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ORIGINAL ARTICLE

Theanine, γ-glutamylethylamide, a unique amino acid in tea leaves, modulates neurotransmitter concentrations in the brain striatum interstitium in conscious rats

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Abstract Theanine (γ-glutamylethylamide) is one of the major amino acid components in green tea and can pass through the blood-brain barrier. Recent studies suggest that theanine affects the mammalian central nervous system; however, the detailed mechanism remains unclear. In this study, we demonstrated the effect of theanine on neurotransmission in the brain striatum by in vivo brain microdialysis. Theanine injection into the rat brain striatum did not increase the concentration of excitatory neurotransmitters in the perfusate. On the other hand, theanine injection increased the concentration of glycine in the perfusate. Because it has been reported that theanine promotes dopamine release in the rat striatum, we investigated the glycine and dopamine concentrations in the perfusate. Co-injection of glycine receptor antagonist, strychnine, reduced theanine-induced changes in dopamine. Moreover, AMPA receptor antagonist, which regulates glycine and GABA release from glia cells, inhibited these effects of theanine and this result was in agreement with the known inhibitory effect of theanine at AMPA receptors.

Keywords L-theanine · Dopamine · Neurotransmission · Glycine · AMPA receptors · Microdialysis

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Introduction

Recently, many studies have shown that green tea and various green tea leaf components, such as catechins, caffeine and γ-aminobutyric acid (GABA), have physiological and pharmacological actions, and more attention has been paid to the effects of green tea ingredients by the public. In particular, GABA is known as an inhibitory neurotransmitter present almost exclusively in the central nervous system (Brambilla et al. 2003), and GABAergic dysfunction causes mood disorders or neurological disorders such as seizures (Wong et al. 2003). Theanine is also a major amino acid component of green tea. Previously, we detected theanine after intragastrical administration in various rat tissues such as serum, liver and brain (Terashima et al. 1999). Some reports suggested that theanine might be degraded via glutamic acid because its structure is similar to glutamic acid, and it was suggested that enzymatic hydrolysis of theanine to glutamic acid and ethylamine was accomplished in the kidney (Unno et al. 1999) (Fig. 1). Therefore, the purpose of many recent studies on theanine was to determine whether the effects of theanine are related to its metabolism to glutamic acid (Sugiyama and Sadzuka 2003; Kakuda et al. 2002a; Tsuge et al. 2003). Using an in vivo brain microdialysis method, we showed that direct administration of theanine into the rat brain striatum enhanced the release of dopamine (Yokogoshi et al. 1998a). Investigations into theanine have

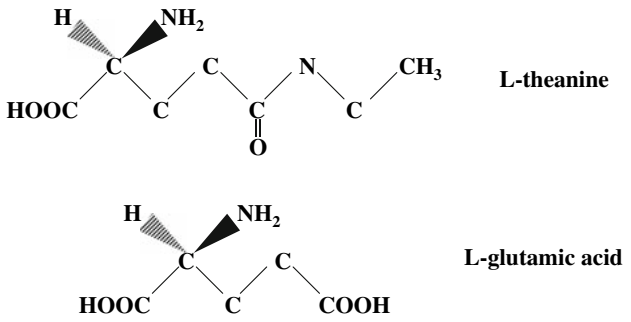

Fig. 1 Chemical structures of theanine (L-theanine) and glutamic acid

occurred later than other green tea ingredients, but recently, various reports on theanine have been published, with other researchers suggesting that theanine altered neurotransmitter concentrations in the rat brain. Intraperitoneal administration of theanine inhibited the convulsive action of caffeine, and increased the intracerebral level of GABA in mice (Kimura and Murata 1971). Theanine decreased the norepinephrine level in the rat brain, and depressed caffeine-induced increases of serotonin and 5-hydroxyindoleacetic acid in rats (Kimura and Murata 1986). Other reports showed that theanine decreased serotonin concentration and increased tryptophan concentration in the rat brain (Yokogoshi et al. 1998b). The administration of theanine into the lateral ventricle prevented ischemia-induced neuronal death in the hippocampal CA1 region in a dose-dependent manner. Moreover, some reports suggested that the neuroprotective effect of theanine occurs via glutamate receptors, with theanine acting as a glutamate receptor antagonist (Kakuda et al. 2000a; Kakuda 2002b). Some of these reports also suggested that theanine is concerned with glutamate receptors, although the affinity of theanine to glutamate receptors was low (Kakuda et al. 2002a). From these results and a report that dopamine release is enhanced when glutamic acid is increased in the interstitium (Globus et al. 1988; Moghaddam et al. 1990), we expected that the effect of theanine would be similar to that of glutamic acid, i.e., enhancing excitatory neurotransmission or increasing glutamic acid release from neurons; however, we showed that theanine did not affect glutamic acid and aspartic acid release from neurons (Yamada et al. 2005). In addition, other reports showed that the effects of theanine differ from those of glutamic acid; for instance, theanine decreased blood pressure in a dose-dependent manner in spontaneously hypertensive rats (SHR) (Yokogoshi et al. 1995), but glutamate did not exhibit antihypertensive action on SHR. From these reports, we suggested that theanine did not stimulate glutamate neurotransmission directly. Thus, next, we supposed that theanine would block glutamate transporters and glutamic acid density would increase in the interstitium and enhance glutamic acid neurotransmission because some reports suggested that theanine affects glutamate transporters (Sazduka et al. 2001); however, our results showed that theanine did not affect glutamate transporters (Yamada et al. 2005). In addition, we found that theanine enhanced glycine perfusate concentrations in the previous study; therefore, we considered that theanine released inhibitory neurotransmitters.

In this study, we demonstrated the effect of theanine on inhibitatory neurotransmission and other neurotransmitters in the central nervous system by in vivo brain microdialysis. In addition, we tried to explain the action mechanism of theanine on brain neurotransmission involving dopamine neurotransmission in the brain striatum.

Materials and methods

Animals

Male Wistar rats (270 g weight; SLC, Hamamatsu, Japan) were kept in individual wire cages in a temperature- and humidity-controlled room (24°C and 55% relative humidity) under regular lightning conditions (12 h light: dark cycle), and given food and water ad libitum. This experiment was carried out in accordance with Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka that refer to the American Association for Laboratory Animals Science. Eight animals were used in all experiments.

Measurement of neurotransmitter concentration by brain microdialysis

The guide cannula of the microdialysis probe in the striatum was stereotaxically implanted 0.2 mm posterior to the bregma, 3.0 mm lateral to the midline, and 3.5 mm ventral to the cortical surface according to the atlas of Paxinos and Watson (Paxinos and Watson 1986). One day after the operation, the dummy cannula was removed from the guide cannula, and the dialysis probe was inserted. This dialysis probe (MI-A-I-8-03, Eicom, Japan) had a dialysis membrane (at molecular weight cut-off of 5,000 and 3 mm protruding from the guide cannula) and a side tube to microinject theanine. The probe was perfused with Ringer's solution (147 mM Na^+, 4 mM K^+, 2.3 mM Ca^{2+}, and 155.6 mM Cl^-, pH 6.0) at a rate of 2 µl/min (ESP-64; Eicom, Japan). L-theanine (0.2 µmol/2 µl perfusate/min) was injected for 1 h and Ringer's solution was re-perfused. In antagonist co-injection cases, each antagonist was injected with perfusate (2 µl perfusate/min) for 1 h before

theanine injection and switched both theanine and each antagonist containing perfusate (2 µl perfusate/min). Theanine and antagonists were infiltrated into the tissue via dialysis membranes and the permeability of the membranes was approximately 10%.

The perfusate from the striatum (40 µl) for measuring dopamine was collected sequentially and was injected into the HPLC every 20 min via an automatic injector (AS-10; Eicom, Japan). Dopamine was separated by a reverse-phase HPLC using an MA-ODS column (5 µm particles, 3.0 × 150 mm; Eicom, Japan) maintained at 25°C by a column oven (ATC-300; Eicom, Japan), and was detected with an electrochemical detector (CB-100; Eicom, Japan). The mobile phase was composed of 0.1 M citric acid buffer, pH 3.9, containing 12% methanol, 20 mM EDTA and 160 mg/l sodium-1-octane-sulfonate. The graphite electrode (WE-3G; Eicom, Japan) was set at +0.65 V (vs. Ag/AgCl). The perfusates for measuring amino acids were collected into collecting tubes automatically every 30 min via the microsampler (Univentor, Malta) maintained at 4°C. The collected perfusates (60 µl) were diluted twice with 0.1N HCl. Ten microliters of the diluted perfusates was mixed with 5 µl o-phthaldialdehyde and 5 µl of 2 mercaptoethanol (OPA Reagent Set; Wako, Japan), and reacted for precisely 3 min. Twenty microliters of the reacted perfusates was injected into the chromatography system. The analytical ODC column (2 µm particles, 4.6 × 100 mm; MERCK, Germany) was maintained at 40°C and the detection wavelength was Ex: 340, Em: 420 (HP1049; Agilent, USA). The mobile phase used for HPLC analysis was composed of two eluants. Eluant A was 0.1 M phosphate buffer containing 10% ethanol, pH 6.0; eluant B was 100% ethanol: elution gradients were: initial, 86% A, 14% B; 0–16 min, and changed to 29% A, 71% B; 16–25 min, maintained for 25–32 min to wash the column, then re-equilibration with 86% A, 14% B for 15 min prior to the next step. The flow rate was kept constant at 1.0 ml/min. After completion of the experiment, the position of the microdialysis probe was histologically examined.

Data presentation and statistical analysis

The concentrations of dopamine and amino acids in the perfusate were expressed as percent change from the averaged basal values. In the measurement of dopamine concentration, basal values were averaged for four samples of perfusate before the theanine injection (pg dopamine/40 µl perfusate/20 min). In the measurement of amino acid concentration, basal values were averaged for three samples of perfusate before the theanine injection (glutamic acid, aspartic acid, glycine, glutamine, asparagine respectively; pg amino acid/60 µl perfusate/30 min). Differences between basal values and each fraction value of perfusates were analyzed using the Tukey-Kramer test. Differences for each reagent were analyzed using Student's *t*-test. In all cases, $P < 0.05$ was considered significant. Results are expressed as the mean ±SEM.

Materials

L-theanine was obtained from Taiyo Kagaku Co., Ltd. (Yokkaichi, Japan). NBQX disodium salt (NBQX), strychnine and 5,7-dichlorokynurenic acid (5,7-DCKA) were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Results

The basal level of dopamine concentration in the perfusate was 133 ± 11.3 pg/40 µl perfusate/20 min. Injection of theanine into the rat brain striatum increased the early response to dopamine concentration and the increase was up to twofold compared with the basal level (Fig. 2a), in agreement with our previous study (Yokogoshi et al. 1998a, b).

Basal levels of glutamic acid, aspartic acid and glycine concentrations in the perfusate were Glu: 20.8 ± 3.3, Asp: 3.86 ± 0.3 and Gly: 23.2 ± 3.1 ng/60 µl perfusate/30 min respectively, before theanine injection. Injection of theanine did not significantly change glutamic acid concentration in the perfusate (Fig. 2b, c). Aspartic acid concentration was reduced approximately 50% from the basal level. Glycine concentration increased about sixfold compared with the basal level. The changes in these amino acid concentrations tended to return to the basal value following re-perfusion of Ringer's solution.

Co-injection theanine with strychnine, the antagonist at the glycine receptor not associated with the NMDA receptor, significantly suppressed the increase of dopamine concentration by theanine injection but the suppression was not complete. The increase of dopamine concentration by theanine injection was not changed by co-injection with 5,7-dichlorokynurenic acid (5,7-DCKA), a potent antagonist at the glycine site of the *N*-methyl-D-aspartate (NMDA) receptor (Fig. 3). In addition, we examined the possible involvement of AMPA receptors in the increase of dopamine dialysate concentrations after theanine injection. NBQX disodium salt (NBQX), a potent, selective and competitive AMPA receptor antagonist, suppressed the increase of dopamine and glycine concentration by theanine injection (Fig. 4).

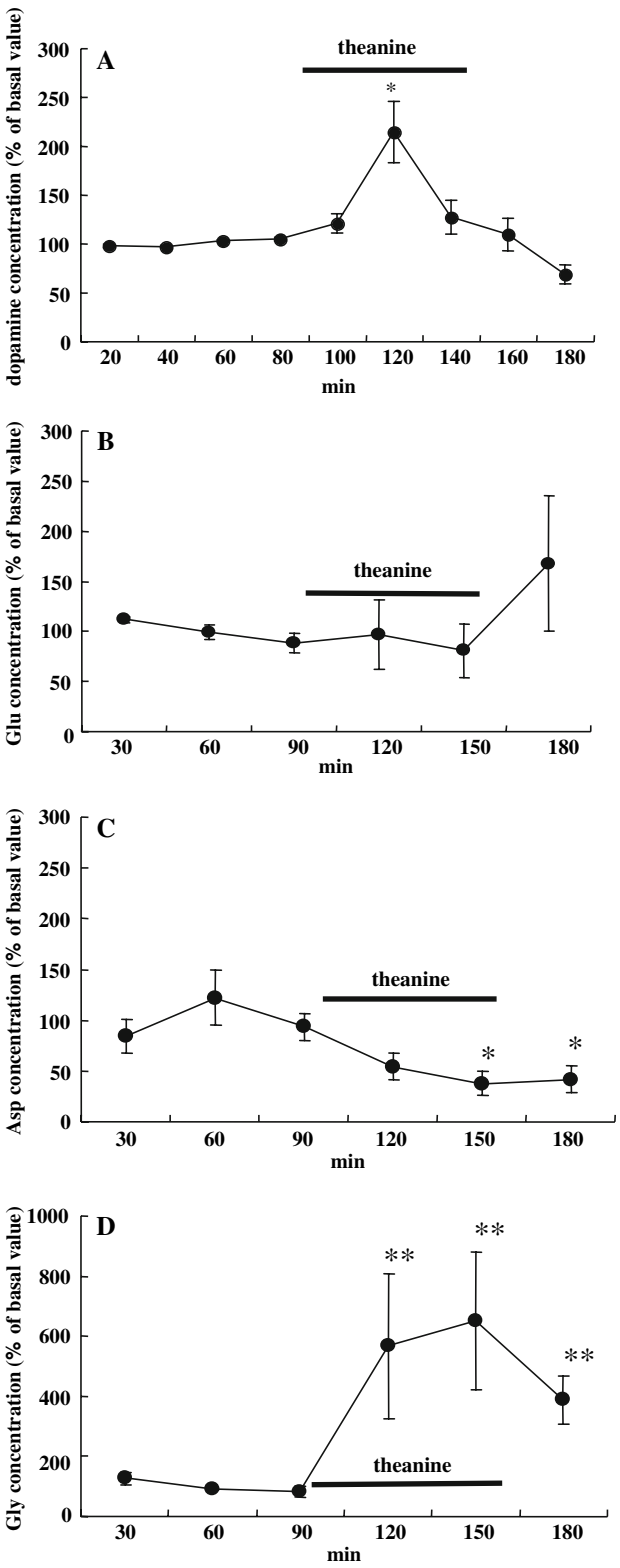

Fig. 2 Changes of neurotransmitter concentration by theanine injection in the rat brain striatum interstitium. Effect of theanine injection on dopamine (**a**), glutamic acid (**b**), aspartic acid (**c**) and glycine (**d**) concentration in the rat brain striatum interstitium. Theanine (0.2 μmol/2 μl perfusion/min) was injected into the striatum for 1 h. The values were measured by in vivo microdialysis, and expressed as the percentage change from the averaged basal value perfused with Ringer's solution ($n = 8$). The period of theanine injection is shown by a *black bar*

Discussion

Dopamine is related to emotion, behavior and brain disorders such as Parkinson's disease (Bosboom et al. 2003; Tessitore et al. 2002). In vivo microdialysis studies suggested that glutamic acid and dopamine were released simultaneously during ischemia (Globus et al. 1988), while others have demonstrated that glutamic acid was required to enhance the extracellular concentration of dopamine in vivo (Moghaddam et al. 1990). In addition, some studies suggested that dopamine could modify glutamate neurotransmission. (Moghaddam et al. 1990; Yadid et al. 1993; Koga and Momiyama 2000). Because the chemical structure of theanine is similar to glutamic acid, some studies of theanine examined its relationship to glutamic acid function and metabolism (Sugiyama et al. 2003; Kakuda et al. 2002a; Tsuge et al. 2003; Oda et al. 1980); however, the glutamate receptor antagonist MK-801 did not affect the increase of dopamine concentration by theanine injection in the rat striatum (Yokogoshi et al. 1998a). This result suggested that theanine did not affect glutamate neurotransmission. On the other hand, some reports suggested that theanine influenced glutamate transporter systems (Sugiyama et al. 1998; Sugiyama et al. 2001; Sadzuka et al. 2001). In a previous study, we showed that injection of L-trans-2, 4-PDC, a glutamate transporter blocker, increased dopamine concentration dramatically and increased glutamic acid concentration in the interstitium. From these results, we expected that L -trans-2,4-PDC injection caused glutamic acid reuptake inhibition, increasing glutamic acid concentration in the interstitium; thus, glutamic acid might promote dopamine neurotransmission. It was observed that aspartic acid concentration was also increased by L-trans-2,4-PDC injection. Aspartic acid is known as an endogenous glutamate receptor agonist, which combines with the NMDA receptor (Olverman et al. 1988).

Theanine injection increased dopamine concentration up to 300% of the basal value in the interstitium and this result was similar to the value obtained in a previous study (Yokogoshi et al. 1998a). Theanine may influence interstitium dopamine concentration either directly or indirectly, e.g., via glutamatergic nerve terminals; however, theanine had no effect on interstitial glutamic acid concentration, and interstitial aspartate was even reduced by theanine. These results suggested that theanine did not increase excitatory neurotransmitter concentrations in the interstitium, such as L-trans-2,4-PDC, and the effect of theanine on dopamine concentration in the interstitium may be independent of the glutamic acid excitatory neurotransmission pathway.

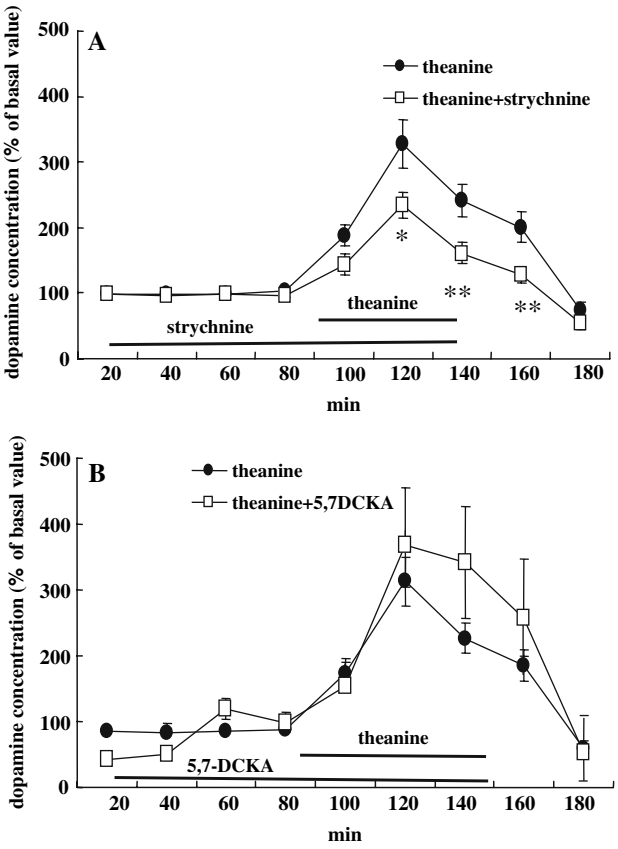

Fig. 3 Effects of glycine receptor antagonists on theanine-changed dopamine concentration in the rat brain striatum interstitium. Effect of strychnine (**a**) or 5,7-dichloroknyrenic acid (5,7-DCKA) (**b**) on the increase of dopamine concentration by theanine injection in the rat brain striatum interstitium. Strychnine (20 μM) or 5,7-DCKA (33.3 μM) was injected into the striatum 1 h before theanine injection and co-perfusised with theanine. Theanine (0.2 μmol/2 μl perfusion/min) was injected for 1 h. Values were measured by in vivo microdialysis, and expressed as the percentage change from the averaged basal value perfused with Ringer's solution ($n = 8$). Values are the means ± SEM. # $P < 0.05$, * $P < 0.01$, significant difference between the theanine-injected group and the 5,7-DCKA-or strychnine-injected group. The period of reagent injection is shown by a *black bar*

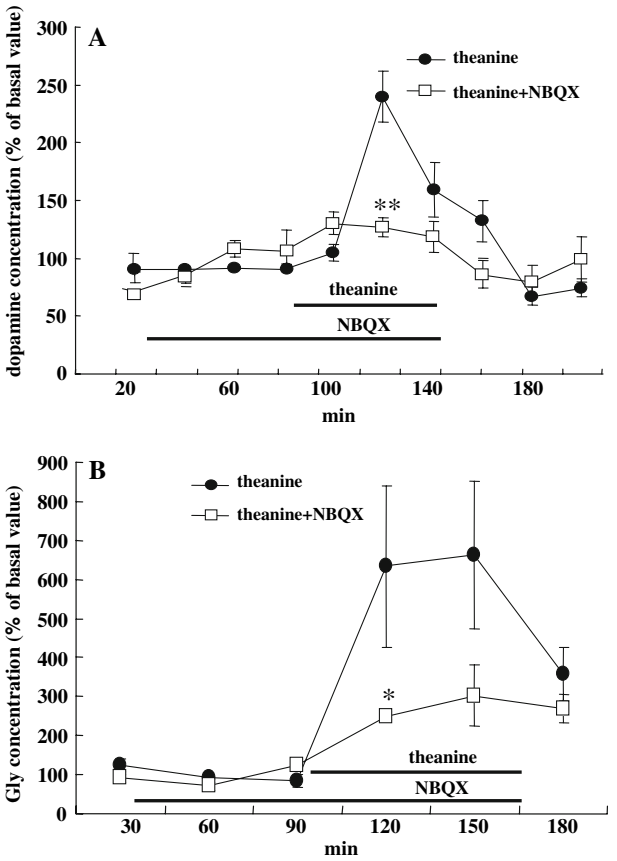

Fig. 4 Effects of glutamate receptor antagonists on theanine-changed neurotransmitter concentrations in the rat brain striatum. Effect of NBQX on the increase of dopamine (**a**) and glycine (**b**) concentrations by theanine injection in the rat brain striatum interstitium. NBQX (100 uM) was injected into the striatum 1 h before theanine injection and co-perfused with theanine. Theanine (0.2 μmol/2 μl perfusion/min) was injected for 1 h. Values were measured by in vivo microdialysis, and expressed as the percentage change from the averaged basal value perfused with Ringer's solution ($n = 8$). Values are the means ± SEM. * $P < 0.05$, ** $P < 0.01$, significant difference between the theanine-injected group and the NBQX-injected group. The period of reagent injection is shown by a *black bar*

Theanine injection increased glycine concentration dramatically in the interstitium. Glycine is known as a major inhibitory neurotransmitter in the spinal cord and brainstem, and is concerned with processing motor and sensory information, and disorders such as epilepsy. Recent studies showed that glycine was released from granule cells and astrocytes, and there are many glycine receptors in the central nervous system. In the central nervous system, glycine and these receptors mediate inhibitory neurotransmission in the same manner as GABA and work simultaneous with GABA (Legendre 2001; Lopez et al. 2001). Moreover, it was revealed that glycine injection increases dopamine concentration in the rat striatum interstitium (Yadid et al. 1993). Thus, we examined whether theanine increased interstitial glycine concentration and whether glycine neurotransmission contributed to the increase in interstitial dopamine.

Strychnine, a glycine receptor antagonist, inhibited the increase of dopamine concentration by theanine injection in the interstitium. This result suggested that the increases of glycine in the interstitium by theanine might have influenced dopamine release via glycine receptors. Although glycine is known as a major inhibitory neurotransmitter, glycine combines with the glycine binding site of the NMDA receptor and enhances excitatory neurotransmission (Xu et al. 1999; Kuhse et al. 1995; Nong et al. 2003). Glycine enhanced NMDA evoked dopamine release, and 7-chloro-kynurenate, an NMDA antagonist, acting on a glycine site, markedly reduced this response

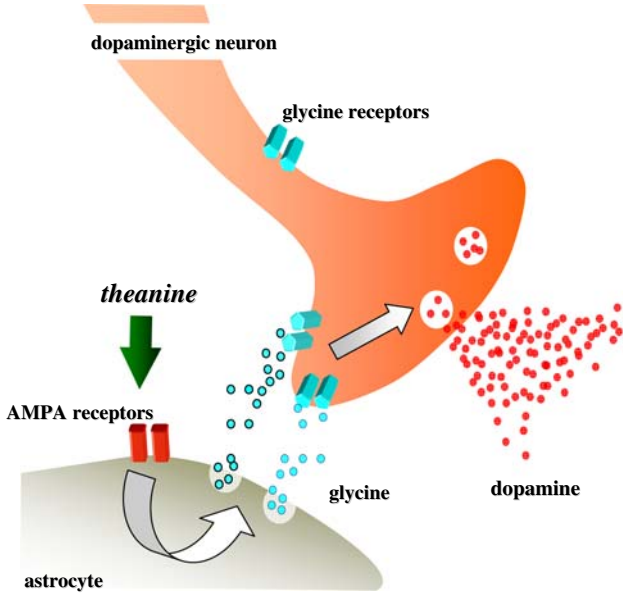

Fig. 5 Hypothesis of the action mechanism of theanine on neurotransmitter concentration in the striatum

(Martinez et al. 1992); however, 5,7-DCKA injection did not prevent the increase of dopamine concentration by theanine injection in this study. These results suggested that theanine-increased glycine influenced interstitial dopamine via glycine receptors, and not NMDA receptors.

In this study, dopamine concentration data have negligible error bars and glycine data have large error bars. From these data, we expected that not only glycine but also other factors were involved in the effect of theanine on dopamine concentration in the perfusate. It was shown that glycine is co-localized with GABA in astrocytes and interneurons (Ross and Soltesz 2001; Bureau and Mulle 1998; Xu et al. 1999; Levi and Patrizio 1992), and glycine and GABA are released simultaneously and work synergistically on different targets (Bohlhalter et al. 1994). We expected that the GABAergic nervous system may also be influenced by theanine injection and is involved in dopamine concentration increase in the perfusate; however we did not examined the effect of theanine on intersutitial GABA concentration and GABA receptors in this study.

It was reported that inhibitory neurotransmitters, such as glycine and GABA, are released by activating α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, ionotropic glutamate receptors (Ross and Soltesz 2001; Bureau and Mulle 1998; Xu et al. 1999), and recent reports showed that activating AMPA/kainate receptors facilitated electrically evoked dopamine efflux in guinea pig striatal slices, and glycine and GABA release from astrocytes and interneurons (Antonelli et al. 1997; Han et al. 2000). In addition, it was shown that theanine bound to AMPA receptors not to NMDA receptors (Kakuda et al. 2002a). Thus, we supposed AMPA receptors to be involved in the effects of theanine on glycine levels in the perfusate. NBQX, an AMPA/kainate receptor antagonist, prevented both theanine-induced increases in perfusate dopamine and glycine levels. This result suggested that AMPA/kainate receptors might be involved in the early phase of theanine-evoked neurotransmitter changes in the perfusate. Moreover, because theanine did not change the glutamate level in the perfusate (Fig. 3a), theanine itself might have acted on AMPA/kainate receptors directly (Kakuda et al. 2002a).

In conclusion, theanine-induced increases in perfusate dopamine levels were not accompanied by significant changes in glutamate levels, and even by decreases in perfusate aspartate, but by pronounced increases in inhibitory neurotransmitter glycine levels. We suggest that theanine-induced increases in perfusate dopamine levels might at least partly be mediated by theanine-induced release of glycine. Moreover, we suggest that theanine-induced changes in perfusate glycine levels involved AMPA/kainate receptors directly addressed by theanine (Fig. 5). Recent research demonstrated the effects of theanine, such as reducing blood pressure in spontaneously hypertensive rats (Yokogoshi et al. 1995), inhibiting caffeine-induced excitatory stimulation (Kakuda et al. 2000b), and a neuroprotective effect on ischemic neuronal death (Kakuda et al. 2000a). These results suggest that theanine has a calming effect on neurotransmission and mood. Our results suggest that these phenomena may be caused by theanine-facilitated inhibitory neurotransmission; however, further studies will be necessary to elucidate the detailed mechanism.

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