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Interaction between the glutamate transporter GLT1b and the synaptic PDZ domain protein PICK1

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Abstract

Synaptic plasticity is implemented by the interaction of glutamate receptors with PDZ domain proteins. Glutamate transporters provide the only known mechanism of clearance of glutamate from excitatory synapses, and GLT1 is the major glutamate transporter. We show here that GLT1 interacts with the PDZ domain protein PICK1, which plays a critical role in regulating the expression of glutamate receptors at excitatory synapses. A yeast two-hybrid screen of a neuronal library using the carboxyl tail of GLT1b yielded clones expressing PICK1. The GLT1b C-terminal peptide bound to PICK1 with high affinity (K_i = 6.5 ± 0.4 μM) in an *in vitro* fluorescence polarization assay. We also tested peptides based on other variants of GLT1 and other glutamate transporters. GLT1b co-immunoprecipitated with PICK1 from rat brain lysates and COS7 cell lysates derived from cells transfected with plasmids expressing PICK1 and GLT1b. In addition, expression of GLT1b in COS7 cells changed the distribution of PICK1, bringing it to the surface. GLT1b and PICK1 co-localized with each other and with synaptic markers in hippocampal neurons in culture. Phorbol ester, an activator of protein kinase C (PKC), a known PICK1 interactor, had no effect on glutamate transport in rat forebrain neurons in culture. However, we found that exposure of neurons to a myristolated decoy peptide with sequence identical to the C-terminal sequence of GLT1b designed to block the PICK1–GLT1b interaction rendered glutamate transport into neurons responsive to phorbol ester. These results suggest that the PICK1–GLT1b interaction regulates the modulation of GLT1 function by PKC.

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Keywords

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Introduction

The extracellular concentration of the excitatory neurotransmitter L-glutamate in the CNS must be kept low to ensure a high signal to noise ratio during synaptic activation (Tanaka *et al*., 1997; Katagiri *et al*., 2001) and to prevent excitotoxicity due to excessive activation of glutamate receptors (Mangano & Schwarcz, 1983; Rosenberg & Aizenman, 1989; Rosenberg *et al*., 1992; Rothstein *et al*., 1996; Tanaka *et al*., 1997; Wang *et al*., 1998a), and this function is served by glutamate transporter proteins. Glutamate transporters help to shape the time-course of glutamate concentrations in the synaptic cleft after release from the presynaptic terminal (Mennerick & Zorumski, 1994; Tong & Jahr, 1994; Tanaka *et al*., 1997; Overstreet *et al*., 1999; Auger & Attwell, 2000; Turecek & Trussell, 2000; Brasnjo & Otis, 2001; Huang *et al*., 2004), and prevent cross-talk between neighboring excitatory synapses (Asztely *et al*., 1997; Rusakov & Kullmann, 1998). Five distinct Na^+-dependent high-affinity glutamate transporters have been cloned (Danbolt, 2001): GLAST, GLT1, EAAC1, EAAT4 and EAAT5. GLT1 is the predominant glutamate transporter in the forebrain, and is expressed in at least two variant forms, GLT1a (Pines *et al*., 1992) and GLT1b (Chen *et al*., 2002; Schmitt *et al*., 2002), both of which are expressed in neurons as well as in astrocytes (Chen *et al*., 2002, 2004; Reye *et al*., 2002). The expression and function of glutamate transporters are modulated by learning (Levenson *et al*., 2002; Pita-Almenar *et al*., 2006), drugs of abuse (Ozawa *et al*., 2001; Mao *et al*., 2002; Xu *et al*., 2003) and pain (Niederberger *et al*., 2003; Sung *et al*., 2003).

All known glutamate transporters terminate in a PDZ domain interaction motif, except GLT1a (Fig. 1A). PDZ domain interactions are important in the regulation of glutamate receptor trafficking and function (Sheng & Sala, 2001; Song & Huganir, 2002). These interactions are dependent upon the C-terminal three–four amino acids of the interacting protein. PICK1 is a PDZ domain protein that was originally discovered as a protein kinase C(PKC)α binding protein (Staudinger *et al*., 1995, 1997). The PKCα–PICK1 interaction was later found to be dependent upon the activation of PKCα, which appears to cause the exposure of the C-terminus of PKCα (Perez *et al*., 2001). PICK1 may provide a means to bring activated PKCα into close proximity with other proteins to promote their phosphorylation, and has been shown to form heteromultimers with activated PKCα and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Perez *et al*., 2001). Here we report that PICK1 interacts with GLT1b, and that this interaction appears to regulate the modulation of GLT1 function by PKC.

Materials and methods

Ethics

All procedures on animals were performed in conformance with Children's Hospital (CH) policy that all research involving animals is conducted under humane conditions, with

appropriate regard for animal welfare. CH is a registered research facility with the United States Department of Agriculture (USDA), and is committed to comply with the Guide for the Care and use of Laboratory Animals (Department of Health and Human Services), the provisions of the Animal Welfare Act (USDA), and all applicable federal and state laws and regulations. CH has developed institutional standards for the humane care and use of animals that are maintained through published policies. An Animal Care Committee has been established to ensure compliance with all applicable federal and state regulations for the purchase, transportation, housing and research use of animals. CH has filed appropriate assurance of compliance with the Office for the Protection of Research Risks of the National Institutes of Health. Discomfort and injury to animals were limited to that which was unavoidable in the conduct of scientifically valuable research. Analgesic, anesthetic and tranquilizing drugs were used where indicated and appropriate to minimize discomfort and pain to animals. Specifically, animals to be used as a source of tissue for tissue culture or immunoblot experiments were anesthetized by CO_2 narcosis and then decapitated.

Antibodies

A polyclonal antibody against the C-terminal peptide NH_2-ECKVPFPFLDIETCI- COOH corresponding to the last 15 amino acids (amino acid 548–562) of GLT1b (anti-cGLT1b) and a polyclonal antibody against the N-terminal peptide NH_2-MASTEGANNMPKQVE-COOH (amino acids 1–15 of GLT1) recognizing both GLT1a and GLT1b (anti-nGLT1) were generated in rabbits and previously characterized (Chen *et al*., 2002, 2004). An anti-PICK1 antibody was raised in chickens against the C-terminal 16 amino acid sequence of PICK1 (401–416; NH_2-TWATGPTDKGGSWCDSCOOH). The polyclonal antibody against the C-terminus of GLT1a (anti-cGLT1a antibody) based on the published sequence (amino acid 559–573 of GLT1) was generously provided by Dr Jeff Rothstein (Johns Hopkins University) and has been previously characterized (Rothstein *et al*., 1994; Chen *et al*., 2002, 2004). A monoclonal antibody against the C-terminus of GLT1a was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). A monoclonal anti-synaptophysin antibody was obtained from Sigma Chemical, St Louis, MO, USA, and a monoclonal anti-NR1 antibody was obtained from Chemicon, Temecula, CA, USA.

Yeast two-hybrid screening using the C-terminus of GLT1b

Yeast two-hybrid screening was performed using the ProQuest Two-Hybrid System (Invitrogen, Carlsbad, CA, USA) with the reporter genes *HIS3*, *URA3* and *lacZ* under the control of upstream GAL4 binding sites (Vidal, 1997). The entire C-terminal cytoplasmic domain of GLT1b (amino acids 469–562) was subcloned in-frame with the GAL4 DNA-binding domain of pDBLeu vector as bait. A rat forebrain neuronal cDNA library was inserted into the GAL4 activation domain vector pPC86. Growth assay was performed by selection on plates free of leucine, tryptophan and histidine. Positive colonies were tested for β-galactosidase activity by transferring them onto filter paper saturated with X-gal. DNA from the positive colonies was isolated and transformed into DH10 bacterial cells by electroporation. Amplified plasmid DNAs were analysed by restriction enzymes and sequenced.

C-terminal deletions were generated by polymerase chain reaction (PCR) and subsequently fused in-frame with the GAL4 DNA-binding domain of the pDBLeu vector. Plasmids expressing GLT1b, GLT1a or GLT1b mutations were co-transformed with PICK1-expressing plasmids into yeast cells, and spread on plates free of leucine and tryptophan. Growth assays on plates free of leucine, tryptophan and histidine, and X-gal assays were used to confirm the interaction or lack of it.

Purification of GST–rPICK1

Rat PICK1 (rPICK1) was purified as previously described (Madsen *et al*., 2005). Briefly, the entire coding region of rPICK1 2–416 was amplified and introduced in-frame with GST in the fusion vector pET41 using the MunI and AvrII sites. The GST–PICK1 fusion was expressed in the BL21DE3 pLysS strain by induction at OD_{600} = 0.6 using 0.5 μM IPTG (Sigma) and grown at 30 °C for 3 h. The bacteria were freeze/thaw lysed in buffer A [Tris, 50 mM, pH 7.4; NaCl, 125 mM; Triton X-100, 0.1%; DNAse I, 20 μg/mL; dithiothreitol (DTT), 1 mM (Sigma); bacterial protease inhibitor cocktail (Sigma)]. Membranes and cellular debris were pelleted, and the supernatant incubated with glutathione-coated Sepharose beads (Pharmacia) under slow rotation. The beads were subjected to three bulk washes in buffer A, and PICK1 was cleaved from GST overnight at 4 °C with thrombin protease in buffer A. The purified protein was routinely analysed by fast performance liquid chromatography (FPLC) size-exclusion chromatography revealing a single protein peak with an estimated molecular mass of approximately 100 kDa corresponding to a homodimer (data not shown). The integrity of the protein was further substantiated by circular dichroism denaturation experiments showing evidence for secondary structure that was markedly reduced upon extended incubation (> 3 days; T.S. Thorsen, K.L. Madsen, M. Gajhede, J. Kastrup and U. Gether, unpublished observation).

Peptide binding using fluorescence polarization

The PICK1 fluorescence polarization assay was performed according to previously described protocols (Madsen *et al*., 2005). The competition binding was done by titrating a fixed concentration of PICK1 (~1 μM) and an Oregon Green-labeled peptide of the 13 C-terminal residues of the dopamine transporter (DAT; ~40 nM) with increasing amounts of different non-labeled peptides [all synthesized and high-pressure liquid chromatography (HPLC)-purified by the Tufts University Core Facility (Boston, MA, USA)]. The assay was performed in 100-μL volumes in microtiter-plates and read with a Chameleon plate-reader (HIDEX) in the FP mode using a 488-nm excitation filter and a 535-nm long-pass emission filter. FP was calculated according to the equation: $FP = (I_V - g \times I_H)/(I_V + g \times I_H)$, where I_V and I_H are the fluorescence measured in the vertical and horizontal plane, respectively, and g is an apparatus-specific correction factor. Equilibrium competition binding isotherms are constructed by plotting FP vs the concentration of unlabeled peptide. To determine K_i, a curve was fitted to the equation $FP = FP_f + ((FP_b - FP_f) \times [R_t])/(K_d \times (1 + X/K_i) + [R_t])$, with FP_f and FP_b being the FP value of the free and bound peptide, $[R_t]$ the concentration of PICK1, and K_d the apparent dissociation constant determined from parallel saturation experiments. K_i, FP_b and FP_f were treated as free parameters. The binding isotherms were constructed from three independent purifications, and data analysis was performed in Prism 4.0 (GraphPad Software).

Expression of recombinant proteins in COS7 cells

COS7 cells were maintained at 37 °C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM; Invitrogen 11960-044) supplemented with 10% fetal bovine serum and non-essential amino acids (Invitrogen 11140-035, diluted 1 : 100) in 24-well plates. The coding sequences of GLT1b and GLT1a were subcloned downstream from the CMV promoter in the mammalian expression vector pcDNA3 (Invitrogen), and the PICK1 coding region was subcloned in-frame at the 3′-end of the DNA encoding EGFP in the EGFP-C1 vector (BD Biosciences Clontech, Palo Alto, CA, USA). A deletion mutant lacking the last four amino acids was generated by PCR mutagenesis. All constructs were confirmed by sequencing. At 60–80% confluence, cells were transfected with GLT1a, GLT1b, GLT1bΔETCI (last four amino acids deleted), EGFP or EGFP–PICK1 using Lipofectamine 2000 reagent (Invitrogen). For each well, 800 ng DNA was mixed with 50 μL OptiMEM medium and, in a separate tube, 2 μL Lipofectamine 2000 with 50 μL OptiMEM. Both tubes were incubated for 5 min at room temperature. Both solutions were combined to obtain the transfection mix and incubated for 20 min at room temperature, then added to each well of a 24-well plate of cells that had been washed once with phosphate-buffered saline (PBS). Cells were then incubated at 37 °C for 6 h before medium was changed back to normal growth medium and cells were incubated for 24 h before immunocytochemistry studies.

Immunocytochemistry on COS7 cells

In brief, COS7 cells were washed twice in PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then rinsed three times with PBS. PBS buffer with 0.1% Triton X-100 containing normal goat serum (5%) and 1% bovine serum albumin (BSA) was used to permeabilize cells and to block non-specific protein binding sites. Cells were incubated in anti-nGLT1 antibody (1.5 μg/mL), washed three times with PBS and detected with goat anti-rabbit IgG conjugated with Alexa594 Red. Coverslips were washed twice with PBS and incubated with bisbenzamide (1 μg/mL) for 10 min. Coverslips were subsequently mounted with Fluoromount G (Fisher) and examined by confocal fluorescence microscopy. The anti-nGLT1 antibody labeled COS7 cells only if they had been transfected with GLT1-expressing plasmids. Occasionally nucleoli staining was observed in COS7 cells.

Primary culture of hippocampal neurons and immunocytochemistry

Cultures of hippocampal neurons were prepared using methods similar to those previously described (Goslin & Banker, 1991), with modification to permit the selective culturing of neurons (Rosenberg, 1991; Wang *et al*., 1998b). Briefly, embryonic day 18 Long–Evans rat hippocampi were removed and dissociated using papain (Worthington, Lakewood, NJ, USA) 10 U/mL for 15 min followed by chicken egg white trypsin inhibitor (Sigma T-9253) 10 mg/mL and trituration in Hank's balanced saline solution (HBSS). Cells were plated in growth medium [Neurobasal medium (Invitrogen) supplemented with 2% B27 supplement (Invitrogen), 500 μM glutamine, 25 μM glutamate and penicillin (100 U/mL) and streptomycin (100 μg/mL)] at a density of 75 000 cells/well in 12-well plates precoated overnight with 37.5 μg/mL poly-D-lysine (Fisher Scientific, Suwanee, GA, USA) and 2.5 μg/mL laminin

(Fisher) in water. On the second day *in vitro*, 5 μM cytosine arabinoside was added for 48 h to inhibit cell proliferation. On the fourth day of culture, the medium was completely removed and replaced with growth medium that had been conditioned by contact with astrocyte cultures for 24 h. Medium was then partially changed (50%) every 3 days with the astrocyteconditioned medium.

At the 14th day *in vitro*, neuronal cells were fixed with 4% paraformaldehyde in HBSS for 10 min at room temperature and then rinsed three times with Tris-buffered saline (TBS buffer) containing 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl. TBS buffer with 0.1% Triton X-100 (TBS-T) containing normal goat serum (4%) was used to permeabilize cells and to block non-specific protein binding sites. Cells were incubated in anti-cGLT1b antibody (3 μg/mL) solution at 4 °C overnight, washed three times with TBS-T and detected with goat anti-rabbit IgG conjugated with Oregon Green (Molecular Probes, Eugene, OR, USA) at 1 : 500 dilution. Then the coverslips were washed three times with TBS-T and blocked again with 4% normal goat serum in TBS-T for 1 h. Anti-PICK1 antibody at 0.64 μg/mL, mouse monoclonal anti-synaptophysin antibody (Sigma Chemical) at 1 : 250 dilution, or mouse monoclonal anti-NR1 antibody (Chemicon) in 2% goat serum at 1 : 200 dilution were then added overnight. Coverslips were then washed three times with TBS-T and detected with goat anti-chicken IgG conjugated with Alexa 488 (for anti-PICK1 antibody) or goat anti-mouse IgG (for anti-synaptophysin or anti-NR1) conjugated with Alexa594 (Molecular Probes) at 1 : 500 dilution. Coverslips were subsequently mounted with Fluoromount G (Fisher) and examined by confocal fluorescent microscopy. No cross-reaction was found between the anti-chicken, anti-mouse or anti-rabbit secondary antibodies and the primary antibodies made from different species.

Primary cerebral neuronal cultures

Neuronal cultures were prepared from embryonic day 16 Sprague–Dawley rat fetuses using methods similar to those previously described (Dichter, 1978), but modified to produce cultures that contained < 1% astrocytes (Rosenberg & Aizenman, 1989; Rosenberg, 1991; Rosenberg *et al*., 1992; Wang *et al*., 1998b). Cultures were initially plated on poly-L-lysine-coated 24-well plastic plates (Costar, Cambridge, MA, USA) using an 80 : 10 : 10 (v/v) mixture of DMEM (Life Technologies 11960-010), Ham's F-12 (Sigma, N-4888), heat-inactivated iron-supplemented calf serum (Hyclone A2151), containing 2 mM glutamine, 25 mM HEPES, 24 U/mL penicillin and 24 μg/mL streptomycin, and maintained in a 5% CO_2 (balance air) incubator at 36 °C. Cell proliferation was inhibited by exposure to 5 μM cytosine arabinoside at 24 h *in vitro* for 72 h. On the fourth day of culture, the medium was completely removed and replaced with 90% MEM, 10% NuSerum IV (Collaborative Research), 2 mM glutamine, 5 mM HEPES, containing 50 units/mL superoxide dismutase (Boehringer Mannheim, Indianapolis, IN, USA), 20 units/mL catalase (Sigma CV-40), total glucose 11 mM, and total sodium bicarbonate 9.3 mM, plus 2% B27 supplement (Life Technologies 17504-036). Medium was not subsequently changed. To prevent evaporation of water, culture dishes were kept on 'wet dishes' containing wet filter paper until they were used.

Immunoprecipitation and immunoblot analysis

Two days after transfection, cells were lysed with RIPA buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS) supplemented with 17 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride and 5 mM DTT. Fresh rat forebrain was homogenized in the same buffer. Cell lysate was shaken at 4 °C for 2 h for protein extraction, and then centrifuged at 60 000 ***g*** at 4 °C for 60 min. Supernatant was then removed and protein concentration measured with a protein assay kit (Pierce Chemical, Rockford, IL, USA). For immunoprecipitation, 30 μL of protein A/G agarose (Oncogene Science, Cambridge, MA, USA) was preincubated with 2 μg of anti-nGLT1 antibody or 2 μg of goat anti-chicken IgG in RIPA buffer for 1 h and, after washing, 2 μg of anti-PICK1 antibody was added to protein A/G with anti-chicken IgG and incubated for another hour. Protein extract (1 mg in 500 μL) from the co-transfected COS7 cells or rat brain tissue was then added to each immunoprecipitation tube and incubated at 4 °C for 4 h to overnight. For the control groups, equal amounts of rabbit or chicken IgG was used in place of anti-nGLT1 or anti-PICK1 antibodies. In control experiments, lysates from COS7 cells transfected either with PICK1 or GLT1b were obtained and mixed prior to immunoprecipitation. Precipitates were washed four times with RIPA buffer and then twice with TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl), solubilized with gel loading buffer containing 62.5 mM Tris, pH 6.8, 10% glycerol, 1.6% SDS and 640 mM β-mercaptoethanol, separated on 7.5% SDS polyacrylamide gels (10 μg per lane) and then transferred to polyvinylidene fluoride membranes (NEN Life Science Products, Boston, MA, USA) by electroblotting. The gels were silver-stained to check for equal loading. Blots were incubated with primary antibodies (cGLT1a at 14 ng/mL, cGLT1b at 1.6 μg/mL and nGLT1 at 1 μg/mL) overnight at 4 °C in 5% non-fat milk, 100 mM Tris, pH 7.5, 306 mM NaCl and 0.01% Tween 20, and then washed three times with Tris-NaCl-Tween buffer, incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ, USA) at 1 : 2500 dilution and washed again. Immunoreactive proteins were detected using enhanced chemiluminescence (NEN Life Science Products).

Uptake studies

Previously published procedures were followed for measuring the uptake of glutamate (Wang *et al*., 1998b) into cultured cells. Cells were exposed to tritiated L-glutamate either in the presence (sodium buffer) or absence (choline buffer) of sodium, and the radioactivity taken up by the cultures in the absence of sodium was subtracted from that taken up by the cultures in the presence of sodium to isolate sodium-dependent transport. The sodium-independent component was always less than 10%. For COS7 cells, uptake in the absence of sodium was 9.6 ± 3.8%; for neurons uptake in the absence of sodium was 1.5 ± 1%. Therefore, in some experiments uptake was measured only in the presence of sodium. Cells grown in 24-well plates were washed twice with sodium or choline buffer at 37 °C before being exposed at 37 °C for 5 min to [^{3}H]-L-glutamate (catalogue #TRK445, Amersham; specific activity 63 Ci/mmol). For uptake studies other than saturation studies, 30 μM L-glutamate was used with selected concentrations of drugs. The uptake assay was stopped by removal of the tracer solution and addition of ice-cold choline buffer containing 1% BSA, followed by three washes in choline/BSA buffer. After stopping uptake with ice-cold

choline-containing buffer, cells were solubilized in 0.1 N NaOH. Aliquots of this lysate were analysed for protein with the Bradford protein assay (Bradford, 1976; Bio-Rad, catalogue #500-0113-5) and radioactivity by liquid scintillation counting. The physiological saline for uptake studies contained (in mM): NaCl or choline chloride, 140; KCl, 2.5; $CaCl_2$, 1.2; $MgCl_2$, 1.2; K_2HPO_4, 1.2; glucose, 10; Tris base, 5; HEPES, 10; pH 7.4; osmolality 300 (Garlin *et al*., 1995).

Myristoylated peptides

N-terminal myristoylated peptides were synthesized by the Tufts University Core Facility (Boston, MA, USA). Myristoylated peptides were purified by SEP-PAK C18 cartridges. Briefly, peptides were first dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mM, then diluted 1 : 100 with 80% acetonitrile and 0.1% trifluoroacetic acid. Each cartridge was loaded with 1 mL of this solution, and five fractions were generated: flow-through; wash 1 and 2 (washed with 80% acetonitrile and 0.1% trifluoroacetic acid); elution 1 and 2 (eluted with 100% acetonitrile and 0.1% trifluoroacetic acid). Fractions were analysed by HPLC and mass spectroscopy, and it was found that most contaminants including small molecules used in the synthesis and short truncated peptides or peptides with deletions were removed in the flow-through and washing steps.

Purified N-terminal myristoylated peptides were dissolved in DMSO to produce a 500–1000 × stock. Cultures were treated with 2.5 μM (purified) peptide, or vehicle (DMSO) in controls, in calcium/magnesium-free Earle's balanced salt solution (CMF-Earle's) containing 10 μM MK-801 for 1.5 h, and then were exposed to 400 nM phorbol 12-myristate 13-acetate (PMA) with or without (2.5 μM) peptide in CMF-Earle's containing 10 μM MK-801 for 2 h. Controls were treated with the vehicle, with or without the peptide, for 2 h.

Biotinylation of cell surface proteins

The biotinylation procedure was performed as described previously (Kalandadze *et al*., 2002), with minor modification. Briefly, plates of neurons were placed on ice after the treatments described above. The cells were rinsed twice with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-Ca/Mg) and then were incubated in this same solution supplemented with 1 mg/mL biotin (EZ-link-sulfo-NHS-LC-Biotin, Pierce cat # 21335) for 20 min on ice at 4 °C in a cold room. After incubation, cells were rinsed three times with PBS-Ca/Mg containing 100 mM glycine and incubated in this buffer for 30 min at 4 °C to quench the unreacted biotin. Cells were then lysed [20 mM CHAPS (Sigma cat # C-9426), 10 mM sodium phosphate buffer pH 7.4, 150 mM NaCl]; or RIPA buffer (Boston Bioproducts cat # BP-115) with protease inhibitor plus EDTA cocktail tablet (Roche cat # 1836153). The cell lysates were collected and sonicated for 5 s. After centrifugation (14 000 ***g*** for 20 min at 4 °C), protein concentration of the supernatant was measured by a protein assay (Bio-Rad, cat # 500-0113-5). The concentrations of all samples were equalized using lysis buffer. Some of these supernatants were then frozen at −20 °C until use. The supernatants were mixed with avidin beads (Immobilized Immunopure Avidin, Pierce cat # 20219) and rotated overnight in the cold room. The biotinylated proteins were then batch-extracted using avidin-coated Sepharose beads. After centrifugation (14 000 ***g*** for 20 min at 4 °C), the beads were collected and washed four times with the same lysate buffer that was used for

solubilization containing protease inhibitors. Biotinylated proteins (cell surface proteins) were released by incubation in SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer at 37 °C for 20 min. The mixture was centrifuged (14 000 ***g***), and the supernatant was transferred to a fresh tube and frozen until analysis.

Results

We sought to identify proteins that interact specifically with GLT1b using the C-terminal cytoplasmic domain of GLT1b (the last 94 amino acids) as bait in a yeast two-hybrid screen (Vidal, 1997) of a neuronal cDNA library. We identified two clones that, when sequenced, were shown to contain the full coding region of rat PICK1. The sequence of rat PICK1 has been deposited in GenBank (#AF542094). PICK1 is a PDZ domain protein discovered as an interactor with PKCα (Staudinger *et al*., 1995). Its function has been best characterized in its role in the clustering and trafficking of glutamate receptors at synapses (Dev *et al*., 1999; Xia *et al*., 1999; Takeya *et al*., 2000; Jaulin-Bastard *et al*., 2001; Duggan *et al*., 2002; Hruska-Hageman *et al*., 2002). PICK1 has also been found to interact with monoamine transporters (Torres *et al*., 2001; Bjerggaard *et al*., 2004) as well as other proteins, including EphB receptors and ephrins (Torres *et al*., 1998), ββ2/HER2 receptors (Jaulin-Bastard *et al*., 2001), prolactin-releasing peptide receptor (Lin *et al*., 2001b), a phorbol ester inducible gene TIS21/PC3/BTG2 (Lin *et al*., 2001c) and ADP ribosylation factors (Takeya *et al*., 2000).

Because the interaction of PICK1 with synaptic proteins is mediated by its PDZ domain, we tested whether the class I type PDZ interaction motif in GLT1b (Fig. 1A) is specific and sufficient for the PICK1–GLT1b interaction. We examined the effect of various mutations of the GLT1b C-terminus on interaction with PICK1 in the yeast two-hybrid system (Fig. 1B), as well as the interaction of PICK1 with the C-terminal cytoplasmic domain of GLT1a. Deletion of the last four amino acids (ETCI) or addition of a tyrosine residue to the C-terminus of GLT1b completely abolished the interaction with PICK1 (Fig. 1B). In addition, we found that PICK1 did not interact with GLT1a. However, substitution of serine or alanine for the glutamate residue at the −3 position, or of isoleucine for the threonine residue at the −2 position, did not interrupt the GLT1b–PICK1 binding. Therefore, the interaction of GLT1b with PICK1 in yeast is dependent upon the amino acid sequence at the C-terminus of GLT1b, with a tolerance for substitutions at the −3 and −2 positions that has been found to be characteristic of PICK1 but atypical of class I PDZ domains (Perez *et al*., 2001; Sheng & Sala, 2001).

To characterize the interaction of PICK1 and GLT1b in further detail and assess the affinity of the interaction, we used a recently developed *in vitro* competitive fluorescence polarization assay (Madsen *et al*., 2005) to determine the binding affinities of PICK1 with the C-termini of GLT1b, GLT1b mutants, other glutamate transporters and known PICK1 interacting proteins. In this assay, with both components in solution, the binding of a fluorescently labeled peptide to a larger protein (in this case PICK1) is detected as an increase in fluorescence polarization because of a decrease in rotational diffusion. We characterized the interaction of peptides with PICK1 by measuring the competition for binding to PICK1 of the peptide of interest with a fluorescently tagged C-terminal peptide of the DAT. Using the fulllength purified PICK1 and a peptide of the C-terminal 13 residues of

GLT1b (GLT1b13) we measured the K_i of the interaction to be 6.5 ± 0.4 μM (Fig. 2; Table 1). A peptide of the C-terminal 10 residues behaved similarly (Fig. 2). The affinity demonstrated is in the range of other PDZ interactions characterized by fluorescence polarization (Niethammer *et al*., 1998; Lin *et al*., 1999; Lim *et al*., 2002; Piserchio *et al*., 2002; Reina *et al*., 2002) and suggests that the 13 C-terminal residues of GLT1b are responsible for a direct interaction with PICK1 via the canonical PDZ binding mode of the ETCI sequence docking in the PDZ binding groove of PICK1. The observed affinity of GLT1b for PICK1 was slightly better than that of GluR2 (9 ± 1 μM; Table 1) and significantly better than that of PKCα (33 ± 2 μM; Fig. 5B and Table 1), but slightly lower than that of the DAT (K_i = 2.3 ± 0.1 μM; Madsen *et al*., 2005). The binding of the GLT1b peptide to PICK1 was almost completely disrupted by adding a tyrosine to the C-terminus of the GLT1b peptide (GLT1b13 + Y; Fig. 2 and Table 1), which presumably prevents the docking of the peptide into the PDZ domain binding groove. Furthermore, the affinity was severely compromised by deletion of the last four residues of the peptide (GLT1ΔDETCI; Fig. 2 and Table 1), demonstrating the critical role played by the C-terminal sequence, characteristic of PDZ domain interactions. The interaction was also disrupted by substituting an amide for a carboxyl group at the C-terminus without any change in sequence (Fig. 2 and Table 1).

Single alanine substitutions of the last three residues [P0 (the position of the last amino acid of the primary sequence), P1 and P2], which are thought to dock into the PDZ domain binding groove, however, were only partially disruptive compared with the deletion of the last four amino acids (GLT1b-ETCA, GLT1b-ETAI and GLT1b-EACI, respectively, in Fig. 3 and Table 1). Given the relative lack of specificity for the three C-terminal residues, we speculated that more distal motifs in the C-terminus might contribute to the binding, as has been reported for other ligands for PICK1 (Dev *et al*., 2000; Hirbec *et al*., 2002). To test this we did N-terminal truncations of the GLT1b13-mer peptide and studied their interaction with PICK1 (Fig. 4 and Table 1). Deleting the two N-terminal residues (P12 and P11, yielding GLT1b11) increased the affinity slightly (Figs 2 and 4, and Table 1); however, deleting the next N-terminal residue (P10) yielding GLT1b10 caused a significant drop in affinity (Fig. 4 and Table 1). Deletion of the next residue (P9) also had a significant effect (GLT1b9), but deletion of P8 had little effect. Another significant drop in affinity was seen with removing the N-terminal residue of GLT1b8 (P7) to yield GLT1b7. These results suggested that residues from P10 to P7 (PFPF) added significantly to the binding.

Finally, we tested C-terminal 11 amino acid peptides from other glutamate transporters (Fig. 5 and Table 1), all of which, except GLT1a, have a PDZ domain interaction motif (Fig. 1A). As illustrated in Fig. 5A, the GLT1a, GLAST, EAAC1 and EAAT4 peptides interacted only with very low affinity, if at all, with PICK1; however, the EAAT5 peptide interacted with an affinity comparable to that of GLT1b (Fig. 5A and Table 1). We also tested peptides based on two other variant forms of GLT1, GLT1c (Rauen *et al*., 2004) and EAAT2d (accession #AI798939). GLT1c but not EAAT2d interacted with PICK1, with an affinity comparable to PKCα (Fig. 5B and Table 1). The results of all studies using fluorescence polarization are summarized in Table 1. For comparison, we have included data obtained with PKCα, GluR2 and DAT C-terminal peptides. As an additional test of the specificity of interaction between

GLT1b and PICK1, we tested the effect of a mutation of PICK1, A87L, that was previously found to not interact with DAT (Madsen *et al*., 2005). This mutant PICK1 failed to interact with GLT1b as well (data not shown).

The next question we addressed was whether GLT1b and PICK1 interact in mammalian cells and in neurons. A peptide-specific antibody was raised in chickens against the C-terminal 16 amino acids of PICK1 (NH_2-CGATGPTDKGGSWCDS-COOH). This antibody recognized a protein that migrated with an apparent molecular mass of ~50 kDa in lysates from neuronal cultures and rat brain tissue (Fig. 6A). The immunolabeling of this band was blocked by preadsorption of the antibody with the peptide against which it was raised (Fig. 6A). COS7 cells were transfected with pcDNA3 expressing GLT1b, or EGFPC1 expressing an EGFP–PICK1 fusion protein, or both plasmids. The anti-PICK1 antibody immunoprecipitated the EGFP–PICK1 fusion protein, represented by a band at ~80 kDa on immunoblot, from COS7 cells co-transfected with EGFP–PICK1 and GLT1b, and GLT1b protein was also immunoprecipitated by the PICK1 antibody (Fig. 6B).

Control experiments with preimmune chicken IgG showed no precipitation of either protein. In COS7 cells co-transfected with GLT1a and EGFP–PICK1 expressing plasmids, GLT1a protein was not present in the anti-PICK1 immunoprecipitated pellet. As a control for non-physiological association in the homogenate, lysates from COS7 cells separately expressing either PICK1 or GLT1b were mixed prior to immunoprecipitation. We found that when lysates of COS7 cells expressing either EGFP–PICK1 or GLT1b were mixed, PICK1 and GLT1b did not co-immunoprecipitate, indicating that the association of PICK1 and GLT1b found in COS7 cells co-expressing these proteins was due to an association occurring in cells, and not simply in the lysate after homogenization. To test whether the interaction between GLT1b and PICK1 in COS7 cells was dependent upon the sequence at the C-terminus, we co-expressed a GLT1b mutant lacking the last four amino acids with PICK1. Deletion of the four amino acids at the C-terminus of GLT1b greatly diminished the amount of GLT1b co-precipitated using the anti-PICK1 antibody (data not shown). These results demonstrate that the interaction between GLT1b and PICK1 found in yeast and in solution also occurs between the full-length recombinant proteins expressed in mammalian cells.

The next question we addressed was whether it could be demonstrated that endogenous GLT1b and PICK1 interact in their sites of expression in the brain. We found that in rat whole brain lysate, anti-PICK1 antibody immunoprecipitated PICK1 protein and pulled down GLT1b protein (Fig. 6C). The antibody directed against the C-terminal sequence of GLT1b, anti-cGLT1b, however, failed to pull down PICK1, although GLT1b protein itself could be precipitated by this antibody (data not shown). The inability of the anti-cGLT1b antibody to immunoprecipitate complexes of GLT1b and PICK1 may be because this antibody was raised against the region of GLT1b with which PICK1 interacts. Therefore, anti-cGLT1b must compete with PICK1 protein for binding to the GLT1b C-terminus. Consistent with this view, PICK1 protein co-immunoprecipitated with GLT1b from the rat brain when an anti-N-terminal GLT1 antibody was used for immunoprecipitation (Fig. 6C). Interestingly, GLT1a protein was also precipitated by both anti-cGLT1b antibody and anti-PICK1 antibody, but appeared only in multimers (size of monomer is about 66 kDa; Fig. 6C). Because GLT1a does not interact with PICK1 directly in yeast, in co-transfected COS7

cells, or in solution, it is likely that GLT1a was present in the GLT1b–PICK1 complex through its interaction with GLT1b. These results, together with previous results demonstrating the tendency of GLT1 to aggregate with itself (Haugeto *et al*., 1996) in trimers (Gendreau *et al*., 2004), and the X-ray crystallographic demonstration of trimer formation by a bacterial glutamate transporter (Yernool *et al*., 2004), suggest the formation of heterotrimers of GLT1a and GLT1b. Another neuronal glutamate transporter, EAAC1, was not detectable in the GLT1b–PICK1 pellet using an anti-EAAC1 antibody (generously provided by Dr Jeff Rothstein; data not shown).

We next asked whether the co-expression of GLT1b and PICK1 might influence the subcellular distribution of either protein. Co-expression of the transporter GLTlb or GLTlbΔ4 with EGFP did not alter the localization of the transporters in the plasma membrane or in the perinuclear compartments in comparison to when the transporters were expressed alone (Fig. 7A and data not shown). No colocalization of GLTlb or GLTlbΔ4 with EGFP was found on the plasma membrane (Fig. 7A). In contrast, when cells were co-transfected with GLTlb and EGFP-PICK1, we found colocalization of both proteins on the plasma membrane (colocalization on the membrane was found in 92% of cells; $n = 36$) and in the perinuclear compartments (yellow labeling, Fig. 7A). In contrast, colocalization was rare in cells that co-expressed GLTlbΔ4 and EGFP-PICK1 (colocalization on the membrane was found in 17% of cells; $n = 42$). The low amount of colocalization found on the plasma membrane with GLTlbD4 and PICK1 co-expression was similar to the fraction of cells with PICK1 found on the plasma membrane when expressed alone (14% of cells; $n = 35$). The majority of cells showed PICK1 in the cytoplasm only (Fig. 7B). To further test whether the effect of GLTlb was specific, we co-transfected COS7 cells with GLTla, which does not possess a PDZ binding sequence at its C-terminus (Fig. 7B). Similar to what was observed with GLTlbΔ4, no alteration of GLTla or PICK1 localization was found with their co-expression (colocalization of GLTla with EGFP-PICK1 on the plasma membrane was found in 15% of cells; $n = 33$). These findings demonstrate that GLT1b and PICK1 interact in cells, and that this effect is dependent upon the last four amino acids of GLT1b, consistent with a PDZ domain interaction.

Next we examined the localization of GLT1b and PICK1 in hippocampal neuronal cultures (Fig. 8A–L). Immunocytochemistry with the rabbit anti-C-terminal GLT1b antibody and the chicken anti- PICK1 antibody demonstrated that both proteins were expressed in a punctate pattern in processes as well as on cell bodies in cultured hippocampal neurons (Fig. 8A–C). At higher magnification, the punctate pattern of labeling with both anti-cGLT1b and anti-PICK1 antibodies is evident, consistent with a synaptic localization (Fig. 8D–F). PICK1 has already been shown to be expressed in cerebral neurons in culture with a punctate distribution that overlaps with synaptic markers (Hruska-Hageman *et al*., 2002). This association of GLT1b with synapses was tested by performing double-label immunocytochemistry using the presynaptic marker synaptophysin (Fig. 8G–I). There was partial co-localization of synaptophysin and GLT1b (yellow in Fig. 8I). For a postsynaptic marker, an antibody against the *N*-methyl-D-aspartate (NMDA) receptor subunit NR1 was used (Fig. 8J–L). Again, as with synaptophysin, partial co-localization of GLT1b with NR1 was demonstrated (yellow in Fig. 8L).

PICK1 was originally discovered as a PKCα binding protein (Staudinger *et al*., 1995), and the PKCα–PICK1 interaction was found to be dependent upon the activation of PKCα (Perez *et al*., 2001). PICK1 contains a BAR (Bin/ Amphiphysin/Rvs) domain, a region that recognizes membrane curvature, located in the C-terminal half of the molecule (Jin *et al*., 2006; Steinberg *et al*., 2006) and through which it may dimerize, bringing two dissimilar PICK1 interacting proteins into close apposition. Therefore, PICK1 has been conceived of as an adaptor to recruit PKCα into complexes with PICK1 binding target proteins to improve the specificity and efficacy of PKC action (Perez *et al*., 2001; Hirbec *et al*., 2002). Deployment of neurotransmitter transporters to the plasma membrane has been shown to be subject to regulation by PKC activation (Qian *et al*., 1997). Although the effects of PKCα activation on GLT1 have been controversial (Casado *et al*., 1993; Tan *et al*., 1999), GLT1 protein (Kalandadze *et al*., 2002), GLT1 function (Fang *et al*., 2002), and GLT1 protein and function (Zhou & Sutherland, 2004) have more recently been shown to be downregulated by PKC activation in stably transfected C6 glioma cells, *Xenopus laevis* oocytes and astrocytes, respectively. We therefore hypothesized that PICK1 regulates the effect of PKCα on GLT1. We first tested this hypothesis in a heterologous expression system using either COS7 cells or MDCK cells, but were not able to obtain consistent results. We then queried whether a functional consequence of the GLT1–PICK1 interaction might be demonstrable in primary forebrain neurons, in which the native environment of these proteins was preserved, unlike in a heterologous expression system. We tested the effect of PKC activation in these cultures, and typically found no significant change in glutamate uptake as a result of exposure to PMA (400 nM, up to 2 h exposure) without (Fig. 9A) or with the phosphatase inhibitor okadaic acid present (data not shown). There was also no consistent effect seen with exposure to an inhibitor of PKC, BISII (data not shown). To account for the lack of response of neuronal cultures to PMA, and based on results of others (see above) showing a downregulation of GLT1 function by treatment with phorbol esters, we hypothesized that in neurons the GLT1b–PICK1 interaction might serve to inhibit the effect of PKC activation on GLT1. If so, then disrupting that interaction might be expected to reveal the effect of PKC action on GLT1-mediated glutamate transport.

Previous studies have shown that myristolation of peptides renders them permeable to plasma membranes (Eichholtz *et al*., 1993; Ward & O'Brian, 1993), and a myristolated peptide based on the C-terminus of GluR2 has been used to disrupt the GluR2–PICK1 interaction *in vivo* (Garry *et al*., 2003). We synthesized a myristoylated decoy peptide designed to compete with the interaction of GLT1b with PICK1 (FPFLDIETCI-COOH), and a peptide identical in sequence but amidated at the C-terminus as a control (FPFLDIETCI-CHO; Fuh *et al*., 2000). Neurons treated with control peptide or vehicle showed no significant decrease in glutamate uptake upon PKC activation (Fig. 9A). In contrast, neurons treated with the active decoy peptide showed a significant decrease, to 63.8 ± 19.6% of control ($P < 0.01$) glutamate uptake. To assess the specificity of the effect of the myristolated peptide, we exploited the fact that both GLT1 and EAAC1 are expressed in cultured rat forebrain neurons (Wang *et al*., 1998b; Chen *et al*., 2002). To determine whether the detected changes in glutamate uptake were a specific change in GLT1 function, we repeated the experiment in the presence and absence of DHK (300 μM), a specific inhibitor of GLT1 at this concentration (Garlin *et al*., 1995; Wang *et al*., 1998b; Kalandadze *et al*., 2002;

Fig. 9B and C). In this series of experiments, PMA significantly decreased glutamate transport only in cells pretreated with the active decoy peptide (Fig. 9B). The DHK-sensitive component of glutamate transport was significantly inhibited (49.2 ± 7.1% decrease with PMA; $n = 5$; $P < 0.01$; Fig. 9C) by PKC activation in neurons treated with the active decoy peptide. In contrast, there was no significant effect on the DHK-insensitive glutamate uptake. There was also no effect using the control amidated peptide. These results showed that in neuronal cultures the changes in glutamate uptake evoked by PMA in the presence of the active decoy peptide were specific to GLT1 and exclude a non-specific effect on glutamate uptake. Saturation analysis showed a significant decrease in the capacity of glutamate uptake (V_{max}) in neurons treated with the active GLT1b decoy peptide upon PKC activation (433 ± 138 vs 1230 ± 291 nmol/min/g; to 34.3 ± 3% of control; $P < 0.01$; $n = 2$; Fig. 9D).

A change in the capacity of glutamate uptake could be due to a change in surface expression of GLT1 or a change in turnover rate of the transporter. Previous studies have demonstrated by biotinylation of surface proteins that PKC-induced downregulation of GLT1 transport in C6 glioma cells is associated with internalization of GLT1 (Kalandadze *et al*., 2002). We used a similar approach to test for subcellular redistribution of GLT1a and GLT1b in neuronal cultures treated with PMA in the presence and absence of the active decoy peptide (Fig. 10). In these experiments, cultures were treated for 90 min with active decoy peptide or vehicle, followed by 400 nM PMA or vehicle for an additional 2 h, all in CMF-Earle's containing 10 μM MK801, just as in the experiments shown in Fig. 9. We found no significant change in the surface expression either of GLT1a (Fig. 10A, C and E) or GLT1b (Fig. 10B, D and F) with PMA alone or with treatment with active decoy peptide and PMA, suggesting that phorbol ester downregulates GLT1 function in primary cerebral neurons in culture by changing rate of turnover of the transport protein.

Discussion

In this study we have found that the glutamate transporter GLT1b interacts directly and specifically with PICK1 *in vitro* as well as *in vivo* in yeast, in heterologous cells and in the brain. Adding a tyrosine or deleting the terminal four amino acids of GLT1b blocked the interaction with PICK1 in yeast, showing the dependence of this interaction on the C-terminus of GLT1b. Heterologous co-expression studies showed that the localization of PICK1 in cells was strongly determined by the presence of GLT1b, inducing a transition from a uniform cytoplasmic distribution to a perinuclear and membraneassociated distribution as seen for other PICK1 interaction partners (Torres *et al*., 1998; Xia *et al*., 1999; Hruska-Hageman *et al*., 2002). This change in distribution was not seen when a mutant form of GLT1b that lacked the last four amino acids was used. Widespread and overlapping patterns of expression are not necessarily evidence of interaction. However, the change in localization in the expression of PICK1 when co-expressed with GLT1b, but not GLT1bΔETCI or GLT1a, producing a distinct pattern of co-labeling at the plasma membrane, strongly suggests that interaction is taking place.

Immunoprecipitation studies further showed that a significant association of GLT1b and PICK1 occurs in the brain. GLT1a was also associated with PICK1 and GLT1b in native

complexes immunoprecipitated from the brain. This most likely occurs through association of GLT1b and GLT1a, because GLT1a itself did not interact with PICK1 in yeast, in COS7 cells or in solution, and because immunoprecipitation of GLT1b pulled down GLT1a. The evidence of interaction between GLT1a and GLT1b shown here is consistent with the emerging view that the functional glutamate transporter unit is a trimer (Haugeto *et al*., 1996; Gendreau *et al*., 2004; Yernool *et al*., 2004). We hypothesize that such an interaction to produce functional trimers is the basis for the observation that although GLT1a is expressed in primary neurons in culture, its activity is not affected by PMA, unless the cells have been treated with the active decoy peptide. That is, it appears that the interactions of GLT1b with PICK1 determine the behavior not only of GLT1b molecules, but also the GLT1a molecules that together form the functional heterotrimer. Otherwise, treatment of these cells with PMA without prior exposure to the myristolated decoy peptide would produce downregulation of glutamate transport function due to an effect on GLT1a, which we know is expressed in these cultures.

Analysis of the interaction of GLT1b and PICK1 by fluorescence polarization assay

In this study we have found that the C-terminus of the glutamate transporter GLT1b interacts directly and specifically with PICK1 by the use of an *in vitro* fluorescence polarization assay. Adding a tyrosine or deleting the terminal four amino acids of GLT1b blocked the interaction with PICK1 in solution, showing the dependence of this interaction on the C-terminus of GLT1b. The interaction of GLT1b with PICK1 was also dependent upon the presence of a carboxyl group at its C-terminus, providing the basis for the design of the control peptide used in the experiments shown in Fig. 9.

The mutational analysis in the GLT1b C-terminus suggests that the GLT1b–PICK1 interaction is only moderately sensitive to alanine substitutions at P0–P2. For the P0 and P1 position this was not surprising, as several ligands for PICK1 have alanines in these positions [PKCα (QSAV; Staudinger *et al*., 1997), ASIC2a (EIAC; Baron *et al*., 2002), BNaC1 (EIAC; Duggan *et al*., 2002), mGluR4a (NHAI; El Far *et al*., 2000) $GluR5_{2b}$ (ETVA; Hirbec *et al*., 2003)], but the tolerance towards the P2 substitution with alanine was unexpected. The E-T-C-I motif constitutes a class I PDZ interaction sequence, and class I interactions are usually highly dependent on the hydrogen bonding of the hydroxyl group of the P2 serine or threonine with the imidazole nitrogen in histidine in the αB1 position in the PDZ domain (Sheng & Sala, 2001). However, the PICK1 PDZ domain is not a traditional class I PDZ domain, as it binds numerous class II sequences (φ-X-φ) [GluR2 (SVKI; Xia *et al*., 1999), EphB2 (SVEV), EphA7 (GIQV), ephrinB1 (YYKV; Torres *et al*., 1998; Madsen *et al*., 2005)], and has a lysine instead of a histidine in the αB1 position in the PDZ domain. Notably, the lysine was recently shown to act much like the hydrophobic residues usually present in domains preferentially recognizing type II sequences (Madsen *et al*., 2005), an observation that is likely to explain the tolerance towards the alanine residue in the P2 position. In addition, our data demonstrate dependence of the GLT1b interaction on the P7–P10 sequence. Interestingly, of the 25 bonafide PICK1 ligands (Madsen *et al*., 2005), 12 have at least one aromatic residue from P7 to P9, whereas the expected frequency for an aromatic residue to be present in a three amino acid stretch is 26%. Furthermore, of the 11 proteins reported to bind PICK1 lacking the preferred type II sequence, seven have at least

one aromatic residue from P7 to P9. This suggests that the aromatic sequence present in GLT1b might reflect a general upstream motif, which is particularly important for ligands that do not have a canonical type II sequence.

All known glutamate transporters except GLT1a terminate in a putative PDZ binding sequence. However, only one other PDZ protein (the Na^+-H^+ exchanger regulatory factor 1) has been shown to interact with any member of this class of transporters, in this case GLAST (Lee *et al*., 2007), although no function has been found for this interaction. Accordingly, we decided to explore whether the interaction of GLT1b with the PICK1 PDZ domain was unique for this transporter or whether additional glutamate transporters could form an interaction. Our data suggest that EAAT5 and the GLT1c variant form also bind PICK1, whereas the C-termini of EAAC1, GLAST and EAAT4, as well as EAAT2d cannot bind. Given the high specificity of the interactions and the fact that the observed affinities are comparable to those observed for other PDZ domain interactions, it is interesting to speculate that the interactions are critical in the regulation of GLT1 and EAAT5 (Arriza *et al*., 1997) function *in vivo*. There are reports of functional interactions between glutamate transporters and other (non- PDZ) proteins (Marie & Attwell, 1999; Jackson *et al*., 2001; Lin *et al*., 2001a; Marie *et al*., 2002).

Localization of PICK1 and GLT1b

Prior immunocytochemical studies in hippocampal cultures have shown that PICK1 is restricted to excitatory synapses, based on its absence from synapses that are labeled with an anti-glutamic acid decarboxylase antibody (Xia *et al*., 1999; Boudin *et al*., 2000), and targeting of recombinant PICK1 exclusively to excitatory synapses (Boudin & Craig, 2001). PICK1 co-localizes with the presynaptic markers synaptophysin (Dev *et al*., 2000) and SV2 (Boudin *et al*., 2000), and biochemical studies have provided evidence for tight association of PICK1 with the PSD (Xia *et al*., 1999). Interestingly, in a recent study characterizing the expression of PICK1 in the brain using light microscopic immunocytochemistry, the protein has been found in a crescent-shaped distribution in the CA3 region of the hippocampus (Duggan *et al*., 2002), in a pattern similar to the distribution of GLT1a and GLT1b transcripts that we have demonstrated by *in situ* hybridization (Chen *et al*., 2004).

Function of the PICK1–GLT1b interaction

Generally, it is believed that PICK1 fulfils its biological role by regulating trafficking of its binding partners and in some cases by recruiting PKCα to facilitate their phosphorylation (Dev *et al*., 2000; Baron *et al*., 2002). Prior studies have in particular revealed an important role of PICK1 in the regulation of glutamate receptor trafficking (Hanley, 2006). It has been suggested that PICK1 is involved in endocytosis of AMPA receptors and thereby for maintaining an intracellular receptor pool, which is believed to be important for induction of long-term depression, a key process in memory and learning (Xia *et al*., 2000; Hanley & Henley, 2005). Notably, this role of PICK1 is likely dependent upon the BAR domain. In relation to other interaction partners, however, PICK1 might enhance their surface expression rather than causing a decrease (Torres *et al*., 2001). This suggests that PICK1 may serve different and distinct roles depending on the nature of its binding partners.

The original hypothesis regarding the role of PICK1 in the function of glutamate receptors was that PICK1 targets them to synapses, based on the ability of PICK1 to induce clustering in heterologous expression systems (Xia *et al*., 1999). Consistent with this view, deletion of the PICK1 binding domain of recombinant mGluR7a prevented presynaptic clustering of the protein in hippocampal neurons (Boudin *et al*., 2000). However, with respect to PICK1's role in the physiology of the AMPA receptor, the view of PICK1 as a targeting protein has given way to the conception that PICK1 is involved critically in the retrieval of AMPA receptors from the plasma membrane (Xia *et al*., 2000; Iwakura *et al*., 2001; Perez *et al*., 2001; Seidenman *et al*., 2003; Terashima *et al*., 2004). Therefore, two possible roles may be envisioned for the interaction of PICK1 with GLT1b: (1) to target and anchor GLT1b at synapses; (2) to provide a mechanism for regulating the trafficking of GLT1 between the plasma membrane and cytoplasmic membranes.

PICK1–PKCα interactions

Our initial hypothesis about the function of the PICK1–GLT1b interaction was that it could provide a mechanism to bring PKC physically close to GLT1b to facilitate phosphorylation of the transporter. Phosphorylation might result in the modulation of transporter activity either by directly affecting its function, or by altering the state of its deployment between the plasma membrane and internal membranes. Deployment of glutamate transporters to the plasma membrane has been shown to be subject to regulation by protein kinases (Robinson, 2002; Gonzalez & Robinson, 2004). In the specific case of GLT1, there has been controversy over the effects of PKC activation (Tan *et al*., 1999). More recent evidence, however, has shown that PKC activation causes internalization of GLT1a in a GLT1a-transfected C6 glioma cell-line, and that this change in surface expression is associated with a downregulation of GLT1a function. In addition, internalization of GLT1a protein with PKC activation has also been shown in primary mixed cultures of rat astrocytes and neurons (Kalandadze *et al*., 2002), although the cell type in which this effect occurs was not identified.

We observed the downregulation of glutamate transporter activity by phorbol ester only in rat cerebral neurons treated with the myristoylated decoy peptide. This downregulation was not associated with a detectable decrease in surface expression of GLT1. Thus, it appears to be inaccurate at this time to refer to the effect of PICK1 on GLT1 function as an effect either on the anchoring of GLT1 in the plasma membrane or to an effect on the trafficking of GLT1 between cytoplasmic membranes and the plasma membrane. This is in contrast to the emerging role of PICK1 in glutamate receptor trafficking, in which the interaction of PICK1 with GluR2 promotes internalization that is dependent upon phosphorylation of S880 (Chung *et al*., 2000, 2003; Xia *et al*., 2000; Perez *et al*., 2001; Seidenman *et al*., 2003; Terashima *et al*., 2004). In some cases, however, PICK1 has been shown to inhibit phosphorylation of its binding partners (Dev *et al*., 2000; Lin *et al*., 2001c), and so it is conceivable that PICK1 interacting with GLT1b blocks the phosphorylation either of GLT1b or an associated protein, and that it is this block of phosphorylation that prevents the downregulation of the transporter. The dichotomous nature of the effects of PICK1 on glutamate receptors and transporters may provide an efficient mechanism for decreasing glutamate receptor expression while preserving glutamate transporter expression at synapses

undergoing activity-dependent changes in synaptic strength. Another glutamate transporter, EAAT5, has a C-terminus that binds to PICK1, and it remains to be determined whether interaction with PICK1 has the same functional significance for this glutamate transporter as we have found for GLT1b.

Our results do not necessarily contradict prior results showing PKC-induced internalization of GLT1, because our results were obtained in a primary neuronal culture system in which GLT1 is the primary functional transporter (Wang *et al*., 1998b; Chen *et al*., 2002). In primary cultures containing astrocytes, GLT1 contributes little to total uptake activity (Swanson *et al*., 1997; Schlag *et al*., 1998), for reasons that are unclear. A precedent for internalization-independent regulation of neurotransmitter transporter function has been demonstrated for the γ-aminobutyric acid (GABA) transporter (GAT) and the serotonin transporter. Regulation of GAT transporter capacity by interaction with syntaxin 1A was shown to be due to changes in turnover rate rather than in surface GAT expression (Deken *et al*., 2000; Quick, 2003; Hansra *et al*., 2004). Previous efforts to identify the site or sites on GLT1a that are phosphorylated as a consequence of PKC activation, and that play a determinative role in the regulation of GLT1 expression, have not produced a clear result (Kalandadze *et al*., 2002). Therefore, it is not known whether the action of PKC that causes downregulation of GLT1 activity is a direct action on GLT1 or on an associated protein.

The active decoy peptide used in these experiments was designed to disrupt the interaction of GLT1b with PICK1. However, it may disrupt the interaction of other proteins that interact with PICK1, for example GluR2. In addition, as the decoy peptide terminates in a PDZ domain interaction sequence, it is possible that it may disrupt other PDZ domain interactions as well, although no other PDZ domain-containing protein has been discovered with the specific C-terminal sequence E-T-C-I. At this point, we can only say that the data obtained using the active decoy peptide in neuronal cultures suggest that the PICK1–GLT1b interaction suppresses the downregulation of GLT1 in neuronal cultures produced by PKCα activation, and that it is disruption of this interaction by the decoy peptide that unmasks the vulnerability to PKCα-induced downregulation.

What is the likely functional significance for the interaction of GLT1b, GLT1c and EAAT5 with PICK1 in the brain? Because PICK1 is predominantly expressed in neurons rather than in astrocytes, it is most likely that it is in neurons that the interactions of these transporters with PICK1 will be of functional importance. *In situ* hybridization studies have shown that: (1) GLT1a mRNA is clearly the predominant GLT1 mRNA in the adult brain; (2) GLT1a and GLT1b isoforms are found in astrocytes throughout the brain; (3) GLT1a and GLT1b are both expressed in neurons in some regions of the brain and that, in general, GLT1a is the predominant form in neurons (Chen *et al*., 2004); (4) GLT1b mRNA is the predominant isoform only in some regions, most notably in the retina (Berger *et al*., 2005). GLT1b protein expression in the retina was found to vary in a species-specific manner; in rats and humans GLT1b was found in photoreceptor terminals and in bipolar cells (Reye *et al*., 2002). A partial sequence (579 bp) of GLT1c was discovered by reverse transcriptase-PCR using rat retina RNA (accession number AY578981; Rauen *et al*., 2004). Light microscopic immunocytochemical studies revealed low-level expression in the brain, but strong expression in ‘synaptic-terminal-like structures in the outer plexiform layer of the retina,

corresponding to the synaptic terminals of rod photoreceptors'. Interestingly, the expression of GLT1c was indistinguishable from that of EAAT5. Because the three transporters that interact with PICK1 are localized in the retina, and in some instances in the same neuronal structures in the retina such as photoreceptor terminals and bipolar cells, it is possible that PICK1– glutamate transporter interactions are involved in the modulation of retinal circuits. This does not preclude the possibility that the PICK1–GLT1b interaction is important in cerebral neurons expressing GLT1b under normal conditions, or under pathological conditions, in which GLTb might be upregulated (Maragakis *et al*., 2004; Rauen *et al*., 2004).

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BSA	bovine serum albumin

CH	Children's Hospital
CMF-Earle's	calcium/magnesium-free Earle's balanced salt solution
DAT	dopamine transporter
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EAAT	excitatory amino acid transporter
GABA	γ-aminobutyric acid
GAT	GABA transporter
GLT1	glutamate transporter 1
HBSS	Hank's balanced saline solution
HPLC	high-pressure liquid chromatography
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDZ	PSD95, discs large, ZO-1
PICK1	protein interacting with C kinase 1
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBS-T	TBS buffer + 0.1% Triton X-100
X-gal	X-galactocerebroside

A

	–3	–2	–1	0
GLAST	E	**T**	K	**M**
GLT1a	K	R	E	K
GLT1b	E	**T**	C	**I**
GLT1c	Q	**S**	W	**V**
EAAT2d	L	**T**	A	**L**
EAAC1	T	**S**	Q	**F**
EAAT4	E	**S**	V	**M**
EAAT5	E	**T**	N	**V**
PDZ I	X	**S/T**	X	**I/V**
PDZ II	X	φ	X	φ

B

Bait clones	Growth	LacZ
***GAL4*DB-cGLT1b-PFPFLDIETCI**	+	+
***GAL4*DB-cGLT1a**	-	-
GAL4*DB-cGLT1b-PFPFLDI	-	-
***GAL4*DB-cGLT1b-PFPFLDIATCI**	+	+
***GAL4*DB-cGLT1b-PFPFLDISTCI**	+	+
***GAL4*DB-cGLT1b-PFPFLDIEICI**	+	+
***GAL4*DB-cGLT1b-PFPFLDIETCIY**	-	-

Fig. 1.

PICK1 interacts with GLT1b in yeast. (A) PDZ domain interaction motifs in glutamate transporter C-terminal sequences are shown. All known glutamate transporters, except GLT1a, have PDZ domain interaction motifs in their C-termini, if any hydrophobic residue is allowed in the P0 position. (B) PICK1 interacted with GLT1b in a yeast two hybrid screen. The C-terminal cytoplasmic domain of the originally cloned GLT1 (GLT1a) and various mutations of the GLT1b C-terminus were inserted into the pDBLeu vector and co-transformed with pPC86–PICK1. Growth and β-galactosidase expression were assessed visually. Deletion of the last four amino acids or addition of an extra tyrosine residue completely abolished the GLT1b interaction with PICK1. GLT1a did not interact with PICK1.

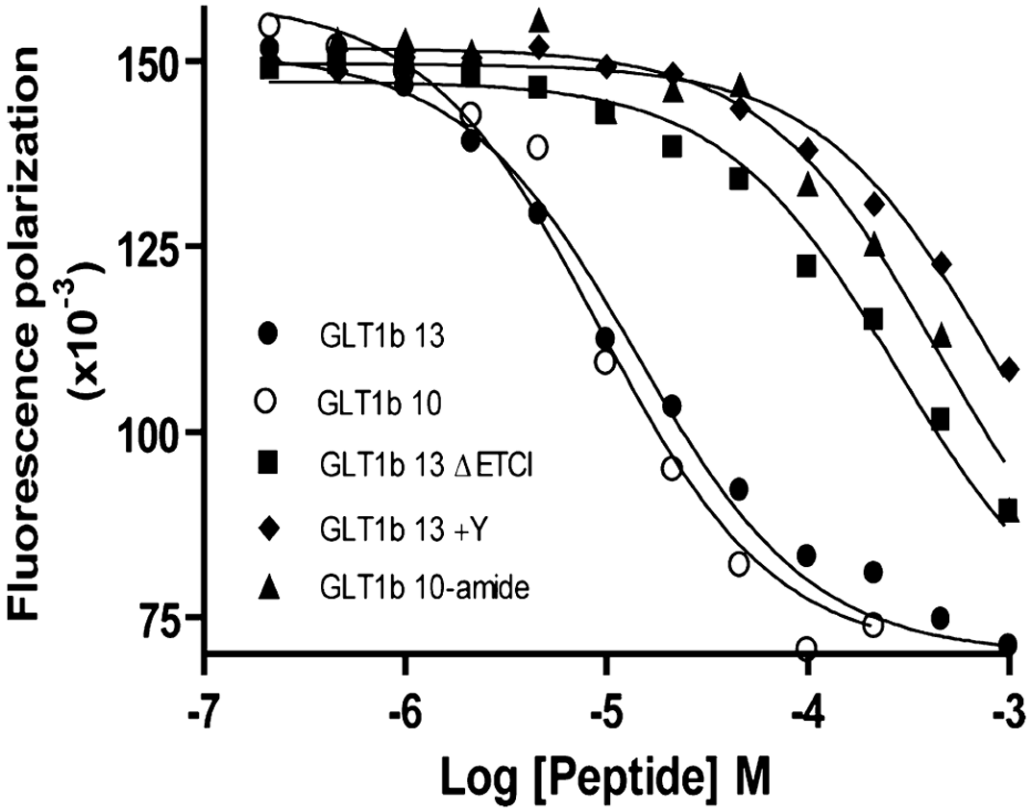

Fig. 2.

The 13 amino acid C-terminus of GLT1b (GLT1b13) interacted with PICK1 with high affinity and specificity in solution. *In vitro* fluorescence polarization assay was used to analyse the interaction of peptides with PICK1. The GLT1b–PICK1 interaction was inhibited by addition of a C-terminal tyrosine (GLT1b13 + Y) or deletion of the C-terminal four amino acids (GLT1b13ΔETCI). Reduction of peptide length to 10 amino acids (GLT1b10) did not significantly affect the interaction compared with the 13 amino acid peptide (GLT1b13). Replacing the C-terminal carboxyl group with an amide group (GLT1b10-amide) blocked the interaction, producing a result similar to that obtained with vehicle (DMSO) alone (see Fig. 5B)

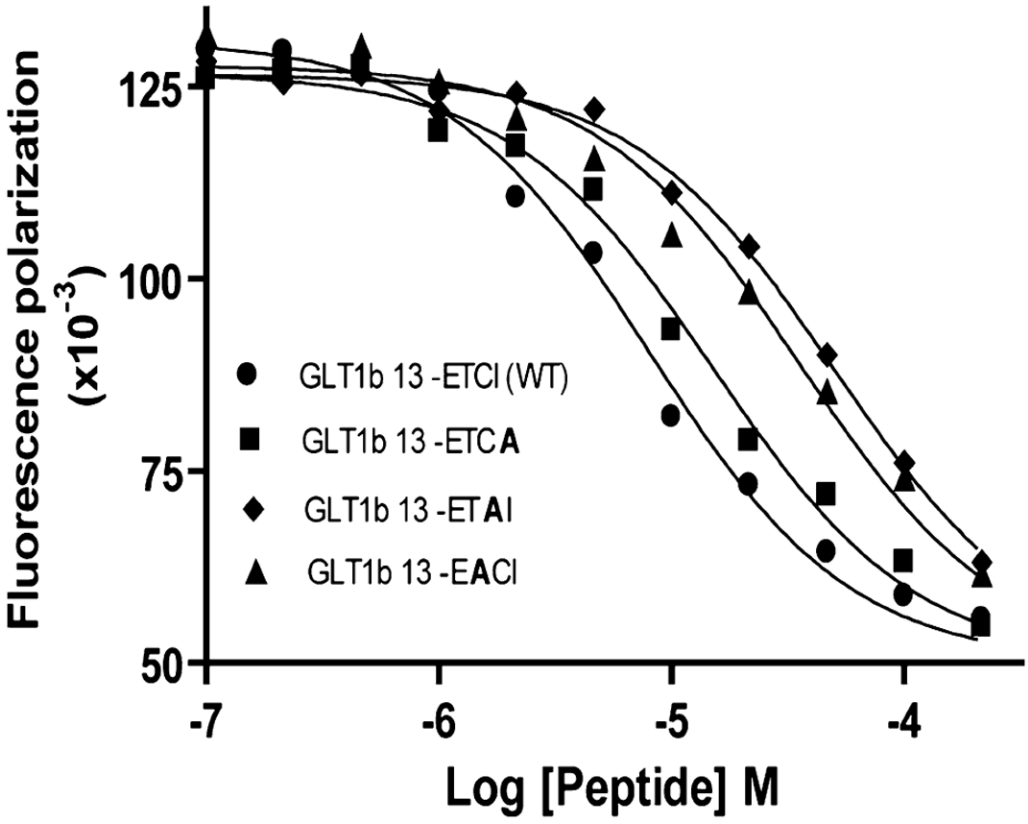

Fig. 3.

The effect of alanine mutagenesis on the interaction of GLT1b peptide with PICK1. Alanine substitution at the P0 position (GLT1b13ETCA) had little effect on the affinity of the GLT1b peptide with PICK1. Alanine substitution at the P1 and P2 (GLT1b13ETAI and GLT1b13EACI) positions produced similar moderate inhibition of the interaction.

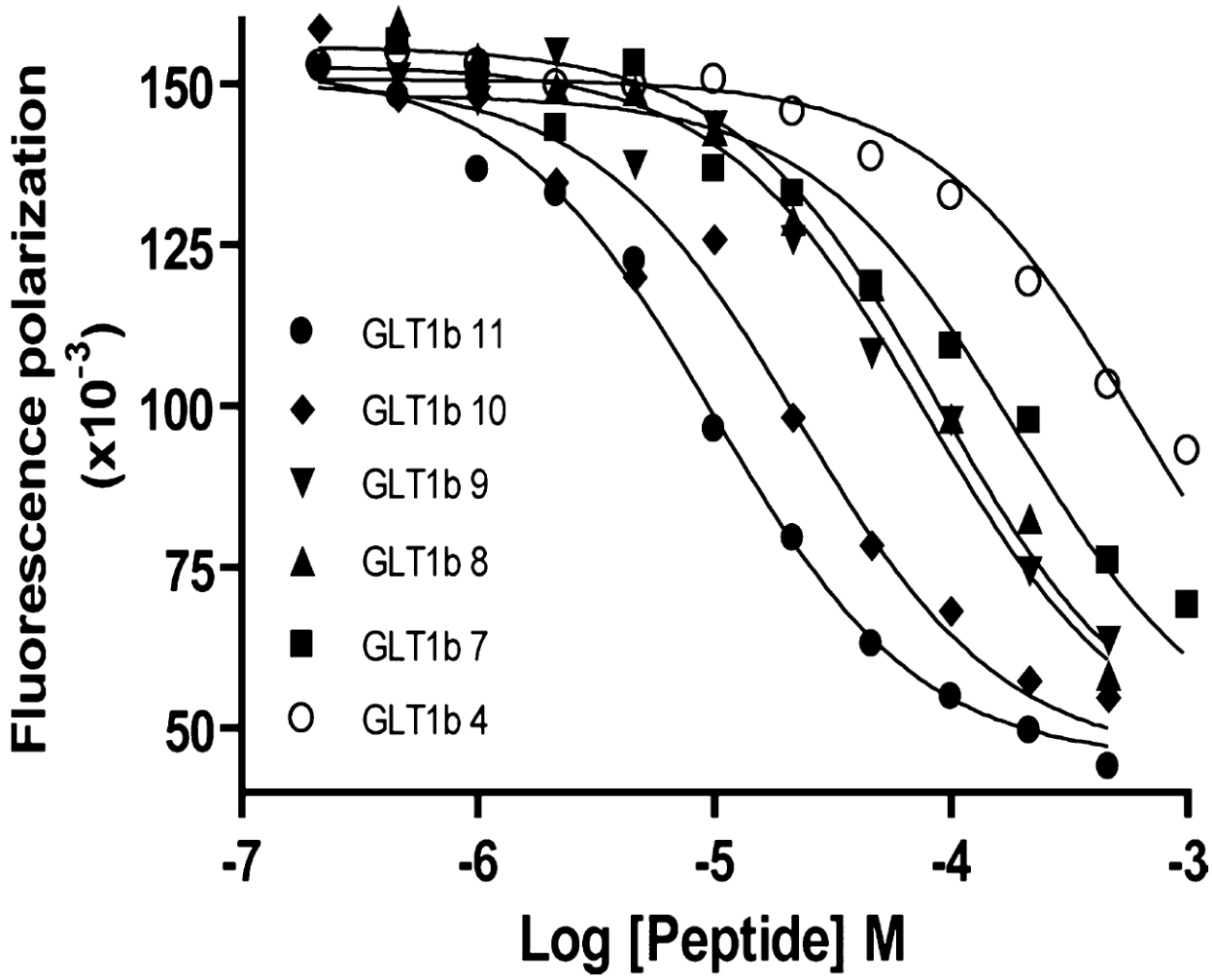

Fig. 4.

Effect of N-terminal truncations on the affinity of GLT1b-based peptides for PICK1. Interaction was greatly decreased with the truncated peptides GLT1b7 (last seven amino acids) and GLT1b4 (last four amino acids).

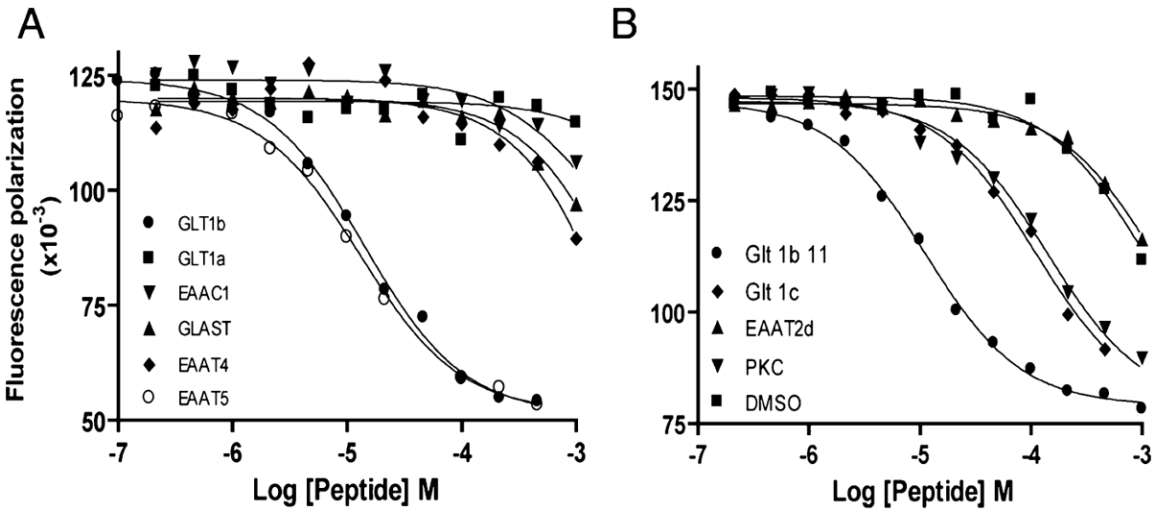

Fig. 5.

Assay of C-terminal peptide interactions of other glutamate transporters (all 11-mers) with PICK1. (A) Only EAAT5, in addition to GLT1b, demonstrated high affinity interaction with PICK1. (B) A peptide based on the variant form GLT1c bound with high affinity to PICK1, comparable to that of protein kinase C (PKC)α. A peptide based on another variant form found in an EST database, 'EAAT2d', did not interact with PICK1. DMSO, dimethylsulfoxide.

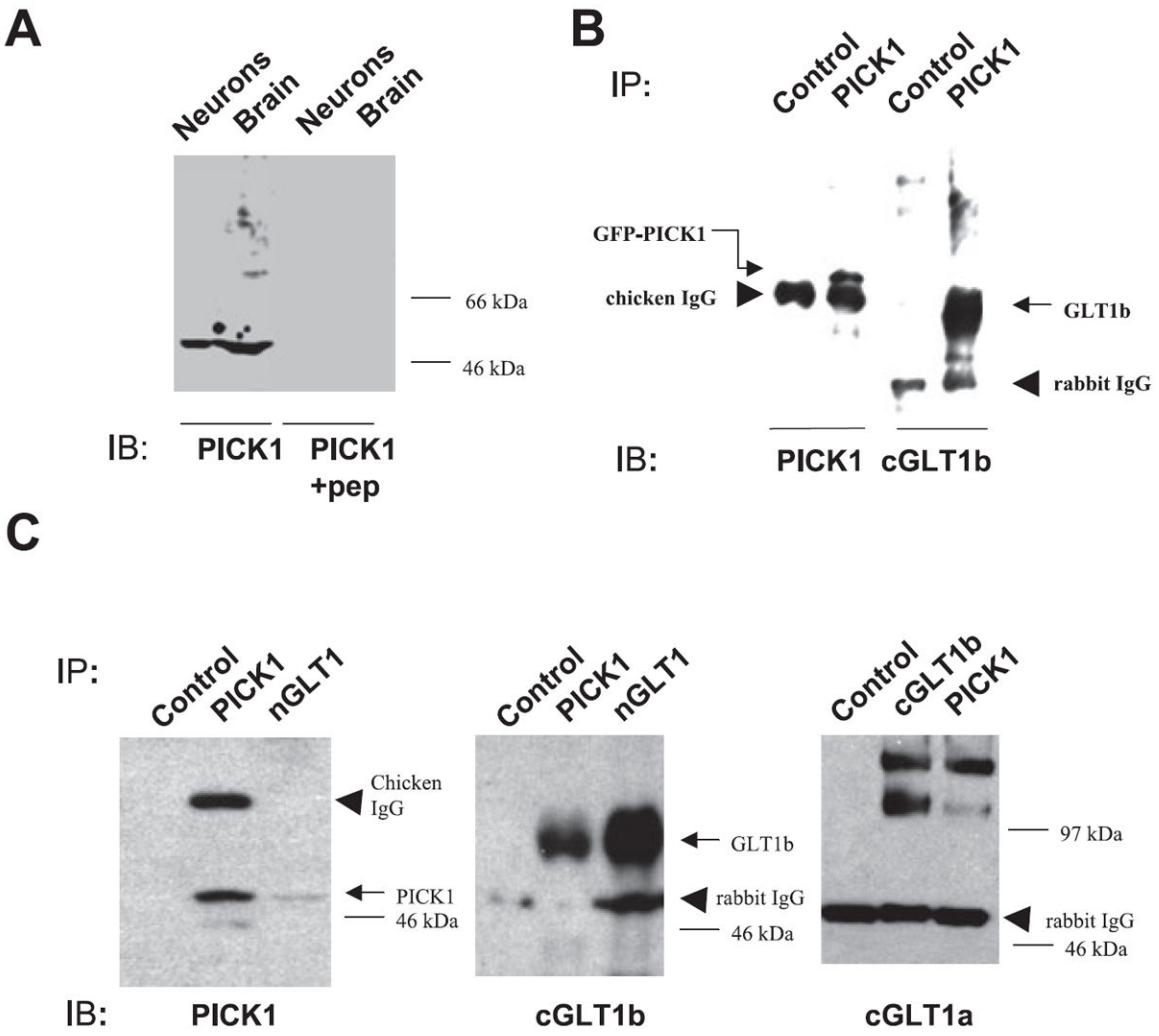

Fig. 6.

PICK1 interacts specifically with GLT1b *in vivo* and *in* vitro. (A) Characterization of an anti-PICK1 antibody. A polyclonal antibody directed against the C-terminus 16 amino acid peptide of PICK1 (RGATGPTDKGGSWCDS) was raised in chickens. The affinity-purified antibody recognized a single band at 55 kDa on immunoblot of rat neuronal and rat brain protein. Co-incubation with the antigenic peptide blocked the immunoreactivity. (B) Co-immunoprecipitation of GLT1b and PICK1 from COS7 cells. The coding sequence of GLT1b was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and the PICK1 coding region was fused at the C-terminus of EGFP in EGFP-C1 vector (Clontech). COS7 cells were co-transfected with these two plasmids. Immunoprecipitation with C-terminal PICK1 antibody, but not chicken IgG (‘Control’), pulled down EGFP–PICK1 fusion protein. A chicken IgG band at about 75 kDa was also detected by the secondary antibody. GLT1b co-immunoprecipitated with PICK1. A 50-kDa band representing the rabbit anti-chicken IgG that was used as an intermediate antibody to pull down chicken IgG and the anti-PICK1 antibody was also recognized by the secondary antibody. (C) Co-immunoprecipitation of GLT1b, GLT1a and PICK1 from rat brain. Rat forebrain lysate was immunoprecipitated with anti-PICK1, anti-nGLT1 or anti-cGLT1b antibody. PICK1 protein

was precipitated with anti-PICK1 antibody and with anti-nGLT1 antibody in much smaller amount. GLT1b was precipitated with anti-nGLT1 antibody as well as anti-PICK1 antibody. GLT1a protein also immunoprecipitated with both anti-cGLT1b and anti-PICK1 antibodies in multimers. The 75-kDa chicken IgG band and the 50-kDa rabbit IgG band that were in the immunocomplex were detectable on the immunoblot.

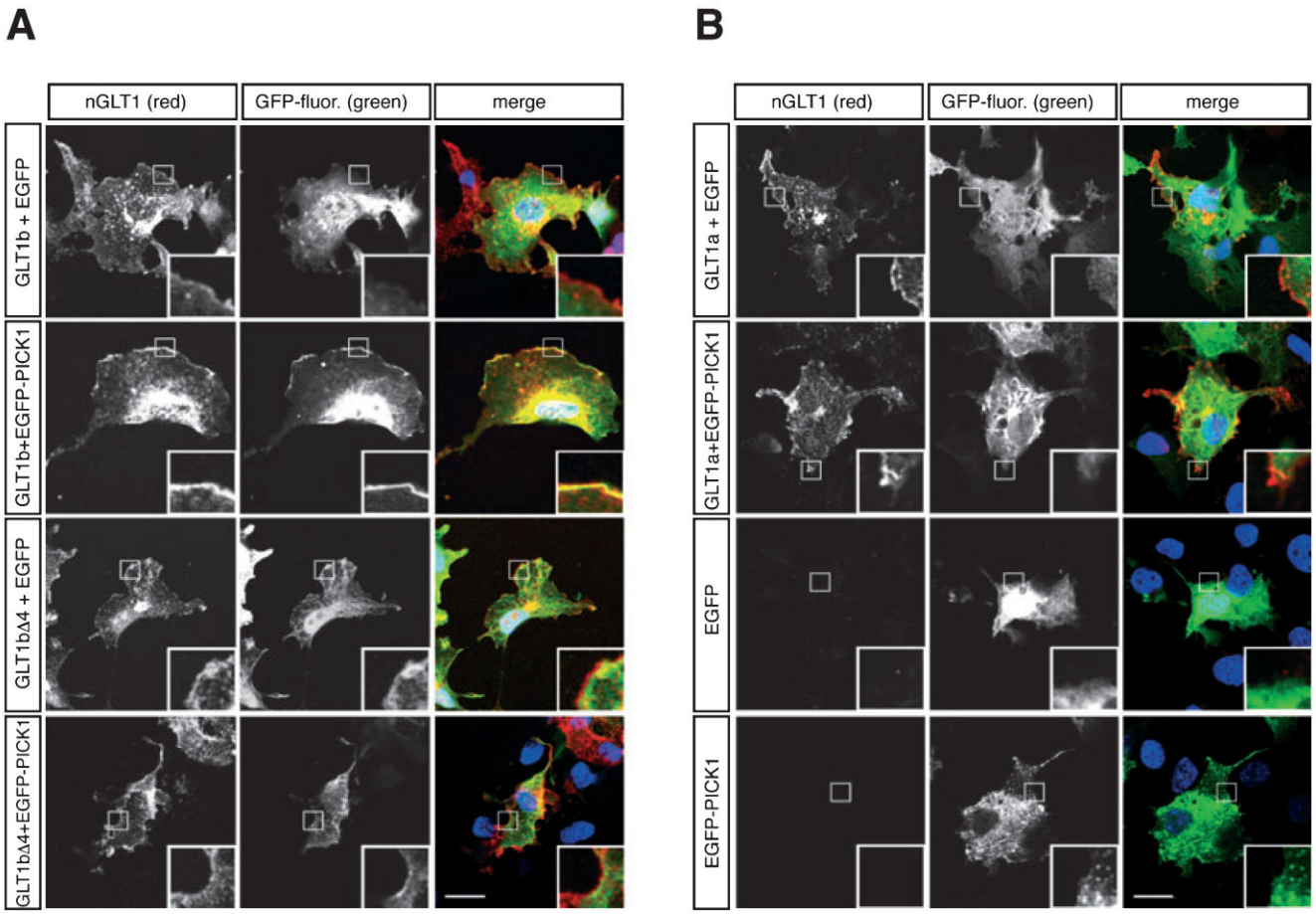

Fig. 7.

Immunocytochemical localization of GLT1 and PICK1 expressed in COS7 cells. (A) GLT1b, but not GLT1bΔETCI (GLT1bΔ4), induced redistribution of PICK1 in COS7 cells. GLT1b and GLT1bΔ4 are present mainly on the plasma membrane, but also in the cytoplasm. Note no co-localization of GLT1b and GLT1bΔ4 with EGFP on the plasma membrane. In contrast, co-expression of GLT1b with PICK1 induced redistribution of the PICK1 fusion protein to the plasma membrane (yellow labeling). Co-localization of EGFP–PICK1 and GLT1b, visualized as yellow labeling, was apparent both at the plasma membrane and in a perinuclear distribution. The deletion of the last four amino acids of GLT1b abolished the co-localization. (B) Distribution of GLT1a co-transfected with EGFP or EGFP– PICK1 in COS7 cells. GLT1a immunoreactivity was detected on the plasma membrane and also intracellularly. The EGFP–PICK1 distribution was not altered when co-expressed with GLT1a (note absence of yellow labeling). EGFP alone was distributed diffusely in the cytoplasm and nucleus. The EGFP–PICK1 fusion protein was primarily intracellular; in about 15% of the cells membrane staining was detected. A similar degree of labeling of the cell membrane by the EGFP–PICK1 fusion protein was detected when co-expressed with GLT1bΔ4. In contrast, when GLT1b was co-expressed with EGFP–PICK1, 92% of the cells showed EGFP–PICK1 at the cell surface, manifest as yellow labeling. The nucleus, detected by bisbenzamide, is shown in blue in the merged pictures only. Small squares depict areas enlarged in the lower right corner. Scale bar: 20 μm.

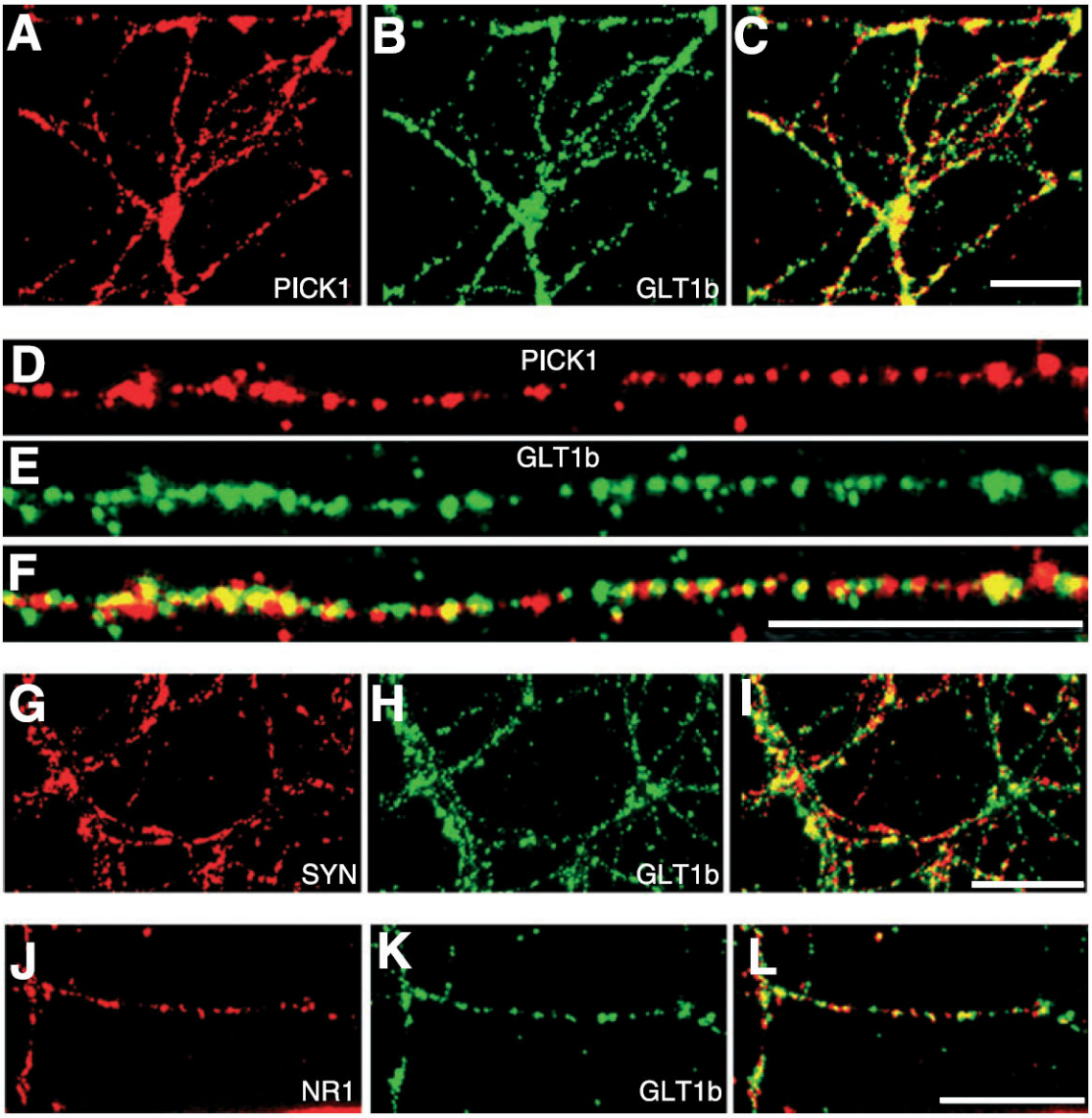

Fig. 8.

Immunocytochemical localization of PICK1, GLT1b and synaptic markers in hippocampal neurons. (A–F) Hippocampal cultures at 14 days *in vitro* were labeled with an anti-cGLT1b (green) antibody raised in rabbit and an anti-PICK1 (red) antibody raised in chicken and then exposed to the appropriate secondary antibodies. (A–C) PICK1 and GLT1b are both present in the cell body as well as in a punctate distribution over neuronal processes. Overlying the two images (C) shows partial overlap of the localization of the two proteins. (D–F) At higher magnification, the localization of immunoreactivity of both PICK1 and GLT1b in discrete roughly spherical units suggestive of synapses is apparent. Overlying the two images shows partial overlap (F). (G–I) Hippocampal cultures were labeled with a mouse monoclonal anti-synaptophysin antibody (G), and anti-cGLT1b (H). Overlying the two images indicates partial overlap (I), indicating close association of GLT1b with the presynaptic marker. (J–L) Hippocampal cultures were labeled with an anti-NR1 antibody (J)

as well as anti-cGLT1b (K). Overlying the two images indicates partial overlap (L), indicating close association of GLT1b with the postsynaptic marker. Scale bars: 20 μm.

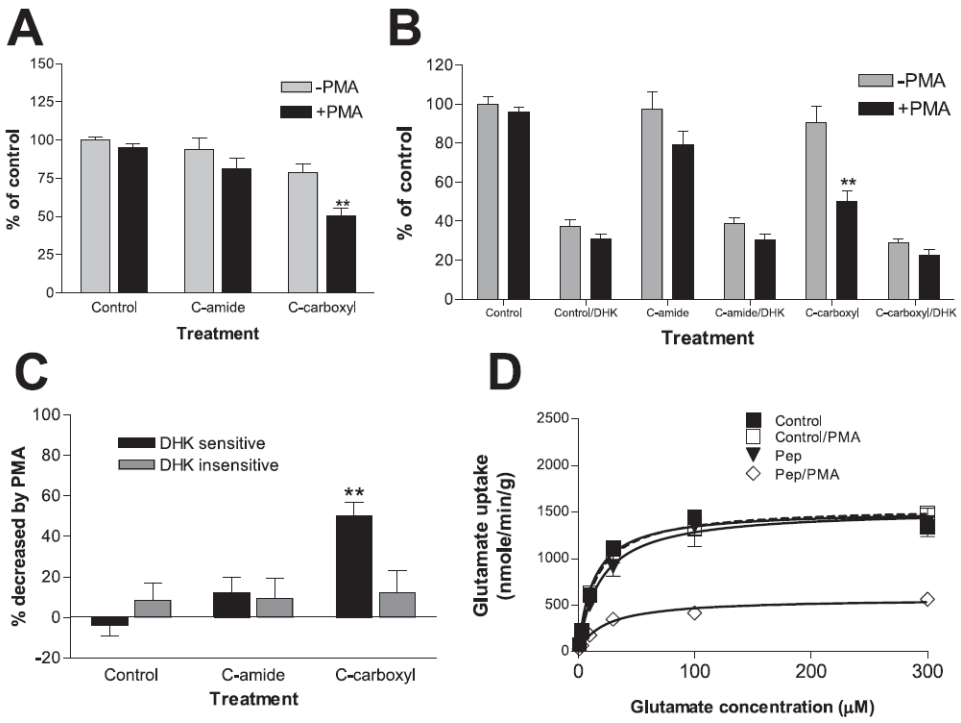

Fig. 9.

Functional interactions of PICK1 and GLT1b in hippocampal neurons. (A) Phorbol 12-myristate 13-acetate (PMA; 400 nM; 2 h) induced downregulation of glutamate uptake in neuronal cultures treated with C-terminal carboxyl myristoylated peptide (3 μM; total time 3.5 h) but not C-terminal amide myristolated peptide. In the presence of the active GLT1b decoy peptide, PMA caused a reduction in glutamate uptake to 63.8 ± 19.6% of the control value (GLT1b decoy treated without PMA; $P < 0.01$). The pooled results of 12 experiments that were performed are shown. Actual uptake data in PMA–control: 32 924 ± 2506 cpm. (B) The effects of PMA plus the C-terminal carboxyl myristolated peptide on DHK-sensitive and DHK-insensitive transport were compared. PMA decreased glutamate transport only in cultures pretreated with the C-terminal carboxyl myristolated peptide, but not in the presence of DHK. The pooled results of five experiments that were performed are shown. Actual uptake data in PMA–control: 32 816 ± 6423 cpm. (C) PMA plus C-terminal carboxyl myristolated peptide decreased the DHK-sensitive component of transport. These results are from the same experiments displayed in B. There was a 49.2 ± 7.1% decrease in the DHK-sensitive component. The DHK-insensitive component did not show an effect that was specific to the active GLT1b decoy peptide. (D) Saturation analysis of glutamate uptake in neuronal cultures was performed after treatment with C-carboxyl myristoylated peptide ('Pep'; 3 μM) treatment (1.5 h) followed by PMA (400 nM with or without peptide, for 2 h). PMA induced a 64.8 ± 4.2% decrease in V_{max}, without effect on K_m. Results from one experiment of two that were performed are shown. $^{**}P < 0.01$.

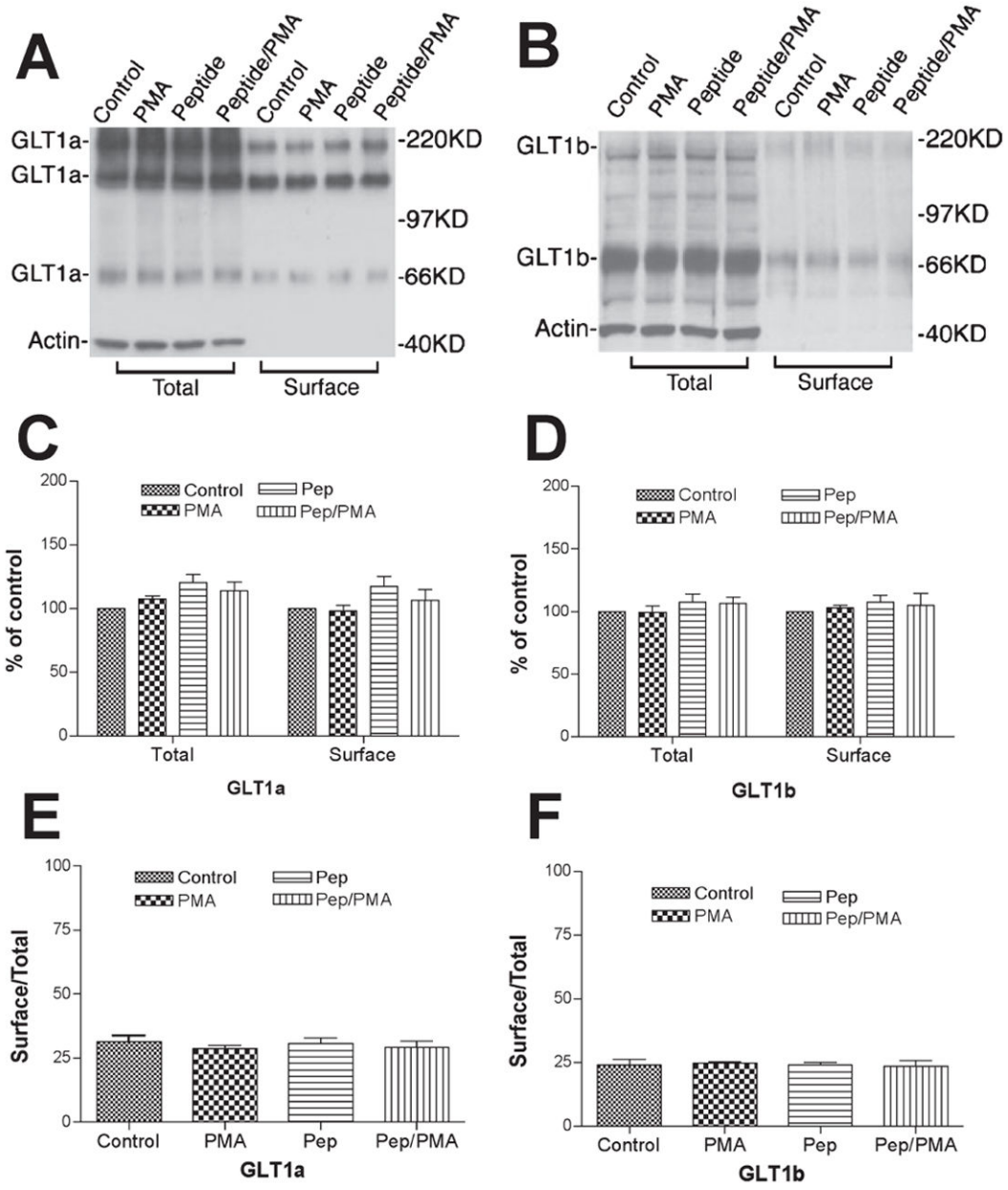

Fig. 10.

PKC activation is not associated with a change in surface expression of GLT1 in cultured forebrain neurons. (A) A representative Western blot showing no effect of phorbol 12-myristate 13-actetate (PMA) with or without myristoylated peptide on cell surface expression of GLT1a. Actin was visualized on the same immunoblot as an internal standard for comparison for protein loading in the 'total' lysates, and to reveal labeling of cytoplasmic proteins in the 'surface' lysates. (B) A representative Western blot showing no effect of 400 nM PMA with or without active decoy peptide on cell surface expression of GLT1b. (C) Densitometry of the GLT1a bands of pooled data from nine experiments that were performed. Bands selected for analysis were monomers (approximately 70 kDa) and multimers (approximately 140 kDa and 210 kDa). The results for each group were similar

and pooled data are shown here. No significant differences were observed. (D) Densitometry of the GLT1b bands pooled data from nine experiments. Bands that were selected for analysis were monomers (approximately 70 kDa) and multimers approximately (210 kDa). No significant differences were observed. (E) Ratio of surface to total immunoreactivity for GLT1a. The ratio was about 31 ± 2% for the control. There were no significant differences among different treatments. (F) Ratio of surface to total immunoreactivity for GLT1b. The ratio was 24 ± 2% for the control. There were no significant differences among different treatments.

Table 1

The affinities of C-terminal glutamate transporter peptides for PICK1

Peptide	K_i (μM)	(*n*)
GLT1bETCI	6.5 ± 0.4	(6)
GLT1bEACI	31 ± 2	(3)
GLT1bETAI	45 ± 2	(3)
GLT1bETCA	12.7 ± 0.8	(3)
GLT1bETCI + Y	360 ± 50	(3)
GLT1bΔETCI	74 ± 6	(3)
GLT1b10CHO	190 ± 50	(3)
GLT1b11	2.3 ± 0.3	(3)
GLT1b10	4.1 ± 1	(3)
GLT1b9	13 ± 3	(3)
GLT1b8	13 ± 4	(3)
GLT1b7	70 ± 7	(3)
GLT1b4	85 ± 8	(3)
EAAC1*	–	–
GLT 1a*	–	–
GLT1c	27 ± 2	(3)
EAAT2d	259 ± 2	(3)
GLAST	350 ± 80	(3)
EAAT4	250 ± 50	(3)
EAAT5	5.5 ± 0.1	(3)
PKCα	33 ± 2	(3)
DAT	2.3 ± 0.1	(3)
GluR2	9 ± 1	(3)

The affinities were determined using a fluorescent polarization assay as described in Materials and methods. Data are means ± SEM of the indicated number (*n*) of independent experiments.

*No interaction.