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Characterization of L-Theanine Excitatory Actions on Hippocampal Neurons: Toward the Generation of Novel *N*-Methyl-D-aspartate Receptor Modulators Based on Its Backbone

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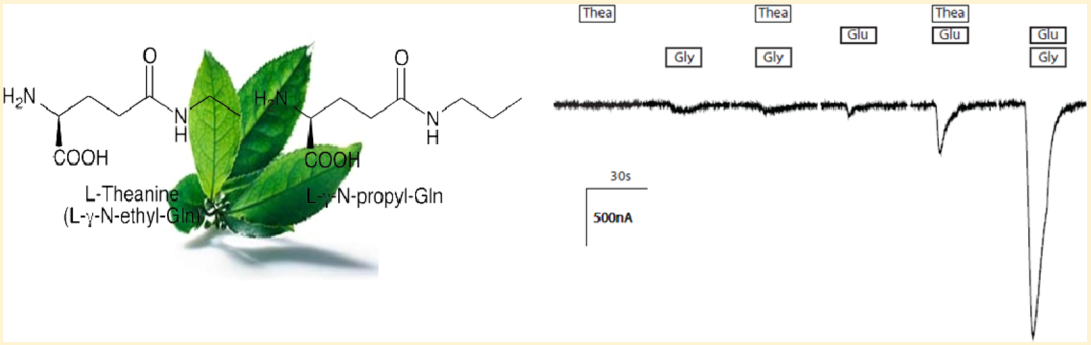

ABSTRACT: L-Theanine (or L-γ-*N*-ethyl-glutamine) is the major amino acid found in *Camellia sinensis*. It has received much attention because of its pleiotropic physiological and pharmacological activities leading to health benefits in humans, especially. We describe here a new, easy, efficient, and environmentally friendly chemical synthesis of L-theanine and L-γ-*N*-propyl-Gln and their corresponding D-isomers. L-Theanine, and its derivatives obtained so far, exhibited partial coagonistic action at *N*-methyl-D-aspartate (NMDA) receptors, with no detectable agonist effect at other glutamate receptors, on cultured hippocampal neurons. This activity was retained on NMDA receptors expressed in *Xenopus* oocytes. In addition, both GluN2A and GluN2B containing NMDA receptors were equally modulated by L-theanine. The stereochemical change from L-theanine to D-theanine along with the substitution of the ethyl for a propyl moiety in the γ-*N* position of L- and D-theanine significantly enhanced the biological efficacy, as measured on cultured hippocampal neurons. L-Theanine structure thus represents an interesting backbone to develop novel NMDA receptor modulators.

KEYWORDS: *L-Theanine, L-γ-N-propyl-glutamine, microwave-assisted organic synthesis (MAOS), NMDA receptors, intracellular Ca^{2+} homeostasis, hippocampal neurons, Xenopus oocytes, expression, GluN2A, GluN2B*

INTRODUCTION

L-Theanine (L-γ-*N*-ethyl-glutamine) is the major amino acid found in green tea (*Camellia sinensis*). It represents, on average, 2% of the weight of dried green tea leaves. The multiple health benefits of this amino acid are widely studied. This is particularly relevant in the central nervous system, as L-theanine easily crosses the blood–brain barrier.[1] In humans, L-theanine has been shown to induce antistress/relaxing actions[2,3] and to improve sleep induction.[4] Further characterizations of its actions on animal models of Alzheimer's disease[5] and stroke[6] indicate that L-theanine also displays neuroprotective action,[7,8] which seems to rely on an antioxidant action[5] or on modulation of neurotransmission, especially the excitatory and inhibitory ones. Indeed, L-theanine shares many structural similarities with glutamate, and ligand-binding studies have established affinities for ionotropic, that is, kainate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and *N*-methyl-D-aspartic acid (NMDA), receptors.[9] Its neuroprotective actions may rely on interference with ionotropic or metabotropic glutamate receptor-mediated neuronal processes.[10,11] The prevention of ischemic damage in the gerbil hippocampus could be attributed to the blockade of AMPA receptors, which actively contribute to

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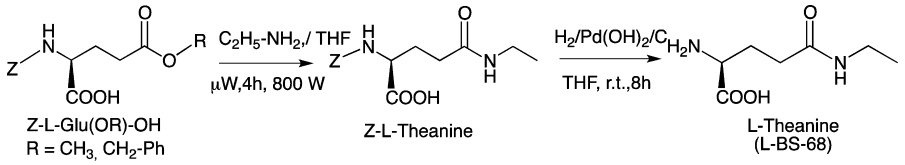
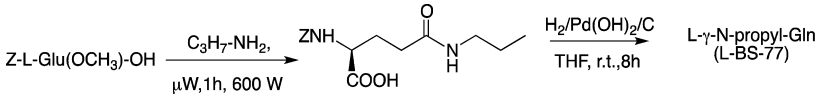
Scheme 1. Chemical Route to L-Theanine (L-BS-68) Synthesis



Scheme 2. Chemical Route to L-γ-*N*-Propyl-Gln (L-BS-77) Synthesis



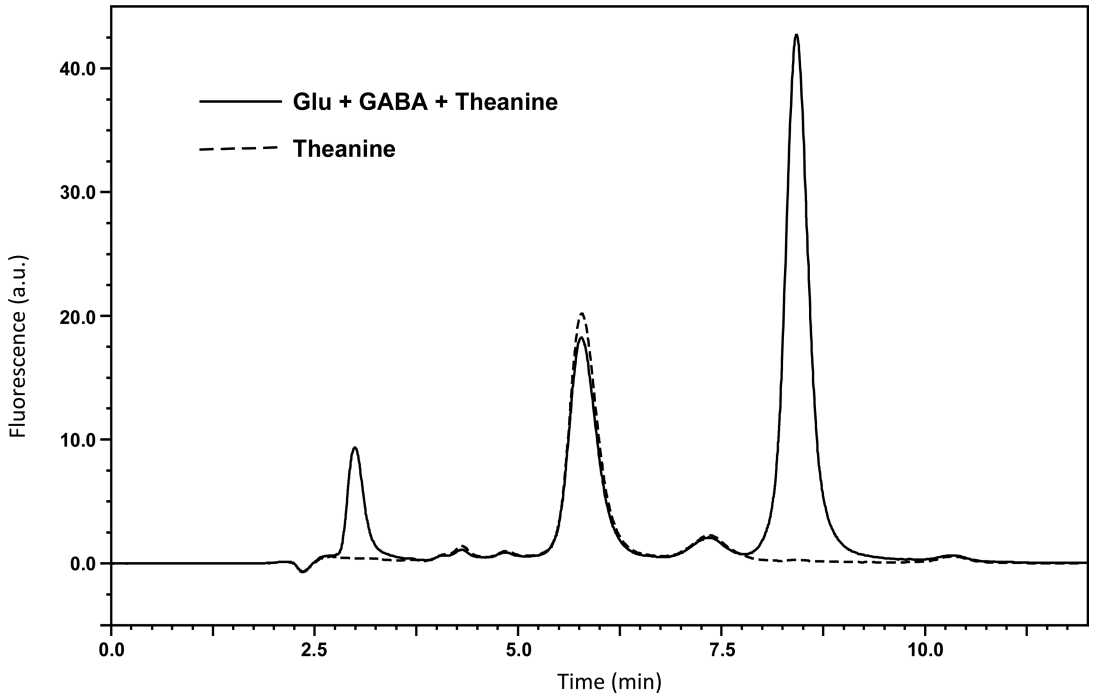


Figure 1. HPLC chromatograms of L-theanine alone (dotted line) and of a mixture of L-theanine, glutamate, and GABA. The fluorescence of the derivatives was measured at 480 nm after excitation at 420 nm and is expressed in arbitrary units.

ischemic injury.[7,12] In addition, L-theanine may also modulate GABAergic transmission to produce neuroprotection under ischemia as evidenced in mice.[6] Other studies uncovered an excitatory action of L-theanine. Indeed, in striatal neurons, L-theanine treatment increases the exocytosis of various neurotransmitters, including γ-amino-butyric acid (GABA), glycine, and dopamine.[13] This could be due to a pleiotropic action on glutamatergic receptors. In this line, it has been shown that the excitatory action of L-theanine on cortical neurons can be blocked by NMDA receptor antagonists.[14] This could explain the beneficial action of L-theanine in a rodent model of schizophrenia induced by NMDA receptor hypofunction. Indeed, NMDA receptors are widely expressed on inhibitory interneurons. Their activation stimulates GABA release and thus regulates the excitability of excitatory cells. This is particularly relevant in the prefrontal cortex where a subset of GABAergic interneurons activated by cortical neurons regulate the excitability of thalamic neurons, themselves exciting cortical neurons (so-called "thalamo-cortical loop"). Preventing the activity of these interneurons, by blocking NMDA receptors, for instance, leads to dysregulation of the thalamo-cortical loop and to the occurrence of pathological conditions such as psychosis.[15] Interestingly, L-theanine treatment reverses sensorimotor gating deficits, which are associated with this pathological state.[14] In addition, partial agonists of the glycine site on NMDA receptors, such as D-cycloserine, have proven to exhibited beneficial effects to alleviate the symptoms of various psychiatric diseases.[16] Therefore, targeting the glycine binding site with partial agonists represents an interesting route to normalize NMDA receptor activity.

NMDA receptors have long been associated with the synaptic plasticity that produces long-term potentiation[17] and long-term depression[18] in the hippocampus. However, the molecular mechanisms responsible for the differential role of NMDA receptors of distinct subunit composition are still poorly understood. L-Theanine could thus be an interesting backbone for the synthesis of new modulators of the NMDA receptor with applications in both fundamental research and therapeutic usage for NMDA receptor-associated pathologies (schizophrenia[19] and neurodevelopmental disorders[20]). We provide here a new, simple, and efficient synthesis of D- and L-theanine and N-alkylated derivatives.

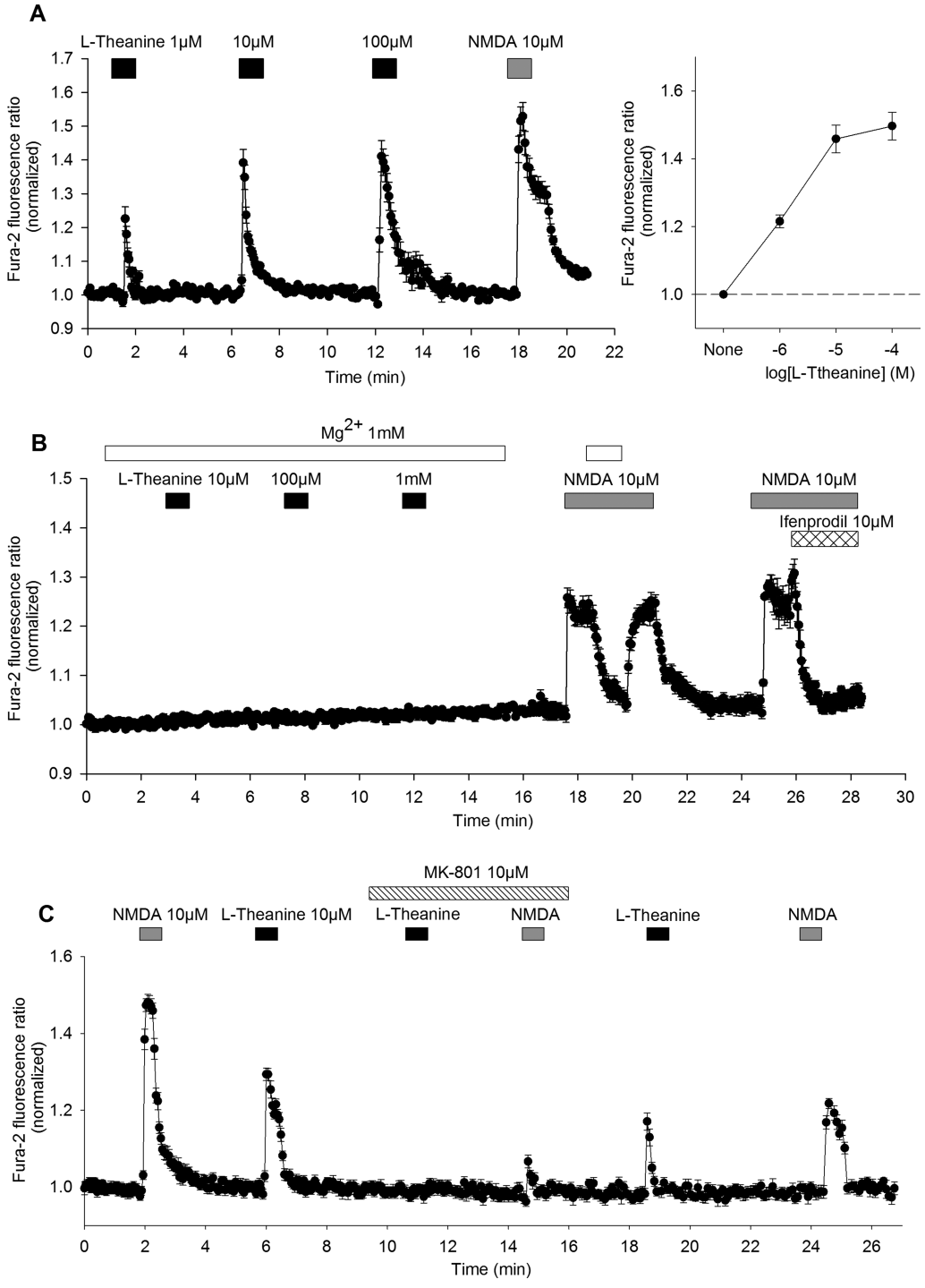


Figure 2. Characterization of the direct effects of L-theanine on $[Ca^{2+}]_i$ in mature (about 2 weeks *in vitro*) cultured hippocampal neurons. (A) Concentration-dependency of L-theanine effect. L-Theanine was tested from 1 to 100 μM. On the left, an illustrative experiment in which 21 cells were recorded is shown. The concentration-dependent curve shown on the right has been obtained by measuring peak intensities of L-theanine responses from n = 45 cells. (B) Inhibitory effect of Mg^{2+} ions on L-theanine-mediated responses. L-Theanine was tested from 10 μM to 1 mM in the presence of Mg^{2+} ions (1 mM) in the extracellular medium. As control, NMDA was tested in Mg^{2+}-free and Mg^{2+}-rich medium, as well as in the presence of the NMDA receptor GluN2B subunit antagonist ifenprodil (10 μM), which completely inhibited NMDA-mediated responses. The graph represents an average of the $[Ca^{2+}]_i$ changes measured in 31 cells. This experiment has been performed on 60 cells in all. (C) Dependency on NMDA receptor activity of L-theanine responses. L-Theanine or NMDA were applied in the presence of the NMDA receptor noncompetitive antagonist MK-801 (10 μM). This antagonist exerted a reversible inhibition of the responses elicited by L-theanine or NMDA. The graph was obtained by averaging F340/F380 ratios from 31 cells. This experiment has been performed on 70 cells in all.

The effects of these compounds are evaluated on cultured hippocampal neurons and on *Xenopus* oocytes expressing NMDA receptors.

RESULTS

Synthesis of Theanine and Alkylated Derivatives. The starting (commercially available) products were N-protected

γ-glutamyl esters: Z-L-Glu(O-CH_2-Ph)-OH or Z-L-Glu(OCH_3)-OH and 2 M ethylamine solution (Z = benzyloxycarbonyl group). Synthetic L-theanine was obtained by a new protocol for specific amidification as described in Scheme 1.

The reaction has been performed using microwave oven (Biotage initiator 60 EXP) in 2 M ethylamine solution in THF (6 equiv). The vessel was heated at different temperatures (for example, for 60 °C power = 600 W). The reactions were followed by high-performance liquid chromatography (HPLC). Absolutely no hydrolysis of newly synthesized Z-theanine in Z-Glu-OH was observed, and this procedure provided approximately 20-fold acceleration (4 h versus 1 day using conventional methods, which led to about 40% of hydrolysis of Z-theanine in Z-glutamic acid, data not shown).

The enantiomeric purity of synthetic Z-L-theanine was controlled by measuring α_D and further chiral HPLC analyses. To confirm that no racemization took place, the amidification with 2 M ethylamine solution in THF has been carried out with the commercially available Z-D-Glu(OCH_3)-OH. Under these conditions, optically pure Z-D-theanine was obtained in the same yield. Both syntheses have been followed by chiral HPLC separately and both compounds appeared at different retention times.

In a second and final step, synthetic L-theanine (L-BS-68) was obtained by removing the benzyloxycarbonyl protecting group Z by catalytic hydrogenation in quantitative yield.

The synthesis of L-theanine congeners by modifying the γ-*N*-alkyl group was then performed by substituting the γ-*N*-ethyl group in L-theanine for other alkyl moieties, in order to enhance the hydrophobicity of the derivatives. We describe here the synthesis of the L-γ-*N*-propylglutamine (or L-BS-77).

With Z-L-Glu(OCH_3)-OH as starting product, in solvent free system, thanks to pure liquid *n*-propylamine (bp = 47−51 °C), and under microwave irradiation, Z-L-γ-*N*-propylglutamine was quantitatively obtained in only 1 h at 60 °C (Scheme 2).

As L-theanine is closely related to glutamate and GABA, we have also analyzed the purity of L-theanine by HPLC. Under the elution conditions used, glutamate, L-theanine, and GABA were well separated as single peaks appearing at 2.99, 5.78, and 8.42 min, respectively. L-Theanine on its own contained neither glutamate nor GABA (Figure 1).

L-Theanine Is a Coagonist of NMDA Receptors. L-Theanine was previously shown to elicit $[Ca^{2+}]_i$ increases when applied in the micromolar range on cultured cortical neurons.[14] We have thus checked whether L-theanine was active in this concentration range (1−100 μM) on cultured hippocampal neurons. In the absence of Mg^{2+} ions, L-theanine elicited $[Ca^{2+}]_i$ increases, which were consistently detected at 1 μM in mature (16 days *in vitro*, DIV) cultured hippocampal neurons (Figure 2A). Under physiological extracellular Mg^{2+} (1 mM; Figure 2B) or in the presence of NMDA receptor antagonist dizolcipine (MK-801, 10 μM; Figure 2C) or without extracellular Ca^{2+} ions (Figure 6D), perfusion of L-theanine had no effect on $[Ca^{2+}]_i$. These data demonstrate that L-theanine produced an influx of Ca^{2+} on mature cultured hippocampal neurons that mainly relied on NMDA receptors. Non-NMDA ionotropic receptors and metabotropic mGlu1/5 receptors did not seem to be activated here by L-theanine, as its effect was fully blocked by MK-801.

Surprisingly, on immature hippocampal neurons (6−8 DIV), L-theanine (range 10 μM to 1 mM) had absolutely no effect on $[Ca^{2+}]_i$, while NMDA could elicit responses sensitive to glycine (Figure 3A). Therefore, we hypothesized that L-theanine might be exerting positive allosteric modulation or coagonist action on NMDA receptors stimulated by the endogenous glutamate release, which occurs only in mature cultures of hippocampal neurons as observed previously.[21] L-Theanine was thus tested in combination with NMDA on immature neurons, where synaptic activation of NMDA receptors did not yet occur, and compared its effects with those elicited by conventional NMDA receptor glycine site agonists, glycine and D-serine. L-Theanine (1 μM to 1 mM) effectively potentiated Ca^{2+} influxes elicited by NMDA (10 μM) in a concentration dependent manner with a maximal effect at a concentration of 100 μM (Figure 3B,E). L-Theanine was significantly less efficient than glycine or D-serine (Figure 3C−E). Nevertheless, the action of L-theanine was rather selective for NMDA receptors. Indeed, we verified whether L-theanine had any effect on non-NMDA ionotropic receptors. For this, L-theanine (1 mM) was applied in combination with kainate (40 μM). We observe that kainate-mediated Ca^{2+} responses were totally insensitive to L-theanine (Figure 3F).

Then, to test whether the presence of endogenous glutamate was a necessary prerequisite to observe intrinsic effects of L-theanine on mature cultured neurons, experiments were performed in the presence of tetrodotoxin (TTX; 500 nM) a Na^+ channel blocker that prevents glutamate release and excitatory transmission in these neurons.[21] TTX completely inhibited the changes in $[Ca^{2+}]_i$ produced by L-theanine (10 μM), glycine (10 μM), or D-serine (100 μM) (Figure 4A) in mature cultured neurons. It is noticeable that NMDA response was also partially inhibited. This could be associated with a decreased coagonist tone obtained by blocking synaptic transmission. However, TTX did not prevent the potentiating effect of L-theanine, glycine, or D-serine on NMDA responses on mature neurons (Figure 4B). Finally, the NMDA glycine binding site antagonist, 7-chlorokynurenic acid (0.1 μM), completely blocked L-theanine-mediated $[Ca^{2+}]_i$ increases in a reversible manner (Figure 4C). It is noteworthy that at the concentration tested, 7-chlorokynurenic acid had a very modest inhibitory effect on NMDA-mediated $[Ca^{2+}]_i$ increases (less than 4%). Taken together, these results confirm that L-theanine behaves as a partial NMDA receptor coagonist acting on the glycine site and therefore strictly requires endogenous release of glutamate to produce intrinsic effects.

In cultured hippocampal neurons, our experimental procedure used to measure intracellular calcium allowed us to principally observe GluN2B-containing NMDA receptor responses as ifenprodil (10 μM), a selective antagonist of GluN2B subunit-containing NMDA receptor, completely blocked the NMDA responses (Figure 2B). The selectivity toward either GluN2A- or GluN2B-containing NMDA receptors was further evaluated on *Xenopus* oocytes where the subunit composition of the NMDA receptors can be easily controlled. The study was restricted to GluN2A- and GluN2B-containing NMDA receptors as these subunits were found to exhibit the highest expression among GluN2 subunit family and we did not evidence the presence GluN3 in our hippocampal cultures. L-Theanine (500 μM) was without effect itself on *Xenopus* oocytes expressing GluN1/GluN2A or GluN1/GluN2B NMDA receptors (Figure 5). However, L-theanine potentiated glutamate-elicited currents by coapplication and to a similar extent on both GluN1/GluN2A and GluN1/GluN2B expressing oocytes (Figure 5C). By itself, glycine (10 μM) elicited a small response, which was not sensitive to L-theanine. Moreover, its coagonist action was evidenced by recording large currents when coapplied with glutamate.

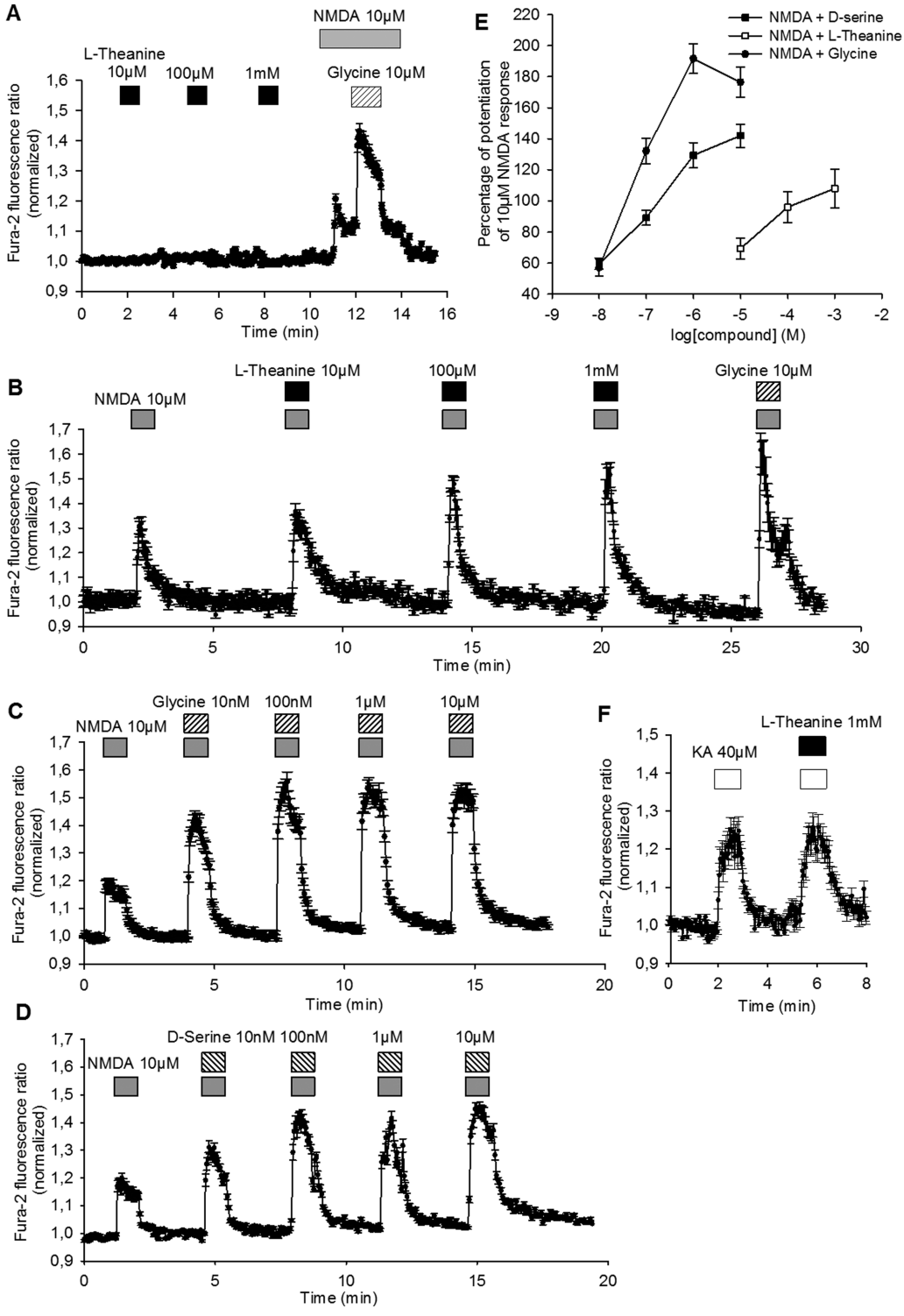


Figure 3. NMDA receptor coagonist action of L-theanine on immature cultured hippocampal neurons. (A) Lack of L-theanine (10 μM to 1 mM) effect on $[Ca^{2+}]_i$ in immature (6 DIV) cultured hippocampal neurons. NMDA (10 μM) in combination with glycine (10 μM) was tested as control. The graph has been obtained by averaging data from 29 cells. The experiment has been performed on 51 cells in all. (B) Concentration-dependent potentiating action of L-theanine (from 10 μM to 1 mM) on the NMDA-mediated $[Ca^{2+}]_i$ increases in immature (6 DIV) hippocampal neurons. Glycine (10 μM) was also tested as control. The graph was obtained by averaging data from 22 individual cells. (C, D) Concentration-dependent potentiating action of glycine (C) and D-serine (D) on NMDA-mediated $[Ca^{2+}]_i$ increases in immature hippocampal neurons. Coagonists were tested in the concentration range of 10 nM to 10 μM. Data are from 24 and 26 cells, for glycine and D-serine, respectively. (E) Recapitulative graph of the potentiating actions of glycine, D-serine, and L-theanine on NMDA-mediated responses, as a function of the concentration of coagonist applied. Data have been obtained from 82, 80, and 71 cells for glycine, D-serine, and L-theanine, respectively. Data are expressed as percentages of increase of NMDA-mediated responses. They were obtained by normalizing the difference of the peak amplitudes of NMDA responses measured either in the presence or in the absence of the coagonist to the amplitude of the NMDA response in each respective cell recorded. (F) Effect of L-theanine (1 mM) on $[Ca^{2+}]_i$ increases elicited by kainate (40 μM) in 6 DIV cultured neurons. The graph was generated by averaging data obtained from 40 individual cells (n = 85 in all).

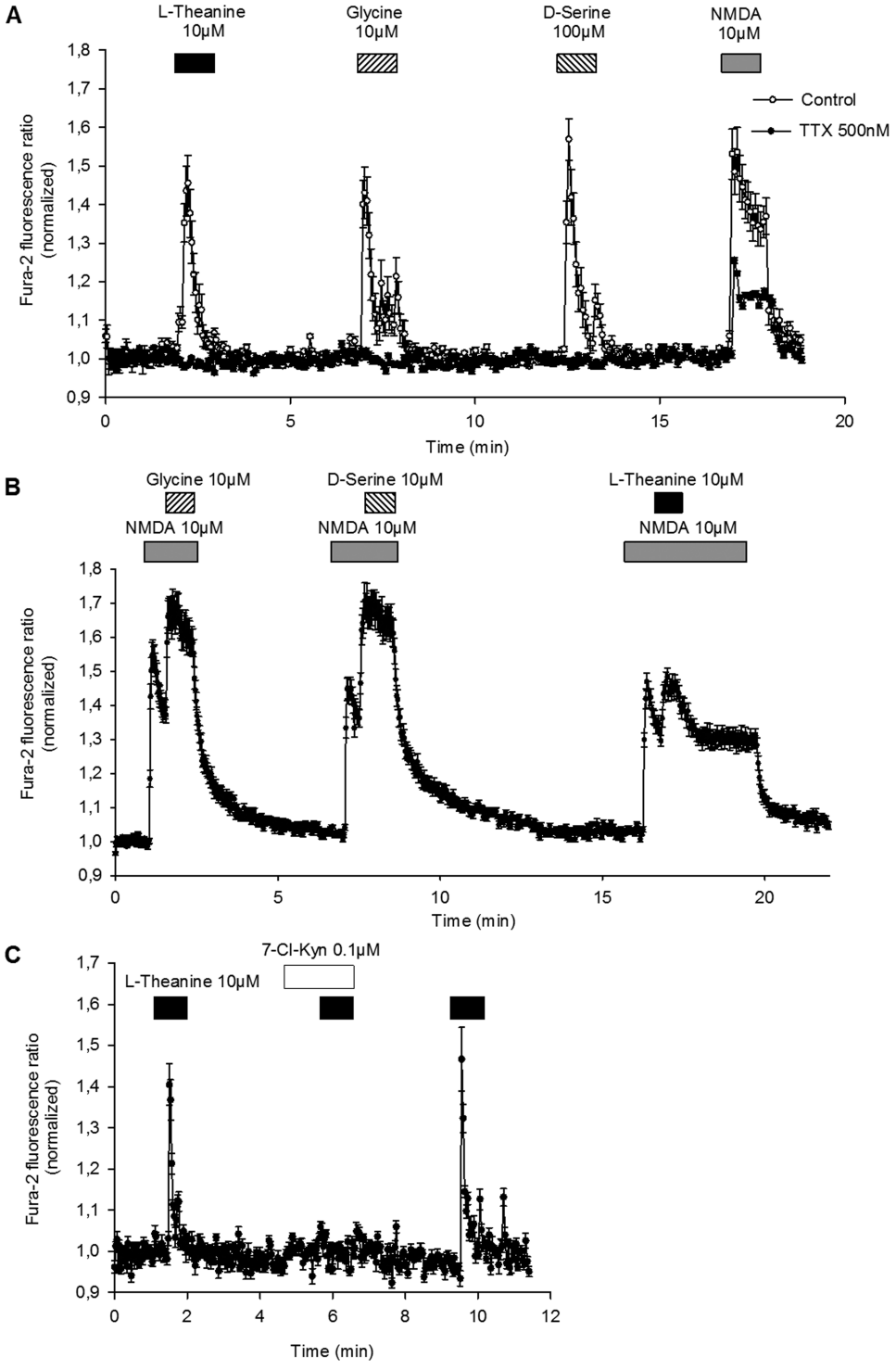


Figure 4. Dependency of the L-theanine-mediated $[Ca^{2+}]_i$ increases on synaptic activity in mature hippocampal cultured neurons. (A) $[Ca^{2+}]_i$ changes elicited by L-theanine and by the NMDA receptor coagonists glycine and D-serine in mature hippocampal cultures (15 DIV) measured either in the absence (open circles, "control", 12 cells) or in the presence of tetrodotoxin (filled circles, 26 cells). TTX (500 nM) was applied throughout the recording. Data obtained with each protocol were superimposed. The experiments with or without TTX were performed on $n = 50$ and 47 cells in all, respectively. (B) Co-agonist action of glycine, D-serine, and L-theanine on NMDA receptors measured in the presence of TTX in mature neurons. The coagonists were applied at 10 μM in combination with 10 μM NMDA, in the presence of 500 nM TTX in the extracellular medium. The graph plots averaged data from 23 individual cells ($n = 48$ in all). (C) Effect of 7-chlorokynurenic acid on the L-theanine-mediated $[Ca^{2+}]_i$ changes. 7-Chlorokynurenic (0.1 μM) was applied 1 min prior to the L-theanine (10 μM) application. The graph plots averaged data from 20 individual cells ($n = 50$ in all).

Nevertheless, the currents obtained by coapplying L-theanine and glutamate represented 19% ± 8% ($n = 12$) and 23% ± 9% ($n = 11$) of those elicited by glycine and glutamate coapplication on GluN1/GluN2A and GluN1/GluN2B expressing oocytes, respectively. The difference between these fractions was not significant.

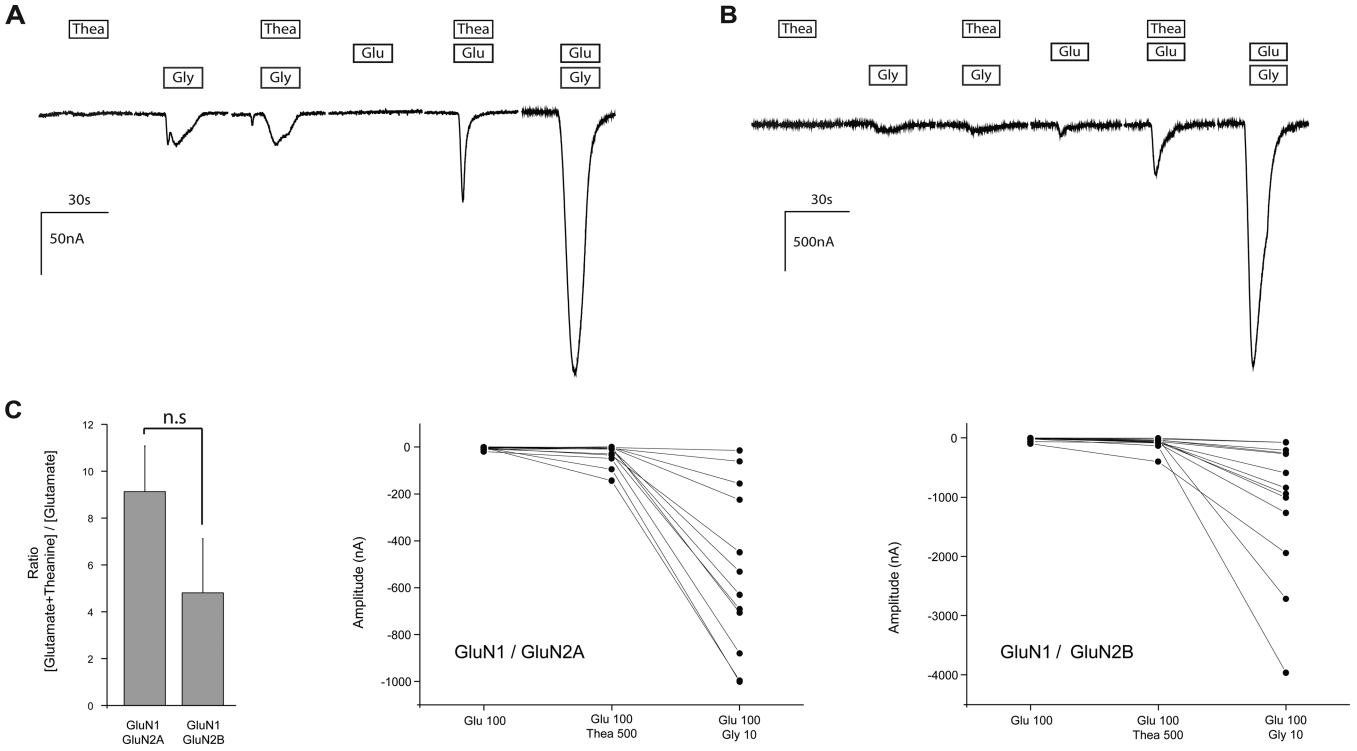


Figure 5. Coagonist action of L-theanine at GluN1/GluN2A and GluN1/GluN2B NMDA receptors. (A, B) Currents evoked by successive applications or coapplications of 500 μM L-theanine, 10 μM glycine, and 100 μM glutamate were measured on *Xenopus* oocyte expressing NMDA receptors. Representative current traces of GluN1/GluN2A (A) and GluN1/GluN2B (B) reveal the coagonist action of L-theanine, as well as that of glycine. (C) Average potentiating effects of L-theanine on GluN1/GluN2A and GluN1/GluN2B (left) and graphs showing the large dispersion of both L-theanine and glycine responses for GluN1/Glu2A (middle) and GluN1/GluN2B (right).

Characterization of the Effects L-Theanine Derivatives: D-Theanine and L- and D-γ-*N*-Propyl-Gln. L-Theanine derivatives were further synthesized by substituting the γ-*N*-ethyl moiety and by changing the chirality from L to D of the asymmetric carbon. Indeed, as observed for D-serine, a D-chirality of the asymmetric carbon is also accepted for coagonist binding on the GluN1 subunit. Activity of derivatives was tested using them at the single concentration of 10 μM on mature cultured hippocampal neurons. Among all the compounds synthesized, it appears that the γ-*N*-propyl derivative exerted excitatory properties on $[Ca^{2+}]_i$ in cultured hippocampal neurons as well as L-theanine did. Therefore, three compounds were further synthesized and tested: D-theanine and L- and D-γ-*N*-propyl-Gln. They all retained the properties of NMDA receptor coagonist as observed for L-theanine, that is, the blockade by NMDA receptor antagonist MK-801 (Figure 6A,B,C) and the dependency on extracellular Ca^{2+} (Figure 6D). In addition, the $[Ca^{2+}]_i$ rises elicited by these L-theanine derivatives were not observed in the presence of TTX (not shown). The direct comparison of the responses induced by these compounds at a single concentration of 10 μM indicated that both the change of chirality and the γ-*N*-propyl substitution significantly enhanced the responses elicited by L-theanine (Figure 6E,F).

DISCUSSION

We demonstrate here that L-theanine exerts an excitatory action on hippocampal neurons mainly by potentiating NMDA responses. L-Theanine had no detectable agonist effect on other glutamate receptors, that is, non-NMDA ionotropic and phospholipase C-associated mGlu receptors. By itself, L-theanine induced $[Ca^{2+}]_i$ only in mature hippocampal neurons. This effect of L-theanine was completely blocked by a NMDA receptor antagonist or by extracellular Mg^{2+} ions, and was dependent on the presence of extracellular Ca^{2+} ions. This strongly suggests that the increase in $[Ca^{2+}]_i$ elicited by L-theanine alone involves the direct or the indirect activation of NMDA receptors. The fact that these effects were only seen in mature cultures of hippocampal neurons with functional excitatory synaptic transmission[21] suggests an indirect action where L-theanine behaves as coagonist of NMDA receptors activated by endogenously released glutamate. Accordingly, blocking synaptic transmission by TTX fully prevented the L-theanine response in mature hippocampal neurons. The fact that TTX partially reduced the NMDA response suggests that glycine or D-serine may also be synaptically released from mature hippocampal neurons. However, the existence of residual intracellular calcium responses induced by NMDA in the presence of TTX in mature hippocampal cultures or by NMDA alone in immature cultures also suggests the presence of extracellular endogenous coagonist during recording, released from the cells by mechanisms independent from the synaptic activity. The coagonist action of L-theanine was directly confirmed by the enhancement of the NMDA induced $[Ca^{2+}]_i$ response when L-theanine and NMDA were coapplied in the presence of TTX and by the inhibitory effect of the glycine NMDA receptor site antagonist, 7-chlorokynurenic acid, on L-theanine-elicited $[Ca^{2+}]_i$ changes.

A similar result was obtained on the NMDA responses recorded on *Xenopus* oocytes expressing GluN1/GluN2A or GluN1/GluN2B NMDA receptors, suggesting that the effects of L-theanine were not restricted to one subtype of the GluN2 subunits. L-Theanine thus behaves as a"conventional" NMDA

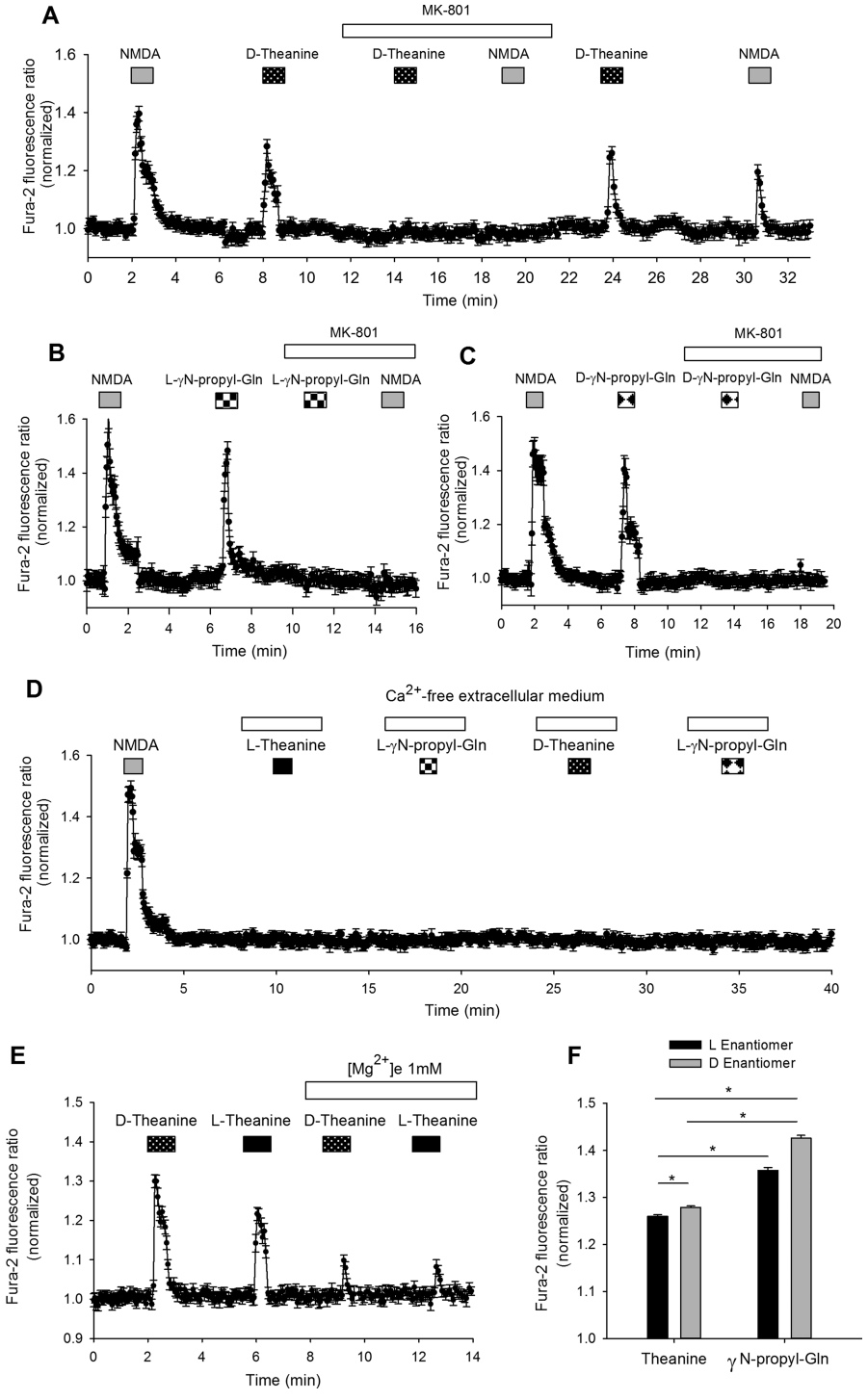


Figure 6. Activity of L-theanine derivatives. (A–C) Dependency on NMDA receptor activity of D-theanine-, L-γ-*N*-propyl-Gln- and D-γ-*N*-propyl-Gln-mediated $[Ca^{2+}]_i$ increases in mature (16 DIV) neurons. The $[Ca^{2+}]_i$ increases mediated by 10 μM D-theanine (A), L-γ-*N*-propyl-Gln (B), or D-γ-*N*-propyl-Gln (C) were challenged with MK-801 (10 μM). NMDA (10 μM) was used as a control. (D) Dependency on the presence of extracellular Ca^{2+} ions of the responses elicited by L-theanine and derivatives. (E) Inhibitory effect of extracellular Mg^{2+} ions on the $[Ca^{2+}]_i$ responses induced by L-theanine and derivatives. (F) Cumulative graph plotting peak responses elicited by 10 μM L-theanine or derivatives on $[Ca^{2+}]_i$. Data were obtained from n = 199, 68, 222, and 114 cells for L-theanine, D-theanine, L-γ-*N*-propyl-Gln, and D-γ-*N*-propyl-Gln, respectively. Bars indicate that the differences between peak amplitudes were statistically significant ($p < 0.001$, one way ANOVA followed by Dunn's test).

receptor coagonist, such as glycine or D-serine, which exert their positive action by acting on the GluN1 subunit. This is in agreement with a previous report showing that L-theanine may bind the glycine binding site of NMDA receptors.[9] Nevertheless, L-theanine was found to be much less potent than glycine or D-serine to exert its coagonist effect at NMDA receptors.

In our hands, L-theanine behaves as a partial agonist at the glycine binding site on NMDA receptors in hippocampal neurons. This action, to some extent, contrasts with previous finding demonstrating the interactions of L-theanine with NMDA receptor-dependent neurophysiological phenomena in the CA1 area of the hippocampus to improve cognition. Indeed,

long-lasting treatments with L-theanine appear to decrease NMDA receptor-dependent LTP, while it increases NMDA receptor-independent LTP.[22,23] The opposite would reasonably be expected from a positive modulation of NMDA receptor. Acute and chronic administrations of L-theanine may thus result in the occurrence of different neuroplastic phenomena.

The L-theanine used for this study was synthesized in a new efficient two-step synthesis with important improvements as compared to previous methods, including extraction from tea leaves[24,25] and enzymatic biosynthesis,[26] which are both expensive and low yield methods.

Indeed, under our optimized conditions, the amidification reaction proceeded smoothly. Good-to-excellent conversion rates, higher yields, and shorter reaction times were observed using microwave irradiation as a source of heating and activation.[27] The reaction occurred in a solvent-free system when the corresponding amines were liquid in an environmentally friendly protocol without any chromatographic purification. This procedure also enabled us to obtain the D-enantiomer of theanine and L- and D-γ-*N*-propyl-Gln with high yields and without any racemization. Moreover, this process can be easily translated for the production of gram scale of these derivatives using, for example, the microwave activation by MiniFlow 200SS model (Sairem). In addition, this reaction enabled the synthesis of derivatives by changing the alkyl moiety on the nitrogen in γ position, leading to L-γ-*N*-alkyl and D-γ-*N*-alkyl derivatives. Among these compounds, the D-γ-propyl-Gln appeared to be more potent than L-theanine itself to elicit $[Ca^{2+}]_i$ changes on hippocampal neurons. These structural modifications of the L-theanine backbone may be helpful for characterizing its binding site on NMDA receptors and testing whether it overlaps the glycine site on GluN1, as suggested here. Studies of the crystal structure of NMDA receptors suggest however the potential presence of many possible allosteric binding sites within the receptor.[28]

Furthermore, we show that novel modulators of NMDA receptors with potential therapeutic activity may be derived from L-theanine. Indeed, glycine site NMDA receptor partial agonists such as D-cycloserine and GLYX13, which bind the GluN1 glycine site, have proven to be beneficial in improving learning during aging[29] and in the course of pathological states such as depression[30] or conditions associated with hypofunction of NMDA receptors, such as psychosis.[16] Many psychiatric diseases resulting from a reduced activity of NMDA receptors, including psychosis,[19] can be mimicked in rodents by a reduction in NMDA receptor function.[31] In this line, L-theanine has proven to alleviate psychotic-like symptoms in a rodent schizophrenia model as observed by the rescue of sensorimotor gating defects assessed by measuring prepulse inhibition (PPI).[14] Moreover, L-theanine seems to enhance PPI in humans,[32] thereby confirming its potential psychotropic effect for curative perspective of neurological diseases. Further behavioral experiments will hopefully confirm the beneficial actions of these L-theanine derivatives *in vivo* and present this novel class of NMDA modulators as therapeutic strategy to alleviate the symptoms of various neurological/psychiatric diseases.

METHODS

Chemistry. Melting points were obtained using a Büchi 510 capillary apparatus and were uncorrected. ^{1}H NMR spectra were recorded at 300 and 75 MHz using a Brüker AC300 instrument and at 600 MHz using a Brüker AC600 instrument. Chemical shifts are quoted in parts per million and were referenced to the residual solvent peak. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants are reported in hertz (Hz). High resolution mass spectra (HRMS) were recorded on micromass electrospray instrument with only molecular ion and other major peaks being reported. LC-MS identification was by electrospray on HPLC Waters Alliance 2690. Flash chromatography was carried out using E-Merck Silica Gel (Kieselgel 60, 230−400 mesh) as stationary phase. Thin layer chromatography was carried out on aluminum plates precoated with Merck Silicagel 60F254 and visualized by quenching of ultraviolet fluorescence or by staining with a 10% methanol phosphomolybdic acid solution followed by heating. Analytic HPLC was performed on a Waters apparatus 717 plus autosampler with Millenium 32 program on SymmetryShield RP_{18} 3.5 μm 2.1 mm × 20 mm column using a linear gradient of ACN in H_2O with 0.1% TFA in 5 min with 3 mL/min flow. Analytical chiral HPLC experiments were performed on a unit composed of a Merck D-7000 system manager, Merck-Lachrom L-7100 pump, Merck-Lachrom L-7360 oven, Merck-Lachrom L-7400 UV-detector, and Jasco OR-1590 polarimeter using Beckman Coulter System Gold 126 Solvent Module HPLC machine with column Chiralcel OD-RH (250 mm × 4.6 mm) and water/acetonitrile solvent system or with Chiralpk AD-H (250 mm × 4.6 mm) and hexane/isopropanol solvent system. Both columns are from Chiral Technologies Europe (Illkirch, France). Optical rotations were determined on a PerkinElmer 341 polarimeter in appropriate solvent (20 °C, sodium ray). THF was distilled from sodium/benzophenone ketyl. Reagents were supplied from commercial sources (Sigma-Aldrich, Fluka).

Z-L-Theanine Synthesis. Substrate (Z-L-Glu(OCH_3)-OH or Z-L-Glu(OBzl)-OH; 2 mmol) was weighed in a 0.5−2.0 mL microwave reactor. Ethylamine (12 mmol, 2 M in THF) was then added. The reactor was sealed and heated under microwave irradiation at 80 °C (800 W) for 4 h. Then, the suspension was dissolved in ethyl acetate and washed two times with concentrated NH_4Cl. The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. Z-L-Theanine precipitated as a white powder in Et_2O (20 mL) and was filtered and lyophilized. ^{1}H NMR (300 MHz, DMSO): δ = 1.02 (t, ^3J = 7.3 Hz, 3 H), 2.08 (m, 2 H), 2.17(m, 2 H), 3.24 (q, ^3J = 7.29 Hz, 2 H), 4.43 (m, 1 H), 5.02 (s, 2 H), 5.71 (d, ^3J = 6.86 Hz, 1H), 7.28 (m, 5 H). ^{13}C NMR (75 MHz, DMSO): δ = 14.27, 25.73, 33.36, 37.22, 53.51, 67.6, 128.1, 128.31, 128.42, 135.09, 156.2, 171.59, 171.84. $[\alpha]_D^{20}$: −11.0 (c = 1, MeOH). MS (ESI): m/z 309.2 $[M + H]^+$, 331.1 $[M + Na]^+$. Analytic chiral HPLC: Chiralcel-OD column, H_2O/ACN (87/13), 0.6 mL/min, 45 min, 214 nm, t_R = 30.15 min.

Z-D-Theanine. $[\alpha]_D^{20}$: 11.2 (c = 1, MeOH). Analytic chiral HPLC: Chiralcel-OD column, H_2O/ACN (87/13), 0.6 mL/min, 45 min, 214 nm, t_R = 34.17 min.

Z-L-γ-*N*-Propylglutamine Characterization. ^{1}H NMR (300 MHz, DMSO): δ = 0.82 (t, ^3J = 7.47 Hz, 3 H), 1.54(m, 2H), 1.90 (m, 2 H), 2.07(m, 2 H), 2.97 (t, ^3J = 7.09 Hz, 2 H), 3.71 (m, 1 H), 5.01 (s, 2 H), 6.61 (d, ^3J = 6.92 Hz, 1H), 7.28 (m, 5 H). ^{13}C NMR (75 MHz, DMSO): δ = 11.32, 22.31, 28.75, 32.10, 40.27, 55.19, 65.04, 127.54, 127.63, 128.27, 137.21, 155.42, 172.05, 174.18. $[\alpha]_D^{20}$: +2.5 (c = 1, DMSO). MS (ESI): m/z 323.2 $[M + H]^+$, 345.1 $[M + Na]^+$.

L-Theanine Characterization. Z-L-Theanine (0.36 mmol, 100 mg) was dissolved in freshly distilled THF (3 mL), and 20% $Pd(OH)_2/C$ (20 mg) was added. The mixture was hydrogenated (H_2) at rt for 8 h. $Pd(OH)_2/C$ was filtered off on Celite, and THF was removed under reduced pressure. L-Theanine was obtained in quantitative yield as a white powder. ^{1}H NMR (300 MHz, D_2O): δ = 1.08 (t, ^3J = 7.32 Hz, 3 H), 2.11 (dd, ^3J = 7.77 Hz, ^3J = 6.16 Hz, 2 H), 2.39(m, 2 H), 3.17 (q, ^3J = 7.32 Hz, 2 H), 3.74 (t, ^3J = 6.16 Hz, 1 H). ^{13}C NMR (75 MHz, D_2O): δ = 13.41, 26.53, 31.73, 34.57, 53.56, 173.95, 174.29. $[\alpha]_D^{20}$: +8.4 (c = 1, H_2O) $[\alpha]_D^{20}$ lit: +8.1 (c = 5, H_2O). Mp = 215−219 °C. MS (ESI): m/z 175.1 $[M + H]^+$, 197.1 $[M + Na]^+$. HRMS calcd for $C_7H_{14}N_2O_3$ m/z $[M + H]^+$ 175.1004, found 175.1039. D-Theanine, $[\alpha]_D^{20}$: −8.3 (c = 1, H_2O).

Commercially available L-theanine from Sigma-Aldrich, ^{1}H NMR (300 MHz, D_2O): δ = 1.10 (t, ^3J = 7.75 Hz, 3 H), 2.13 (dd, ^3J = 8.0 Hz, ^3J = 6.5 Hz, 2 H), 2.4(td, ^3J = 8.0 Hz, ^3J = 4.2 Hz 2 H), 3.2 (q, ^3J = 7.75 Hz, 2 H), 3.76 (t, ^3J = 8.0 Hz, 1 H).

L-γ-*N*-Propylglutamine Characterization. ^{1}H NMR (300 MHz, D_2O): δ = 0.82 (t, ^3J = 7.43 Hz, 3 H), 1.45 (td, ^3J = 7.24 Hz, ^3J = 6.96 Hz, 2 H), 2.05 (m, 2H), 2.31(m, 2 H), 3.08 (t, ^3J = 6.96 Hz, 2 H), 3.64 (t, ^3J = 6.1 Hz, 1 H). ^{13}C NMR (75 MHz, D_2O): δ = 10.63, 21.79, 27.21, 31.83, 41.33, 54.43, 174.75, 175.04. $[\alpha]_D^{20}$: +13.9 (c = 1, H_2O). Mp = 207−209 °C. MS (ESI): m/z 189.2 $(M + H)^+$, 211.2 $[M + Na]^+$. HRMS calcd for $C_7H_{14}N_2O_3$ m/z $[M + H]^+$ 189.1239, found 189.1239. D-γ-*N*-Propylglutamine, $[\alpha]_D^{20}$: −14.0 (c = 1, H_2O).

HPLC Measurement of Glutamate, GABA, and L-Theanine. Determination of glutamate, GABA, and L-theanine involved the HPLC separation of precolumn derivatized amino-acids, detected and quantified via fluorescence detection. The chromatographic system consisted of a Dionex ASI-100 cooled autosampler, a Dionex P-680 pump, a Dionex TCC-100 oven, and a Dionex RF-2000 fluorescence detector. Chromeleon software 6.80 SP4 was used to drive the HPLC system and to integrate the signals obtained.

Derivatization protocol was based on the method previously reported[33] with slight modifications. Briefly summarized, 100 μL of sample or standards in borate buffer (0.1 M; pH 9.3) was added to 20 μL of potassium cyanide (KCN; 10 mM in borate buffer) and 20 μL of naphtalene-2,3-dicarboxaldehyde (NDA; 6 mM in HPLC-grade methanol), either freshly prepared or stored at 4 °C in the dark (for less than a week). Reagents were thorougly mixed in the autosampler, and the reaction was allowed to proceed, protected from light, at a temperature of 6 °C. After 20 min, 20 μL of the derivative was loaded into the HPLC system. Separation was obtained using a Strategy C18 (150 mm length × 3 mm diameter, 3 μm granulometry) column (octadecyl 100 Å porosity, 425 m^2/g, C18 end-capped silica with 19% C) eluted and separated using an isocratic mobile phase contining Na_2HPO_4 (100 mM), EDTA (50 μM), and HPLC grade MeOH (50:50) that was thoroughly filtered (Millipore 0.45 μm durapore) and continuously degassed. Elution was performed at a rate of 0.8 mL·min^{-1} at a constant 20 °C in less than 12 min. Emission of derivatives was measured at 480 nm after excitation at 420 nm.

Materials for Biological Characterizations. All experiments were carried out in accordance with the European Community Council Directive of 24th November 1986 (86/609/ECC). Sprague−Dawley rats were from Janvier Laboratories (France). Culture media (DMEM/Ham F12 with HEPES and 4.5 g/L glucose), Dulbecco's phosphate-buffered saline (Dulbecco's PBS), Versene, antibiotics, and fetal calf serum (FCS) were purchased from Invitrogen. Culture dishes were from Nunc. All chemicals were from Sigma.

Hippocampal Neuron-Enriched Cultures. Primary neuronal cultures were established from 18-day-old embryonic rat hippocampi, as previously described,[34,35] with minor modifications. After preincubation with Versene, hippocampal cells were mechanically dissociated and plated at a density of 2 × 10^6 cells/dish, on 8-well plates containing square (10 × 10 mm^2) glass coverslips, previously coated with poly(D-lysine) (50 μg/mL) and then with DMEM/HAM F12 containing 10% FCS. Cells were grown in a defined medium containing DMEM/HAM F12, supplemented with 33 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 13 mM sodium bicarbonate, 5.5 μg/mL transferrin, 10 μg/mL insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 5 ng/mL sodium selenite, and 100 μM putrescine. Experiments were performed on cell cultures grown from 6 to 16 DIV.

Measurements of Cytosolic-Free Ca^{2+} Concentration. Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with the fluorescent indicator fura-2.[36] For this purpose, hippocampal cells grown on glass coverslips were loaded with fura-2 by a 30 min incubation at 37 °C with 5 μM fura-2-AM and 0.02% Pluronic in the extracellular solution comprising 124 mM NaCl, 3.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2-PO_4, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM D-glucose, and 10 mM HEPES (bubbled with O_2/CO_2, 95/5), pH 7.4. $[Ca^{2+}]_i$ was monitored by videomicroscopy. After rinsing, the glass coverslip was transferred to the recording chamber mounted on an inverted microscope (Leica, DMIRB). Fura-2 emission was obtained by exciting alternatively at 340 and 380 nm with a rotating filter wheel (Sutter Instruments) and by monitoring emissions (F340 and F380) at 510 nm. The ratio of emissions at 510 nm (F340/F380) was recorded in cells every second. Fluorescent signals were collected with a CCD camera (Hamamatsu), digitized, and analyzed with image analysis software (Acquacosmos, Hamamatsu). The coverslips were continually superfused with the extracellular solution thermostated at 37 °C. Unless otherwise stated, experiments were carried out in the absence of Mg^{2+} ions in the extracellular medium. Drug application was performed with a gravity-fed system and lasted 1 min for each compound tested. Data are expressed as averages (±SEM) of the ratio between the fura-2 fluorescence values of 340/380 nm excitation wavelengths ratios (F340/F380) normalized to the corresponding basal F340/F380 measured prior to any drug application. Graphs presenting time-courses of F340/F380 ratio changes have been obtained by averaging data from a population of cells recorded individually during one single experiment. For a given determination, three individual experiments were at least performed on three independent cell cultures. Therefore, the "n" values represent the entire population of cells recorded from at least three independent cultures. One-way ANOVA followed by Dunn's t test was used for multiple comparisons to determine significant differences between the experimental determinations. Statistical analysis was performed with SigmaPlot 12.0 software. Values of $p < 0.05$ were considered significant.

***Xenopus* Oocyte Injection and Oocyte Current Recording.** *Xenopus* oocytes were prepared and injected with *in vitro* transcribed RNA at 1 μg/μL (20−40 nL) of rat GluN1-1a (named GluN1 herein) and rat GluN2A or mouse ε2 (named GluN2B herein) with a stoichiometry of 1:1, as already described.[32] All constructions of NMDA receptor subunits have been kindly provided by Pierre Paoletti (IBENS, ENS, Paris). Macroscopic currents were recorded under two electrode voltage-clamp using a GeneClamp 500 amplifier (Axon Instruments) and analyzed in the ND96 HERG recording solution (in mM, 96 NaCl, 3 KCl, 0.5 $CaCl_2$, 5 HEPES, pH = 7.2). Current and voltage electrodes (less than 1 MΩ) were filled with 3 M KCl. Currents were filtered (20 Hz) and digitized (66 Hz) using a Digidata-1200 interface (Axon Instruments). Data acquisition was done using version 7 of the pClamp software (Axon Instruments).

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M.V. and V.R. have shared seniority. M.R., S.B., M.R., J.G., P.C., M.V., and V.R designed experiments. F.S., M.R., M.R., M.-C. de J.F., J. G., C. C.-S., G.B., T.C., M.A., A.T., M.G., C.M., P.C., M.V., and V.R. performed experiments. M.R., M.R., J.G., P.C., M.V., and V.R wrote the paper.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Zhang, L., Zhang, Z.-Z., Lu, Y.-N., Zhang, J.-S., and Preedy, V. R. (2013) L-Theanine from Green Tea: Transport and Effects on Health, in *Tea in Health and Disease Prevention*, Chapter 35, pp 425−435, Academic Press.

(2) Juneja, L. R., Chu, D.-C., Okubo, T., Nagato, Y., and Yokogoshi, H. (1999) L-theanine—a unique amino acid of green tea and its relaxation effect in humans. *Trends Food Sci. Technol. 10*, 199−204.

(3) White, D. J., de Klerk, S., Woods, W., Gondalia, S., Noonan, C., and Scholey, A. B. (2016) Anti-Stress, Behavioural and Magnetoencepha-

lography Effects of an l-Theanine-Based Nutrient Drink: A Randomised, Double-Blind, Placebo-Controlled, Crossover Trial. *Nutrients 8*, 53.
(4) Hidese, S., Ota, M., Wakabayashi, C., Noda, T., Ozawa, H., Okubo, T., and Kunugi, H. (2017) Effects of chronic l-theanine administration in patients with major depressive disorder: an open-label study. *Acta Neuropsychiatr. 29*, 72.
(5) Kim, T. I., Lee, Y. K., Park, S. G., Choi, I. S., Ban, J. O., Park, H. K., Nam, S.-Y., Yun, Y. W., Han, S. B., Oh, K. W., and Hong, J. T. (2009) l-Theanine, an amino acid in green tea, attenuates beta-amyloid-induced cognitive dysfunction and neurotoxicity: reduction in oxidative damage and inactivation of ERK/p38 kinase and NF-kappaB pathways. *Free Radical Biol. Med. 47*, 1601−1610.
(6) Egashira, N., Hayakawa, K., Osajima, M., Mishima, K., Iwasaki, K., Oishi, R., and Fujiwara, M. (2007) Involvement of GABA(A) receptors in the neuroprotective effect of theanine on focal cerebral ischemia in mice. *J. Pharmacol. Sci. 105*, 211−214.
(7) Kakuda, T. (2002) Neuroprotective Effects of the Green Tea Components Theanine and Catechins. *Biol. Pharm. Bull. 25*, 1513−1518.
(8) Kakuda, T. (2011) Neuroprotective effects of theanine and its preventive effects on cognitive dysfunction. *Pharmacol. Res. 64*, 162−168.
(9) Kakuda, T., Nozawa, A., Sugimoto, A., and Niino, H. (2002) Inhibition by theanine of binding of [3H]AMPA, [3H]kainate, and [3H]MDL 105,519 to glutamate receptors. *Biosci., Biotechnol., Biochem. 66*, 2683−2686.
(10) Nagasawa, K., Aoki, H., Yasuda, E., Nagai, K., Shimohama, S., and Fujimoto, S. (2004) Possible involvement of group I mGluRs in neuroprotective effect of theanine. *Biochem. Biophys. Res. Commun. 320*, 116−122.
(11) Di, X., Yan, J., Zhao, Y., Zhang, J., Shi, Z., Chang, Y., and Zhao, B. (2010) L-theanine protects the APP (Swedish mutation) transgenic SH-SY5Y cell against glutamate-induced excitotoxicity via inhibition of the NMDA receptor pathway. *Neuroscience 168*, 778−786.
(12) Kakuda, T., Yanase, H., Utsunomiya, K., Nozawa, A., Unno, T., and Kataoka, K. (2000) Protective effect of gamma-glutamylethylamide (theanine) on ischemic delayed neuronal death in gerbils. *Neurosci. Lett. 289*, 189−192.
(13) Yamada, T., Terashima, T., Kawano, S., Furuno, R., Okubo, T., Juneja, L. R., and Yokogoshi, H. (2009) Theanine, gamma-glutamylethylamide, a unique amino acid in tea leaves, modulates neurotransmitter concentrations in the brain striatum interstitium in conscious rats. *Amino Acids 36*, 21−27.
(14) Wakabayashi, C., Numakawa, T., Ninomiya, M., Chiba, S., and Kunugi, H. (2012) Behavioral and molecular evidence for psychotropic effects in L-theanine. *Psychopharmacology (Berl.) 219*, 1099−1109.
(15) Coyle, J. T. (2012) NMDA receptor and schizophrenia: a brief history. *Schizophr. Bull. 38*, 920−926.
(16) Schade, S., and Paulus, W. (2016) D-Cycloserine in Neuropsychiatric Diseases: A Systematic Review. *Int. J. Neuropsychopharmacol. 19*, pyv102.
(17) Volianskis, A., France, G., Jensen, M. S., Bortolotto, Z. A., Jane, D. E., and Collingridge, G. L. (2015) Long-term potentiation and the role of N-methyl-D-aspartate receptors. *Brain Res. 1621*, 5−16.
(18) Collingridge, G. L., Peineau, S., Howland, J. G., and Wang, Y. T. (2010) Long-term depression in the CNS. *Nat. Rev. Neurosci. 11*, 459−473.
(19) Steullet, P., Cabungcal, J. H., Monin, A., Dwir, D., O'Donnell, P., Cuenod, M., and Do, K. Q. (2016) Redox dysregulation, neuroinflammation, and NMDA receptor hypofunction: A "central hub" in schizophrenia pathophysiology? *Schizophr. Res. 176*, 41−51.
(20) Escobar, M., Crouzin, N., Cavalier, M., Quentin, J., Roussel, J., Lanté, F., Batista-Novais, A. R., Cohen-Solal, C., De Jesus Ferreira, M.-C., Guiramand, J., Barbanel, G., and Vignes, M. (2011) Early, time-dependent disturbances of hippocampal synaptic transmission and plasticity after in utero immune challenge. *Biol. Psychiatry 70*, 992−999.
(21) Vignes, M. (2001) Regulation of spontaneous inhibitory synaptic transmission by endogenous glutamate via non-NMDA receptors in cultured rat hippocampal neurons. *Neuropharmacology 40*, 737−748.
(22) Takeda, A., Tamano, H., Suzuki, M., Sakamoto, K., Oku, N., and Yokogoshi, H. (2012) Unique induction of CA1 LTP components after intake of theanine, an amino acid in tea leaves and its effect on stress response. *Cell. Mol. Neurobiol. 32*, 41−48.
(23) Tamano, H., Fukura, K., Suzuki, M., Sakamoto, K., Yokogoshi, H., and Takeda, A. (2014) Advantageous effect of theanine intake on cognition. *Nutr. Neurosci. 17*, 279−283.
(24) Baudouin, S. X. (2008) Process for purifying theanine. US Patent US7303773B2.
(25) Ekanayake, A., and Li, J. J. (2003) Process for enriching extracts of natural theanine. US Patent US7303773B2.
(26) Zhang, F., Zheng, Q.-Z., Jiao, Q.-C., Liu, J.-Z., and Zhao, G.-H. (2010) Enzymatic synthesis of theanine from glutamic acid γ-methyl ester and ethylamine by immobilized Escherichia coli cells with γ-glutamyltranspeptidase activity. *Amino Acids 39*, 1177−1182.
(27) Kappe, C. O., and Stadler, A. (2005) *Microwaves in Organic and Medicinal Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, FRG.
(28) Zhu, S., and Paoletti, P. (2015) Allosteric modulators of NMDA receptors: multiple sites and mechanisms. *Curr. Opin. Pharmacol. 20*, 14−23.
(29) Burgdorf, J., Zhang, X., Weiss, C., Matthews, E., Disterhoft, J. F., Stanton, P. K., and Moskal, J. R. (2011) The N-methyl-D-aspartate receptor modulator GLYX-13 enhances learning and memory, in young adult and learning impaired aging rats. *Neurobiol. Aging 32*, 698−706.
(30) Burgdorf, J., Zhang, X., Nicholson, K. L., Balster, R. L., Leander, J. D., Stanton, P. K., Gross, A. L., Kroes, R. A., and Moskal, J. R. (2013) GLYX-13, a NMDA receptor glycine-site functional partial agonist, induces antidepressant-like effects without ketamine-like side effects. *Neuropsychopharmacology 38*, 729−742.
(31) Jones, C. A., Watson, D. J. G., and Fone, K. C. F. (2011) Animal models of schizophrenia. *Br. J. Pharmacol. 164*, 1162−1194.
(32) Ota, M., Wakabayashi, C., Matsuo, J., Kinoshita, Y., Hori, H., Hattori, K., Sasayama, D., Teraishi, T., Obu, S., Ozawa, H., and Kunugi, H. (2014) Effect of L-theanine on sensorimotor gating in healthy human subjects. *Psychiatry Clin. Neurosci. 68*, 337−343.
(33) Clarke, G., O'Mahony, S., Malone, G., and Dinan, T. G. (2007) An isocratic high performance liquid chromatography method for the determination of GABA and glutamate in discrete regions of the rodent brain. *J. Neurosci. Methods 160*, 223−230.
(34) Blanc, E. M., Jallageas, M., Recasens, M., and Guiramand, J. (1999) Potentiation of glutamatergic agonist-induced inositol phosphate formation by basic fibroblast growth factor is related to developmental features in hippocampal cultures: neuronal survival and glial cell proliferation. *Eur. J. Neurosci. 11*, 3377−3386.
(35) de Jesus Ferreira, M. C., Crouzin, N., Barbanel, G., Cohen-Solal, C., Récasens, M., Vignes, M., and Guiramand, J. (2005) A transient treatment of hippocampal neurons with alpha-tocopherol induces a long-lasting protection against oxidative damage via a genomic action. *Free Radical Biol. Med. 39*, 1009−1020.
(36) Crouzin, N., de Jesus Ferreira, M.-C., Cohen-Solal, C., Aimar, R.-F., Vignes, M., and Guiramand, J. (2007) Alpha-tocopherol-mediated long-lasting protection against oxidative damage involves an attenuation of calcium entry through TRP-like channels in cultured hippocampal neurons. *Free Radical Biol. Med. 42*, 1326−1337.