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Specificity Controls for Immunocytochemistry: The Antigen Preadsorption Test Can Lead to Inaccurate Assessment of Antibody Specificity

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Summary

The biomedical research community relies directly or indirectly on immunocytochemical data. Unfortunately, validation of labeling specificity is difficult. A common specificity test is the preadsorption test. This test was intended for testing crude antisera but is now frequently used to validate monoclonal and affinity purified polyclonal antibodies. Here, the authors assess the power of this test. Nine affinity purified antibodies to different epitopes on 3 proteins (EAAT3, slc1a1; EAAT2, slc1a2; BGT1, slc6a12) were tested on samples (tissue sections and Western blots with or without fixation). The selected antibodies displayed some degree of cross-reactivity as defined by labeling of samples from knockout mice. The authors show that antigen preadsorption blocked all labeling of both wild-type and knockout samples, implying that preadsorption also blocked binding to cross-reactive epitopes. They show how this can give an illusion of specificity and illustrate sensitivity-specificity relationships, the importance of good negative controls, that fixation can create new epitopes, and that cross-reacting epitopes present in sections may not be present on Western blots and vice versa. In conclusion, they argue against uncritical use of the preadsorption test and, in doing so, address a number of other issues related to immunocytochemistry specificity testing. (J Histochem Cytochem 60:174–187, 2012)

Keywords

antibody dilution, glutamate transporter, GABA transporter, knockout mice, blot fixation test, blocking peptide, neoantigens

Immunochemical techniques have been in widespread use for several decades for identifying individual proteins in complex biological samples (e.g., tissue extracts and sections), and the principles of immunocytochemistry are well established (e.g., Pool and Buijs 1988). Nevertheless, the field of immunocytochemistry is still troubled by spurious results due to insufficient controls of antibody specificity. Inaccurate immunocytochemical data are a major concern, considering the widespread use of this method and the considerable effort required to correct inaccurate results. Several recent publications have addressed these issues and have proposed guidelines for inclusion of immunocytochemical data (e.g., Saper and Sawchenko 2003; Saper 2005; Holmseth et al. 2006; Rhodes and Trimmer 2006; Fritschy 2008; Lorincz and Nusser 2008; Burry 2011). The arguments for improvements in quality control are strong, but it is hard to define the exact tests that should be performed. One important step in this direction is to demand detailed descriptions of antibodies (e.g., Saper and Sawchenko 2003; Saper 2005; Holmseth et al. 2006; Rhodes and Trimmer 2006; Fritschy 2008). Another would be to

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motivate commercial antibody producers to test their antibodies more rigorously before selling them to scientists who often lack the resources or expertise to evaluate acquired antibodies (Boenisch 2006; Pradidarcheep et al. 2008; Couchman 2009; Kalyuzhny 2009). However, not all testing can be done in advance because the overall labeling specificity is affected by so many parameters that antibodies have to be tested for each application (e.g., Ottersen 1987; Holmseth et al. 2006; Rhodes and Trimmer 2006; Lorincz and Nusser 2008). Virtually all assay conditions can affect antibody binding, including protein conformation and hydrophobic interactions (e.g., pH, buffer composition, and ionic strength), tissue handling steps (e.g., time to fixation, type of fixation, fixative composition, fixation time, storage after fixation), and antigen retrieval techniques (e.g., Josephsen et al. 1999; Willingham 1999; Burry 2000; Boenisch 2006; Holmseth et al. 2006; Lorincz and Nusser 2008; Saper 2009; Webster et al. 2009; Hoffman et al. 2010; Paavilainen et al. 2010; Xie et al. 2011).

The scope of the present report is not to provide a comprehensive overview of all aspects of immunocytochemical specificity testing but to compare the power of the antigen preadsorption test with other tests. Antigen preadsorption was originally introduced to validate antisera (e.g., Swaab et al. 1977; Pool and Buijs 1988; Burry 2000, 2011), but it is still considered mandatory by many investigators although it is now commonly used to validate labeling obtained with monoclonal or affinity purified antibodies. Here, we tested the specificity of several antibodies to 2 glutamate transporters (EAAT2 and EAAT3; for review, see Danbolt 2001) and the betaine-GABA transporter (Zhou et al. 2012) by (a) performing the antigen preadsorption test, (b) doing immunoblotting, (c) using several antibodies to the same antigen, and (d) using tissue from knockout mice as negative controls. We show that antigen preadsorption blocks all binding of the affinity purified antibodies, regardless of whether this binding is to the proteins under study or to cross-reacting epitopes. These data also illustrate a number of other issues such as sensitivity-specificity relationships and that there is no absolute correlation between the specificity of the labeling of immunoblot and of sections.

Materials and Methods

Materials

N,N′-methylenebisacrylamide, acrylamide, ammonium persulfate, tetramethylene ethylenediamine, and alkaline phosphatase substrates (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) were from Promega (Madison, WI). Sodium dodecyl sulfate (SDS) of high purity (>99% C12 alkyl sulfate), and electrophoresis equipment was from Hoefer Scientific Instruments (San Francisco, CA). Molecular mass markers for SDS polyacrylamide gel electrophoresis (SDS PAGE), biotinylated anti-rabbit and anti-sheep immunoglobulins, streptavidin-biotinylated horseradish peroxidase complex, and nitrocellulose sheets (0.22 µm pores, 100% nitrocellulose) were from Amersham (Buckinghamshire, UK). Paraformaldehyde and glutaraldehyde were from TAAB (Reading, UK). Alkaline phosphatase conjugated secondary antibodies (Sigma A2556) and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

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 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. The C57BL/6 mice (4 weeks old) lacking EAAT3 (Peghini et al. 1997) were raised at the animal facility at the Johns Hopkins University (Baltimore, MD). The BGT1-fKO mice lacking the BGT1 (slc6a12) gene were in a mixed (C57BL/6 × 129) background (Lehre et al. 2011), whereas the EAAT2-KO mice (Tanaka et al. 1997) lacking the EAAT2 gene (GLT1, slc1a2) were in a pure C57Bl/6 background. These mice and adult male Wistar rats (10–12 weeks old) were kept in the animal facility at the Governmental Institute of Public Health (Oslo, Norway). The rats were obtained from B&K Universal (Sollentuna, Sweden). The 3 genetically modified mouse lines were maintained by crossing heterozygote mice with each other. This was done to obtain paired wild-type and knockout mice from the same litters. Thus, potential differences in rearing conditions, genetic background, and age were minimized. Animals (both rats and mice) for immunocytochemistry were killed by lethal injection of pentobarbital and fixed by cardiac perfusion as described previously (Danbolt et al. 1998) with 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Glutaraldehyde (0.1%) was included when not stated otherwise. The tissue was processed as described later (“Light Microscopic Immunocytochemistry”). To obtain fresh tissue, mice were killed by cervical dislocation, and the relevant tissues were rapidly collected and processed as described later (see “Electrophoresis and Immunoblotting”). New Zealand White rabbits obtained from B&K Universal were kept in the animal facility at the Institute of Basic Medical Sciences. Rabbits were immunized and bled as described (Danbolt et al. 1998) but using subcutaneous rather than intracutaneous injections.

Antibodies to Excitatory Amino Acid Transporters (EAATs)

Over the years, our laboratory has produced 239 different batches of antibodies to EAAT2 using 51 different animals and 46 different synthetic peptides, as well as purified

Table 1. Primary Antibodies to Transporter Proteins

Antibody ID	Purification Date	Host Species	Antibody Name	Animal No.	Protein	Antigen Peptide Name	Antigen Sequence	Ligand on Affinity Column	Specificity
48	19 June 1993	Rb	Anti-B2	81024	EAAT2	B2–11	ASTEGANNMP-(amide)	2–11	EAAT2
360	10 July 2002	Rb	Anti-B12	26970	EAAT2	B12–26	KQVEVRMHDSHLSSE-(amide)	12–26	EAAT2
63	5 June 1994	Rb	Anti-B372	82898	EAAT2	B372–382	RCLEDNLGIDK-(amide)	372–382	EAAT2
95	29 May 1994	Rb	Anti-B493	84946	EAAT2	B493–508	YHLSKSELDTIDSQHR-(amide)	493–508	EAAT2
355	5 September 2002	Rb	Anti-B563	1B0707	EAAT2	B563–573	SVEEEPWKREK-(free acid)	563–573	EAAT2
359	19 September 2002	Rb	Anti-C480	0B0721	EAAT3	C480–499	IVNPFALEPTILDNEDSDTK-(amide)	480–499	EAAT3
547	19 September 2002	Rb	Anti-C480-tub	0B0721	EAAT3	C480–499	IVNPFALEPTILDNEDSDTK-(amide)	Tubulin	EAAT3 and tubulin
371	3 January 2003	Rb	Anti-C491	1B0683	EAAT3	C491–523	CLDNEDSDTKKSYVNGGFSVDK SDTISFTQTSQF-(free acid)	491–523	EAAT3
565	31 October 2005	Sh	Anti-C510	4131	EAAT3	C491–523	CLDNEDSDTKKSYVNGGFSVDK SDTISFTQTSQF-(free acid)	509–523	EAAT3
323	28 April 1999	Rb	Anti-BGT1(599)	8D0156	BGT1	BGT1(599–614)	SPAKQELIAWEKETHL-(free acid)	599–614	BGT1

Antibodies to transporter proteins were made by immunizing animals with synthetic peptides coupled to carrier proteins (keyhole limpet hemocyanin) with glutaraldehyde and then isolating the antipeptide antibodies by affinity chromatography using columns with immobilized peptide (coupled to N-hydroxysuccinimide activated agarose) as described previously (Danbolt et al. 1998). The antigenic sequences correspond to rat glutamate transporter 2 (EAAT2; NP_058911.2), rat glutamate transporter 3 (EAAT3; NP_037164.3), and mouse betaine-GABA transporter (BGT1; P31651.1). The numbers in the peptide names and in the ligand names correspond to residue numbers in the sequences. The peptides were synthesized as C-terminal amides or free acids as indicated. A cysteine (C) residue was added to the N-terminal of the C491–523 peptide to facilitate directional coupling, but the successful immunizations resulted from coupling by adding glutaraldehyde to a mixture of peptide and carrier protein. Antibody no. 547 was raised against the C491–523 peptide and then affinity purified using immobilized tubulin.

EAAT2 protein. For the present study, we selected 5 affinity purified rabbit antibodies (see Table 1) already known to recognize EAAT2 (Pines et al. 1992) both before and after aldehyde fixation. These antibodies were from the same purified batches as described previously (Furness et al. 2008; Holmseth et al. 2009). Similarly, out of the 87 antibodies we have made to EAAT3 (Holmseth et al. 2005), we selected 3 for the present study: sheep anti-C510 (Ab#565), rabbit anti-C480-Tub (Ab#547), and rabbit anti-C491 (Ab#371). Briefly, these antibodies were made by immunizing animals with peptides (Table 1) corresponding to residues 480–499 or 491–523 of rat EAAT3 (NP_037164.3). The antibodies are named after the peptide immobilized on the affinity columns. Thus, the anti-C480 and the anti-C491 antibodies were affinity purified using the same peptide as that used to immunize the animals, whereas the anti-C510 antibodies were collected on a column with a shorter peptide (corresponding to residues 509–523).

When immunizing rabbits with the C480–499 peptide, it was noted (Holmseth et al. 2005) that the ensuing antisera cross-reacted with tubulin, despite absence of primary sequence, similarly with EAAT3. One of these antisera (from rabbit 0B0721, see Table 1) was passed through a column with glutaraldehyde treated proteins (bovine serum albumin and keyhole limpet hemocyanin) to remove polyreactive antibodies, then through a column containing immobilized tubulin (to remove tubulin-binding antibodies), and finally through a column with immobilized EAAT3 peptide. As expected, antibodies detached from the latter column were devoid of tubulin reactivity (Ab#359, not shown here; see Holmseth et al. 2005). The surprise was that the antibodies captured on the column with immobilized tubulin (anti-C480-tub; Ab#547) were able to recognize both the EAAT3 peptide and tubulin (Holmseth et al. 2005).

We have also made a number of antibodies to BGT1 (Zhou et al. 2012). The rabbit anti-BGT599 (Ab#323) antibodies selected for the present study have not been previously published. These antibodies were made in the same way (Danbolt et al. 1998) as that used for the other antibodies. A New Zealand White rabbit (no. 8D0156) was immunized with a peptide corresponding to residues 599–614 (Table 1) of mouse BGT1 (slc6a12; accession number P31651; Liu et al. 1993) coupled to keyhole limpet hemocyanin with glutaraldehyde. The ensuing antiserum was affinity purified on a column with immobilized BGT599–614 peptide using our standard procedure (Danbolt et al. 1998).

Preadsorption of Antibodies

The preadsorption test was performed both on immunoblots and on sections. In both cases, antibodies were diluted in blocking solution to the desired concentrations. The compositions of the blocking solutions and the final antibody concentrations were as stated below. Each of these antibody solutions was then divided in 2 aliquots to obtain pairs of identical solutions. Peptide antigen was added in the stated amounts to 1 aliquot from each pair. The paired aliquots were then incubated overnight (room temperature) before being used for immunolabeling as described in the following.

Electrophoresis and Immunoblotting

Electrophoresis and immunoblotting were performed as described previously (Lehre et al. 1995). Proteins were separated by SDS PAGE and electroblotted onto nitrocellulose membranes. Then, blots were immunolabeled. Briefly, the blots were washed in phosphate buffered saline, incubated in blocking solution (blocking agent in Tris-HCl buffered saline unless stated otherwise), followed by primary antibodies (as stated) and alkaline phosphatase conjugated secondary antibodies (1:5000). When not stated otherwise, the blocking agent was 1% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20. This procedure was chosen because it has relatively low contrast and therefore reveals cross-reactivity better than do enhanced chemoluminescence-based procedures. The latter procedures typically have higher contrast and therefore give "cleaner" pictures. Omission of the primary antibody led to a virtually complete loss of labeling. This implied both that the secondary antibody did not cross-react with tissue components to any significant degree (secondary antibody control) and that the immunoblots did not contain any functional enzymes able to convert the substrates (label control).

When stated, the tissue was homogenized in water and subjected to high-speed centrifugation (18000 rpm, 39000 × g, 20 min, 4C) to separate the water soluble components ("supernatant") from water insoluble ones ("pellet"). The pellets were solubilized in SDS. Brain tissue contains about 100 mg protein per gram wet weight (Lowry et al. 1954). When homogenizing brain tissue in water (S. Holmseth and N.C. Danbolt, unpublished), about half of the proteins will remain in the supernatant, which is virtually devoid of integral membrane proteins. Thus, this method is an easy way to increase sensitivity by a factor of about 2 for detection of transporter proteins.

The Blot Fixation Test

After SDS PAGE and electroblotting had been done as described previously, but before the start of immunolabeling, the blots were washed (1 × 5 min) in 0.1 M sodium phosphate buffer and then incubated with fixatives of the same composition as used to fix tissue for immunocytochemistry. After fixation, the blots were rinsed (1 × 1 min) in 0.1 M sodium phosphate buffer, incubated (30 min) with 1 M ethanolamine-HCl (pH 7.4) in sodium phosphate buffer or 2 M Tris-HCl buffer (pH 7.4) to quench aldehyde groups, and then immunolabeled as described earlier.

Light Microscopic Immunocytochemistry

Immunoperoxidase labeling was done as described previously (Holmseth et al. 2009). Briefly, free-floating vibratome sections (40 µm thick) were treated with 1 M ethanolamine-HCl (pH 7.4) in sodium phosphate buffer, blocked with 10% newborn calf serum 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 blocking solution. The antibodies were used at fairly high concentrations, and Triton X-100 was included to enhance antibody penetration. Bound antibodies were detected with biotinylated donkey anti-rabbit IgG (1:100) and streptavidin-biotinylated horseradish peroxidase complex (1:100). Diaminobenzidine was used as substrate. The sections were examined and photographed on Zeiss Axioskop 2 plus equipped with AxioCam MRc r1.2 camera (Zeiss, Jena, Germany). Omission of the primary antibody led to a virtually complete loss of labeling. This implied both that the secondary antibody did not cross-react with tissue components to any significant degree (secondary antibody control) and that the tissue did not contain any functional enzymes able to convert the substrates (label control). Brain tissue and heart tissue were chosen as examples to test EAAT2 antibodies because EAAT2 is expressed at very high levels in the brain (Lehre and Danbolt 1998) and is not expressed in the heart (S. Holmseth and N.C. Danbolt, unpublished). BGT1 is present in the kidney and in the liver but not in the brain (Zhou et al. 2012).

Results

The preadsorption test did not reveal cross-reactivity of antibodies to glutamate transporter 2 (EAAT2, slc1a2). We first tested the selected EAAT2 antibodies (Table 1) on tissue sections from wild-type mice and EAAT2 knockout mice. All the antibodies labeled sections of brain tissue from wild-type mice strongly, but only 2 of them (anti-B493 and anti-B372) labeled sections of heart tissue (Fig. 1). The lack of labeling of heart sections with 3 of the antibodies needed an explanation. One possibility could be that heart and brain expressed different splice variants. Alternatively, the anti-B493 and anti-B372 antibodies might cross-react with other molecules. Testing of the antibodies on tissue from EAAT2-deficient mice showed that the reason was

cross-reactivity. The preadsorption test was not helpful because preadsorption of the anti-B493 with the B493 peptide eliminated all of the labeling in both brain and heart sections.

The anti-B2 and the anti-B12 to the N-terminal part of EAAT2 and the anti-B563 to the C-terminus hardly produced any labeling at all in the EAAT2-deficient tissue, except that the anti-B12 antibodies gave a fairly strong labeling in the cerebellum (molecular layer). The labeling observed in the EAAT2-deficient tissue could not be due to EAAT2 because this is a conventional knockout, and EAAT2 was completely absent in the rest of the brain. Note that preadsorption of the anti-B12 antibodies with the antigenic peptide (B12) did not help to resolve this issue, as preadsorption before incubation with the sections eliminated all labeling in both wild-type and knockout tissue (Fig. 1).

Preadsorption of antibodies to glutamate transporter 3 (EAAT3, slc1a1) prevented binding to tubulin. All the selected EAAT3 antibodies labeled all brain regions in wild-type mice and rats (Figs. 2 and 3), but the anti-C491 and the anti-C480-tub antibodies also labeled sections from the EAAT3 knockout mice (Fig. 2). The widespread and fairly uniform labeling obtained with anti-C480-tub is consistent with the widespread distribution of tubulin. Preadsorption of the anti-C491 antibodies with the C491–523 peptide and preadsorption of the anti-C480-tub antibody with the C480–499 peptide eliminated all labeling, including labeling of cross-reactive epitopes seen in the sections from the EAAT3 knockout mice (Fig. 2). Thus, the EAAT3 peptide (C480–499) blocked the interactions of the anti-C480-tub antibodies with both EAAT3 and tubulin. Antibodies to EAAT2 were used as a positive control and were unaffected by the addition of EAAT3 peptides.

Interaction with cross-reactive epitopes can be highly specific and localized. The data obtained with the anti-B12 antibodies (Fig. 1) illustrate that cross-reactivity can be highly specific and limited to one tissue or region. Also, the anti-C491 antibodies gave a nonuniform and distinct labeling pattern in tissue sections from the knockout mice (Figs. 2 and 3). These antibodies labeled hippocampus CA1–3 and striatum strongly, whereas neocortex and thalamus were virtually unlabeled. This raised the question of whether the knockout mice express some EAAT3 protein, but this possibility was ruled out by the observation that the anti-C510 antibodies did not label the same structures in the EAAT3-deficient tissue (Fig. 3).

Also, the anti-BGT599 antibodies to the betaine-GABA transporter (BGT1, slc6a12) gave rise to distinct labeling of cross-reacting epitopes. These antibodies (Table 1) labeled tubules in the outer renal medulla in wild-type mice (Figs. 4A and 4G) but not in BGT1 knockout mice (Figs. 4B and 4I). Although this immunoreactivity represents BGT1 (Zhou et al. 2012), the most prominent signal obtained with these antibodies in kidneys was associated with the capillary endothelium, in particular the glomeruli. Because this labeling was seen in both wild-type (Figs. 4A and 4C) and BGT1 knockout mice (Figs. 4B and 4E), it does not represent BGT1. This conclusion is supported by the fact that other BGT1 antibodies labeled medullary tubules and not endothelium (not shown here, but see Zhou et al. 2012).

Again, the preadsorption test failed to distinguish between labeling of the epitope of interest and cross-reacting epitopes. Addition of excess antigen (peptide) to anti-BGT599 led to complete loss of glomerular labeling (Figs. 4D and 4F). Also note that there is labeling in the cerebellum of both the wild-type (Fig. 4K) and the knockout (Fig. 4M) mice but not after antigen preadsorption (Figs. 4L and 4N).

The Usefulness and Limitations of Western Blots

Testing of antibodies to EAAT2. All of the EAAT2 antibodies (Fig. 1, Table 1) gave rise to strong labeling of a band at around 70 kDa in forebrain samples from wild-type mice (Fig. 5, not fixed, lane 1). This band was not seen in forebrain samples from the EAAT2 knockout mice (Fig. 5, not fixed, lane 2) in agreement with the notion that these antibodies recognize EAAT2. In fact, 4 of the antibodies looked highly specific on blots of forebrain (Fig. 5, not fixed) and cerebellum (not shown). In contrast, the anti-B493 antibodies labeled an additional band with lower molecular mass (below the 45 kDa marker) fairly strongly. This may explain why this antibody gave some labeling of sections of brain tissue from the EAAT2 knockout mice. Thus, in the case of anti-B2, anti-B12, anti-B493, and anti-B563 antibodies, there were good correlations between the labeling seen on blots and in sections (Fig. 1). In contrast, the anti-B372 antibodies looked highly specific on blots (Fig. 5) despite the strong labeling of tissue sections from the knockout mice (Fig. 1).

The anti-B493 and the anti-B2 antibodies labeled bands (Fig. 5, not fixed, arrowheads) that could be mistaken for EAAT2 in the heart samples. The presence of these bands in the samples from the knockout mice, and the fact that the other antibodies did not label them, proved that these bands did not represent EAAT2. Note that preadsorption of the anti-B493 antibodies blocked the labeling of the extra bands and that preadsorption therefore was not helpful in distinguishing labeling representing EAAT2 from labeling representing cross-reactivity (Fig. 5).

One of the differences between Western blots and tissue sections (Figs. 1–4) is that the latter tissue has been exposed to aldehyde fixatives. We then exposed immunoblots to aldehydes before blocking and immunolabeling to get an indication whether this could matter (Figs. 5 [fixed], 6, and 7D). The labeling obtained with the anti-B12 and the anti-B563 antibodies appeared virtually unaffected (compare

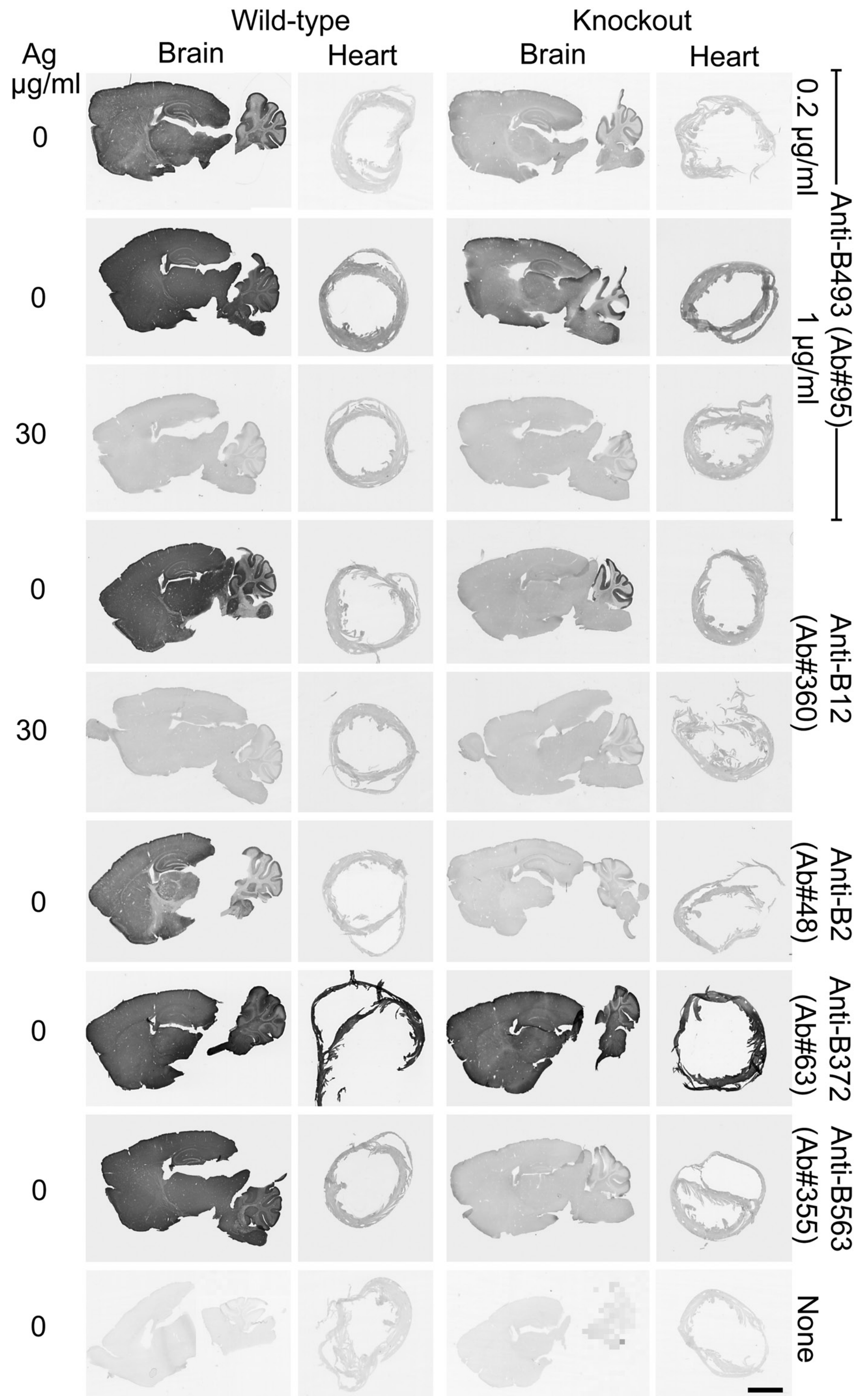

Figure 1. Antibodies to 5 different EAAT2 epitopes were tested in brain and heart sections from wild-type and EAAT2 knockout mice. Note that some of the antibodies gave rise to labeling in the EAAT2-deficient tissue. This labeling cannot represent EAAT2 but was nevertheless blocked when the antibodies were preadsorbed with 30 µg/ml of their respective peptide antigens (Ag), as indicated. Also note that the assay conditions were deliberately chosen to reveal cross-reactivity (high antibody concentrations and inclusion of Triton X-100 to enhance antibody penetration). Antibodies: anti-B2 (Ab#48), 0.5 µg/ml; anti-B12 (Ab#360), 0.3 µg/ml; anti-B372 (Ab#63), 0.5 µg/ml; anti-B493 (Ab#95), 0.2 µg/ml; anti-B563 (Ab#355), 0.3 µg/ml. Some sections ("none") were developed without primary antibodies to control for the secondary antibodies and the label. Scale bar = 2 mm.

Fig. 5 not fixed with fixed). In contrast, fixation created several epitopes that cross-reacted with the anti-B493, the anti-B2, and in particular with the anti-B372 antibodies (compare Fig. 5 not fixed with fixed). This may, at least in part, explain why anti-B493 and anti-B372 labeled cardiomyocytes (Fig. 1). But also note that the cross-reaction observed with the anti-B2 antibodies on blots of heart proteins did not occur in sections as the latter were unlabeled (Fig. 1). Furthermore, the reaction of the anti-B2 antibodies was stronger after fixation than before fixation.

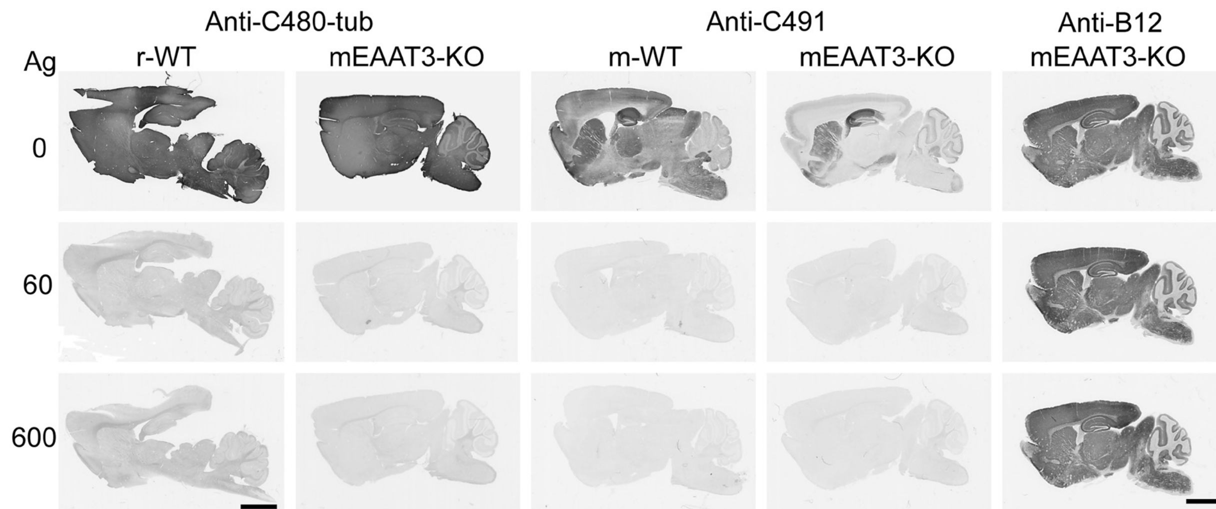

Figure 2. Antigen preadsorption blocks all labeling, including labeling of cross-reacting epitopes. The anti-C480-tub (Ab#547) and anti-C491 (Ab#371) to EAAT3 were preadsorbed overnight with 0, 60, or 600 µg/ml of the peptide-antigens (Ag) used to generate them (the C480–499 and the C491-523 peptides, respectively) before being used to label tissue sections from wild-type rats (r-WT), wild-type mice (m-WT), and EAAT3 knockout mice (mEAAT3-KO). The fact that the 2 EAAT3 antibodies label tissue devoid of EAAT3 shows that these antibodies cross-react with non-EAAT3 epitopes. Then, note that preadsorption with the peptide-antigens blocks the interaction with both the EAAT3 and the non-EAAT3 epitopes. As expected, the binding of the anti-B12 (Ab#360) antibodies to EAAT2 was not affected by the presence of EAAT3 peptides. Also note that the epitope cross-reacting with the anti-C491 antibodies is not uniformly distributed, showing that an antibody may be specific in one brain region and not in another depending on the distribution of cross-reacting molecules. Scale bar = 4 mm in r-WT and 2 mm in m-WT and m-EAAT3-KO.

Testing of antibodies to BGT1. The anti-BGT599 antibodies to BGT1 were also tested on Western blots. Tissue was collected from both the outer renal medulla and the renal cortex of both wild-type and BGT1 knockout mice. As shown in the left panel of Fig. 6, the BGT1 band was the predominant band (asterisk) and was only observed in the water-insoluble fraction from the outer medulla from wild-type mice (lane 4). Thus, the antibodies appeared to be specific, although some lower molecular mass species were labeled in all lanes containing water-insoluble proteins (lanes 2, 4, 6, and 8), and there was more background in lanes from the cortex (lanes 5–8) than from the medulla (lanes 1–4). Consequently, this did not explain the labeling observed in the kidney sections (Fig. 4). However, fixation of the blots (Fig. 6, middle and right panels) caused a substantial increase in non-BGT1 labeling. This cross-reactivity was so strong that the BGT1 band was not visible when 1% bovine serum albumin was used as blocking agent (Fig. 6, middle panel). The stronger blocking (10% newborn calf serum) used for immunocytochemistry (Fig. 4) reduced unwanted labeling sufficiently to allow identification of the BGT1 band (Fig. 6, lane 4 asterisk). Nevertheless, even with this blocking condition, a strongly labeled band (arrowhead) appeared in the renal cortex from both wild-type (lanes 5 and 6) and BGT1 knockout mice (lanes 7 and 8). This labeling may represent the molecular species that gives rise to the glomerular labeling in the sections (Fig. 4).

Testing of antibodies to EAAT3. The anti-C491 antibodies recognized EAAT3 in brain extracts from both rat and wild-type mice but not from EAAT3 knockout mice (Fig. 7A). Thus, this antibody looked specific when tested on Western blots, and the blots therefore did not explain the cross-reactivity observed in sections (Figs. 2 and 3). We then dissected brains from wild-type and EAAT3 knockout mice to enable blotting (Fig. 7CD) of hippocampus, neocortex, and cerebellum separately. Furthermore, 10% to 20% gradient gels were used to improve detection of lower molecular mass proteins. Two identical blots were made. Fixation of one of the blots (Fig. 7D) showed that fixation enhanced binding to cross-reactive epitopes, but there was in this case no clear correlation between blots and sections. Although the immunocytochemistry showed particularly strong cross-reactivity in the hippocampus, the blots did not reveal any hippocampus-specific cross-reactive molecular species.

The anti-C480-tub antibodies (Fig. 7B) recognized a band (arrowhead) with slightly lower apparent molecular mass than EAAT3. This band was present in all 3 lanes, including the 1 with the extract from the EAAT3 knockout, in agreement with the immunocytochemistry (Fig. 3). This band became visible after very short development times, whereas longer development times were needed to visualize EAAT3. The arrow in Fig. 7B points to the location of EAAT3. The reason is that tubulin is one of the most abundant proteins in the brain (Shelanski et al. 1973), whereas

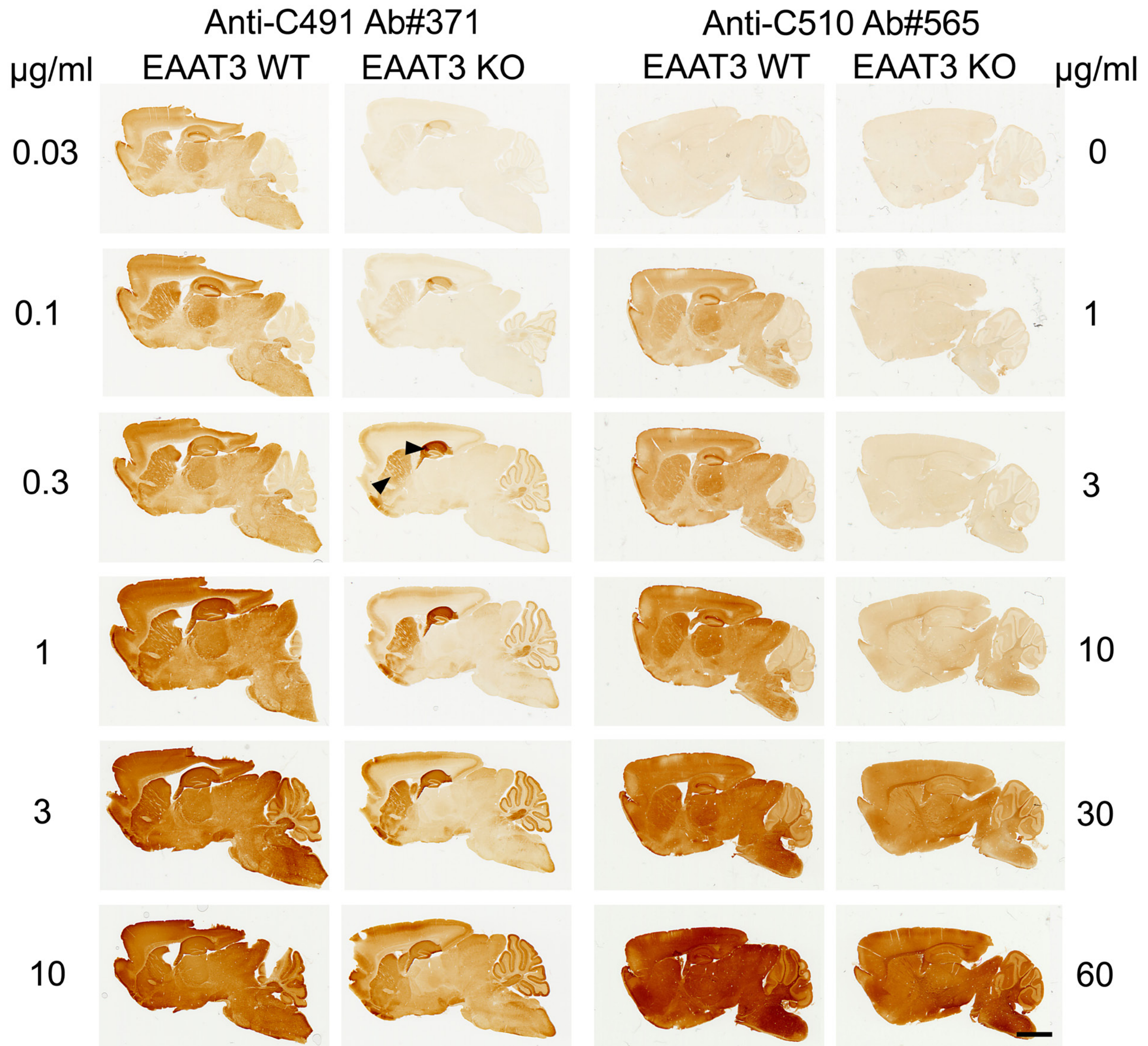

Figure 3. Determination of optimal antibody concentrations is straight forward when tissues from knockout animals are available as negative controls. Sections from wild-type (WT) and EAAT3-deficient mice (KO) were incubated with either anti-C491 (Ab#371) or anti-C510 (Ab#565) antibodies in concentrations as indicated. Note that anti-C491 cross-react with something else in some regions (arrowheads: hippocampus and striatum). Scale bar = 2 mm.

EAAT3 represents less than 0.01% (Furness et al. 2008). Thus, the labeling of this blot matches the labeling seen in the sections.

Discussion

Burry (2011) introduced a new classification of immunocytochemical controls. According to this system, there are 3 groups of controls: primary antibody controls, secondary antibody controls, and label controls. The focus of the present study is primary antibody controls, which are usually the most difficult ones.

Value of Genetically Modified Tissue

As illustrated here, genetically modified animals are very powerful controls. Such animals are costly, but they allow specificity issues to be resolved faster. Nevertheless, it is always a good idea to be observant because not even genetically modified organisms are perfect specificity controls (e.g., Holmseth et al. 2006; Burry 2011). For instance, when a gene is deleted, then this may affect expression of other genes. Cross-reactive molecules may be downregulated or upregulated. Another problem can be other genes containing the same sequence or residual expression of the deleted gene, unless the deleted sequence includes the part

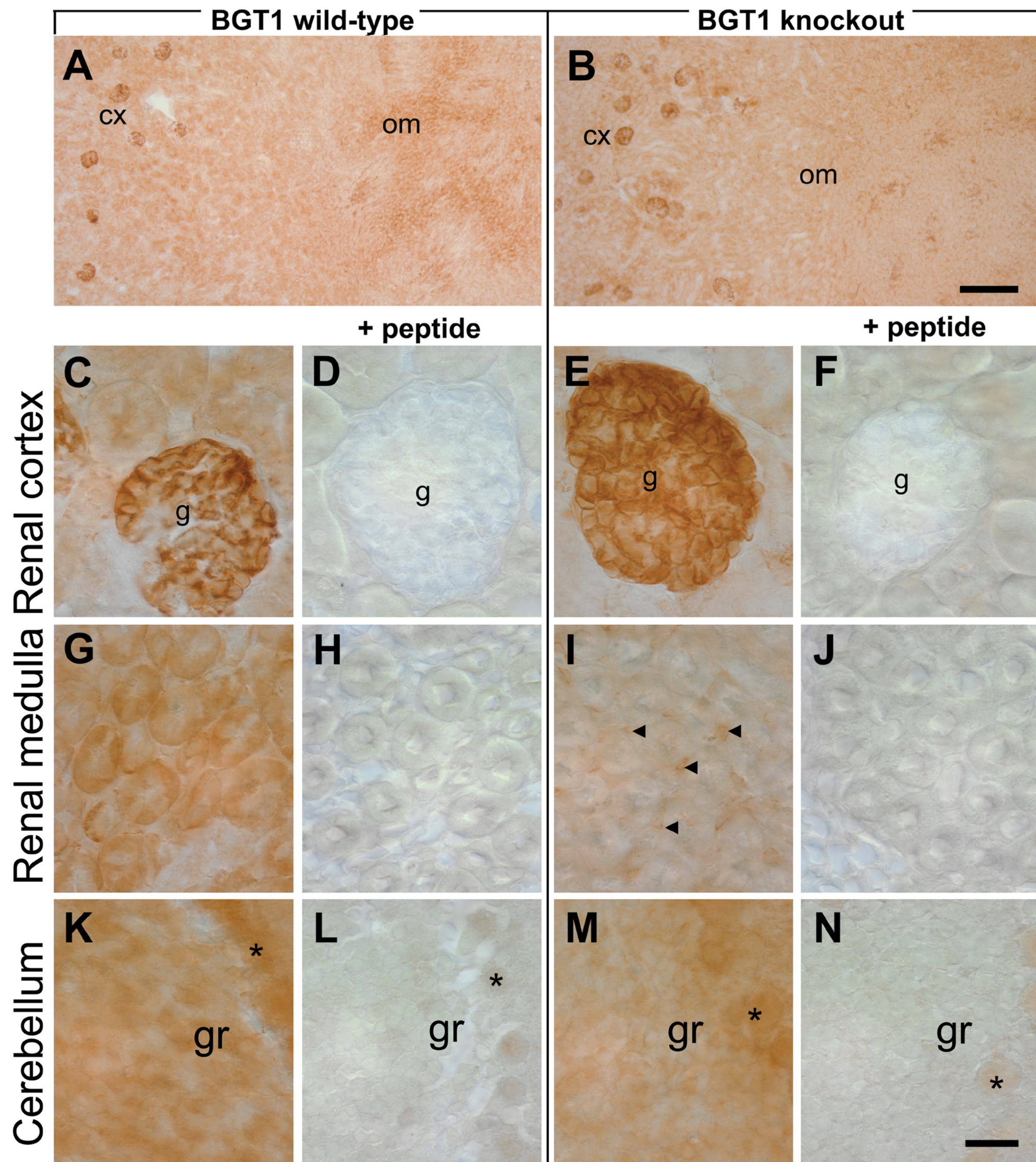

Figure 4. Preadsorption test gives a false sense of specificity. Kidney sections from wild-type (A, C, D, G, H, K, L) and BGT1 knockout (B, E, F, I, J, M, N) mice were labeled with anti-BGT599 (Ab#323) antibodies (3 µg/ml) to BGT1. Note that there are labeled glomeruli in the renal cortex (cx) in sections from both the wild-type and the knockout mice. This is shown at higher magnification in C and E. A closer look reveals that the outer medulla (om) is more strongly labeled in A than in B. This is confirmed at higher magnification (compare G and I). This is the labeling that truly represents BGT1 (Zhou et al. 2012). The only remaining labeling in I is due to capillaries (arrowheads). Also note that there is labeling in the cerebellar granule cell layer (gr) in both wild-type (K) and knockout mice (M). The preadsorption test is not helpful. Panels D, H, L, F, J, and N show that virtually all labeling, regardless of whether it represents BGT1 or not, is abolished if the antibodies are preadsorbed with the peptide (1 mg/ml BGT599–614) to which they have been raised and affinity purified. The only remaining labeling (*) seen in panels L and N is due to reactivity of the secondary antibody. Fixative, 4% formaldehyde without glutaraldehyde. Scale bars A and B = 200 µm; C-N = 20 µm.

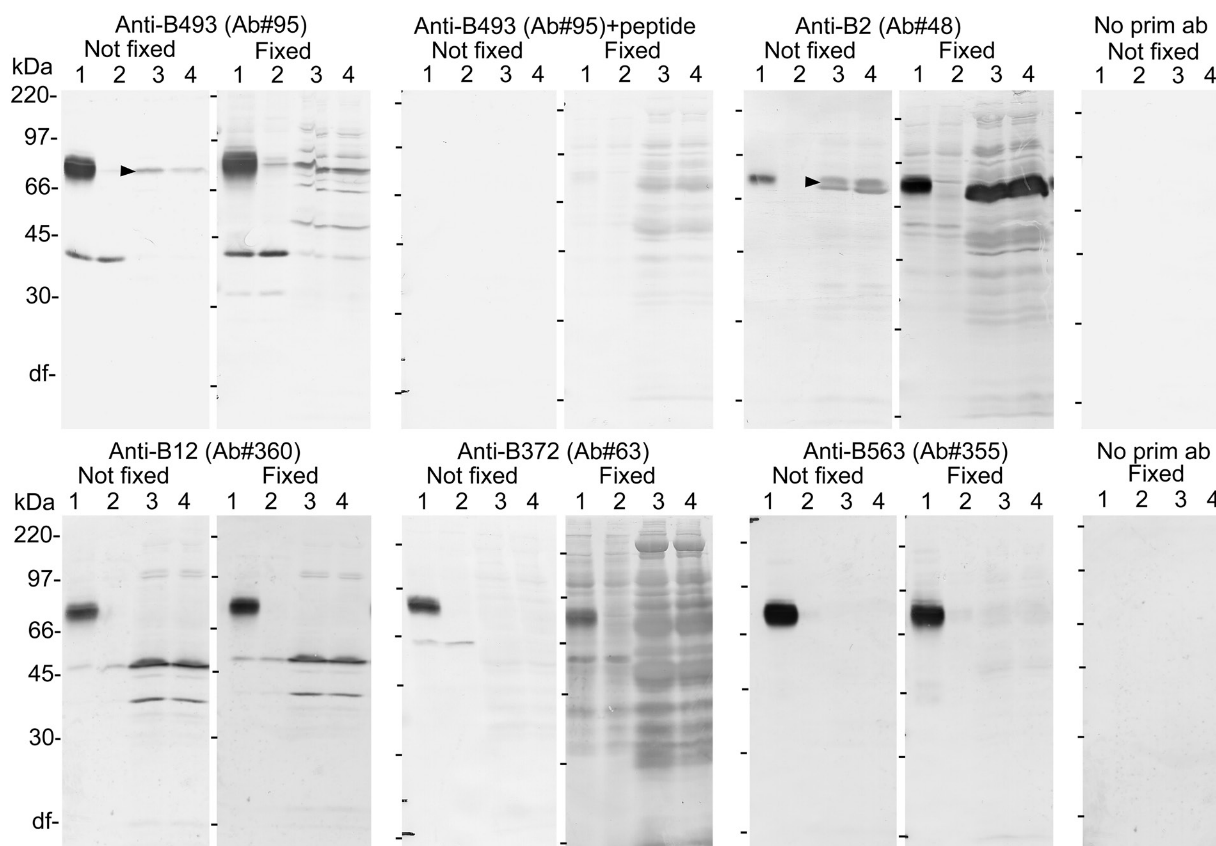

Figure 5. Forebrain (lanes 1 and 2) and heart (lanes 3 and 4) tissue from wild-type (lanes 1 and 3) and EAAT2 knockout (lanes 2 and 4) mice were solubilized and immunoblotted with the same antibodies as were used in Fig. 1. Fourteen identical blots were prepared. Half of them were fixed (3 hr, 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M NaPi) before being developed with the antibodies as indicated. One of the antibodies, anti-B493 (Ab#95), was preadsorbed overnight with 10 µg/ml peptide-antigen (Ag). This low antigen concentration was sufficient to abolish both the labeling representing EAAT2 and the cross-reactivity seen on unfixed blots. Two of the antibodies (anti-B2 and anti-B493) label heart (arrowheads) at the same molecular mass as EAAT2 (lane 1), but note that this band is also seen in the knockout (lane 4). Also note that the anti-B2 antibodies reacted better with the fixed EAAT2 than with the unfixed protein, whereas other antibodies showed weaker reaction after blot fixation (not shown). Antibody concentrations: anti-B2 (Ab#48), 1 µg/ml; anti-B12 (Ab#360), 0.2 µg/ml; anti-B372 (Ab#63), 1 µg/ml; anti-B493 (Ab#95), 0.2 µg/ml; anti-B563 (Ab#355), 0.2 µg/ml. The lanes contained either 5 µg of forebrain protein or 20 µg heart protein extracted with sodium dodecyl sulfate.

containing the epitope or care has been taken to carry out the deletion in such a way that it causes a shift of the reading frame. Nevertheless, if an antibody gives rise to labeling in knockout tissue, then this should not be taken lightly.

Even Monoclonal Antibodies May Display Cross-Reactivity

As pointed out (Saper and Sawchenko 2003; Holmseth et al. 2005), an antibody molecule is not a "magic bullet" with absolute specificity but a protein molecule that recognizes the antigen much like a receptor protein recognizes the ligand or an enzyme recognizes the substrate. Antibodies can adhere to other molecules, according to their respective concentrations and affinities (Rhodes and Trimmer 2006; Fritschy 2008). A good antibody binds to the desired target with high affinity, allowing it to be used at a concentration well below the concentration where it starts to bind to other targets. It should be recalled how medicinal chemists are able to develop new molecules that bind to the same receptor as endogenous ligand in spite of having a very different chemical structure. From this perspective, it is not surprising that antibodies often cross-react with seemingly unrelated molecules. For instance, many lupus erythematosus-like anti-DNA antibodies do not bind only to DNA but also to peptide sequences (Sibille et al. 1997; James et al. 1999) and may even cross-react with the glutamate receptor subtype NR2 (DeGiorgio et al. 2001).

It should also be taken into account that tissue processing (postmortem delay, fixation, embedding, and antigen retrieval) chemically modifies the tissue, leading to the creation and elimination of epitopes as shown here (Figs. 5–7)

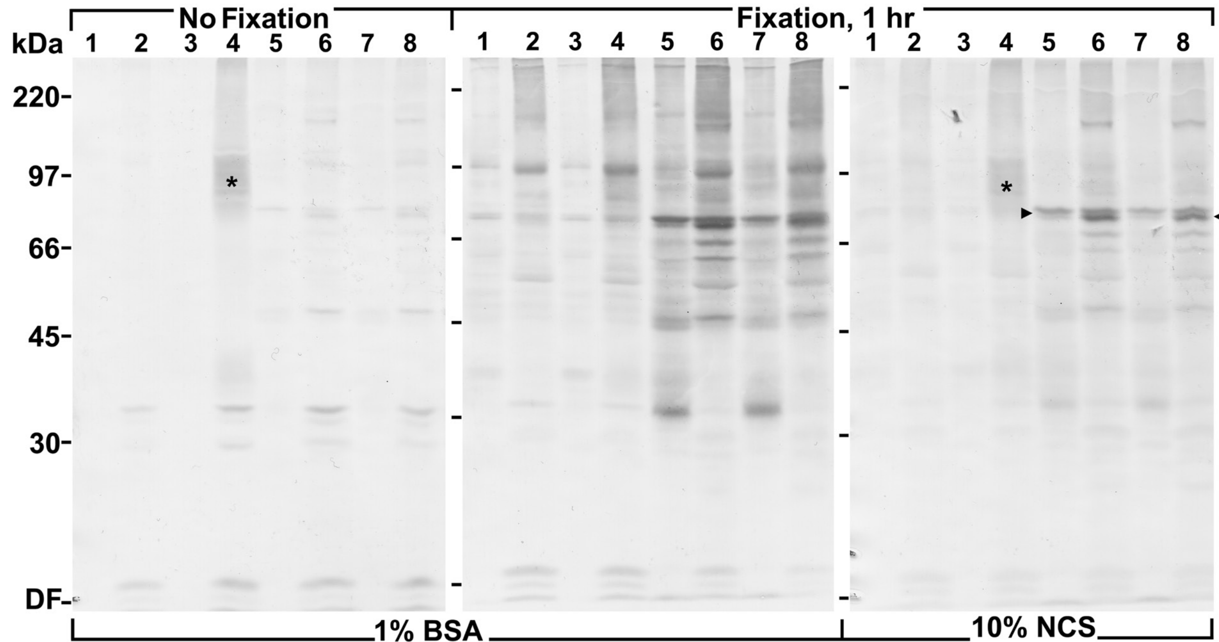

Figure 6. Fixation creates new epitopes cross-reacting with the anti-BGT1 antibodies. Tissue from the renal medulla (lanes 1–4) and cortex (lanes 5–8) from both BGT1 knockout (lanes 1, 2, 5, and 6) and wild-type (lanes 3, 4, 7, and 8) mice was homogenized in water to yield water-soluble (lanes 1, 3, 5, and 7) and water-insoluble fractions containing membrane proteins (lanes 2, 4, 6, and 8). Three identical blots were made. Before development with the anti-BGT1 antibodies (Ab#323. 0.5 µg/ml), 2 of the blots (as indicated) were incubated (at room temperature) in the same fixative as was used to fix the tissue for immunocytochemistry (Fig. 4). The unfixed (left) and one of the fixed blots (middle) were developed using 1% bovine serum albumin (BSA) as blocking agent, whereas the last was blocked with the same blocking agent as was used for immunocytochemistry (Fig. 4: 10% newborn calf serum). The band representing the BGT1 protein is indicated (lane 4, asterisk). Note that many extra bands are seen after fixation (arrowheads).

and by others (e.g., Josephsen et al. 1999). Thus, it is important to realize that cross-reactivity does not have to be due to the presence of contaminating antibodies but can be due to the antigen-recognizing antibodies themselves, as shown here with the anti-C480-tub antibodies (Figs. 2 and 7) and shown previously with monoclonal antibodies (Danbolt et al. 1998).

Correct Use of the Preadsorption Control

In the early days of immunocytochemistry, only crude sera were used to label sections. A serum contains a huge number of different antibodies, and only a small fraction of these will be antibodies to the injected antigen. Therefore, when a serum is used for labeling of tissue sections, it is important to determine if the labeling is due to antibodies directed toward the antigen or to other antibodies. A first indication can be obtained by comparing serum collected after immunization (immune-serum) with serum collected before immunization (preimmune serum). This tells if the immunoreactivity of the serum was there before immunization started or came after the immunization (and therefore may be a consequence of it), but this does not tell if the labeling is due to antibodies to the target antigen or to antibodies to other substances (e.g., components of the adjuvant used to enhance the immune response). This is where the preadsorption control comes in. If the addition of the target antigen to the serum prevents the serum from labeling the sections, then it follows that the labeling is due to those antibodies that are able to bind the added antigen. As illustrated in the present report with several examples, this does not test if the antibodies also can bind other antigens. Thus, when crude sera are being used, then the preadsorption test does add valuable information. If the antigen can be readily obtained in pure form, and in particular if tissue from knockout animals is unavailable, this test should be carried out. A common situation today, however, is to work with antibodies that are already selected for their ability to bind to the antigen (monoclonal or affinity purified polyclonal antibodies). In this situation, the preadsorption test does not give us much additional information as should be evident from the present study.

Specificity and Sensitivity

The labeling is sensitive if low concentrations of the antibodies give rise to labeling, and it is specific if the antibodies bind only to the target antigen. At low antibody

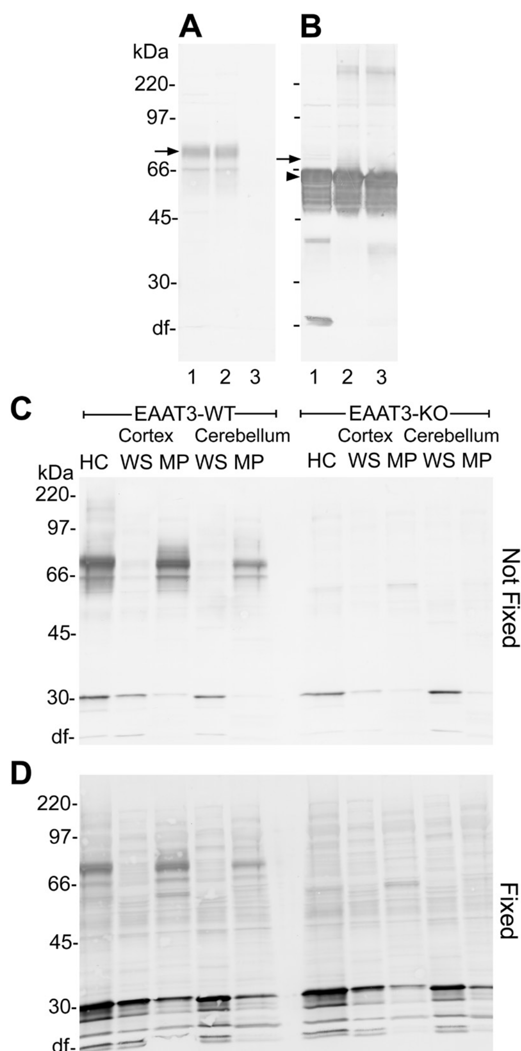

Figure 7. Immunoblots showing the specificity of the anti-C491 antibodies (A, C, and D) and the anti-C480-tub antibodies (B) to EAAT3. Panels A and B: obtained from wild-type Wistar rats (lane 1), wild-type mice (lane 2), and EAAT3 knockout mice (lane 3). Note that the anti-C491 antibodies (Ab#371, 0.5 µg/ml) clearly visualize the EAAT3 protein (arrow) in extracts from wild-type mice and rats (panel A, lanes 1 and 2). There is no labeling of proteins from the EAAT3 knockout (panel A, lane 3). In contrast, the anti-C480-tub antibodies (Ab#547; 1 µg/ml) label the EAAT3 band weakly (too weak to be easily seen on this blot; arrow), but they label another protein (tubulin) strongly (arrowhead). This protein is present in very high concentrations in all 3 protein extracts (panel B, lanes 1, 2, and 3). Note that the labeling has reached saturation. The identities of the lower bands have not been determined but may represent partly proteolysed tubulin. Panels C and D represent 2 identical blots of 10% to 20% gradient gels. Gradient gels were used to detect low molecular mass proteins. The blot in C is unfixed, whereas the blot in D was fixed before incubation with the antibodies. The blots contain extracts from mouse hippocampus (HC, whole tissue), mouse neocortex (WS, water soluble; MP, membrane pellet), and mouse cerebellum (WS, MP) separately. Each lane contained 20 µg of sodium dodecyl sulfate–extracted tissue proteins.

concentrations, only the antigens that have the highest affinities will be labeled. Antigens interacting with lower affinities will be labeled at higher antibody concentrations. Thus, if the antigen of interest binds the antibodies with much higher affinity than other antigens, then the labeling will be specific at low concentrations. One problem is that cross-reactive antigens can sometimes bind with high affinity, as illustrated here (Fig. 3, anti-C491, upper arrowhead) and by others (e.g., Josephsen et al. 1999; Dolman et al. 2004; Holmseth et al. 2005; Lorincz and Nusser 2008). Another problem is that labeling can usually be obtained by adjusting the assay conditions to increase sensitivity (e.g., Fig. 3). The labeling of sections from knockout mice mimics situations where a protein is not present or present below detection limit. It is not easy to distinguish a true positive signal from a false positive signal. Several antibodies to the same protein can help, but as shown with the selected antibodies to EAAT2 (Fig. 1), several antibodies may have the same reactivity. In particular, we have frequently observed cross-reactivity with mitochondria, postsynaptic densities, cell nuclei, and the cerebellar molecular layer. Thus, immunocytochemical labeling is not in itself a proof of expression.

Notes on Immunoblots

As explained earlier, the antibodies shown here have not been randomly selected but have been selected to illustrate a number of points. Together, they may give the impression that there is poor correlation between labeling of immunoblots and sections. But that is not the message we want to send. Our experience with immunoblots is that they are informative. If antibodies look specific on blots, then they are often also specific in sections. Nevertheless, exceptions like those shown here are so common that immunoblots should be supplemented with other tests whenever possible.

It is not surprising that antibodies may display different degrees of specificity when tested on immunoblots and on sections considering that the former is based on molecules that have been solubilized. The molecules may have different conformations and are likely to be separated from their natural molecular neighbors. Furthermore, the smallest and the largest molecules are lost, and the three-dimensional structure of the tissue is destroyed. In contrast, the three-dimensional structure is preserved in sections, but the tissue is often chemically modified, and some of the components may be lost depending on the type of tissue processing used.

When interpreting immunoblots, it is important to keep in mind that 1 band may contain more than 1 protein. The anti-B493 antibodies (Fig. 5) illustrate this point. A weak non-EAAT2 band is present in lane 2 (brain from the knockout mice) and in the heart samples (arrowhead). The most likely interpretation is therefore that the EAAT2 band in

lane 1 is a mixture of (mostly) EAAT2 and this other molecular species.

Conclusions

The primary focus of this study has been to evaluate the preadsorption test. We rediscover and illustrate that this test is not a specificity test in the true sense but a test for identifying the population of antibodies that is responsible for the labeling. This test does not tell if the observed labeling represents a specific visualization of the antigen under study or if it is due to cross-reaction with other molecules. This is old knowledge and has been discussed, albeit not illustrated, in several publications (e.g. Swaab et al. 1977; Pool and Buijs 1988; Burry 2000; Holmseth et al. 2005; Fritschy 2008; Burry 2011). Despite this, the preadsorption test is still regarded by many as an obligate control for the verification of immunocytochemical labeling—even labeling obtained with monoclonal and affinity purified antibodies. As shown here, the preadsorption test can give a misleading impression of specificity. Compounding this problem, it is often costly to obtain enough free antigen to perform the test, diverting time and resources from more definitive experiments.

Another conclusion from this study is that labeling in sections may be due to cross-reactivity even if the blots look perfect, and labeling in sections may be specific even if extra bands are visible on the blots. Extra bands on a blot represent a warning but are in themselves not an absolute indicator.

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