Degeneration of Neural Cells in the Central Nervous System of Mice Deficient in the Gene for the Adhesion Molecule on Glia, the β2 Subunit of Murine Na,K-ATPase

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Abstract. We generated mice, null mutant in the adhesion molecule on glia (AMOG), the β2 subunit of the murine Na,K-ATPase gene. These mice exhibit motor incoordination at 15 d of age, subsequently tremor and paralysis of extremities, and die at 17-18 d after birth. At these ages, the mutants have enlarged ventricles, degenerating photoreceptor cells, and swelling and degeneration of astrocytic endfeet, leading to vacuoles adjoining capillaries of brain stem, thalamus, striatum, and spinal cord. In tissue homogenates from entire brains of 16-17-d-old mutants, Na,K-ATPase activity and expression of the β1 subunit of the Na,K-ATPase and of the neural adhesion molecules L1, N-CAM, and MAG appear normal. We suggest that the mutant phenotype can be related primarily to reduced pump activity, with neural degeneration as a possible consequence of osmotic imbalance.

The adhesion molecule on glia (AMOG) was first described as a Ca2+-independent murine recognition molecule that mediates neuron-to-astrocyte interaction in vitro and that is functionally involved in the migration of granule cells along Bergmann glial fibers in cerebellar explant cultures (Antonicek et al., 1987). Subsequently, AMOG has been termed AMOG/β2, since sequence analysis revealed AMOG to be a close homologue of the β1 subunit of the Na,K-ATPase (Gloor et al., 1990). A β2 subunit of the Na,K-ATPase was also identified in rats and humans by low stringency hybridization using a β1 subunit probe (Martin-Vasello et al., 1989).

The Na,K-ATPase is a membrane-bound ion pump consisting of one catalytic α (α1, α2 or α3) subunit and one β (β1 or β2) subunit, the function of which has not yet clearly been established. AMOG/β2 is tightly associated with the α subunit of the Na,K-ATPase, since α2, to a lesser extent, also α3 copurifies with AMOG/β2 during stringent immunoaffinity chromatography isolation procedures from adult mouse brain (Gloor et al., 1990). Furthermore, expression of AMOG/β2 in Xenopus laevis oocytes in the presence of endogenous or coexpressed α subunits reconstitutes a functional Na,K-ATPase (Schmalzing et al., 1992). Last, a monoclonal antibody to AMOG/β2 that blocks adhesion, triggers Na,K-ATPase-specific Rb+ uptake of cultured astrocytes by its ability to increase pump activity (Gloor et al., 1990).

AMOG/β2, which carries the oligomannosidic L3 carbohydrate structure (Schmitz et al., 1993) expressed also by other neural recognition molecules (Kücherer et al., 1987; Fahrig et al., 1990; Bollensen and Schachner, 1987; Pesheva et al., 1987; Horstkorte et al., 1993), has also been characterized as a recognition molecule by several operational criteria. Liposomes containing immunoaffinity purified AMOG/β2 bind specifically to cell surfaces of distinct neuronal cell types (Antonicek and Schachner, 1988). Furthermore, elimination of pump activity during the adhesion assay either with ouabain or by reducing the temperature to 4°C does not abolish AMOG/β2-dependent neuron-to-astrocyte adhesion (Gloor et al., 1990). Finally, expression of AMOG/β2, but not that of β1 subunit of the Na,K-ATPase on the cell surface of fibroblasts, enhances neurite outgrowth of cocultured small cerebellar neurons (Müller-Husmann et al., 1993). Monoclonal AMOG/β2 antibodies or recombinantly expressed AMOG/β2 protein inhibit this increase in neurite
outgrowth on AMOG/β2 transfected fibroblasts, but not on nontransfected fibroblasts or on fibroblasts transfected with the β1 subunit (Müller-Husmann et al., 1993).

The characterization of AMOG/β2 as a cell recognition molecule and as a subunit of the Na,K-ATPase introduces a new concept in the link between cell recognition and signal transduction: coupling of cell recognition with ion transport implicates cell–cell interactions in the regulation of the ionic milieu. Concentrations of both Na⁺ and K⁺ determine the transduction: coupling of cell recognition with ion transport implicates cell-cell interactions in the regulation of the ionic symbiosis particularly in cerebellum and hippocampus (Pagliusi et al., 1990), suggests that cell contacts between neurons and astrocytes may be instrumental in regulating the ionic symbiosis between neural cells.

To analyze how the two functions of AMOG/β2 are combined to influence development and maintenance of nervous tissue, we have generated a mouse deficient in the expression of the AMOG/β2 gene (Magyar and Schachner, 1990). Here we show that the null mutation in the AMOG/β2 gene affects the morphological integrity of several, but not all neural cell types which express AMOG/β2. We suggest that most of the histological abnormalities observed shortly before the mutant's death result from alterations of ionic homeostasis.

Materials and Methods

AMOG/β2 Gene Targeting and PCR Mock Construct

A mutation was inserted into the first exon of the AMOG/β2 genomic clone G7SH (Magyar and Schachner, 1990) by subcloning the PvuII fragment of G7SH into the PvuII site of vector pSP72 (Promega, Madison, WI). An oligonucleotide (5′-TATGACTCGAGCTAGCGTAGC-Y′ hybridized with 5′-TACGCTAGCTGATTACG-3′ hybridized with 5′-TACGCTAGCTGATTACG-3′) was inserted into the SacII site of this fragment generating a unique XhoI site and two in frame stop codons. After reintroduction of this mutated PvuII fragment into G7SH, the XhoI-SalI fragment of pIC19R/MC-1-tk (Mansour et al., 1988) containing the HSV-tk cassette was then inserted into the SaclI site of AMOGtk. NotI linearized AMOGtk was used for electroporation of embryonic stem cells. To construct a mock vector for PCR screening, the G7SHneo was linearized with HindIII and used for optimization and control of the PCR reaction.

Growth, Transfection, and Selectin of Embryonic Stem Cells

Culture, transfection with NotI linearized AMOGtk, and double selection of the embryonic stem cell line D3 (Doetschman et al., 1985) was carried out as described (Soriano et al., 1991; Giese et al., 1992).

PCR Screening of Recombinant Clones and Southern Blot Analysis

Picking of G418 and FLA1 (1-2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracyl) resistant clones and lysis of pools were performed as described previously (Soriano et al., 1991). Half of the lysate was amplified in a final volume of 25 μl in 1 × PCR buffer (16.5 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8), 67 mM MgCl₂, 5 mM 2-mercaptoethanol, and 67 μM ethylenediaminetetraacetic acid; Kogan et al., 1987), 1 mM each 2′-deoxy-nucleotide (5′-triphosphate (Pharmacia LKB Biotechnology, Piscataway, NJ), 120 nM primer each, 10% dimethyl sulfoxide, 80 μg/ml BSA and 25 U/ml Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). Amplifications were carried out for 40 cycles of 30 s at 93°C, 30 s at 56°C, and 2 min at 65°C. Primers for the reaction were derived from AMOG/β2 genomic sequences upstream of the targeting vector homologous region (5′-TACATTCGACCCTCTTCTTC-3′) and from the neo cassette (5′-TGC-AAACCACTGTCGCA-3′).

PCR conditions were established from amplifications of dilution series of the PCR construct (G7SHneo) in the presence or absence of 300 ng of Na⁺ and K⁺ genomic DNA per reaction. Under the conditions used, the amplification product of 1 fg template was detectable on an ethidium bromide–stained agarose gel.

For Southern blot analysis, 5–10 μg genomic DNA from the PCR-positive clones or from mouse tail biopsies was digested with BamHI or EcoRI. Obtained fragments were separated on an agarose gel and transferred with 0.4 M NaOH to Hybond N⁺ membrane (Amersham Corp., Arlington Heights, IL). Hybridization probes were labeled by random priming to a specific activity of at least 10⁶ cpm/μg DNA. As probes, the 5′-external probe (416 bp Sty1-SnaBl fragment of the AMOG/β2 gene lying upstream of the construct) or the neo' cassette were used. Hybridization, washing, and stripping conditions were according to the manufacturer's recommendations.

Blasto cyst Injections and Mating of Chimeric Mice

C57BL/6J blastocysts were injected with AMOG/β2-targeted ES cells as described previously (Wang et al., 1991). Male chimeras were mated with C57BL/6J females. The heterozygous offspring were pair-fed to obtain homozygous mice. Genotypes of mice were determined by Southern blot analysis using the 5′-external probe.

Determination of Na,K-ATPase Activity

The Na,K-ATPase activity was determined by the timed test tube assay according to Müller-Husmann et al. (1993).

Antibodies

Production and purification of polyclonal rabbit antibodies to immuno-affinity purified L1 (Rathjen and Schachner, 1984), N-CAM (Kinbauer et al., 1985), MAG (Pylvorak et al., 1987), and to the β1 subunit of the Na,K-ATPase (Schmalzing et al., 1991) have been described. Polyclonal antibodies against L1 and N-CAM were immuno-affinity purified on L1 and N-CAM coupled to Sepharose 4B (Martini and Schachner, 1986). Additionally, polyclonal rabbit AMOG/β2 antibodies (Antonieck et al., 1987), the rat monoclonal AMOG/β2 antibodies 426, 614 (prepared according to Antonieck et al., 1987), and B8 (Schmidt, C., personal communication), the mouse monoclonal antibody gp 50 (Beesley et al., 1987) reacting with AMOG/β2 (Glocer, S., and G. Müller-Husmann, personal communication), and the monoclonal rat antibodiy B8-3 (Gorvel et al., 1984) specific for the β1 subunit of the Na,KATPase were used.

For Western blot analysis, polyclonal and monoclonal antibodies were visualized by horseradish peroxidase–conjugated antibodies to mouse IgG and IgM, to rat IgG and to rabbit IgG (all from Dianova, Hamburg, Germany). For immunocytochemistry, primary antibodies were detected using fluorescein isothiocyanate–conjugated goat anti-rat antibodies (Dianova). Digoxigenin-labeled cRNA probes for in situ hybridization were visualized by alkaline phosphatase–conjugated Fab fragments to digoxigenin (Boehringer Mannheim Corp., Indianapolis, IN).

Western Blot Analysis

Brains from 16 to 18-d-old mice were homogenized with a Dounce homogenizer in homogenization buffer (50 mM NaHPO₄, 100 mM NaCl, 0.2 mM MgCl₂, 0.2 mM CaCl₂, 1 mM spermidine). After sonification, membranes were isolated by centrifugation and washed twice with homogenization buffer (Antonieck et al., 1987). The protein content was determined (according to Bradford, 1976). Proteins (200 μg) were analyzed by Western blotting under nonreducing conditions using the polyclonal antibodies to AMOG/β2 (dilution 1:500), to L1 (dilution 1:2,000), to N-CAM (dilution 1:5,000), to MAG (dilution 1:10,000), and to the β1 subunit of Na,K-ATPase (10 μg/ml) or using the AMOG/β2 monoclonal antibodies 614 (dilution 1:1,000), 426 (dilution 1:500), gp 50 (dilution 1:500), and B8 (dilution 1:500). Horseradish peroxidase–conjugated secondary antibodies (dilution 1:1,000) were detected by ECL (Amersham, Buckinghamshire, GB).
sequence beginning with amino acid 23; Gloor et al., 1990) was inserted into the first exon of the AMOG/\(\beta\)2 gene to generate 2 in frame stop codons and a new and unique Xhol restriction endonuclease site. (A3) Restriction map of the AMOG/\(\beta\)2 gene-targeting construct, AMOGtk, containing homologous sequences of 1 kb on the 5' and 4.7 kb on the 3' side of the neo insertion. Transcriptional orientation of the neo and HSV-tk genes is indicated by arrows. \(N\) represents a cleavage site for NotI originating from the cloning site of the vector. (A4) Observed 5' and L1 replacement event of the AMOG/\(\beta\)2 gene (for nomenclature see Hasty et al., 1991). In such an event, the indicated PCR product will be formed. (B) Southern blot analysis of DNA samples from ES cells (lanes 1, 3, 5, and 7 not targeted; lanes 2, 4, 6, and 8 targeted) and from AMOG\(^{+/+}\) (lane 9), AMOG\(^{+/0}\) (lane 10), and AMOG\(^{+/-}\) (lane 11) mouse tail biopsies. The DNA was digested either with BamHI (lanes 1, 2, 5, 6, and 9-11) or with EcoRI (lanes 3, 4, 7, and 8) and was hybridized either with the 5'-external probe (lanes 1-4 and 9-11) or with the neo probe (lanes 3-8). (C) Western blot analysis of crude membrane fractions of brains from 18-d-old AMOG\(^{+/0}\) (lanes with odd numbers) and AMOG\(^{+/+}\) (lanes with even numbers) mice. The blot was developed with a monoclonal AMOG/\(\beta\)2 antibody (lanes 2 and 3), polyclonal antibodies to the \(\alpha1\) subunit of the Na,K-ATPase (lanes 4 and 5), and with polyclonal antibodies directed against MAG (lanes 7 and 8), L1 (lanes 9 and 10), and N-CAM (lanes 11 and 12). Lanes 1 and 6 are controls in which first antibodies were omitted for lanes 2-3 and 4-5, respectively.

**Light and Electron Microscopy**

For light and electron microscopy, animals were deeply anaesthetized and perfused through the left ventricle with 4% paraformaldehyde and 2% glutaraldehyde in phosphate buffer (pH 7.4). Tissue was removed and postfixed in the same fixative. Eyes were fixed by immersion in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Vibratome sections, 100-200 \(\mu\)m in thickness, were incubated in 1% OsO\(_4\) for 30 min, dehydrated in an ascending series of acetone and embedded in Spurr's epoxy resin. For light microscopy, 1-3-\(\mu\)m thick sections were stained with Toluidine blue and examined with a Zeiss Axiophot. For electron microscopy, ultrathin sections were counterstained with uranyl acetate and lead citrate and examined with a Zeiss EM 10.

**Indirect Light Microscopic Immunocytochemistry**

For indirect light microscopic immunocytochemistry, eyes, optic nerves, and cerebella were removed, embedded in OCT (Miles, Elkhart, IN), and frozen in liquid nitrogen-cooled 2-methylbutane. Cryostat sections, 14 \(\mu\)m, were fixed with 3% formaldehyde in 0.1 M PBS (pH 7.4) for 20 min, dehydrated in an ascending series of aceton and embedded in Spurr's epoxy resin. For light microscopic localization, 1-3-\(\mu\)m thick sections were counterstained with uranyl acetate and lead citrate. Even low levels of mRNA could be unequivocally detected (for comparison see Pagliusi et al., 1990). The 625-bp Apal–SacII fragment of the AMOG/\(\beta\)2 genomic clone G7SH (Magyar and Schachner, 1990), encompassing the region of the first exon of AMOG/\(\beta\)2 situated upstream of the mutation in the AMOG gene, was subcloned into the Apal–SacII site of BluescriptKS+ and BluescriptSK+. These plasmids were linearized with SacII and Apal, respectively, and used for transcription with T3-DNA polymerase to produce digoxigenin-labeled riboprobes (Bartsch et al., 1992a; Dörres et al., 1993).

The 548-bp HindIII–SnaBI fragment of the Na,K-ATPase \(\beta\) subunit cDNA (Gloor, 1989), encoding the COOH-terminal part and 91 bp noncoding sequence of the \(\beta\) subunit, was subcloned in the CMV promoter and used for transcription with T3-DNA polymerase.

**Results**

**Generation of AMOG\(^{+/0}\) Mice**

The AMOG/\(\beta\)2-targeting construct AMOGtk (Fig. 1 A) includes 5.5 kb of homologous sequence and a neo gene expression cassette inserted in the first coding exon of the AMOG/\(\beta\)2 gene in the same transcriptional orientation as AMOG/\(\beta\)2. Since alternative splice sites in the AMOG/\(\beta\)2 gene have not been observed (Magyar and Schachner, 1990; Shyjan et al., 1991), replacement of the endogenous gene by
Figure 2. Immunocytochemical localization of AMOG/β2 (a–c, e) and β1 (f and g) in the cerebellar cortex (a, f, and g), retina (b), and optic nerve (c and e) of 17-d-old AMOG/β2 (a–c, and f) and AMOG/β1 (e and g) littermates using monoclonal antibodies. AMOG/β2 is strongly expressed in the molecular layer (mol) and internal granular layer (igl; a). In the retina, AMOG/β2 immunoreactivity is detectable in the nerve fiber layer (1), the inner (2) and outer (4) plexiform layers, and the inner (3) and outer (5) nuclear layers (b). Strongest immunoreactivity is detectable between the apical border of the outer nuclear layer and the outer segments of photoreceptor cells (asterisk in b). In the myelinated part of the optic nerve, AMOG/β2 immunolabeling is homogeneously distributed (c). Sections from optic nerves of AMOG/β1 animals which were incubated with secondary antibodies only (d) showed a weak background labeling similar to that observed in sections from AMOG/β2 littermates (e) incubated with primary and secondary antibodies. In the cerebellar cortex β1 is homogeneously distributed in the molecular layer (mol) and internal granular layer (igl) with a similar intensity in expression in AMOG/β2 (f) and AMOG/β1 (g) animals. Bars: (a–g) 100 μm.

The gene-targeting construct AMOGtk should result in a null mutation.

Linearized AMOGtk was electroporated into D3 embryonic stem cells, and seven potential recombinants were identified by PCR among 184 clones resistant to both G418 and FIAU. Targeted integration into the AMOG/β2 gene (Fig. 1 A) was verified by Southern blot analysis using a hybridization probe located 5' upstream to the targeting construct (Fig. 1 B; lanes 1–4). The endogenous 8.4-kb BamHI and 13.9-kb EcoRI fragments were found to be reduced to 2.9 and 3.4 kb, respectively, as expected for homologous recombination of the short 5'-arm of AMOGtk. Using the neo cassette as a hybridization probe, the EcoRI digested DNA gave bands of 3.4 and 11.5 kb (Fig. 1 B; lanes 7 and 8) indicating that homologous recombination of both arms of AMOGtk had occurred. The single band of 2.9 kb observed in the BamHI digested DNA using the neo probe (Fig. 1 B; lanes 5 and 6) verified the absence of non-homologous recombination events. All seven recombinants identified by PCR showed only one homologous recombination event.

Three AMOG/β2 targeted embryonic stem cell clones were injected into C57BL/6J blastocysts and all clones gave germline transmitting male chimeras with a high degree of chimerism. Heterozygous mice showed no obvious abnormal behavioral phenotype. Approximately 25% of the offspring (N = 52) from crosses between heterozygous mice were homozygous for the mutation as identified by Southern blot analysis (Fig. 1 B; lanes 9–11), indicating that AMOG/β2 mice did not die during embryonic development.

To confirm the generation of a null allele, we analyzed proteins from brains of AMOG/β1 mice by immunoblotting (Fig. 1 C). Using either polyclonal or different monoclonal antibodies, no AMOG/β2 protein could be detected in crude membrane preparations of brains of AMOG/β1 mice (Fig. 1 C; lane 1), whereas AMOG/β2 was easily detected in AMOG/β2 mice (Fig. 1 C, lane 2) even at 100-fold less protein concentration (not shown). Immunocytochemical analysis of sections from different regions of the central nervous system of AMOG/β2 mice using the two monoclonal AMOG/β2 antibodies 614 and 426 showed no immunoreactivity in comparison to AMOG/β1 mice (Fig. 2, e, and not shown). In addition, in situ hybridization experiments were performed on tissue sections from AMOG/β1 and AMOG/β2 mice using an AMOG/β2 specific cRNA probe hybridizing with the region of the first exon of AMOG/β2 located upstream of the
in situ hybridization analysis of the localization of AMOG/β2 (c, e, and f) and β1 (a, b, and d) mRNA in the cerebellar cortex (a–e) and retina and optic nerve (d–f) of 17-d-old AMOG+/+ (a, c–e) and AMOG−/− (b and f) littermates. In the cerebellar cortex of AMOG+/+ (a) and AMOG−/− (b) animals, β1 mRNA is present in Golgi and granule cells of the internal granular layer (igl), large-sized Purkinje cells of the Purkinje cell layer (pcl), and stellate and basket cells of the molecular layer (mol). AMOG/β2 mRNA is detectable in granule cells and astrocytes located in the internal granular layer and small-sized Bergmann glial cell bodies located in the region of the Purkinje cell layer (c). β1 mRNA is not visible in the optic nerve (on), but is present in retinal ganglion cells (f) and cells located in the inner nuclear layer (d). AMOG/β2 mRNA is weakly expressed by retinal ganglion cells and cells of the inner nuclear layer and strongly by photoreceptor cells (arrowheads in e). In the optic nerve, AMOG/β2 is present in glial cells located throughout the nerve (e). Using the AMOG/β2 cRNA anti-sense probe, no hybridization signal was detectable in the optic nerve or retina of AMOG−/− mice (f). The pigment epithelium is labeled by white asterisks. Bars: (a–f) 100 μm.

Behavioral Phenotype of AMOG−/− Mice

No overt behavioral phenotype could be observed until the mice were 15-d-old. At this time, AMOG−/− mice showed reduced righting behavior and orientation when lifted by the tail and dropped gently into the cage. While wild-type or heterozygous mice landed on their feet and immediately moved around, AMOG−/− mice fell onto their side and paused for a few seconds before they stood up on their feet and started moving. This motor incoordination rapidly worsened within a few days. AMOG−/− mice appeared to develop paralysis in their forelimbs and were unable to hold their heads in a normal position. The hind limbs started to become tremorous and animals were no longer able to remain in an upright position. AMOG/β2 deficient mice opened their eyes at the correct age, but most of them kept them closed when the abnormal behavior became apparent. Mutant animals lying on their side still showed normal grasping reflexes and responded to sound. AMOG−/− mice died at postnatal days 17 or 18, 2–3-d after the first appearance of the subtle abnormal behavioral phenotype. However, as the motor incoordination increased, animals were no longer able to feed and drink. Efforts to keep the animals viable by artificial feeding failed.

Na,K-ATPase Activity

The Na,K-ATPase pump activity was determined in homogenates of brains of 16-17-d-old animals. Na,K-ATPase activities were not significantly different between AMOG−/− mice (1.67 ± 0.19 μmol/mg/h; n = 4) and wild-type mice (1.64 ± 0.17 μmol/mg/h; n = 4) (p = 0.83; according to Student's unpaired two-tailed t test).

Expression of the β Subunits of Na,K-ATPase

To characterize the expression of the β subunits at the protein level, we performed Western blot analysis of crude brain membrane preparations of 17–18-d-old homozygous AMOG-deficient, heterozygous, and wild-type mice (Fig. 1 C). Different mono- and polyclonal AMOG antibodies revealed that wild-type (Fig. 1 C; lane 2) and heterozygous (not shown) mice expressed AMOG at similar levels. In contrast, AMOG/β2 was not detectable in AMOG−/− mice (Fig. 1 C; lane 3). Expression levels of the β1 subunit were identical in AMOG−/− and AMOG+/+ littermates (Fig. 1 C, lanes 4 and 5).
Expression of the Recognition Molecules N-CAM, L1, and MAG

The expression levels of N-CAM, L1, or MAG were similar in AMOG°°°, AMOG°°+ (not shown), and AMOG°+/+ littermates as determined by Western blot analysis of crude membrane preparations of 17-18-d-old brains (Fig. 1 C; lanes 7-12).

Analysis of AMOG°°° Mice by Light and Electron Microscopy, Immunocytochemistry, and In Situ Hybridization

At the light microscope level, the lateral and third ventricles of AMOG°°° mice (Fig. 4 b) were significantly enlarged when compared to wild-type (Fig. 4 a) or heterozygous (not shown) littermates.

In the brain stem of AMOG°°° mice, swollen cellular processes and vacuoles were detectable, usually closely associated with blood vessels (Fig. 4, d-f). Swollen cellular processes and vacuoles were not detectable in corresponding regions of the brain stem of wild-type (Fig. 4 c) or heterozygous (not shown) littermates. For a more detailed analysis, brain stems of AMOG mutants were studied at the ultrastructural level. Confirming the light microscopic observations, swollen cellular processes (Fig. 5, a and c) and electron-lucent vacuoles (Fig. 5, b and c) were found in direct association with blood vessels. The close association between degenerating cellular processes and blood vessels suggests that degenerating cells represent astrocytes. The electron-lucent vacuoles thus most likely result from swelling and subsequent degeneration of astrocytic processes. Similar vacuolated structures were visible in the thalamus, striatum, and spinal cord (not shown).

Cerebellum. The cytoarchitecture of the cerebellar cortex of AMOG°°° (Fig. 6 b) mice appeared normal and the thickness of different cortical layers were similar to that of AMOG°°+ (Fig. 6 a) littermates. In particular, the thickness of the internal and external granular layers of AMOG°°° animals and AMOG°+/+ littermates were similar, indicating that granule cell migration is not disturbed in the absence of AMOG/β2. In contrast to the brain stem, blood vessels of the cerebellar cortex were not associated with degenerating cellular elements. However, some degenerating Purkinje cells and some small-sized degenerating cells in the internal granular layer were present in AMOG°°° mice (not shown). In AMOG°+/+ mice, AMOG/β2 immunoreactivity was detectable in all cortical layers of the cerebellum (Fig. 2 a). Strong immunoreactivity was found in the internal granular layer. The molecular layer was homogeneously labeled, suggesting that the cell surfaces of parallel fibers are im-

Figure 4. Light microscopic analysis of the ventricles (a and b) and brainstem (c-f) of AMOG°°° (b, d-f) and AMOG°°+ (a and c) littermates. The lateral ventricles and the third ventricle of AMOG°°° mice (b) are significantly enlarged when compared to AMOG°°+ littermates (a). In the brainstem of AMOG°°° (d-f) mice, swollen cellular structures (d, arrowheads in e) or vacuoles (d, arrowheads in f) are visible, usually in close association with blood vessels (bv; arrows in d). These morphological abnormalities were not detectable in corresponding regions of the brainstem of AMOG°°+ animals (c). Bars: (a and b) 2.5 mm; (c and d) 50 μm; (e and f) 20 μm.
Figure 5. Electron microscopic analysis of the brainstem of 18-d-old AMOG°°° mice. Some blood vessels (bv) are surrounded by swollen and electron-lucent cellular processes (filled asterisks in a and c). In addition, blood vessels are also associated with electron-lucent intracellular vacuoles (open asterisks in b and c). Bars: (a-c) 4 μm.

Figure 6. Histological analysis of the cerebellar cortex (a and b) and optic nerve (c–e) of 17-d-old AMOG°°° (a and c) and AMOG°°° (b, d, and e) littermates. The cerebellar cortex of AMOG°°° (a) and AMOG°°° (b) animals shows a similar cytoarchitecture, with a comparable thickness of the different cortical layers. The external granular layer of both animals has almost disappeared at this developmental age. Similarly, the optic nerve of AMOG°°° animals (d) shows no morphological abnormalities when compared with AMOG°°° littermates (c). Ultrastructurally, myelinated retinal ganglion cell axons (a) and astrocytes (as) and their processes abutting optic nerve blood vessels (bv) appear morphologically unaffected in AMOG°°° animals (e). Bars: (a–d) 100 μm; (e) 2 μm.
munoreactive (Fig. 2 a). In situ hybridization analysis showed AMOG/β2 transcripts in cells located in the white matter (not shown). The internal granular layer was homogeneously labeled with the AMOG/β2 cRNA probe (Fig. 3 c), indicating that granule cells synthesize the molecule. Some cells, scattered throughout the internal granular layer, showed an increased labeling intensity. The number and distribution of these intensely labeled cells suggest that they correspond to astrocytes. AMOG/β2 mRNA was also detectable in the region of the Purkinje cell layer (Fig. 3 c). An analysis of these sections at higher magnification or of sections counterstained with methylene blue (not shown) revealed that the labeled cells corresponded to Bergmann glial cells. Purkinje cells, in contrast, contained no detectable levels of AMOG/β2 mRNA and no AMOG/β2 transcripts were detectable in the molecular layer (Fig. 3 c). β1 immunoreactivity was homogenously distributed in the molecular layer and internal granular layer of AMOG<sup>−/−</sup> (Fig. 2 f) and AMOG<sup>+/+</sup> (Fig. 2 g) animals. No differences in the intensity of immunolabeling were detectable (compare Fig. 2, f and g). Cells containing β1 mRNA were visible in the internal granular layer, Purkinje cell layer, and molecular layer (Fig. 3, a and b). As judged from the position and size of the cell bodies, β1-positive cells corresponded to granule and Golgi cells in the internal granular layer, Purkinje cells in the Purkinje cell layer, and stellate and basket cells in the
molecular layer. The intensity of the hybridization signal was similar in AMOG+/÷ and AMOG0/0 animals (compare Fig. 3, a and b).

Optic nerve. Light microscopic analysis of optic nerves of AMOG0/0 (Fig. 6 d) mice revealed no detectable histological changes in comparison to AMOG+/÷ (Fig. 6 c) littermates. No signs of degeneration were visible in the vicinity of blood vessels (Fig. 6, d and e). Compacted myelin, retinal ganglion cell axons, and astrocytic endfeet abutting onto blood vessels (Fig. 6 e) or forming the glial limiting membrane at the outer surface of the nerve (not shown) appeared ultrastructurally normal in AMOG0/0 mice. In AMOG+/÷ mice, strong AMOG/B2-immunoreactivity was found in the distal myelinated part of the optic nerve (Fig. 2 c). In the proximal unmyelinated part of the nerve, AMOG/β2-immunoreactivity was homogeneously distributed (not shown), suggesting that along with optic nerve glial cells, retinal ganglion cell axons are AMOG/β-positive. By in situ hybridization, a subpopulation of glial cells was labeled by the AMOG/β2 cRNA probe in AMOG+/÷ animals (Fig. 3 e). Since labeled cells were not restricted to the myelinated distal part, but were also detectable in the unmyelinated retinal end of the optic nerve, these cells most likely represent astrocytes. Sections from AMOG0/0 animals incubated with AMOG/β2 antibodies (Fig. 2 e) showed a weak background labeling, similar to that of AMOG+/÷ mice incubated with secondary antibodies only (Fig. 2 d). Similarly, sections from AMOG0/0 mice hybridized with the AMOG/β2 anti-sense cRNA probe (Fig. 3 f) showed only weak background labeling comparable to that of AMOG+/÷ animals hybridized with the AMOG/β2 sense cRNA probe (not shown). Hybridization with the β1 cRNA probe showed no signal in the optic nerve (Fig. 3 d).

Retina. In AMOG0/0 mice, the outer nuclear layer appeared to be reduced in thickness and contained numerous degenerating cells (Fig. 7 b). Moreover, the lengths of inner and outer segments of photoreceptor cells (Fig. 7, b and d) were significantly reduced when compared to that of AMOG+/÷ littermates (Fig. 7, a and c). Furthermore, degenerating photoreceptor cell bodies were observed at the ultrastructural level (Fig. 7 d). In contrast to the brain stem, retinal blood vessels were not associated with degenerating cellular elements. In the retinae of AMOG+/÷ animals, the nuclear layers, plexiform layers, and the nerve fiber layer were AMOG/β2-immunoreactive (Fig. 2 b). Strongest immunoreactivity was visible in association with the inner segments of photoreceptor cells (Fig. 2 b). AMOG/β2 transcripts were found in retinal ganglion cells, cells of the inner nuclear layer, and in photoreceptor cells (Fig. 3 e). β1 mRNA was detectable in retinal ganglion cells and a subpopulation of cells located in the inner nuclear layer, but not in photoreceptor cells (Fig. 3 d).

Discussion

We have generated mice deficient for the adhesion molecule on glia (AMOG/β2 subunit of Na,K-ATPase) by targeted disruption of its gene in embryonic stem cells. The position of the AMOG0/0 mutation within the first exon generates a null allele as verified by immunochecmic, immunocytochemical, and in situ hybridization experiments. AMOG0/0 mice exhibit a characteristic behavioral phenotype that becomes apparent at postnatal day 15 by abnormalities in motor coordination, tremors, and paralysis with extremely rapid progression of symptoms leading to death at days 17-18. Mice heterozygous for the mutation appear unaffected.

In view of the abnormal phenotype of the mutant, it is worthwhile to review the expression of AMOG/β2 as a function of developmental stage and cell type in AMOG+/÷ mice. AMOG/β2 is hardly detectable outside the central nervous system (Antonicek et al., 1987; Antonicek and Schachner, 1988; Glooor et al., 1990; Martin-Vasallo et al., 1989; Pagliusi et al., 1990). The molecule is mainly expressed by glial cells, although expression by certain types of neurons could never be rigorously excluded and has, in fact, recently been demonstrated (Antonicek et al., 1987; Pagliusi et al., 1990; Schneider et al., 1991; this study). Its expression is first detectable in the brain at late fetal stages, increases during the first two postnatal weeks, and reaches highest levels in the adult (Pagliusi et al., 1990).

Histological analysis of AMOG0/0 mice shortly after the onset of behavioral abnormalities reveals that several brain regions display a normal histological phenotype at the light microscopic level. For example, the cytoarchitecture of the cerebellar and cerebral cortices and hippocampus (unpublished observations) appears to be normal. The question thus arises whether AMOG/β2 plays a profound morphogenetic role during formation of these brain regions. In difference to the apparently normal differentiation of the cerebellar cortex of AMOG0/0 mice, AMOG/β2 antibodies have been demonstrated to interfere with granule cell migration in vitro (Antonicek et al., 1987). This discrepancy might result from the fact that other recognition molecules known to be functionally involved in migration of granule cells, such as the neural recognition molecules tenacin (Chuong et al., 1987; Husmann et al., 1992), thrombospondin (O'Shea et al., 1990), L1 (Lindner et al., 1983), and possibly others, are able to compensate for the lack of AMOG/β2 expression in the mutant. It is noteworthy in this respect that the expression of the recognition molecules L1, N-CAM, and myelin-associated glycoprotein was found to be normal in the AMOG mutant. To resolve whether AMOG/β2 plays a subtle role in morphogenetic cell interactions, the differentiation, and, in particular, the migration of granule cells will have to be analyzed systematically in a developmental time sequence.

We suggest that the abnormal histological phenotype observed in some brain regions of the mutant can be explained on the basis of altered Na+ pump activity, as AMOG/β2 is an integral component of the Na,K-ATPase. For instance, degeneration of some neural cells in the AMOG mutant could be due to the failure of pump activity, being in particular demand in highly active cells, such as the photoreceptor cells, the primary sensory neurons of the retina. The swelling and consecutive disintegration of astrocytic endfeet, particularly near capillaries, is another indication of the failure in pump activity at locations requiring tight regulation of ionic homeostasis. The resulting osmoregulatory imbalance may lead to spongiform encephalopathy which is characterized by intracellular vacuoles in the brain tissue as observed in other neurodegenerative disorders of various etiologies (Eisiri and Kennedy, 1992; Kamin and Petito, 1991; Prusiner, 1991). Attempts to directly relate the histological
phenotype of the AMOG/β2 deficient mice to a decreased pump activity failed, since we did not detect a significant decrease of Na,K-ATPase activity in tissue homogenates of brains of AMOG-deficient mice. This finding may result from the fact that tissue from the entire brain was used for this analysis. However, only circumscribed small areas of the brain are morphologically affected in the mutant. Furthermore, although exact values of Na,K-ATPase activity are available that relate to the relative abundance of the individual α subunits in the brain at the protein level, Northern blot analysis and our own in situ hybridization experiments (Molthagen, M., and U. Bartsch, unpublished observations) show that at the mRNA level, the α3 subunit is the most abundant α subunit in differentiated rodent brain tissue. Since the α3 subunit predominantly associates with β1, it is likely that a possible decrease in ATPase activity contributed by the complex between AMOG/β2 and α2 would not have been detectable due to the relative abundance of the complex between β1 and α3. The present observations, therefore, do not yield conclusive interpretations as to the correlation between pump activity and morphologically detectable degeneration of neural cell types.

The spongiform status of the AMOG/β2 mutant is confined to the brain stem, thalamus and striatum, and, to a lesser degree, to the spinal cord, but is not observed in cerebellum and hippocampus, where AMOG/β2 is most prominently expressed (Pagliusi et al., 1990). It is presently not known why some brain regions show spongiform abnormalities while others do not. However, it is conceivable that compensatory mechanisms at the individual cell level have to be considered. This possibility is supported by the observation that only some, but not all cells that express AMOG/β2 in the wild-type degenerate in the mutant. The two β subunits presently known are both able to interact with more than one α subunit to yield a fully functional Na+ pump (Schmalzing et al., 1992). It is therefore possible that the few cells expressing both β subunits, such as the cerebellar granule cells or retinal ganglion cells, are able to survive in the AMOG/β2 mutant, because the β1 subunit may take over at least some of the functions of AMOG/β2. However, some cell types, such as glial cells in the optic nerve, express only AMOG/β2 in the wild-type animal but are also unaffected in the mutant. These cells may express a yet unidentified β subunit and thus a functionally active Na+ pump. Alternatively, the β1 subunit may be present in these cell types at earlier developmental ages and might then be substituted by AMOG/β2 during the third postnatal week. Evidence for a developmental switch in the expression of the β subunits, i.e., downregulation of the β1 subunit and concomitant upregulation of AMOG/β2 in several neural cell types has recently been obtained (Molthagen, M., and U. Bartsch, unpublished observations). According to these observations, the time of degeneration of cells in the AMOG/β2 mutant may correspond to the time of the switch in expression of β subunits: neural cell types in which AMOG/β2 substitutes β1 early during development are morphologically affected, whereas cells in which AMOG/β2 substitutes β1 late in development appear morphologically unaffected at the time of the mutant's death.

A failure in the ionic homeostasis similar to that of the AMOG/β2 mutant has been observed in the mec-4 mutant of Caenorhabditis elegans (Driscoll and Chalfie, 1991). The mec-4 gene product is related to the Na+ channel of intestinal epithelia in mammals (Canessa et al., 1993). Neurons in the mec-4 mutant swell and reach several times their normal diameter before degeneration. The cellular defects of the mec-4 mutation are probably due to an impaired regulation of the ion channel resulting in an enhanced entry of Na+ and water into the cell. These observations are in agreement with our interpretation of the abnormal phenotype of the AMOG/β2 mutant.

Finally, the question remains as to the cause of the mutant's death. We can presently not exclude that other organs in which AMOG/β2 is negligibly expressed may be involved. However, we presently consider it more likely that the animal's death is due to the dysfunction of vitally important brain structures, such as the brain stem and spinal cord. Morphological analysis of other organs and physiological analysis of the cellular functions of neurons and glia in vitro and in vivo should clarify these questions.

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