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The authors declare no competing financial interests

Author Contributions

All authors contributed to the manuscript with drafting, writing, and revision.

Abstract

Enhancing glutamate (Glu) uptake by positive allosteric modulation of the excitatory amino acid transporter subtype 2 (EAAT2) is an attractive strategy to enable neuroprotection. However, while the EAAT field is rich in reports on inhibitors, enhancing EAAT2 protein dynamics is a much more difficult objective. A natural product approach reported a spider venom HPLC fraction number 10 to show neuro-protective effects. It was referred to as parawixin10 and later the structure of the key component responsible for the neuroprotective action was disclosed to be an *N*-acylamine, compound **2**.

We have resynthesized the *N*-acylamine **2** (parawixin10) and show here that this compound does not enhance Glu uptake in a wide range of radioligand binding assays, nor does it in our hands show any neuroprotective effect in a dose-dependent statistically significant way.

Keywords

Glutamate

Transporters

Positive allosteric modulators

Natural products

Neuroscience

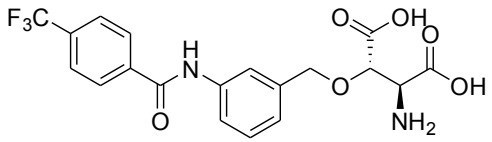
Neuroprotection

Introduction

The excitatory amino acid transporters (EAATs), also referred to as glutamate (Glu) transporters, are membrane-bound proteins that take up Glu. Five EAAT subtypes have been identified and named EAAT1-5 in humans, while the rodent nomenclature differs for EAAT1-3 due to historical reasons (GLAST, GLT2, EAAC1). The transport capacity varies amongst the five EAAT subtypes, with EAAT2 holding the highest transport capacity and being expressed predominantly on glial cells.[1] Over the years, accounts by several research groups have disclosed selective competitive EAAT inhibitors as well as non-competitive ones (negative allosteric modulators, NAMs) (Figure 1). The aspartate analog TBOA opened the field, and several potent analogs, such as TFB-TBOA, were reported. Screening of a compound library and subsequent medicinal chemistry efforts led to the disclosure of **UCPH-101** as the first selective EAAT1 NAM,[2] followed by its brain-penetrable analog **UCPH-102**.[3] A class of selective inhibitors of EAAT3 has also been disclosed, although only with micromolar potencies.[4] The development of new tool compounds for the EAAT has for long been stalling, so the review from 2009[5] and 2015[6] still holds actuality.

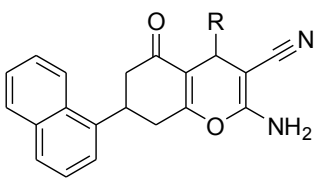
Figure 1. Chemical structures of reported competitive inhibitors, and negative allosteric modulators (NAM) of the EAATs.

Selective, competitive EAAT inhibitor



TFB-TBOA
EAAT1: IC_{50}= 22 nM
EAAT2: IC_{50}= 17 nM
EAAT3: IC_{50}= 300 nM

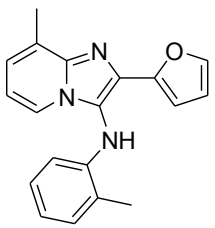
Selective EAAT1 NAM



UCPH-101: R= p-MeO-Ph-
IC_{50}= 0.66 µM

UCPH-102: R= Me-
IC_{50}= 0.43 µM

Selective EAAT3 NAM



1a
IC_{50}= 13 µM

In 2011, Santos et al. reported a study on neurobiological effects of a venom, isolated from *Parawixia bistriata* spider.[7] The 10th HPLC fraction (in the following designated parawixin10) showed anti-convulsant activity and neuro-protective effects in rodent models, however the mechanism of action was not elucidated at the molecular level.[7]

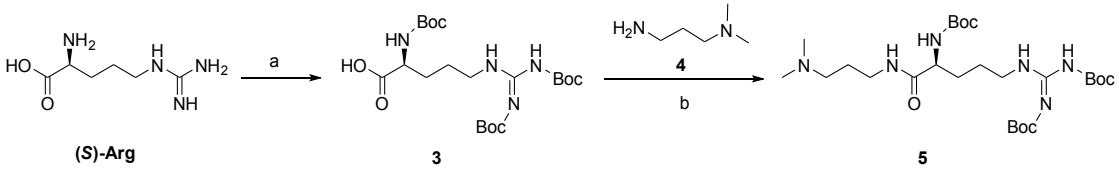
While it is generally accepted by medicinal chemists that it is much easier to design a molecule that stalls a biological process (a NAM or antagonist), it is much more difficult to design a molecule that accelerates a biological process (a PAM). Thus, it attracted our attention when Fontana et al. reported the structural elucidation of *N*-acylpolyamine **2** as the key constituent in fraction 10 of the spider venom, which they named Parawixin10. Following, Fontana and coworkers synthesized compound **2** and reported EAAT2 uptake enhancement in a radioligand uptake assay and data showing neuroprotective effects in rats.[8]

In this paper, we report the re-synthesis of *N*-acylpolyamine **2**, following the route published by Fontana et al.[8], and its comparative pharmacological characterization in radioligand uptake assays and neuronal cultures.

Chemistry

The synthesis of *N*-acylpolyamine **2** was carried out according to the convergent route reported by Fontana et al. (Scheme 1 and 2).[8] Tri-*N*-Boc protected (*S*)-Arg **3** was readily obtained from (*S*)-Arg, and coupled with *N*,*N*-dimethylpropane-1,3-diamine (**4**) by the use of the coupling reagent propanephosphonic acid anhydride (T3P) to give amide **5** (Scheme 1) ready for subsequent alkylation.

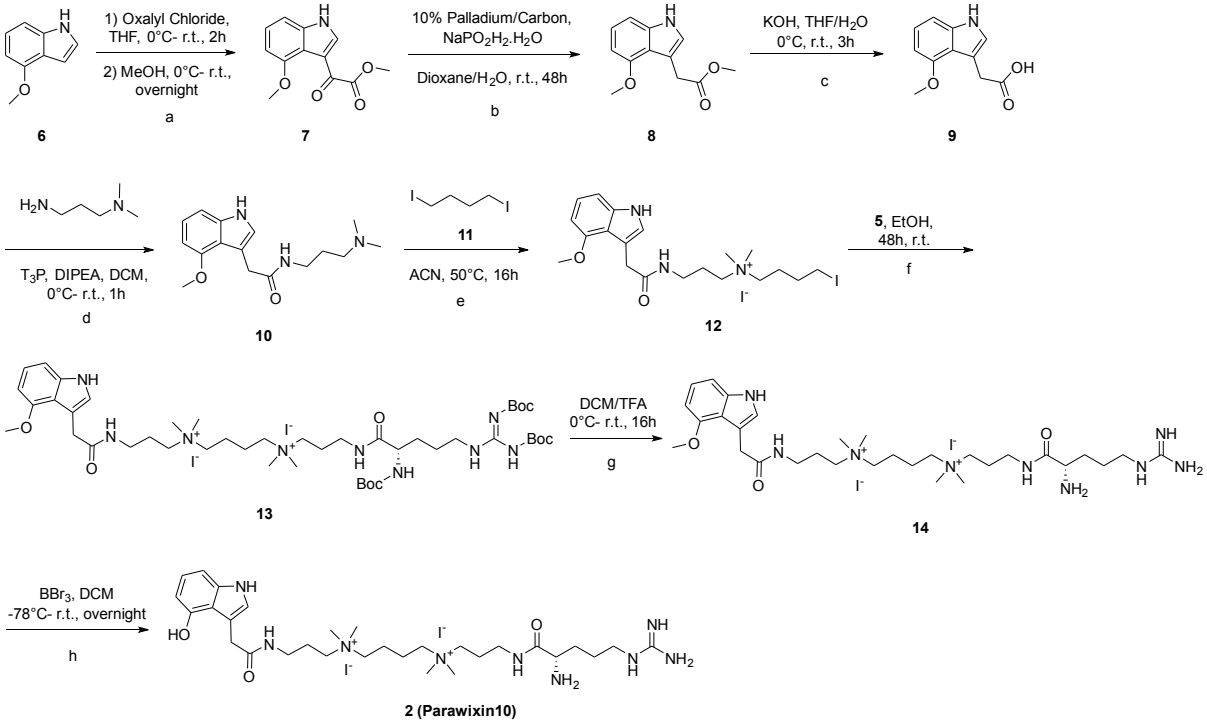
Scheme 1. Synthetic route for *N*, *N*-dimethylpropane tri-*N*-Boc-arginine amide **5**.



Reagents and reaction conditions: a) Di-*tert*-butyl dicarbonate, NaOH, THF/H_2O, 0°C, 48h, 52%. b) *N*,*N*-Dimethylpropane-1,3-diamine, propanephosphonic acid anhydride (T_3P), DIPEA, DCM, 0°C to rt, 1h, 73%.

The synthesis of compound **11** (Scheme 2) commenced with reacting commercially available 4-methoxy-1*H*-indole (**6**) with oxalyl chloride to give keto-ester **7**. Reduction over Pd/C hydrogenation provided the corresponding ester **8**, which underwent basic hydrolysis to give free carboxylic acid **9**. Amide coupling with *N*, *N*-dimethylpropane-1,3-diamine in the presence of T3P as coupling reagent afforded amide **10**. *N*-Alkylation with 1,4-diiodobutane (**11**)[9] then gave compound **12,** ready for alkylation with prepared arginine amide **5**. Polyamine product **13** was formed following deprotecting its *N*-Boc groups by use of trifluoroacetic acid (TFA) to give **14**, which was fully deprotected with BBr_3 in DCM to obtain the desired *N*-acylpolyamine **2**.

Scheme 2. Synthesis of *N*-acylpolyamine **2** (compound 12 in ref [8]).



Reagents and reaction conditions: a) Oxalyl chloride, THF, 0 °C to rt, 2h, 57%. b) 10% Pd/C, $NaPO_2H_2.H_2O$, dioxane/H_2O (2:1), rt, 48h, 37%. c) KOH, THF/H_2O (2:1), 0°C to rt, 3h, 92%. d) *N*,*N*-Dimethylpropane-1,3-diamine, T_3P, DIPEA, DCM, 0°C to rt, 1h, 84%. e) 1,4-Diiodobutane, ACN, 50 °C, 16h, 87%. f) **5**, EtOH, 48h, r.t., 63%. g) DCM/TFA (1:1), 0°C to rt, 16h, 62%. h) BBr_3, DCM, -78°C, 16h, 32%.

In summary, we could reproduce the yields reported for each step, and our synthesized compound **2** matched an authentic sample of Parawixin10, kindly provided by Fontana (NMR tube mixing proved identical compounds, see supporting information). On this basis, we were therefore fully confident in our synthetic work and proceeded to the pharmacological characterization of **2**.

Initially, the concentration-modulation relationships for **2** at human EAAT1, EAAT2, and EAAT3 were studied at three previously developed stable HEK293 cell lines expressing the transporters[10] in previously reported [^{3}H]-D-aspartate ([^{3}H]-D-Asp) and [^{3}H]-L-glutamate ([^{3}H]-L-Glu) uptake assays[10,11] (outlined as "*Setup I*" in Experimental Section). In both assays, no significant modulation of EAAT1-, EAAT2-, or EAAT3-mediated radiosubstrate uptake

was observed for **2** when tested in the concentration range 0.3 nM-10 μM (Figure 2). Moreover, **2** (10 μM concentration level) did not mediate significant modulation of the uptake through EAAT1, EAAT2, and EAAT3 measured at six different radiosubstrate concentrations in the two assays (Figure 3).

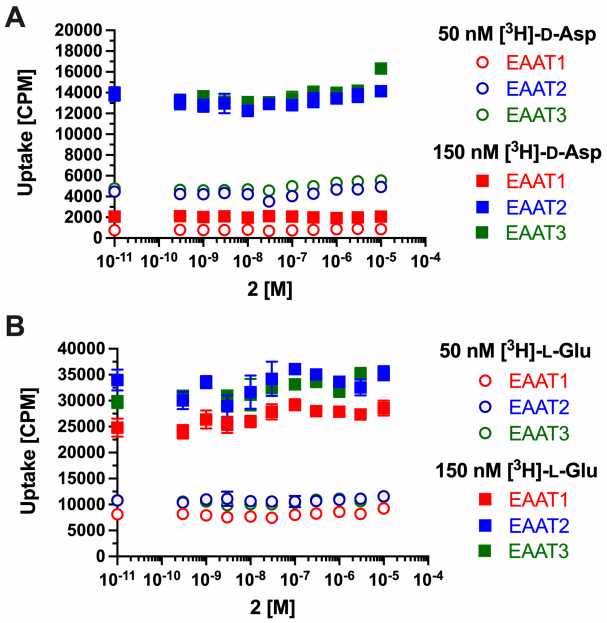


Figure 2. Pharmacological characterization of **2** at stable EAAT1-, EAAT2- and EAAT3-HEK293 cell lines in [^{3}H]-D-Asp (**A**) and [^{3}H]-L-Glu (**B**) uptake assays performed essentially as previously described[10,11] (outlined as "*Setup I*" in Experimental Section). The concentration-modulation relationship for **2** were determined using two different radiosubstrate concentrations in the assays. Data are from individual representative experiments performed in duplicate or triplicate (out of a total of three individual experiments) and are given as mean ± S.D. in CPM.

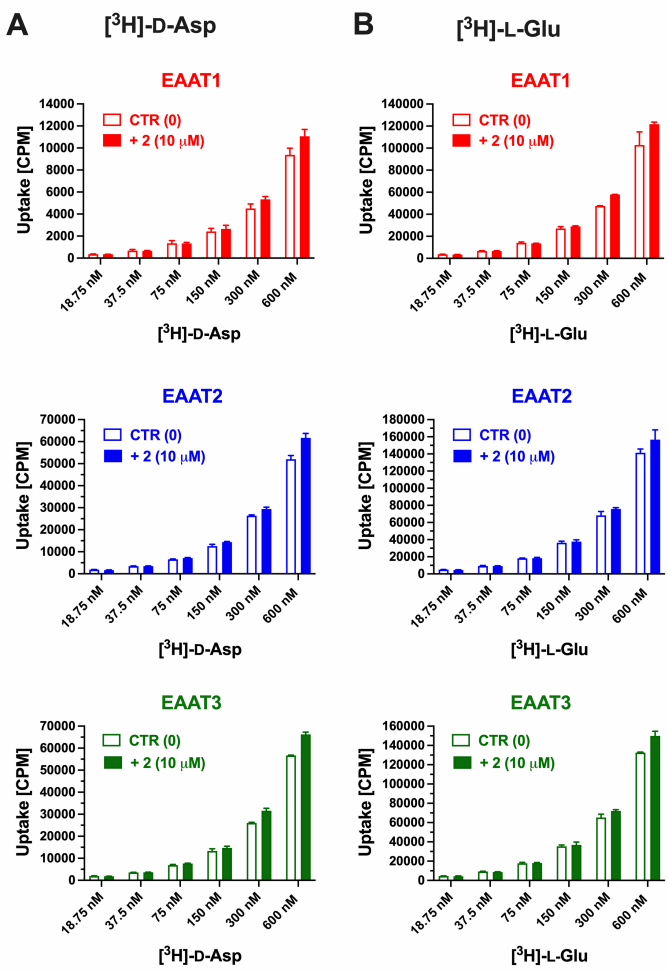


Figure 3. Pharmacological characterization of the modulation exerted by **2** (10 μM) at stable EAAT1-, EAAT2- and EAAT3-HEK293 cell lines in [^{3}H]-D-Asp (**A**) and [^{3}H]-L-Glu (**B**) uptake assays performed essentially as previously described[10,11] (outlined as "*Setup I*" in Experimental Section) using six different radiosubstrate concentrations. Data are from individual representative experiments performed in triplicate (out of a total of three individual experiments) and are given as mean ± S.D. in CPM. Data from the two other individual experiments are provided in SI (Figure S1).

The lack of apparent modulatory effect of **2** at the uptake through the three EAATs in these assays prompted us to study its putative effects at the transporters in an uptake assay more comparable to that employed by Forster et al.[8] (outlined as "*Setup II*" in Experimental Section). Also in this assay, **2** (1 nM-10 μM) did not mediate significant modulation of [^{3}H]-D-Asp or [^{3}H]-L-Glu uptake in stable EAAT1- and EAAT2-HEK293 cells (Figure 4). Finally, we investigated whether the lack of modulatory effects of **2** could be rooted in our

use of HEK293 cell lines (in contrast to the COS-7 cells used by Forster et al.[8]) by testing the modulatory activity of **2** on [^{3}H]-D-Asp uptake in COS-7 cells transiently transfected with EAAT2. **2** was found to be devoid of significant modulatory effect at the EAAT2-expressing COS-7 cells in this assay (Figure 5).

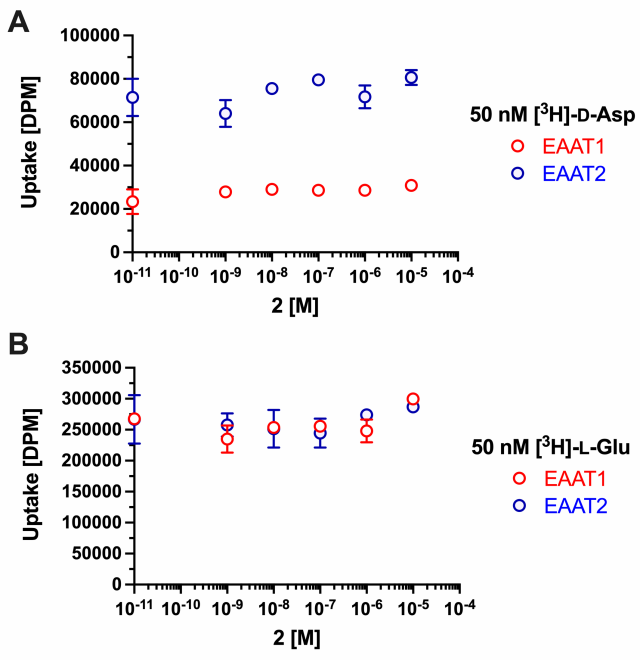


Figure 4. Pharmacological characterization of **2** at stable EAAT1- and EAAT2-HEK293 cell lines in [^{3}H]-D-Asp (**A**) and [^{3}H]-L-Glu (**B**) uptake assays comparable to that used by Forster et al.[8] (outlined as "*Setup II*" in Experimental Section). Data are from individual representative experiments performed in duplicate (out of a total of three individual experiments) and are given as mean ± S.D. in DPM.

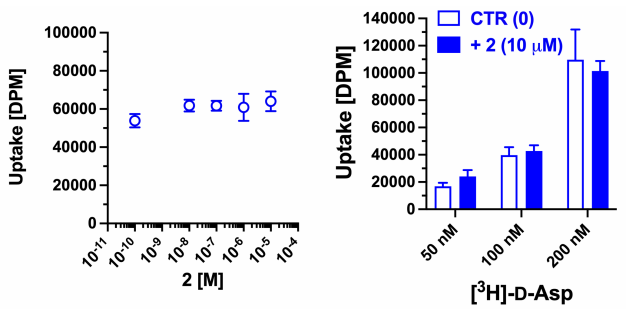


Figure 5. Pharmacological characterization of **2** at EAAT2 transiently expressed in COS-7 cells in the [^{3}H]-D-Asp uptake assay comparable to that used by Forster et al.[8] (outlined as "*Setup II*" in Experimental Section). The concentration-modulation relationship for **2** at EAAT2 using 100 nM [^{3}H]-D-Asp (**A**) and the modulation exerted by **2** (10 µM) at EAAT2 using three different [^{3}H]-D-Asp concentrations (**B**) are given. Data are from

individual representative experiments performed in duplicate (out of a total of three individual experiments) and are given as mean ± S.D. in DPM.

In summary, we did not observe **2**-mediated modulation of radiosubstrate ([^{3}H]-D-Asp and [^{3}H]-L-Glu) uptake in stable EAAT1-3-HEK293 cells in assay setup I[10,11] (Figs. 2 and 3). We also did not detect **2**-mediated modulation of [^{3}H]-D-Asp and [^{3}H]-L-Glu uptake in stable EAAT1-2-HEK293 cells (Fig. 4) or of [^{3}H]-D-Asp uptake in EAAT2-expressing COS-7 cells (Fig. 5) in assay setup II, which was more similar to the assay applied by Forster et al.[8]

Neuronal cultures

Parawixin10 (compound 2) has been reported to exert neuroprotective effects in mixed neuronal-glia cultures (rats) in a dose-dependent manner (1, 10, and 100 nM).[8] We investigated the potential neuroprotective effect of **2** (compound 12 in ref [8]) and analog **14** (compound 11 in ref [8]) in primary mixed neuron–glia cultures (mice) exposed to glutamate excitotoxicity (Figure 6). Under our experimental conditions, neither compound **2** nor analog **14** prevented glutamate-induced neuronal death in a clear dose-dependent manner, as measured by the Incucyte® Cytotox Red assay (Figure 6A, B). Glutamate exposure significantly increased cell death over 24 h, and treatment with either compound 2 or 14 did not significantly modify this response, as quantified by the area under the curve (AUC). Consistent with this, MAP2 immunostaining showed a marked reduction in neuronal area after glutamate insult, which was not rescued by **2** or analog **14** (Figure 6C, D). In summary, we see a clear species difference between mice (here) and previously published positive neuro-protective effects in rat neuro-glial cultures.

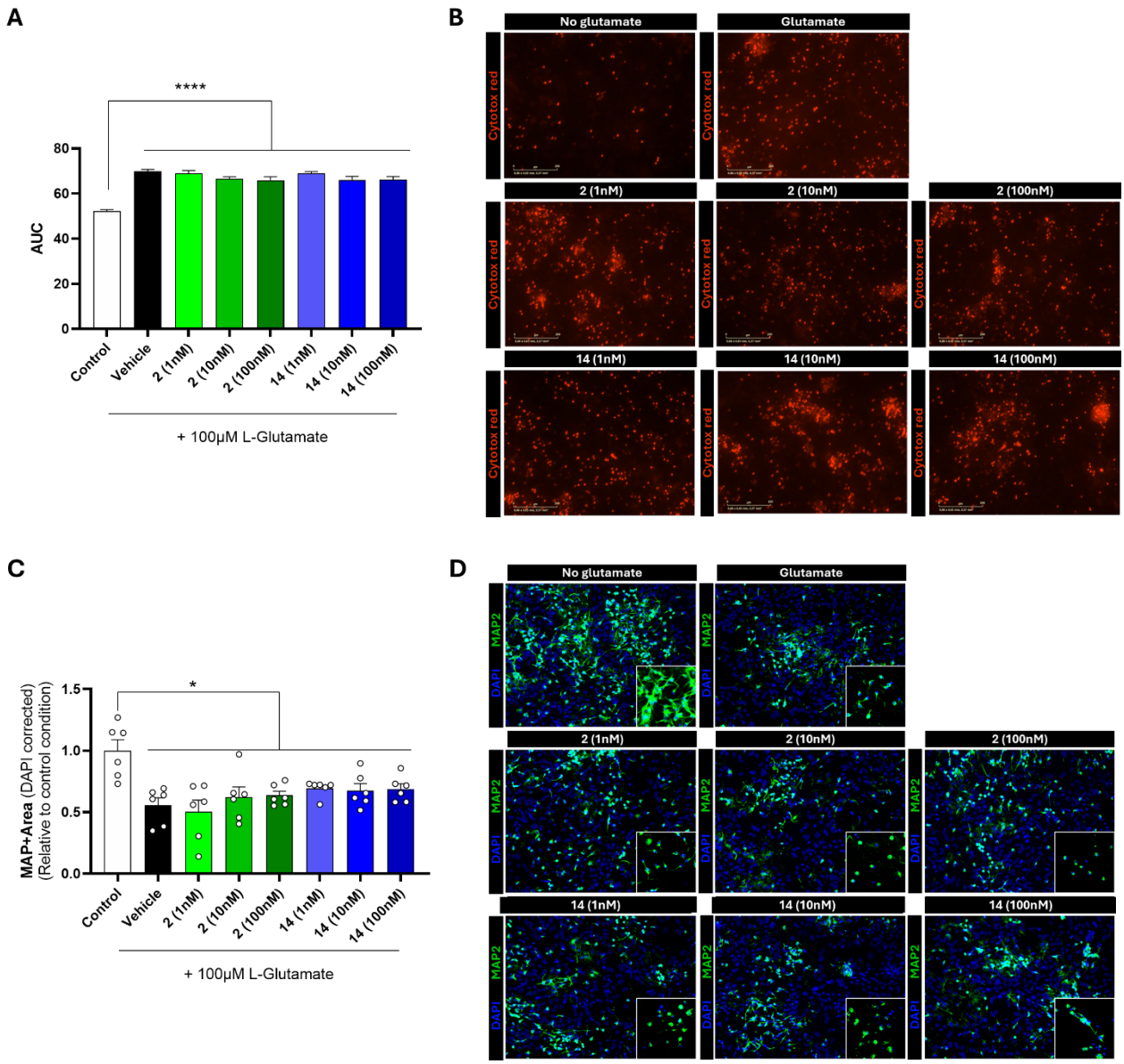


Figure 6. (A) Neither **2** (compound 12 in ref [8]) nor its analog **14** (compound 11 in ref [8]) protects primary mouse neurons from cell death induced by glutamate excitotoxicity. Cell viability was assessed using the Incucyte® Cytotox Red assay. Red signal intensity was monitored for 24 h following glutamate exposure, normalized to baseline (time 0), and quantified as area under the curve (AUC). Glutamate excitotoxicity significantly increased cell death, which was not rescued by **2** or **14** treatments. **(B)** Representative images are shown. **(C)** MAP2+ neuronal area was significantly reduced by glutamate excitotoxicity, and neither **2** nor **14** rescued this effect. (**D**) Representative MAP2 images are shown at 20 x magnification (40 x magnified insets shown), with three random fields analyzed per condition. Tukey's multiple comparisons test. Data are presented as mean ± SEM (n = 6). *p ≤ 0.05; ****p ≤ 0.001.

Conclusion

We have re-synthesized **2** (structure assigned as Parawixin10) and compared it with an authentic sample prepared and kindly provided by Fontana. In our following *in vitro* studies we were not able to detect significant **2**-mediated modulation of radiosubstrate (([^{3}H]-D-Asp and [^{3}H]-L-Glu)) transport through the human Glu transporters EAAT1-3 expressed in HEK293 or COS-7 cells in two uptake assay formats. Nor could we see a dose-dependent and statistical significance neuroprotective effect in a neuronal survival assay (mouse). These results add important data to the field of research of PAMs for the EAATs.

In 2020 the small molecule **GT949** was reported as a selective EAAT2 PAM exerting neuroprotective effects in rat.[12] However, later studies employing radioligand uptake and impedance-based assays did not show enhancement of EAAT-mediated glutamate transport by GT949.[13] The discrepancies found in this and the previous study[13] indicate that the reported EAAT PAM activities may depend on subtle methodological variables, potentially limiting the compounds' reliability as tool compounds for mechanistic investigations of EAAT2 function.

EXPERIMENTAL SECTION

Chemistry

All reactions involving sensitive reagents or anhydrous solvents were performed under an argon atmosphere and with flame-dried glassware or dried under vacuum prior to use. Commercially available chemicals were used without further purification. SG WATER solvent purification system (commercialized by Pure Process Technology) was used to dry organic solvents including DCM, THF and DMF. Other organic solvents were dried by standing over 4Å molecular sieves for a minimum of 48h. Analytical thin-layer

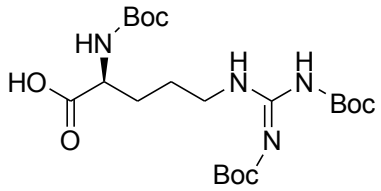
chromatography (TLC, Merck silica gel 60 F254 aluminum sheets) or HPLC were used to monitor the reactions mixture. Purification was performed using flash chromatography on Merck silica gel 60Å (40-63 μm).

^{1}HNMR spectra were acquired using a 400 MHz Bruker Avance II or 600 MHz Bruker Avance III HD. ^{13}CNMR spectra were recorded at 101 or 151 MHz on a Bruker Avance II and Bruker Avance III respectively. Chemical shifts (δ) relative to TMS are reported in ppm.

HPLC-MS spectra were recorded using an Agilent 1200 series solvent delivery system equipped with an autoinjector coupled to an Agilent 6400 series triple quadrupole mass spectrometer equipped with an electrospray ionization source. In addition to HPLC-MS, UPLC-MS was carried out on Waters Aquity UPLC-MS with dual UV detector with electrospray ionization. Gradients of 5% aqueous MeCN (+ 0.1% HCO_2H) as eluent A and 95% aqueous MeCN (+ 0.05% HCO_2H) as eluent B were used.

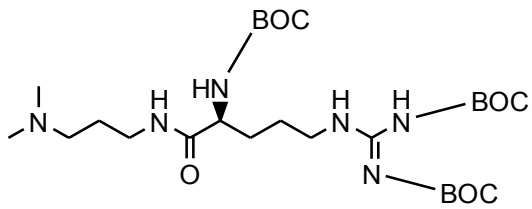
Analytical HPLC was equipped with a Dionex UltiMate 3000 pump and photodiode array detector at 210 and 254 nm respectively, using an XTerra MS C_{18} (3.5 μm, 4.6 mm × 150 mm) column. The method was adjusted on 1 mL/min flow rate using 5 to 95% MeCN gradient in H_2O containing 0.1% TFA. For HPLC analysis, data collection and control, Chromeleon software v.6.80 was employed. Preparative HPLC was carried out on an Agilent Prep HPLC systems with Agilent 1100 series pump, Agilent 1200 series diode array with multiple wavelength detector (G1365B), and Agilent PrepHT High Performance. It was equipped with Preparative Cartridge Column (Zorbax, 300 SB-C18 Prep HT, 21.2 × 250 mm, 7 μm).

Compounds were freeze dried over a Holm & Halby, Heto LyoPro 6000 freeze drier or dried under high vacuum.



(Z)-5- (2,3-Bis (*tert*-butoxycarbonyl) guanidino)-2-(*tert*-butoxycarbonylamino) pentanoic acid (3). (*S*)-Arginine (1eq., 3 mmol, 523 mg) was added into a solution of THF (10 mL) and water (10 mL) in a 100 mL round-bottom flask. The mixture was cooled to 0 °C in an ice bath and NaOH (4 eq., 12 mmol, 480 mg) was added. The solution was stirred for 5 min at 0 °C and di-*tert*-butyl dicarbonate (3.1 eq., 9.3 mmol, 2030 mg) in portions was added. The reaction mixture was stirred for 48h at room temperature and monitored by TLC. After the completion of the reaction, THF was removed under reduced pressure, and the aqueous layer was extracted with diethyl ether (2* 5 mL). The aqueous layer was acidified with HCl (1M) to pH 3-4 and then extracted with DCM (3* 10 mL). The organic phase was dried over anhydrous magnesium sulfate and the solvent was evaporated under reduced pressure to obtain the product as white solid (52%).

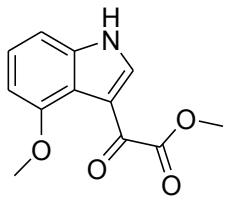
^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.81 (s,1H), 4.36 (s, 1H), 3.86 (t, *J*= 7.2 Hz, 2H), 1.84-1.68 (m, 5H), 1.53 (s, 9H),1.50 (s, 9H), 1.45 (s, 9H) ppm. MS: *m/z* 475.5 ([M + H]$^+$).



(*S*,*Z*)-*tert*-Butyl-2,17,17-trimethyl-7,15-dioxo-16-oxa-2,6,12,14-tetraazaoctadecan-8-yl-13-ylidenedicarbamate (5). *N*,*N*-Dimethylpropane-1,3-diamine (1.2 eq., 0.4 mmol, 40 mg,) and (*Z*)-5-(2,3-bis(*ter*t-butoxycarbonyl)guanidino)-2-(*tert*-butoxycarbonylamino)pentanoic acid (3) (1 eq., 0.32 mmol, 155 mg) were added to 6.2 mL of dry CH_2Cl_2 at 0°C with stirring. Then diisopropylethylamine (4 eq., 1.3 mmol, 232 µL) was added followed by the dropwise addition of 50% solution of propanephosphonic acid anhydride (T3P) in CH_2Cl_2 (1.3 eq., 0.42

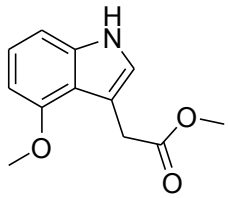
mmol, 270 mg). The reaction mixture was then stirred for 1 h at 0 °C. Completion of the reaction was confirmed by LC-MS, then the reaction mixture was quenched with water, and the product was extracted with CH_2Cl_2 and dried over anhydrous magnesium sulfate. The solvent was then evaporated under reduced pressure to obtain the crude product residue, which was purified by silica gel flash column chromatography to afford the desired compound as a white solid (132 mg, 73%).

^{1}H NMR (400 MHz, $CDCl_3$) δ= 9.32 (s, 1H), 7.45 (t, *J*= 5.8 Hz, 1H), 5.78 (d, *J*= 8.3 Hz, 1H), 4.22 (d, *J* = 8.3 Hz, 1H), 3.98−3.92 (m, 1H), 3.77−3.70 (m, 1H), 3.43−3.22 (m, 3H), 2.51−2.42 (m, 2H), 2.32 (s, 6H), 1.79−1.73 (m, 5H), 1.51 (s, 9H), 1.50 (s, 9H), 1.44 (s, 9H). MS: *m/z* 559.6 ($[M + H]^+$).



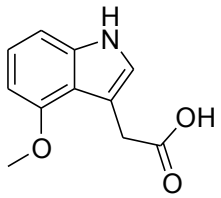
Methyl 2-(4-methoxy-1*H*-indol-3-yl)-2-oxoacetate (7). To a stirring solution of 4-methoxy-1*H*-indole (1eq., 3.8 mmol, 560 mg) dissolved in 11 mL of anhydrous THF at 0 °C was added oxalyl chloride (1.1 eq., 4.2 mmol, 359 µL) dropwise, and then the reaction mixture was allowed to stir for 2 h at room temperature. The reaction mixture was then cooled to 0 °C, and 5 mL of MeOH was added dropwise. Then the reaction mixture was stirred overnight at room temperature until the formation of the product was confirmed by TLC. The reaction mixture was diluted with diethyl ether, dried over magnesium sulfate, filtered, and evaporated under reduced pressure to afford the crude, which was further purified using flash column chromatography to afford the desired compound as an orange solid (505 mg, 57%).

^{1}H NMR (400 MHz, $CDCl_3$) δ= 9.02 (s, 1H), 8.06 (d, *J*= 3 Hz, 1H), 7.23 (t, *J*= 8 Hz, 1H), 7.06 (d, *J* = 8 Hz, 1H), 6.69 (d, *J*= 8 Hz, 1H), 3.93 (s, 3H), 3.92 (s, 3H) ppm.



Methyl 2-(4-methoxy-1*H*-indol-3-yl)acetate (8). To a stirring solution of methyl 2-(4-methoxy-1*H*-indol-3-yl)-2-oxoacetate (1.1eq., 1 mmol, 233 mg) in 6 mL of dioxane/ H_2O (2:1) were added 10% palladium on activated carbon (1 eq., 0.88 mmol, 94 mg) and sodium hypophosphite monohydrate (11.3 eq, 10 mmol, 1069 mg) simultaneously. Then the mixture was stirred for 48 h at room temperature. Completion of the reaction was monitored by TLC. Then the reaction mixture was diluted with 4 mL of H_2O and filtered through a celite pad. The aqueous mixture was then extracted with EtOAc and dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure to provide a crude residue which was purified by silica gel flash column (72 mg, 37%).

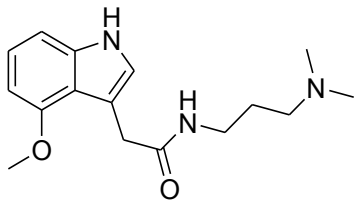
^{1}H NMR (400 MHz, $CDCl_3$) δ= 8.03 (s, 1H), 7.08 (t, *J*= 8 Hz, 1H), 6.98 (d, *J*= 2.3 Hz, 1H), 6.94 (d, *J*= 8 Hz, 1H), 6.47 (d, *J* = 8 Hz, 1H), 3.95 (s, 2H), 3.88 (s, 3H), 3.72 (s, 3H) ppm. MS: *m/z* 220.2 ($[M + H]^+$).



2-(4-Methoxy-1*H*-indol-3-yl)acetic acid (9). To a stirring solution of methyl 2-(4-methoxy-1*H*-indol-3-yl) acetate (1eq, 2.1 mmol, 471 mg) in 16.2 mL of THF/ H_2O (2:1) at 0 °C was added KOH (3 eq., 6.4 mmol, 362 mg) and the reaction mixture was allowed to warm to room temperature and stirred for 6 h. Completion of the reaction was monitored by TLC and LC-MS. Then THF was evaporated under reduced pressure, and the crude product was neutralized with HCl (1M aqueous solution) and extracted with EtOAc, dried over anhydrous

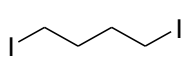
magnesium sulfate and the solvent was evaporated under reduced pressure to afford the desired product as a white salt (406 mg, 92%).

^{1}H NMR (400 MHz, *d6*-DMSO) δ= 11.92 (s, 1H), 10.81 (s, 1H), 7.03 (d, *J*= 2.3 Hz, 1H), 6.95 (t, *J*= 8 Hz, 1H), 6.93 (d, *J*= 8 Hz, 1H), 6.42 (dd, *J*= 8, 1.6 Hz, 1H), 3.78 (s, 3H), 3.73 (s, 3H) ppm. MS: *m/z* 206.2 ([M + H]$^+$).



***N*-(3-(Dimethylamino)propyl)-2-(4-methoxy-1*H*-indol-3-yl)acetamide (10).** To a stirring solution of 2-(4-methoxy-1*H*-indol-3-yl) acetic acid (1eq., 1.6 mmol, 328 mg) and *N*1,*N*1-dimethylpropane-1,3-diamine (2.8 eq., 4.5 mmol, 461 mg) in 11.5 mL of anhydrous CH_2Cl_2 at 0°C were added diisopropylethylamine (5eq., 8 mmol, 1.03 mL) and T3P 50% solution in EtOAc (1.3 eq., 2.1 mmol, 1357 mg) dropwise. Then the reaction mixture was stirred for 30 min at 0 °C. The completion of the reaction was monitored by TLC. After the reaction completion, the mixture was quenched with cold water, extracted with CH_2Cl_2, and then dried over anhydrous magnesium sulfate and the solvent was evaporated under reduced pressure to afford the crude product which was then purified by flash column chromatography to afford the desired compound (390 mg, 84%).

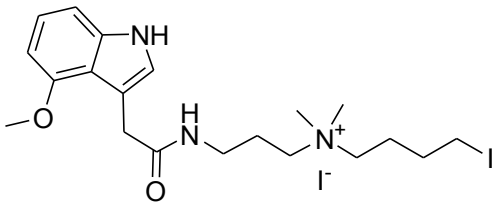
^{1}H NMR (400 MHz, $CDCl_3$) δ= 8.41 (s, 1H), 7.09 (t, *J*= 8 Hz, 1H), 7.00-6.98 (m, 2H), 6.75 (s, 1H), 6.50 (d, *J*= 8 Hz,1H), 3.91 (s, 3H), 3.80 (s, 2H), 3.25 (q, *J*= 6.5 Hz, 2H), 2.18 (t, *J*= 6.5 Hz, 2H), 1.93 (s, 6H), 1.55 (quintet, *J*= 6.5 Hz, 2H) ppm.



1,4-Diiodobutane (11). Tetrahydrofuran (1.4 eq., 100 mmol, 8.1 mL) is added to a mixture of potassium iodide (6.9 eq., 500 mmol, 83 g), 85% orthophosphoric acid (6.9 eq., 500 mmol,

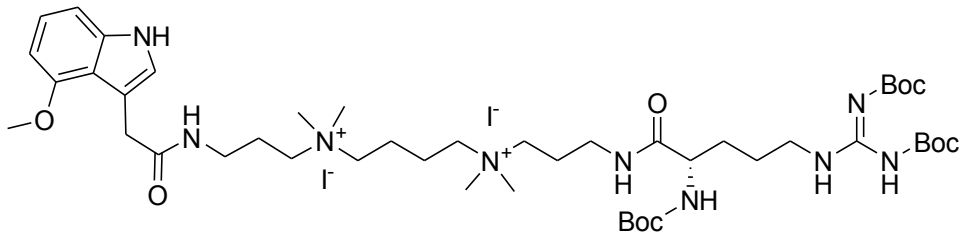
34.2 mL), and phosphorus pentoxide (1 eq., 72 mmol, 20.3 g.) in a three-necked flask equipped with a sealed mechanical stirrer, a reflux condenser, and a thermometer. The mixture is stirred and heated at its reflux temperature for 3 hours. The stirred mixture is cooled to room temperature, and 30 mL of water and 50 mL of diethyl ether are added. The organic layer is separated, decolourized with dilute aqueous sodium thiosulfate solution, washed with cold saturated sodium chloride solution, and dried over anhydrous magnesium sulfate. The solvent is removed by distillation on a steam bath, and the residue is distilled under reduced pressure from a modified Claisen flask. The portion boiling at 108–110°/10 mm. is collected to afford the desired white product (20g ,65%).

^{1}H NMR (400 MHz, $CDCl_3$) δ= 3.20 (quintet, *J*= 6 Hz, 4H), 1.95 (quintet, *J*= 6 Hz, 4H) ppm.



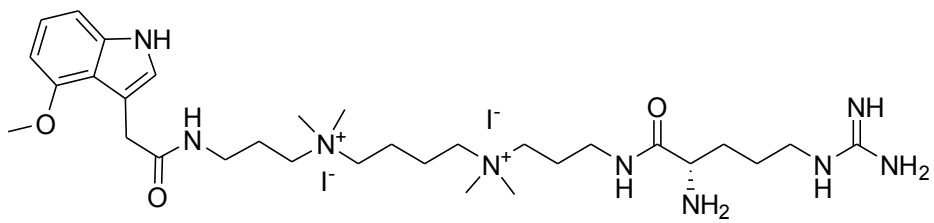
4-Iodo-*N*-(3-(2-(4-methoxy-1*H*-indol-3-yl)acetamido)propyl)-*N*,*N*-dimethylbutan-1-aminium (12). To a stirred solution of *N*-(3-(dimethylamino)propyl)-2-(4-methoxy-1*H*-indol-3-yl) acetamide (**10**) (1eq, 0.6 mmol, 286 mg) in butyronitrile (3 mL) at room temperature was added 1,4-diiodobutane (**11**) (10 eq, 6 mmol, 797 µL). The temperature of the reaction mixture was then raised to 50 °C and stirred for 16 h. Completion of the reaction was monitored by LC-MS. The reaction mixture was then diluted with 6 mL of diethyl ether, and the resulting precipitate was filtered and washed with diethyl ether to obtain the desired compound (249 mg, 87%).

^{1}H NMR (600 MHz, MeOD) δ= 7.12-7.03 (m, 3H), 6.57 (d, *J*= 8 Hz, 1H), 3.96 (s, 3H), 3.78 (s, 2H), 3.33 (quintet, *J*= 2 Hz, 2H), 3.22 (3, *J*= 6.5 Hz, 2H), 3.12-3.05 (m, 4H), 2.89 (s, 6H), 1.87 (septet, *J*= 6.5 Hz, 2H), 1.78 (quintet, *J*= 6.5 Hz, 2H), 1.60-1.55 (m, 2H) ppm.



(*S*,*Z*)-*N*^{1}-(6,11-Bis(*tert*-butoxycarbonylamino)-2,2-dimethyl-4,12-dioxo-3-oxa-5,7,13-triazahexadec-5-en-16-yl)-*N*^{4}-(3-(2-(4-methoxy-1*H*-indol-3-yl)acetamido)propyl)-*N*^{1},*N*^{1},*N*^{4},*N*^{4}-tetramethylbutane-1,4-diaminium (13). To a stirred solution of 4-iodo-*N*-(3-(2-(4-methoxy-1*H*-indol-3-yl)acetamido)propyl)-*N*,*N*-dimethylbutan-1-aminium iodide (**12**) (1 eq., 0.33 mmol, 200 mg) in 2 mL of ethanol was added (*S*,*Z*)-*tert*-butyl-2,17,17-trimethyl-7,15-dioxo-16-oxa-2,6,12,14-tetraazaoctadecan-8-yl-13-ylidenedicarbamate (1 eq., 0.33 mmol, 187 mg) at room temperature, and the reaction temperature was raised to 45 °C and the reaction stirred for 48 h. Completion of the reaction was monitored by LC-MS. Solvent were removed under reduced pressure to obtain crude product that was purified by reversed phase HPLC to afford the title compound (240 mg, 63%).

^{1}H NMR (600 MHz, MeOD) δ= 7.11−7.03 (m, 3H), 6.56 (d, *J* = 7.0 Hz, 1H), 3.97 (m, 1H), 3.95 (s, 3H), 3.78 (s, 2H), 3.40−3.23 (m, 8H), 3.29−3.11 (m, 14H), 3.02 (s, 6H), 2.93 (s, 6H), 1.97−1.87 (m, 5H), 1.77−1.62 (m, 9H), 1.54 (s, 12H), 1.45 (s, 14H) ppm. MS: *m/z* 452 ([M]$^{2+}$).

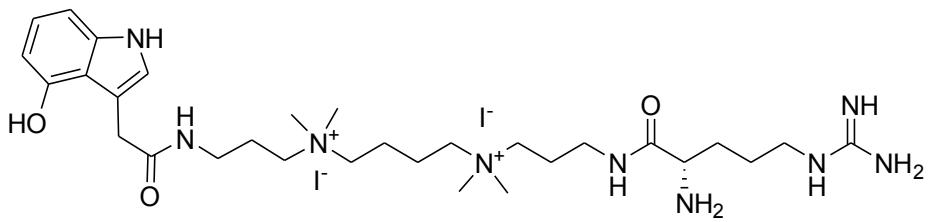


(*S*)-*N*^{1}-(3-(2-Amino-5-guanidinopentanamido)propyl)-*N*^{4}-(3-(2-(4-methoxy-1*H*-indol-3-yl)acetamido)propyl)-*N*^{1},*N*^{1},*N*^{4},*N*^{4}-tetramethylbutane-1,4-diaminium (14)

To a stirring solution of (*S*,*Z*)-*N*^{1}-(6,11-bis(*tert*-butoxycarbonylamino)-2,2-dimethyl-4,12-dioxo-3-oxa-5,7,13-triazahexadec-5-en-16-yl)-*N*^{4}-(3-(2-(4-methoxy-1*H*-indol-3-

yl)acetamido)propyl)-N^1,N^1,N^4,N^4-tetramethylbutane-1,4-diaminium (**13**) (1 eq, 0.052 mmol, 60 mg) in 2 mL dry CH_2Cl_2 at 0°C, 2 mL of trifluoroacetic acid was added dropwise, and then the reaction mixture was allowed to stir at room temperature overnight. Completion of the reaction was monitored by UPLC-MS. The organic solvents were evaporated under vacuum to obtain crude product that was purified by reversed phase HPLC to obtain the desired product (27 mg, 0.03 mmol, 62%).

^{1}H NMR (400 MHz, MeOD) δ= 7.10−7.02 (m, 3H), 6.57 (d, *J* = 7.0 Hz, 1H), 3.94 (s, 3H), 3.91 (t, *J* = 6.5 Hz, 1H), 3.77 (s, 2H), 3.47 (t, *J*= 6.5 Hz, 2H), 3.24- 3.10 (m, 14H), 3.04 (s, 6H), 2.92 (s, 6H), 1.97−1.83 (m, 6H), 1.72−1.63 (m, 4H), 1.51-1.43 (m, 2H) ppm. MS: *m/z* 301.9 ($[M]^{2+}$).



(*S*)-N^1-(3-(2-Amino-5-guanidinopentanamido)propyl)-N^4-(3-(2-(4-hydroxy-1*H*-indol-3-yl)acetamido)propyl)-N^1,N^1,N^4,N^4-tetramethylbutane-1,4-diaminium Iodide (2):

To the solution of (*S*)-N^1-(3-(2-amino-5-guanidinopentanamido)propyl)-N^4-(3-(2-(4-methoxy-1*H*-indol-3-yl)acetamido)propyl)-N^1,N^1,N^4,N^4-tetramethylbutane-1,4-diaminium (**14**) (1 eq, 0.024 mmol, 21 mg) in 2 mL of anhydrous CH_2Cl_2 at −78 °C, 0.2 mL of BBr_3 was added dropwise. The reaction mixture was gradually heated up to room temperature over 1−2 h and stirred overnight. Completion of the reaction was monitored by UPLC-MS. The organic solvents were evaporated under vacuum to obtain crude product. At -78 °C, 1.5 mL of MeOH was added dropwise to the mixture and was slowly heated to room temperature. The crude product was purified by reversed phase HPLC to obtain the desired compound (6.5 mg, 0.008 mmol, 32%).

^{1}H NMR (400 MHz, MeOD) δ= 7.09 (s, 1H), 6.99−6.93 (m, 2H), 6.45 (dd, *J* = 7.0, 1.5 Hz, 1H), 3.94 (s, 3H), 3.94-3.88 (m, 2H), 3.75 (s, 2H), 3.49-3.45 (m, 2H), 3.26-3.22 (m, 7H), 3.12-0.06 (m, 8H), 3.03 (s, 6H), 2.87 (s, 6H), 1.99−1.83 (m, 8H), 1.73−1.56 (m, 7H), 1.46-1.36 (m, 3H) ppm. MS: *m/z* 294.7 ($[M]^{2+}$).

In vitro Pharmacology

Materials: All chemicals for buffers were purchased from Sigma-Aldrich (St. Louis, MO), and culture medium, serum, antibiotics and trypsin for cell culture were obtained from Invitrogen (Paisley, UK). [^{3}H]-D-Asp, [^{3}H]-L-Glu and the Opti-Fluor and Microscint^{20} scintillation fluids were obtained from PerkinElmer (Boston, MA), and DL-TBOA was purchased from Tocris Cookson (Bristol, UK). The generation of the stable EAAT1-, EAAT2- and EAAT3-HEK293 cells has been described previously.[10]

[^{3}H]-D-Asp and [^{3}H]-L-Glu uptake assays

The pharmacological characterization of **2** at EAATs in [^{3}H]-D-Asp and [^{3}H]-L-Glu uptake assays were performed in two different assay setups: Setups I and II:

Setup I (HEK293 cells). The [^{3}H]-D-Asp and [^{3}H]-L-Glu uptake assays were performed essentially as described previously.[10,14] The day before the assay, stable EAAT1-, EAAT2- and EAAT3-HEK293 cells were split into poly-D-lysine-coated white 96-well plates (PerkinElmer, Boston, MA) in Dulbecco's Modified Eagle Medium Glutamax™-I supplemented with 5% dialyzed fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 mg/mL G-418. After 16−24 h, the culture medium was removed, and cells were washed twice with 100 µL assay buffer (Hank's Buffered Saline Solution supplemented with 20 mM HEPES, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4). In a preincubation step, 100 µL assay buffer with or without various concentration of **2** were then added to the cells, and the plate was incubated at 37 °C for 10 min after which the solution

was removed. Then 70 µL assay buffer supplemented with radiosubstrate ([^{3}H]-D-Asp or [^{3}H]-L-Glu) and various concentrations of **2** or reference ligands were added to the wells, and the plate was incubated at 37 °C for 4 min. Nonspecific [^{3}H]-D-Asp or [^{3}H]-L-Glu uptake/binding in the cells was determined in the presence of 300 µM DL-TBOA. The assay mixtures were quickly removed from the wells, and the cells were washed with 2 x 100 µL ice-cold assay buffer, after which 150 µL Microscint^{20} scintillation fluid was added to each well. Then the plate was shaken for at least 1 h and counted in a TopCounter (PerkinElmer, Boston, MA). The background levels of counts measured in wells where no radiosubstrate had been added to the cells were negligible (in the 30-50 CPM range) compared to the levels of counts (in CPM) measured in wells where the uptake assay was performed (see Figs. 2 and 3 for examples of these counts). The actual radiosubstrate concentrations used for the experiments were determined by scintillation counting in a TriCarb scintillation counter (PerkinElmer, Boston, MA), and these never differed more than 20% from the targeted concentrations. The experiments were performed in duplicate or triplicate a total of three times for each transporter, and data analysis was performed in GraphPad Prism 10.0 (GraphPad Software).

Setup II (HEK293 and COS-7 cells). The [^{3}H]-D-Asp and [^{3}H]-L-Glu uptake assays were performed in a setup comparable to that used by Forster et al.[8] This assay was performed in 24-well plates using both stable EAAT1- and EAAT2-HEK293 cells and COS-7 cells transiently expressing EAAT2.[10,14] The three cell lines were cultured in Dulbecco's Modified Eagle Medium Glutamax™-I supplemented with 5% dialyzed fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (for the EAAT1- and EAAT2-HEK293 cells the medium was also supplemented with 1 mg/mL G-418). The COS-7 cells were transiently transfected with EAAT2-pCDNA3.1 cDNA by use of Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) in a 10-cm tissue culture dish. The following day, the cells were

split into poly-D-lysine-coated 24-well plates, and they were assayed the day after (i.e. two days after the transfection). The stable EAAT1- and EAAT2-HEK293 cells were split into poly-D-lysine-coated 24-well plates and assayed the following day.

On the day of the experiment, the culture medium was removed, and cells were washed twice with 400 µL assay buffer (Hank's Buffered Saline Solution supplemented with 20 mM HEPES, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4). In a preincubation step, 200 µL assay buffer with or without various concentration of **2** were added to the cells, and the plate was incubated at 37 °C for 10 min after which the solution was removed. Then 200 µL of assay buffer supplemented with radiosubstrate ([^{3}H]-D-Asp or [^{3}H]-L-Glu) and various concentrations of **2** or reference ligands were added to the wells, and the plate was incubated at 37 °C for 7 min. Nonspecific [^{3}H]-D-Asp or [^{3}H]-L-Glu uptake/binding in the cells was determined in the presence of 300 µM DL-TBOA. The assay mixtures were quickly removed from the cells, which then were washed with 2 x 400 µL ice-cold assay buffer and then lysed by addition of 300 µL 1% SDS/0.1 M NaOH solution and incubation for 20 min while shaking. The lysates were transferred to scintillation vials containing 4 mL Opti-Fluor scintillation fluid, and radioactivity was counted in a TriCarb scintillation counter. The background levels of counts measured in vials with cell lysate from wells where no radiosubstrate had been added were negligible (in the 50-150 DPM range) compared to the levels of counts (in DPM) measured in wells with cell lysate from wells where the uptake assay was performed (see Figs. 4 and 5 for examples of these counts). The actual radiosubstrate concentrations used for the experiments were also determined by scintillation counting, and these never differed more than 20% from the targeted concentrations. The experiments were performed in duplicate a total of three times for each transporter/cell line, and data analysis was performed in GraphPad Prism 10.0 (GraphPad Software).

Neuronal cultures

Primary cortical mixed cultures were prepared from embryonic day 18 (E18) C57BL/6J mouse embryos. Cortices were dissected in ice-cold HBSS, mechanically dissociated, and plated onto poly-L-lysine–coated coverslips (24-well plates, 20,000 cells/coverslip). Cultures were maintained without the addition of AraC to allow astrocyte growth. Cells were plated in Neurobasal medium (NB; Thermo Fisher) supplemented with 2% B27 (Sigma-Aldrich), 1% GlutaMAX (Thermo Fisher), and 1 % penicillin–streptomycin (Life technologies). Two hours after plating, media was replaced with NB containing 2% B27, 2% horse serum (Thermo Fisher), 1% GlutaMAX, 25 µM L-glutamate (Sigma Aldrich), and 1% penicillin–streptomycin. At 4 days *in vitro* (DIV), media was changed to NB with 2% B27, 1% GlutaMAX, and 1% penicillin–streptomycin, and replenished again at 8 DIV. Experiments were performed at 14 DIV, when cultures display well-differentiated neurons and stable glial populations.

At 14 DIV, cultures were exposed to 100 µM L-glutamate for 24 h to induce excitotoxicity. Compound **2** (parawixin10, PW in ref) or analog **14** (compound C11 in ref) were co-applied to the glutamate at 1, 10 and 100 nM. Vehicle-treated cultures were included as controls. Cytotox Red reagent (Sartorius) was added simultaneously with glutamate and compound treatments. Red fluorescence, indicating dead cells, was monitored in real time for 24 h using an Incucyte® live-cell imaging system. Signal intensity was normalized to baseline (time 0) and quantified as area under the curve (AUC).

After 24h of glutamate insult, cultures were fixed with 4% paraformaldehyde for 15 min, washed with PBS, and blocked with 10% protein block solution in PBS-T (DAKO). MAP2 primary antibody incubation was done overnight (1:1000, ab32454, ABCAM) and incubated for 1h in the dark with Alexa 488-conjugated secondary antibody (1:600, Invitrogen). Nuclei were counterstained with DAPI (Invitrogen). Images were acquired at 20× and 40× magnification using the Leica DM2000 LED microscope. For each condition, three random fields per coverslip were analyzed. Cytotox Red and MAP2 signals were quantified using

Incucyte analysis software and ImageJ, respectively. Data are expressed as mean ± SEM. Statistical significance was determined using Tukey's multiple comparisons using GraphPad Prism 10.0 (GraphPad Software).

Supporting information available. ^{1}H-NMR spectra of compounds **2,3,5,7-14** and ^{13}C NMR spectra of compounds **2** and **14**.

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Abbreviations

Excitatory Amino Acid Transporters (EAAT)

Propanephosphonic acid anhydride (T3P)

Counts per minute (CPM)

Disintegrations per minute (DPM)

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The *N*-Acylpolyamine Structure Assigned as Parawixin10 does not display Positive Allosteric Modulatory Activity in EAAT1-3 Radioligand Uptake Assays, nor does it Enhance Neuronal Survival in Mice in a Dose-Dependent and Statistically Significant Way

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