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Full Paper

Evaluation of β-Aminocarboxylic Acid Derivatives in Hippocampal Excitatory Synaptic Transmission

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β-Aminocarboxylic acid derivatives (LINS04 series) were screened with the aim to explore their potential functional role in excitatory synaptic transmission in the central nervous system. We used field recordings in rat hippocampal slices to investigate the effects of the LINS04 series on the synaptic transmission at hippocampal CA1 synapses. We found that LINS04008 and LINS04009 increase the size of the evoked field excitatory postsynaptic potential (EPSP) in a dose-dependent manner. The concentration–response curve shows that the efficacy of LINS04008 is highest in the series ($EC_{50} = 91.32\ \mu M$; maximum fEPSP 44.97%). The esters LINS04006 and LINS04005 did not affect the synaptic evoked activity. These data provide the first evidence of synaptic activity enhancement by these compounds and the importance of the acidic group to the activity. This set of data may provide direction for a strategic procedure to restore the glutamate synaptic transmission; however, further studies are needed to establish a more complete picture of how these molecules act on the glutamate transmission, which are in our mind for the next steps.

Keywords: β-Amino acids / β-Amino esters / Excitatory postsynaptic potential / Hippocampus / Synaptic transmission

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Introduction

β-Amino acids are molecules closely related to the corresponding natural α-amino acids in that they contain an ionizable amino group and a carboxy group; however, two carbons separate these groups instead of one [1]. The main advantage in designing β-amino acids to obtain bioactive molecules (such as peptidomimetics or other kind of molecules) is that they are non-proteinogenic, i.e., they are not inserted in protein synthesis, and also they can evade the metabolic pathway to the natural α-amino acids, being then metabolically resilient [1, 2].

L-Glutamic acid (Glu, Fig. 1) is the most abundant α-amino acid in the brain which mediates the most of excitatory transmission, and it is involved in most aspects of normal brain functions, such as motor behavior, fear and learning and memory [3–5]. Glu is synthesized in the cytoplasm and packaged into synaptic vesicles in the presynaptic terminal by vesicular glutamate transporters (VGLUTs). Following an action potential, it activates two families of receptors: the ionotropic glutamate receptors (iGluRs), *N*-methyl-D-aspartic acid, kainic acid and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (respectively NMDARs, KARs, and

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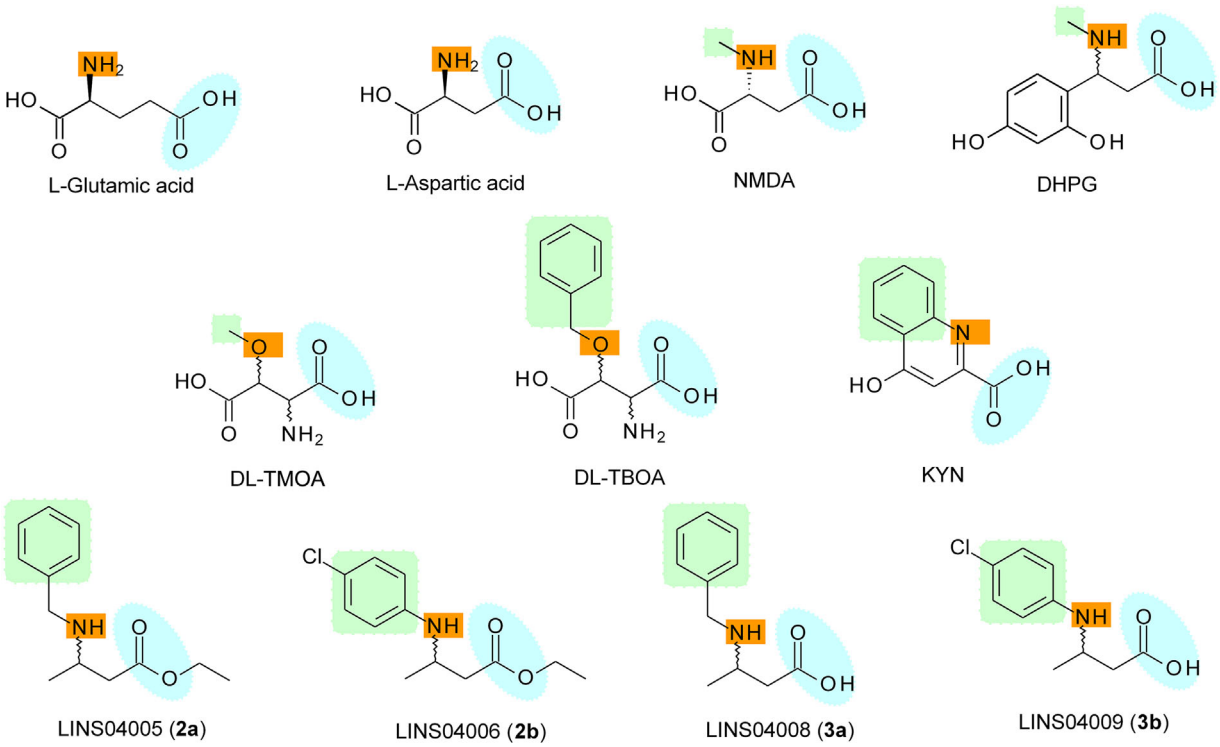


Figure 1. Structures of several molecules that interfere in Glu transmission and the LINS04 compounds (**2**–**3**). The characteristics considered in the design are colored; blue: carboxy group; orange: polar group; green: additional lipophilic moiety.

AMPARs), and metabotropic receptors (mGluRs) groups I, II, and III. Once completed, glutamatergic transmission is mainly finished by the excitatory amino acid transporters (EAATs), which take up synaptic Glu into neurons, astrocytes, and oligodendrocytes. Glutamate reuptake into the brain prevents neuronal excitotoxicity and regulates synaptic transmission avoiding the establishment of acute CNS injury (e.g., stroke, trauma, hypoglycemia) [3, 5–8]. Dysfunctions of Glu transmission affects normal brain functions with devastating consequences which could trigger several neurological (e.g., epilepsy, Alzheimer's disease, and Parkinson's disease) and psychiatric disorders (e.g., schizophrenia and mood disorders) [3–6]. There is a great interest for the development of novel therapeutic agents that interfere in Glu transmission by binding to the post-synaptic Glu receptors, or by interfering with membrane transporters.

Traditionally, iGluRs have received much attention as targets to interfere in Glu-mediated processes [9–12]. Molecules as NMDA (and also the α-amino acid L-aspartic acid (Asp), Fig. 1), AMPA, and KA are classical agonists of such receptors closely related to the amino acids. mGluRs are also a target for several bioactive compounds, such as dihydroxyphenylglycine (DHPG), a group I mGluRs agonist that shows that an additional carboxy group is not necessary for glutamatergic activity (Fig. 1). More recently, Glu transporters have also emerged as possible targets to halt or slow the course of neurological and psychiatric disorders mediated by Glu, by regulating transmitter release and restoring synaptic activity [7–10]. Several molecules have been developed as ligands of Glu transporters. DL-*threo*-β-Benzyloxyaspartate (DL-TBOA) [9] is a potent competitive EAATs blocker (IC_{50} = 2–70 μM) and has been shown to depress the size of the evoked field excitatory postsynaptic potential (fEPSP) (10 μM) *in vitro* [9–12]. DL-*threo*-β-Methoxyaspartate (DL-TMOA) is also an EAAT ligand, but it acts mainly as substrate of such transporter [10]. Kynurenic acid (KYN) is a selective VGluTs inhibitor (K_i 1.3 mM) and depresses synaptic transmission at hippocampal CA1 synapses (IC_{50} = 130–400 μM) [7, 13–15]. It is important to stress that Asp itself is not a substrate for VGluTs, but it is also substrate for EAATs [13–15].

Considering the chemical similarities between the molecules cited above with amino acids, we have screened a first set of substituted β-amino acids (LINS04 series, Fig. 1) to investigate their effects on Glu transmission. To the best of our knowledge, these molecules were not tested yet for this purpose. Therefore, a preliminary evaluation on synaptic transmission by LINS04 compounds at hippocampal synapses from CA1 area was performed.

Results and discussion

The LINS04 compounds were synthesized as shown in Scheme 1. The β-enamino esters were prepared by reacting ethyl acetoacetate with corresponding amine [16]. The yields for compounds **1a** and **1b** were 97 and 60%, respectively. The lower yield of the latter can be attributed to the low availability of the electrons of aniline to attack the ketone carbonyl group of ethyl acetoacetate. As can be observed in the experimental procedure, the synthesis of compound **1a** was carried out in ultrasonic conditions at 25°C by simply mixing the reagents in equimolar amount with catalytic (10%) of AcOH. This procedure did not show convenient to prepare compound **1b**, and then classical reflux method was used.

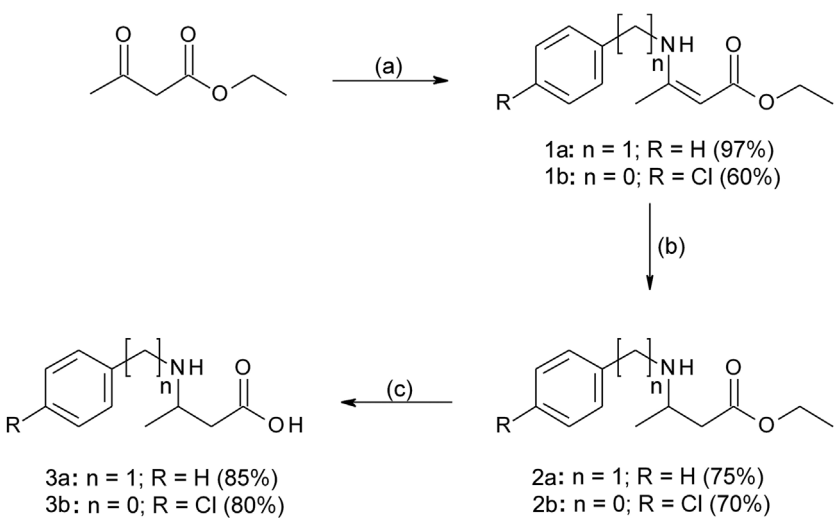
The β-enamino esters were then converted to the corresponding β-amino esters LINS04005 (**2a**) (75%) and LINS04006 (**2b**) (70%) using STAB as reducing agent, prepared *in situ* [17]. As STAB is a moderate and highly selective reducing agent for imine/enamine group, the ester group could be easily preserved. Finally, these compounds were hydrolyzed by refluxing with 10% NaOH in THF [18], to give the compounds LINS04008 (**3a**) and LINS04009 (**3b**) with 85 and 80% yields, respectively.

To investigate the effects of β-amino acid derivatives on synaptic transmission at hippocampal CA1 synapses, we used field recordings in rats hippocampal slices. Application of **3a** increased the size of of evoked field excitatory postsynaptic potential (fEPSP) in the hippocampal-CA1 synapses in a concentration-dependent manner, producing a curve with a maximal increase of 44.97% (Table 1) and EC_{50} value of 91.32 μM (Fig. 2A). Compound **3a** has the greatest potency on the dose–response curve. The effects of **3b** also increased fEPSP in hippocampal-CA1 synapses; the curve displayed a maximal increase of 32.72% (Table 1) and EC_{50} value of 29.97 μM (Fig. 2B). Interestingly, application of compounds **2a** and **2b** did not affect the transmission (Table 1).

The results provide the first evidence that these β-amino acid derivatives increase synaptic activity at the hippocampus. Although this study must continue, the preliminary collected data brought important findings regarding structure–activity relationships (SAR) data of the compounds. The presence of an acidic group (i.e., carboxylic acid) seems to be mandatory to produce an increase in the size of evoked field excitatory postsynaptic potential, whereas the ester group showed to be detrimental to the activity. This finding shows the importance of the acidic group to the interaction to the proteins involved in Glu transmission, the receptors (mainly iGluRs) or the membrane transporters (mainly EAATs). However, considering that the ester group can be biologically hydrolyzed to the acidic molecules that will be active, they can be considered as prodrugs [19] for *in vivo* activation and possibly better cross the blood–brain barrier. Future pharmacokinetic studies would define which compounds can adequately reach the CNS *in vivo*.

In addition, the 4-chloroaniline group produced less potent stimulation than the compound with benzylamine group. The characteristic of the group linked to the β-carbon atom seems to be important to the activity. Considering the Asp analogs, NMDA is a potent agonist of NMDAR, while DL-TBOA and DL-TMOA are not. Moreover, DL-TBOA is an EAAT blocker, whereas DL-TMOA is a substrate [13, 14]. The role of *N*-benzyl or *N*-(4-chloro)phenyl groups should be better explored yet, but it may be related to the differences observed in the activity of **3a** and **3b**.

To investigate some of the possible mechanisms involved in the enhancement of synaptic activity by **3a**, we performed some initial assays in the presence and absence of DL-TBOA and KYN (Fig. 3). Several works have shown that DL-TBOA and KYN depress the size of fEPSP in hippocampal-CA1 synapses, mainly by acting as iGluRs antagonists [9–15]. We observed that when 100 μM of **3a** was added along with 10 μM of DL-TBOA (Fig. 3A), significant reduction on fEPSP was



Scheme 1. Synthesis of compounds **1**–**3**. Reagents and conditions. (a) $BnNH_2$ (1 eq.), AcOH (0.1 eq.), US 0.5 h or 4-chloroaniline (1 eq.), AcOH (0.1 eq.), EtOH, reflux, 3 h; (b) STAB (3 eq.), AcOH, 2 h; (c) NaOH 40%, THF, reflux, 12 h.

Table 1. Activity of tested compounds in fEPSP recording in CA1 hippocampal slices.

Compounds	EC_{50} μM (95%CI)	fEPSP max. (% ±SD)
2a	NA	2 (±2)
2b	NA	2 (±2)
3a	91.32 (63.51–131.30)	44.97 (±2.50)
3b	29.97 (16.71–53.74)	32.12 (±3.12)

EC_{50}, effective concentration on 50% of maximum fEPSP observed; NA, not active.

observed. On the other hand, after DL-TBOA washing out, **3a** increased again the size of fEPSP, as observed previously in Fig. 2A. Considering that DL-TBOA acts as inhibitor of EAATs in addition to some iGluRs blocker activity [14], our data suggest that **3a** can also be ligand of the EAATs, acting as substrate, which competed by these transporters leading to higher DL-TBOA concentration in the synapses. This effect led to increased activity on iGluRs by DL-TBOA, and consequent reduction on fEPSP recordings.

Conversely, **3a** (100 μM) did not affect the depression produced by 100 μM of KYN (Fig. 3B). After KYN washing out, **3a** increased again the size of fEPSP by the same way observed in Fig. 2B. Though, considering that DL-TBOA and KYN are iGluRs antagonists [7, 14, 15], the obtained data suggest that the effects of **3a** on fEPSP may be attributed to possible agonism on iGluRs, albeit more specific experiments must be done to confirm this hypothesis.

The most important contribution of this work is the investigations in the effects of LINS04 series on synaptic transmission at hippocampal CA1 synapses which were unpublished until this report. Our study has revealed that **3a** and **3b** increased the synaptic activity, similar to those seen in the natural amino acids (Glu and Asp) [20, 21]. These data provide information from novel prototypes for a strategic design of molecules to restore synaptic activity, especially mediated by AMPAR and NMDAR. Clearly, further studies are needed to establish a more complete picture of how these molecules contribute to synaptic activity in both health conditions and neurological/psychiatric disorders, which are in our mind for the next steps.

Experimental

Chemistry

General

The chemicals were provided by Sigma–Aldrich, Merck (Darmstadt, Germany) and LabSynth (Diadema, Brazil) in

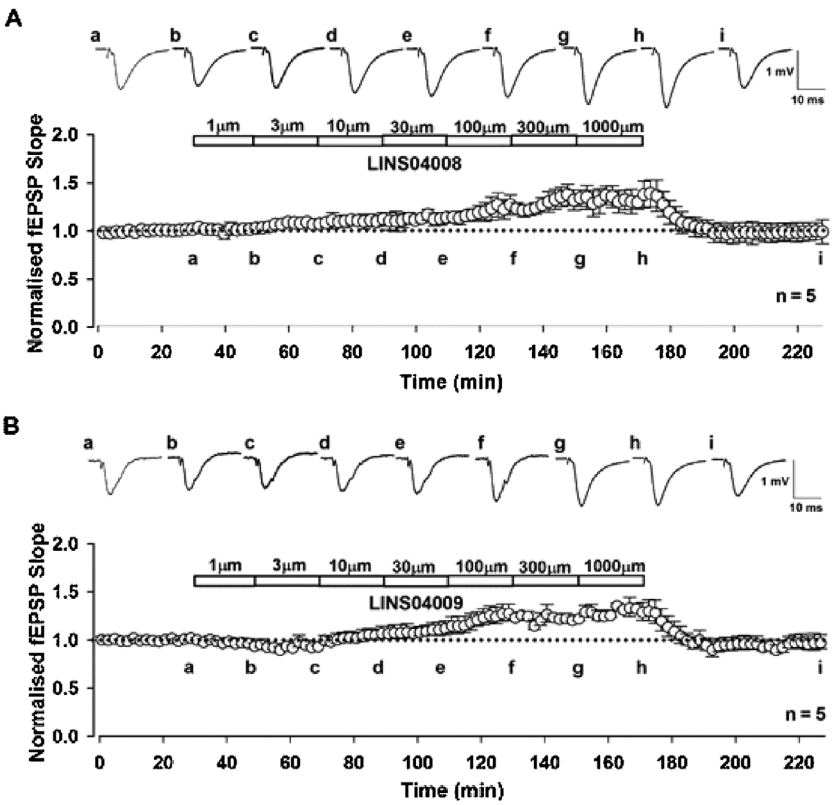


Figure 2. Effects of **3a** and **3b** at hippocampal synapses from CA1 area. Application of **3a** (A) and **3b** (B) (1.0–1000 μM) causes a significant and reversible increase in the size of the evoked field excitatory postsynaptic potential (fEPSP) ($n = 5$). The experiments were conducted in presence of picrotoxin (50 μM).

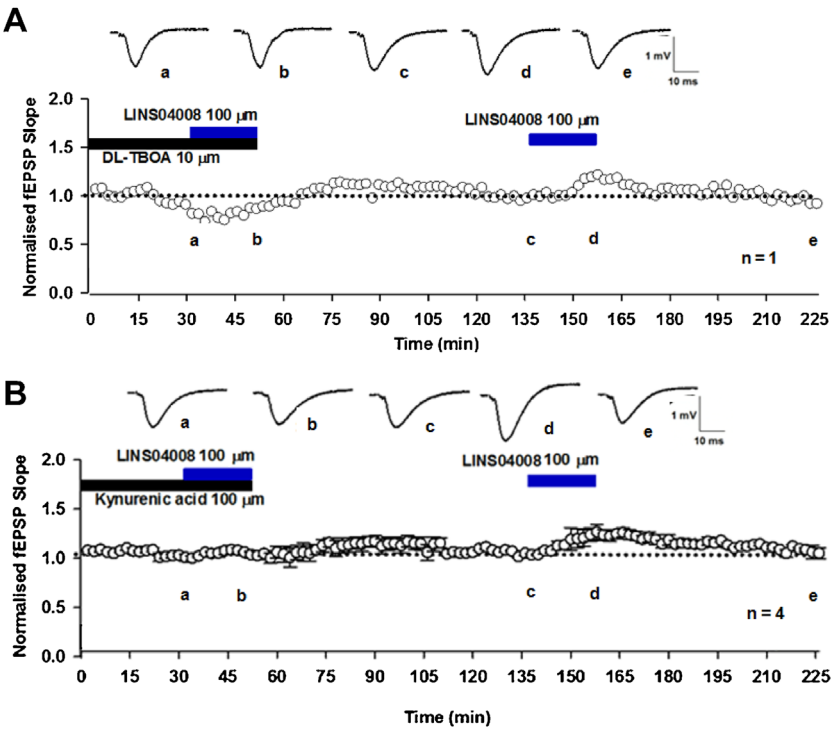


Figure 3. Effects of **3a** at hippocampal synapses from CA1 area in presence of DL-TBOA (A, single experiment) and KYN (B, $n = 4$). Application of **3a** (100 μM) together with DL-TBOA (10 μM) causes a reduction of the size of the evoked fEPSP, while no influence was observed for its application with KYN (100 μM). The experiments were conducted in presence of picrotoxin (50 μM).

adequate purity grade, and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded in a Bruker Ultrashield 300 spectrometer, operating at 300 and 75 MHz, respectively, using the indicated deuterated solvent and internal standard. Chemical shifts are reported in parts per million (ppm, δ units). Coupling constants (*J*) are reported in units of hertz (Hz), if applicable. The high resolution mass spectra (HRMS) were obtained through direct injection after electron-spray ionization in positive mode (ESI+) in a MicroTOF from Bruker Daltonics mass spectrometer. Stock solutions of the tested compounds were prepared by dissolving in purified water prior to the experiments.

The InChI codes of the investigated compounds together with some biological activity data are provided as Supporting Information. The ^{1}H NMR spectra of compounds **3a** and **3b** are also provided as Supporting Information.

*Synthesis of compounds **1***

In a flask, 5 mmol (0.65 g) of ethyl acetoacetate, 5 mmol of the corresponding amine (benzylamine or 4-chloroaniline), and 0.5 mmol (0.03 g) of acetic acid were added. The reaction was maintained in the conditions presented in Table 2. Ethanol (10 mL) was added to the final mixture, which was dried over anhydrous Na_2SO_4, and the solvent evaporated. Crude products were purified by column chromatography in silica gel, using hexane/ethyl acetate (8:1) as eluent. Spectral data were in agreement with previous report from literature [16].

*Synthesis of compounds **2***

In a flask, 4 mL of acetic acid and 4 mmol (0.15 g) of sodium borohydride were added and stirred at 0°C until the final of hydrogen evolution. After this, 2 mmol of compound **1** was added to the reaction mixture, and the reaction was kept at 25°C for 2 h. The solvent was evaporated under reduced pressure, and 10 mL of saturated K_2CO_3 solution was added. The mixture was extracted with 2×10 mL of dichloromethane, and the organic layer was dried over anhydrous Na_2SO_4, and the solvent evaporated. Crude products were purified by column chromatography in silica gel, using hexane/ethyl acetate (1:1) as eluent.

2a: Yellowish liquid (75%); ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm) δ 7.35–7.21 (m, 5H), 3.82–3.75 (m, 2H), 3.16 (sext, 1H, $J = 6.4$ Hz), 2.49 (dd, 1H, $J = 15.2$, 7.0 Hz), 2.37 (dd, 1H, $J = 15.2$, 5.9 Hz), 1.37 (br.s, 1H), 1.25 (t, 3H, $J = 7.2$ Hz), 1.16 (d, 3H, $J = 6.3$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$, TMS, ppm) δ 171.3, 140.6, 128.2, 127.2, 127.1, 60.8, 51.9, 48.0, 40.7, 19.7, 14.0; HRMS calculated 221.1415; found 222.1517 [M+H$^+$].

2b: Yellowish liquid (70%); ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm) δ 7.15–7.08 (m, 2H), 6.58–6.51 (m, 2H), 4.14 (q, 2H, $J = 7.2$ Hz), 3.95–3.82 (m, 1H), 2.63 (dd, 1H, $J = 15.0$, 5.5 Hz), 2.42 (dd, 1H, $J = 15.0$, 6.8 Hz), 1.26 (d, 3H, $J = 7.0$ Hz), 1.25 (t, 3H, $J = 7.2$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$, TMS, ppm) δ 171.5, 143.9, 128.9, 126.2, 119.3, 60.7, 45.9, 40.8, 20.7, 14.1; HRMS calculated 241.0869; found 242.0971 [M+H$^+$].

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Table 2. Conditions to prepare compounds 1a and 1b.

Compound	Condition	Temperature (°C)	Time (h)	Yield (%)
1a	Ultrasound	25	0.5	97
1b	Conventional	90	3	60

*Synthesis of compounds **3***

To 2 mmol of compounds **2** in 5 mL of tetrahydrofuran, was added 2 mL of aqueous NaOH 40%. The reaction was maintained under vigorous stirring for 12 h at 90°C. The solution was then neutralized with aqueous HCl, and the solvent was removed under reduced pressure to dryness. The crude solid was extracted with ethyl acetate, dried over anhydrous Na_2SO_4, and the solvent evaporated again. The product was purified by precipitation in ethyl acetate/hexane mixture.

3a: White solid (85%); ^{1}H NMR (300 MHz, D_2O, DSS, ppm) δ 7.43–7.36 (m, 5H), 4.26–4.09 (m, 2H), 3.56–3.43 (m, 1H), 2.48 (d, 2H, $J = 6.4$ Hz), 1.30 (d, 3H, $J = 6.9$ Hz); ^{13}C NMR (75 MHz, D_2O, DSS, ppm) δ 176.1, 140.5, 128.1, 127.0, 126.9, 51.9, 47.6, 42.3, 19.5; HRMS calculated 193.1103; found 192.1205 [M−H$^+$].

3b: Yellowish liquid (80%); ^{1}H NMR (300 MHz, D_2O, DSS, ppm) δ 7.25–7.15 (m, 2H), 6.83–6.73 (m, 2H), 3.81–3.65 (m, 1H), 2.49 (dd, 1H, $J = 14.1$, 5.1 Hz), 2.10 (dd, 1H, $J = 14.1$, 8.4 Hz), 1.13 (d, 3H, $J = 6.4$ Hz); ^{13}C NMR (75 MHz, D_2O, DSS, ppm) δ 176.1, 144.0, 128.9, 126.2, 119.3, 45.9, 42.3, 20.6; HRMS calculated 213.0556; found 212.0658 [M−H$^+$].

Biological assays

Animals

Male Wistar rats 4 to 5-week-old were obtained from Charles River, UK. All animals were housed under 12 h light/dark cycle, and experiments were performed in the light phase of the cycle. Animals were allowed access to food and water *ad libitum*, and all procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

Hippocampal slice preparation

Rats were killed by cervical dislocation and decapitation, and the brains were rapidly removed and placed into ice-cold artificial cerebrospinal fluid (aCSF; in mM: 124 NaCl, 3 KCl, 26 $NaHCO_3$, 1.4 NaH_2PO_4, 1 $MgSO_4$, 10 D-glucose, 2 $CaCl_2$) continuously oxygenated with 95% O_2 and 5% CO_2. Parasagittal slices (400 μm) containing the hippocampal formation were prepared in ice-cold, oxygenated aCSF using Vibroslicer and placed in a Petri dish containing fresh-oxygenated aCSF. The hippocampus was dissected from these slices and area CA3 was removed to reduce the risk of contaminating the field excitatory post-synaptic potential (fEPSP) recording via indirect pathway to area CA1. Hippocampal slices were left to rest for ≥2 h before being transferred to the recording chamber maintained at 27–30°C, where they were continuously perfused with oxygenated aCSF at a rate of 2 mL/min [22].

Extracellular field recordings

Field excitatory postsynaptic potentials (fEPSPs) were evoked at 0.033 Hz using bipolar stimulating electrodes (0.05 mm diameter) and recorded using glass micropipettes filled with 3 M NaCl (resistance 3–6 MΩ). Stimulating electrode was placed in the Schaffer collateral and the recording electrode was placed in the stratum radiatum (SR) of CA1 area. Once responses were found, slices were fully submerged in oxygenated aCSF perfused in a rate of 2 mL/min. A total of 50 μM of picrotoxin was also added to the perfusate in all experiments. Stimulation intensity was selected that produced responses that were around 50% maximal, and experiments began after stable baseline response was recorded for a period of at least 30 min. Responses were measured and recorded online using WinLTP software (version 2.20a; WinLTP Ltd. and The University of Bristol, Bristol, UK). Compounds were applied by addition to perfuse by 20 min.

Data analysis

Data were normalized to the mean slope recorded during the 30 min baseline. All data are presented as mean ± standard error of the mean (SEM) and EC_{50} are presented with 95% confidence intervals in parentheses. Concentration–response were plotted using GraphPad Prism software (version 6.0). Standard agonist concentration–response curves were fitted with the following equation: $Y = Y_{min} + (Y_{max} - Y_{min})/(1 + 10^{(logEC50-X)})$, where X is logarithm of the concentration of the agonist and Y is the percentage inhibition. Y_{min} was constrained to zero for curves.

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The authors have declared no conflict of interest.

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