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Title page

Enhancing glutamate transport: mechanism of action of Parawixin1, a neuroprotective compound from *Parawixia bistriata* spider venom

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Running title page

a) Running title: Characterization of a glutamate transport enhancer

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d) List of abbreviations: CNS, Central Nervous System; EAAT1-3, human excitatory amino acid transporter subtypes 1-3; ED_{50}, Dose of drug that produces effect under investigation in 50% of the population; EDTA, ethylenediaminetetraacetic acid, GLAST, glutamate and aspartate transporter; GLT-1, rat glutamate transporter 1; EAAT2, human glutamate transporter 2, homologous to GLT-1; HPLC, High Performance Liquid Chromatograph; $[K^+]i$, internal potassium; $[K^+]e$, external potassium; $[Na^+]e$, external sodium; NaSCN, Sodium thiocyanate; OD, Optical Density; PMSF, phenylmethylsulphonyl fluoride; TBOA, DL-*threo-beta*-benzyloxyaspartate; U, unit of toxin based on $\lambda=215_{nm,}$ 1 O.D. being 1000 U.

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Abstract

Previous studies have shown that a compound purified from the spider *Parawixia bistriata* venom stimulates the activity of glial glutamate transporters and can protect retinal tissue from ischemic damage. To understand the mechanism by which this compound enhances transport, we examined its effects on the functional properties of glutamate transporters after solubilization and reconstitution in liposomes and in transfected COS-7 cells. Here we demonstrate in both systems that Parawixin1 promotes a direct and selective enhancement of glutamate influx by the EAAT2 transporter subtype through a mechanism that does not alter the apparent affinities for the co-substrates, glutamate or sodium. In liposomes we observed maximal enhancement by Parawixin1 when extracellular sodium and intracellular potassium concentrations are within physiological ranges. Moreover, the compound does not enhance the reverse transport of glutamate under ionic conditions that favor efflux, when extracellular potassium is elevated and the sodium gradient is reduced. Nor does it alter the exchange of glutamate in the absence of internal potassium. These observations suggest that Parawixin1 facilitates the reorientation of the potassium-bound transporter, the rate-limiting step in the transport cycle, a conclusion further supported by experiments showing that Parawixin1 does not stimulate uptake by an EAAT2 transport mutant (E405D) defective in the potassium-dependent reorientation step. Thus, Parawixin1 enhances transport through a novel mechanism targeting a step in the transport cycle distinct from substrate influx or efflux, and provides a basis for the design of new drugs that act allosterically on transporters to increase glutamate clearance.

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Introduction

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system (CNS). Excitatory amino acid transporters (EAATs) limit the actions of extracellular glutamate and are crucial to maintain low extracellular concentrations of glutamate and prevent neurotoxicity (for review see Danbolt, 2001). Extracellular concentrations of glutamate are regulated by a family of five subtypes of sodium dependent transporters: GLAST or EAAT1 (Storck *et al.,* 1992) in astroglia, GLT-1 or EAAT2 (Pines *et al.,* 1992) predominantly in astrocytes (Danbolt *et al*.; 1992; Lehre *et al*., 1995) and at low levels in synaptic terminals (Furness *et al.,* 2007), EAAC1 or EAAT3 in neurons (Kanai and Hediger, 1992), EAAT4 in cerebellar Purkinje cells (Fairman *et al.,* 1995), and EAAT5 in the retina (Arriza *et al.,* 1997).

Gene deletion, immunoprecipitation and pharmacological studies have revealed that the GLT-1/EAAT2 subtype may account for more than 90% of glutamate uptake in forebrain (reviewed in Danbolt, 2001). Because GLT-1/EAAT2 is the predominant CNS glutamate transporter subtype, substances that enhance its activity would be powerful tools for increasing the clearance of glutamate in pathological conditions. Increased extracellular levels of glutamate have been implicated in pathological conditions like cerebral ischemia (Oechmichen and Meissner, 2006), amyotrophic lateral sclerosis (Rothstein *et al.,* 1992), epilepsy (Coutinho-Netto *et al.,* 1981), schizophrenia (Carlsson *et al*., 1999) and others.

There have been several reports on substances that appear to acutely stimulate glutamate uptake, by indirect modulation of the glutamate transporter activity. MS-153 (R)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline) reduces KCl-stimulated

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efflux from hippocampal slices by a mechanism proposed to involve GLT-1 (Shimada *et al*., 1999). The anticonvulsant agent riluzole enhances glutamate uptake in rat spinal cord synaptosomes, perhaps by acting on pertussis toxin-sensitive G proteins (Azbill *et al*., 2000). Nicergoline has been proposed to increases glutamate uptake in rat cortical synaptosomes by modulating a transporter-associated protein (Nishida *et al*., 2004).

Several substances also have been shown to increase GLT-1 expression. In a molecular library screen Rothstein *et al.* (2005) found many beta-lactam antibiotics to be potent stimulators of GLT-1 expression and that one of them, ceftriaxone, increased both gene expression and activity of GLT-1, preventing neurotoxicity in models of ischemic injury and motor neuron degeneration. GPI-1046, a synthetic, nonimmunosuppressive derivative of FK506-binding protein immunophilin exert neuroprotective and neuroregenerative actions in several systems by inducing selective expression of GLT-1 *in vitro* and *in vivo* (Ganel *et al*., 2006). Guanosine also appears to enhance glutamate uptake in cultured astrocytes and slices by increasing transporter expression (Frizzo *et al*., 2001 and 2005). Finally, expression of constitutively active Akt (protein kinase B) induces the expression of GLT-1 through increased transcription (Li *et al.,* 2006).

Additional data demonstrate that glutamate uptake can be modulated by direct action on the transporter proteins by cis-polyunsaturated fatty acids (Trotti *et al.,* 1995) and by reduction and oxidation (Trotti *et al.,* 1996). Despite these observations, and numerous reports on compounds that modulate glutamate uptake,

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there are as yet no compounds that have been unequivocally shown to enhance glutamate uptake activity by a direct action on the transporter proteins themselves.

Because L-glutamate is the primary neurotransmitter at the insect neuromuscular junction and spiders paralyze their prey by interfering with glutamate neurotransmission (see reviews by Beleboni *et al.,* 2004 and Estrada *et al*., 2007) we previously tested the venom of a spider, *Parawixia bistriata* for its potential effects on glutamate receptors, transporters, and other modulators of glutamate transmission. In these studies we isolated a compound from this spider venom (Parawixin1, previously referred to as PbTx1.2.3) that could enhance glutamate uptake into cortical synaptosomes (Fontana *et al*., 2003).

In the present study, we show, using recombinant transporters expressed in COS-7 cells and native transporters reconstituted in liposomes, that Parawixin1 enhances glutamate uptake by a selective and direct action on the EAAT2 glutamate transporter protein. We demonstrate that the compound accelerates the activity of the glutamate transporter at a step in the transport cycle that is not directly associated with glutamate influx, efflux or exchange. The reorientation of the carrier to the outward facing state is linked to counter transport of internal K^+, which completes the transport cycle. Glutamate uptake by a mutant carrier defective in this potassium coupling is not enhanced in the presence of Parawixin1. Thus, we propose that Parawixi1 acts to facilitate the major rate-limiting step in the transport cycle, a potassium-dependent step associated with the reorientation of the carrier to the extracellular surface.

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Materials and Methods

Reagents

L-[^{3}H]-glutamate (22.5 Ci/mmol), was obtained from New England Nuclear (Boston, MA, USA). Cell culture media, DMEM, FCS, horse serum, penicillin/streptomycin, glutamine and Dulbecco´s PBS (D-PBS) were from Invitrogen Life Technologies (Carlsbad, CA, USA). Fugene6 transfection reagent was from Roche (Indianapolis, IN, USA). Unlabelled L-glutamate, Sephadex G-25 fine, cholic acid (purified with activated charcoal and by recrystallization from 70% ethanol) and reagents for buffers were from Sigma-RBI (St. Louis, MO, USA). BCA™ Assay kit was from Pierce (Rockford, IL, USA). All other reagents were of analytical grade.

Spider Collection and purification of active fraction

The collection of spiders and purification procedures for obtaining Parawixin1 were done as previously described (Fontana *et al*., 2003). For measurement of Parawixin1, a standard Optical Density (OD) of 1.0 at λ_{215nm} (path length of the cuvette was 10 mm) was defined as 1,000 units (U). For reference, a single gland in 1.0 ml of water has a concentration of approximately 1,500 U.

Cell transfection and transport assays in COS-7 cells

COS-7 cells were maintained in DMEM with 10% fetal calf serum at 5% CO_2. Each EAAT subtype was expressed independently with the appropriate expression plasmid (pCMV-EAAT1, pCMV-EAAT2 or pCMV-EAAT3, as described in Arriza *et al*., 1994) and E405D mutant that prevents the translocation by K^+ (described in

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Kavanaugh *et al.,* 1997). Transfection with empty vector pCMV-5 was used to control for the level of endogenous uptake of radiolabeled L-glutamate in each experimental condition. Subconfluent COS-7 cells were plated at a density of ~100,000 cells per well in 24-well tissue culture plates and transfected with 0.2 μg of plasmid DNA per well using Fugene6 according to the manufacturers protocol. Two days post-transfection the cells were washed with 0.5 ml of room temperature D-PBS (2.7 mM KCl; 1.2 mM KH_2PO_4, 138 mM NaCl; 8.1 mM Na_2HPO_4, added 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4).

The residue E404 in GLT-1 (Kavanaugh *et al.,* 1997) corresponds to E405 in EAAT2. The E405D (pCMVEAAT2-E405D) mutant was generated from the wild type pCMV5-EAAT2 vector (Arriza *et al*., 1994), using the QuickChange® site directed mutagenesis kit (Stratagene, La Jolla, CA) to produce a GAA to GAV codon conversion using the following oligo pair :

Sense: GGTACAGCCCTTTATGACGCGGTGGCCGCCATCTT

Anti-sense: AAGATGGCGGCCACCGCGTCATAAAGGGCTGTACC

(Substituted bases underlined). Positive mutants were verified by partial DNA sequencing (approximately 300 bp including the region of the codon substitution) and function initially verified (> 40% of control wild type) in HEK293 cells by L-[^{3}H]-glutamate transport assays as previously described (Arriza *et al*., 1994) before being used in the subsequent studies.

To determine the specificity of action of Parawixin1 for different glutamate transporter species, COS-7 cells were transfected with EAAT1, EAAT2, EAAT3 or the mutant E405D and incubated with Parawixin1 (0.1 U/ml) or buffer alone at room

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temperature for 10 min and uptake was carried out for 10 min in the presence of 100 nM of L-[^{3}H]-glutamate.

For dose response experiments, increasing concentrations of Parawixin1 (from 0.01 to 10 U/ml) were added to the cells expressing EAAT2 in 0.5 ml of D-PBS and pre-incubated at room temperature for 10 min. Uptake was carried out for 10 min in the presence of 100 nM L-[^{3}H]-glutamate.

For kinetic analysis of glutamate uptake, EAAT2 (or empty vector) expressing COS-7 cells were pre-incubated in the absence or presence of 0.1 U/ml Parawixin1 for 10 min. Assays were initiated by the addition of unlabeled L-glutamate and L-[^{3}H]-glutamate (1 to 1000 μM, final concentration, 90% unlabeled and 10% labeled compound) to cells, and uptake was carried out for 10 min. Background from cells expressing empty vector was subtracted.

Sodium dependence of the stimulation of glutamate uptake by Parawixin1 was investigated as follows: cells transfected with EAAT2 or empty vector were pre-incubated in buffer containing 15, 60 or 140 mM sodium (with choline chloride to 140 mM added as needed to maintain constant osmolarity), in the presence or absence of 0.1 U/ml Parawixin1. Glutamate transport assays were performed as described above.

To terminate the uptake assays, cells were washed three times with ice cold D-PBS or the appropriate buffer and solubilized in 0.5 ml of 0.1% SDS prior to scintillation counting in 5 ml of ScintiVerse (Fisher Scientific, Pittsburgh, PA). Radioactivity collected was counted with Packard 1900 TR Liquid scintillation analyzer.

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Glutamate transport in liposomes

Liposomes were prepared as described previously (Danbolt *et al.*, 1990, Trotti *et al*., 1995). Sephadex G-25 fine was swollen in buffer overnight and packed in plastic syringes (1 ml) from which the pistons have been removed and the outlets closed by cotton fiber. The columns were then centrifuged (140 x g; 2 min; Sorvall RT 6000D) to remove the void volume.

Rat brain homogenates were prepared by homogenizing and solubilizing whole brains (including cerebellum) with cholate [1.2% final concentration (w/v) plus 5 mM EDTA and 1 mM PMSF to inhibit proteases] followed by centrifugation (10000x g for 20 min). The supernatant (containing 0.1-0.2 mg protein/ml, measured using BCA kit, Pierce, USA) was mixed with 1.5 volumes of a phospholipid, cholate and salt mixture, incubated on ice for 15 min. This mixture was applied to the G25 column (0.2 ml per syringe) and columns were centrifuged (2 min, 500 x g).

Liposomes form spontaneously during this gel filtration and the column equilibration buffer becomes the internal medium of the liposomes. To change the external buffer, liposomes were gel filtrated once more in columns equilibrated with the desired buffer, which became the external medium of the liposomes.

Glutamate transport experiments with liposomes were done essentially as described (Danbolt *et al*., 1990; Trotti *et al.*, 1995).

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Time dependence and reversibility of the actions of Parawixin1

For a time course experiment liposomes were prepared with internal potassium buffer (140 mM KCl, 15 mM potassium phosphate and 1% glycerol) and external sodium buffer (140 mM NaCl, 15 mM sodium phosphate, 1% glycerol). 1 U/ml Parawixin1 was added to the preparation together with 50 nM L-[^{3}H]-glutamate in uptake buffer (similar to external sodium buffer) and samples were collected at different times (from 1 to 10 min), and the reactions terminated.

To determine the reversibility of the action of Parawixin1, liposomes with internal potassium buffer (140 mM KCl, 15 mM potassium phosphate and 1% glycerol) and external sodium buffer (140 mM NaCl, 15 mM sodium phosphate, 1% glycerol) were pre-incubated for 2 min with 25 U/ml Parawixin1 or buffer alone. Half of the samples were then gel filtrated to remove the external media and uptake (see above) was carried out for up to 70 seconds, with a final concentration of Parawixin1 of 1 U/ml.

ED_{50} determination for Parawixin1 on glutamate uptake in liposomes

To determine the ED_{50} for the action of Parawixin1, liposomes were prepared with internal medium of 140 mM KCl, 15 mM KPi and 1% glycerol, and external medium of 140 mM NaCl, 15 mM KPi and 1% glycerol. Liposomes were pre-incubated with varying concentrations (0.001 to 10 U/ml) of Parawixin1 or buffer alone for 2 min at room temperature. Liposomes (20 µl) were diluted into 500 µl of phosphate buffer (140 mM NaCl, 15 mM sodium phosphate buffer (NaH_2PO_4), 1%

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(v/v) glycerol, pH 7.4) and 50 nM of L-[^{3}H]-glutamate. Uptake reactions were carried out for 1 min for dose-response experiments.

Liposome are less useful for saturation kinetic experiments as they are small [most of them are between 0.1 and 0.3 μm in diameter (Drs. C. Granum and N.C. Danbolt, unpublished data)], and cannot replenish their potassium content, thus rapidly depleting their ion gradients and making it difficult to obtain values that reflect the initial rates of transport. For these reasons, our measurements of the effects of Parawixin1 on saturation kinetics were examined only in transfected COS-7 cells.

Experiments examining the ionic requirements for Parawixin1 action

To determine if the enhancement of transport by Parawixin1 requires external sodium during the pre-incubation step (normal transport conditions) or whether it can still occur when potassium is substituted for external sodium, liposomes were prepared with internal 140 mM KCl, 15 mM potassium phosphate and 1% glycerol and external 140 mM NaCl, 15 mM sodium phosphate, 1% (v/v) glycerol, pH 7.4) or with 140 mM KCl, 15 mM potassium phosphate and 1% glycerol on both sides of the membrane. Both liposome sets were pre-incubated for 2 min with 25 U/ml Parawixin1 or buffer alone. Reactions were initiated by the addition of 25 volumes of 50 nM L-[^{3}H]-glutamate in sodium phosphate buffer, resulting in a final concentration of Parawixin1 of 1 U/ml in the uptake buffer. Reactions were terminated after 1 min.

To investigate the role of internal potassium or external sodium in the stimulation of glutamate uptake by Parawixin1, liposomes were prepared with different concentrations of internal potassium (from 105 to 215 mM). Several ions

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were tested as osmotic substitutes for sodium in the external buffer (gluconic acid, N-methyl-D-glucamine, cesium chloride and rubidium chloride) but all had additional effects that precluded their utility as substitutes. Thus, to maintain osmotic balance, external sodium and internal potassium concentrations were varied in parallel. Liposomes were pre-incubated with Parawixin1 or buffer alone and reactions performed as above.

Glutamate efflux in liposomes

Efflux experiments were done according to Volterra *et al.* (1996). Liposomes were prepared with internal potassium and actively loaded by incubation with 0.2 µM L-[^{3}H]-glutamate in Sodium thiocyanate (NaSCN) buffer (100 mM NaSCN, 50 mM Na-HEPES, pH 7.5). Permeant anions such as SCN^- result in higher uptake rates than anions that are known to be relatively impermeant, as phosphate (Sips *et al.,* 1982). After 3 min of pre-loading efflux was started: liposomes were gel filtrated on columns containing NaSCN buffer. Buffer alone or glutamate (1 µM) were added and reactions stopped at different times. We determined that reaction times of 10 seconds were within the linear portion of the time course determined experimentally. Actively loaded liposomes were incubated for 10 seconds in NaSCN buffer alone or buffer plus glutamate (1 µM) in the presence of Parawixin1 (final concentration 10 U/ml) or Parawixin1 alone, and the remaining radioactivity was determined.

Glutamate exchange and uptake plus exchange in liposomes

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Finally, the effect of Parawixin1 was tested on pure exchange of glutamate (with sodium only) and under conditions that permit exchange plus net uptake of glutamate experiments (with sodium and potassium).

For the pure exchange experiments, liposomes were prepared in sodium buffer (140 mM NaCl, 10 mM sodium phosphate buffer, 1% (v/v) glycerol, pH 7.4) with 1 or 10 mM unlabeled L-glutamate or glycine as the internal medium. Glycine was used as ion substitute in these experiments because it did not alter the action of Parawixin1, as opposed to gluconic acid that inhibit the stimulation by Parawixin1 (data not shown).

External L-glutamate was removed by gel-filtration of the liposomes in sodium buffer (140 mM NaCl, 1% glycerol plus the inert ion glycine at the same concentrations for ion compensation). In this system potassium is completely absent. Liposomes were pre-incubated for 2 min with 25 U/ml Parawixin1, uptake reactions were initiated by the addition of 50 nM L-[^{3}H]-glutamate, the final concentration of Parawixin1 being 1 U/ml, and reactions were terminated after varying times from 5 to 180 seconds.

For exchange plus net uptake experiments, liposomes were prepared in potassium buffer (130 mM KCl, 10 mM NaCl, 1% glycerol) with 1 or 10 mM unlabeled L-glutamate or glycine as the internal medium. External L-glutamate was removed by gel-filtration of the liposomes in sodium buffer (140 mM NaCl, 1% glycerol plus the inert ion glycine at the same concentrations for ion compensation). Liposomes were pre-incubated for 2 min with Parawixin1 as above. Uptake reactions were performed as above for pure exchange experiments.

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All liposome reactions were finished by dilution in 2 ml of ice-cold phosphate buffer and filtration through Millipore HAWP filters (0.45 µm pores). The filters were rinsed 3 times, dissolved in ScintiVerse scintillation fluid and counted.

Data analysis

ED_{50s} are given as mean ± SEM of at least three independent experiments done on different days. Apparent affinity constant (K_M) and V_{max} were determined by measuring uptake at 8 substrate concentrations and compared for statistical significance between treatments using t-test (*P <0.05, **P<0.01, ***P<0.001). Bar graphs, XY graphs and time-courses represent average ± SEM of independent experiments performed in triplicate on multiply days, analyzed using t-test (*P <0.05, **P<0.01, ***P<0.001) or ANOVA ($^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001) as appropriate.

Data were analyzed with GraphPad Prism version 4.

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Results

Parawixin1 selectively enhances L-glutamate uptake by EAAT2

We previously established that Parawixin1 enhanced the maximal uptake of glutamate without changing the K_M in a rat cortical synaptosome preparation in which the EAAT2/GLT-1 glutamate transporter is the most abundant subtype. To find out if the compound acts on all glutamate transporter isoforms we examined the effects of Parawixin1 on cloned carriers expressed in COS-7 cells. Figure 1A shows the effect of Parawixin1 on glutamate uptake mediated by the EAAT1, EAAT2 and EAAT3 glutamate transporter subtypes. Parawixin1 (0.1 U/ml) significantly (**P<0.01) increased glutamate uptake through EAAT2, but it had no effect on the other isoforms even with concentrations as high as 100 U/ml (data not shown). DHK (200 μM) strongly inhibited EAAT2, but had little effect on EAAT1 or EAAT3. The K_i for the effect of DHK on EAAT2 in this system is 23 ± 6 μM, with no effects on the activity of EAAT1 and EAAT3 observed at concentrations of DHK less than 1 mM, consistent with the K_is between 24 and 79 μM previously reported for DHK in cells expressing EAAT2 (Arriza *et al*., 1994).

Parawixin1 causes a dose-dependent increase in transport in EAAT2-expressing cells without altering the apparent K_Ms for substrate or sodium

In assays in EAAT2-transfected COS-7 cells using a fixed concentration of L-[^{3}H]-glutamate (100 nM) and varying concentrations of Parawixin1, glutamate transport was progressively enhanced by increasing concentrations of the compound which had an ED_{50} of 0.3 ± 0.28 U/ml (data not shown). The results of experiments

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examining the effects of Parawixin1 on the saturation kinetics of glutamate uptake are presented in Figure 1B. Although the K_M values determined under control conditions and in the presence of Parawixin1 were similar (91.7 ± 10 μM and 97 ± 10 μM, respectively, means were not significantly different using t-test), the V_{max} value was increased by the compound (from 631.8 ± 18.4 pmol/well/min to 821 ± 25 pmol/well/min, Student *t-test* ****P*<0.001), thus demonstrating that Parawixin1 causes an increase in transport velocity (23%) without shifting the apparent affinity for substrate.

Because the transport process is driven by and coupled to ion gradients (Zerangue and Kavanaugh, 1996; Amara and Fontana, 2002) we examined the influence of sodium concentration on the stimulation of glutamate uptake by Parawixin1 (Figure 1C). We observe that 0.1 U/ml Parawixin1 was able to enhance glutamate uptake in the presence of all concentrations of external sodium used (15, 60 or 140 mM). The Parawixin1-induced enhancement is not mediated through a change in the sodium dependence of the transport process, as EC_{50}s for sodium were not significantly different in presence or absence of the compound. Data from two independent experiments performed in triplicate generated values of 35.20 ± 18 mM for the EC_{50} and 385.2 ± 66.3 pmol/well/min for the V_{max} under control conditions and 50.9 ± 27 mM for the EC_{50} and 813.8 ± 166 pmol/well/min for V_{max} in the presence of Parawixin1.

Dose response curve for the Parawixin1 stimulation of L-[^{3}H]-glutamate uptake in liposomes

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To examine the effects of Parawixin1 on different steps in the transport cycle we prepared proteoliposomes, a preparation that enables the manipulation of substrate and ion concentrations on both sides of the membrane. Glutamate transport activity is primarily from EAAT2, as this is the predominant transporter in this preparation. The dose response relationship for the effects of Parawixin1 on transport of L-[^{3}H]-glutamate (50 nM) into liposomes under standard ionic conditions (140 mM external NaCl, 140 mM internal KCl) is shown in Figure 2A. Parawixin1 produced a dose-dependent stimulation of transport, with an ED_{50} of 1.3 $\pm$ 0.6 U/ml. This ED_{50} value is slightly higher than we obtained in the COS-7 cells (0.3 ± 0.28 U/ml), but likely reflects the many differences between transport measurements in intact transfected cells and in the artificial liposome preparation.

Previous work has indicated that the uptake measured in the liposome preparation reflects the total transport activities of the various isoforms but more than 90 % of the transport activity is due to EAAT2 when the preparation is derived from adult forebrain (reviewed in Danbolt, 2001). This is supported by our observation that 100 μM DHK, a dose that selectively blocks the activity of the GLT-1/EAAT2, but not the other isoforms (Arriza *et al*., 1994), completely abolishes both basal and Parawixin1-stimulated transport of glutamate in our liposome preparations (data not shown). Thus, these results are consistent with those from our studies using cloned carriers that demonstrate that Parawixin1 acts selectively on GLT-1/EAAT2.

The effect of Parawixin1 is saturable and reversible

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A time-course of the effect of 1 U/ml Parawixin1 on glutamate uptake in liposomes with internal potassium buffer (140 mM KCl, 15 mM potassium phosphate and 1% glycerol) and external sodium buffer (140 mM NaCl, 15 mM sodium phosphate, 1% glycerol) showed that the stimulating effect appears within 1 min, but disappears after 10 min (data not shown). The internal volume of the liposomes has been established to be on the order of 20 µl/ml of liposome suspension (Trotti *et al.,* 1995), so at this substrate concentration it is reasonable to expect the ion gradient would be dissipated after 10 min in the presence of Parawixin1. We decided to pre-incubate liposomes for 2 min with Parawixin1 in all the following experiments.

To examine the reversibility of the Parawixin1 action, liposomes prepared as above were pre-incubated with Parawixin1 or buffer alone for 2 min following which half of each sample was gel filtrated again to remove the external media. Uptake was carried out as described in the Methods section. The actions of Parawixin1 are completely reversible within the time required to remove the external medium, and must be present in the medium to maintain its enhancing effect.

We conclude that the stimulating effect of Parawixin1 is fast and reversible. This agrees with the observation that Parawixin1 is hydrophilic according to the HPLC profile (Fontana *et al.,* 2003) and does not seem to dissolve into the membrane.

Effects of ionic conditions on the ability of Parawixin1 to stimulate glutamate uptake

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Next we examined the ionic conditions in the pre-incubation buffer required for the modulation of transport by Parawixin1. Figure 2B shows liposomes prepared with potassium inside and outside, or with sodium outside and potassium inside and pre-incubated for 2 minutes with 25 U/ml Parawixin1. To initiate assays of transport activity, the liposomes are then diluted 25-fold in sodium-containing transport buffer with 50 nM L-[^{3}H]-glutamate. In this experiment we observe enhancement of transport only when the external pre-incubation medium contains sodium and not under conditions where sodium is replaced by potassium.

Figure 2C shows the result of an experiment examining the effect of Parawixin1 on liposomes prepared with different concentrations of internal potassium and external sodium. In this experiment in which the concentrations of the two ions were varied in parallel to maintain osmotic balance, we found that 1 U/ml Parawixin1 can stimulate glutamate uptake only within a physiological concentration range between 120-145 mM at internal potassium and external sodium.

The enhancement of uptake is not caused by alteration of substrate efflux

Liposomes with 140 mM internal potassium were pre-loaded with substrate (0.2 μM L-[^{3}H]-glutamate in NaSCN buffer (100 mM NaSCN, 50 mM Na-HEPES, pH 7.5). Figure 3 shows the efflux of glutamate in the presence of 1 μM unlabeled L-glutamate, plus or minus 10 U/ml Parawixin1, or 10 U/ml Parawixin1 alone. We observe that 1 μM unlabeled L-glutamate elicits efflux of labeled substrate. This efflux was not significantly different when compared with 1 μM L-glutamate in the presence

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of Parawixin1. In addition, Parawixin1 did not produce efflux of glutamate by itself. We conclude that Parawixin1 does not alter efflux of glutamate in pre-loaded liposomes.

Parwixin1 does not alter the exchange rate of L-glutamate

Figure 4 shows the accumulation of externally-added L-[^{3}H]-glutamate over a time course of 3 min by liposomes containing either 1 (Figure 4A) or 10 mM (Figure 4B) internal glutamate (in sodium phosphate buffer, no potassium present). Under these conditions the presence of substrate inside facilitates the exchange of radiolabel across the membrane by a process known as trans-stimulation (Borghetti *et al*., 1981). In this experiment, in which the potassium-dependent reorientation of the carrier cannot occur, we observe no differences between the amount of substrate exchanged in the presence or absence of 1 U/ml Parawixin1. Glycine loaded liposomes (at the same concentrations of glutamate used) were prepared as controls, and as expected, show no accumulation of glutamate.

Parawixin1 needs the full cycle of transport to affect glutamate uptake

Figure 5 shows the accumulation of external L-[^{3}H]-glutamate over a period of 3 min by liposomes containing either 1 (Figure 5B) or 10 mM (Figure 5C) internal glutamate. The internal buffer contained potassium and sodium and the external buffer contained sodium, allowing the liposomes to accumulate glutamate by both exchanger and net-uptake modes. Under these conditions Parawixin1 stimulated accumulation of radio labeled glutamate into liposomes containing either 1 or 10 mM glutamate. Liposomes containing 1 or 10 mM glycine were prepared as controls, and

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showed only modest accumulation of radio labeled external glutamate when compared with the liposomes containing glutamate. These results stand in contrast to Parawixin1's lack of effect when it was added under exchange conditions as shown in Figure 4 and indicate that the action of Parawixin1 requires the full cycle of transport (potassium dependent, see figure 5A).

Parawixin1 cannot enhance glutamate uptake in a transporter mutant defective in potassium coupling

To further explore the possibility that Parawixin1 enhances glutamate transport by facilitating the potassium-dependent reorientation steps in the transport cycle, we examined its actions on a mutated EAAT2 carrier shown previously to predominantly operate in a homoexchange mode with a dramatically reduced rate of potassium dependent reorientation (Kavanaugh *et al.,* 1997; Grewer and Rauen, 2005). Figure 6 shows uptake of glutamate by wild type EAAT2 transporter and mutant E405D. Parawixin1 increased glutamate uptake by the wild type transporter (Students *t*-test, $^{**}P < 0.01$, see also Figure 1) but it failed to increase glutamate uptake by the E405D mutant. Under control conditions, uptake of radiolabeled glutamate by wild type transporter and E405D were comparable, as previously noted for this mutant (Kavanaugh *et al*., 1997). Because this mutant is locked in an obligatory exchange mode (Kavanaugh *et al*., 1997), this result indicates that enhancement by Parawixin1 occurs only when carriers have an intact transport cycle and can transition through the steps required for return of unoccupied carrier to the outward facing configuration.

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Discussion

Although compounds from spider and wasp venoms that *inhibit* neurotransmitter transport have been identified (for examples see Pizzo *et al.*, 2004; Beleboni *et al*., 2006; Lovelace *et al.,* 2006), Parawixin1 is the first agent that appears to act directly on a glutamate transporter to *increase* uptake (Fontana *et al.,* 2003). To establish the transporter subtype targeted by Parawixin1 and its mechanism of action, we evaluated its effects on glutamate uptake in COS-7 cells transfected with each of the three major CNS EAAT subtypes, as well as on native carriers reconstituted into proteoliposomes. This latter assay measures transport activity in the absence of more complex and potentially confounding cellular processes (for example, the pathways involved in signal transduction, membrane potential regulation, and trafficking). When brain tissue is used as the source of transporter proteins for liposomes reconstitution, the activity measured primarily reflects the activity of EAAT2, in agreement with its abundance relative to the EAAT1 and EAAT3 subtypes (for reviews see Danbolt, 2001; Grewer and Rauen, 2005). In addition, this system allows manipulation of both external and internal concentrations of ions and substrates.

Addition of Parawixin1 results in a saturable and dose-dependent stimulation of L-glutamate transport by EAAT2 in transfected COS-7 cells. This selectivity of Parawixin1 for EAAT2 (Figure 1A) agrees with previous work demonstrating enhanced glutamate uptake in synaptosomes rat cortex (Fontana *et al*., 2003) where the majority of transporters are GLT-1/EAAT2. This enhancement of uptake by Parawixin1 involves an increase in the $V_{max.}$ of transport with no change in apparent substrate affinity (Figure 1B). These effects on the kinetic parameters for substrate

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transport are consistent with our previous observations that Parawixin1 increases the rate of glutamate uptake in synaptosomes (Fontana *et al*., 2003). Moreover, we show here that Parawixin1 does not alter the apparent affinity for the co-substrate sodium (Figure 1C), a finding that led us consider the possible significance of potassium-dependent steps of the cycle.

To resolve which steps in the transport cycle might be critical for the effects of the Parawixin1, we undertook a series of experiments in liposomes. Pre-incubations of Parawixin1 with liposomes increased the uptake of glutamate in a dose dependent manner (Figure 2A). In experiments to define the optimal pre-incubation conditions, Parawixin1 could enhance transport only when sodium was present in the external pre-incubation medium, but not when potassium served as the external monovalent cation (Figure 2B). These findings support the idea that the compound may not bind effectively to the cytoplasmic-facing carrier, a state favored when external sodium is replaced by potassium. We also found that the effect of Parawixin1 is fast and reversible (data not shown), consistent with the observation that Parawixin1 is hydrophilic (Fontana *et al*., 2003) and may not bind to the transporter within the hydrophobic membrane environment.

To further examine the ionic requirements for the action of Parawixin1, but still maintain osmotic balance, we performed experiments in which external sodium and internal potassium were varied in parallel (as several ions tested for their ability to serve as inert ionic substitutes for internal potassium had additional effects that precluded their utility). Figure 2C shows that the enhancement of uptake occurs at

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concentrations within the physiological range of external sodium and internal potassium concentrations (120-145 mM internal potassium and external sodium).

To rule out the possibility that the enhancement of glutamate uptake was caused indirectly by the inhibition of glutamate efflux, we tested the effect of Parawixin1 under conditions that favor reverse transport. In liposomes pre-loaded with radiolabeled glutamate we did not observe differences in the rate of efflux when unlabeled glutamate was added in the presence or absence of Parawixin1. Moreover, Parawixin1 alone did not alter efflux of glutamate from liposomes (Figure 3) or from pre-loaded COS-7 cells (data not shown).

Next, we investigated the effect of Paraxiwin1 under conditions that favor the exchange of glutamate in the presence of sodium. Glutamate influx is coupled to the inward movement of 3 sodium ions and a proton, and to the counter-transport of a potassium ion, which facilitates the reorientation of the empty carrier. Thus, the full cycle is driven by the electrochemical gradients across the cell membrane. (Kanner and Sharon, 1978). The transport cycle is reversible at all stages, a property that can lead to incomplete transport cycles and exchange. When substrate is present on both sides of the membrane, substrate molecules can undergo exchange between internal and external compartments in a process that is sodium-dependent. In the absence of potassium, the carrier cannot catalyze net uptake, but can exchange substrates on a 1:1 basis between external and internal media (Danbolt, 2001). As illustrated in Figure 4, Parawixin1 had no effect on the accumulation of L-[^{3}H]-glutamate by liposomes containing either 1 or 10 mM internal glutamate under conditions where the transporter is operating in exchange mode. These findings indicate that Parawixin1

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does not increase the rate of the exchange reaction and suggest that it may act on a step distinct from the sodium-coupled steps in the cycle.

In a population of cycling transporters exposed to substrate on both side of the membrane, exchange and net uptake can occur simultaneously. Thus, when liposomes containing both substrate and potassium are added to a medium containing both sodium and substrate, net uptake takes place, but some of the internal substrate appears on the outside, likely the result of exchange caused by incomplete transport cycles (Volterra *et al*., 1996). Accumulation of glutamate by liposomes under conditions that allow both exchange and net uptake is shown in Figure 5. Parawixin1 stimulated the accumulation of labeled glutamate in liposomes containing either 1 or 10 mM glutamate.

The glutamate transport cycle consists of two hemicycles. On the translocation side sodium ions are coupled to the inward movement of glutamate by sequential binding of sodium ions and glutamate with each sodium ion binding in distinct steps to both the glutamate-free and glutamate-bound conformation of the carrier. In the second hemicycle transport of a potassium ion is coupled to the reorientation of the unoccupied transporter to the outward facing state (Grewer and Rauen, 2005). A kinetic model for GLT-1 was developed to simulate the behavior of components of the transporter current and to estimate the capture efficiency of GLT-1 (Bergles *et al.,* 2002). The K^+ counter-transport step was proposed to be rate-limiting based in part on work from several groups showing that the substitution of internal K^+ with Cs^+ or Na^+ resulted in a significant slowing of the transporter cycling. We show a simplified version of this model in Figure 5A.

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Because Parawixin1 can enhance glutamate uptake at all concentrations of sodium examined (Figure 1C), and had no effect in assays measuring efflux or exchange, a compelling explanation for these observations is that Parawixin1 acts to accelerate the potassium-dependent translocation step T_iK. To more closely link the action of Parawixin1 to the potassium-dependent reorientation steps of the glutamate cycle, wild type EAAT2, and an EAAT2 single-substitution mutant E405D mutant, which has been shown to operate predominantly in a homoexchange mode, were tested for their sensitivity to Parawixin1. Figure 6 shows that although Parawixin1 significantly enhanced transport by the wild-type EAAT2, it did not affect transport by the E405D transporter. This suggests that the compound will only stimulate transport when the potassium-dependent steps in the transport cycle are intact and carriers can progress through the full forward transport cycle. Because it does not compete directly with the primary substrate, glutamate, we speculate that Parawixin1 likely acts allosterically to change the dynamics of the transport cycle by facilitating potassium-dependent conformational changes that allow the unoccupied glutamate binding site to return to reorient to an externally facing configuration.

Regulation of extracellular glutamate levels in the brain is critical for normal brain function and is implicated in numerous neurological diseases. Tools like Parawixin1 may serve as a basis for designing therapeutic drugs that increase glutamate clearance in pathological conditions where increased concentrations of glutamate lead to excitotoxicity, such as during stroke and ischemia (Oechmichen and Meissner, 2006). A compound such as Parawixin1, which has the ability to enhance glutamate transport and does not enhance reverse transport, could be an

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effective means for limiting excitatory signaling and neurotoxicity under these conditions.

Although the chemical nature of the compound is still unknown, its action to increase the rate of glutamate transport by facilitating the return of the unoccupied carrier is unique among all drugs known to act on cation-coupled cotransporters. Thus, this spider venom component, Parawixin1 provides proof of principle for the development of drugs that act through this novel mechanism and may also serve as useful tool for exploring specific conformational transitions in the glutamate transport cycle.

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Footnotes

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Figure 1

A. Parawixin1 acts selectively on the EAAT2 subtype of glutamate transporter

Glutamate uptake by EAAT subtypes (EAAT1-3) expressed in transfected COS-7 cells. Cells were pre-incubated with 0.1 U/ml Parawixin1 for 10 min. Uptake was started by the addition of 100 nM L-[^{3}H]-glutamate. Results are expressed as mean ± S.E.M. of three independent experiments. Determinations of significance were made using Student's *t* test (***P*<0.01).

B. Parawixin1 alters the V_{max} of the transporter in COS-7 cells

Kinetic analysis of L-glutamate uptake by EAAT2 expressing COS-7 cells pre-incubated in absence (circles) or presence (squares) of 0.1 U/ml Parawixin1. Data from three independent experiments using eight substrate concentrations generated values of 91.7 ± 10 μM for the K_M and 631.8 ± 18.4 pmol/well/min for the V_{max} under control conditions and 97 ± 10 μM for K_M and 821 ± 25 pmol/well/min for V_{max} in the presence of Parawixin1. V_{max}´s between treatments (with or without addition of Parawixin1) were significantly different (****P*<0.001).

C. Parawixin1 does not alter the sodium dependence of glutamate uptake

EAAT2 expressing COS-7 cells were pre-incubated in absence (squares) or presence (triangles) of 0.1 U/ml Parawixin1 in buffers containing 15, 60 or 140 mM sodium. Uptake was performed as described in the Methods section. The graphic is representative of one experiment. Data from two separate experiments performed in triplicate for each point generated values of 35.20 ± 18 mM for EC_{50} for sodium and

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385.2 ± 66.3 pmol/well/min for the V_{max} under control conditions and 50.9 ± 27 mM for EC_{50} and 813.8 ± 166 pmol/well/min for V_{max} in the presence of Parawixin1.

Figure 2

A. Parawixin1 produces a dose-dependent increase in glutamate transport

Dose response curve for the effect of Parawixin1 on L-[^{3}H]-glutamate uptake in liposomes. Liposomes with internal potassium (140 mM internal KCl, 15 mM KPi and 1% glycerol) and external sodium (140 mM NaCl, 15 mM KPi and 1% glycerol) were pre-incubated with varying concentrations of Parawixin1 (from 0.001 to 10 U/ml) or buffer alone for 2 min at room temperature. The uptake assays (20 μl liposomes in 500 μl final volume with 50 nM L-[^{3}H]-glutamate in sodium phosphate buffer per vial) were initiated and terminated after a 1 min incubation. Data from three independent experiments generated an ED_{50}= 1.3 ±0.6 U/ml.

B. Parawixin1 requires external sodium to stimulate glutamate uptake.

Liposomes were prepared with internal and external 140 mM internal KCl, 15 mM potassium phosphate and 1% glycerol. In another set of liposomes the external media was replaced by sodium buffer (140 mM NaCl, 15 mM sodium phosphate, 1% (v/v) glycerol, pH 7.4). Both sets of liposomes were pre-incubated for 2 min in the presence or absence of Parawixin1 (1 U/ml) and uptake assays were perfomed as described above. Comparison of data with external potassium and external sodium by Student's *t* test showed significant difference (***P*<0.01) and also performing 1 way ANOVA followed by Dunnett's Multiple Comparison test ($^{###}P$<0.001).

[K^+]i= internal potassium, [K^+]e= external potassium, [Na^+]e= external sodium.

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C. The enhancement of uptake only occurs at physiological concentrations of sodium and potassium

Liposomes were prepared with the indicated concentrations of internal potassium buffer and external sodium buffer (90 mM to 250 mM), pre-incubated for 2 min in presence or absence of Parawixin1. Reactions were performed as described above. Data represent the average ± SEM of three (b) and two (c) independent experiments performed in triplicate (Student´s *t* test ***P*<0.01, ****P*<0.001).

Figure 3. Parawixin1 does not alter efflux of glutamate from liposomes

Liposomes with 140 mM internal potassium were actively loaded by incubation with 200 nM L-[^{3}H]-glutamate in NaSCN buffer for 3 min. The loading step was terminated by gel filtration through columns containing NaSCN buffer. Next, the liposomes were incubated for 10 seconds in NaSCN buffer plus glutamate (1 mM) in presence or absence of Parawixin1 (10 U/ml) or Parawixin1 alone, and the remaining within the liposomes was subtracted from the total radioactivity to determine the percentage of glutamate efflux. Data represent the average ± SEM of four independent experiments performed in triplicate. No statistical difference was observed between treatments of addition of 1 μM L-glutamate and 1 μM L-glutamate plus Parawixin1 (Student's *t* test and 1 way ANOVA followed by Dunnett's Multiple Comparison test).

Figure 4

A, B. Pure exchange of glutamate is not altered by Parawixin1

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Accumulation of external L-[^{3}H]-glutamate by glutamate-containing liposomes in absence of potassium. Liposomes were prepared with internal medium (140 mM NaCl, 10 mM sodium phosphate buffer, 1% (v/v) glycerol) with 1 or 10 mM unlabeled L-glutamate or glycine. External L-glutamate was removed by gel-filtration in 140 mM NaCl, 1% glycerol plus glycine at the same concentrations for ion compensation. In this system potassium is completely absent. Liposomes were pre-incubated for 2 min with Parawixin1, uptake reactions were initiated by the addition of 50 nM L-[^{3}H]-glutamate and terminated after varying times from 5 to 180 seconds.

Data represent the average ± SEM of three independent experiments performed in triplicate (*P <0.05, **P<0.01, ***P<0.001, Student's t test between control group and Parawixin1´s additions group).

Figures 5A, B, C. Parawixin1 acts under conditions that allow the full transport cycle to occur

A. Simplified state diagram of the EAAT2 transport cycle.

To= transporter on the outside, Ti=transporter on the inside,

B, C. Accumulation of external L-[^{3}H]-glutamate by glutamate-containing liposomes in presence of potassium and sodium.

Liposomes were prepared in potassium buffer with sodium (130 mM KCl, 10 mM NaCl, 1% glycerol) with 1 (B) or 10 mM (C) unlabeled K-L-glutamate or K-glycine as the internal medium. External L-glutamate was removed by gel-filtration of the liposomes in sodium buffer (140 mM NaCl, 1% glycerol plus the Na^+-glycine at the same concentrations for ion compensation).

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Liposomes were pre-incubated for 2 min with Parawixin1, uptake reactions were initiated by the addition of 50 nM L-[^{3}H]-glutamate and 1 μM unlabelled L-glutamate, terminated after varying times from 5 to 180 seconds.

Data represent the average ± SEM of three independent experiments performed in triplicate (*P <0.05, **P<0.01; Student's t test between control group and Parawixin1´s additions group).

Figure 6. Parawixin 1 does not increase glutamate uptake by a mutant defective in potassium translocation

EAAT2, E405D or empty vector expressing COS-7 cells were pre-incubated with D-PBS buffer alone (open bars) or with buffer and 0.1 U/ml Parawixin1 (black bars) for 10 min. Uptake was performed for 10 min with 100 nM L-[^{3}H]-glutamate. The same experiments were performed in parallel with cells expressing empty vector to obtain the background (values were subtracted). Data represent the average ± SEM of three independent experiments performed in triplicate (Students *t-test* **P<0.01; comparison between buffer alone and Parawixin1 addition; 1 way ANOVA using Dunnett's Multiple Comparison test showed differences between uptake in transport by wild type with or without Parawixin1 (*P < 0.05) but not between transport by E405D mutant with or without Parawixin1 (P > 0.05).

Figure 1

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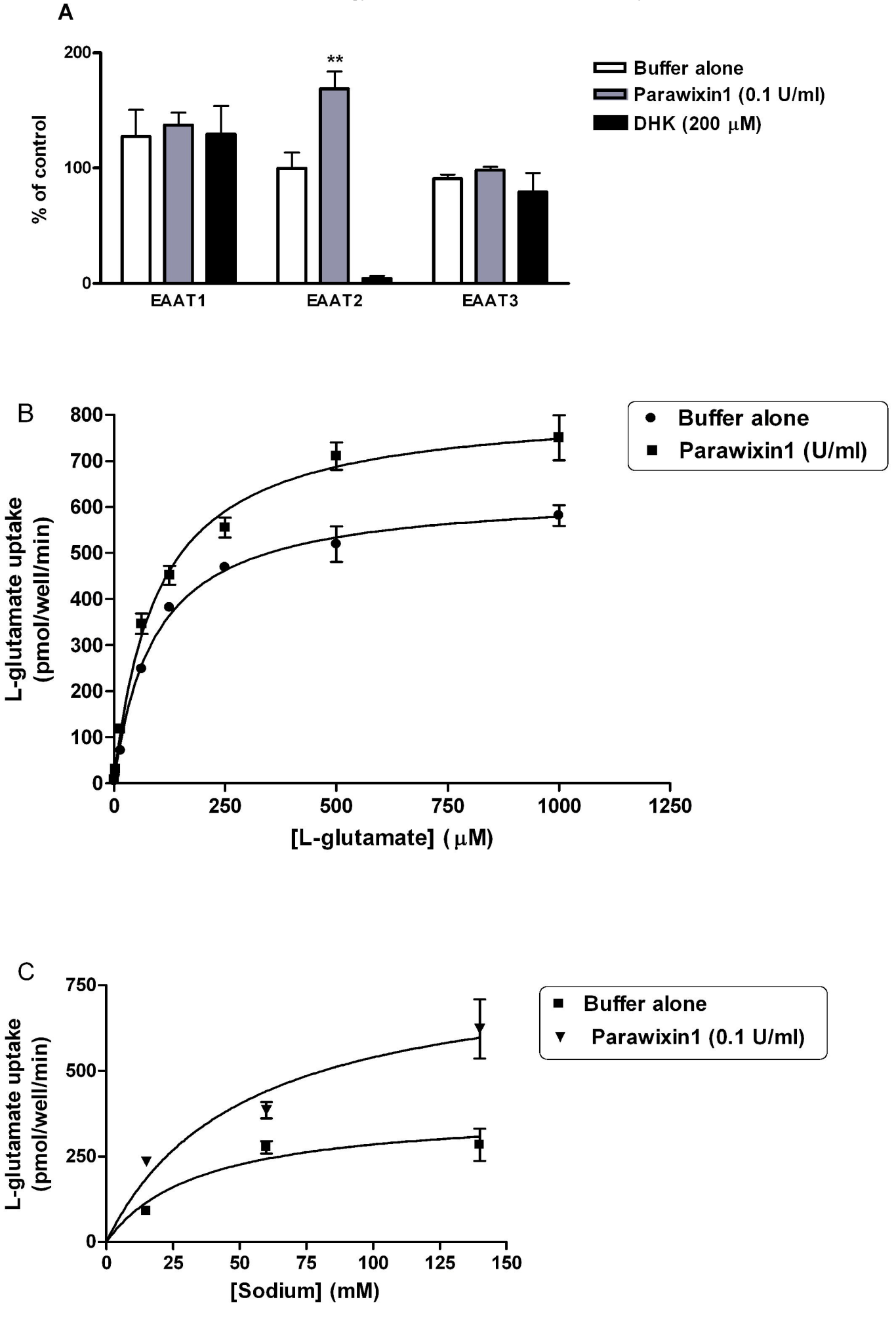

Figure 2

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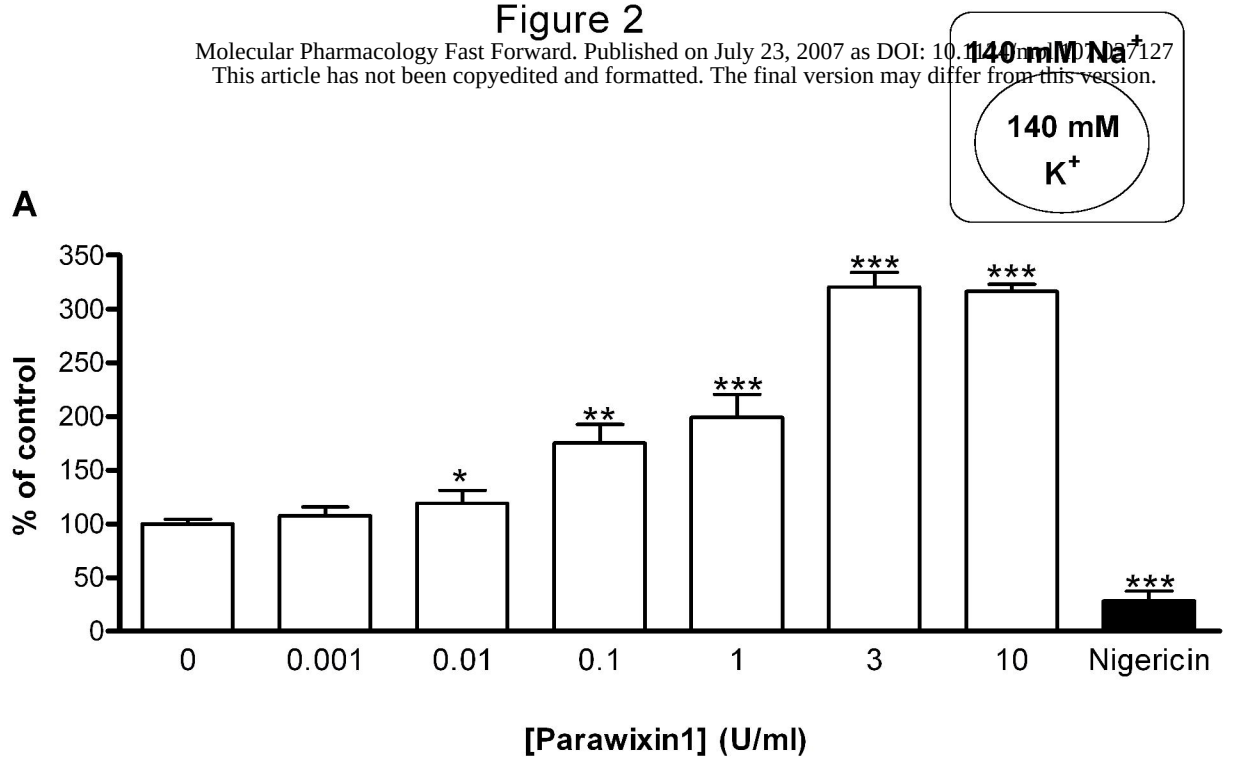
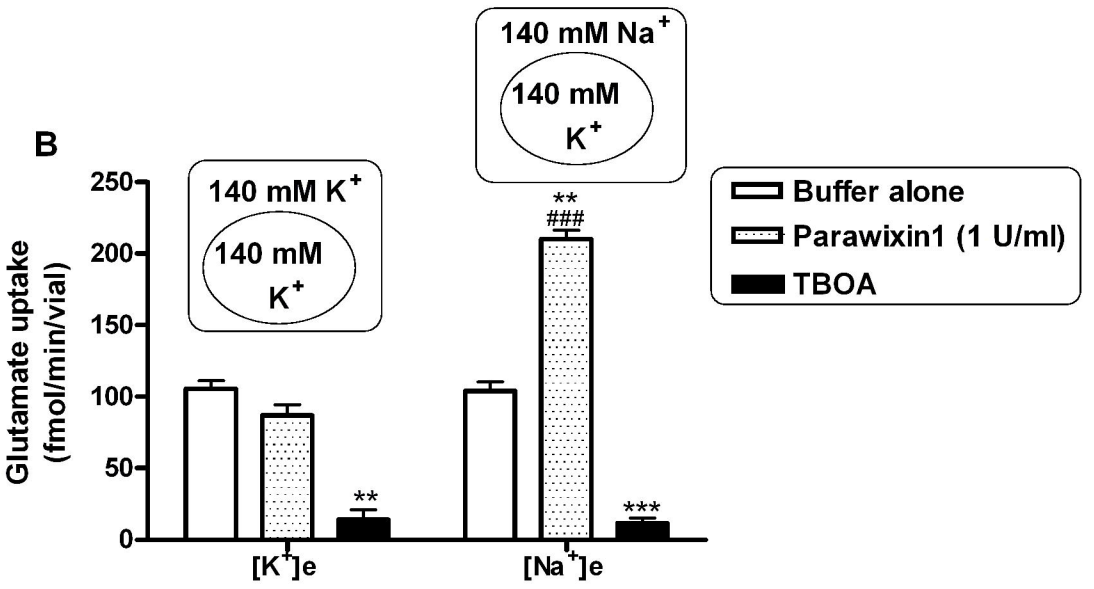




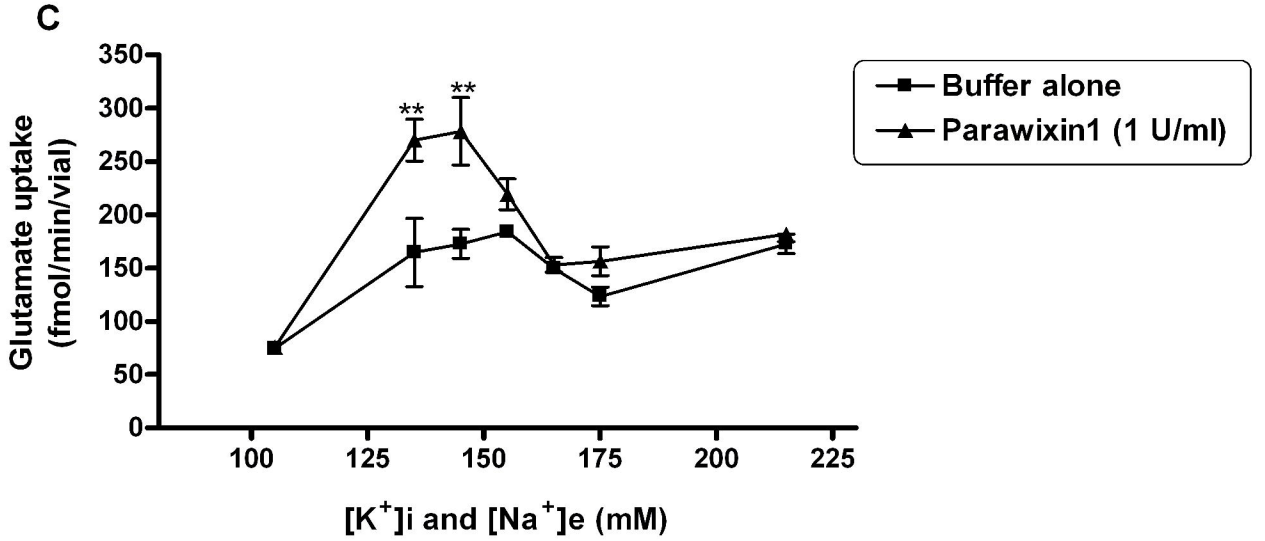


Figure 3

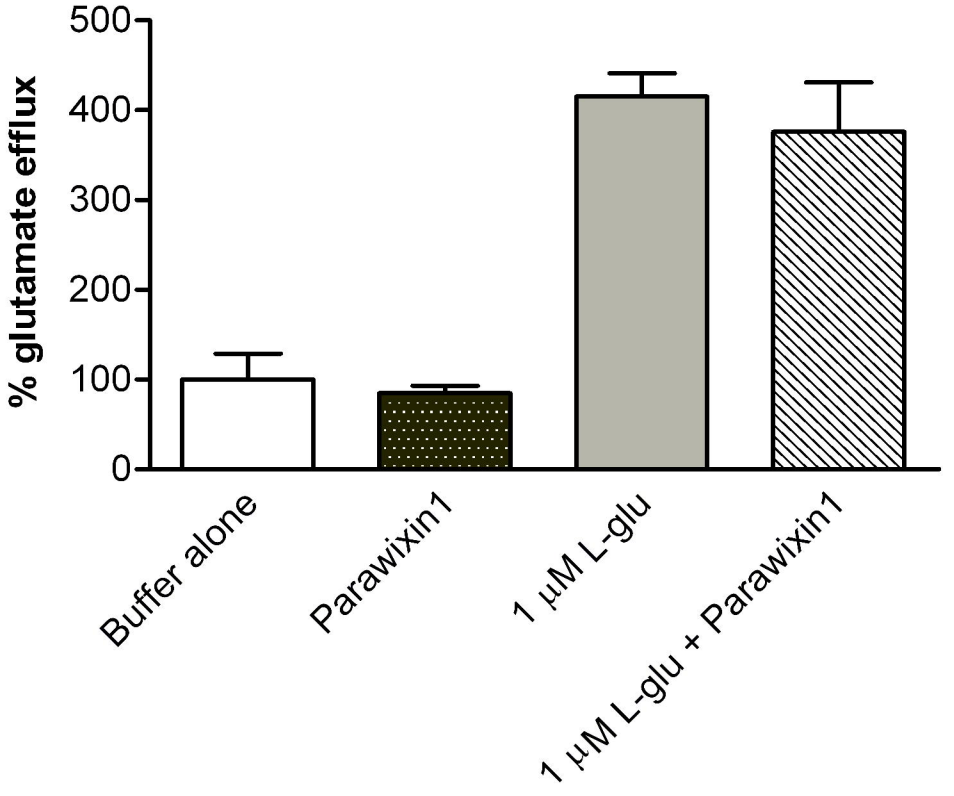
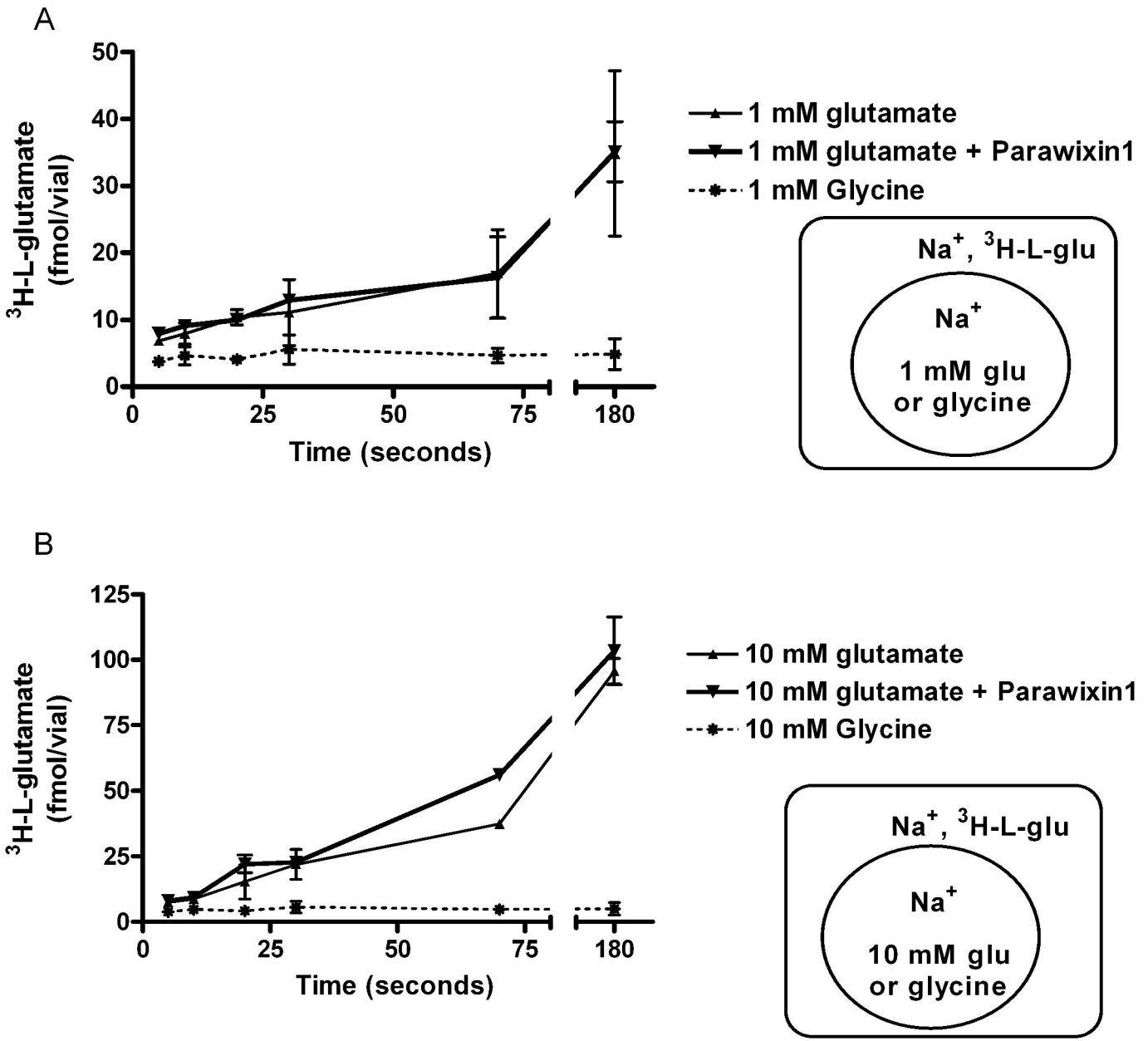
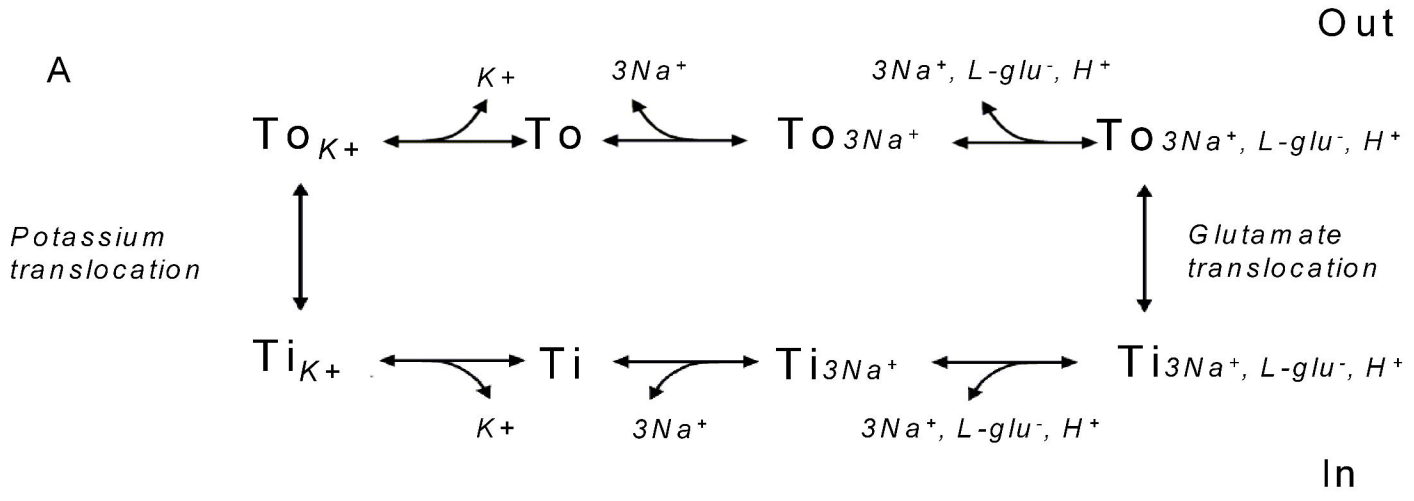
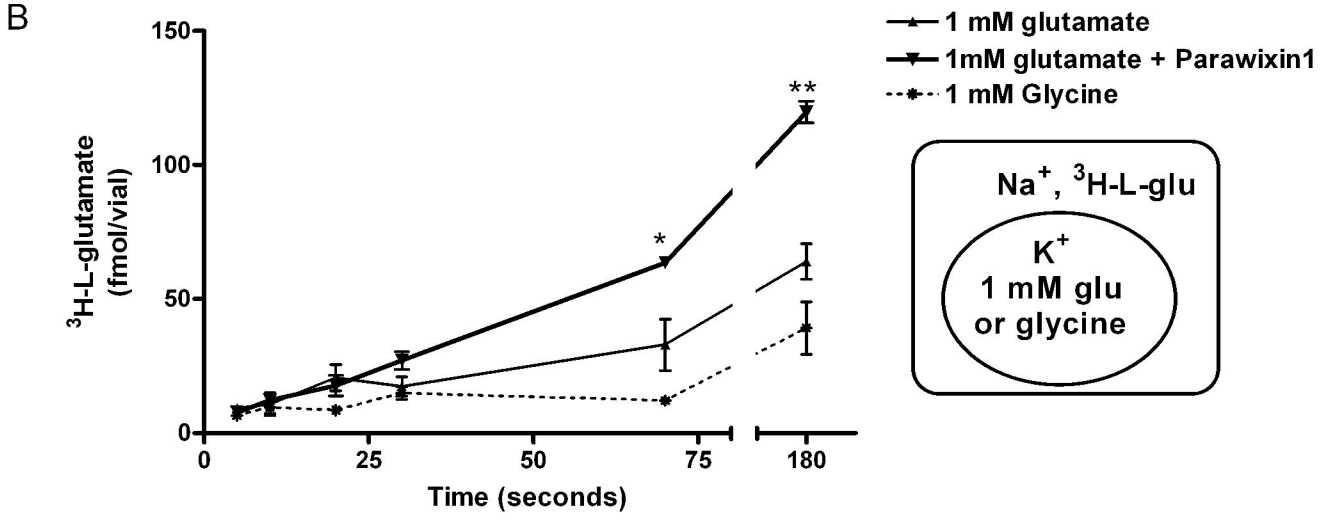

% glutamate efflux
500
400
300
200
100
0
Buffer alone
Parawixin1
1 μM L-glu
1 μM L-glu + Parawixin1

Figure 4


A
^{3}H-L-glutamate (fmol/vial)
50
40
30
20
10
0
0
25
50
75
180
Time (seconds)
1 mM glutamate
1 mM glutamate + Parawixin1
1 mM Glycine
Na$^+$, ^{3}H-L-glu
Na$^+$
1 mM glu or glycine
B
^{3}H-L-glutamate (fmol/vial)
125
100
75
50
25
0
0
25
50
75
180
Time (seconds)
10 mM glutamate
10 mM glutamate + Parawixin1
10 mM Glycine
Na$^+$, ^{3}H-L-glu
Na$^+$
10 mM glu or glycine


Out
A
K+
3Na+
3Na+, L-glu-, H+
To K+
To
To 3Na+
To 3Na+, L-glu-, H+
Potassium translocation
Glutamate translocation
Ti K+
Ti
Ti 3Na+
Ti 3Na+, L-glu-, H+
K+
3Na+
3Na+, L-glu-, H+
In


B
1 mM glutamate
1mM glutamate + Parawixin1
1 mM Glycine
Na+, 3H-L-glu
K+
1 mM glu
or glycine
3H-L-glutamate (fmol/vial)
Time (seconds)
0
25
50
75
100
150
180
*
**

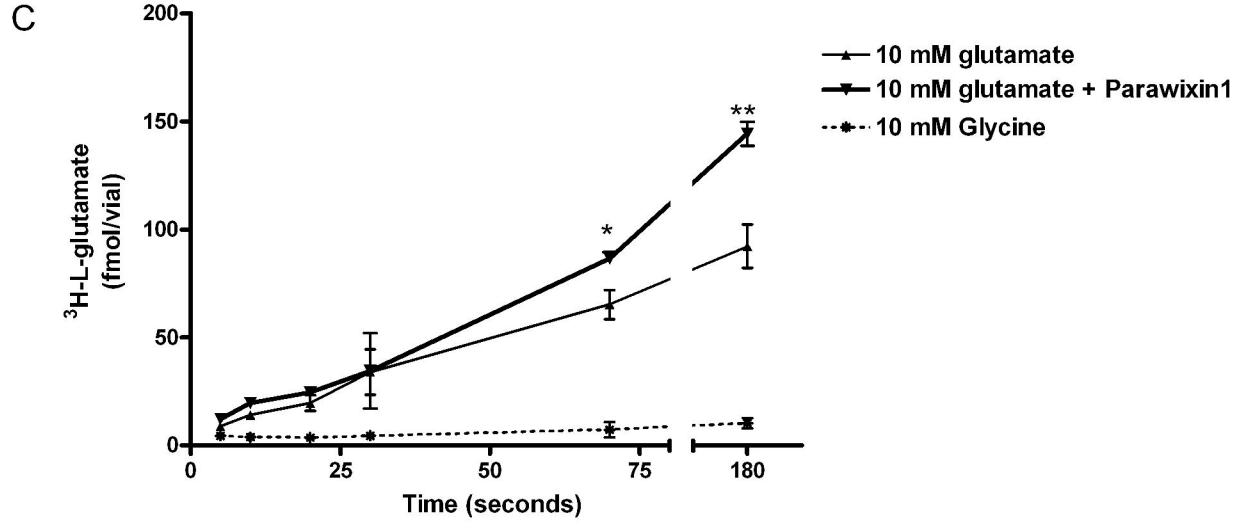
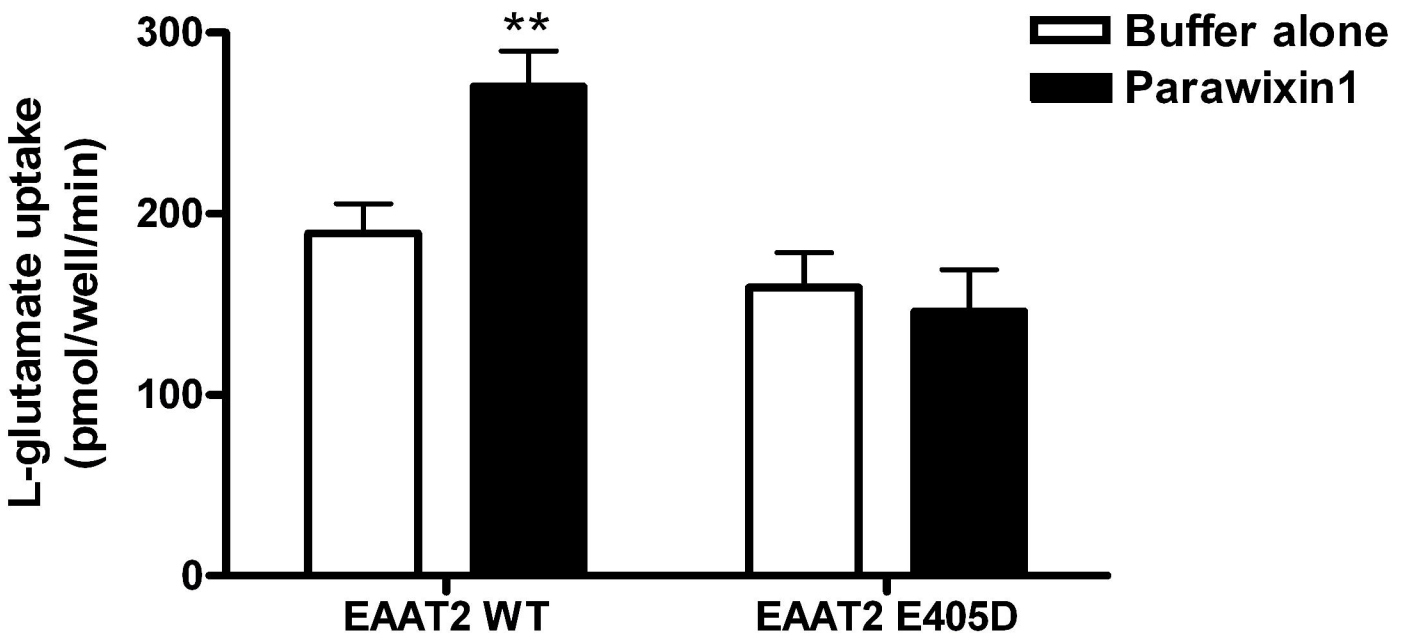

C
10 mM glutamate
10 mM glutamate + Parawixin1
10 mM Glycine
3H-L-glutamate (fmol/vial)
Time (seconds)
0
25
50
75
100
150
180
200
*
**

Figure 6


**
Buffer alone
Parawixin1
L-glutamate uptake
(pmol/well/min)
300
200
100
0
EAAT2 WT
EAAT2 E405D