

Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins

Mandy Jackson*, Wei Song*, Mu-Ya Liu†, Lin Jin*, Margaret Dykes-Hoberg‡, Chien-liang G. Lin*, William J. Bowers‡, Howard J. Federoff‡, Paul C. Sternweis† & Jeffrey D. Rothstein*

* Department of Neurology and Neuroscience, Johns Hopkins University, Baltimore, Maryland 21287, USA

† Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235, USA

‡ Department of Neurology, and the Center for Aging and Developmental Biology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, USA

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system and is removed from the synaptic cleft by sodium-dependent glutamate transporters. To date, five distinct glutamate transporters have been cloned from animal and human tissue: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5 (refs 1–5). GLAST and GLT-1 are localized primarily in astrocytes^{6,7}, whereas EAAC1 (refs 8, 9), EAAT4 (refs 9–11) and EAAT5 (ref. 5) are neuronal. Studies of EAAT4 and EAAC1 indicate an extrasynaptic localization on perisynaptic membranes that are near release sites^{8–10}. This localization facilitates rapid glutamate binding, and may have a role in shaping the amplitude of postsynaptic responses in densely packed cerebellar

terminals^{12–15}. We have used a yeast two-hybrid screen to identify interacting proteins that may be involved in regulating EAAT4—the glutamate transporter expressed predominately in the cerebellum—or in targeting and/or anchoring or clustering the transporter to the target site. Here we report the identification and characterization of two proteins, GTRAP41 and GTRAP48 (for glutamate transporter-4-associated protein) that specifically interact with the intracellular carboxy-terminal domain of EAAT4 and modulate its glutamate transport activity.

To identify proteins that interact with the C terminus of the EAAT4 protein, we used the last 77 amino acids of EAAT4 as bait to screen a rat brain complementary DNA library. We isolated two independent cDNA clones and called them GTRAP41 and GTRAP48 (for glutamate transporter-4-associated protein). Isolation of the full-length cDNAs by a series of 5' and 3' rapid amplification of cDNA ends (RACE) polymerase chain reactions (PCRs) showed that the largest open reading frame (ORF) for GTRAP41 is 7,164 base pairs (bp), which encodes a 2,388 amino-acid protein with a predicted relative molecular mass (M_r) of 270,958 (accession no. AF225960).

A BLAST search of the GenBank database showed that GTRAP41 possesses 87% identity with β -spectrin III (accession no. AB008567). GTRAP41 possesses seventeen 16-amino-acid spectrin repeats, two α -actinin domains and a pleckstrin homology (PH) domain (Fig. 1a). The largest ORF identified for GTRAP48 (accession no. AF225961) is 4,581 bp, which encodes a 1,527-amino-acid protein with a predicted M_r of 168,698. A BLAST search of the GenBank database showed that GTRAP48 is unique, but it possesses significant homology to the KIAA0380 cDNA encoded protein (90% identity) and RhoGEF, p115 (ref. 16). GTRAP48 possesses a PDZ domain, a regulatory G-protein-signalling sequence (LH),

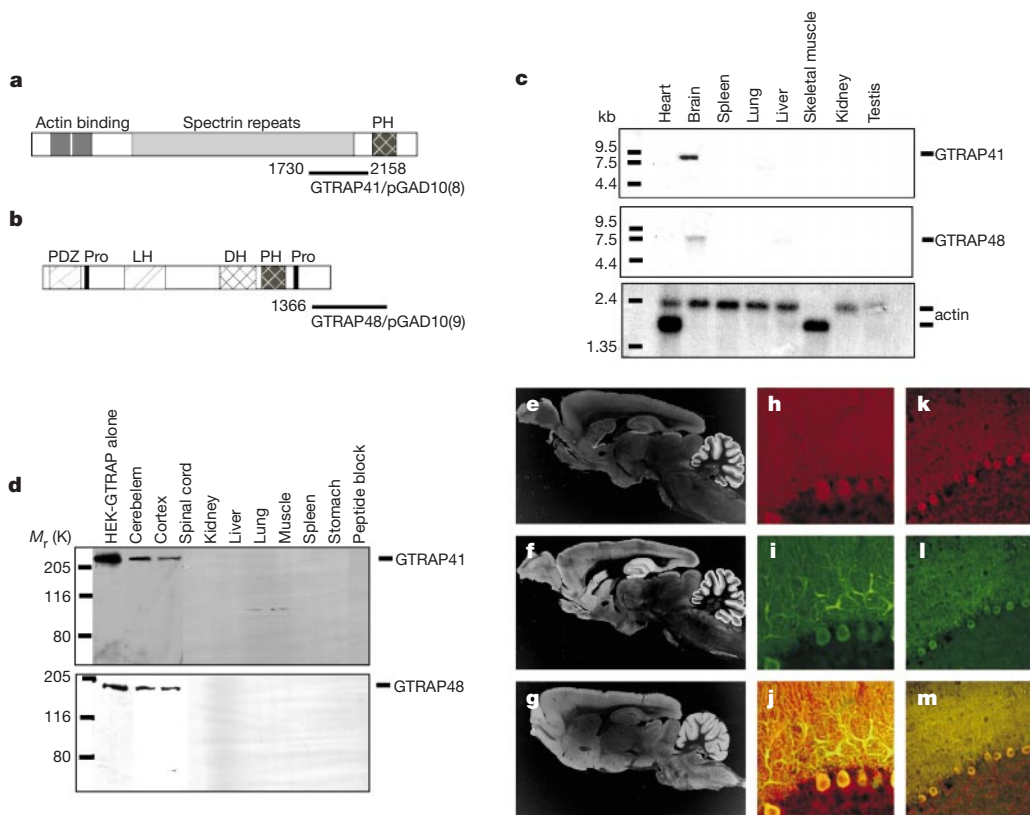


Figure 1 Structure and distribution of GTRAP41 and GTRAP48. **a, b**, cDNA clones GTRAP41/pGAD10 and GTRAP48/pGAD10 isolated from the yeast two-hybrid screen are shown aligned below representations of full-length GTRAP41 and GTRAP48, respectively. The number of times the clones were isolated is shown in parentheses. **c**, Multiple tissue northern (MTN; Clontech) blot probed with 3' PCR probes of GTRAP41 and GTRAP48.

d, Western analysis of GTRAP41 and GTRAP48. **e–g**, Rat brain sections stained with anti-EAAT4 (**e**), anti-GTRAP41 (**f**) and anti-GTRAP48 (**g**) antibodies. **h–m**, EAAT4 (**h, k**), GTRAP41 (**i**) and GTRAP48 (**l**) are all predominately expressed in the cell bodies and dendrites of Purkinje cells. The overlaps of GTRAP41 and GTRAP48 with EAAT4 are shown in **j** and **m**, respectively.

tandem dbl homology (DH) and pleckstrin homology (PH) domains characteristic of guanine nucleotide exchange factors for the Rho family of G proteins, and two proline-rich sequences (Fig. 1b).

Northern blot analysis detected a 8.3-kilobase (kb) GTRAP41 and a 7.5-kb GTRAP48 messenger RNA in brain tissue (Fig. 1c). Longer exposure revealed a low level of expression in liver and kidney for both GTRAP41 and GTRAP48. Anti-peptide antibodies were raised and the affinity-purified antibodies recognized a M_r 270,000 (270K) protein and a 170K protein, respectively, in HEK 293T cells transfected with full-length GTRAP41 and GTRAP48 cDNA (Fig. 1d). GTRAP41 and GTRAP48 were both selectively localized to brain, consistent with the northern blot analysis (Fig. 1d). EAAT4 is selectively localized to cerebellar Purkinje cells, although low-level expression is observed in cerebral cortex, hippocampus and striatum⁹. We found that GTRAP41 and GTRAP48 were expressed predominately in the cerebellum (Fig. 1e–g), with low-level immunoreactivity in striatum, hippocampus and thalamus. Immunofluorescence microscopy revealed that all three proteins are expressed in cerebellar Purkinje cell soma and dendrites, with little axonal staining (Fig. 1h–m).

We first confirmed the biochemical interaction between GTRAP41/GTRAP48 and EAAT4 by an *in vitro* binding assay. Full-length Myc-tagged GTRAP41 and GTRAP48 were expressed in HEK 293T cells. The solubilized cell extracts were then mixed with bead-linked glutathione *S*-transferase (GST)–EAAT4 or GST alone, and the bound proteins were eluted. GTRAP41 and GTRAP48 were retained specifically by the GST–EAAT4 fusion protein, but not by GST alone (Fig. 2a). A stable, rat EAAT4-expressing cell line was generated in HEK 293T cells (HEK-rEAAT4) and transfected with cDNAs encoding Myc-tagged GTRAP41 and GTRAP48. We used antibodies directed at the amino terminus of EAAT4 to immunoprecipitate the antigen plus any associated protein. Western blot analysis using an anti-c-Myc antibody showed that GTRAP41 and GTRAP48 co-immunoprecipitated with EAAT4 (Fig. 2b).

Similarly, when the anti-c-Myc antibody was used, EAAT4 was co-immunoprecipitated with GTRAP41 and GTRAP48 (Fig. 2c). *In vivo*, GTRAP41 (Fig. 2d) and GTRAP48 (Fig. 2f) were co-immunoprecipitated with EAAT4 from brain by antibodies directed at the N terminus of EAAT4 but not by antibodies directed at the C terminus (Fig. 2d, f). As the site of interaction is within the C terminus of EAAT4, however, it is likely that the C-terminal antibodies disrupt the protein–protein interaction. Furthermore, GTRAP41 and GTRAP48 seem to interact specifically with EAAT4, as we could not co-immunoprecipitate GTRAP41 and GTRAP48 from brain with antibodies directed at other glutamate transporters (Fig. 2d, f).

Western blot analysis confirmed that the immunoprecipitating antibodies pulled down their corresponding antigen (see Supplementary Information). No co-immunoprecipitation was observed when the precipitating antibody was omitted or pre-absorbed (Fig. 2b, e, g). We next determined the region of EAAT4 that binds GTRAP41 and GTRAP48. We used a series of successively larger C-terminal deletions of the original, 77-amino-acid, C-terminal EAAT4 bait in a yeast two-hybrid screen. Residues 555–561 and 527–534 appear to be required for GTRAP41 and GTRAP48 binding, respectively.

As GTRAP48 possesses areas of homology to p115 and PDZRhogEF, two RhoGEFs that selectively activate Rho^{16,17}, we investigated whether GTRAP48 interacts with the Rho family of GTPases. We measured the amount of GTP- γ S that bound to GST–RhoA, GST–Cdc42 and GST–Rac in the presence of full-length GTRAP48 or p115, and found that GTRAP48, like p115, shows a specific guanine nucleotide exchange activity for Rho (Fig. 3a, b). Co-immunoprecipitation assays also showed that GTRAP48 interacts with the active form (in the presence of aluminium fluoride) but not the inactive form of the $G\alpha_{13}$ subunit and therefore, may act as a link between G-protein-coupled receptors and their downstream targets (Fig. 3c). Specificity of the $G\alpha_{13}$ antibody has been previously described¹⁸.

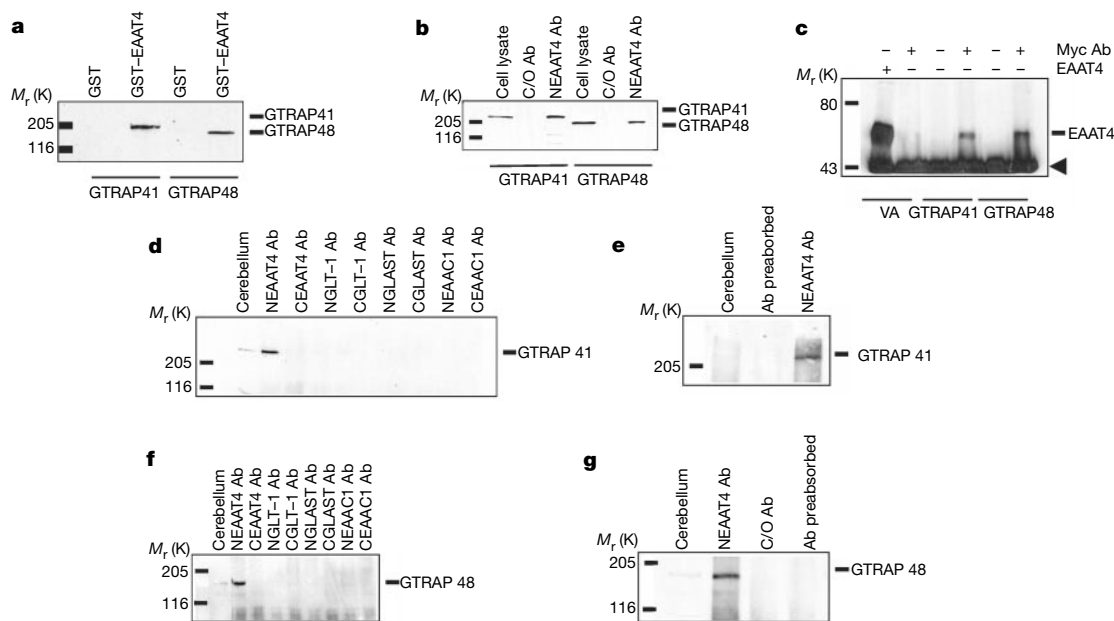


Figure 2 Interaction of GTRAP41 and GTRAP48 with EAAT4. **a**, Binding of Myc–GTRAP41 and Myc–GTRAP48 to GST–EAAT4. **b**, **c**, GTRAP41, GTRAP48 or vector alone (VA) were expressed (as indicated by the bars) in HEK-rEAAT4 cells. Immunoprecipitations were performed either with the N-terminal anti-EAAT4 antibody (**b**), or with the antibodies (Ab) indicated above the lanes (**c**). Immunoprecipitates were analysed by immunoblotting using anti-c-Myc (**b**) or C-terminal anti-EAAT4 (**c**; arrowhead indicates protein-A–

Sepharose band) antibodies. **d–g**, Extracts of rat brain were immunoprecipitated with antibodies (**d**, **f**) directed at the N terminus and C terminus of the glutamate transporters (as indicated above the lanes), no antibody or antibody pre-absorbed with peptide (**e**, **g**). Immunoblots were probed with the anti-GTRAP41 (**d**, **e**) and the anti-GTRAP48 (**f**, **g**) antibodies.

Rho regulates the remodelling of the actin cytoskeleton through various actin-binding proteins, although the mechanism is not well characterized¹⁹. As GTRAP48 can activate Rho, we next determined whether the expression of GTRAP48 induced reorganization of the actin cytoskeleton and whether it altered the distribution of GTRAP41, a possible actin-binding protein. When GTRAP41 was expressed alone there was a close relationship between actin and GTRAP41 at the cell membrane, but there were very few organized

actin filaments (Fig. 3d, e). Conversely, when GTRAP41 and GTRAP48 were co-expressed, GTRAP41 colocalized with actin in structures that resembled actin-stress fibres (Fig. 3f, g), a typical Rho-dependent effect. We noted that the overexpression of GTRAP48 also induced the formation of membrane ruffling and filopodia (Fig. 3h, i), suggesting some degree of cross-talk between the small GTPases, as these are typical Rac- and Cdc42-dependent effects.

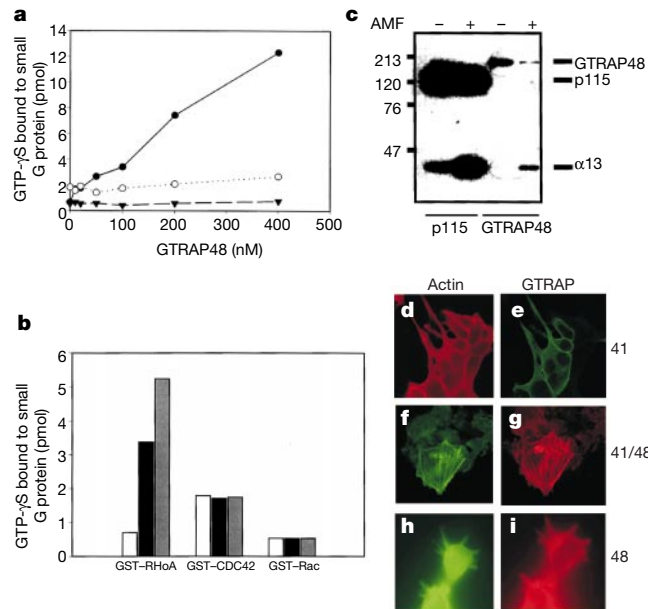


Figure 3 Guanine nucleotide exchange activity of GTRAP48. **a**, Binding of GTP- γ S to GST-RhoA (filled circles), GST-CDC42 (open circles) and GST-Rac (filled triangles) was measured after 10 min at 30 °C in the presence of the indicated concentrations of full-length GTRAP48 as described in Methods. **b**, Binding of GTP- γ S to the indicated GTPases in either the absence (white), or the presence of 100 nM GTRAP48 (black) or 100 nM

p115-RhoGEF (grey). **c**, Binding of active $G\alpha_{13}$ to Glu-tagged (EE) GTRAP48 and p115 RhoGEF. **d-i**, HEK-REAT4 cells transfected with either vector alone (**d**, **e**), Myc-tagged GTRAP41 and GTRAP48 (**f**, **g**) or Myc-tagged GTRAP48 (**h**, **i**). Actin filaments were visualized with FITC- or rhodamine-conjugated phalloidin.

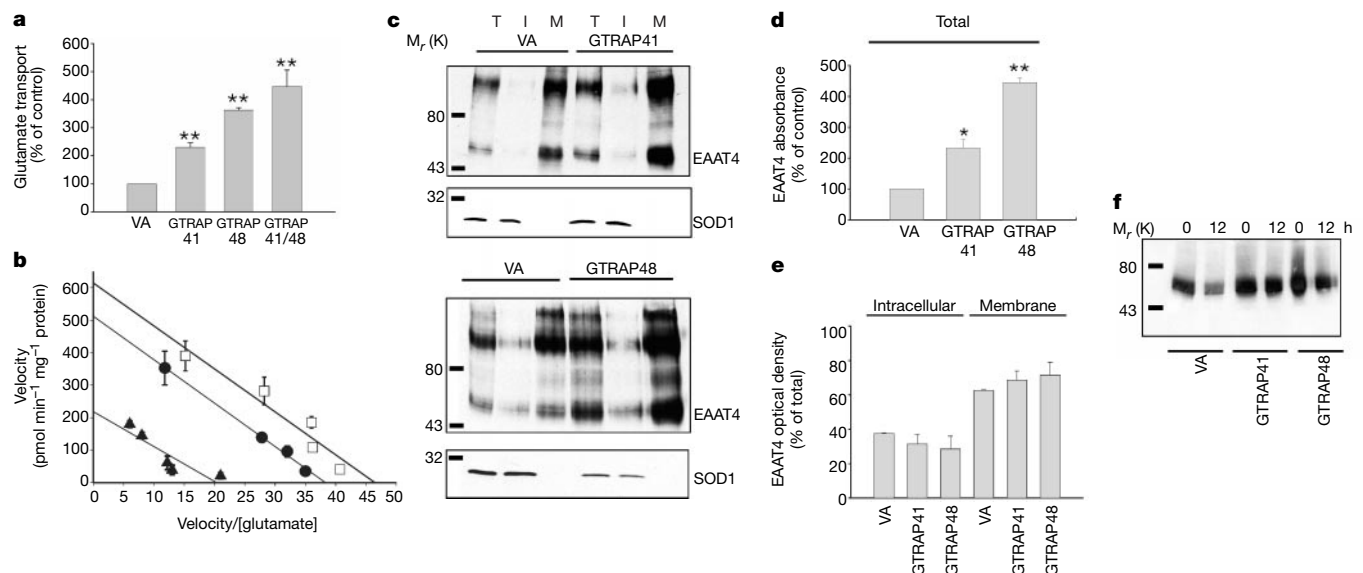


Figure 4 Effect of GTRAP41 and GTRAP48 on Na^+ -dependent [3H]-glutamate uptake. **a**, GTRAP41 and GTRAP48 expression increased glutamate uptake (5 μ M) significantly over cells transfected with vector alone (VA; $n = 4$; $**P < 0.005$). **b**, Kinetic data showed that GTRAP41 (open squares) increased the V_{max} from 222 to 605 $pmol\ mg^{-1}\ min^{-1}$ and increased the K_m slightly from 7 to 11 μ M, as compared with EAAT4 alone (filled triangles). GTRAP48 increased the V_{max} from 208 to 512 $pmol\ mg\ min^{-1}$ (filled circles) and

increased the K_m from 10 to 13 μ M. **c**, Immunoblots of total (T), intracellular (I), and biotinylated fractions (M) of HEK-REAT4 cells transfected with GTRAP41 and GTRAP48. **d**, Quantitation of immunoblots for total EAAT4 protein ($n = 3$; $**P < 0.005$, $*P < 0.05$). **e**, Ratio of membrane-bound to intracellular EAAT4. **f**, Cells transfected with GTRAP41, GTRAP48 or vector alone incubated with cycloheximide (10 μ g ml^{-1}) for 12 h.

We determined whether there was a functional association between GTRAP41, GTRAP48 and EAAT4. We measured the sodium-dependent glutamate transport activity of HEK-rEAAT4 cells that had been transfected with one or both of the interacting proteins. GTRAP41 and GTRAP48 produced respective twofold and fourfold increases in glutamate transport (Fig. 4a), and their co-expression resulted in a further increase in glutamate uptake. Kinetic analysis indicated that GTRAP41 and GTRAP48 produced an increase in the V_{max} of glutamate transport activity (Fig. 4b). GTRAP41 and GTRAP48 may therefore enhance glutamate transport either through an increase in the catalytic rate of the transporter or through an increase in cell-surface availability.

To examine changes in the cell-surface levels of EAAT4, we used a cell-membrane-impermeant biotinylation reagent to label cell-surface proteins selectively. Figure 4c shows western analysis of a representative biotinylation experiment for GTRAP41 and GTRAP48. The total amount of EAAT4 increased when GTRAP41 and GTRAP48 were co-expressed (Fig. 4d); in contrast, the total amount of SOD1, a control for the total amount of protein loaded, was unaltered or decreased in the GTRAP41 or GTRAP48 samples, respectively. The percentage of total EAAT4 that was biotinylated remained the same when GTRAP41 and GTRAP48 were co-expressed, showing that most of the increase in total EAAT4 protein was located at the cell surface and not in an intracellular

pool (Fig. 4e).

These results indicated that GTRAP41 and GTRAP48 stabilize and/or anchor EAAT4 at the cell membrane, making it less likely to be internalized and subsequently degraded; however, we could not rule out the possibility of increased expression of the cell's native gene. To address this issue, we treated cells with cycloheximide, an inhibitor of protein synthesis, 48 h after transfection (Fig. 4f). Densitometry showed that 12 h after treatment the EAAT4 protein in HEK-rEAAT4 cells was reduced to $54 \pm 0.6\%$ of its level before cycloheximide treatment. In contrast, $81 \pm 2\%$ and $74 \pm 1.7\%$ of the EAAT4 protein remained after 12 h when GTRAP41 and GTRAP48 were co-expressed, respectively. These results provide compelling evidence that GTRAP41 and GTRAP48 do stabilize EAAT4 at the membrane.

To determine whether the EAAT4/GTRAP48 interaction is required to mediate the increase in EAAT4 activity, we transfected HEK-rEAAT4 cells with GTRAP48 constructs lacking the last 155 amino acids, which were pulled out by EAAT4 in the yeast two-hybrid screen. The C-terminally truncated GTRAP48 had only a modest effect on stimulating EAAT4 activity, indicating that the protein-protein interaction is responsible for most of the increase in uptake activity (Fig. 5a). We also co-transfected HEK-rEAAT4 cells with GTRAP48 and a Myc-tagged cDNA construct encoding the last 77 amino acids of EAAT4, to disrupt the interaction of GTRAP48 with full-length EAAT4. We found that the co-expression of this construct inhibited the GTRAP48-mediated effect by about 25%, but co-expression of a smaller construct (residues 1,452–1,578), lacking the GTRAP48-binding domain, had no effect (Fig. 5b). Together, these results indicate that the EAAT4/GTRAP48 interaction is important in modulating EAAT4 uptake activity.

The physiological relevance of GTRAP41 and GTRAP48 on EAAT4 uptake activity *in vivo* was subsequently examined by the intra-cisternal injection of HSV amplicon vectors expressing GTRAP41 and GTRAP48. Cerebellar glutamate uptake was measured 48 h after injection, and was elevated when GTRAP41 and GTRAP48 were expressed but not when the control HSVlac amplicon vector was injected (Fig. 5c). Dihydrokainic acid (DHK), an inhibitor of GLT-1-mediated glutamate transport, had no effect on cerebellar glutamate uptake, ruling out any involvement of GLT-1. There is no method to distinguish functionally between GLAST, EAAC1 and EAAT4; but, as GTRAP41 and GTRAP48 do not interact directly with any other transporter, it is likely that the observed increase in uptake is due to an increase in EAAT4 activity. Western blot analysis confirmed an increased expression of GTRAP41 and GTRAP48 in the cerebellum after the injections. Preliminary studies of primary cultures of rat Purkinje cells indicate that EAAT4 and GTRAP41 may colocalize perisynaptically at 70% of synapses.

Glutamate transporters, through their rapid and efficient removal of glutamate from the synaptic cleft, are critical in glutamatergic plasticity and the prevention of glutamate-mediated excitotoxicity. Identifying and characterizing the glutamate transporter associated proteins GTRAP41 and GTRAP48 has begun to unravel the complex mechanism of glutamate uptake. In the modulation of EAAT4 glutamate transport, our findings implicate a role for G-protein signalling, a pathway that may involve Rho activation, and anchoring to the actin cytoskeleton (Fig. 5d). These proteins may also modulate the perisynaptic distribution of EAAT4 at glutamatergic synapses. Future studies are required to delineate the signalling pathway of GTRAP41 and GTRAP48, and how their interaction may be relevant to normal and abnormal glutamatergic neurotransmission, as altered EAAT4 function may be involved in the neurodegenerative disease spinocerebellar ataxia (SCA1)²⁰. By understanding the physiological regulation of the EAAT4 transporter, we may be able to identify possible therapies for SCA1 or other toxic insults that lead to the degeneration of Purkinje cell neurons. □

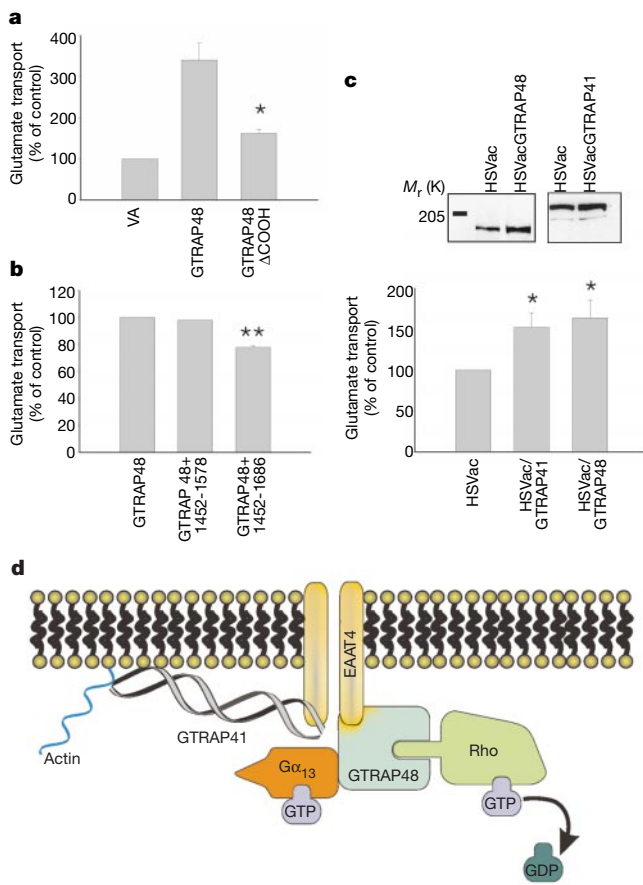


Figure 5 Modulation of EAAT4 activity. **a**, GTRAP48 Δ COOH results in a very small increase in EAAT4 uptake activity compared with full-length GTRAP48 ($*P < 0.05$). **b**, Overexpression of the EAAT4 C-terminus reduced the GTRAP48-mediated effect on EAAT4 activity ($**P < 0.005$). **c**, Intra-cisternal injection of HSVlac amplicons expressing GTRAP41 and GTRAP48 increased glutamate uptake *in vivo* ($n = 6$; $*P < 0.05$). Western blot analysis demonstrates increased expression of GTRAP41 and GTRAP48. **d**, A possible model of the coupling of EAAT4 to a Rho GTPase signal transduction cascade and to the actin cytoskeleton via GTRAP48 and GTRAP41. GDP, guanosine diphosphate; GTP, guanosine triphosphate.

Methods

Yeast two-hybrid screen

We screened a rat brain cDNA library (Clontech) using the final 77 amino acids of EAAT4 as bait (pGBT9). For the EAAT4 C-terminal domain analysis, different regions of the final 77 amino acids of EAAT4 were subcloned in-frame into the pGBT9 vector.

Cloning of full-length GTRAP41 and GTRAP48 cDNAs

Marathon cDNA amplification (CLONTECH) was used to perform both 5' and 3'-RACE on cDNA synthesized from rat brain poly(A)⁺ RNA.

Antibodies

Affinity-purified polyclonal antisera to EAAT4, GTRAP41 and GTRAP48 were generated as described⁶. We synthesized peptides corresponding to epitopes of EAAT4 (C-terminal, EKGASRGRGGNESA; N-terminal, KNSLFLRESGAGGGCL), rat GTRAP41 (KRGAPSPMPQSRSSSE) and rat GTRAP48 (KTPERTSPSHHRQPSD). The anti-c-Myc monoclonal antibody was from Boehringer Mannheim. For visualization of intracellular F-actin organization, the cells were probed with Texas-red-conjugated or fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma).

Transfection and maintenance of HEK-rEAAT4 cells

The EAAT4 cDNA was subcloned into pcDNA3.1/Hygro(+) (Invitrogen) using the EcoRI restriction site. The plasmid was linearized with SspI and transfected into HEK 293T cells. Forty-eight hours after transfection, the cells were split to 50% confluency, and hygromycin (Invitrogen) was added at a concentration of 50 µg ml⁻¹. After about 2–3 weeks of selection, a serial dilution was carried out and cells were plated out, without selection, in a 96-well plate to obtain one cell per well. Several colonies were picked, expanded in selective medium and checked for expression by western blotting.

Fusion proteins and *in vitro* binding

Full-length EAAT4 was subcloned into the EcoRI site of the GST–fusion vector pGEX-6P-1 (Pharmacia). Synthesis of recombinant proteins in BL21 cells (Novagen) was induced by 0.1 mM isopropyl β-D-thiogalactoside for 2 h at 30 °C and purified according to the protocol provided by Pharmacia. HEK 293T cells were transfected with Myc-tagged GTRAP41 or GTRAP48 and gathered in ice-cold immunoprecipitation (IP) buffer. The cellular lysate was incubated with GST or GST–EAAT4 immobilized on glutathione–Sepharose-4B, and washed to remove nonspecifically bound proteins. Specifically bound proteins were eluted with 2× SDS loading buffer and analysed by immunoblotting using an anti-c-Myc antibody.

Co-immunoprecipitation

Full-length GTRAP41 and GTRAP48 cDNAs were subcloned into the NotI site of a Myc-tagged pRK5 vector and used to transfect HEK-rEAAT4 cells. After transfection (48–72 h), cells were solubilized with 1 ml of ice-cold IP buffer for 2 h at 4 °C with rotation and centrifuged to remove cellular debris. Antibody was added to 0.5 ml of supernatant and incubated overnight at 4 °C. We dissected and prepared the cerebellum from a Sprague-Dawley rat as described²¹. For each immunoprecipitation, 500 µg of the Triton-lysate was incubated overnight at 4 °C with 5 µg of antibody. Protein-A–Sepharose (Pharmacia) was then added for 2 h at 4 °C, washed once with IP buffer and three times with IP buffer minus Triton X-100. Bound protein was eluted by boiling in 2× SDS loading buffer, and analysed by immunoblotting.

Immunohistochemistry

Rat brain sections were stained as described⁹, using the following antibodies: C-terminal anti-EAAT4 (1.5 µg ml⁻¹), anti-GTRAP41 (127 ng ml⁻¹) or anti-GTRAP48 (132 ng ml⁻¹) antibodies. Texas-red and FITC-conjugated secondary antibodies were used at dilutions of 1:200.

Guanine nucleotide exchange assay

Small G proteins GST–RhoA, GST–CDC42 and GST–Rac were expressed in bacterial cells and affinity purified to ~80% purity using a glutathione column. We incubated 20 pmoles of each protein with 100 pmoles GTP-γS for 10 min at 30 °C with varying concentrations of full-length GTRAP48 or p115. The binding reactions were filtered through BA-85 nitrocellulose and the amount of GTP-γS bound to small G protein was quantified by scintillation counting of the dried filters. Binding of Gα₁₃ to GTRAP48 was assayed as described¹⁸.

Na⁺-dependent glutamate transport activity

HEK-rEAAT4 cells transfected with GTRAP41 and GTRAP48 were grown in a monolayer on six-well plates, and assays were conducted 72 h after transfection as described²². We subcloned GTRAP41 and GTRAP48 into the EcoRI site of HSVPrPUC amplicon parent vector²³. Amplicon vector DNA (3.6 µg) and pBAC-V2 DNA (25 µg) were used to transfect 2 × 10⁷ baby hamster kidney cells as described²⁴. Virus was collected 72 h after transfection, and titred as described²⁵. Expression particles (2 × 10⁷) were injected intracisternally into male Sprague-Dawley rats (250 g) obtained from Zivic Miller. Forty-eight hours after injection the rats were killed, synaptosomal preparations of the cerebellum were prepared using a polytron, and glutamate uptake was measured.

Biotinylation

Biotinylation of cell-surface proteins was done as described²⁶. We used SOD1 to control for total protein and to determine whether the biotinylation reagent labels proteins in the intracellular compartment.

Statistics

Statistical differences were determined by Student's *t*-test for two-group comparisons.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Correspondence and requests for materials should be addressed to J.D.R. (e-mail: jrothste@welchlink.welch.jhu.edu). The GenBank accession numbers are AF225960 (GTRAP41) and AF225961 (GTRAP48).