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ATP-Binding Cassette Transporter ABC2/ABCA2 in the Rat Brain: A Novel Mammalian Lysosome-Associated Membrane Protein and a Specific Marker for Oligodendrocytes But Not for Myelin Sheaths

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We recently cloned a full-length cDNA of the rat ATP-binding cassette transporter 2 (ABC2, or ABCA2) protein, a member of the ABC1 (or ABCA) subfamily (-ABC1/ABCA1 is a causal gene for Tangier disease) and found it to be strongly expressed in the rat brain. In this study, we identified ABC2 as a lysosome-associated membrane protein that is being localized specifically in oligodendrocytes. The ABC2-immunolabeled cells were detected mainly in the white matter but were also scattered in gray matter throughout the whole brain. In addition, these cells were found to be colocalized with 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase) immunoreactivity when the marker antibody for oligodendrocytes was used. However, no such colocalization was observed with markers for other kinds of glial cells. Unlike the CNP antibody, which also intensely stains myelin sheaths in the white matter, ABC2 immunoreactivity was detected only in the cell bodies of oligodendrocytes. At the ultrastructural level, ABC2 immunoreactivity was detected mostly around lysosome and partly in Golgi apparatus by electron microscopy. This was confirmed by immunocolocalization of ABC2 and lysosomal markers in a neuroblastoma cell line. Immunoblotting analysis of ABC2 from the whole brain and the ABC2-transfected cell line revealed bands at ~260 kDa. The result of *in situ* hybridization with a riboprobe for ABC2 matched the results obtained from immunostaining. These findings strongly suggest that ABC2 is a specific marker for oligodendrocytes but not for myelinsheaths and that it is as a novel mammalian lysosome-associated membrane protein involved in myelinization or other kinds of metabolism in the CNS.

Key words: ATP-binding cassette (ABC) transporter; ABC1 (or ABCA) subfamily; ABC2/ABCA2; oligodendrocytes; myelinization; lysosomes; marker protein; CNPase; lysosome-associated membrane protein LAMP1/LAMP2; rat brain

To date, hundreds of evolutionarily conserved membrane proteins have been identified as members of the ATP-binding cassette (ABC) transporter (or traffic ATPase) superfamily. These proteins have been implicated in the energy-dependent transport of a wide variety of substrates across the organellar or plasma membranes of both prokaryotic and eukaryotic cells (Higgins, 1992; Doige and Ames, 1993; Allikmets et al., 1996; Linton and Higgins, 1998; Saurin et al., 1999). Typical eukaryotic ABC transporters (full-molecule) consist of two transmembrane domains and two ATP-binding cassettes. Half-molecule transporters have only a single transmembrane domain and ATP-binding cassette, whereas quarter-molecule transporters have only an ATP-binding cassette (Hyde et al., 1990; Dean and Allikmets, 1995; Decottignies and Goffeau, 1997).

Many mammalian ABC transporters have recently been found to be associated with clinically relevant phenotypes, including neurodegenerations. X-linked adrenoleukodystrophy (ALD), which results in a fatal demyelinating disease, has been linked to mutations in a 70 kDa peroxisomal ABC transporter (Kamijo et al., 1990; Mosser et al., 1993; Dubois-Dalcq et al., 1999), and an autosomal recessive retinal disorder, Stargardt's macular dystrophy, has been connected to a retina-specific ABC transporter (ABCR/ABCA4) gene (Allikmets et al., 1997; Illing et al., 1997; Weng et al., 1999). Other genetic diseases reported to be linked to mutations of ABC transporters include cystic fibrosis (Sheppard and Welsh, 1999), persistent hyperinsulinemic hypoglycemia of infancy (Thomas et al., 1995), congenital jaundice (Paulusma et al., 1996), intrahepatic cholestasis (Strautnieks et al., 1998), and pseudoxanthoma elasticum (Ringpfeil et al., 2000).

We have cloned and characterized a full-length rat ATP-binding cassette transporter cDNA for ABC2/ABCA2, which encodes for a glycoprotein of 2434 amino acids with 44.5, 40.0, and 40.8% identity with mouse ABC1/ABCA1, human ABC3/ABCA3, and human ABCR/ABCA4, respectively (Zhao et al., 2000). ABC2 belongs to the ABC1 (ABCA) subfamily, which is

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structurally distinct from other subfamilies. Four members of this subfamily have been identified and named ABC1 (Luciani et al., 1994), ABC2 (Luciani et al., 1994; Zhao et al., 2000), ABC3 (ABC-C) (Klugbauer and Hofmann, 1996), and ABCR (Illing et al., 1997). This subfamily has recently received much attention because it has been proposed that ABC1 and ABCR are flippases (Higgins, 1994; Weng et al., 1999), and they have also been identified as the causal genes for Tangier disease (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999; Orso et al., 2000) and Stargardt's macular dystrophy.

Although it has been reported that ABC2 is expressed abundantly in rat and mouse brains (Luciani et al., 1994; Zhao et al., 2000) and it has been hypothesized that ABC2 may play a very important role in the mammalian CNS, knowledge of the morphology and physiological functions of this protein remains poor. The present study aims to identify the ABC2-expressing cell types in the brain and to determine its ultrastructural localization.

MATERIALS AND METHODS

Animals

Two or four male adult rats (2- or 3-month-old Sprague Dawley; Saitama Experimental Animal Center, Saitama, Japan) were used for each experiment. Rats were housed in a temperature- and light-controlled room (22°C, lights on at 6:00 A.M. and off at 8:00 P.M.) and fed standard Oriental Chow and water *ad libitum*. Two to five animals were used in each experiment. During the experiments, all efforts were made to minimize suffering of the animals, in accordance with the *National Institutes of Health Guidelines for the Care and Use of Laboratory Animals*.

Primary antibodies

The specific antibody for ABC2 was raised in rabbits against the synthetic peptide corresponding to 20 C-terminal amino acid residues (GLISFEEERAQLSFNTDTLC) of rat ABC2, which constructively differs from the other members of the ABC1 subfamily or any other protein from the sequence databases. The antibody was purified using affinity chromatography (HiTrap Protein G, Amersham Pharmacia Biotech). To identify the ABC2-expressing cell types in this study, double immunofluorescence was performed using the monoclonal mouse anti-human 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase; Sigma C5922) as a marker for oligodendrocytes (Sprinkle et al., 1987) and the monoclonal mouse anti-rat CD11b (Serotec, Kidlington, Oxford, England) as a marker for microglial cells in the brain (Milligan et al., 1991). In addition, the rabbit anti-myelin basic protein (MBP) (Dako, Carpinteria, CA) and the rabbit anti-glial fibrillary acidic protein (GFAP; Sigma G9269), a marker for astrocytes, were also used. Furthermore, two affinity-purified goat polyclonal antibodies against human lysosome-associated membrane proteins LAMP1 (N-19; Santa Cruz Biotechnology, Santa Cruz, CA) and LAMP2 (C-20; Santa Cruz Biotechnology) were used to identify the lysosomal colocalization (Karlsson and Carlsson, 1998).

Western blot analysis

Cell culture and transfection. To demonstrate the specificity of the antibody for ABC2 used in the present study, the rat ABC2 expression vector (pCMVrABC2) was transfected into cultured COS-1 cells. Culture and transfection of COS-1 cells was performed as previously described (Zhao et al., 2000). Briefly, cells were plated on 35 mm culture dishes at a density of 2×10^5 cells per dish 24 hr before transfection and cultured in DMEM (450 mg/dl glucose) supplemented with 10% fetal calf serum. Two micrograms of pCMVrABC2 were transfected into cells with Lipofectamine and Opti-MEM I (Life Technologies), according to the manufacturer's instructions.

Crude membrane preparation. Crude membrane from COS-1 cells was prepared as described previously (Zhao et al., 2000). Briefly, for immunoblot analysis, 3 d after transfection with pCMVrABC2 or pCMV vector alone, the COS-1 cells were washed three times with PBS, suspended in buffer A consisting of 50 mM Tris, pH 7.5, and 1 mM EDTA containing protease inhibitor mixture (10 μl/ml) (Sigma), homogenized, and then centrifuged at 100,000 × *g* for 1 hr at 4°C. The pellets were resuspended in 500 μl of buffer A and stored at −80°C until used. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). For preparation of crude membrane from the rat brain, the whole brain were immediately dissected into buffer A containing protease inhibitor mixture (10 μl/ml) (Sigma), and the tissue suspension was homogenized in a Teflon pestle–glass homogenizer on ice and centrifuged at 800 × *g* for 7 min at 4°C. The supernatant was subjected to ultracentrifugation at 100,000 × *g* for 1 hr at 4°C, and the collected pellets were resuspended in 500 μl of buffer A and stored at −80°C until immunoblot analysis.

Immunoblot analysis. The crude membrane proteins from brain tissue (60 μg) or from the COS-1 cells (3 μg) were boiled in SDS reducing sample buffer for 10 min and electrophoresed on a 7% SDS-polyacrylamide gel, then transferred electrophoretically to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) at 4°C at 200 mA overnight. The membrane was blocked in 5% defatted milk in 0.1% Tween 20-PBS (PBS-T) for 1 hr at room temperature. After washing with PBS-T, the membrane was incubated with 1:500 diluted ABC2 antibody for 2 hr at room temperature and washed with PBS-T. The membrane was then incubated with 1:5000 diluted horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech) for 1 hr at room temperature. After washing with 0.3% Tween 20-PBS and then with PBS-T, protein bands were detected using an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech), according to the manufacturer's instructions.

Immunohistochemistry

Tissue preparation. The animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfusion-fixed with 4% paraformaldehyde and 0.1% glutaraldehyde buffer, pH 7.4. Then the brains were removed quickly and post-fixed in 4% paraformaldehyde for 2 hr or more. After the brains were permeated with 20% sucrose for 1 or 2 d, they were frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at −70°C until used. Frozen 7-μm-thick coronal or sagittal sections were cut with a cryostat (Microm HM500, Heidelberg, Germany) and thaw-mounted on poly-L-lysine-coated glass slides.

Immunofluorescence. This procedure was performed as described previously (Zhou et al., 1999), although with some modification in relation to double labeling. The brain sections were blocked in 10% normal goat serum (NGS) and 1% bovine serum albumin, as well as 0.3% Triton X-100, in 0.1 M PBS for 1 hr at room temperature. Except for the MBP antibody that was used directly, each primary antiserum was diluted 1:500 in PBS containing 1% NGS or diluted together for double staining and was incubated overnight at 4°C. The antisera were then rinsed and incubated with one or two secondary antibodies for 1 hr at room temperature. For ABC2/CNP double labeling, Alexa 546-conjugated goat anti-rabbit IgG (for ABC2) and Alexa 488-conjugated goat anti-mouse IgG (for CNPase) were diluted 1:200 in PBS. In the case of ABC2/CD11b double labeling, Alexa 488-conjugated goat anti-rabbit IgG (for ABC2) and Alexa 546-conjugated goat anti-mouse IgG (for CNPase) were used. For MBP or GFAP single staining, Alexa 546-conjugated goat anti-rabbit IgG was used as the second antibody. After washing in PBS, the sections were counterstained with Hoechst 33258 (1 μg/ml, Sigma) for 1 min and rinsed. In the case of ABC2/LAMP1 double staining, Alexa 546-conjugated donkey anti-goat IgG (for LAMP1) was used first, and then Alexa 488-conjugated goat anti-rabbit IgG (for ABC2) was used. For ABC2/LAMP2, Alexa 488-conjugated donkey anti-goat IgG (for LAMP2) was used first, and then Alexa 546-conjugated goat anti-rabbit IgG (for ABC2) was used. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA).

Fluoromicroscopy and confocal microscopy. Fluorescence immunolabeling was detected using a U-MNIBA filter cube (Olympus, Tokyo, Japan) for Alexa 488, a U-MWIG filter cube for Alexa 546, and a U-MNU filter cube for Hoechst 33258. A U-MDA/FI/TR cube was used for triple-fluorescence immunolabeling with a fluoromicroscope (AX-70, Olympus). In the case of ABC2/LAMP1 or LAMP2 double staining, the confocal microscope (Leica TCS SP2, Leica, Microsystems, Heidelberg, Germany) was used.

Immunoelectron microscopy

Free-floating immunolabeling. Vibratome sections (30 μm) from the perfusion-fixed brain tissue were used to perform a preembedding immunoperoxidase method. After blocking and permeation as described above, the sections were floated in the primary antibody buffer (rabbit anti-ABC2, 1:1000) on ice overnight. A biotinylated anti-rabbit IgG (Vector Labs, Burlingame, CA) (diluted 1:400) was used as the secondary

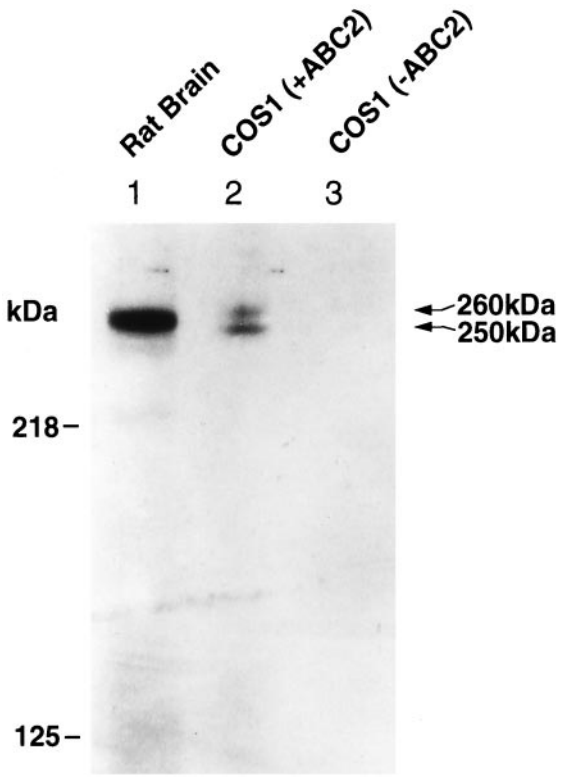

Figure 1. Immunoblot analysis of ABC2 protein. Membrane proteins from rat whole brain (60 μg) (*lane 1*) and COS-1 cells transfected with ABC2 expression vector (3 μg) (pCMVrABC2) (*lane 2*) and pCMV vector alone (3 μg) (*lane 3*) were electrophoresed on a 7% SDS-polyacrylamide gel. For immunoblot analysis, 1:500 diluted anti-ABC2 rabbit antibody and 1:5000 diluted horseradish peroxidase-conjugated anti-rabbit IgG were used as the primary and secondary antibodies, respectively. Proteins were detected using an enhanced chemiluminescence system. Molecular weights of markers are indicated on the *left*, and the detected 260 and 250 kDa bands are indicated by *arrows*.

antibody, with a 1 hr incubation at room temperature. The sections were then labeled with avidin—biotin–peroxidase complex (ABC Kit, Vector) and color-developed with a 3,3′-diaminobenzidine tetrahydrochloride (DAB)-substrate kit (Vector) for light-microscope or direct electron microscope observation.

Silver-gold intensification. To obtain clearer electron micrographs (or for double staining to detect a secondary antigen), the peroxidase–DAB reaction product was intensified as described previously (Guan et al., 2000). Briefly, the sections (after DAB reaction) were treated with 10% thioglycolic acid for 2 hr at room temperature to inhibit nonspecific binding of silver in tissue compartments and rinsed with 2% sodium acetate. Then the sections were transferred to a physical developer (Gallyas et al., 1982) (mixture of 0.2 ml 37% formalin, 50 ml solution containing 0.2% ammonium nitrate, 0.2% silver nitrate, 1% silicotungstic acid, and 50 ml 5% sodium carbonate) for 3–10 min for silver intensification, after which the reaction was stopped with 1% acetic acid, and the sections were rinsed with 2% sodium acetate. Finally, the sections were transferred into a 0.05% gold chloride solution for 10 min to enhance the stability of the metallic particles and the electron density, then rinsed and recovered in 0.1 M PBS, pH 7.4, for use in the next step.

Electron microscopy. The sections were post-fixed with 1% osmium tetroxide (in 0.1 M PBS), dehydrated with a graded series of ethanol, and flat-embedded in Epon-Araldite mixture. Ultrathin sections were cut with a Reichert Ultracut Microtome, from the corpus callosum, anterior commissure, optic chiasm, and the medulla of cerebellum, and observed using a Hitachi H7000 electron microscope without counterstaining. The morphological identification for oligodendrocytes and other glial cells as well as the organellar structures were consulted in Peters et al. (1976).

In situ *hybridization.* The first half of this experiment was performed as described previously (Zhao et al., 2000). The fresh-frozen brain sections and a [^{35}S]-labeled 400 bp ABC2 riboprobe were used.

Slide emulsion and signal development. As a continuous procedure (Shioda et al., 1997), the slides of hybridized brain sections (after exposure to x-ray film) were dipped in a nuclear track emulsion (NTB2; Kodak, Rochester, NY) at 42°C, air-dried, and then left in the dark to expose for 1 week. They were then developed in a Kodak D19 developer (20°C, 2 min), fixed in Kodak Rapid Fixer, counterstained with hematoxylin and eosin, and then analyzed under dark- and bright-field microscopy.

RESULTS

Immunoblot analysis of rat brain and ABC2-transfected cells

Immunoblot analysis for both brain tissue and the transfected cell samples was performed using the specific antibody for ABC2. A single band between molecular weight 260 and 250 kDa was detected from the total membrane fraction of the rat whole brain (Fig. 1, *lane 1*), and two bands of 260 and 250 kDa were detected from the total membrane fraction of COS-1 cells transfected with ABC2 expression vector (pCMVrABC2) (Fig. 1, *lane 2*). However, no band was detected from those transfected with pCMV vector only (Fig. 1, *lane 3*).

Cellular identification of ABC2 in the cerebellum and the anterior commissure

In the cerebellum of rats considered in this study, intense ABC2 immunoreactivity was clearly observed in those cell bodies that were mainly distributed in the medulla and scattered in the granular layer (Fig. 2*A*,*B*,*D*,*E*, *red* labeling for ABC2). In the medulla, the ABC2-immunolabeled cells showed oval-shaped and rare dendrite morphological characteristics (Fig. 2*E*, *red* labeling, *G*, *green* labeling for ABC2) and can therefore be considered to be oligodendrocytes. Further evidence to this effect was obtained in two subsequent double-immunofluorescence staining experiments (Fig. 2*A-I*). All ABC2-immunolabeled cell bodies in both the medulla and the granular cell layer (Fig. 2*B*,*E*, *red* labeling for ABC2) were found to be co-immunostained in the same tissue sections with an antibody against CNP, a marker protein that exists both in oligodendrocytes and in the myelin sheath (Fig. 2*C*,*F*, *green* labeling for CNP).

To confirm that ABC2 immunoreactivity was not localized in other kinds of glial cells, we chose an antibody against CD11b, a marker for microglial cells in brain sections, for double immunostaining with ABC2. This time, ABC2 immunoreactivity was labeled with green fluorescence and found to be restricted to oval-shaped cell bodies (Fig. 2*G*). The red-labeled CD11b immunoreactivity, on the other hand, was localized in another kind of cell, identified as the microglia cell type (Fig. 2*I*), and there was no observable overlap between the nuclei of these two immunolabeled cell types (Fig. 2*H*).

Further confirming staining was showed in the anterior commissure (Fig. 2*J-L*). The intense ABC2-immunoreactived cell bodies were clearly observed in the white matter region (Fig. 2*J*, *red* labeling), and this region from a neighboring section stained with MBP antibody showed intense positive labeling for myelin basic protein in whole white matter (Fig. 2*K*, *red* labeling). However, with an antibody for GFAP, the marker for astrocytes, most positive labeling was observed in the gray matter at the anterior commissure, and rare positive immunoreactivity was detected in the white matter (Fig. 2*L*, *red* labeling).

Distribution of ABC2 in various brain regions

In addition to its distribution in the cerebellum as well as the anterior commissure, the distribution of ABC2 immunoreactivity in both the gray and white matter of other brain regions was also

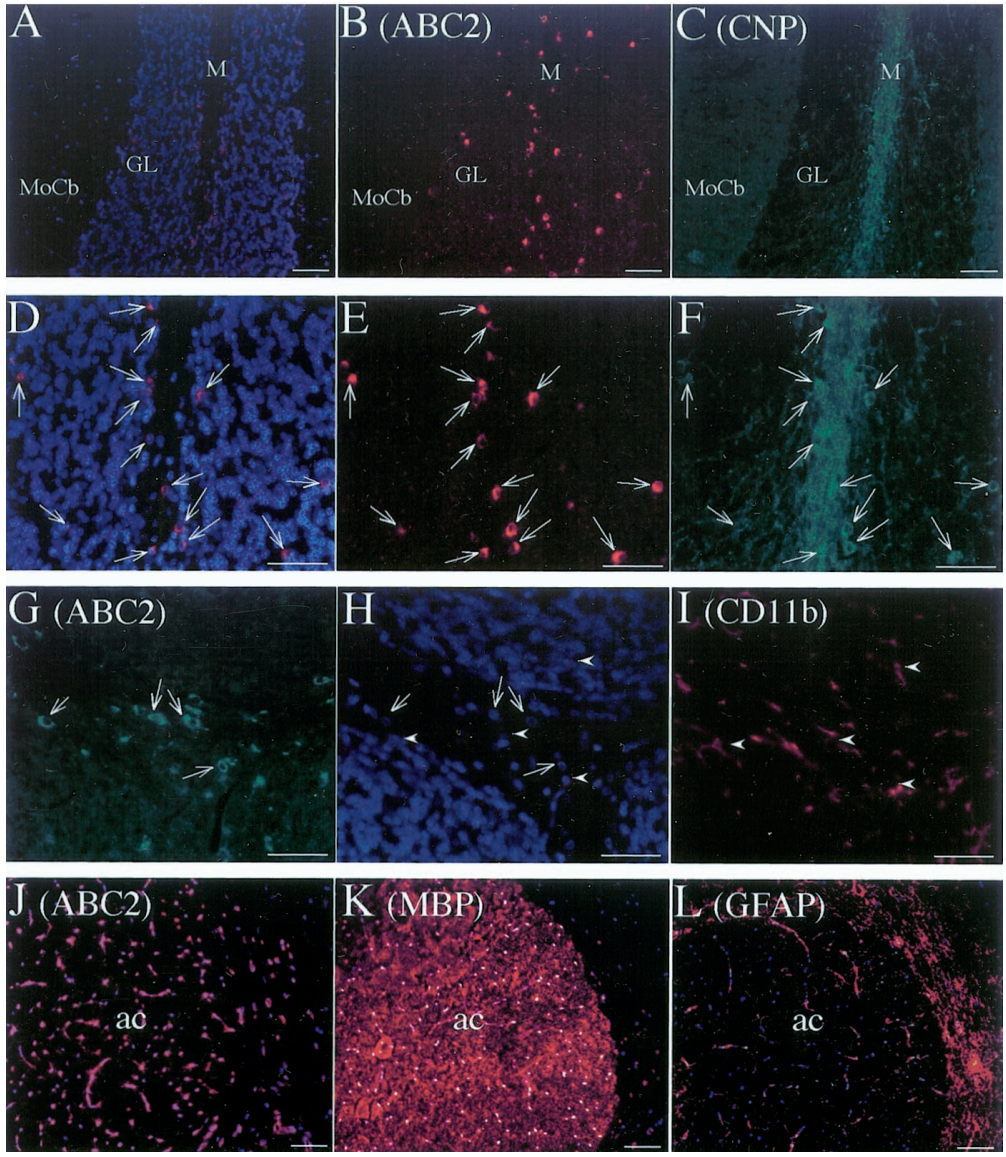

Figure 2. Identification of the ABC2-immunolabeled cell type in the brain section. *A–F*, ABC2 immunoreactivity is restricted to cell bodies that have been identified by CNP marker as being oligodendrocytes. *A–C*, Low-magnification micrographs indicate that the immunoreactivities of both ABC2 (*red*) and CNP (*green*) are localized mainly in the medulla and apparently rarely in the granular cell layer. *D–F*, Zoom-in micrographs from *A* to *C*, respectively, show the precise colocalization of ABC2-immunolabeled cells with CNP; the *arrows* indicate the same location of the immunolabeled cells under different fluorescence microscopes. *A*, *D*, The *red* fluorescence (Alexa 546) represents ABC2 immunoreactivity and the *blue* (Hoechst 33258) indicates all nuclei, including a large number of nonimmunolabeled cells. *B*, *E*, Only ABC2 immunoreactivity is visible in the positive cell bodies. *C*, *F*, The *green* fluorescence labeling (Alexa 488) that represents the marker protein of CNP exists in both the oligodendrocytes and the myelin sheath. It is of note that all *red* ABC2 labeling surrounding the nuclei overlapped with *green* CNP labeling. *MoCb*, Molecular layer; *GL*, granular layer; *M*, medulla of the cerebellum. *G–I*, ABC2-immunoreactivity does not colocalize with marker labeling of CD11b for microglial cells. *G*, For comparison with *I*, ABC2 is labeled using *green* fluorescence (Alexa 488) is labeled for ABC2, and the oval-shaped positive cells (*arrows*) are observed. *H*, All cell nuclei are stained *blue* by Hoechst 33258; the *arrows* (the same as those used in *G*) showing the nuclei locations of ABC2-immunolabeled cells in the medulla of the cerebellum and the *arrowheads* showing the nuclei of CD11b-immunolabeled cells (in *I*) indicate that there was no overlap with these two kinds of immunolabeling. *I*, The microglial cell bodies, which are immunolabeled with a marker antibody against CD11b (Alexa 546, *red*), are morphologically distinct from ABC2-immunolabeled cells (*G*) in the same tissue sections of the cerebellum. *J–L*, ABC2-immunoreactivity in the anterior commissure (*ac*) compared with MBP and GFAP immunoreactivities. All positive immunoreactivities by above antibodies in the neighboring brain sections are labeled using *red* fluorescence (Alexa 546) and counterstained with Hoechst 33258. *J*, Most of the ABC2-immunolabeled cell bodies are distributed in the white matter region. *K*, The MBP immunoreactivity is observed intensely in the white matter, especially in the myelin sheaths and the oligodendrocytes, but the cell bodies are difficult to distinguish. *L*, The distribution of the GFAP immunolabeling, which specifically for astrocytes clearly shows a different localization pattern from oligodendrocytes immunolabeled by ABC2 or MBP antibodies. Scale bars, 50 μm.

investigated in this study by double-immunofluorescence labeling with CNP (Fig. 3). In the gray matter of the olfactory bulb (Fig. 3*A*, *OB*), cerebral cortex (Fig. 3*B*,*J*, *CX*), dentate gyrus (Fig. 3*C*, *DG*), corpora quadrigemina (Fig. 3*L*, *CQ*), thalamus, and hypothalamus, as well as in other parts, the double-immunolabeled (for ABC2 and CNP; overlapped as the *yellow* labeling) cells were found to be scattered within neurons and other kinds of glial cells.

In white matter throughout the entire brain, ABC2-immunostained cells were found to be in the majority. Intense double-immunolabeled cells were observed especially in the corpus callosum (Fig. 3*D*,*E*,*J*, *cc*), from the forceps minor part (Fig. 3*D*, *fmi*) to the forceps major part, the cingulum (Fig. 3*J*, *cg*), the ventral (Fig. 3*E*, *vhc*) and dorsal (Fig. 3*F*, *dhc*) hippocampal commissure, the anterior commissure (Fig. 3*H*, *ac*), the stria medullarias thalamus (Fig. 3*K*, *sm*), the optic chiasm (Fig. 3*I*, *ox*), the olfactory tubercle (Fig. 3*G*, *Tu*), the olfactory tract, and the fibers pons, as well as in other regions.

Ultrastructural localization of ABC2 in oligodendrocytes

The white matter of brain tissues was cut ultrathin for the purposes of electron microscopy. In the medulla of the cerebellum, most of silver–gold particles that intensified the peroxidase–DAB reaction product for ABC2 immunoreactivity were found to be mostly distributed around the lysosomes of the oligodendrocytes (Fig. 4*A*). Using high magnification, the dense silver–gold particles, which were morphologically characterized as lysosomes (Fig. 4*B*), were clearly observed in the organellar membrane. The dense particles were also observed particularly in the *trans* face of the Golgi apparatus, and no specific particles were observed in all

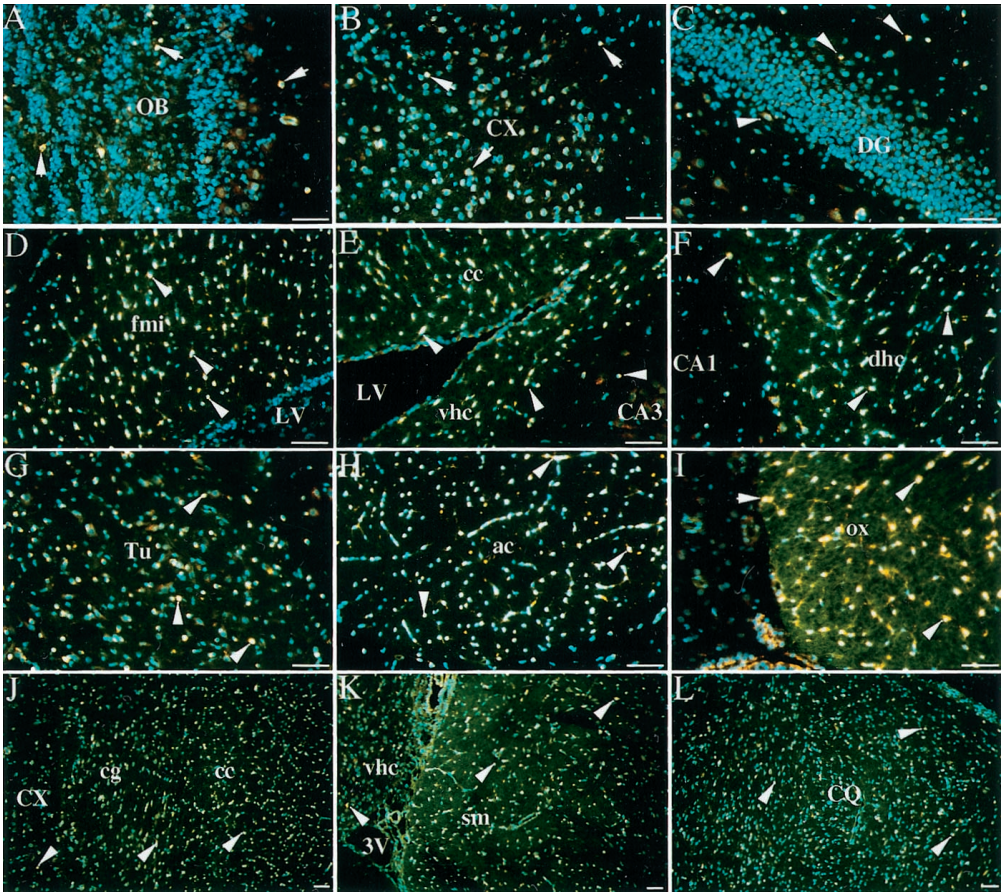

Figure 3. Distribution of ABC2 immunoreactivity, co-immunolabeled with CNP, in the gray matter and white matter from different brain sections. The *yellow* positive labeling (some cells are indicated by *arrowheads*), which indicates colocalization of double fluorescence of Alexa 546 (*red*, for ABC2) and Alexa 488 (*green*, for CNP) in the same cells, was observed through a triple fluorescence filter cube. All cell nuclei in each brain section were labeled *blue* by Hoechst 33258. It is clear that the double-immunolabeled cells are rarely found in the gray matter (*A–C*) but occur much more frequently in the olfactory tubercle (*G*) and corpora quadrigemina (*L*) and are distributed mainly in the white matter (*D–F*, *H–K*). *3V*, *Third* ventricle; *ac*, anterior commissure; *CA1*, *CA3*, the CA1 or CA3 field of the hippocampus; *cc*, corpus callosum; *cg*, cingulum; *CQ*, corpora quadrigemina; *CX*, cerebral cortex; *DG*, dentate gyrus of the hippocampal formation; *dhc*, dorsal hippocampal commissure; *fmi*, forceps minor corpus callosum; *LV*, lateral ventricle; *OB*, olfactory bulb; *ox*, optic chiasm; *sm*, stria medullaris thalamus; *Tu*, olfactory tubercle; *vhc*, ventral hippocampal commissure. Scale bars, 50 μm.

mitochondria and most endoplasmic reticulum or nucleus (Fig. 4*B*). At least 20 oligodendrocytes in cerebellum or other brain regions were examined. No specific ABC2 immunoreactivity was detected in the myelin sheath or the axon around the oligodendrocytes (Fig. 4*A*).

Comparison of the histochemical distribution of ABC2 mRNA and protein

The x-ray film autoradiograms obtained from brain sections using an isotope-labeled riboprobe for ABC2 mRNA has been described in our previous work (Zhao et al., 2000). Both dark- and bright-field micrographs were made, and results are partly shown in Figure 5. Brain regions showing intense labeling for ABC2 mRNA coincided well with those demonstrating ABC2 immunoreactivity (described in this paper); however, a detailed description has been omitted here.

Colocalization of ABC2 and LAMP1 or LAMP2 in the neuro-2a cell line

It has been reported that mouse ABC2 mRNA was detected in the neuro-2a (N2a), a mouse neuroblastoma cell line, by Northern blot (Luciani et al., 1994). We used this cell line to confirm the lysosomal localization of ABC2 by double immunostaining with LAMP1 and LAMP2. As shown in Figure 6, most ABC2-immunolabeled orgenelles (Fig. 6*A*, *green*, *D*, *red*) were found to colocalize with lysosomal markers, either LAMP1 (Fig. 6*B*, *red*) or LAMP2 (Fig. 6*E*, *green*), and they were overlapped with each other by use of the confocal microscope (Fig. 6*C*,*F*, *yellow*).

DISCUSSION

This study has demonstrated that ABC2 is a novel lysosome-associated membrane protein and is localized specifically in oligodendrocytes in the rat brain. A number of ABC transporter proteins are specific to a particular tissue or organelle and function specifically by coupling the hydrolysis of ATP to the transmembrane flux of molecules such as ions, sugars or polysaccharides, steroids, phospholipids, amino acids or peptides, and proteins, as well as of chemotherapeutic drugs. However, to date, only a couple of these transporters have been detected and investigated at length in the CNS. For example, sulfonylurea receptors, the regulator subunits of ATP-sensitive potassium channels (Inagaki et al., 1995, 1996), have been shown to be widely expressed in the rodent brain (Karschin et al., 1997). The present study has provided the first identification of a mammalian ABC membrane protein that is localized in oligodendrocytes in the mammalian brain and associated with lysosomes. Our findings should lead to the discovery of currently unknown aspects of both lysosomal membrane transport processes in oligodendrocytes and the physiological functions performed by ABC2 in the mammalian CNS.

Specificity of the primary antibody for ABC2

A polyclonal antibody was generated in a rabbit against the C-terminal domain of rat ABC2, which showed low homology with other peptides from the sequence databases on line. The antibody was affinity-purified to investigate the distribution of ABC2 protein in the rat brain. The specificity of the antibody was assessed by Western blot analysis. The present study revealed two bands of 260 and 250 kDa from the COS-1 cells transfected with rat ABC2 expression vector (pCMVrABC2), whereas no any band was revealed from those transfected with pCMV vector alone. This is consistent with our previous report that both 260 and 250 kDa proteins also were detected by anti-HA antibody in

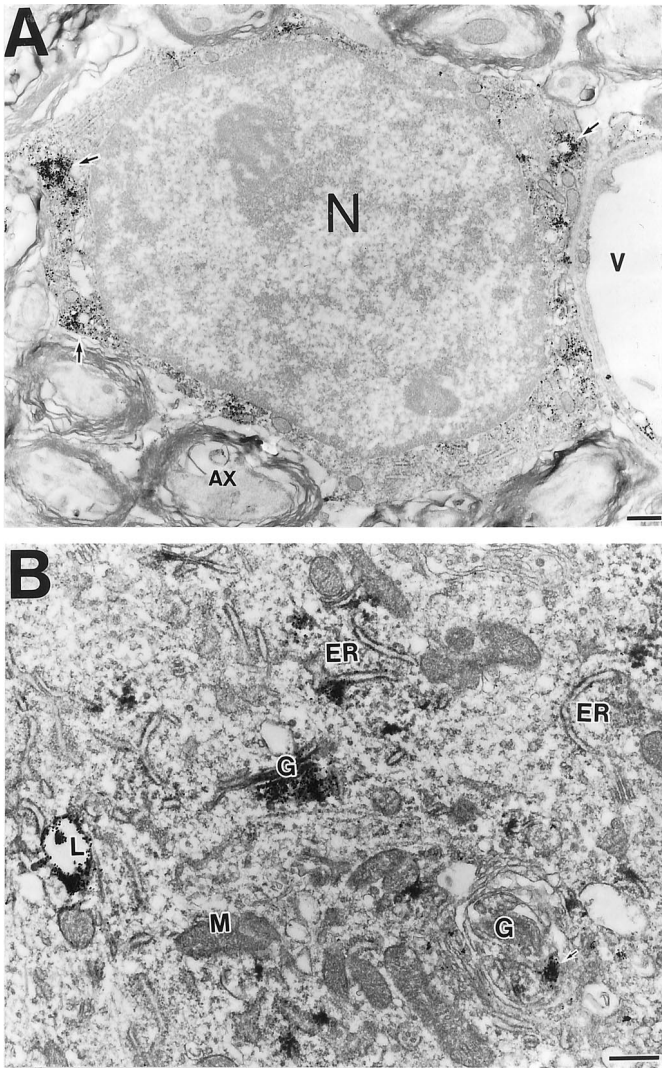

Figure 4. ABC2 in oligodendrocytes by immunoelectron microscopy. The ultrathin section was cut from the medulla of the cerebellum. The peroxidase–DAB reaction product from ABC2 immunohistochemistry was intensified with the silver–gold particles. *A*, Most of the intense labeling was observed around lysosomes (*arrows*), but only particular labeling or none at all was observed in other organelles at a magnification of 7000×. Note that there is no specific labeling in the myelin sheath or the axon around the cell body. *AX*, Axon; *N*, nucleus; *V*, blood vessel. *B*, High-magnification (10,000×) graphs for organellar localization of the ABC2 in oligodendrocytes. The silver–gold particles representing ABC2 immunoreactivities localized densely in lysosome membranes and particularly in the *trans* face of the Golgi apparatus (*arrow*). *ER*, Endoplasmic reticulum; *G*, Golgi body; *L*, lysosome; *M*, mitochondria. Scale bar, 500 nm.

COS-1 cells transfected with an expression vector carrying ABC2-HA, in which a hemagglutinin epitope (YPYDVPDYA) was introduced into the C terminus of the ABC2 protein (Zhao et al., 2000). Furthermore, the present study revealed a single band from rat brain between 260 and 250 kDa. The small difference of the mobilities between ABC2 protein expressed in COS-1 cells and in the brain might be attributable to the difference of glycosylation, because ABC2 is a glycoprotein (Zhao et al., 2000).

Colocalization of ABC2 and CNP in the brain

We have demonstrated that all ABC2-immunolabeled cell bodies in this study were also colocalized with CNP immunoreactivity. CNP is the main component of the Wolfgram protein fraction (Drummond and Dean, 1980; Sprinkle et al., 1980) and has been widely used as a marker for myelin oligodendrocytes in the CNS. CNP is present in the cytoplasm-containing compartments of the oligodendrocytes and myelin sheaths but not in lamellae or cellular membranes (Braun et al., 1988). In contrast, we found that ABC2 immunoreactivity occurred only in cell bodies and was specifically localized to oligodendrocyte organelles. We observed that the ABC2-immunostained cells were mainly distributed within the white matter of the brain, in the interfascicular oligodendroglia that produce multilamellar myelin sheaths around nerve axons. In addition, many immunostained oligodendrocytes were observed in the gray matter, within neurons as so-called satellite cells, or adjacent to blood vessels. However, the functions of these latter cells remain unclear. The distribution pattern of immunostained oligodendrocytes coincided well with those of CNP immunohistochemistry (Braun et al., 1988).

ABC2 as a specific marker for oligodendrocytes but not for myelin sheaths

Numerous tissue-specific chemical components have been isolated and identified from myelin oligodendrocytes (as well as from Schwann cells) and may have been used as specific markers for cell identification. To date, >40 enzymes, including CNP, ceramide galactosyltransferase, glutathione *S*-transferase, and carbonic anhydrase, are actively expressed at specific stages during myelinization (Newman et al., 1995; Wiesinger, 1995). However, CNP is widely used as the marker in experiments involving the adult CNS, as was the case in this study. Other proteins found in the myelin sheath are galactocerebroside, a kind of typical myelin lipid also used as a specific marker in cultured cells (Benjamins et al., 1987), sulfatide, cholesterol, ethanolamine phosphatide, lecithin, and plasmalogen (Morell et al., 1994). Furthermore, 60–80% of the protein mass in the myelin sheath is made up of proteolipid protein and MBP. Other glycoproteins, such as myelin-associated glycoprotein, myelin-oligodendrocyte glycoprotein (MOG), and oligodendrocyte-myelin glycoprotein, have also been isolated (Newman et al., 1995). Although these components exist both in the myelin-sheath and in oligodendrocytes, a number of them, such as CNP, MBP, and MOG, are localized in different regions at the ultrastructural level (Brunner et al., 1989). In the present study, we compared not only the immunocolocalizaiton of ABC2 with CNP but also its colocalization with MBP, and obtained a similar result. We therefore propose that, although further investigation is needed into the expression of ABC2 in the peripheral nervous system and during development, ABC2 can be used as a specific marker for oligodendrocytes. We also have compared ABC2 with markers for other glial cells such as CD11b, in the case of microglial cells, and GFAP, in the case of astrocytes (Debus et al., 1983), and they shown no cross-immunostaining with each other.

ABC2 as a lysosomal membrane protein

The colocalization of ABC2 with lysosome-associated membrane proteins (LAMP1 and LAMP2) and the immunoelectron microscopy as well as crude membrane-immunoblotting results in the present study have clearly shown ABC2 to be a novel lysosome-associated membrane protein. Lysosomes are membrane-bound organelles with an acidic internal milieu containing hydrolytic enzymes for degrading for various substances. The major structural components of lysosomal membrane are glycoproteins with molecular weights between 90 and 110 kDa (Peters and von Figura, 1994).

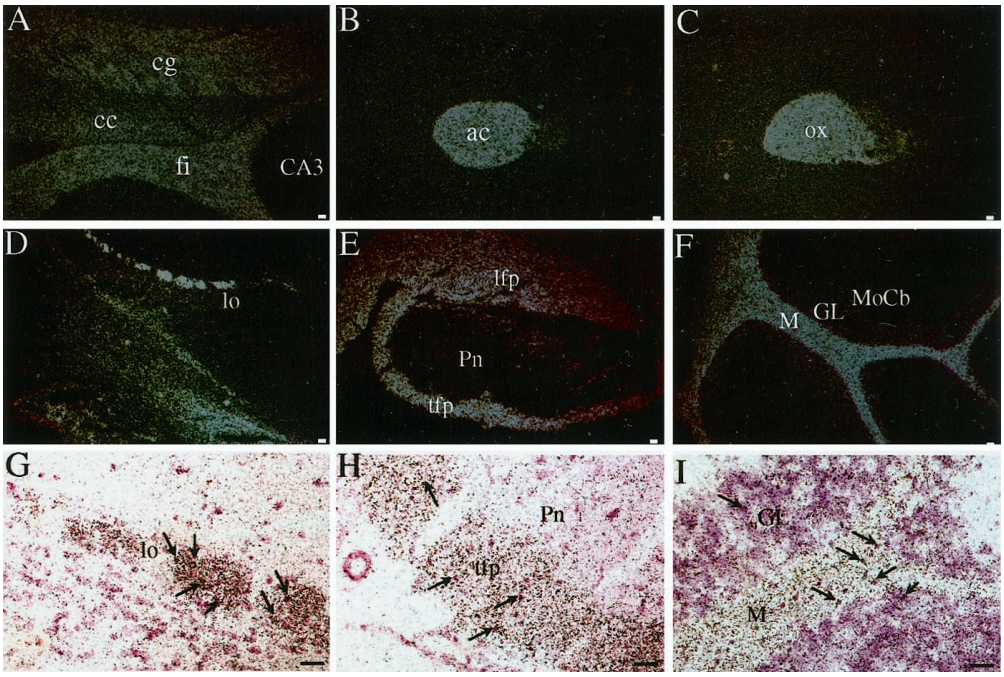

Figure 5. Dark- and bright-field micrographs of the distribution of ABC2 mRNA in brain tissues. Sections, after hybridization with [^{35}S]-labeled riboprobe for ABC2, were dipped in a nuclear track emulsion and exposed for 1 week. Intense labeling was observed in the white matter and weak labeling in the gray matter throughout the whole brain. The areas around the corpus callosum (*A*, coronal section), anterior commissure (*B*, sagittal), optic chiasm (*C*, sagittal), olfactory bulb (*D*, sagittal), pontine nuclei (*E*, sagittal), and the lobe of cerebellum (*F*, sagittal) showed up in the dark-field. The bright-field micrographs (*G–I*) are magnified from the relative regions (*E, F*), and the dense silver particles representing ABC2 mRNA are observed over many cell bodies within the white matter (*arrows*). *ac*, Anterior commissure; *CA3*, the CA3 field of hippocampus; *cc*, corpus callosum; *cg*, cingulum; *fi*, fimbria hippocampus; *GL*, granular layer of cerebellum; *lfp*, longitudinal fasciculus pons; *lo*, lateral olfactory tract; *M*, medulla of cerebellum; *MoCb*, molecular layer of cerebellum; *ox*, optic chiasm; *Pn*, pontine nuclei; *tfp*, transverse fibers pons. Scale bars, 50 μm.

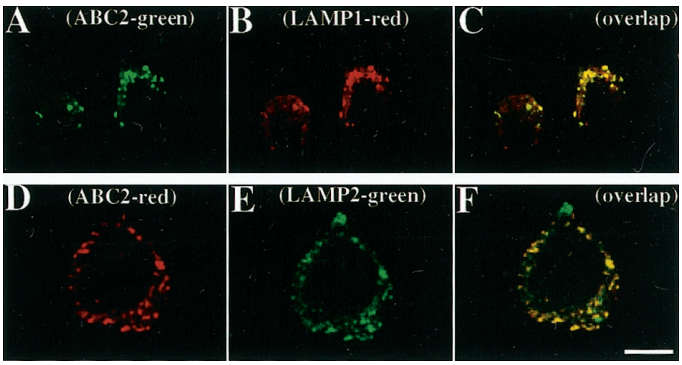

Figure 6. Colocalization of ABC2 and lysosomal markers LAMP1 or LAMP2 with confocal microscopy in neuro-2a cells, an ABC2-expressing mouse neuroblastoma cell line. The *green* labeling for ABC2-immunoreactivity (*A*) and the *red* labeling for LAMP1 immunoreactivity (*B*) are mostly overlapped as *yellow* in the lysosomes (*C*). In contrast, the *red* labeling for ABC2 immunoreactivity (*D*) and the *green* labeling for LAMP2 immunoreactivity (*E*) are also overlapped well as *yellow* in lysosomes of the neuro-2a cells (*F*). Scale bars, 20 μm.

The lysosomal membrane regulates the transport activities of substances such as proteins, lipids, nucleic acids, and saccharides of the cellular components as well as toxins, drugs, and heavy metals from the cytoplasm into the lysosome, as well as the release of digested molecules from the lysosomal lumen into the cytoplasm. These processes usually consume energy. Therefore, ABC transporter proteins were potential candidates to be performing some of these transporter activities. In this study, we have identified ABC2 as being specifically localized in the lysosome membranes of oligodendrocytes. Although the ABC protein HMT1, involved in heavy-metal tolerance (Ortiz et al., 1992), and the yeast cadmium factor protein, a glutathione *S*-conjugate pump (Li et al., 1996), have been found in the yeast vacuolar membrane (equivalent to the lysosomal compartment), until this study no ABC transporter had as yet been identified as a mammalian lysosomal membrane protein. It should be noted that almost at the same time as the completion of this manuscript, one paper did describe a half-molecule ABC transporter, ABCB9, which was mainly expressed in rodent testes and was localized in the lysosomes in transfected cells (Zhang et al., 2000). Although data are needed that directly demonstrate the intracellular localization of ABCB9 (this half-transporter belongs to a different subfamily than the full-length ABC2 transporter) in testis, this *in vitro* data supports our present *in vivo* findings. Further studies should be performed to investigate whether ABC2 also plays the lysosome-specific role in the testis (we have detected ABC2 in testis but further studies are needed), whether ABCB9 plays a role in both testis and brain, or whether either protein plays a role in other tissues. Several other organelle-specific ABC proteins, however, have been reported: four murine peroxisomal ABC half-transporters (Berger et al., 1999), the ALD protein (ALDP) (Mosser et al., 1993), ADL-related protein (ALDRP) (Holzinger et al., 1997a), 70 kDa peroxisomal membrane protein (PMP70), and PMP70-related protein (P70R or PMP69) (Holzinger et al., 1997b); the mitochondrial ABC protein (M-ABC1) (Hogue et al., 1999) and ABC7 (Csere et al., 1998); and the Tap1 and Tap2 as a heterodimer associated with ABC transporters in the endoplasmic reticulum (Gileadi and Higgins, 1997).

Significance of ABC2 expressed in the brain

In the ABC1 subfamily, ABC1 and ABCR have been proposed to be flippases for intracellular cholesterol and protonated *N*-retinylidene-phosphatydylethanolamine, respectively (Weng et al., 1999). The N-terminal 40-amino acid sequences including the first hydrophobic segment, the membrane-spanning domains, and the nucleotide-binding domains of the four identified members of this subfamily (ABC1, ABC2, ABC3, and ABCR) between murine and human are strongly conserved (Zhao et al., 2000); however, the fundamental function of ABC2 remains unknown. The ABC2 gene is located at chromosome 9q34 (Luciani et al.,

1994), which is a homogeneous staining region for an estramustine-resistant human ovarian carcinoma cell line. One research group has in fact used this cell line to show that amplification and overexpression of ABC2 contributes to estramustine resistance (Laing et al., 1998). The current findings that ABC2 is expressed in oligodendrocytes and associated with lysosomes provides evidence that this transporter should be studied further in the CNS. Investigation of cultured oligodendrocytes may potentially reveal an essential cellular role for ABC transporters. Oligodendrocytes are well known as the myelinating glial cells, and hypomyelination or demyelination diseases such as genetic leukodystrophy, multiple sclerosis, and multisystem atrophy are all closely linked to the intracellular metabolism connected with oligodendrocyte lysosomes. This strongly suggests that ABC2, as a lysosomal membrane transporter of oligodendrocytes, may be involved in normal myelination and therefore may be associated in some way with these degeneration processes. As with the other members of ABC1 subfamily, ABC1 and ABCR have been identified as the causal genes for certain genetic diseases. Further investigation of ABC2 will provide more insight into its functional significance.

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