculated. Detailed descriptions of the behavioral analyses are available in the Supplemental Information.

Collection of CSF and determination of amino acid levels

On the day of sampling for rats subjected to behavioral and CSF assessments (day 11, Fig. 1), the animals were anesthetized with sevoflurane and euthanized via exsanguination, following which the CSF was immediately collected from the cisterna magna. Samples were then placed on ice, followed by centrifugation at 1000×*g* for 5 min. The supernatant was aliquoted and stored at − 80 °C until measurement. One of the 13 CSF samples of the L-theanine-treated group was omitted from the analysis due to blood contamination. Levels of amino acids and related molecules were measured using high-performance liquid chromatography (HPLC), as previously described (Ogawa et al. 2015). Thawed CSF samples were each mixed with an equivalent volume of 4% 5-sulfosalicylic acid dihydrate (WAKO, Tokyo, Japan) solution for deproteinization, following which they were centrifuged for 10 min at 4 °C and 12,000×*g*. Each supernatant was filtered, transferred to a microtube, and analyzed using the LC-2000 Li-LG amino acid measurement system (JASCO, Tokyo, Japan), which utilizes the post-column *O*-phthalaldehyde derivatization method. Amino acid levels were measured using the LC-2000 Li-LG system in accordance with the manufacturer's instructions. We prepared 40 standards for the absolute calibration curve using the Amino Acids Mixture Standard Solution (Type B and Type AN-2, WAKO). An additional standard solution was prepared for tryptophan, glutamine, theanine, proline, and asparagine (all purchased from WAKO). We excluded amino acids from the data analysis if at least one sample was under the detection limit, which enabled us to compare 24 molecules in the rat CSF between the L-theanine and saline groups.

PET experiment

We prepared [^{18}F]FDG using a fully automated synthesis system (F300, Sumitomo Heavy Industry, Tokyo, Japan). The ^{18}O(p, n)^{18}F reaction was performed using a cyclotron (HM-12, Sumitomo Heavy Industry) in the Animal Imaging Center at the NCNP (Tokyo, Japan). PET images were acquired using a Clairvivo PET instrument (Shimadzu Corp., Kyoto, Japan). This scanner contained depth of interaction (DOI) detector modules with an axial field of view (FOV) of 151 mm, yielding 213 contiguous slices, a transaxial FOV of 100 mm (matrix, 128 × 128), and a transaxial spatial resolution of 1.5 mm at the center. A 15-min transmission scan, which was acquired using retractable rotating ^{68}Ge sources, was used for attenuation correction. Rats were initially anesthetized with 3% isoflurane vaporized in oxygen, following which they were gently placed in the PET gantry with their brains positioned at the center of the FOV. Anesthesia was maintained at 2% during PET scanning, while body temperature was maintained using a heating blanket. The prepared [^{18}F]FDG (24.5 ± 3.5 MBq) was injected as a bolus via the tail vein.

Previous research has indicated that radioactivity in the rat brain rises rapidly and remains constant between 30 and 60 min following injection (Shimoji et al. 2004). Therefore, we obtained data in the list-mode format for 45 to 60 min after [^{18}F]FDG injection. Summation images with corrected scattering were reconstructed using an iterative three-dimensional (3D) dynamic raw-action maximum likelihood algorithm (Tanaka and Kudo 2003).

Magnetic resonance imaging procedure and image preprocessing

Magnetic resonance imaging (MRI) was used to determine the locations of intracranial brain regions for processing FDG-PET data. Detailed descriptions of the MRI procedure and image preprocessing are available in the Supplemental Information.

Statistical analysis

Changes in body weight were analyzed via repeated measures analysis of variance (ANOVA). Differences in anxiety-like behavior between the L-theanine- and saline-treated groups during the EPM test were assessed using unpaired Student's *t* tests. Unpaired Student's *t* tests were also used to investigate between-group differences in immobility time during the FST, which reflects the severity of depression-like behaviors. Total time immobile was scored along with swimming time and climbing time. Levels of amino acids and related molecules in the CSF were compared between the two groups using unpaired Student's *t* tests. To investigate differences in hippocampal glucose uptake, differences in the standardized uptake value ratio (SUVr) between WKY rats treated with and without L-theanine in the FDG-PET experiment were examined using unpaired two-sample *t* tests. The level of statistical significance was set at $P < 0.05$ (two-tailed). These analyses were conducted using the Statistical Package for the Social Sciences (SPSS) version 22 (Japan IBM, Tokyo, Japan).

Results

Effects of L-theanine on body weight and behavior

We assessed changes in body weight and behavior following administration of L-theanine in WKY rats (Fig. 2). There was no significant difference in the rate of body weight increase between the L-theanine and saline groups (Fig. 2a). In the EPM test, the time spent in the open arms was significantly longer in the L-theanine group than in the saline group

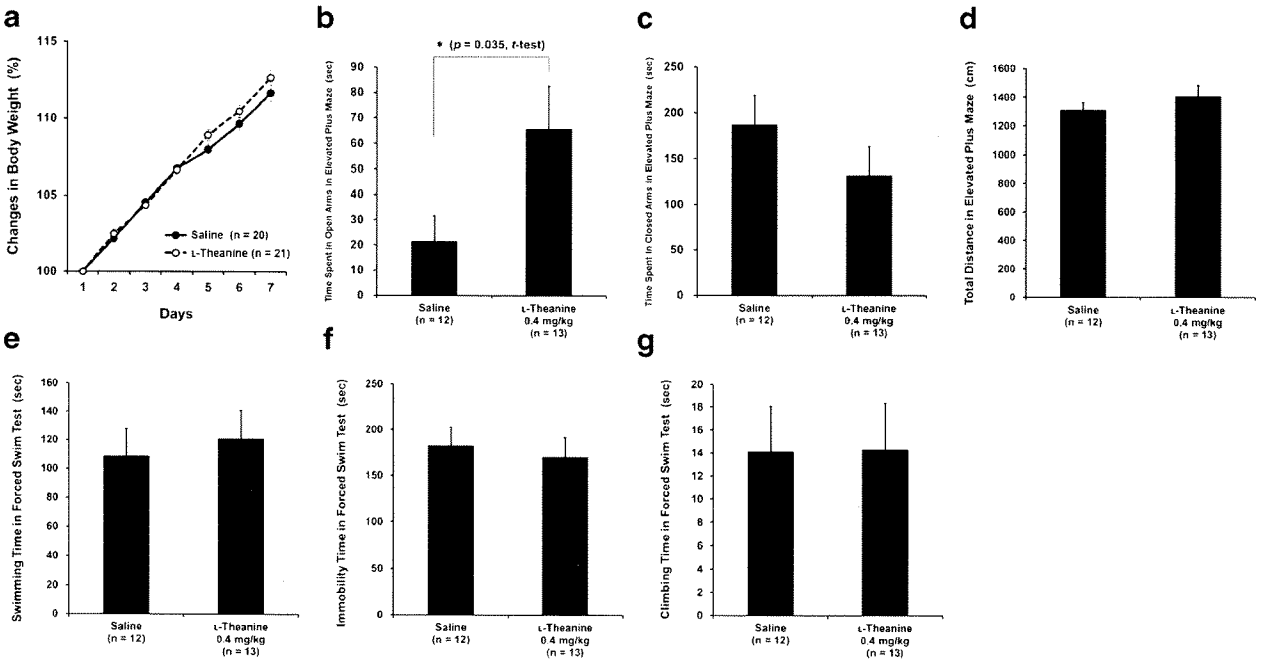

Fig. 2 Comparison of changes in body weight and behavioral test results between L-theanine- and saline-treated groups. **a** Percentage of change from baseline (day 1) for body weight (%) over 7 days in the L-theanine- ($n = 21$) and saline-treated ($n = 20$) groups. **b** Time spent in open arms (s) during the elevated plus maze (EPM) test, which was used to evaluate anxiety-like behavior (L-theanine group: $n = 13$, saline group: $n = 12$). **c** Time spent in closed arms (s) during the EPM test, for comparison. **d** The total distance (cm) traveled during the EPM test was used to assess differences in spontaneous locomotor activity. **e** Swimming time (s) during the forced swimming test (FST) (L-theanine group: $n = 13$, saline group: $n = 12$). **f** Immobility time (s) during the FST was used to assess differences in depression-like behaviors. **g** Climbing time (s) during the FST, which reflected the time rats spent attempting to climb the cylinder wall. $^*P < 0.05$ (unpaired Student's *t* test, vs. saline group)

($t = -2.26$, $df = 19.42$, $P = 0.035$, Fig. 2b), while there were no significant differences in the time spent in closed arms ($t = 1.22$, $df = 23$, $P = 0.24$, Fig. 2c) or distance traveled ($t = -1.023$, $df = 23$, $P = 0.32$, Fig. 2d). In the FST, no significant differences in swimming ($t = -0.42$, $df = 23$, $P = 0.67$, Fig. 2e), immobility ($t = 0.41$, $df = 23$, $P = 0.68$, Fig. 2f), or climbing ($t = -0.034$, $df = 23$, $P = 0.97$, Fig. 2g) time were observed between the two groups.

Effects of L-theanine on CSF amino acid levels

As shown in Table 1, we successfully determined the levels of 24 amino acids and related molecules in the rat CSF (Table 1). We observed that CSF glutamate levels were significantly lower ($t = 2.22$, $df = 18$, $P = 0.039$) in the L-theanine group than in the saline group, while methionine levels were significantly higher in the L-theanine group than in the saline group ($t = -2.43$, $df = 22$, $P = 0.024$).

[^{18}F]FDG-PET experiment

We examined the effect of L-theanine on hippocampal glucose uptake to investigate the potential association between emotional behavior and hippocampal function. The volumes of interest (VOIs) in the lateral hippocampi and cerebellum on the background images are described in Fig. 3. A significant increase in the SUVr was observed in the hippocampi of rats in the L-theanine group, relative to that observed in saline-treated rats (mean ± standard deviation, 0.95 ± 0.06 vs. 0.83 ± 0.06, $t = -4.2$, $df = 14$, $P < 0.001$).

Discussion

In the present study, we examined the effects of repeated L-theanine administration on behavior, levels of amino acids in the CSF, and hippocampal activity in WKY rats. Our findings

Table 1 Comparison of amino acid and related molecules in cerebrospinal fluid (CSF) of Wistar Kyoto (WKY) rats

Molecules	Control ($n = 12$)	L-Theanine ($n = 12$)	Statistics		
			t	*df*	*P*
Taurine	27.5 ± 1.0	27.1 ± 0.5	0.34	16.024	0.74
Phosphoethanolamine	7.0 ± 0.1	6.9 ± 0.1	0.67	22	0.51
Hydroxyproline	1.9 ± 0.1	1.7 ± 1.0	1.30	22	0.21
Asparagine	2.5 ± 0.2	2.4 ± 0.2	0.19	22	0.85
Glutamate	*3.7 ± 0.6*	*2.2 ± 0.4*	*2.22*	*18.00*	*0.039*
Glutamine	370.7 ± 17.7	397.5 ± 10.3	− 1.31	17.72	0.21
Glycine	4.7 ± 0.2	4.6 ± 0.2	0.20	22	0.84
Alanine	31.02 ± 0.7	31.06 ± 0.7	− 0.040	22	0.97
Citrulline	2.9 ± 0.1	3.0 ± 0.1	− 0.41	22	0.69
α-Amino-n-butyric acid	0.6 ± 0.04	0.7 ± 0.02	− 0.46	22	0.65
Valine	3.1 ± 0.1	3.03 ± 0.07	0.58	22	0.57
Cystathionine	1.8 ± 0.1	1.8 ± 0.1	0.23	22	0.82
Methionine	*2.2 ± 0.08*	*2.5 ± 0.08*	*− 2.43*	*22*	*0.024*
Isoleucine	1.4 ± 0.05	1.3 ± 0.04	0.71	22	0.49
Leucine	3.5 ± 0.09	3.4 ± 0.07	0.84	22	0.41
Tyrosine	3.5 ± 0.1	3.6 ± 0.08	− 0.79	22	0.44
Phenylalanine	3.4 ± 0.05	3.3 ± 0.1	0.82	22	0.42
β-Alanine	0.4 ± 0.05	0.3 ± 0.03	1.39	22	0.18
Tryptophan	0.6 ± 0.1	0.7 ± 0.1	− 0.67	22	0.51
Ethanolamine	38.5 ± 0.8	37.7 ± 0.6	0.87	22	0.39
Lysine	68.6 ± 1.3	69.7 ± 1.2	− 0.65	22	0.52
Histidine plus 1-methylhistidine	3.3 ± 0.08	3.3 ± 0.08	0.14	22	0.89
Arginine	25.3 ± 0.6	25.8 ± 0.9	− 0.50	22	0.62
Carnosine	5.3 ± 0.1	5.5 ± 0.1	− 1.86	22	0.077
Total	1325.0 ± 44.2	1420.7 ± 32.5	− 1.75	22	0.095

Statistically significant results are shown in italic

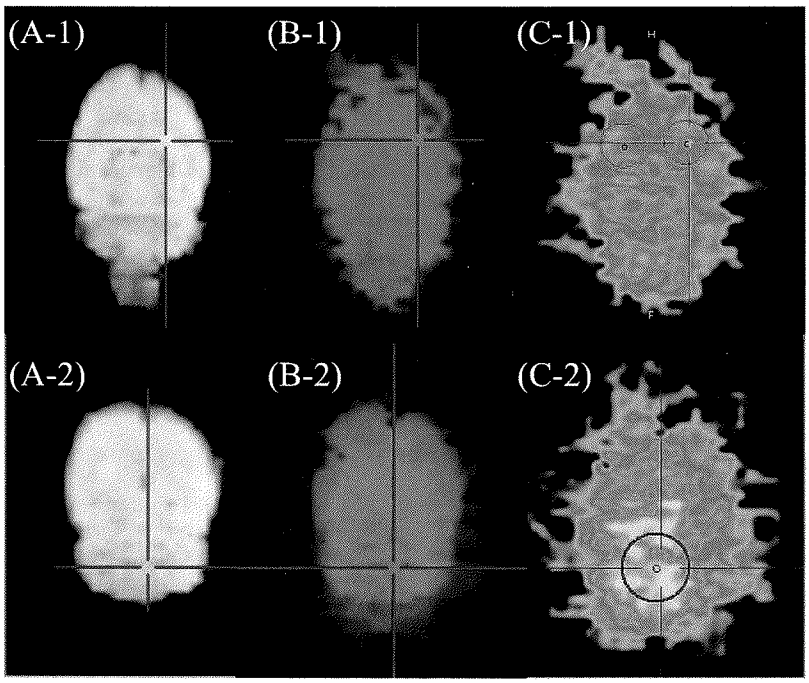

Fig. 3 Volume of interest (VOI) in brain hippocampi of WKY rats for [^{18}F]FDG-PET scanning. Imaging was performed in both L-theanine- (n = 8) and saline-treated (n = 8) rats. VOIs are drawn over the hippocampi (red circle, upper row) and cerebellum (blue circle, lower row) in each PET summation image. Background image of (A) resliced three-dimensional (3D)-T2 weighted image (gray scale), (B) resliced 3D-T2 weighted image (green scale) and summation image of 45–60 min data (x_rain scale), (C) summation image of 45–60 min data (cold scale). Images (A) and (B) were generated in "MRIcron" (http://people.cas.sc.edu/rorden/mricron/index.html), while (C) was generated in "Pmod." Abbreviations: WKY, Wistar Kyoto; FDG, fluorodeoxyglucose; PET, positron emission tomography

indicated that WKY rats treated with L-theanine spent a significantly greater amount of time in open arms on the EPM test, suggesting that L-theanine exerts anxiolytic effects. These effects were accompanied by significant reductions in CSF levels of glutamate, as well as significant increases in CSF levels of methionine. In the PET experiment, significantly elevated hippocampal [^{18}F]FDG uptake was observed in L-theanine-treated animals relative to that in saline-treated animals, suggesting that L-theanine enhances hippocampal activity.

Consistent with the findings of previous studies (Kardashev et al. 2015; Ritsner et al. 2011; Rogers et al. 2008; Unno et al. 2013b; Wise et al. 2012; Yoto et al. 2012), we observed anxiolytic effects of L-theanine in the present study. However, we failed to obtain evidence for antidepressant-like effects of L-theanine in WKY rats, possibly because the WKY rat has been regarded as an animal model of refractory depression. Indeed, previous reports have revealed that fluoxetine—one of the most widely prescribed selective serotonin reuptake inhibitors (SSRIs)—exerts no effect on depression-like behavior in WKY rats (Griebel et al. 1999; Will et al. 2003). Thus, further investigation using other animal strains is necessary to clarify whether L-theanine exerts antidepressant-like effects. Alternatively, the dose of L-theanine used in the current study (0.4 mg kg^{-1}) may have been insufficient, and a higher dose may be required to induce antidepressant-like effects.

In the present study, rats treated with L-theanine exhibited decreased levels of CSF glutamate relative to control animals, in contrast to the findings of a previous study, which reported no difference in glutamate levels obtained via hippocampal microdialysis between L-theanine- and water-treated groups (Schallier et al. 2013). However, the dose of L-theanine used in this previous study was 4000–5000 mg kg^{-1} day^{-1} (Kim et al. 2009), which is approximately 10,000 times higher than that used in our study. Such differences in experimental design may account for this inconsistency. However, in accordance with our findings, previous studies have demonstrated that other anxiolytic agents such as guanosine (Almeida et al. 2017), JM-20 (Nunez-Figueredo et al. 2014), and vigabatrin (Halonen et al. 1991) are associated with decreased levels of glutamate in the rat CSF, suggesting that decreases in CSF glutamate levels may be associated with anxiolytic effects.

In addition, L-theanine-treated rats exhibited increased levels of methionine in the CSF. Two research groups, including our own, have reported that patients in MDD remission exhibit higher levels of methionine in the CSF than patients with current MDD (Kaddurah-Daouk et al.

2012; Ogawa et al. 2015), suggesting that increased levels of central methionine play a role during recovery from depression and anxiety disorders. Indeed, S-adenosylmethionine (SAMe), an activated form of methionine, has been shown to exert both antidepressant and anxiolytic effects (Di Pierro et al. 2015; Mischoulon et al. 2014; Sarris et al. 2014), although a recent meta-analysis reported negative results concerning the antidepressant effect (Galizia et al. 2016). Although the mechanism underlying increases in methionine levels remains to be elucidated, neuronal activity results in extracellular K+ accumulation (Ransom et al. 2000), which in turn suppresses methionine uptake in the nervous system (Cummins et al. 1979, 1982). Therefore, increased neuronal activity induced by L-theanine as demonstrated in our PET experiment may explain the observed elevation in CSF methionine levels.

The present study is the first to demonstrate that enhanced hippocampal glucose uptake occurs following repeated administration of L-theanine, suggesting that L-theanine treatment increases neuronal activity in the hippocampus. Consistent with our findings, anxiolytic agents such as RD-1 (Yang et al. 2016) and LY379268 (Lam et al. 1999) have been associated with elevated glucose metabolism in the hippocampus in animal studies. Riluzole—a drug commonly used in the treatment of amyotrophic lateral sclerosis—inhibits K+-evoked glutamate release from presynaptic sites and has been shown to exert anti-depressive and anxiolytic properties (Zarate and Manji 2008). Additional studies have revealed that such changes are accompanied by increased glucose metabolism in the rat prefrontal cortex and hippocampus (Chowdhury et al. 2008). Furthermore, one previous study reported that rats with brain injury exhibit anxiety-like behavior and decreased [^{18}F]FDG metabolism in the hippocampus (Liu et al. 2010). In rat models of brain injury, glutamatergic signaling mediates decreased glucose metabolism in the prefrontal cortex and hippocampus (Omata et al. 2003). Taken together, these findings indicate that L-theanine may increase glucose metabolism by reducing glutamatergic signaling.

In conclusion, our findings demonstrate that repeated systemic L-theanine administration at a relatively low dose elicits significant anxiolytic effects in WKY rats, which are accompanied by decreased glutamate and increased methionine levels in the CSF. Our findings further indicated that L-theanine treatment produces increases in hippocampal activity as measured using FDG-PET. Collectively, our results suggest that L-theanine enhances hippocampal activity and exerts anxiolytic effects, which may be mediated by changes in glutamate and methionine levels in the brain. However, further study is required to elucidate the mechanisms underlying the effects of L-theanine.

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Compliance with ethical standards

Conflict of interest This research study was funded by an unrestricted research grant provided by Taiyo Life Insurance Himawari Foundation, Tokyo, Japan. This funding agency had no role in the design, methods, analysis, or preparation of the paper.

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