

## SPECIFICITY OF ANTIBODIES: UNEXPECTED CROSS-REACTIVITY OF ANTIBODIES DIRECTED AGAINST THE EXCITATORY AMINO ACID TRANSPORTER 3 (EAAT3)

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**Abstract**—Specific antibodies are essential tools for identifying individual proteins in biological samples. While generation of antibodies is often straightforward, determination of the antibody specificity is not. Here we illustrate this by describing the production and characterization of antibodies to excitatory amino acid transporter 3 (EAAT3). We synthesized 13 peptides corresponding to parts of the EAAT3 sequence and immunized 6 sheep and 30 rabbits. All sera were affinity purified against the relevant immobilized peptide. Antibodies to the peptides were obtained in almost all cases. Immunoblotting with tissue extracts from wild type and EAAT3 knockout animals revealed that most of the antibodies did not recognize the native EAAT3 protein, and that some recognized other proteins. Several immunization protocols were tried, but strong reactions with EAAT3 were only seen with antibodies to the C-terminal peptides. In contrast, good antibodies were obtained to several parts of EAAT2. EAAT3 was only detected in neurons. However, rabbits immunized with an EAAT3-peptide corresponding to residues 479–498 produced antibodies that labeled axoplasm and microtubules therein particularly strongly. On blots, these antibodies recognized both EAAT3 and a slightly smaller, but far more abundant protein that turned out to be tubulin. The antibodies were fractionated on columns with immobilized tubulin. One fraction contained antibodies apparently specific for EAAT3 while another fraction contained antibodies recognizing both EAAT3 and tubulin despite the lack of primary sequence identity between the two proteins. Addition of free peptide to the incubation solution blocked immunostaining of both EAAT3 and tubulin. **Conclusions:** Not all antibodies to syn-

thetic peptides recognize the native protein. The peptide sequence is more important than immunization protocol. The specificity of an antibody is hard to predict because cross-reactivity can be specific and to unrelated molecules. The antigen preabsorption test is of little value in testing the specificity of affinity purified antibodies. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

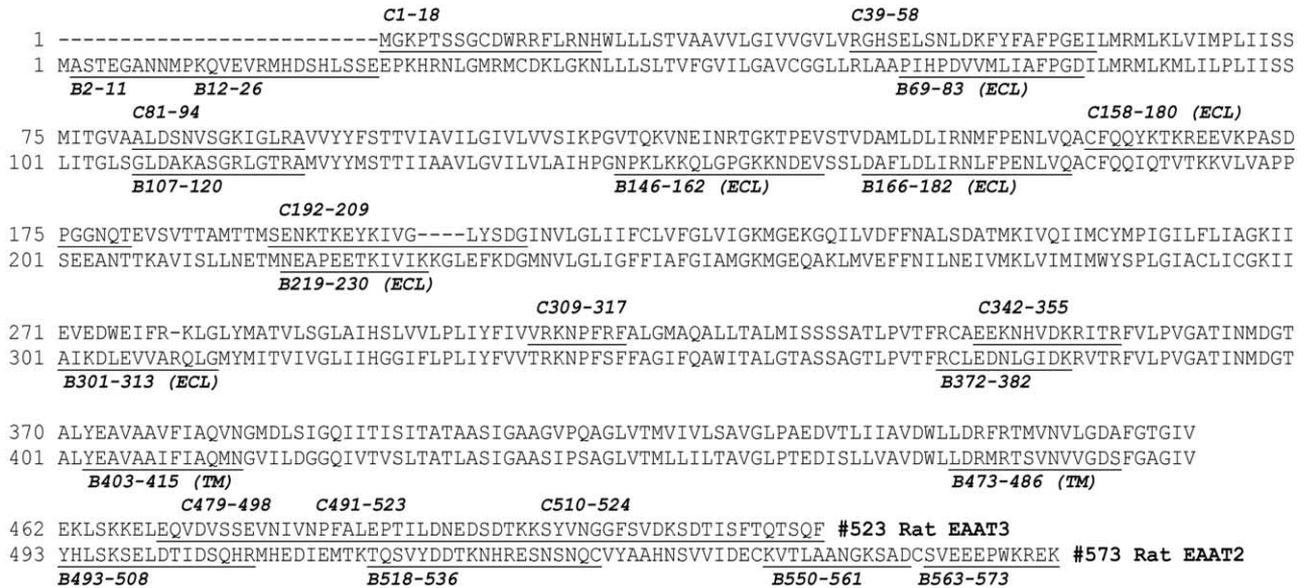
**Key words:** glutamate uptake, immunocytochemistry, polyreactive, antibodies, tubulin, specificity testing, oligodendrocyte.

The amino acid glutamate is the major excitatory neurotransmitter in the mammalian CNS. The only significant mechanism for inactivation of extracellular glutamate appears to be cellular uptake mediated by a family of five glutamate (excitatory amino acid) transporter proteins (EAAT1–5; for review see: Danbolt, 2001). EAAT3 is expressed in neurons (Kanai and Hediger, 1992; Rothstein et al., 1994; Shashidharan et al., 1997; He et al., 2001), including GABAergic ones, in most parts of the nervous system. EAAT3 is concentrated in the neuronal cell bodies (somata) and dendrites apparently avoiding the nerve terminals. Later studies (Conti et al., 1998; Kugler and Schmitt, 1999) have confirmed these findings, but have reported that astrocytes of the cerebral cortex and white matter also express EAAT3 (Conti et al., 1998). Kugler and Schmitt (1999) detected the protein in oligodendrocytes and noted co-localization with tubulin using an antibody directed to a synthetic peptide corresponding to residues 480–499 of rat EAAT3.

We have previously produced antibodies to EAAT1, EAAT2 and EAAT4, and used them to identify the transporter proteins in tissue sections and protein extracts (e.g. Danbolt et al., 1992; Levy et al., 1993; Lehre et al., 1995; Dehnes et al., 1998; Lehre and Danbolt, 1998). In parallel with this work, we have also generated antibodies to EAAT3 by immunizing animals with synthetic peptides corresponding to different parts of the EAAT3 protein sequence. Here we describe the production and testing of the latter antibodies in order to demonstrate some of the difficulties in determining the specificity of an antibody. We show that rabbits immunized with a peptide corresponding to residues 479–498 of rat EAAT3 gave rise to antibodies recognizing both EAAT3 and tubulin. Using antibodies specific to EAAT3, no EAAT3 immunoreactivity was detected in oligodendrocytes in contrast to the previous report based on antibodies to EAAT3 residues 480–499 (Kugler and Schmitt, 1999).

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**Abbreviations:** BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; EAAC1, rabbit glutamate transporter (Kanai and Hediger, 1992); EAAT, excitatory amino acid transporter (=glutamate transporter); EDTA, sodium ethylenediamine tetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; Map, multiple antigenic peptide; MBP, myelin basic protein; MBS, *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester; NaPi, sodium phosphate buffer with pH 7.4; NSC, newborn calf serum; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with 0.1% Triton X-100.



**Fig. 1.** Sequence alignment of rat EAAT2 and rat EAAT3. The amino acid sequences used for peptide synthesis are underlined and the peptide names given either above (EAAT3) or below (EAAT2) the sequences. Some peptides represent parts of putative extracellular (ECL) or transmembrane (TM) domains as indicated. All the other peptides are selected from putative intracellular domains. The peptides C468–482 and C486–499 correspond to parts of rabbit EAAT3 differing from rat EAAT3 and are therefore not shown in this figure. Peptide C510–524 is also from rabbit, but this sequence is identical to rat, corresponding to rat amino acids 509–523. To avoid confusion, we have kept this peptide's original (rabbit) numbering, as it is used throughout this paper. Peptide C1–13 is not shown because of the overlap with C1–18.

## EXPERIMENTAL PROCEDURES

### Materials

Sodium dodecyl sulfate (SDS) of high purity (>99% C12 alkyl sulfate) and bis(sulfosuccinimidyl) suberate were obtained from Pierce (Rockford, IL, USA). *N,N'*-methylene-bisacrylamide, acrylamide, ammonium persulfate, TEMED and alkaline phosphatase substrates (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) were from Promega (Madison, WI, USA). Biotinylated anti-rabbit, anti-sheep and anti-mouse immunoglobulins, streptavidin-biotinylated horseradish peroxidase complex, and colloidal gold-labeled anti-rabbit and anti-mouse immunoglobulins, electrophoresis equipment, molecular mass markers for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), nitrocellulose sheets (0.22 m pores, 100% nitrocellulose), Protein A-Sepharose Fast Flow and Sephadex G-50 fine were from Amersham Biosciences (Buckinghamshire, UK). Alexa fluor goat anti-rabbit 555 and goat anti-mouse 488 were from Molecular Probes (Eugene, OR, USA). Paraformaldehyde and glutaraldehyde EM grade were from TAAB (Reading, UK). Fluoromount G and Lowicryl HM20 were from Electron Microscopy Sciences (Fort Washington, PA, USA). Alkaline phosphatase-conjugated monoclonal antibodies to rabbit and sheep IgG, anti-beta-tubulin, bovine serum albumin (BSA), 3-[(3-cholamido-propyl)dimethylammonio]-1-propanesulphonate (CHAPS), dithiothreitol (DTT), EDTA, guanosine-5'-triphosphate (GTP), HEPES, human serum albumin (HSA), keyhole limpet hemocyanin (KLH), *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester (MBS), phenylmethanesulfonyl fluoride (PMSF), rabbit serum albumin, thyroglobulin, Trizma base, Trisma-HCl and tubulin were obtained from Sigma (St. Louis, MO, USA). Other reagents were obtained from Fluka (Buchs, Switzerland). Anti-myelin basic protein (MBP) and anti-CNPase were from Sternberger Monoclonals (Lutherville, MD, USA).

### Peptides

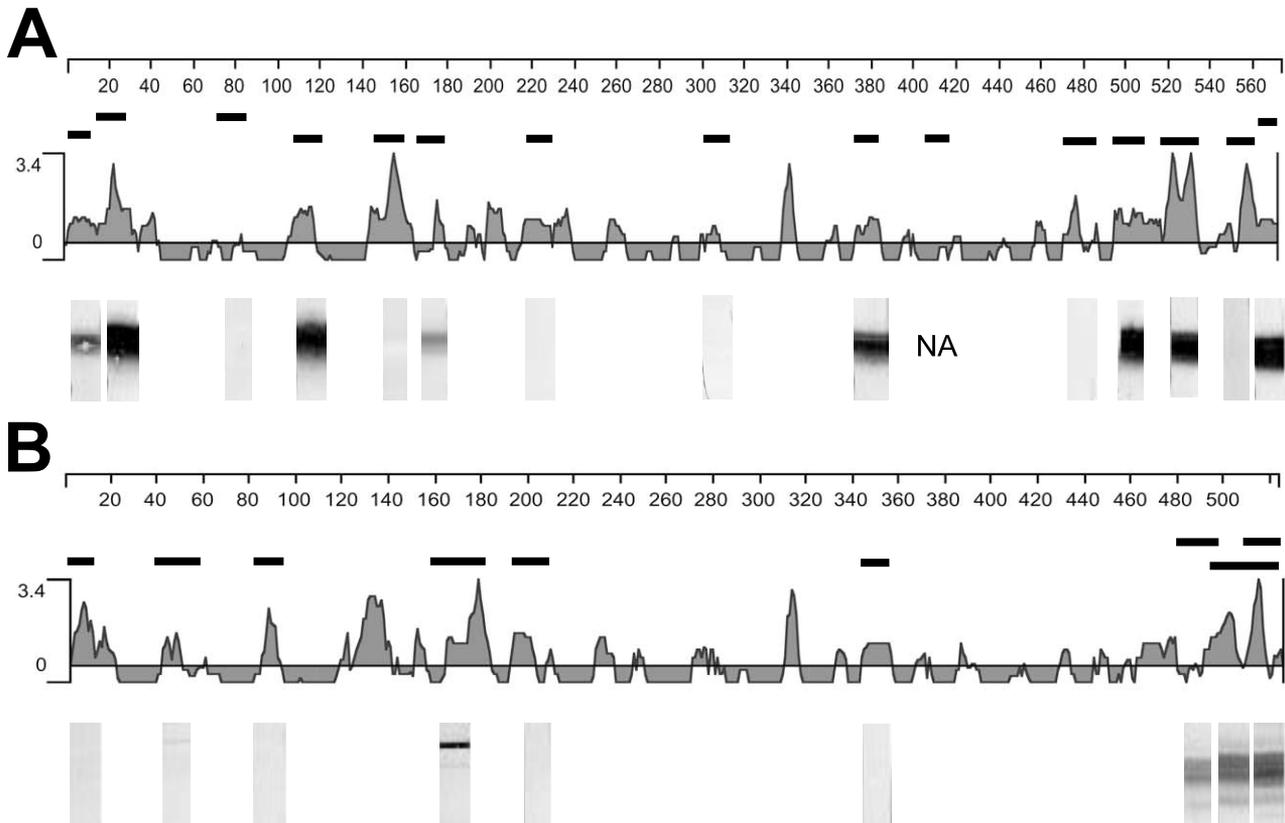
Peptides representing parts of EAAT2 (Pines et al., 1992; 573 amino acid residues) and EAAT3 are referred to by capital letters "B" and "C," respectively, followed by numbers indicating the corresponding amino acid residues in the sequences (given in parentheses). The first EAAT3-peptides were made based on the rabbit sequence which is 524 amino acid residues long (Kanai and Hediger, 1992). The rat sequence was used when it became available (Bjørås et al., 1996) and is 523 residues long (lacking residue 191 in the rabbit sequence). The peptide sequences are shown in Fig. 1. Note that the C510–524 peptide is numbered according to the rabbit sequence although identical to the rat 509–523. The following two rabbit peptides are not shown in Fig. 1 because the sequences are different: C468–482 (KELEQMD-VSEVNIV-*amide*) and C486–499 (ALESATLDNEDSDT-*amide*).

Only the peptides representing the C-termini of the native proteins were synthesized as free C-terminal acids (B563–573, C491–523 and C510–524). The remaining peptides shown were synthesized as C-terminal amides. B301–313 and C1–13 were also synthesized as multiple antigenic peptides (map). Map-peptides were used for immunization without coupling to carrier protein while the other peptides were coupled to either KLH, rabbit serum albumin or thyroglobulin with either glutaraldehyde (with or without reduction with sodium borohydride) or MBS as described previously (Danbolt et al., 1998). The production of gold particles (Frens, 1973) and the conjugation of gold to immunogens (Pow and Crook, 1993) were performed as described.

Antigenicity profiles (Fig. 2) were calculated for rat EAAT2 and EAAT3 according to Jameson and Wolf (1988) using the Protean program (DNASTAR, Inc., Madison, WI, USA).

### Animals, immunizations and collection of tissue

All animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and



**Fig. 2.** Comparison of antibody production to EAAT2 (A) and EAAT3 (B). Antigenicity profiles (Jameson and Wolf, 1988) were used to help find the most antigenic amino acid sequences in the proteins. The amino acid sequences of the two transporters are represented by numbered lines above the profiles. Peptides corresponding to parts of the sequences were synthesized as indicated by short horizontal black lines between the numbered lines and the antigenicity profiles. Sequence information is given in Fig. 1. All peptides shown (except B403–415) gave rise to antibodies as determined by the amount of protein isolated by affinity purification. The purified antibodies were subsequently tested by immunoblotting (see Table 2 for antibody concentrations) as shown below the profiles. The nitrocellulose blots represent PAGE separated SDS extracts of rat hippocampus, and the blots were cut into identical strips. (A) Antibodies from left to right: anti-B2 (+); anti-B12B (+); anti-B69, anti-B107 (+); anti-B146; anti-B166 (+); anti-B219; anti-B301; anti-B372 (+); anti-B473; anti-B493 (+); anti-B518 (+); anti-B550 and anti-B563 (+). Each strip contained 1.75  $\mu$ g protein rat hippocampus protein except the strip for anti-B166 which had 17.5  $\mu$ g. (B) Antibodies from left to right: anti-C1; anti-C39; anti-C81; anti-C158; anti-C192; anti-C342; anti-C479P (+); anti-C491B (+) and anti-C510A (+). All the strips had 35  $\mu$ g rat hippocampus protein. Please note: (1) Lack of labeling reaction does not imply lack of antibody, but lack of reactivity toward the native proteins. (2) EAAT2 was recognized by antibodies to eight of the 15 EAAT2-peptides, but EAAT3 by only three of 13 EAAT3-peptides. NA, no antibody; (+), positive reaction with native proteins.

the European Communities Council Directive of 24 November 1986 (86/609/EEC). Formal approval to conduct the experiments described was obtained from the animal subjects review board of our institutions. Care was taken to minimize the number of animals used and avoid suffering.

**Rabbits and sheep.** Chinchilla rabbits (Chbb:CH) and New Zealand rabbits (obtained from B&K Universal, Sollentuna, Sweden) were kept in the animal facility at the Institute of Basic Medical Sciences (University of Oslo, Oslo, Norway). The sheep were kept at the Governmental Institute of Public Health (SIFF) or at the School of Veterinary Medicine (Oslo, Norway). The animals (Table 1) were immunized and bled as described previously (Danbolt et al., 1998), but using subcutaneous rather than intracutaneous injections.

**Rats and mice.** Adult male Wistar rats (10–12 weeks old) were obtained from B&K Universal. Mice lacking EAAT3 (Peghini et al., 1997) were bred and kept in the animal facility at the John Hopkins University (Baltimore, USA) until they reached 4 weeks of age. Fresh tissue for biochemical studies was obtained from rats and mice killed humanely using approved procedures. Brain tissue for immunocytochemistry was obtained from animals that had

been killed by injection of pentobarbital followed by perfusion fixation (see Immunocytochemistry below).

### Glutamate transporter antibody purification and nomenclature

Antibodies against the peptides could be isolated from the anti-sera in most cases, albeit in highly variable amounts (0–300  $\mu$ g/ml serum; data not shown). Testing of crude antisera was usually done (data not shown), but all antibodies presented here have been affinity purified as described previously on columns containing covalently immobilized antigen (Lehre et al., 1995; Danbolt et al., 1998). Sera from rabbits immunized with multiple peptides were passed through one affinity column for each of the peptides used for immunization. Rabbit 82356 (Table 1) may serve as an example. This rabbit was immunized with three peptides (C468–482, C486–499 and C510–524), and the serum was passed through three affinity columns which were eluted separately. This resulted in three different antibody fractions which were named according to the antigen immobilized on the respective affinity column: anti-C468 (Ab,50), anti-C486 (Ab,51) and anti-C510 (Ab,52). (The parentheses contain the database identification

**Table 1.** Overview EAAT3 antibody production

Animal no.	Peptide(s)	Carrier	Coupling reagent	Antibody [ID no.]
20580*	C1-18+C39+C81+C468+C486+C510	KLH, Map	GA-F, MBS	
26693	C1-18+C39+C81+C158+C192+C342+C468+C486+C510	TG	GA-R	Anti-C510 [Ab,243] Anti-C158+C192 [Ab,158]
26697*	C1-18+C39+C81+C158+C192+C342+C468+C486+C510	RSA, liposomes	GA-R, NHS	Anti-C158+C192 [Ab,161]; Anti-C510 [Ab,240]
26699	C1-18+C39+C81+C158+C192+C342+C468+C486+C510	KLH	GA-R	Anti-C510 [Ab,239]
26719	C1-18+C39+C81+C158+C192+C342+C468+C486+C510	RSA	GA-R	Anti-C510 [Ab,238]
69738*	C510	RSA	GA-R	Anti-C510 [Ab,136]
80820	C510			
82356	C468+C486+C510	KLH	GA-R	Anti-C510 [Ab,52]
84172	C468+C510		GA-R	Anti-C510 [Ab,234]
89058	C1-13+C39	Map		
89350	C1-13	Map		
89780	C39	KLH		
0B0620	C479	KLH	GA-R	
0B0715	C479	KLH	GA-R	Anti-C479 [Ab,334]
0B0717	C479	KLH	GA-F	Anti-C479 [Ab,333]
0B0721	C479	KLH	GA-F	Anti-C479 [Ab,335], [Ab,359], [Ab,545], [Ab,547]
1B0683	C491	KLH	GA-F	Anti-C491 [Ab,10], [Ab,371]
1B0696*	C1-13+C39+C81+C158+C192	Gold-TG	GA-F	
1B0716*	C1-13+C39+C81+C158+C192	Gold-TG	GA-F	
1B0764*	C1-13+C39+C81+C158+C192	Gold-TG	GA-F	
1B0784	C491	KLH	GA-F	
1B0853*	C1-13+C39+C81+C158	Gold-TG	GA-F	
1B1012*	C1-13+C39+C81+C158	Gold-TG	GA-F	
1B1225*	C1-13+C39+C81+C158	Gold-TG	GA-F	
7D0988	C491	KLH	GA-F	Anti-C491 [Ab,236]
7D0993	C491	KLH	GA-F	Anti-C491 [Ab,237]
JQ51	C491	KLH	GA-F	
JR26	C491	KLH	GA-F	
MB303	C81	KLH	GA-R	
MB3459	C1-18+C39+C81+C468+C486+C510		GA-R	
Sh3016	C1-13+C39+C510	Map, KLH	MBS, GA-R	
Sh4131	C491	KLH	GA-F	Anti-C491 [Ab,256]; Anti-C510 [Ab,340]
Sh4430	C158+C192+C510	KLH	MBS	Anti-C158 [Ab,209]; Anti-C192 [Ab,211]
Sh I	C510	KLH	GA-R	
Sh II	C468	KLH	GA-R	
Sh III	C486	KLH	GA-R	

Most of the EAAC peptide sequences gave rise to antibodies recognizing the peptides they were directed against, but only a few of these labeled EAAC protein (listed in "Antibody [ID no.]"). Although some very weak antibodies were obtained for C158–180 and C192–209, by far the best antibodies were obtained from sequences close to the C-terminal (C479–498, C491–523 and C510–524; see also Fig. 1). Changing the carrier protein or coupling reagent did not seem to change this trend, and this was true whether the animals were immunized with one peptide or a mix of different ones. Peptide-conjugates were mixed with Freund's Complete Adjuvant (FCA) at the first immunization and Freund's Incomplete Adjuvant (FIA) at the subsequent ones, except for those animals marked with asterisk (\*). These animals received FIA supplemented with muramyl dipeptide at all immunizations. Abbreviations: GA-F, glutaraldehyde in free form; GA-R, glutaraldehyde reduced after coupling with NaBH<sub>4</sub>; NHS, N-hydroxysuccinimide; Map, multiple antigenic peptide; MBS, m-maleimido benzoyl-N-hydroxysuccinimide ester; RSA, rabbit serum albumin; TG, thyroglobulin.

numbers). Only the latter antibody is listed in Table 1 because it was the only one which recognized EAAT3.

An overview of the antibodies used in the present report is given in Table 2. To make this manuscript easier to read, the antibodies have been given short systematic and informative names. However, these names do not contain sufficient information to identify them unequivocally in our records. Therefore

we have also included the unique database identification numbers.

The antiserum from rabbit (Rb) 0B0721 to C479–498 (19.09.2002) was subjected to a four stage purification process (see Results) which included absorption against tubulin. Purified antibodies were quantified spectrophotometrically at 280 nm using bovine IgG as the standard.

**Table 2.** Primary antibodies used

Antibody ID no.	Animal number	Antibody names used in the present report	Ligand on affinity column	Reference date	Conc. used for blot labeling ( $\mu\text{g/ml}$ )
Ab,109	Rb 89350	Anti-C1	C1–18	1994-07-16	3
Ab,125	Rb 89780	Anti-C39	C39–58	1994-07-16	3
Ab,206	Rb 26693	Anti-C81	C81–94	1996-07-08	3
Ab,245	Rb 26693	Anti-C158	C158–180	1997-12-17	3
Ab,166	Rb 26699	Anti-C342	C342–355	1996-05-27	3
Ab,50	Rb 82356	Anti-C468	C468–482	1993-06-20	3
Ab,336	Rb 0B0620	Anti-C479A	C479–498	2001-07-26	1
Ab,334	Rb 0B0715	Anti-C479B	C479–498	2001-07-26	1
Ab,333	Rb 0B0717	Anti-C479C	C479–498	2001-07-26	1
Ab,335	Rb 0B0721	Anti-C479D	C479–498	2001-07-26	1
Ab,545	Rb 0B0721	Anti-C479-KLH	KLH	2002-09-19	
Ab,547	Rb 0B0721	Anti-C479-Tub	Tubulin	2002-09-19	
Ab,359	Rb 0B0721	Anti-C479P	C479–498	2002-09-19	3
Ab,371	Rb 0B0683	Anti-C491B	C491–523	2003-01-03	1
Ab,237	Rb 7D0993	Anti-C491A	C491–523	1997-12-14	1
Ab,126	Rb 69738	Anti-C510A	C510–523	1993-04-04	1
Ab,340	Sh 4131	Anti-C510B	C510–523	2001-08-16	1
Ab,48	Rb 81024	Anti-B2	B2–11	1993-06-15	1
Ab,152	Rb 68518	Anti-B12A	B12–26	1995-09-14	0.2
Ab,360	Rb 26970	Anti-B12B	B12–26	2002-07-10	0.2
Ab,130	Rb 89606	Anti-B107	B107–120	1995-04-23	1
Ab,528	Rb 8D0155	Anti-B146	B146–162	1998-08-01	1
Ab,311	Rb 84204	Anti-B166	B166–182	1998-08-01	10
Ab,42	Rb 68550	Anti-B219	B219–230	1993-01-30	1
Ab,132	Rb 89330	Anti-B301	B301–313	1995-07-25	1
Ab,63	Rb 82898	Anti-B372	B372–384	1994-06-05	1
Ab,64	Rb 82898	Anti-B473	B473–486	1993-08-09	1
Ab,97	Rb 84946	Anti-B493	B493–508	1994-05-29	0.5
Ab,94	Rb 84932	Anti-B518	B518–536	1993-12-28	1
Ab,356	Rb 1B0707	Anti-B550	B550–561	2002-09-05	3
Ab,355	Rb 1B0707	Anti-B563	B563–573	2002-09-05	0.5

### Electrophoresis and immunoblotting

Brain and kidney tissues were rapidly dissected out from rats and mice and directly homogenized in five to 15 volumes of 20 mM sodium phosphate buffer (NaPi) pH 7.4 containing 1% (w/v) SDS and 1 mM PMSF. The mixture was sonicated (30 s; dr. Hielscher UP 50H<sup>®</sup>) to reduce viscosity (by breaking up DNA). Brain tissue was homogenized in a Dounce glass–glass homogenizer while kidney tissue was first homogenized by means of a Polytron PT1200<sup>®</sup> homogenizer (which is able to break up connective tissue) and then further treated in a Dounce glass–glass homogenizer. Undissolved kidney tissue components were sedimented by centrifugation (3000 r.p.m., 41 °C, 5 min). These extracts are referred to below as brain or kidney SDS-extracts. Protein concentrations were determined with the bicinchoninic acid assay (BCA assay; Smith et al., 1985).

The SDS-extracts were diluted in SDS-sample buffer (Laemmli, 1970) to 1 mg/ml and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) which was performed as described before (Laemmli, 1970; Lehre et al., 1995) with separating gels consisting of 7.5 or 10% acrylamide. The molecular mass markers were used in non-reduced form. After electrophoresis the proteins were either silver stained (Danbolt et al., 1990) or electroblotted onto nitrocellulose membranes (Towbin et al., 1979; Lehre et al., 1995). The blots were immunostained with alkaline phosphatase-conjugated secondary antibodies (Lehre et al., 1995).

### Light microscopical immunocytochemistry

This was performed as described previously (Danbolt et al., 1998; Boulland et al., 2004). Briefly, animals were deeply anesthetized and fixed by transcardiac perfusion with 0.1 M NaPi containing either 4% formaldehyde or 4% formaldehyde and 0.05% glutaraldehyde. Free floating vibratome sections (40  $\mu\text{m}$  thick) were treated with 1 M ethanalamine–HCl (pH 7.4), blocked with 10% newborn calf serum and 3% (w/v) BSA in TBST (300 mM NaCl, 0.5% Triton X-100 and 100 mM Tris–HCl pH 7.4), and incubated overnight with primary antibodies diluted in TBST with 3% newborn calf serum (NCS) and 1% BSA, followed by secondary antibodies diluted in blocking solution. Anti-glutamate transporter antibodies were used in different concentrations as indicated. The mouse anti-CNPase and anti-MBP from Sternberger monoclonals Inc. (Lutherville, MD, USA) were both used at 1:500 dilutions. The secondary antibodies (biotinylated anti-rabbit, anti-sheep and anti-mouse, and fluorescently tagged GAM Alexa 468 and GAR Alexa 555) were all used at 1:1000 dilutions. When fluorescently marked secondary antibodies were used, the sections were mounted in Fluoromount G water base, and observed in a Zeiss Axioplan 2 microscope equipped with a Zeiss LSM 5 Pa confocal scanner head. Pinhole size was around 1 area unit, optimized for each wavelength to ensure confocality. When biotinylated secondary antibodies were used, then the sections were developed with the biotin–streptavidin–peroxidase system and diaminobenzidine as described (Danbolt et al., 1998). Control sections incubated with preimmune IgG instead of anti-peptide antibodies, or with antibod-

ies preabsorbed with the peptide used for immunization, showed no labeling.

### Postembedding

Postembedding immunogold labeling was performed on freeze-substituted low temperature resin-embedded tissue, from rats perfusion fixed as above with 4% formaldehyde and 0.05% glutaraldehyde, as described previously (Dehnes et al., 1998). Ultrathin sections were cut, collected on nickel grids and labeled by sequential immersion for 10 min each at room temperature unless otherwise stated, in small drops of the following solutions: Tris-buffered saline with 0.1% (w/v) Triton X-100 (TBST), 2–3% HSA in TBST, primary antibody diluted as appropriate in HSA–TBST (4 °C overnight), three times in TBST, gold-conjugated secondary antibody in HSA–TBST diluted 1:20 (1–2 h), three times in TBST and two times in distilled water. They were stained with uranyl acetate and lead citrate and examined in a Tecnai 12 transmission electron microscope.

### ELISA-procedure for antibody testing

The procedure was performed by a Tecan Genesis 200 Workstation robot. The microtiterplates were kept on a horizontal shaker at room temperature during all incubations. Each well in the 96 well microtiterplate was first incubated (2 h) with 50  $\mu$ l TBS (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% NaN<sub>3</sub>) containing 3  $\mu$ g antigen per ml and then washed with TBS (4 cycles, 50 s) to remove unbound antigen. To block free binding sites, the wells were incubated with TBS (380  $\mu$ l/well) containing 20% NCS (when not stated otherwise) for 2 h with agitation and washed in TBS with 0.05% (v/v) Tween 20 (TBST) (4 cycles, 50 s). Antibody fractions to be tested were diluted in blocking solution to the desired concentration. 50  $\mu$ l was added to each well and was incubated for 60 min and then washed with TBST (eight cycles, 50 s). The wells were incubated for 60 min with 50  $\mu$ l TBST containing 20% NCS and alkaline phosphatase-conjugated anti-rabbit diluted 1:1000. A final washing with TBST (eight cycles, 50 s) was followed by addition of 100  $\mu$ l *p*-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethanolamine–HCl buffer (pH 9.8) with 1 mM MgCl<sub>2</sub>. The OD405 was measured after 60 min. Background levels in each assay were determined by using BSA as the coating antigen.

## RESULTS

### Anti-peptide antibodies are usually obtained, but they often fail to recognize the parent protein

Animals were immunized with synthetic peptides corresponding to parts of the EAAT3-sequence (Fig. 1, Table 1). The amounts of antibodies which could be isolated by antigen affinity chromatography varied greatly. For example, rabbit 80886 which was immunized with B403–415 did not produce any detectable amounts of anti-peptide antibodies while about 0.2 mg anti-C491 antibodies (Ab<sub>237</sub>) was isolated from each ml of serum from rabbit 7D0993 (data not shown).

Because the antibodies were affinity purified, it follows that all the antibodies shown here (Table 2) did recognize the peptides used to generate them. In spite of this, only a minority of the affinity purified antibodies recognized the EAAT3 protein on immunoblots (Fig. 2B). Only peptides in the C-terminal region generated antibodies recognizing EAAT3. The two peptides from the putative second extracellular loop (C158 and C192) also generated antibodies to the EAAT3 proteins, but their reactions were too weak to

be seen in Fig. 2B and too weak to be useful. The other peptide antibodies showed no detectable signal.

### The peptide sequence is the main factor, but is hard to predict

It is interesting to note that the ability of a peptide antibody to recognize both the peptide and the parent protein seemed to be a property of the peptides and not of the immunization protocol used. The last column to the right in Table 1 lists the peptides giving rise to antibodies recognizing EAAT3-protein. It can be seen that even when animals were immunized with mixtures of peptides, it was the same peptides that gave rise to the good antibodies.

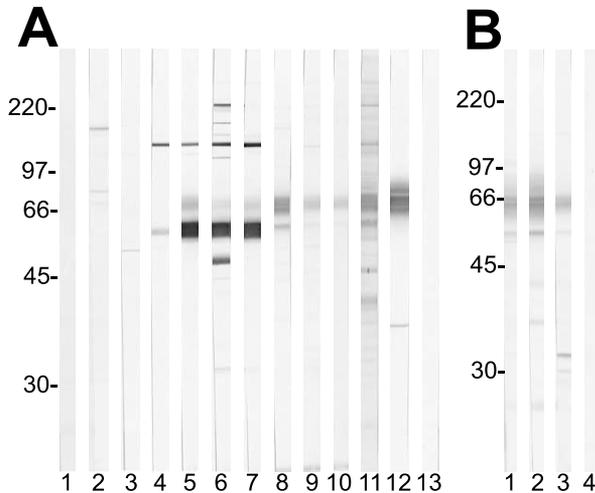
Consequently, in order to produce good anti-peptide antibodies, the key factor is to select the right parts of the sequence for peptide synthesis. Unfortunately, this is difficult as shown in Fig. 2. The EAAT3 and EAAT2 proteins are about 60% identical and the predicted antigenicity profiles are similar. Like EAAT3, peptides selected from the C-terminal region of EAAT2 were excellent immunogens while, similarly, weak antibodies were obtained to the second extracellular loop (B166), but not to the first one (B69 and C39). But in contrast to EAAT3, peptides from the N-terminus (B2 and B12) and from both the first (B107) and the third (B372) intracellular loops gave rise to good antibodies. This could not be predicted prior to immunization and testing.

### Antibodies recognizing unrelated proteins are frequently obtained

Because the purpose of the immunoblotting was to maximize the probability of detecting possible immunoreactivity toward non-EAAT3-proteins, the samples were made from whole tissue directly homogenized in SDS to ensure that the immunoblots would contain as many of the tissue antigens as possible. Examples of labeling patterns are shown in Fig. 3. The antibodies generated by immunization and purification with five of the peptides (C1–13, C1–18, C39–58, C81–94 and C468–482) did not recognize EAAT3, but did frequently bind to other proteins (examples are shown in Fig. 3A, strips 1–4) and are therefore not discussed further. Strong reaction with the EAAT3-protein was observed with the majority of the antibodies obtained after immunization with the C479–498, C491–523 and C510–523 (Fig. 3A, strips 5–11; Fig. 3B, strips 1–3).

The anti-C491 and the anti-C510 antibodies labeled one relatively broad fuzzy band at around 70 kDa on immunoblots of brain (Fig. 3A, strips 8–11) and kidney (Fig. 3B, strips 2 and 3). The labeling intensity of this band was weak compared with the band immunopositive for antibodies to EAAT2 (Fig. 3A, strip 12). The weak labeling was due to the low amounts of EAAT3-protein in brain tissue and not the result of low affinity of the antibodies, because high labeling intensities were obtained when they were tested on immunoblots of transfected HeLa cells (data not shown) and on blots containing purified EAAT3-protein (data not shown).

The anti-C479 antibodies labeled the same band as the anti-C491 and the anti-C510 antibodies, but also a



**Fig. 3.** Specificity testing of EAAT3 antibodies by immunoblotting. Whole rat tissue was solubilized with SDS, subjected to SDS-PAGE and blotted onto nitrocellulose. The nitrocellulose sheets were cut into identical strips (each with 16  $\mu$ g protein) which were labeled with the following antibodies: A (hippocampus): (1) anti-C1; (2) anti-C39; (3) anti-C158; (4) anti-C479A; (5) anti-C479B; (6) anti-C479C; (7) anti-C479D; (8) anti-C491B; (9) anti-C491A; (10) anti-C510A; (11) anti-C510B; (12) anti-B12A (positive control); (13) no primary antibody (negative control). (B) (kidney): (1) anti-C479P; (2) anti-C491B; (3) anti-C510A; (4) no primary antibody (negative control). Note the strong labeling of an extra band just below the EAAT3 labeling in panel A (strips 5–7). For antibody concentrations see Table 2.

broad band just below the 66 kDa marker (Fig. 3A, lanes 5–7). This band was labeled with higher intensity than that of the upper band.

#### Uncovering of the identity of the lower anti-C479 positive band

It was important to uncover the identity of the protein represented by the lower band recognized by the anti-C479 antibodies because the strong labeling suggested it is abundant, and expression of such high concentrations of an EAAT3 variant would be a major discovery. We therefore attempted to immunoprecipitate the molecular species using procedures we have successfully applied to transporter proteins (Dehnes et al., 1998; Lehre and Danbolt, 1998) in order to subject the purified protein to partial protein sequencing. However, we found that the water solubility of the unknown protein varied with the buffer composition during homogenization, in contrast to EAAT3, which was always found in the pellet, and always soluble with CHAPS (data not shown).

The variable water solubility suggested reversible attachment to cytoskeletal proteins. To obtain information about the protein's localization, the different EAAT3-antibodies were used to label vibratome sections of brain tissue. All the anti-C491 and anti-C510 antibodies and three of the anti-C479 antibodies labeled neuronal cell bodies and dendrites in tissue sections. Examples using anti-C491B, anti-C479D and anti-C479P antibodies are illustrated (Fig. 4). One particularly striking difference between the three anti-C479 antibodies and the rest was the

dense labeling of axons. The labeling of dendritic cytoplasm was also somewhat stronger. Axonal labeling was particularly evident in white matter tracts (Fig. 4D). At the electron microscopical level, label was found to be associated with axonal and dendritic microtubules (Fig. 4E).

The antibodies were then tested in a robotic ELISA assay for reactivity toward proteins present in high concentrations in axons. A strong and specific reaction to tubulin was observed, but not to any of the other proteins tested, including another abundant cytoskeletal protein, actin. This result indicated that the anti-C479 antibodies recognized both tubulin and EAAT3 despite being affinity purified against the C479–498 peptide.

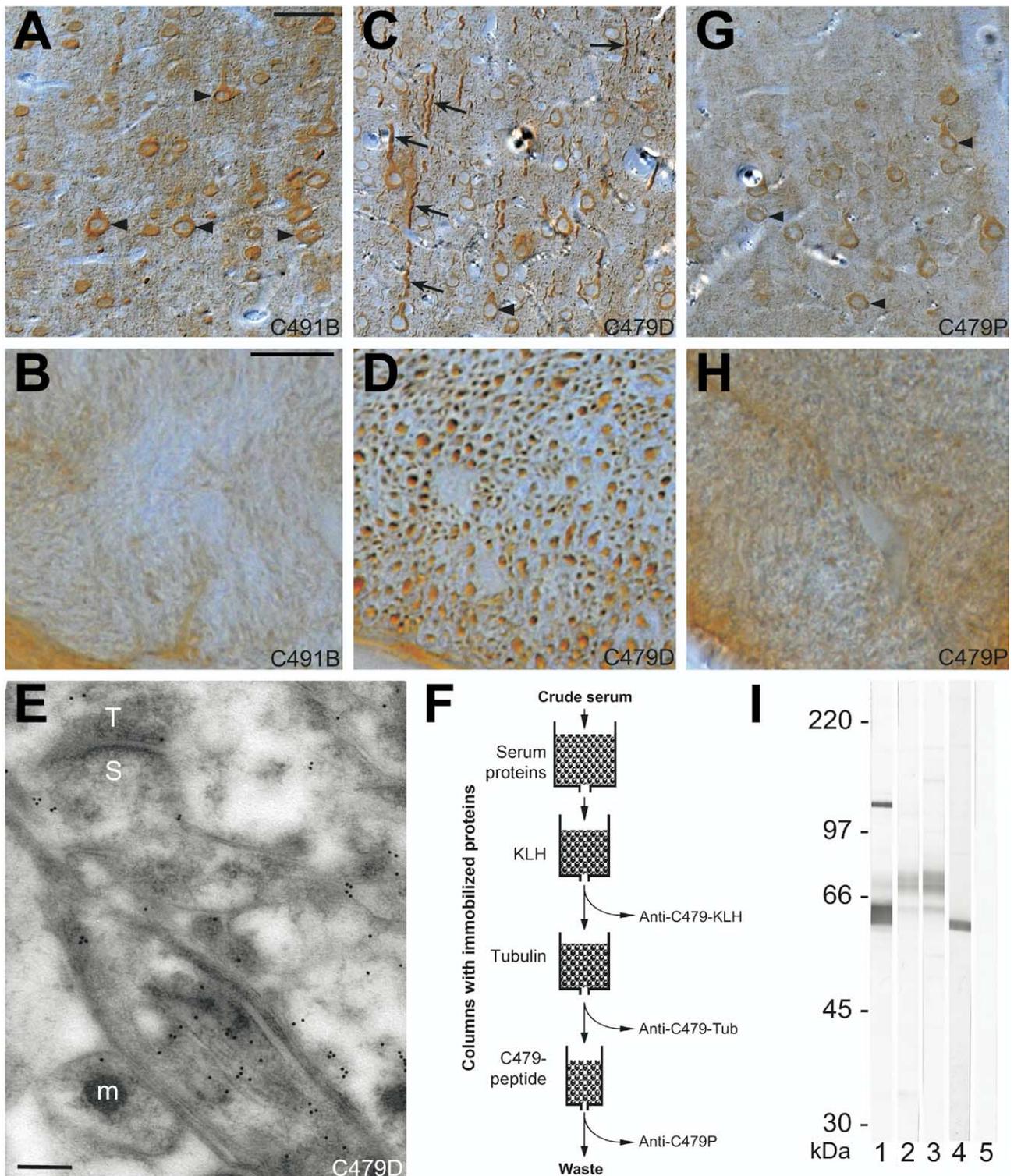
#### Fractionation of the anti-C479 antiserum

In order to separate antibodies to EAAT3 from the antibodies to tubulin, another aliquot of crude serum from one of the same rabbits (OB0721) was first fractionated by absorption on a column containing glutaraldehyde-treated bovine serum proteins to remove polyreactive antibodies and antibodies recognizing aldehyde-treated proteins in general (Fig. 4F). Then it was passed through a column with immobilized KLH (the carrier protein to which the peptide was conjugated during immunization), and subsequently through columns containing immobilized tubulin and the C479–498 peptide. The antibodies that were retained on the various columns were eluted with low pH-buffer and tested in an ELISA assay. The immunoreactivities of the antibody fractions obtained are shown in Table 3. The antibodies eluted with low pH from the KLH-column (referred to as “anti-C479KLH”) reacted both with the C479–498 peptide and with KLH. The antibodies eluted from the tubulin-column (“anti-C479-Tub”) reacted both with tubulin and with the C479–498 peptide, while the antibodies collected from the peptide-column reacted only with the peptide and neither with KLH nor with tubulin. These latter antibodies are referred to below as the “anti-C479P.”

The anti-C479P antibodies were then tested on both immunoblots and tissue sections (Figs. 3, 4, 5, 6 and 7). As can be seen in Fig. 4I (strip 2) these absorbed antibodies displayed the same labeling profile as the anti-C491 antibodies (strip 3). They did not recognize the lower band labeled by the non-absorbed anti-C479 antibodies (strip 1). Consequently, absorption against tubulin removed the antibodies labeling the lower band. The absorption also removed the antibodies giving rise to labeling of axons (Figs. 4G, 4H and 7D).

#### Reaction of the antibodies with proteins from wild-type and EAAT3 knockout mice

To verify that the band expected to represent EAAT3 really did so, the antibodies were tested by immunoblotting with protein extracts from wild-type (Fig. 5A) and EAAT3 knockout mice (Fig. 5B). The bands detected in the wild type (Fig. 5A, strips 1–5) were exactly as would be observed in rat tissue. In contrast, neither the absorbed anti-C479 nor anti-C491 antibodies showed detectable reaction with blots of tissue from genetically modified mice deficient in



**Fig. 4.** Immunocytochemical labeling of rat brain sections (A, C and G: neocortex layer 4; panels B, D and H: white matter of the pyramidal tract; panel E: hippocampus CA1) using 3  $\mu\text{g}/\text{ml}$  anti-C491B (panels A and B), 1  $\mu\text{g}/\text{ml}$  anti-C479D (panels C, D and E) and 10  $\mu\text{g}/\text{ml}$  anti-C479P (G and H). The latter antibody was purified as shown in panel F: 10 ml anti-C479 serum (bleeding 26.09.2001 of rabbit 0B0721; database ID: serum, 70). The serum was passed through a column with aldehyde-treated bovine serum proteins to remove polyreactive antibodies and antibodies to aldehyde-treated proteins in general. Then the absorbed serum was first passed through a column with immobilized carrier protein (KLH), then on a column with tubulin and finally on a column with the C479-peptide in order to collect the desired antibodies. The antibodies bound to the last three columns were eluted with low pH-buffer and neutralized. The amounts of anti-C479-KLH, anti-C479-Tub and anti-C479P antibodies collected were 4.26 mg, 0.7 mg and 0.8 mg, respectively. Their specificity was tested by ELISA (see Table 3), by immunocytochemistry (panels G and H) and by immunoblotting (panel I: rat hippocampus, 16  $\mu\text{g}$  protein

**Table 3.** Testing of fractionated anti-C479 antiserum (0B0721) by ELISA

	Antigen coating in the microtiterplate wells		
	C479	Tubulin	KLH
Anti-C479-KLH (14 $\mu\text{g/ml}$ )	3.96	0.05	3.86
Anti-C479-KLH (1.4 $\mu\text{g/ml}$ )	3.97	0.00	3.63
Anti-C479-Tub (4 $\mu\text{g/ml}$ )	3.95	3.86	0.06
Anti-C479-Tub (0.4 $\mu\text{g/ml}$ )	0.83	0.52	0.00
Anti-C479P (4 $\mu\text{g/ml}$ )	3.96	0.03	0.03
Anti-C479P (0.4 $\mu\text{g/ml}$ )	3.94	0.00	0.00
Anti-C491B (1 $\mu\text{g/ml}$ )	0.31	0.01	0.01

C479–498 peptide, purified tubulin and KLH were immobilized in the wells of microtiter plates. The plates were used to test the immunoreactivities of the various antibody fractions from the separation experiment described in Fig. 4. Table shows the absorbance values obtained (average of duplicate determinations). Note that the antibodies collected on the last column (containing C479-peptide) were devoid of tubulin reactivity. The anti-C491B antibodies did not react with tubulin. The slight reactivity towards the C479–498 peptide shows that some of the antibodies directed to the C491–523 peptide react with the overlapping part of the sequence.

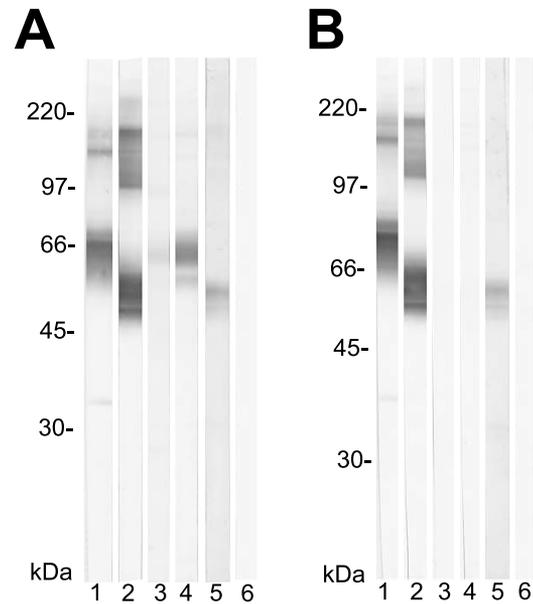
EAAT3 (Fig. 5B, strips 3 and 4) while tubulin labeling only was present with the non-absorbed anti-C479 (strip 2) and EAAT2 was detected, as expected, with anti-B12 (strip 1). Labeling with anti-tubulin antibody (strip 5) produced a band consistent with that observed with the non-absorbed anti-C479.

#### Screening of antibodies for reactivity toward tubulin

Tubulin was purified from rabbit brain according to the method of Weisenberg (1980) and immunoblotted with a number of antibodies. Some of these tests are shown in Fig. 6. Of the anti-glutamate transporter antibodies tested, only the unabsorbed anti-C479 antibodies recognized tubulin. No reaction was observed with any of the anti-B12 or anti-C491 antibodies.

#### Preabsorption of the anti-C479D and the anti-C479Tub antibodies

As shown in Table 3, the tubulin-reactive antibodies in the anti-C479 antisera bound to both tubulin and the C479–498 peptide. This indicated that the antiserum contained a mixture of antibodies. Some of these were specific for the peptide (anti-C479P) and some had a dual specificity in that they could bind both the peptide and tubulin. To test this further, anti-C479D and anti-C479Tub antibodies were preincubated with free C479–498 peptide prior to incubation with immunoblots and sections. As expected, the free



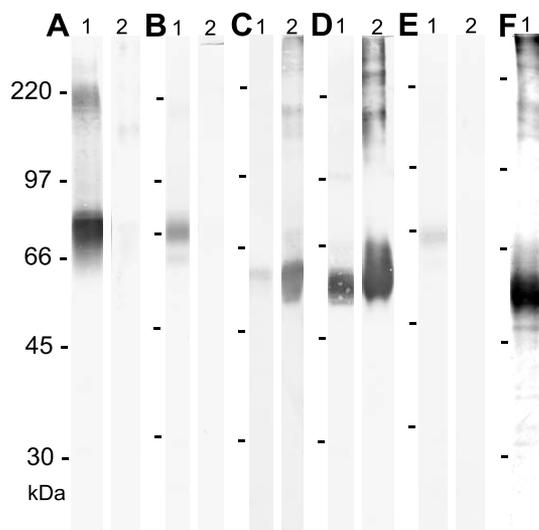
**Fig. 5.** Immunoblotting of antibodies with brain protein from wild type (panel A) and EAAT3-knockout mice (panel B): (strip 1) anti-B12 antibodies to EAAT2; (strip 2) unabsorbed anti-C479D; (strip 3) absorbed anti-C479P; (strip 4) anti-C491B; (strip 5) monoclonal anti-tubulin (Sigma-Aldrich) 1:200; (strip 6) no primary antibody. Note that the labeling of the EAAT3-band is absent on the Western blot of proteins from the EAAT3-knockout. Also note the difference in labeling intensity obtained with the anti-C479P and anti-C491B in mice. The anti-C479 antibodies show almost no reaction, while they label the rat EAAT3 almost as strongly as the anti-C491 antibodies (compare strips 1 and 2 in Fig. 3B or strips 2 and 3 in Fig. 4I). The unabsorbed anti-C479D antibodies recognize tubulin in both wild-type and EAAT3-knockout.

peptide was able to block all binding of the antibodies to all tissue proteins. Thus, the peptide also abolished the binding of the antibodies to tubulin (data not shown).

#### Double labeling with anti-EAAT3 antibodies and oligodendrocyte markers

It has been reported (Kugler and Schmitt, 1999) that EAAT3 is expressed in oligodendrocytes. This study is based on antibodies to a peptide corresponding to EAAT3 residues 480–499. Because our peptide (C479–498), covers almost the same sequence, it is natural to ask if their antibodies also cross-react with tubulin. This has not been tested, and the antibody has not been available to us. However, the authors show in their article that they have performed double labeling with a monoclonal anti-tubulin antibody and observe colocalization of labeling. On this background, we wanted to check if our antibodies also labeled oligodendrocytes. Vibratome sections were double

per strip): anti-C479D (not absorbed; strip 1), anti-C479P (after tubulin absorption; strip 2), anti-C491B (strip 3), a monoclonal anti-tubulin antibody (1:200) from Sigma-Aldrich (strip 4) and negative control (no primary antibody; strip 5). Note that both EAAT3 antibodies labeled neurons (arrowheads), but that the latter antibody labeled axons and dendrites stronger than the former and labeled microtubules at the electron microscopical level (E). Absorption against tubulin removes both the tubulin reactivity and the axonal labeling in tissue sections. There was no evidence of myelin labeling. Scale bars=50  $\mu\text{m}$  in panels A, C and G, 10  $\mu\text{m}$  in panels B, D and H and 250 nm in panel E. Letters (T, S, m) in panel E indicate nerve terminal, dendritic spine and mitochondrion, respectively. The animals were perfusion fixed with 0.1 M NaPi containing 4% formaldehyde (panels A–D, H) or 4% formaldehyde and 0.05% glutaraldehyde (panel E).



**Fig. 6.** Immunoblotting (panels A–E) of antibodies with brain proteins and purified tubulin. Tubulin was purified from rabbit brain according to a published procedure (Weisenberg, 1980). The purity was checked by SDS-PAGE and silver staining (panel F). Lane 2 in panels A–E contains each 5  $\mu$ g of the purified tubulin, while lane 1 contains 5  $\mu$ g total rat forebrain protein. The blots were immunolabeled with anti-B12 (panel A), anti-C491B (panel B), anti-tubulin (1:200; Sigma-Aldrich) (panel C), anti-C479D (panel D) and anti-C479P (panel E). Note that no reaction with tubulin is seen with the anti-B12, anti-C491B and the anti-C479P, while strong reaction is seen with the anti-tubulin and anti-C479D antibodies.

labeled with rabbit antibodies to EAAT3 and with mouse antibodies to oligodendrocyte markers (CNPase and MBP). No co-localization between oligodendrocyte markers and EAAT3 was detected (Fig. 7A–D). The antibody produced by Kugler and Schmitt (1999) must be different from our anti-C479D, because none of our anti-EAAT3 antibodies label myelin or oligodendrocyte cell bodies.

## DISCUSSION

This paper illustrates that immunization with an antigen may lead to the generation of antibodies that recognize not only the antigen, but also molecules that appear completely unrelated to the antigen. While it might be expected that two molecules which share some sequence similarity could be recognized by antibodies raised against one of them, in this case the cross-reactivity between anti-EAAT3 antibodies and tubulin could not have been predicted from existing knowledge.

Clearly, as reported here, animals frequently produce antibodies that have the ability to bind to the antigen-columns and also to bind to unrelated proteins on immunoblots. Consequently, the generation of antibodies with oligo- or poly-reactivity is something that frequently occurs. We have seen this also in connection with the production of antibodies to other transporter proteins, e.g. a monoclonal polyreactive IgG antibody (Danbolt et al., 1998). This is in line with studies of autoantibodies in systemic lupus erythematosus where certain peptide sequences bind anti-DNA antibodies (Sibille et al., 1997; James et al., 1999).

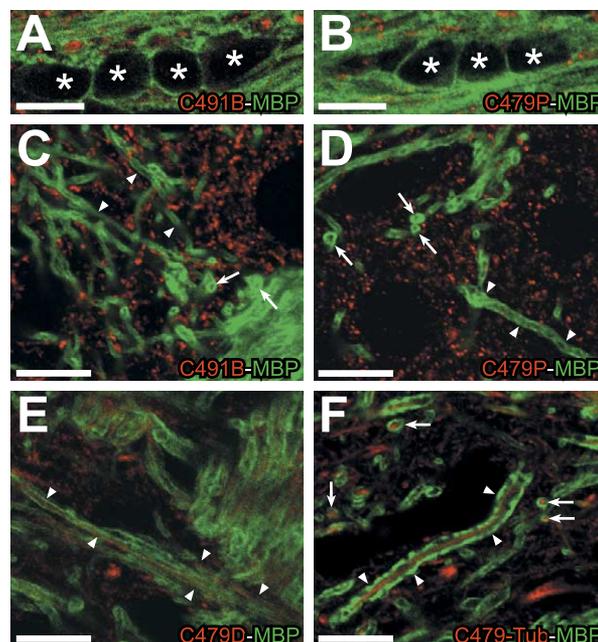
Another issue this raises relates to preabsorption with the relevant antigen which is considered by many to be a key test of antibody specificity. The results presented here show that this test is of little value when the antibody is already affinity purified against the immobilized antigen, because only the antibodies recognizing the antigen have been collected and the rest eliminated. Since antibodies have a finite number of binding sites (IgG molecules have two), it follows that antigen added in excess, will always saturate the antibody binding sites and thereby completely block the labeling of tissue sections, even when the antibody has affinity for other tissue antigens. As we show here, the anti-C479-Tub fraction labels both EAAT3 and tubulin in sections. Preabsorption with C479-peptide blocks all labeling of the sections, including that directed against tubulin.

A third point illustrated here is that it is hard to predict in advance if sequence differences between species are going to matter for antibody binding. The rat 479–498 sequence and the rat 491–523 sequence both differ with one amino acid from the corresponding mouse sequences:

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479 NIVNPFALPTILDNEDSDTK                               498 Rat
491                               LDNEDSDTKKSYVNGGFVVDKSDTISFTQTSQF 523 Rat
479 NIVNPFALPTILDNEDSDTKKSYVNGGFVVDKSDTISFTQTSQF 523 Mouse
  
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The anti-C491 antibodies detect mouse and rat EAAT3 with about the same strength, while anti-C479 antibodies



**Fig. 7.** Double labeling with anti-EAAT3 antibodies (red) and MBP (green). (A, B) Neither 1  $\mu$ g/ml anti-C491B nor 10  $\mu$ g/ml anti-C479P labels oligodendrocytes (asterisks) in corpus callosum. Panels C and D show that neither anti-C491B nor anti-C479P labels axons in rat thalamus. There is no red color inside longitudinally (arrowheads) or transversally cut (arrows) myelin sheets (green). Panels E and F show that both the anti-C479D and the anti-C479Tub antibodies label axons inside myelin sheets. Rat tissue perfusion fixed with 4% formaldehyde in 0.1 M NaPi. Scale bars=10  $\mu$ m.

do not recognize the mouse protein to any significant degree.

A fourth point illustrated is that generation of good anti-peptide antibodies mainly depends on the selection of the best part of the protein sequence. This, however, is hard as shown in Fig. 2. The difference between EAAT2 and EAAT3 was not predicted in advance. Our conclusion is that the most efficient way to produce anti-peptide antibodies is to use a “shotgun” approach: synthesize several peptides, mix them together before conjugation to carrier protein and inject them all into the same rabbits. By separating the various antibodies from the ensuing antisera, it is easy to find out which are the best peptides. Then these can be injected alone into new rabbits if larger amounts of antibody are needed. This approach conserves the number of animals used. Further, if good antibodies are not obtained, it is better to try new peptides rather than trying unsuccessful peptides in new rabbits.

### Concluding remarks

Polyreactivity is a well-known phenomenon which comes in various guises. As this paper demonstrates, antibody specificity is no trivial matter. Because the cross-reactivity can be highly specific, it may be hard to discover. It also follows from this that cross-reactivity depends on the presence of the cross-reacting molecular species. Thus, an antibody may be specific in one organ and not in another due to differences in the expression of proteins cross-reacting with the antibody. Often, antibodies tested in one organ in animals of a certain age and species are used to study other organs in animals of different ages or species using different immunocytochemical protocols. On this background it is unacceptable that immunocytochemical papers are published with little or no information on the antibodies used.

If sharp and beautiful pictures are obtained, investigators often tend to believe that the antibodies are specific. The main concern is that the cost involved in disproving spurious results from other laboratories is huge, and often much higher than the costs of proper testing in the first place. This problem is recognized, and the *Journal of Comparative Neurology* lists requirements that must be met in order to make a paper acceptable for publication (Saper and Sawchenko, 2003). Data presented in this paper suggest that these requirements should be taken seriously and perhaps be made stricter. The main difficulty is not to distinguish between antibodies that recognize the desired antigen and those antibodies that recognize other antigens, but to find out whether or not antibody molecules derived from a single clone recognize both the desired antigen and something else. This is costly and a solution to this problem may be to establish web-based database systems in which all antibodies used in scientific publications are listed (for general consideration on neuroscience databases, see Amari et al., 2002; Koslow and Subramaniam, 2005). Then it would be possible to track each antibody, and thereby make it possible to accumulate knowledge on the specificity of each antibody. This would be particularly valuable for monoclonal antibodies, but if

such a system is established, then it could just as well include all antibodies because polyclonal ones are often produced in sufficient quantities to be used in a number of studies.

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