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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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 anti-stress/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 γ-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-γ-L-Glu(O-CH₂-Ph)-OH or Z-γ-L-Glu(OCH₃)-OH and 2 M ethylamine solution (Z = benzylxycarbonyl 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 d₉ 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-γ-L-Glu(OH)-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 (1-BS-68) was obtained by removing the benzylxycarbonyl 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 γ-N-propylglutamine (or L-βS-77).

With Z-γ-L-Glu(OH)-OH as starting product, in solvent free system, thanks to pure liquid n-propylamine (bp = 47–51 °C), and under microwave irradiation, Z-γ-L-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 NMRA Receptors. L-Theanine was previously shown to elicit [Ca²⁺], 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 NMRA 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 NMRA receptors. Non-NMRA ionotropic receptors and metabotropic mGlu1/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 NMRA could elicit responses sensitive to glycine (Figure 3A). Therefore, we hypothesized that l-theanine might be exerting positive allosteric modulation or coagonist action on NMRA 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 NMRA on immature neurons, where synaptic activation of NMRA receptors did not yet occur, and compared its effects with those elicited by conventional NMRA receptor glycine site agonists, glycine and D-serine. L-Theanine (1 μM to 1 mM) effectively potentiated Ca²⁺ influxes elicited by NMRA (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 NMRA receptors. Indeed, we verified whether l-theanine had any effect on non-NMRA 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 NMRA 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 NMRA responses on mature neurons (Figure 4B). Finally, the NMRA 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 NMRA-mediated [Ca²⁺]ᵢ increases (less than 4%). Taken together, these results confirm that l-theanine behaves as a partial NMRA 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 NMRA receptor responses as ifenprodil (10 μM), a selective antagonist of GluN2B subunit-containing NMRA receptor, completely blocked the NMRA responses (Figure 2B). The selectivity toward either GluN2A- or GluN2B-containing NMRA receptors was further evaluated on Xenopus oocytes where the subunit composition of the NMRA receptors can be easily controlled. The study was restricted to GluN2A- and GluN2B-containing NMRA 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 NMRA 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⁺] 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⁺] 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 L-serine (D) on NMDA-mediated [Ca²⁺] 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 L-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⁺] 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.

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).
Characterization of the Effects \(\alpha\)-Theanine Derivatives: \(\alpha\)-Theanine and \(\alpha\) and \(\alpha\)\(\gamma\)-N-Propyl-Gln. \(\alpha\)-Theanine derivatives were further synthesized by substituting the \(\gamma\)-N-ethyl moiety and by changing the chirality from \(\alpha\) to \(\alpha\) of the asymmetric carbon. Indeed, as observed for \(\alpha\)-serine, a \(\alpha\)-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 \(\mu\)M in mature cultured hippocampal neurons. Among all the compounds synthesized, it appears that the \(\gamma\)-N-propyl derivative exerted excitatory properties on \([\text{Ca}^{2+}]_i\) in cultured hippocampal neurons as well as \(\alpha\)-theanine did. Therefore, three compounds were further synthesized and tested: \(\alpha\)-theanine and \(\alpha\) and \(\alpha\)\(\gamma\)-N-propyl-Gln. They all retained the properties of NMDA receptor coagonist as observed for \(\alpha\)-theanine, that is, the blockade by NMDA receptor antagonist MK-801 (Figure 6A,B,C) and the dependency on extracellular \([\text{Ca}^{2+}]_i\) (Figure 6D). In addition, the \([\text{Ca}^{2+}]_i\) rises elicited by these \(\alpha\)-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 \(\mu\)M indicated that both the change of chirality and the \(\gamma\)-N-propyl substitution significantly enhanced the responses elicited by \(\alpha\)-theanine (Figure 6E,F).

**DISCUSSION**

We demonstrate here that \(\alpha\)-theanine exerts an excitatory action on hippocampal neurons mainly by potentiating NMDA responses. \(\alpha\)-Theanine had no detectable agonist effect on other glutamate receptors, that is, non-NMDA ionotropic and phospholipase C-associated mGlu receptors. By itself, \(\alpha\)-theanine induced \([\text{Ca}^{2+}]_i\), only in mature hippocampal neurons. This effect of \(\alpha\)-theanine was completely blocked by a NMDA receptor antagonist or by extracellular \(\text{Mg}^{2+}\) ions, and was dependent on the presence of extracellular \(\text{Ca}^{2+}\) ions. This strongly suggests that the increase in \([\text{Ca}^{2+}]_i\), elicited by \(\alpha\)-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 \(\alpha\)-theanine behaves as coagonist of NMDA receptors activated by endogenously released glutamate. Accordingly, blocking synaptic transmission by TTX fully prevented the \(\alpha\)-theanine response in mature hippocampal neurons. The fact that TTX partially reduced the NMDA response suggests that glycine or \(\alpha\)-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 \(\alpha\)-theanine was directly confirmed by the enhancement of the NMDA induced \([\text{Ca}^{2+}]_i\) response when \(\alpha\)-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 \(\alpha\)-theanine-elicited \([\text{Ca}^{2+}]_i\) 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 \(\alpha\)-theanine were not restricted to one subtype of the GluN2 subunits. \(\alpha\)-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,

**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+}] increases in mature (16 DIV) neurons. The [Ca^{2+}] 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+}] responses induced by L-theanine and derivatives. (F) Cumulative graph plotting peak responses elicited by 10 μM L-theanine or derivatives on [Ca^{2+}], 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).
Among these compounds, the D- enantiomer using a Bruker AC300 instrument and at 600 MHz was obtained. The mixture was hydrogenated (H2) at rt for 12 h. D-Theanine (20 mg) was added. The product was purified by suction chromatography on silica gel (Kieselgel 60, 230–400 mesh) as stationary phase. Thin layer chromatography was carried out on aluminum plates precoated with Merck Silica gel 60F254 and visualized by quenching with ultraviolet fluorescence or by staining with a 10% methanol phosphomolybdic acid solution followed by heating. Analytical HPLC was performed on a Waters apparatus 717 plus autosampler with Millenium 32 program on SymmetryShield RP3, 3.5 μm × 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-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/acetoni trol 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).

Z-L-Theanine Synthesis. Substrate (Z-L-Glu(OCH3)-OH or Z-L-Glu(Obz)-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 twice with concentrated NH4Cl. The organic phase was dried over MgSO4 and concentrated under reduced pressure. Z-L-Theanine precipitated as a white powder in Et2O (20 mL) and was filtered and lyophilized. 1H NMR (300 MHz, DMSO): δ = 1.02 (t, J = 7.3 Hz, 3 H), 2.08 (m, 2 H), 2.17 (m, 2 H), 3.24 (q, J = 7.29 Hz, 2 H), 4.43 (m, 1 H), 5.02 (s, 2 H), 5.71 (d, J = 6.86 Hz, 1 H), 7.28 (m, 5 H). 13C 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. Analytic chiral HPLC: Chiralcel-OD column, H2O/ACN (87/13), 0.6 mL/min, 45 min, 214 nm, 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-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/acetoni trol 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).

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**L-γ-N-Propylglutamine Characterization.** ¹H NMR (300 MHz, D₂O): δ = 0.82 (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, 17.19, 27.21, 31.83, 41.33, 54.43, 174.75, 175.04. (α-γ-N-Propylglutamine, δ = 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 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. Chromereon 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 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 naphthalene-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 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 column (150 mm length × 3 mm diameter, 3 µm granulometry) (octadecyl 100 Å porosity, 425 mg/g, C18 end-capped silica with 19% C) eluted and separated using an isocratic mobile phase containing Na₂HPO₄ (100 mM), EDTA (50 mM), and HPLC grade MeOH (50:50) that was thoroughly filtered (Millipore 0.45 µm) 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, supplemented with 33 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 13 mM sodium bicarbonate, 5.5 mg/mL transferrin, 10 µg/mL insulin, 1 pM β-estradiol, 3 mM 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 Ca²⁺ Concentration.** Intra-cellular calcium concentration ([Ca²⁺]ᵢ) was measured with the fluorescent indicator fura-2. 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 NaHCO₃, 1.25 mM NaH₂PO₄, 1 mM CaCl₂, 2 mM MgSO₄, 10 mM d-glucose, and 10 mM HEPES (bubbled with 95%/5% O₂/CO₂, pH 7.4. [Ca²⁺]), was monitored by videomicroscopy. After rinsing, the coverslip was transferred to the recording chamber mounted on an inverted microscope (Leica, DMIRE). 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 (Acutacosmos, 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 Mg²⁺ ions in the extracellular medium. Drug application was performed with a gravity-fed syringe 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 ε2 (named GluN2B herein) with a stoichiometry of 1:1, as already described. 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 Genclamp 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).

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**Notes**

The authors declare no competing financial interest.

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