

10. Goujet, D. *Les Poissons Placodermes du Spitsberg. Arthroires Dolicho thoraci de la Formation de Wood Bay (Dévonien Inférieur)* (Cahiers de Paléontologie, Section Vertébrés, Paris, 1984).

11. Maisey, J. G. in *Major Events in Early Vertebrate Evolution* (ed. Ahlberg, P. E.) Ch. 16 (Taylor & Francis, London, in press).

12. Ahlberg, P. E. A re-examination of sarcopterygian interrelationships, with special reference to the Porolepiformes. *Zool. J. Linn. Soc.* **103**, 241–287 (1991).

13. Rosen, D. E., Forey, P. L., Gardiner, B. G. & Patterson, C. Lungfishes, tetrapods, paleontology and plesiomorphy. *Bull. Am. Mus. Nat. Hist.* **167**, 159–276 (1981).

14. Schultze, H.-P. & Cumbaa, S. L. in *Major Events in Early Vertebrate Evolution* (ed. Ahlberg, P. E.) Ch. 18 (Taylor & Francis, London, in press).

15. Cloutier, R. in *Devonian Fishes and Plants of Miguasha, Quebec, Canada* (eds Schultze, H.-P. & Cloutier, R.) 227–247 (Dr Friedrich Pfeil, Munich, 1996).

16. Forey, P. L. *et al.* A new coelacanth from the Middle Devonian of Latvia. *J. Vert. Paleont.* **20**, 243–252 (2000).

17. Jessen, H. L. Lower Devonian Porolepiformes from the Canadian Arctic with special reference to *Powichthys thorstenssoni* Jessen. *Palaeontographica A* **167**, 180–214 (1980).

18. Chang, M. M. *The braincase of Youngolepis, a Lower Devonian Grossopterygian from Yunnan, south-western China*. Thesis, Stockholm Univ. (1982).

19. Chang, M. M. *Diabolepis* and its bearing on the relationships between porolepiforms and dipnoans. *Bull. Mus. Natl. Hist. Nat.* **17**, 235–268 (1995).

20. Zardoya, R. & Meyer, A. Evolutionary relationships of the coelacanth, lungfishes, and tetrapods based on the 28S ribosomal RNA gene. *Proc. Natl. Acad. Sci. USA* **93**, 5449–5454 (1996).

21. Zardoya, R. *et al.* Searching for the closest living relative(s) of tetrapods through evolutionary analyses of mitochondrial and nuclear data. *Mol. Biol. Evol.* **15**, 506–517 (1998).

22. Janvier, P. & Phuong, T. H. Les vertébrés (Placodermi, Galeaspidia) du Dévonien inférieur de la coupe de Lung Cò—Mia Lé, Province de Hà Giang, Viêt Nam, avec des données complémentaires sur les gisements à vertébrés du Dévonien inférieur du Bac Bo oriental. *Geodiversitas* **21**, 33–67 (1999).

23. Long, J. A. (ed.) *Palaeozoic Vertebrate Biostatigraphy and Biogeography* (Belhaven, London, 1993).

24. Swofford, D. L. *PAUP: phylogenetic analysis using parsimony, ver.3.1.1* (Illinois Natural History Survey, Campaigne, Illinois, 1993).

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.

**Acknowledgements**

We thank M. M. Chang, P. Janvier and M. I. Coates for useful discussions, U. Samuelson and Zhang Jie for photographic work, Lu Xiufen for specimen preparation and Hu Huiqing for artwork. M.Z. & X.Y. acknowledge the support from the Chinese Foundation of Natural Sciences, Ministry of Science & Technology (China), and National Geographic Society (US). X.Y. thanks Kean University for faculty research and development support.

Correspondence and requests for materials should be addressed to M.Z.

# Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18

Chien-liang Glenn Lin\*, Irina Orlov\*, Alicia M. Ruggiero, Margaret Dykes-Hoberg, Andy Lee, Mandy Jackson & Jeffrey D. Rothstein

Johns Hopkins University, Department of Neurology and Neuroscience, Meyer 6-109, 600 North Wolfe Street, Baltimore, Maryland 21287, USA

\* These authors contributed equally to this work

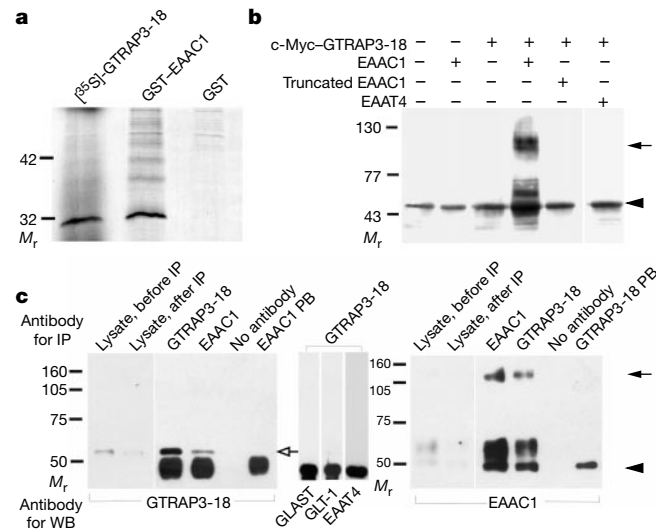
Excitatory amino-acid carrier 1 (EAAC1) is a high-affinity Na<sup>+</sup>-dependent L-glutamate/D, L-aspartate cell-membrane transport protein<sup>1</sup>. It is expressed in brain as well as several non-nervous tissues. In brain, EAAC1 is the primary neuronal glutamate transporter<sup>2,3</sup>. It has a polarized distribution in cells and mainly functions perisynaptically to transport glutamate from the extracellular environment<sup>2-4</sup>. In the kidney it is involved in renal acidic amino-acid re-absorption and amino-acid metabolism<sup>5-7</sup>. Here we describe the identification and characterization of an EAAC1-associated protein, GTRAP3-18. Like EAAC1, GTRAP3-18 is expressed in numerous tissues<sup>8,9</sup>. It localizes to the cell membrane and cytoplasm, and specifically interacts with carboxy-terminal intracellular domain of EAAC1. Increasing the expression of GTRAP3-18 in cells reduces EAAC1-mediated glutamate transport by lowering substrate affinity. The expression of GTRAP3-18

can be upregulated by retinoic acid, which results in a specific reduction of EAAC1-mediated glutamate transport. These studies show that glutamate transport proteins can be regulated potently and that GTRAP can modulate the transport functions ascribed to EAAC1. GTRAP3-18 may be important in regulating the metabolic function of EAAC1.

Using the C-terminal intracellular domain of EAAC1 (the last 87 amino acids) as bait in a yeast two-hybrid screen of an adult rat brain complementary DNA library, we isolated 78 clones displaying β-galactosidase activity. Restriction and sequencing analyses revealed that 10 of the clones with the strongest β-galactosidase activity were identical. This clone, designated E18, was completely sequenced and was found to be unique after GenBank analysis. JWA protein, (GenBank NP006398) a human, differentially displayed, vitamin-A-responsive gene, is 95% identical to E18, suggesting that E18 is a JWA protein homologue of rat.

E18 is a full-length complementary DNA containing an initiation methionine and a poly(A) tail. We named the protein glutamate transporter EAAC1-associated protein (GTRAP3-18). GTRAP3-18 encodes a protein of 188 amino acids (see Supplementary Information), with a calculated relative molecular mass of 22,500 (M<sub>r</sub> 22.5K). Protein analysis indicated that it is a very hydrophobic protein with four possible transmembrane domains. Both the C-terminal and amino-terminal domains contain protein kinase C motifs and may be intracellular.

To confirm the yeast two-hybrid results, we examined the interaction of GTRAP3-18 with EAAC1 using *in vitro* and *in vivo* methods. For *in vitro* cell-free binding, EAAC1 was expressed as a fusion protein with glutathione S-transferase (GST), and GTRAP3-18 was produced and labelled with [<sup>35</sup>S]methionine by *in vitro* transcription and translation. Purified GST or GST-EAAC1 fusion proteins immobilized on glutathione-sepharose were incubated with [<sup>35</sup>S]labelled GTRAP3-18 protein. GTRAP3-18 bound specifically to immobilized GST-EAAC1 (lane 2) but not to GST alone (lane 3), indicating that they interact *in vitro* (Fig. 1a).

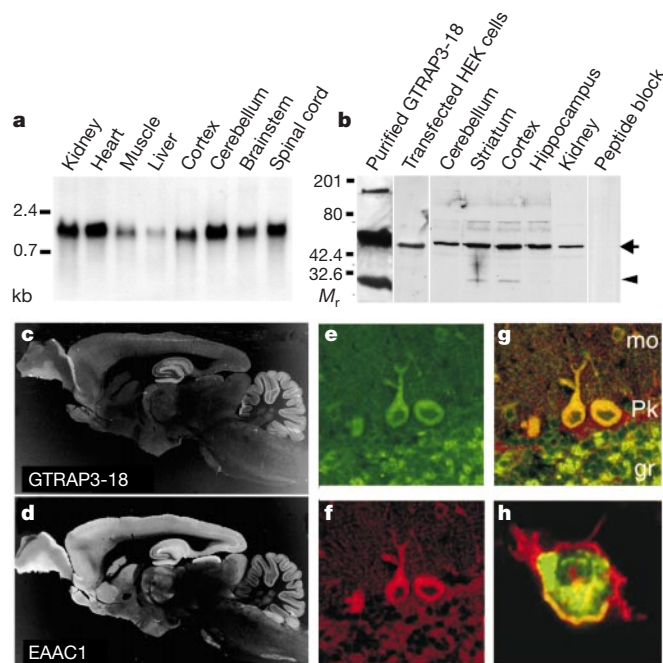


**Figure 1** GTRAP3-18 interacts with EAAC1 *in vitro* and *in vivo*. **a**, SDS-PAGE analysis of cell-free *in vitro* binding. *In vitro* synthesized [<sup>35</sup>S]-labelled GTRAP3-18 bound specifically to immobilized GST-EAAC1 and not to the negative control GST. **b**, Western blot analyses of immunoprecipitations from transfected HEK293 cells extracts, using anti-c-Myc antibodies for immunoprecipitation and anti-EAAC1 or EAAT4 antibodies for western blot. EAAC1 (monomer, arrowhead; dimer, arrow) was specifically co-immunoprecipitated with c-Myc-GTRAP3-18. An immunoprecipitation artifact is present in each lane at about 50–60K. **c**, Western blot (WB) analyses of immunoprecipitations (IP) from rat brain extracts. GTRAP3-18 (open arrowhead) was specifically co-immunoprecipitated with EAAC1. EAAC1 (monomer, arrowhead; dimer, arrow) was co-immunoprecipitated with GTRAP3-18. PB, peptide block.

We carried out immunoprecipitation experiments to test whether EAAC1 and GTRAP3-18 interact *in vivo*. Initial experiments were performed in transfected HEK293 cells using N-terminal c-Myc-tagged GTRAP3-18. As shown in Fig. 1b, EAAC1 co-immunoprecipitated with c-Myc-GTRAP3-18 in the cell extract prepared from co-expression cells (lane 4), but not from EAAC1 (lane 2) or c-Myc-GTRAP3-18 (lane 3) single-expression cells, which eliminated the possibility of artefacts arising from nonspecific immunobead binding or an antibody cross-reaction, respectively. A truncated EAAC1 lacking the interacting C-terminal domain (see below) did not co-immunoprecipitate with c-Myc-GTRAP3-18 (lane 5), further indicating an interaction between EAAC1 and GTRAP3-18. This interaction was specific, as EAAT4, another neuronal glutamate transporter subtype, was not immunoprecipitated with c-Myc-GTRAP3-18 (lane 6). Identical results were obtained using other cell lines (COS-7 and C6 glioma; data not shown).

To study the protein interaction *in vivo*, we used anti-EAAC1 or GTRAP3-18 polyclonal antibodies to immunoprecipitate EAAC1 or GTRAP3-18 from rat brain extract. EAAC1 co-immunoprecipitated specifically with GTRAP3-18, but not GLAST, GLT-1 or EAAT4 (Fig. 1c). Similarly, GTRAP3-18 co-immunoprecipitated with EAAC1. These studies suggest that EAAC1 and GTRAP3-18 can interact *in vivo*.

GTRAP3-18 messenger RNA was widely expressed in brain regions and body organs, consistent with the distribution of EAAC1 (Fig. 2a)<sup>8,9</sup>. Similarly, GTRAP3-18 protein was expressed in many neural and non-neural tissues, when examined using a polyclonal oligopeptide antibody to the N terminus of GTRAP3-18 (Fig. 2b). GTRAP3-18 protein seemed to aggregate as multimers.



**Figure 2** Tissue and cellular distribution of GTRAP3-18 protein and mRNA. **a**, Northern blot analysis of GTRAP3-18 mRNA. **b**, Western blot analyses of GTRAP3-18 protein. Pure GTRAP3-18 (~30K, arrowhead) monomer tended to form multimers including the dimer (arrow). Dimeric GTRAP3-18 was found in HEK293 cells transfected with GTRAP3-18 cDNA and tissue homogenates. **c**, **d**, Immunostaining of GTRAP3-18 and EAAC1 proteins in rat brain. **e–h**, Confocal microscopy of cellular colocalization of GTRAP3-18 and EAAC1. In the cerebellum, GTRAP3-18 (**e**, green), EAAC1 (**f**, red) reveals prominent cytosolic colocalization (**g**, yellow). mo, molecular layer; pk, Purkinje cell layer; gr, granular layer. In transfected HEK293 cells (**h**), EAAC1 (red) tends to localize to the cell surface whereas GTRAP3-18 was found typically cytosolic and co-localized with EAAC1 (yellow) at the cell membrane.

The dimeric form of GTRAP3-18 was the predominant species in tissue homogenates, and it was also observed when purified GTRAP3-18 protein was detected using the N-terminal GTRAP3-18 antibody (Fig. 2b, lane 1), and when c-Myc-GTRAP3-18 protein was detected using anti-c-Myc antibodies (Fig. 2b, lane 2).

Immunohistological analysis of rat brain revealed that GTRAP3-18 protein was expressed widely (Fig. 2c) and primarily localized to neurons such as cerebellar Purkinje cells (Fig. 2e, g), identical to the expression and localization<sup>4</sup> of EAAC1 (Fig. 2f, g)<sup>2</sup>. In transfected HEK293 cells, EAAC1 protein (red) seemed to be aggregated at the cell membrane (Fig. 2h), whereas GTRAP3-18 protein (green) was typically localized to the cell membrane and cytosol, and co-associated with EAAC1 protein (yellow) at the cell membrane.

We tested whether GTRAP3-18 modulates EAAC1 function by studying, 72 h after transfection, sodium-dependent [<sup>3</sup>H]glutamate transport<sup>3,10</sup> in HEK293 cells co-expressing both proteins. Total glutamate transport decreased progressively with increasing expression of GTRAP3-18 protein (Fig. 3a). This effect was specific for EAAC1; co-expression of GTRAP3-18 with EAAT4 had no effect on transport activity. The inhibition of transport was not caused by a decrease of EAAC1 protein level by the co-expression of GTRAP3-18, as quantitated by western blot (Fig. 3b). Similarly, the loss of EAAC1 activity was not due to altered protein trafficking. Even at high levels of GTRAP3-18 expression—when little EAAC1-mediated transport was observed—surface biotinylation and confocal microscopy showed that EAAC1 surface expression was unaltered (Fig. 3b).

To evaluate the biochemical nature of altered transport, we carried out kinetic analyses with HEK293 cells co-expressing EAAC1 and GTRAP3-18. EAAC1 and GTRAP3-18 co-expressing cells showed a decrease in affinity (Michaelis constant,  $K_m = 40 \mu\text{M}$ ,  $n = 4$ ,  $P < 0.01$ ) without a shift in maximal velocity ( $V_{max} = 0.99 \text{ nmol min}^{-1}$  per mg protein), when compared with cells only expressing EAAC1 ( $K_m = 9 \mu\text{M}$ ;  $V_{max} = 1.02 \text{ nmol min}^{-1}$  per mg protein; Fig. 3c). Similar results were observed with other cell lines (COS7 and C6 glioma; data not shown).

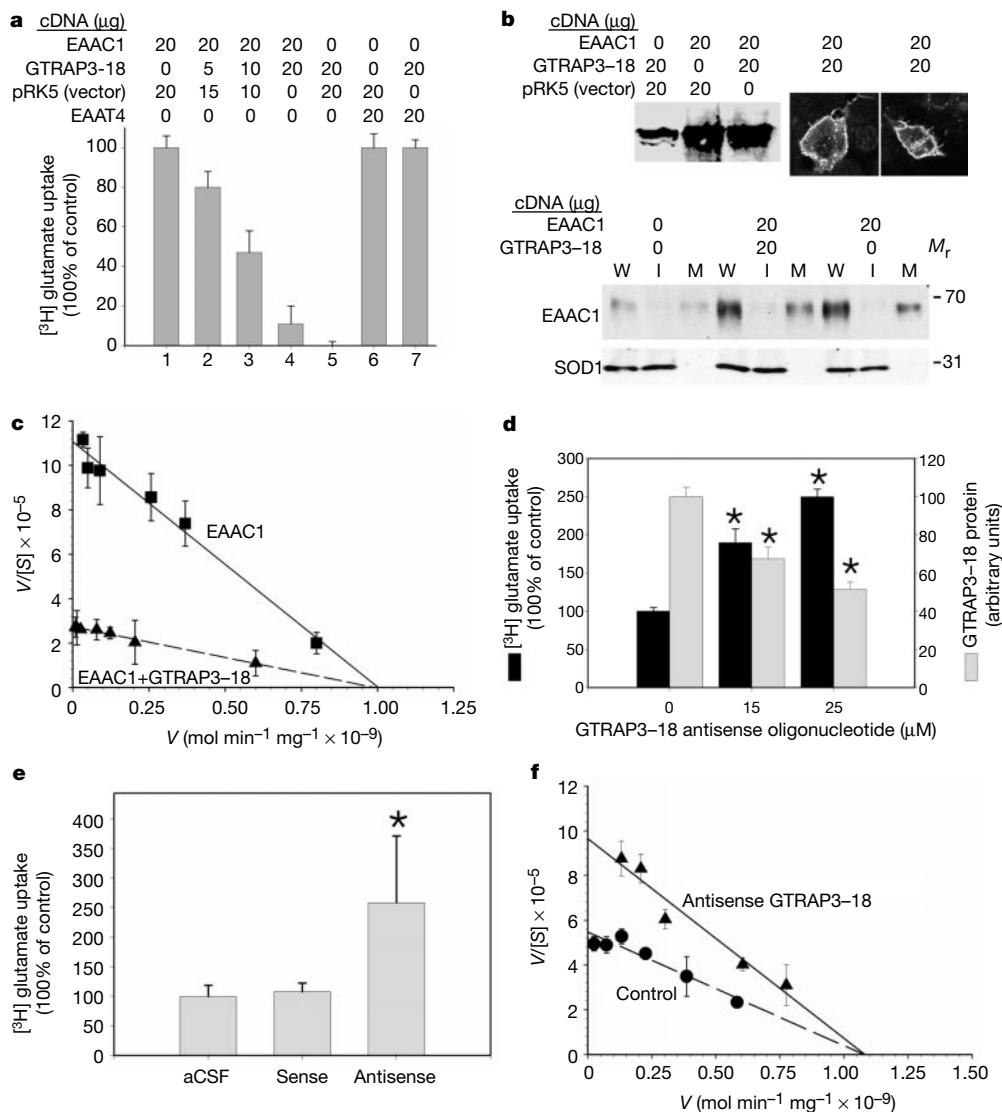
These studies indicate that GTRAP3-18 modulates EAAC1 transport activity by decreasing its affinity for glutamate. On the basis of these results, we thought that GTRAP3-18 might tonically modulate EAAC1 activity. To test this, we used antisense oligomers to lower GTRAP3-18 expression in HEK293 cells. Notably, western blot analyses and glutamate uptake assays revealed that HEK293 cells endogenously express some EAAC1 and GTRAP3-18 protein, but do not express the transporter subtypes GLAST, GLT-1 or EAAT4. We transfected antisense oligomers, targeted to the 5' GTRAP3-18 transcript, into HEK293 cells. Antisense oligomers specifically reduced endogenous GTRAP3-18 protein level (Fig. 3d, grey bars); EAAC1 protein level was not affected. Significantly, glutamate transport activity was elevated concomitantly with the reduction of GTRAP3-18 protein level (black bars).

We determined whether the *in vitro* modulation of EAAC1 by GTRAP3-18 is physiologically relevant, by administering GTRAP3-18 antisense oligomers intraventricularly. Eleven days of antisense treatment resulted in a reduction of GTRAP3-18 protein level and a significant increase in cortical glutamate uptake, whereas glutamate uptake was not altered in animals treated with the sense oligomer (Fig. 3e). The effect was due to increased EAAC1-mediated transport because it was not altered by dihydrokainic acid (DHK), an inhibitor of GLT-1-mediated glutamate transport<sup>11</sup>. In kinetic studies of DHK-insensitive, cortical glutamate uptake from antisense-treated animals, the apparent affinity for glutamate was increased (antisense  $K_m = 10 \mu\text{M}$ ;  $V_{max} = 1.08 \text{ nmol min}^{-1}$  per mg protein) compared with artificial cerebrospinal-fluid (CSF)-treated or sense-treated control animals (control  $K_m = 19.7 \mu\text{M}$ ;  $V_{max} = 1.08 \text{ nmol min}^{-1}$  per mg protein; Fig. 3f). These results suggest that GTRAP3-18 negatively modulates EAAC1 glutamate transport activity *in vivo*.

Human GTRAP3-18 (JWA protein), as described above, was originally identified as a retinoic-acid-responsive gene. We therefore tested whether retinoic acid could upregulate GTRAP3-18 expression and consequently inhibit EAAC1-mediated glutamate transport in HEK293 cells. Retinoic acid induced a large increase in GTRAP3-18 expression, over a non-toxic dose range from 1 to 10  $\mu\text{M}$  (Fig. 4a). A significant decrease in glutamate transport activity paralleled the increase of GTRAP3-18 protein level (Fig. 4a). Fluorescence microscopy indicated that this loss of transport activity was not due to changes in EAAC1 protein level (Fig. 4a) or in the cellular membrane localization of EAAC1 protein induced by retinoic acid (Fig. 4b).

To confirm that loss of transport activity was specifically due to GTRAP3-18 and not by other factors induced by retinoic acid or direct effects on EAAC1, we constructed a truncated EAAC1 cDNA,

lacking the last 93 amino acids. The truncation corresponded to the region used as bait in yeast two-hybrid screening, and was not able to interact with GTRAP3-18 (Fig. 1b, lane 5). Nevertheless, after transient expression in HEK293 cells, the truncated EAAC1 transported glutamate. Retinoic acid treatment did not alter activity of the truncated EAAC1 protein; even though GTRAP3-18 protein expression was elevated markedly (Fig. 4c). Thus, the loss of transport activity caused by retinoic acid was the result of GTRAP3-18 induction. Notably, truncated EAAC1 had increased glutamate transport activity as compared with wild type. Truncated EAAC1 had a  $K_m$  of 5.4  $\mu\text{M}$ , which was more than a threefold increase in affinity over that of wild-type EAAC1 ( $K_m = 17 \mu\text{M}$ ; Fig. 4d). This might mean that endogenous EAAC1 is normally inhibited by GTRAP3-18—an effect that was eliminated in a truncated EAAC1, and that was mimicked by GTRAP3-18 antisense



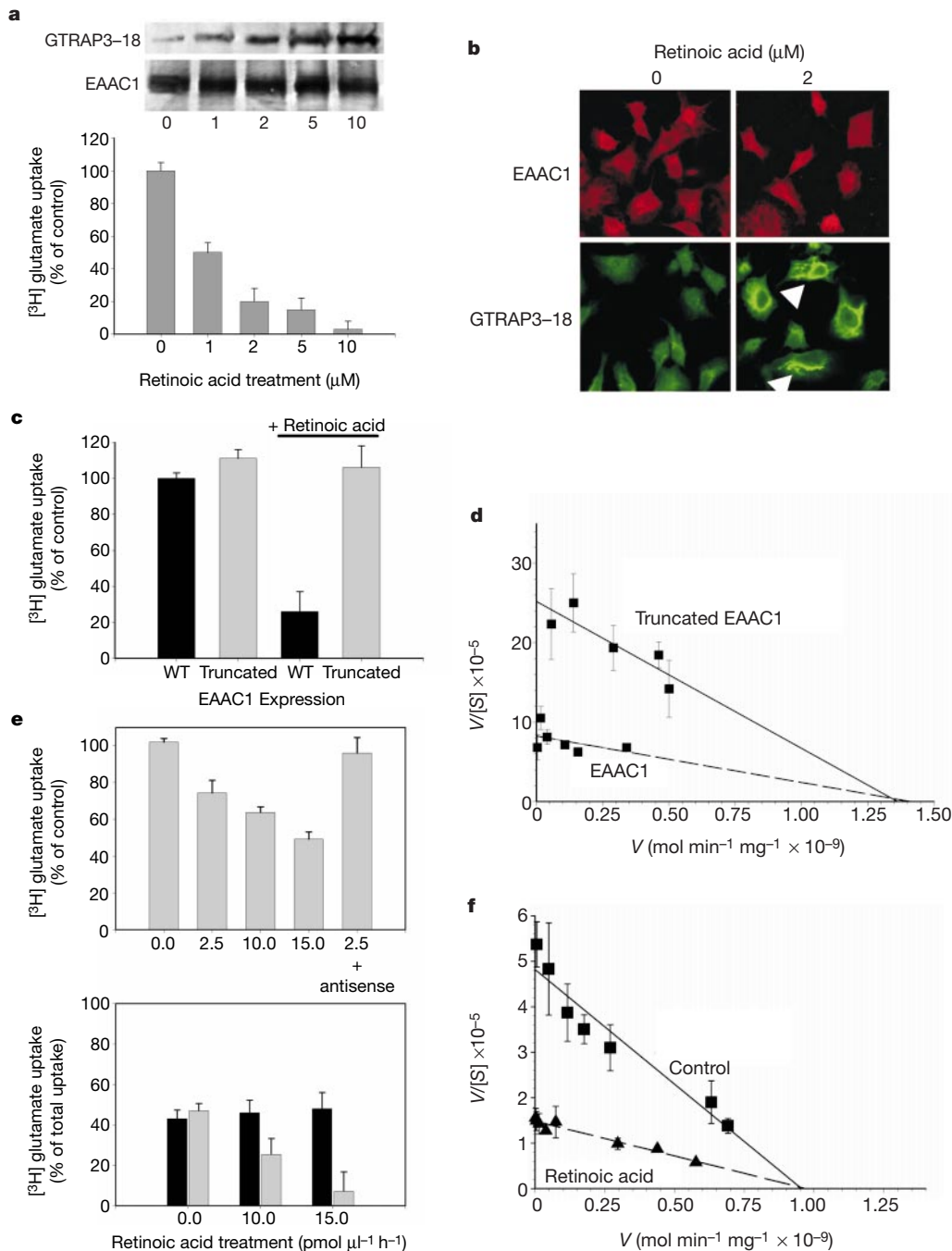
**Figure 3** GTRAP3-18 negatively modulates EAAC1-mediated glutamate transport. **a**, Glutamate transport in HEK293 cells transfected with the indicated plasmids. GTRAP3-18 inhibited EAAC1-mediated transport, but had no effect on EAAT4 ( $n = 6$ ). **b**, Western blot showed that the co-expression of GTRAP3-18 had no effect on total EAAC1 protein expression (top, left). Analysis of HEK293 cells by confocal microscopy (top panel, right) and surface biotinylation (bottom) revealed no alteration in the membranous localization of EAAC1. I, intracellular fraction; M, cellular membrane fraction; W, whole cell. Superoxide dismutase (SOD1) was used as a control. **c**, Edie-Scatchard plot of glutamate transport in transfected HEK293 cells showed a 4–10-fold decrease in affinity ( $n = 4$ ). **d**, Antisense

GTRAP3-18 oligomers reduced endogenous GTRAP3-18 expression in HEK293 cells and produced an increase in EAAC1 transport. Asterisks indicate statistical significance (Students  $t$ -test;  $P < 0.01$ ) versus untreated control ( $n = 4$ ). **e**, Intraventricular antisense delivery led to a 50% loss of cortical GTRAP3-18 levels and a significant increase in dihydrokainate-insensitive glutamate uptake. Asterisk indicates statistical significance (Students  $t$ -test;  $P < 0.05$ ) versus artificial CSF (aCSF) control ( $n = 7$ ). **f**, Kinetic analysis of cortical tissue from rats treated with antisense GTRAP3-18 shows an increase in apparent affinity for glutamate. All errors bars represent standard deviation.

treatment (Fig. 3f).

To test this hypothesis *in vivo*, retinoic acid was infused intravenicularly. After 4 d of treatment, cortical GTRAP3-18 protein expression was increased in a dose-dependent manner, and this was associated with a significant decrease of total glutamate uptake (Fig. 4e, top panel). This effect was specifically due to decreased EAAC1-mediated transport because it was not altered by the glutamate transport inhibitor dihydrokainic acid, at a concentra-

tion that predominantly effects GLT-1 (Fig. 4e, bottom panel)<sup>11</sup>. Kinetic analysis of DHK-insensitive, cortical glutamate transport from animals treated 4 d with intraventricular retinoic acid revealed a fourfold decrease in affinity over control transport (Fig. 4f)—very similar to that seen *in vitro* (Fig. 3c). In addition, retinoic acid inhibition of glutamate transport could be reversed *in vivo*; chronic intraventricular treatment with antisense GTRAP3-18 oligomer (50–100 ng d<sup>-1</sup>, for 7–10 d) blocked the retinoic acid (2.5 μM)



**Figure 4** Retinoic acid upregulates GTRAP3-18 expression and consequently inhibits EAAC1 transport in HEK293 cells in brain. **a**, After 14 d, retinoic acid induced GTRAP3-18 expression and inhibited transport ( $n = 5$ ) in HEK293 cells. **b**, Membrane localization of EAAC1 protein was not changed by retinoic acid, as visualized by fluorescent microscopy. GTRAP3-18 expression, particularly in a subcellular compartment, was greatly induced by retinoic acid (arrow). **c**, Glutamate transport by HEK293 cells expressing truncated EAAC1 (lacking a C-terminal interacting domain) was not inhibited by 14 d of treatment with retinoic acid (5 μM;  $n = 3$ ). **d**, Truncated EAAC1 expressed by HEK293 cells has

increased apparent affinity for glutamate compared with wild type (WT) EAAC1. **e**, Intraventricular delivery of retinoic acid (3–5 d) inhibited glutamate uptake; the effect was blocked by concomitant treatment with GTRAP3-18 antisense oligomers (5 μg μl<sup>-1</sup>; 1 μl h<sup>-1</sup>; top panel). This effect was specific for DHK-insensitive transport (bottom panel, grey bars)—a crude partial estimate of EAAC1 activity *in vivo*—but not for DHK-sensitive transport (EAAT2-mediated) (black bars;  $n = 5$ ). **f**, Kinetic analysis of cortical uptake from rats treated with retinoic acid (2.5 pmol l<sup>-1</sup> h<sup>-1</sup> for 4 d) showed a large decrease in apparent affinity for glutamate ( $n = 3$ ). All errors bars represent standard deviation.

induction of GTRAP3-18, and also blocked the inhibition of glutamate transport seen with retinoic acid treatment (Fig. 4e, top panel). Retinoic acid had no effect on glutamate transport by cells expressing GLT-1 or EAAT4.

In brain, glutamate serves as an excitatory neurotransmitter, a metabolic substrate for other neurotransmitters (that is, GABA ( $\gamma$ -amino butyric acid)), and an amino acid for general cellular metabolism. In brain, glutamate transporters maintain low extracellular glutamate and influence the kinetics of glutamate receptor activation<sup>12–14</sup>. Little is known about the molecular and protein regulation of glutamate transporters, or for neurotransmitter transporters in general. In tumour cell lines, for example, the cell-surface expression of EAAC1 appears to be regulated by pathways mediated by both protein kinase C and phosphatidylinositol-3-OH kinase, albeit through as yet unidentified proteins<sup>7,15</sup>. GTRAP3-18 appears to be an endogenous inhibitory regulator of EAAC1. EAAC1 is found throughout the brain on somas and dendrites of small and large pyramidal neurons<sup>2,4</sup>. EAAC1 is also localized to pre-synaptic GABA-containing terminals, and may have a metabolic role in providing glutamate for GABA metabolism<sup>4,16</sup>. Loss of brain EAAC1 expression interferes with GABA synthesis and results in epilepsy<sup>3,17</sup>. In kidney, EAAC1 may contribute to renal acidic amino-acid reabsorption, acid/base balance, cell volume regulation, and amino-acid metabolism<sup>5,6</sup>. GTRAP3-18 may have a critical role in the regulation of the neurotransmitter and metabolic functions of EAAC1. □

## Methods

### Yeast two-hybrid screening

The MATCHMAKER Two-Hybrid System (Clontech) was used for screening. We screened a rat brain cDNA library with a bait protein corresponding to C-terminal intracellular domain of EAAC1 (last 87 amino acids, cDNA position 1,458–1,719). The positive clones were selected and assayed for  $\beta$ -galactosidase activity. Plasmid DNAs were isolated from positive clones and co-transformed with bait cDNA back into yeast to reconfirm the interaction.

### GST fusion proteins

The GST Gene Fusion System (Pharmacia) was used to construct and generate GST–EAAC1 and GST–GTRAP3-18 fusion proteins using pGEX–6P-1 vector.

### In vitro cell-free binding assay

The TnT Coupled Reticulocyte Lysate System (Promega) was used to generate [<sup>35</sup>S]methionine-labelled GTRAP3-18 protein using the pRK5–GTRAP3-18 plasmid DNA as a template, driven by SP6 RNA polymerase. GST–EAAC1 fusion proteins were expressed in *Escherichia coli* and recovered on glutathione–sepharose beads as described above. The beads were incubated with lysate which containing [<sup>35</sup>S]labelled GTRAP3-18 protein for 1 h at 4 °C and then washed five times with NETN buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). The beads were boiled in sample buffer (SB) (2% SDS, 10% glycerol, 62 mM Tris pH 6.8) and bound proteins resolved in SDS–PAGE.

### Gene constructions and mammalian cell expression system

We used the eukaryotic expression vectors pcDNA3 and pRK5 for expression of cDNAs in mammalian cell lines. Full-length EAAC1 cDNA was subcloned into *NotI*, *EcoRI* sites of the pcDNA3 vector. For the truncated EAAC1, the last 279 nucleotides were removed by *AatII* and *EcoRI* digestion in a pcDNA3/EAAC1 construct and blunt ended. For tagging GTRAP3-18 with c-Myc, GTRAP3-18 cDNA was first subcloned into downstream of c-myc gene (*NotI* site) in the pTYGL vector. The *EcoRI* /*BamHI* fragment containing Myc–GTRAP3-18 fusion gene was then subcloned into pRK5. The constructed plasmid DNA was transfected into HEK293, COS-7 or C6 glioma cell lines by standard electroporation, and 72 h later cells were collected for various assays.

### Immunoprecipitation, biotinylation and transport assays

For the transfected cells, cells were solubilized in 1 ml of IPB buffer (10 mM Tris pH 7.6, 5 mM EDTA, 5 mM EGTA, 1 mM sodium orthovanadate, aprotinin 15  $\mu$ g ml<sup>-1</sup>, 0.1 mM PMSF) with 1% Triton at 4 °C for 30 min with gentle rotation. Cell debris was removed (15,000g, 15 min), and supernatants were incubated (4 °C, 16 h) with protein A/sepharose beads (150  $\mu$ l, CL-4B, Amersham) and primary antibodies (1.5 mg ml<sup>-1</sup>). The immunobead-bound protein complexes were washed three times (IPB buffer in 1% Triton X-100, 100 mM NaCl) followed by three IPB buffer washes. Immunoprecipitated proteins were boiling in SB and analysed by western blotting.

For the rat brain, coronal sections of brain were sliced at 1–2-mm intervals from the cerebellum to the olfactory bulbs. The cortex region was excised from the brain and placed in cold buffer A (50 mM Tris pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.5 mM dithiothreitol).

The tissue was washed three times in buffer A and then weighed. The tissue was then homogenized using a blender in 2.5 volumes of buffer B (50 mM Tris pH 7.5, 10% glycerol, 5 mM magnesium acetate, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM PMSF). The particulate material was removed by centrifugation at 15,000g for 30 min at 4 °C. The supernatant fraction was incubated with protein A/sepharose beads and primary antibodies as above.

Biotinylation was performed as described<sup>15</sup> with modifications. Glutamate uptake assays were done as described on either cells grown on six-well plates (up to 1 mg protein) or brain tissues (100–150  $\mu$ g protein). To block GLT-1 transport activity, homogenates were pre-incubated with dihydrokainic acid (300  $\mu$ M, Sigma) for 20–60 min before assay.

### Blotting, immunostaining and antibodies

The methods for isolation of RNA and northern blotting, western blotting and immunostaining have been described<sup>18</sup>. We synthesized a synthetic peptide corresponding to an N-terminal domain of GTRAP3-18 protein NH<sub>2</sub>-K<sup>14</sup>FFPGSDRFARPDFRD<sup>28</sup>-COOH and used this to generate polyclonal, affinity-purified antibodies from New Zealand White rabbits<sup>2,19</sup>. Confocal microscopy of transfected cells of brain sections was performed with a Zeiss LSM 510 laser scanning microscope using fluorescein (Vector, FI1000) and Texas red (Vector, TI2000) fluorochromes.

### Intraventricular antisense/drug administration

We used the following sequences for the new phosphodiester oligonucleotides: sense GTRAP3-18, 5'-GTGAACCTTGCCCGCTC-3'; antisense GTRAP3-18, 5'-GAGCGG GGCAAGGTTTACAC-3'. Oligonucleotides (5  $\mu$ g  $\mu$ l<sup>-1</sup>), retinoic acid (1–20  $\mu$ M; 0–20 pmol  $\mu$ l<sup>-1</sup>) separately or in combination were administered intraventricularly over 3–11 d, by mini-osmotic pumps (Alza Corporation, Palo Alto, CA) as described<sup>3</sup>.

Received 5 October; accepted 20 November 2000.

1. Kanai, Y. & Hediger, M. A. Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* **360**, 467–471 (1992).
2. Rothstein, J. D. et al. Localization of neuronal and glial glutamate transporters. *Neuron* **13**, 713–725 (1994).
3. Rothstein, J. D. et al. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* **16**, 675–686 (1996).
4. He, Y., Janssen, W. G. M., Rothstein, J. D. & Morrison, J. H. Differential synaptic localization of the glutamate transporter EAAC1 and glutamate receptor subunit GluR2 in the rat hippocampus. *J. Comp. Neurol.* **418**, 255–269 (2000).
5. Shayakul, C. et al. Localization of the high-affinity glutamate transporter EAAC1 in rat kidney. *Am. J. Physiol. Renal Physiol.* **42**, F1023–F1029 (1997).
6. Peghini, P., Janzen, J. & Stoffel, W. Glutamate transporter EAAC-1-deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. *EMBO J.* **16**, 3822–3832 (1997).
7. Sims, K. D., Straff, D. J. & Robinson, M. B. Platelet-derived growth factor rapidly increases activity and cell surface expression of the EAAC1 subtype of glutamate transporter through activation of phosphatidylinositol 3-kinase. *J. Biol. Chem.* **275**, 5228–5237 (2000).
8. Hediger, M. A. & Welbourne, T. C. Introduction: glutamate transport, metabolism, and physiological responses. *Am. J. Physiol.* **277**, F477–F480 (1999).
9. Hediger, M. A. Glutamate transporters in kidney and brain. *Am. J. Physiol.* **277**, F487–F492 (1999).
10. Lin, C. G. et al. Aberrant RNA processing in a neurodegenerative disease: The cause for absent EAAT2 a glutamate transporter, in amyotrophic lateral sclerosis. *Neuron* **20**, 589–602 (1998).
11. Robinson, M. B. The family of sodium-dependent glutamate transporters: a focus on the GLT-1/EAAT2 subtype. *Neurochem. Int.* **33**, 479–491 (1998).
12. Bergles, D. E. & Jahr, C. E. Synaptic activation of glutamate transporters in hippocampal astrocytes. *Neuron* **19**, 1297–1308 (1997).
13. Bergles, D. E., Dzuba, J. A. & Jahr, C. E. Glutamate transporter currents in Bergmann glial cells follow the time course of extrasynaptic glutamate. *Proc. Natl. Acad. Sci. USA* **94**, 14821–14825 (1997).
14. Diamond, J. S. & Jahr, C. E. Transporters buffer synaptically released glutamate on a submillisecond time scale. *J. Neurosci.* **17**, 4672–4687 (1997).
15. Davis, K. E. et al. Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma. *J. Neurosci.* **18**, 2475–2485 (1998).
16. Conti, F., DeBiasi, S., Minelli, A., Rothstein, J. D. & Melone, M. EAAC1, a high-affinity glutamate transporter, is localized to astrocytes and GABAergic neurons besides pyramidal cells in the rat cerebral cortex. *Cereb. Cortex* **8**, 108–116 (1998).
17. Sepkuty, J., Eccles, C. U., Lesser, R. P., Dykes-Hoberg, M. & Rothstein, J. D. Molecular knockdown of neuronal glutamate transporter EAAT3 produces epilepsy and dysregulation of GABA metabolism. *Soc. Neurosci. Abstracts* **23**, 1484 (1997).
18. Lin, C. L. G. et al. Molecular cloning and expression of the rat EAAT4 glutamate transporter subtype. *Mol. Brain Res.* **63**, 174–179 (1998).
19. Furuta, A., Martin, L. J., Lin, C. L. G., Dykes-Hoberg, M. & Rothstein, J. D. Cellular and synaptic localization of the neuronal glutamate transporters excitatory amino acid transporter 3 and 4. *Neuroscience* **81**, 1031–1042 (1997).

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.

### Acknowledgements

We thank R. Haganir for the pRK5 vector; J. Sepkuty, R. Ganel, and W. Song for helpful suggestions and discussions; and L. Jin, C. Coccia and B. Kim for technical support.

Correspondence and requests for materials should be addressed to J.D.R. (e-mail: jrothste@welchlink.welch.jhu.edu). GenBank accession number for GTRAP3-18 is AF240182.