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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 Ca²⁺ 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. In humans, L-theanine has been shown to induce antistress/relaxing actions and to improve sleep induction. Further characterizations of its actions on animal models of Alzheimer’s disease and stroke indicate that L-theanine also displays neuroprotective action, which seems to rely on an antioxidant action 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. Its neuroprotective actions may rely on interference with ionotropic or metabotropic glutamate receptor-mediated neuronal processes. 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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ischemic injury. In addition, l-theanine may also modulate GABAergic transmission to produce neuroprotection under ischemia as evidenced in mice. Other studies uncovered an excitatory action of l-theanine. Indeed, in striatal neurons, l-theanine treatment increases the exocytosis of various neurotransmitters, including \( \gamma \)-amino-butyric acid (GABA), glycine, and dopamine. 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. 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. Interestingly, l-theanine treatment reverses sensorimotor gating deficits, which are associated with this pathological state.

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. 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 and long-term depression 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 and neurodevelopmental disorders). We provide here a new, simple, and efficient synthesis of D- and l-theanine and N-alkylated derivatives.
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-CH₂-Ph)-OH or Z-L-Glu(OCH₃)-OH and 2 M ethylamine solution (Z = benzyloxy carbonyl 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 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 αD 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(OCH₃)-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 benzyloxy carbonyl 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(OCH₃)-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 [Ca²⁺]ᵢ increases when applied in the micromolar range on cultured cortical neurons. We have thus checked whether L-theanine was active in this concentration range (1–100 μM) on cultured hippocampal neurons. In the absence of Mg²⁺ ions, L-theanine elicited [Ca²⁺]ᵢ increases, which were consistently detected at 1 μM in mature (16 days in vitro, DIV) cultured hippocampal neurons (Figure 2A). Under physiological extracellular Mg²⁺ (1 mM; Figure 2B) or in the presence of NMDA receptor antagonist dizocilpine (MK-801, 10 μM; Figure 2C) or without extracellular Ca²⁺ ions (Figure 6D), perfusion of l-theanine had no effect on [Ca²⁺]ᵢ. These data demonstrate that L-theanine produced an influx of Ca²⁺ on mature cultured hippocampal neurons that mainly relied on NMDA receptors. Non-NMDA ionotropic receptors and metabotropic mGlu/S 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 [Ca²⁺]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. 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 Ca²⁺ 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 Ca²⁺ 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. TTX completely inhibited the changes in [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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).
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.
Characterization of the Effects L-Theanine Derivatives: D-Theanine 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 [Ca2+]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 5A,B,C) and the dependency on extracellular Ca2+ (Figure 5D). In addition, the [Ca2+]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 5E,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 [Ca2+]i, only in mature hippocampal neurons. This effect of L-theanine was completely blocked by a NMDA receptor antagonist or by extracellular Mg2+ ions, and was dependent on the presence of extracellular Ca2+ ions. This strongly suggests that the increase in [Ca2+]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 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 synthetically 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 [Ca2+]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 [Ca2+]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.
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. 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.\textsuperscript{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\textsuperscript{24,25} and enzymatic biosynthesis,\textsuperscript{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.\textsuperscript{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-N-propyl-Gln appeared to be more potent than l-theanine itself to elicit \( [\text{Ca}\text{²⁺}] \), 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.\textsuperscript{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 GLXY13, which bind the GluN1 glycine site, have proven to be beneficial in improving learning during aging\textsuperscript{29} and in the course of pathological states such as depression\textsuperscript{30} or conditions associated with hypofunction of NMDA receptors, such as psychosis.\textsuperscript{31} Many psychiatric diseases resulting from a reduced activity of NMDA receptors, including psychosis,\textsuperscript{32} can be mimicked in rodents by a reduction in NMDA receptor function.\textsuperscript{33} 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).\textsuperscript{34} Moreover, l-theanine seems to enhance PPI in humans,\textsuperscript{35} 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.\textsuperscript{75}\textsuperscript{1H} NMR spectra were recorded at 300 and 75 MHz using a Bruker AC300 instrument and at 600 MHz using a Bruker 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 Symmetry Shield RP18 3.5 μm 2.1 mm × 20 mm column using a linear gradient of ACN in H₂O 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-7300 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 Chiralpak 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 in 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).

### L-γ-N-Propylglutamine Characterization

\( ^{1}H \) NMR (300 MHz, D₂O): \( \delta = 0.82 \text{ (t, } J = 7.47 \text{ Hz, 3 H}), 1.54 \text{ (m, 2H), } 1.90 \text{ (m, 2H), } 2.07 \text{ (m, 2H), } 2.97 \text{ (t, } J = 7.09 \text{ Hz, 2H}), 3.71 \text{ (m, 1H), } 5.01 \text{ (s, 2H), } 6.61 \text{ (d, } J = 6.92 \text{ Hz, 1H), } 7.28 \text{ (m, 5H). } \)

\( ^{13}C \) NMR (75 MHz, D₂O): \( \delta = 11.32, 22.31, 28.75, 32.10, 40.27, 55.19, 65.04, 127.54, 127.63, 128.27, 137.21, 155.42, 172.05, 174.18. [\alpha]^{20}_{D}: +2.5 \text{ (c = 1, DMSO). MS (ESI): m/z } 323.2 \text{ [M + H]^{+}}, 345.1 \text{ [M + Na]^{+}}. \)

### L-Theanine Characterization

\( L \)-z-Theanine (0.36 mmol, 100 mg) was dissolved in freshly distilled THF (3 mL), and 20% PD(OH)<sub>C</sub> (20 mg) was added. The mixture was hydrogenated (H₂) at rt for 8 h. PD(OH)<sub>C</sub> 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, D₂O): \( \delta = 1.08 \text{ (t, } J = 7.32 \text{ Hz, 3 H), } 2.11 \text{ (dd, } J = 7.77 \text{ Hz, } J = 6.16 \text{ Hz, 2H), } 2.39 \text{ (m, 2H), } 3.17 \text{ (q, } J = 7.32 \text{ Hz, 2H), } 3.74 \text{ (t, } J = 6.16 \text{ Hz, 1H). } \)

\( ^{13}C \) NMR (75 MHz, D₂O): \( \delta = 13.41, 26.53, 31.73, 34.57, 53.56, 173.95, 174.29. [\alpha]^{20}_{D}: +8.4 \text{ (c = 1, H₂O). \ [\alpha]^{20}_{D}: +8.1 \text{ (c = 5, H₂O). Mp = 215–219 °C. MS (ESI): m/z } 175.1 \text{ [M + H]^{+}}, 197.1 \text{ [M + Na]^{+}, } 175.1004, \text{ found 175.1039. t-Theanine, [\alpha]^{20}_{D}: -8.3 \text{ (c = 1, H₂O).} \)

Commercially available l-theanine from Sigma-Aldrich, \( ^{1}H \) NMR (300 MHz, D₂O): \( \delta = 1.10 \text{ (t, } J = 7.75 \text{ Hz, 3 H), } 2.13 \text{ (dd, } J = 8.00 \text{ Hz, } J = 6.50 \text{ Hz, 2H), } 2.40 \text{ (td, } J = 8.00 \text{ Hz, } J = 4.20 \text{ Hz, 2H), } 3.2 \text{ (q, } J = 7.75 \text{ Hz, 2H), } 3.76 \text{ (t, } J = 8.00 \text{ Hz, 1H).} \)
L-γ-N-Propylglutamine Characterization. 1H NMR (300 MHz, D2O): δ = 8.82 (t, J = 7.43 Hz, 3 H), 1.45 (td, J = 7.24 Hz, J = 6.96 Hz, 2 H), 2.05 (m, 2 H), 2.31(m, 2 H), 3.08 (t, J = 6.96 Hz, 2 H), 3.64 (t, J = 6.1 Hz, 1 H). 13C NMR (75 MHz, D2O): δ = 10.63, 21.79, 27.21, 31.83, 41.33, 54.43, 174.75, 175.04. [α]D 20° = +13.9 (c = 1, H2O). Mp = 207–209 °C. MS (ESI); m/z 189.2 (M + H)2, 211.2 [M + Na]+. HRMS calcd for C11H16N2O5+ [M + Na]+ 189.1239, found 189.1239. γ-N-Propylglutamine, [α]D 20° = –14.0 (c = 1, H2O).

HPLC Measurement of Glutamate, GABA, and l-Theanine. Determination of glutamate, GABA, and l-theanine involved the HPLC separation of precolumns 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. Chromatone 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 reported33 with slight modifications. Brieﬂy, standardized, 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 (for less than a week). Reagents were thoroughly 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 (octacycl 100 Å porosity, 425 μg/g silica with 19% C) eluted and separated using an isocratic mobile phase containing NaH2PO4, 1 mM CaCl2, 2 mM MgSO4, 10 mM MD-glucose, 1.25 mM NaH2PO4, 1 mM CaCl2, 2 mM MgSO4, 10 mM Na-glucose, and 10 mM HEPEs (buffered with Na2CO3/NaOH, pH 7.4. [Ca2+]i), was measured at 490 nm after excitation at 420 nm. The ratio of emissions at 510 nm (F340/F380) was recorded in cells treated with the extracellular solution thermostated at 37 °C. Unless otherwise stated, experiments were carried out in the absence of Mg2+ ions in the extracellular medium. Drug application was performed with a gravity-fed delivery 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 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 e2 (named GluN2B herein) with a stoichiometry of 1:1, as already described.33 All constructions of NMDA receptor subunits have been kindly provided by Pierre Paolelli (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 CaCl2, 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).

Author Contributions

References
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