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Theanine, an Ingredient of Green Tea, Inhibits [^{3}H]Glutamine Transport in Neurons and Astroglia in Rat Brain

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We have previously shown that theanine (=γ-glutamylethylamide), an ingredient of green tea, has a protective effect against ischemic neuronal death in the hippocampal CA1 region of the gerbil brain without affecting ligand binding to ionotropic receptor subtypes of the neurotransmitter glutamate structurally related to theanine. The neurotransmitter pool of glutamate is thought to be fueled by the entry of the other structural analog glutamine (Gln) and subsequent cleavage by glutaminase. Although theanine did not inhibit [^{3}H]glutamate accumulation, [^{3}H]theanine was actively accumulated in a temperature-dependent and saturable manner in rat brain synaptosomal fractions. The accumulation of [^{3}H]theanine was markedly inhibited by Gln in a concentration-dependent manner, whereas [^{3}H]Gln accumulation was inhibited by theanine vice versa. Both [^{3}H]theanine and [^{3}H]Gln accumulations were decreased after the replacement of sodium chloride with choline chloride, along with similarly high distribution profiles in telencephalic structures. A similar equilibrium was observed within 30 min at 30°C for the accumulations of both [^{3}H]theanine and [^{3}H]Gln in cultured rat neocortical astroglia as well as neurons, whereas theanine inhibited [^{3}H]Gln accumulation in a concentration-dependent manner at 0.1–10 mM. Furthermore, sustained exposure to 10 mM theanine led to a significant decrease in the level of extracellular glutamate released from cultured neurons. These results suggest that the green tea ingredient theanine would be an inhibitor of different transporters capable of transporting Gln across plasma membranes toward the modulation of the glutamate/Gln cycle required for the neurotransmitter pool of glutamate in neurons.

Key words: green tea; theanine; glutamate/glutamine cycle; glutamine transporter; [^{3}H]Gln accumulation

Previously, we have shown that a prior intracerebroventricular injection of the green tea ingredient L-theanine (=γ-glutamylethylamide; Fig. 1A) leads to significant protection of the hippocampal CA1 pyramidal neurons against delayed neuronal cell death in gerbils with bilateral forebrain global ischemia (Kakuda et al., 2000). However, theanine is a poor inhibitor of ligand binding to three different ionotropic receptor subtypes of the excitatory amino acid neurotransmitter L-glutamate (Fig. 1B), which is a structural analog of theanine, in rat cortical neurons compared with that of glutamate itself (Kakuda et al., 2002). In contrast, theanine is metabolized by phosphate-independent glutaminase responsible for the degradation of another theanine structural analog, L-glutamine (Gln; Fig. 1C), in the rat kidney, but not by phosphate-dependent glutaminase (Tsuge et al., 2003). Although glutamate is shown to play a pivotal role in mechanisms underlying delayed neuronal cell death after ischemia (Szatkowski and Attwell, 1994), a structural evaluation clearly reveals a greater similarity of theanine to Gln than to glutamate with regard to the amide moiety, suggesting that theanine could elicit its neuroprotective action through particular mechanisms relevant to Gln rather than to glutamate in the brain.

Indeed, in the central nervous system, Gln is believed to be the principal substrate for the glutamine transporter (GlnT) (=ATA1/SAT1/SNAT1), with a precursor role for the neurotransmitters glutamate and γ-aminobutyric acid (Schousboe et al., 1979). In neurons, Gln is hydrolyzed to glutamate by the catalytic action of phosphate-dependent glutaminase (Schousboe et al., 1979) toward the condensation of glutamate into synap-

The first two authors contributed equally to this work.

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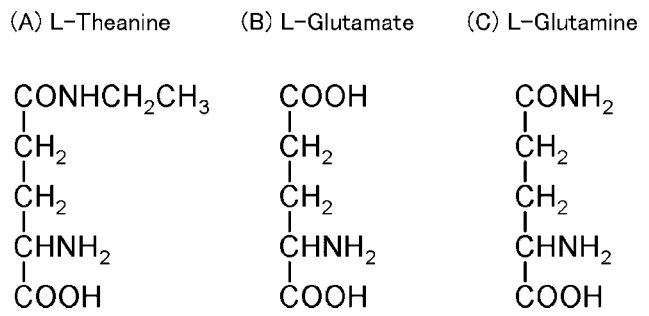

Fig. 1. Chemical structures of L-theanine (**A**; γ-glutamylethylamide), L-glutamate (**B**) and L-glutamine (**C**).

tic vesicles by vesicular glutamate transporters expressed at vesicular surfaces for subsequent exocytotic release into synaptic clefts upon stimulation. Extracellular glutamate is then removed from synaptic clefts by excitatory amino acid transporters expressed by adjacent cells, including neurons and astroglia. It is widely accepted that this active transport is crucial for preventing the accumulation of extracellular glutamate to a neurotoxic level in addition to terminating synaptic glutamatergic neurotransmission. In astrocytes, incorporated glutamate is then converted by glutamine synthetase to Gln. This Gln is exported to extracellular spaces for the reentry through GlnT into neurons to fuel the aforementioned glutamate/Gln cycle required for the neurotransmitter pool at nerve terminals in glutamatergic neurons (Schousboe et al., 1979; Broer, 2002; Dolinska et al., 2004). As described above, however, theanine is not metabolized by phosphate-dependent glutaminase enriched in the brain (Tsuge et al., 2003). In this study, therefore, we have determined the incorporation of [^{3}H]glutamate, [^{3}H]Gln, and [^{3}H]theanine in order to evaluate the action site in the brain for neuroprotection by theanine.

MATERIALS AND METHODS

Materials

Amino acids were all purchased from Wako (Tokyo, Japan). Theanine was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). [^{3}H]Theanine (1.41 TBq/mmol) was synthesized by Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan). [^{3}H]Gln (1.89 TBq/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA). Other chemicals used were of the highest purity commercially available.

Preparation of Synaptosomal (P2) Fractions

This study was conducted in compliance with the Guideline for Animal Experimentation at Kanazawa University, with an effort to minimize the number of animals used and their suffering. Unless otherwise indicated, crude synaptosomal fractions were prepared from the whole brains of male Wistar rats of 8–10 weeks old, with minor modifications of the method described by Takarada et al. (2004). Each discrete rat brain structure was also dissected according to the procedures described by Glowinski and Iversen (1966). In brief, brains were removed from the cranial cavity and homogenized in 0.32 M sucrose with a Teflon/glass homogenizer. Homogenates were centrifuged at 800*g* for 10 min, followed by further centrifugation of the supernatants at 20,000*g* for 20 min and subsequent suspension of the sediment of P2 fractions in a 20-fold volume of 0.32 M sucrose. Suspensions were again centrifuged at 20,000*g* for 10 min for washing. This washing process was repeated three times. The final pellets were suspended in a tenfold volume of Krebs-Ringer buffer (KRB) [10 mM HEPES buffer (pH 7.4) containing 125 mM NaCl, 3.5 mM KCl, 1.25 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.5 mM $CaCl_2$, and 10 mM glucose] for the use as the synaptosomal fractions in uptake experiments on the same day.

Preparation of Synaptic Membranes

The crude synaptosomal fractions were subjected to osmotic shock by the use of ice-cold water, followed by centrifugation at 8,000*g* for 20 min and subsequent suspension of the upper sediments with supernatants by gentle shaking as described previously (Ogita and Yoneda, 1986). The suspensions thus obtained were again centrifuged at 50,000*g* for 30 min, followed by suspension of the pellets in 40 vol 5 mM Tris-acetate buffer (pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride, 0.01% bacitracin, and 1 mM EDTA using a Physcotron homogenizer at setting 6 for 2 min at 4°C and subsequent centrifugation at 50,000*g* for 30 min. The resulting pellets were washed three times by suspension in the same volume of buffer with centrifugation at 50,000*g* for 30 min. The final pellets were suspended in 8 vol 0.32 M sucrose and frozen at −80°C for no longer than 1 week. On the day of the experiments, frozen suspensions were thawed at room temperature, followed by washing twice as mentioned above. In addition, the suspensions were treated with 0.08% Triton X-100 at an approximate protein concentration of 0.32 mg protein/ml at 2°C for 30 min with gentle stirring (Ogita and Yoneda, 1988). This treatment was terminated by the centrifugation as described above, and the resulting pellets were washed once more to obtain Triton-treated membranes for use in receptor binding assays.

Preparation of Astroglial Culture

Astrocytes were prepared as described previously (Murakami et al., 2003). In brief, cerebral neocortex (CX) from 20-day-old embryonic Wistar rats was cleared of meninges and treated with 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing 5.5 mM glucose for 20 min at 37°C with gentle shaking. An equal volume of horse serum supplemented with 0.1 mg/ml of DNase I was added to the medium to inactivate the trypsin. Then, the tissues were centrifuged at 500*g* for 5 min. The tissue sediments were triturated through a fire-narrowed Pasteur pipette with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 U/ml penicillin. After the suspensions were filtered through a lens-cleaning paper (Fuji Photo Co., Tokyo, Japan), cells were plated on 90-mm-diameter polyethyleneimine-coated plastic dishes (Nunc, Roskilde, Denmark) at a density of 1.0–

1.3×10^5 cells/cm^2. Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C with the medium changed twice per week. After 1 week, cells were replated to remove the neurons. On days 12–14, they were replated onto a six- or 24-well dish using an ordinary trypsin treatment technique at a density of 0.75×10^5 cells/cm^2 and cultured for an additional 1–2 days.

Preparation of Neuronal Culture

Primary neuronal cultures were prepared from 18-day-old embryonic rat CX as originally described by di Porzio et al. (1980), with minor modifications (Nakamichi et al., 2002). In brief, embryonic rat CX was dissected and incubated with Versene at room temperature for 12 min. After the cells were counted with a Trypan blue exclusion test, cells were mechanically dissociated with a fire-narrowed Pasteur pipette in the culture medium and plated at a density of 2.5×10^5 cells/cm^2 in a six- or 24-well dish (Nunc). Prior to use, the dishes were sequentially coated with 7.5 μg/ml poly-L-lysine and 10% FCS. The culture medium contained basal DMEM/F-12 supplemented with 10% FCS, 33 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES, and 0.11% sodium bicarbonate. Cells were cultured in DMEM/F-12 with the supplement described above for an initial 3 days, treated with 10 μM cytosine arabinoside for 24 hr over 2–3 days, and then cultured in basal DMEM containing 33 mM glucose, 2 mM Gln, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES, 0.11% sodium bicarbonate, 50 μg/ml transferrin, 500 ng/ml insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/ml sodium selenite, and 100 μM putrescine for 3–12 days. The culture medium was replaced every 3 days with freshly prepared culture medium of the same composition. Cultures were maintained at 37°C in a 5% CO_2/95% air-humidified incubator.

Determination of Transport Activities

Freshly prepared P2 fractions were incubated with [^{3}H]theanine, [^{3}H]glutamate, or [^{3}H]Gln at 1 μM in either the presence or the absence of a variety of test drugs at 4°C or 30°C for different periods in 0.5 ml KRB. Incubation was terminated by the addition of 3 ml ice-cold buffer and subsequent filtration through a Whatman GF/B glass fiber filter under a constant vacuum of 15 mmHg. The filter was further rinsed four times with buffer, and the amount of radioactivity trapped on the filter was measured with a liquid scintillation spectrometer using 5 ml Triton-toluene scintillation cocktail at a counting efficiency of about 40%. Nonspecific binding was calculated by the amount of radioactivity detected in the presence of each unlabeled substrate at 1 mM, which was similar to that found with the incubation at 4°C. Assays were performed in triplicate at intervals of 20 sec.

Determination of [^{3}H]Theanine Binding

Crude synaptic membranes were incubated with 10 nM [^{3}H]theanine in 0. 5 ml 50 mM Tris-acetate buffer (pH 7.4) at 4°C or 30°C for different periods up to 30 min. The incubation was similarly terminated by the addition of 3 ml ice-cold buffer and subsequent filtration through a Whatman GF/B glass fiber filter under a constant vacuum of 15 mmHg. The filter was further rinsed four times with buffer, and the amount of radioactivity trapped on the filter was measured with a liquid scintillation spectrometer using 5 ml Triton-toluene scintillation cocktail. Nonspecific binding was calculated by the amount of radioactivity in the presence of 1 mM unlabeled theanine. Assays were performed in triplicate at intervals of 20 sec.

Determination of [^{3}H]Gln Transport in Cultured Cells

Cultured CX neurons and astrocytes were washed twice with HEPES Krebs-Ringer (HKR; 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 50 mM HEPES, and 5.6 mM D-glucose, pH 7.4) buffer, followed by incubation in HKR buffer at 37°C for 30 min in a 5% CO_2 incubator. The cells were then incubated with 1 μM [^{3}H]Gln at 37°C for 10 min in either the absence or the presence of theanine at different concentrations. Reaction was terminated by gentle aspiration of the buffer, followed by rinsing three times with ice-cold HKR buffer containing 5 mM unlabeled Gln at 4°C and subsequent solubilization with 0.1 M NaOH for liquid scintillation spectrometry using 3 ml scintillation cocktail (clear sol I), as described previously (Takarada et al., 2004). The protein concentration was determined with a Bio-Rad Protein Assay Kit.

Measurement of Endogenous Glutamate

Rat cortical neurons were cultured in DMEM/F-12 supplemented as described above in either the presence or the absence of 10 mM theanine without cytosine arabinoside for 3 days. The concentration of the extracellular glutamate was measured in the culture medium using HPLC with the o-phthalaldehyde derivatization method. The culture medium was collected from the primary cultured neurons, followed by the addition of perchloric acid at a final concentration of 0.2 M and subsequent centrifugation for 5 min at 20,000*g*. The supernatant was neutralized by 1 M NaOH, followed by the reaction for 2 min at room temperature with a derivatization reagent [5 mg/ml o-phthalaldehyde, 1% β-mercaptoethanol, and 0.36 M potassium borate (pH 10.4)] and subsequent injection into an analysis column (Symmetry C_{18} 3.5 μm, 4.6 × 75 mm). Elution was conducted using a programmable pump (Gilson model 305 + 306) with a linear gradient to 70% acetonitrile and 20 mM sodium phosphate (pH 6.0) at a flow rate of 1.0 ml/min. Derivatized amino acids were detected with a fluorometer (Gilson model 21).

Data Analysis

Results are expressed as mean ± SEM, and the statistical significance was determined by two-tailed and unpaired Student's *t*-test or one-way ANOVA followed by Bonferroni/Dunnett post hoc test.

RESULTS

[^{3}H]Theanine Accumulation

To investigate the possible affinity of theanine for particular membrane receptors, crude synaptic membranes

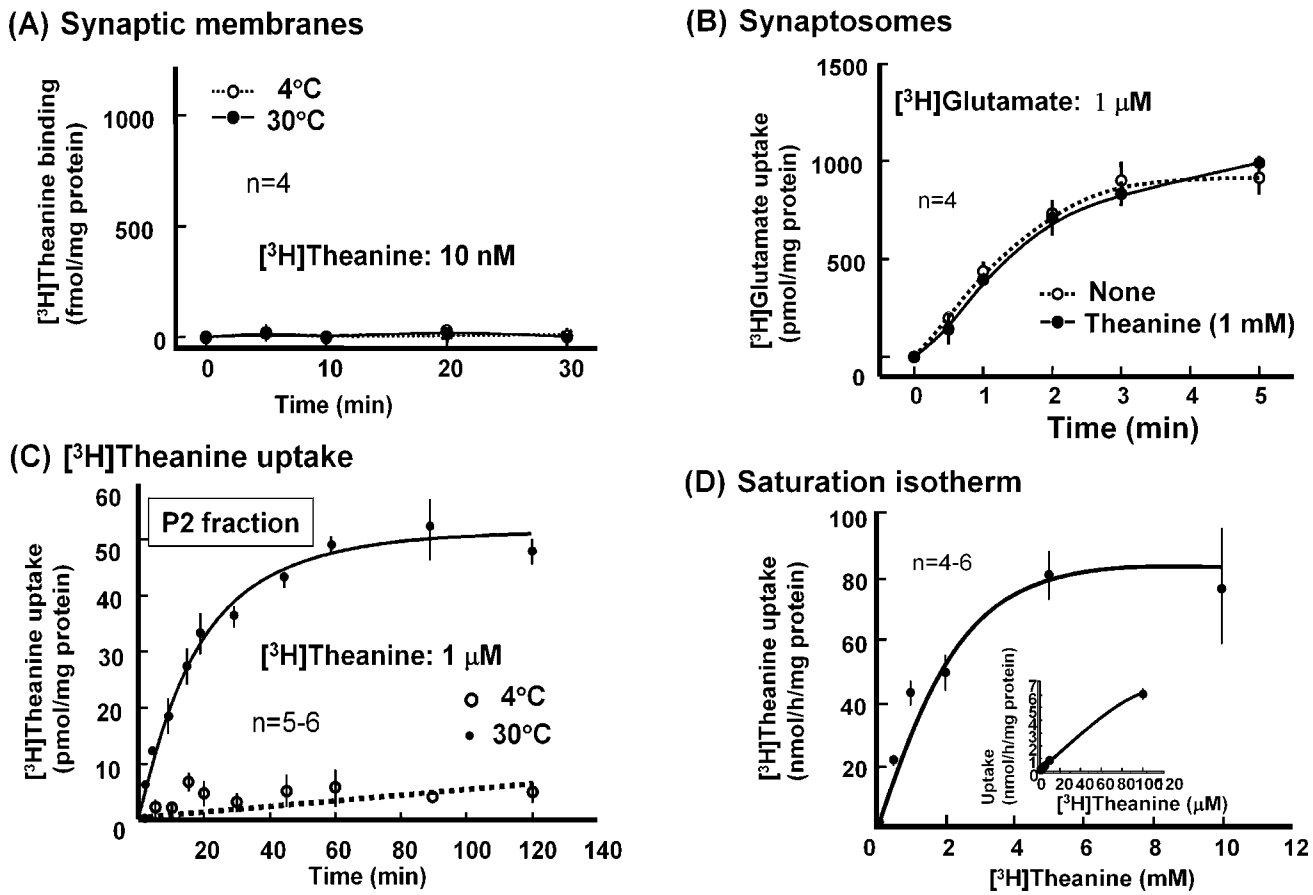

Fig. 2. [^{3}H]Theanine binding and uptake. **A:** Synaptic membranes were incubated with 10 nM [^{3}H]theanine at 4°C and 30°C for a period up to 30 min. **B:** Synaptosomal fractions were incubated with 1 μM [^{3}H]glutamate in either the presence or the absence of 1 mM theanine at 30°C for 5 min. **C:** Synaptosomal fractions were incubated with 1 μM [^{3}H]theanine at 4°C and 30°C for a period up to 120 min. **D:** Synaptosomal fractions were incubated with [^{3}H]theanine at a concentration range of 1 μM to 10 mM at 30°C for 20 min. Values are the mean ± SE in three independent sets of experiments done in triplicate.

were incubated with 10 nM [^{3}H]theanine at 4°C and 30°C for different periods. However, no marked binding for [^{3}H]theanine was detectable in synaptic membranes irrespective of the incubation time and temperature (Fig. 2A). Consequently, an attempt was made to determine whether theanine affects the incorporation of [^{3}H]glutamate in synaptosomal fractions. [^{3}H]Glutamate accumulation reached a plateau within 5 min after a linear increase up to 2 min at 30°C, whereas the addition of 1 mM theanine did not markedly affect [^{3}H]glutamate accumulation throughout the incubation (Fig. 2B). In contrast, [^{3}H]theanine was highly accumulated in a temperature-dependent manner in synaptosomal fractions (Fig. 2C). [^{3}H]Theanine accumulation was linearly increased with incubation time up to 30 min, with a plateau within 120 min at 30°C in synaptosomal fractions. Saturation isotherms revealed that the [^{3}H]theanine accumulation consisted of at least two different components with Km values of 42.3 μM and 1,88 mM, and Vmax values of 3.92 and 104.2 nmol/hr/mg protein, respectively (Fig. 2D). Therefore, theanine could be incorporated into rat brain synaptosomes through a mechanism not related to glutamate transporters.

Pharmacological Profiles

Actually, [^{3}H]theanine accumulation was markedly inhibited by the addition of the other structural analog, Gln, in a concentration-dependent manner in a concentration range of 10 nM to 1 mM, which occurred to a greater extent than did the inhibition by theanine itself (IC_{50} values: Gln, 329.2 ± 59.5 μM; theanine, >1,000 μM; Fig. 3A, left panel). However, glutamate was rather ineffective at markedly inhibiting [^{3}H]theanine accumulation at the concentrations used, whereas asparagine markedly inhibited [^{3}H]theanine accumulation in synaptosomal fractions, with aspartate being ineffective, at a concentration range of 10 nM to 1 mM (IC_{50} value = 577.2 ± 88.1 μM; Fig. 3A, right panel).

To examine the possible inhibition by theanine of [^{3}H]Gln accumulation, synaptosomal fractions were incubated with 1 μM [^{3}H]Gln in either the presence or

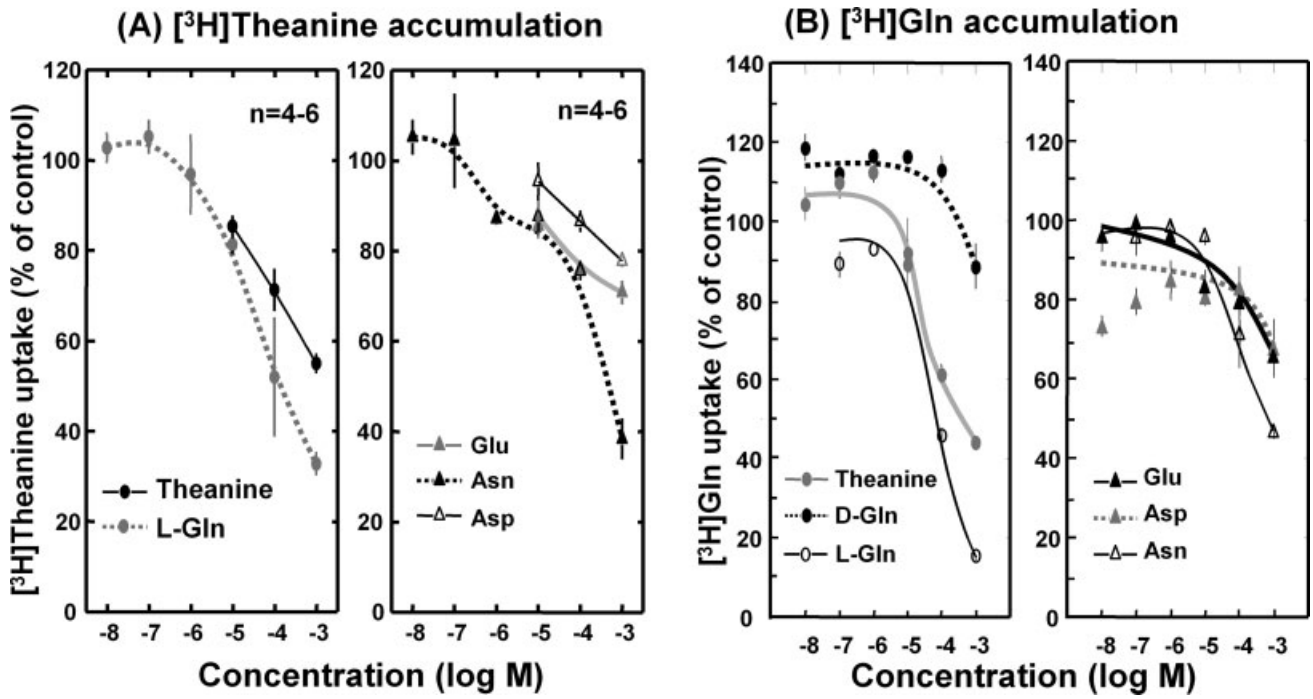

Fig. 3. Effects of structural analogs on accumulations of [^{3}H]theanine and [^{3}H]Gln in synaptosomal fractions. Synaptosomal fractions were incubated with 1 μM [^{3}H]theanine (**A**) or [^{3}H]Gln (**B**) at 30°C for 20 min in either the absence or the presence of L-theanine, L-Gln, D-Gln, glutamate (Glu), aspartate (Asp), and asparagine (Asn) at different concentrations from 10 nM to 1 mM. Values are the mean ± SE in four to six independent sets of experiments done in triplicate.

the absence of theanine analogs at different concentrations. In synaptosomal fractions, [^{3}H]Gln was highly accumulated in a temperature-dependent and saturable manner, with a plateau within 60 min at 30°C (data not shown). The addition of theanine markedly inhibited [^{3}H]Gln accumulation in a concentration-dependent fashion at concentrations of 10 nM to 1 mM, whereas L-Gln was more efficient than theanine at inhibiting [^{3}H]Gln accumulation, with less inhibition by D-Gln [IC_{50} values (μM): L-Gln 85.1 ± 11.8; theanine 479.8 ± 132.2; D-Gln, >1,000; Fig. 3B, left panel]. Similarly, asparagine was more effective at inhibiting the accumulation of [^{3}H]Gln than glutamate or aspartate [IC_{50} values (μM): asparagine 719.1 ± 75.3; glutamate, >1,000; aspartate, >1,000; Fig. 3B, right panel].

In addition to the theanine analogs described above, a variety of amino acids markedly inhibited the temperature-dependent accumulation of [^{3}H]theanine (Fig. 4A) as well as [^{3}H]Gln (Fig. 4B) in synaptosomal fractions at 1 mM. These included tyrosine, methionine, tryptophan, phenylalanine, isoleucine, leucine, and cysteine for [^{3}H]theanine accumulation and Gln for [^{3}H]Gln accumulation. Moderate inhibition was seen with theanine, histidine, lysine, threonine, serine, Gln, asparagine, glycine, proline, valine, and alanine for [^{3}H]theanine accumulation and with theanine, histidine, and cysteine for [^{3}H]Gln accumulation. Rather weak inhibition was found with GABA, arginine, glutamate, and aspartate for [^{3}H]theanine accumulation and with GABA, arginine, lysine, glutamate, aspartate, tyrosine, threonine, serine, asparagine, glycine, methionine, proline, tryptophan, phenylalanine, isoleucine, leucine, valine, and alanine for [^{3}H]Gln accumulation. Accordingly, [^{3}H]theanine accumulation shows pharmacological profiles similar to those of [^{3}H]Gln accumulation in rat brain synaptosomal fractions.

Distribution Profiles

To evaluate the requirement for sodium ions, NaCl was replaced with choline chloride in KRB for the incubation with [^{3}H]Gln and [^{3}H]theanine. Removal of sodium ions led to a significant decrease in [^{3}H]Gln accumulation by 50% (Fig. 5A, left columns) and in [^{3}H]theanine accumulation by 30% (Fig. 5A, right columns) in synaptosomal fractions.

To analyze the distribution profile in the brain, discrete brain regions were excised from rat brains, followed by the preparation of P2 fractions and subsequent determination of the accumulation of [^{3}H]Gln and [^{3}H]theanine. [^{3}H]Gln was highly accumulated in synaptosomal fractions of telencephalic structures, including CX, hippocampus, and corpus striatum, with progressively less potent activities in midbrain, hypothalamus, medulla-pons, and cerebellum (Fig. 5B, left panel; [^{3}H]Gln accumulation (pmol/mg protein/20 min): hippocampus, 541.9 ± 23.2; striatum, 477.2 ± 9.9; CX, 448.0 ± 21.4; hypothalamus, 222.5 ± 10.9; midbrain,

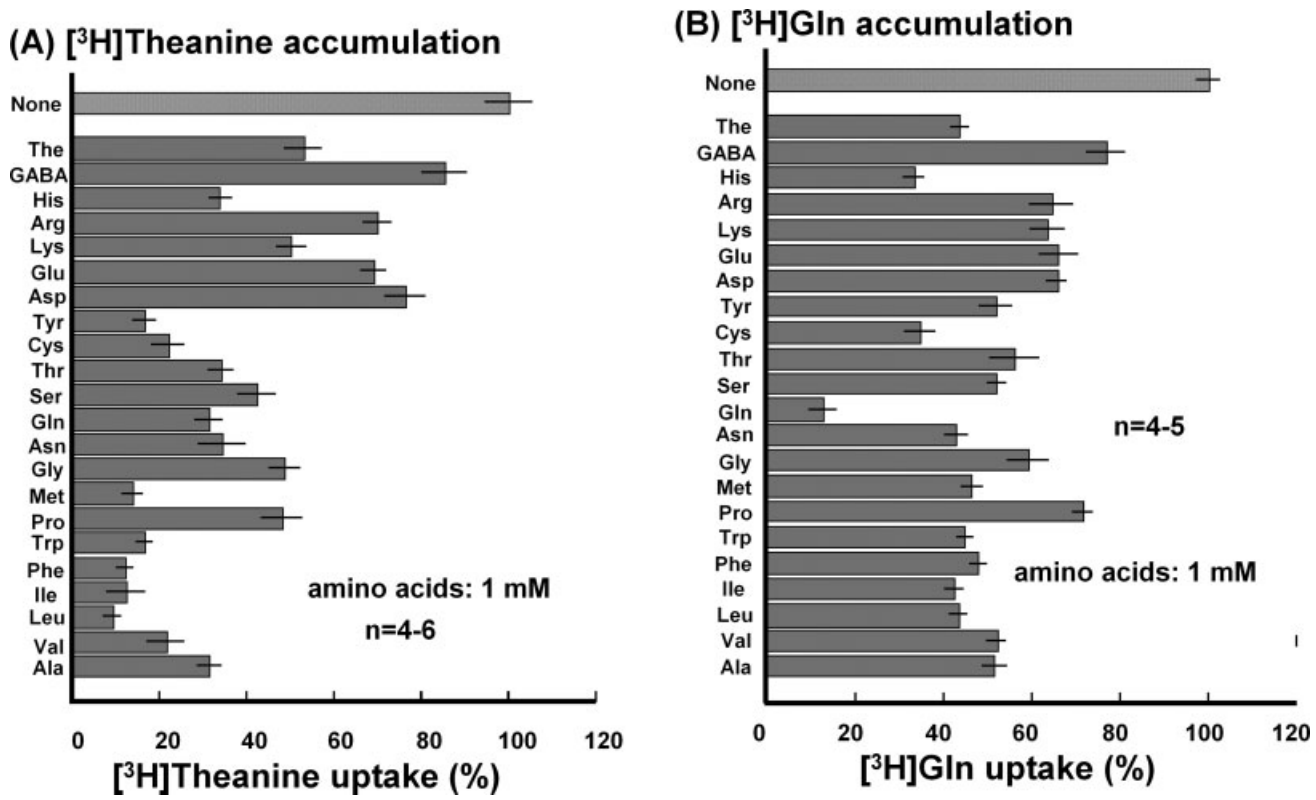

Fig. 4. Pharmacological evaluation of accumulations of [^{3}H]theanine and [^{3}H]Gln in synaptosomal fractions. Synaptosomal fractions were incubated with 1 μM [^{3}H]theanine (**A**) or [^{3}H]Gln (**B**) at 30°C for 20 min in either the presence or absence of different amino acids at 1 mM. Alan, alanine; Arg, arginine; Asn, asparagine, Asp, aspartate; Cys, cysteine; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; The, theanine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

286.0 ± 10.4; medulla-pons, 128.7 ± 5.0; cerebellum, 69.9 ± 8.6). Similarly, high [^{3}H]theanine accumulation was seen in synaptosomal fractions of telencephalic structures followed by medulla-pons, midbrain, hypothalamus, and cerebellum in order of decreasing activity (Fig. 5B, right panel; [^{3}H]theanine accumulation (pmol/mg protein/20 min): striatum, 41.8 ± 1.5; CX, 36.0 ± 1.7; hippocampus, 34.3 ± 0.85; medulla-pons, 30.9 ± 2.2; midbrain, 28.7 ± 2.3; hypothalamus, 27.1 ± 1.1, cerebellum, 14.8 ± 0.85). Thus, [^{3}H]theanine accumulation shows distribution profiles similar to those of [^{3}H]Gln accumulation in discrete rat brain structures.

Accumulation in Primary Cultured Cells

We next investigated the accumulation of [^{3}H]Gln and [^{3}H]theanine in primary cultured neurons and astrocytes prepared from rat cerebral cortex, in place of synaptosomal fractions. Cultured cells were incubated with either [^{3}H]Gln or [^{3}H]theanine at 1 μM for different periods at 30°C. [^{3}H]Gln accumulation increased almost linearly with incubation time up to 20 min and reached a plateau within 60 min in primary cultured neurons, whereas considerably high [^{3}H]Gln accumulation was also seen in primary cultured astrocytes (Fig. 6A). Similarly, [^{3}H]theanine was highly accumulated, with a plateau within 60 min at 30°C in primary cultured neurons and astrocytes (Fig. 6B). Therefore, both [^{3}H]Gln and [^{3}H]theanine would be actively incorporated into astrocytes in addition to neurons.

To analyze the inhibition of [^{3}H]Gln accumulation by theanine, cultured neurons and astrocytes were incubated with [^{3}H]Gln in either the absence or the presence of theanine at a concentration range of 0.1–10 mM. In both cell cultures, the addition of theanine induced a significant decrease in [^{3}H]Gln accumulation in a concentration-dependent manner at the concentrations used (Fig. 7A). Because the incorporation of Gln is required for the Gln/glutamate cycle to fuel the neurotransmitter pool of glutamate at glutamatergic synapses, neurons were cultured in either the presence or the absence of 10 mM theanine for 3 days, followed by the collection of culture medium and subsequent determination of endogenous glutamate released during culture. As shown in Figure 7B, sustained exposure to theanine led to a slight but statistically significant decrease in the extracel-

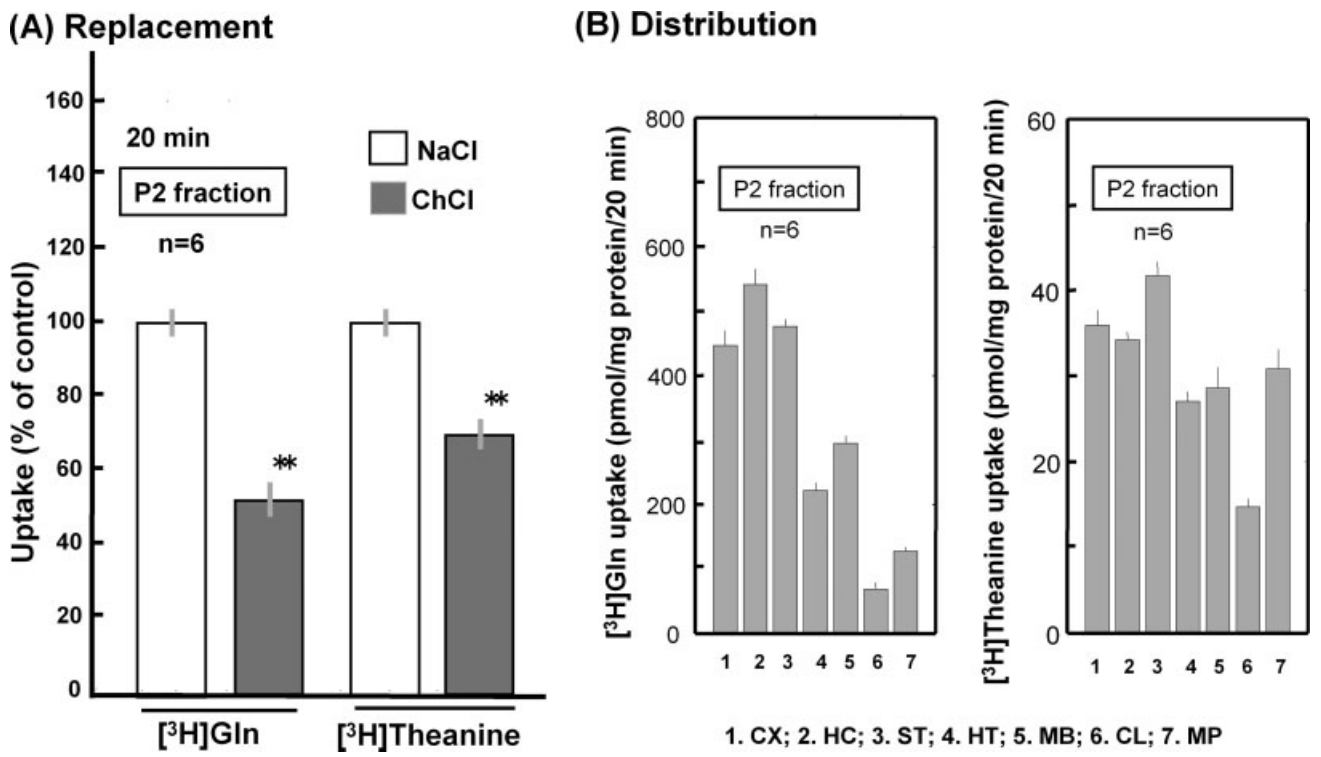

Fig. 5. Comparison between accumulations of [^{3}H]Gln and [^{3}H]theanine. **A:** Synaptosomal fractions were incubated with 1 μM [^{3}H]Gln or [^{3}H]theanine at 30°C for 20 min in KRB containing sodium chloride (NaCl) or choline chloride (ChoCl) at 130 mM. $\star P < 0.05$, $\star\star P < 0.01$, significantly different from each control value obtained in the presence of 130 mM NaCl. **B:** Each brain structure was dissected for preparation of synaptosomal fractions, followed by incubation with 1 μM [^{3}H]Gln or [^{3}H]theanine for 20 min at 30°C. Values are the mean ± SE in six independent sets of experiments done in triplicate. CL, cerebellum; HC, hippocampus; HT, hypothalamus; MB, midbrain; MP, medulla-pons; ST, striatum.

lular glutamate level in the cultured neurons (1.0 ± 0.03 μM vs. 0.88 ± 0.04 μM; $P < 0.05$). These results suggest that theanine could suppress the exocytotic release of glutamate derived from the Gln/glutamate cycle through the inhibition of Gln incorporation in glutamatergic neurons in a particular situation (Fig. 8).

DISCUSSION

The essential importance of the present findings is that the green tea ingredient theanine markedly inhibited [^{3}H]Gln accumulation in rat brain synaptosomal fractions and cultured rat neocortical neurons, without affecting [^{3}H]glutamate accumulation. Furthermore, [^{3}H]theanine binding sites were not detectable in synaptic membranes at all, although [^{3}H]theanine was highly accumulated in a temperature- and sodium-dependent manner with a saturable profile in synaptosomal fractions. Because the chemical structure of theanine is analogous to the neuronal excitotoxin glutamate, we first thought that the neuroprotection by theanine (Kakuda et al., 2000) would be mediated by an antagonistic profile of this green tea ingredient against particular glutamate receptor subtypes responsible for delayed neuronal cell death after ischemia. These include ionotropic glutamate receptor subtypes such as N-methyl-D-aspartate (NMDA) and DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors (Lodge and Collingridge, 1991). In our previous study on receptor binding, however, theanine has a very low affinity for either the NMDA or the AMPA receptor subtype in cortical membranes (Kakuda et al., 2002). By contrast, the present findings give rise to the idea that theanine would elicit its neuroprotective action through a mechanism relevant to Gln transporters rather than receptors and transporters for glutamate. To our knowledge, this is the first direct demonstration of temperature- and sodium-dependent and saturable [^{3}H]theanine accumulation in rat brain synaptosomal fractions and cultured rat cortical neurons. High-quality green tea leaves have been shown to contain more than 2% theanine (Goto et al., 1996), and Japanese people have been ingesting about 20 mg of theanine per day according to an estimate on the use of 1 g of powdered green tea (Matcha) for the traditional tea ceremony for years. Theanine is also shown to reach the brain through the blood–brain barrier (Yokogoshi et al., 1998) and to prevent of psychological and physiological stress responses (Kimura et al., 2007).

In fact, Gln is actively incorporated into intracellular spaces in rat brain slices (Balcar and Johnston, 1975),

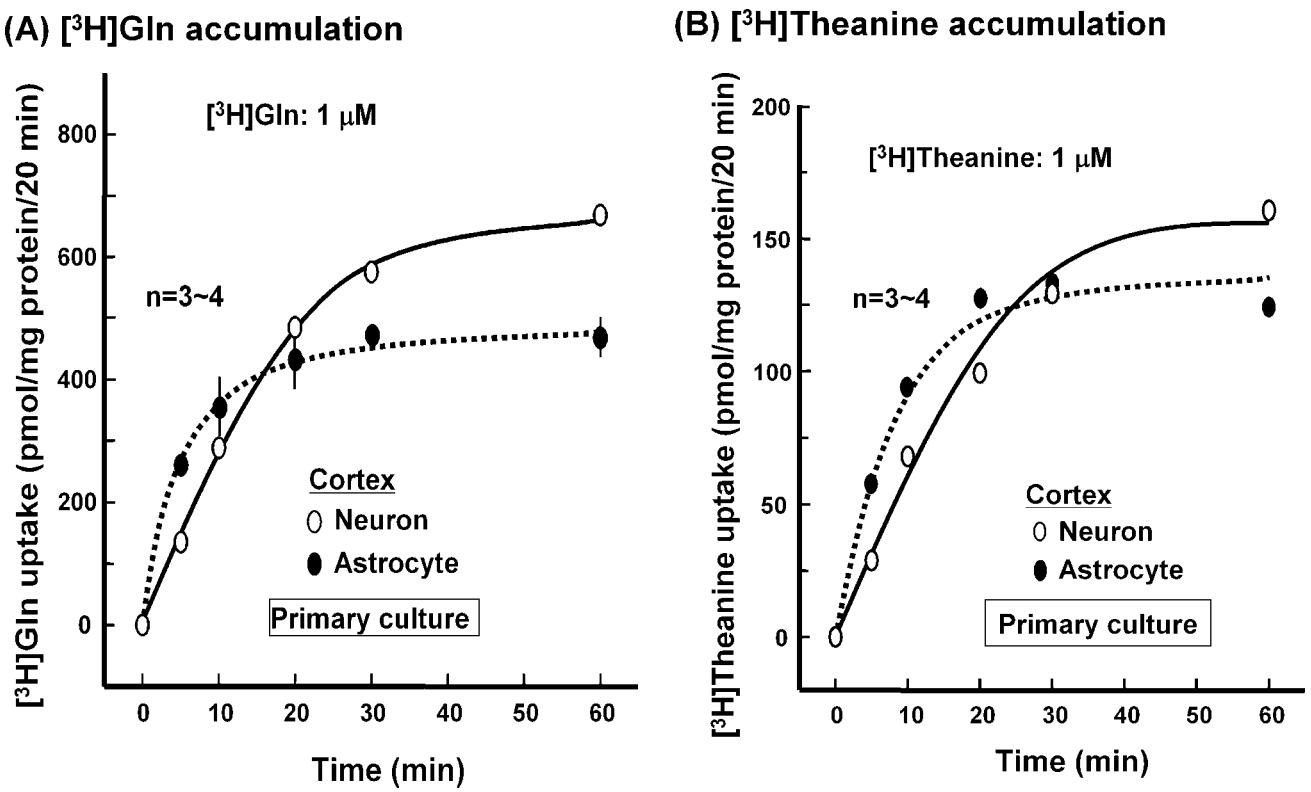

Fig. 6. Accumulations of [^{3}H]Gln and [^{3}H]theanine in cultured astrocytes and neurons. Cultured CX astrocytes and neurons were incubated with 1 μM [^{3}H]Gln (**A**) or [^{3}H]theanine (**B**) for 1–60 min at 30°C. Values are the mean ± SE from three or four independent sets of experiments done in triplicate.

and several independent lines of evidence indicate the involvement of different amino acid transporters in active transmembrane trafficking of Gln in the brain. For example, Gln transport is mediated by at least three sodium-dependent transport systems in the brain. These include system A (Reimer et al., 2000; Sugawara et al., 2000; Varoqui et al., 2000), system ASC (Utsunomiya-Tate et al., 1996; Broer et al., 1999), and system N (Chaudhry et al., 1999; Broer et al., 2002). In addition, Gln is also transported through the sodium-independent system L composed of heteromeric assemblies of different subunits (Chillaron et al., 2001; Wagner et al., 2001; Broer, 2002). Among these different transporters capable of transporting Gln across plasma membranes, only the glutamine transporter (GlnT; = ATA1/SAT1/SNAT1) belonging to the system A family is believed to be exclusively expressed by neurons with high and selective affinity for Gln in the brain (Nagaraja and Brookes, 1996; Albers et al., 2001; Chaudhry et al., 2002). Nevertheless, pharmacological evaluation as well as the sodium dependence is suggestive of the participation of diverse isoforms of membrane transporters with affinity for Gln in the accumulations of [^{3}H]theanine and [^{3}H]Gln in this study. This assumption could account for the differential pharmacological and distribution profiles between [^{3}H]Gln and [^{3}H]theanine accumulations. Theanine could have affinities different from Gln for particular isoforms of membrane transporters capable of transporting Gln.

The prevailing view is that Gln exported to extracellular spaces is taken up through GlnT expressed by neurons to fuel the glutamate/Gln cycle required for the neurotransmitter pool of glutamate at nerve terminals in glutamatergic neurons. The present inhibition supports the assumption that theanine could at least in part alter extracellular Gln levels under the delicate control through GlnT expressed by astrocytes adjacent to glutamatergic synapses in a particular pathological situation such as brain ischemia. Indeed, a higher level of Gln is shown in the cerebrospinal fluid of patients with Alzheimer's disease than in normal subjects (D'Aniello et al., 2005). Theanine markedly inhibited the incorporation of extracellular Gln, so a decrease would be induced in the amount of intracellular glutamate required for the condensation into synaptic vesicles as a neurotransmitter responsible for the subsequent exocytotic release upon stimuli in CX neurons cultured with theanine. The absence of depolarizing stimuli could thus account for the slight but statistically significant decrease in the extracellular level of glutamate accumulated during the culture of neurons with theanine at a relatively high concentration in this study. It is conceivable that theanine is rather

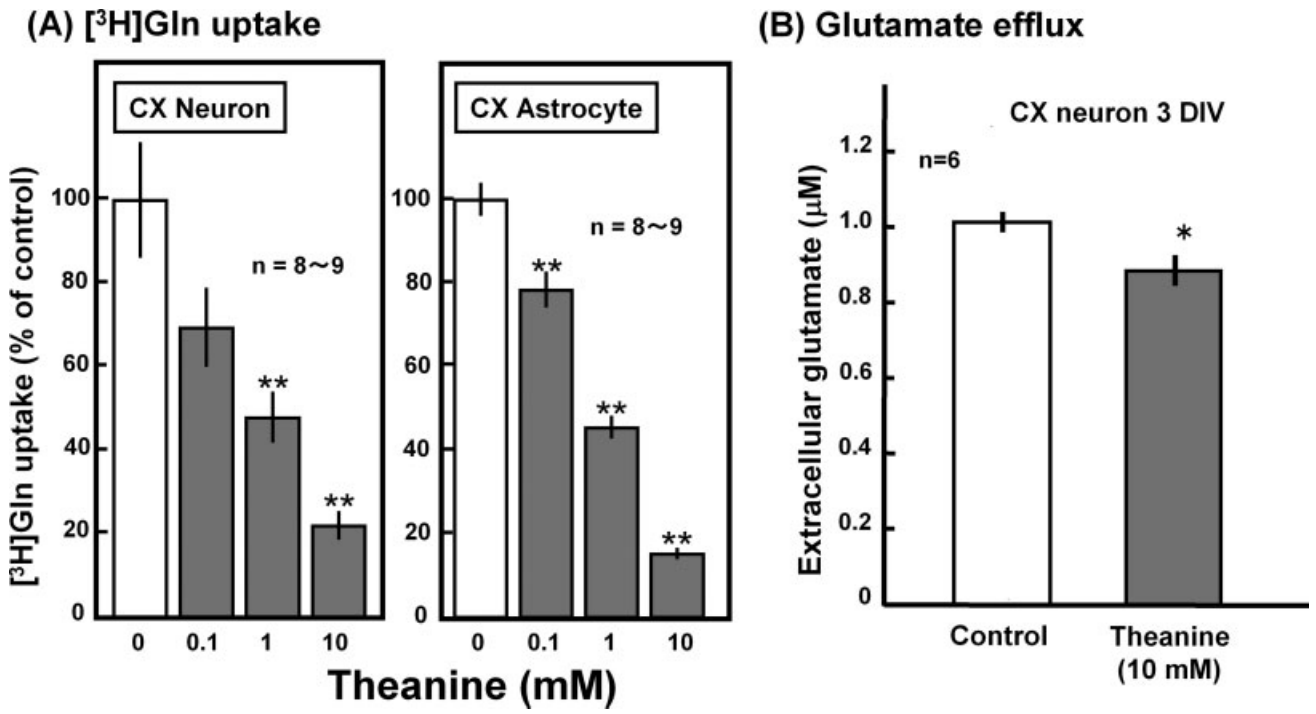

Fig. 7. Effects of theanine on [^{3}H]Gln accumulation and glutamate efflux. **A:** CX neurons and astrocytes were incubated with 1 μM [^{3}H]Gln for 20 min at 30°C in the presence of theanine at a concentration range of 0.1–10 mM. **B:** CX neurons were cultured for 3 days in either the presence or the absence of 10 mM theanine, followed by the collection of culture medium for the determination of glutamate. Values are the mean ± SE from the separate measurements indicated. ⋆$P < 0.05$, ⋆⋆$P < 0.01$, significantly different from each control value obtained in the absence of theanine.

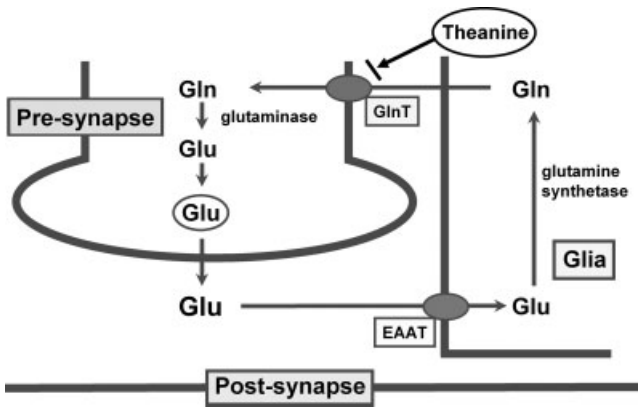

Fig. 8. Schematic representation. Theanine could inhibit the incorporation of extracellular Gln into neurons, which in turn suppresses the conversion by glutaminase of Gln to glutamate required for condensation into synaptic vesicles as a neurotransmitter pool responsible for subsequent exocytotic release upon stimuli. Extracellular glutamate would be incorporated into adjacent astroglia through excitatory amino acid transporters (EAATs), whereas the glutamine transporter (GlnT) would mediate the import of extracellular Gln derived from the synthesis by glutamine synthetase enriched in astrocytes into neighboring neurons.

inefficient at affecting the spontaneous efflux of intracellular glutamate condensed into the neurotransmitter pool in neurons. Furthermore, extracellular glutamate could be not only derived from the spontaneous and evoked release from the neurotransmitter pool but also originated in energy production, protein synthesis, and/or amino acid metabolism pools. From this point of view, the exocytotic release of endogenous glutamate from neurons cultured with theanine in response to depolarization remains to be elucidated in future studies. Taken together, theanine would suppress glutamatergic neurotransmission through the inhibition of Gln incorporation across plasma membranes into neurons toward subsequent neuroprotection against delayed neuronal death after ischemia. Although the present study employed embryonic rat CX for preparation of primary neuronal and astroglial cultures toward the evaluation of mechanisms underlying the protection by theanine of hippocampal CA1 pyramidal neurons in ischemic gerbils (Kakuda et al., 2000), it is rather unrealistic to prepare primary cultures of CA1 pyramidal neurons from the gerbil hippocampus because of a variety of experimental limitations. Moreover, the ischemic damage has been selectively seen in the vulnerable CA1 pyramidal neurons,

but not in the resistant dentate granule neurons (Kakuda et al., 2000). Even in cultured gerbil hippocampal neurons, accordingly, the number of vulnerable CA1 pyramidal neurons would be quite limited compared with those of CA3 pyramidal and dentate granule neurons resistant to ischemic insults. The possible species and structural differences in the pharmacological action of theanine, however, have not been completely ruled out so far.

One of the interesting findings obtained in this study is that [^{3}H]Gln was also accumulated in cultured rat cortical astrocytes in a manner similar to that of neurons. For instance, GlnT is the first member of the system A neutral amino acid transporter superfamily identified at the molecular level. Among the different transporters cloned to date, GlnT has the highest affinity for Gln (Km = 0.3–0.5 mM) with a sodium-amino acid cotransport mechanism (Mackenzie et al., 2003). Another typical feature of GlnT is the selective inhibition by the amino acid analog N-methylaminoisobutyric acid (MeAIB; Christensen, 1990; McGivan and Pastor-Anglada, 1994). On reverse transcription polymerase chain reaction analysis, constitutive expression is not detected at all for GlnT mRNA in primary cultured mouse neocortical and cerebellar astrocytes, with marked expression in cultured neocortical neurons and cerebellar granular cells (Dolinska et al., 2004). Moreover, in these cultured astrocytes, [^{3}H]Gln accumulation is insensitive to several excess system A substrates, including MeAIB, proline, and glycine. In neurons, therefore, the system A transporter isoform GlnT is believed primarily to mediate transmembrane Gln transport. By contrast, recent studies have shown marked expression of GlnT mRNA in primary cultured astrocytes prepared from rat whole brain with [^{3}H]Gln uptake and efflux activities insensitive to MeAIB (Heckel et al., 2003; Deitmer et al., 2003). Double-labeling immunohistochemistry has also demonstrated the colocalization of immunoreactivities for GlnT and the glial marker protein glial fibrillary acidic protein in the cerebral cortex of adult rat and human brains (Melone et al., 2004). More recently, we have shown the constitutive expression of GlnT mRNA with MeAIB-sensitive [^{3}H]Gln accumulation in cultured rat neocortical astrocytes (Ogura et al., 2006), whereas overexpression of GlnT leads to the exacerbation of vulnerability to the oxidative cytotoxicity in cultured astrocytes (Ogura et al., 2007). The present and previous findings thus argue in favor of the idea that transmembrane Gln transport would at least in part be mediated by GlnT belonging to the system A superfamily, in addition to the system ASC, system N, and system L transporter families capable of transporting Gln across plasma membranes, even in astrocytes. Astrocytes might play a role in mechanisms relevant to regulation of the extracellular concentration of Gln for the glutamate/Gln cycle in glutamatergic neurons.

It therefore appears that the green tea ingredient theanine is an inhibitor of different transporters capable of transporting Gln across plasma membranes in neurons and astroglia. Daily intake of theanine could thus be beneficial for the prophylaxis of a variety of neurodegenerative and/or neuropsychiatric disorders relevant to overactivation of glutamatergic neurotransmission and neurotoxicity in the brain.

REFERENCES

Albers A, Broer A, Wagner CA, Setiawan I, Lang PA, Kranz EU, Lang F, Broer S. 2001. Na^+ transport by the neural glutamine transporter ATA1. Pflugers Arch 443:92–101.

Balcar VJ, Johnston GAR. 1975. High affinity uptake of L-glutamine in rat brain slices. J Neurochem 24:875–879.

Broer S. 2002. Adaptation of plasma membrane amino acid transport mechanisms to physiological demands. Pflugers Arch 444:457–466.

Broer A, Brookes N, Ganapathy V, Dimmer KS, Wagner CA, Lang F, Broer S. 1999. The astroglial ASCT2 amino acid transporter as a mediator of glutamine efflux. J Neurochem 73:2184–2194.

Broer A, Albers A, Setiawan I, Edwards RH, Chaudhry FA, Lang F, Wagner CA, Broer S. 2002. Regulation of the glutamine transporter SN1 by extracellular pH and intracellular sodium ions. J Physiol 539:3–14.

Chaudhry FA, Reimer RJ, Krizaj D, Barber D, Storm-Mathisen J, Copenhagen DR, Edwards RH. 1999. Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. Cell 99:769–780.

Chaudhry FA, Schmitz D, Reimer RJ, Larsson P, Gray AT, Nicoll R, Kavanaugh M, Edwards RH. 2002. Glutamine uptake by neurons: interaction of protons with system a transporters. J Neurosci 22:62–72.

Chillaron J, Roca R, Valencia A, Zorzano A, Palacn M. 2001. Heteromeric amino acid transporters: biochemistry, genetics, and physiology. Am J Physiol Renal Physiol 281:F995–F1018.

Christensen HN. 1990. Role of amino acid transport and countertransport in nutrition and metabolism. Physiol Rev 70:3–77.

D'Aniello A, Fisher G, Migliaccio N, Cammisa G, D'Aniello E, Spinelli P. 2005. Amino acids and transaminases activity in ventricular CSF and in brain of normal and Alzheimer patients. Neurosci Lett 388:49–53.

Deitmer JW, Broer A, Broer S. 2003. Glutamine efflux from astrocytes is mediated by multiple pathways. J Neurochem 87:127–135.

di Porzio U, Daguet MC, Glowinski J, Prochiantz A. 1980. Effect of striatal cells on in vitro maturation of mesencephalic dopaminergic neurones grown in serum-free conditions. Nature 288:370–373.

Dolinska M, Zablocka B, Sonnewald U, Albrecht J. 2004. Glutamine uptake and expression of mRNA's of glutamine transporting proteins in mouse cerebellar and cerebral cortical astrocytes and neurons. Neurochem Int 44:75–81.

Glowinski J, Iversen LL. 1966. Regional studies of catecholamine in the rat brain. I. The disposition of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]DOPA in various regions of the brain. J Neurochem 13:655–669.

Goto T, Yoshida Y, Amano I, Horie H, 1996. Chemical composition of commercially available Japanese green tea. Foods Food Ingred J 170:46–51.

Heckel T, Broer A, Wiesinger H, Lang F, Broer S. 2003. Asymmetry of glutamine transporters in cultured neural cells. Neurochem Int 43:289–298.

Kakuda T, Yanase H, Utsunomiya K, Nozawa A, Unno T, Kataoka K. 2000. Protective effect of γ-glutamylethylamide (theanine) on ischemic delayed neuronal death in gerbils. Neurosci Lett 289:189–192.

Kakuda T, Nozawa A, Sugimoto A, Niino H. 2002. Inhibition by theanine of binding of [^{3}H]AMPA, [^{3}H]kainate, and [^{3}H]MDL 105,519 to glutamate receptors. Biosci Biotechnol Biochem 66:2683–2686.

Kimura K, Ozeki M, Juneja LR, Ohira H. 2007. L-theanine reduces psychological and physiological stress response. Biol Psychol 74:39–45.

Lodge D, Collingridge G. 1991. The pharmacology of excitatory amino acids: a TiPS special report. Trends Pharmacol Sci 1–89.

Mackenzie B, Schafer MKH, Erickson JD, Hediger MA, Weihe E, Varoqui H. 2003. Functional properties and cellular distribution of the system A glutamine transporter SNAT1 support specialized roles in central neurons. J Biol Chem 278:23720–23730.

McGivan J, Pastor-Anglada M. 1994. Regulatory and molecular aspects of mammalian amino acid transport. Biochem J 299:321–334.

Melone M, Quagliano F, Barbaresi P, Varoqui H, Erickson JD, Conti F. 2004. Localization of the glutamine transporter SNAT1 in rat cerebral cortex and neighboring structures, with a note on its localization in human cortex. Cereb Cortex 14:562–574.

Murakami K, Nakamura Y, Yoneda Y. 2003. Potentiation by ATP of lipopolysaccharide-stimulated nitric oxide production in cultured astrocytes. Neuroscience 117:37–42.

Nagaraja TN, Brookes N. 1996. Glutamine transport in mouse cerebral astrocytes. J Neurochem 66:1665–1674.

Nakamichi N, Ohno H, Kuramoto N, Yoneda Y. 2002. Dual mechanisms of Ca^{2+} increases elicited by N-methyl-D-aspartate in immature and mature cultured cortical neurons. J Neurosci Res 67:275–283.

Ogita K, Yoneda Y. 1986. Characterization of Na^{+}-dependent binding sites of [^{3}H]glutamate in synaptic membranes from rat brain. Brain Res 397:137–144.

Ogita K, Yoneda Y. 1988. Disclosure by Triton X-100 of NMDA-sensitive [^{3}H]glutamate binding sites in brain synaptic membranes. Biochem Biophys Res Commun 153:510–517.

Ogura M, Nakamichi N, Takano K, Oikawa H, Kambe Y, Ohno Y, Taniura H, Yoneda Y. 2006. Functional expression of a glutamine transporter responsive to down-regulation by lipopolysaccharide through reduced promoter activity in cultured rat neocortical astrocytes. J Neurosci Res 83:1447–1460.

Ogura M, Taniura H, Nakamichi N, Yoneda Y. 2007. Upregulation of the glutamine transporter through transactivation mediated by cAMP/protein kinase A signals toward exacerbation of vulnerability to oxidative stress in rat neocortical astrocytes. J Cell Physiol 212:375–385.

Reimer RJ, Chaudhry FA, Gray AT, Edwards RH. 2000. Amino acid transport system A resembles system N in sequence but differs in mechanism. Proc Natl Acad Sci U S A 97:7715–7720.

Schousboe A, Hertz L, Svenneby G, Kvamme E. 1979. Phosphate activated glutaminase activity and glutamine uptake in primary cultures of astrocytes. J Neurochem 32:943–950.

Sugawara M, Nakanishi T, Fei YJ, Huang W, Ganapathy ME, Leibach FH, Ganapathy V. 2000. Cloning of an amino acid transporter with functional characteristics and tissue expression pattern identical to that of system A. J Biol Chem 275:16473–16477.

Szatkowski M, Attwell D, 1994. Triggering and execution of neuronal death in brain ischemia: two phases of glutamate release by different mechanisms. Trends Neurosci 17:359–365.

Takarada T, Balcar VJ, Baba K, Takamoto A, Acosta GB, Takano K, Yoneda Y. 2003. Uptake of [^{3}H]L-serine in rat brain synaptosomal fractions. Brain Res 983:36–47.

Takarada T, Hinoi E, Fujimori S, Tsuchihashi Y, Ueshima T, Taniura H, Yoneda Y. 2004. Accumulation of [^{3}H]glutamate in cultured rat calvarial osteoblasts. Biochem Pharmacol 68:177–184.

Tsuge H, Sano S, Hayakawa T, Kakuda T, Unno T. 2003. Theanine, gamma-glutamylethylamide, is metabolized by renal phosphate-independent glutaminase. Biochim Biophys Acta 1620:47–53.

Utsunomiya-Tate N, Endou H, Kanai Y. 1996. Cloning and functional characterization of a system ASC-like Na^{+}-dependent neutral amino acid transporter. J Biol Chem 271:14883–14890.

Varoqui H, Zhu H, Yao D, Ming H, Erickson JD. 2000. Cloning and functional identification of a neuronal glutamine transporter. J Biol Chem 275:4049–4054.

Wagner CA, Lang F, Broer S. 2001. Function and structure of heteromeric amino acid transporters. Am J Physiol Cell Physiol 281:C1077–C1093.

Yokogoshi H, Kobayashi M, Mochizuki M, Terashima T. 1998. Effect of theanine, γ-glutamylethylamide, on brain monoamines and striatal dopamine release in conscious rats. Neurochem Res 23:667–673.