Regulation of gene expression in experimental autoimmune encephalomyelitis indicates early neuronal dysfunction

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Summary
Multiple sclerosis is an inflammatory, demyelinating disease of the CNS. Whereas oligodendrocytes have been considered the primary neural cell type most affected, recent evidence indicates that axonal and neuronal degeneration also occurs in both multiple sclerosis and experimental autoimmune encephalomyelitis (EAE), an animal model reproducing many features of multiple sclerosis. The molecular mechanisms underlying neuronal deficits in multiple sclerosis and EAE remain elusive. To address this issue, we have analysed the expression of genes encoding proteins that play critical roles in ion homeostasis, exocytosis, mitochondrial function and impulse conduction in the Lewis rat lumbar spinal cord during the clinical course of acute EAE. Transcript and protein levels of plasma membrane Ca²⁺ ATPase 2 (PMCA2), an essential ion pump expressed exclusively in grey matter and involved in Ca²⁺ extrusion, synapsin IIa and syntaxin 1B, important regulators of vesicular exocytosis, were dramatically decreased coincident with the onset of clinical symptoms. In contrast, changes in the expression of several other ion pumps, vesicular proteins, mitochondrial enzymes and sodium channels occurred at more advanced disease stages. Moreover, exposure of spinal cord slice cultures to kainic acid significantly reduced PMCA2 mRNA levels. Taken together, our findings suggest that glutamate, which recently has been implicated in EAE pathogenesis, suppresses neuronal PMCA2 expression leading to Ca²⁺ dyshomeostasis at initial clinical phases. Consequently, perturbations in Ca²⁺ balance and neurotransmitter exocytosis may partially underlie aberrant neuronal function and communication at onset of symptoms. Altered mitochondrial function and impulse conduction may exacerbate neurological deficits at subsequent disease stages.

Keywords: EAE; exitotoxicity; synaptic protein; neuronal injury; ATP2B2

Abbreviations: DIG = digoxigenin; EAE = experimental autoimmune encephalomyelitis; PMCA2 = plasma membrane Ca²⁺ ATPase 2; SERCA2 = sarcoplasmic and endoplasmic reticulum Ca²⁺-activated ATPase 2; SNAP-25 = synaptosome-associated protein of 25 kDa

Introduction
Multiple sclerosis is a CNS disease leading to progressive neurological deficits and permanent disability (Steinman, 1996; Prineas and McDonald, 1997; Antel, 1999; Hickey, 1999). Experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis, is induced by immunization of rodents with immunogenic myelin components or by adoptive transfer with T cells reactive against CNS myelin antigens (Wekerle et al., 1994; Steinman, 1999; van der Goes and Dijkstra, 2001). The clinical symptoms and histopathology of EAE may be diverse depending on the antigen and the animal strain employed. It has been hypothesized that distinct EAE models reproduce different features of multiple sclerosis, a complex disease with variable clinical course (Wekerle et al., 1994). Immunization of adult Lewis rats with myelin basic protein results in acute, monophasic EAE manifested by progressive
ascending weakness leading to hindlimb paralysis and quadriplegia.

Classically, demyelination of structurally intact axons and the resulting impairment in saltatory nerve conduction have been considered as the causes of functional deficits in multiple sclerosis and EAE (McFarlin and McFarland, 1982a, b; Raine, 1997). However, the lack of correlation between myelin loss and symptom severity suggests that EAE and multiple sclerosis are more complex than initially believed (Rivera-Quinones et al., 1998; Antel, 1999). Indeed, studies reporting axonal dysfunction and damage (Ferguson et al., 1997; Trapp et al., 1998, 1999; De Stefano et al., 1999; Bitsch et al., 2000; Bjartmar and Trapp, 2001; Bjartmar et al., 2001), neuronal loss or apoptosis (Smith et al., 2000; Peterson et al., 2001) indicate that multiple sclerosis and EAE are not only demyelinating diseases but neuronal disorders as well (Waxman, 2000a, b; Filippi, 2001).

The triggers that cause aberrant neuronal axonal function and destruction of myelin are not well defined. A correlation between inflammatory reaction and axonal injury has been observed (Ferguson et al., 1997; Trapp et al., 1998). Activated resident microglia and macrophages have also been implicated in myelin and oligodendrocyte damage (for a review see Sriram and Rodriguez, 1997). Recent studies indicate that glutamate excitotoxicity may be a factor contributing to axonal, neuronal and oligodendrocyte injury during EAE (Pitt et al., 2000; Smith et al., 2000). Moreover, it has been suggested that increased calcium influx through voltage-dependent calcium channels induces axonal degeneration and neuronal injury (Kornek et al., 2000, 2001). Calcium dyshomeostasis, which has been implicated in many pathological conditions, may also result from the abnormal function of pumps that extrude Ca\textsuperscript{2+} from cells. This possibility has not been investigated in EAE or multiple sclerosis.

In summary, in contrast to the well-defined histopathology, the molecular events underlying neuronal impairment contributing to neurological deficits and persistent disability in EAE and multiple sclerosis remain elusive. To delineate molecular mechanisms responsible for the onset and progression of symptoms in EAE, we defined the expression pattern of genes encoding proteins that play essential roles in neuronal function during the clinical course of the disease. Our findings suggest that abnormal Ca\textsuperscript{2+} extrusion and vesicular exocytosis may initiate neuronal dysfunction. At later clinical stages, changes in the expression of other genes that encode proteins involved in mitochondrial function and impulse conduction may contribute further to disease progression.

Material and methods

Induction of EAE

Female adult Lewis rats (200–250 g body weight) were immunized in hindlimb footpads with 100 μg of guinea pig myelin basic protein (Sigma, St Louis, MO, USA, or generously provided by Dr A. Ben-Nun) in 50 μl of saline emulsified in 50 μl complete Freund’s adjuvant (Difco, Detroit, MI, USA). Control rats received only saline/adjuvant. Animals were monitored daily for clinical symptoms. A very stereotyped clinical onset and disease course was observed. Neurological impairment was scored as: 0, no neurological symptoms; 1, limp tail [stage E1, observed on day 10 post-injection (PI)]; 2, hindlimb weakness (stage E2, day 11 PI); 3, hindlimb paralysis (stage E3, day 12–13 PI); 4, quadriplegia (stage E4; day 14–15 PI); 5, moribund (past day 16 PI). During the observation period, rats were given food and water ad libitum. Some animals were sacrificed by exposure to CO\textsubscript{2}/O\textsubscript{2}. Others were anaesthetized with a mixture of ketamine/xylazine and perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.5. Lumbar spinal cords were dissected out and frozen immediately on dry ice. The tissue was kept at −80°C until further use. All animal procedures were performed according to IACUC and institutional guidelines.

Isolation of RNA

Total RNA was isolated from 100–150 mg of tissue obtained by pooling 2–3 lumbar spinal cords. The Trizol One Step Isolation method was utilized according to the manufacturer’s instructions (Life Technologies, Grand Island, NY, USA). All RNA samples were treated with DNase I to remove any trace of contaminating genomic DNA and repurified by phenol/chloroform extraction. RNA was quantified by measurement of optical density. Samples were stored at −20°C in the presence of RNase inhibitor.

cDNA microarray analysis

This was performed utilizing the Atlas Rat 1.2 microarrays according to the manufacturer’s instructions (Clontech, Palo Alto, CA, USA). The microarray includes 1176 rat cDNAs immobilized on nylon membranes, nine housekeeping cDNAs and negative controls. The list of the cDNAs can be found at the Clontech web site (http://www.clontech.com/\atlas/genelists/7854-1_Ra12.txt). Total RNA was purified from lumbar spinal cords of control and EAE rats (three rats per group; EAE clinical score 3, E3). A 25 μg aliquot of pooled RNA was reverse transcribed in the presence of MMLV reverse transcriptase, gene-specific primer mix, 0.5 mM each of dCTP, dGTP and dTTP, and 35 μCi of [α-\textsuperscript{33}P]dATP (2500 Ci/mmol; Amersham, Piscataway, NJ, USA) in a 30 μl reaction volume at 50°C for 25 min using the Atlas pure total RNA labelling system (Clontech). The arrays were pre-hybridized in Express Hyb hybridization buffer (Atlas hybridization kit) containing heat-denatured salmon testes DNA (Sigma) for 30 min at 68°C. Subsequently, the probe mixture (2–10 × 10\textsuperscript{6} c.p.m.) and 5 μl of human Cot-1DNA were added to the pre-hybridization buffer. Hybridization was performed overnight at 68°C. The arrays were then washed and hybridization was determined with a phosphorimager ( carnagezeichner.de).
Table 1 Differentially expressed genes in the lumbar spinal cord during EAE

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression changes</th>
<th>Ratio of spot intensity (E/C)</th>
<th>GeneBank accession No.</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane Ca²⁺ ATPase 2</td>
<td>Decrease</td>
<td>0.10</td>
<td>J03754</td>
<td>Ion homeostasis</td>
</tr>
<tr>
<td>Na⁺/K⁺ transporting ATPase β2 subunit</td>
<td>Decrease</td>
<td>&lt;0.10</td>
<td>J04629</td>
<td>Ion homeostasis</td>
</tr>
<tr>
<td>SERCA2</td>
<td>Decrease</td>
<td>0.50</td>
<td>J04022</td>
<td>Ion homeostasis</td>
</tr>
<tr>
<td>Synapsin IIa</td>
<td>Decrease</td>
<td>0.15</td>
<td>M27925</td>
<td>Vesicular function</td>
</tr>
<tr>
<td>Syntaxin 1B</td>
<td>Decrease</td>
<td>0.40</td>
<td>M95735</td>
<td>Vesicular function</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Decrease</td>
<td>0.50</td>
<td>AB003991</td>
<td>Vesicular function</td>
</tr>
<tr>
<td>Rab12</td>
<td>Increase</td>
<td>6.30</td>
<td>M83676</td>
<td>Vesicular function</td>
</tr>
<tr>
<td>Rab14</td>
<td>Increase</td>
<td>8.30</td>
<td>M83680</td>
<td>Vesicular function</td>
</tr>
<tr>
<td>Rab16</td>
<td>Increase</td>
<td>3.30</td>
<td>M83681</td>
<td>Vesicular function</td>
</tr>
<tr>
<td>Cytochrome c oxidase subunit Vb</td>
<td>Increase</td>
<td>13.0</td>
<td>D10952</td>
<td>Mitochondrial function</td>
</tr>
<tr>
<td>Mitochondrial cytochrome c subunit IV</td>
<td>Increase</td>
<td>4.80</td>
<td>X14209</td>
<td>Mitochondrial function</td>
</tr>
<tr>
<td>Na⁺ channel β1 subunit</td>
<td>Decrease</td>
<td>&lt;0.10</td>
<td>M91808</td>
<td>Impulse conduction</td>
</tr>
<tr>
<td>Na⁺ channel β2 subunit</td>
<td>Decrease</td>
<td>0.30</td>
<td>U37026</td>
<td>Impulse conduction</td>
</tr>
<tr>
<td>Na⁺ channel II</td>
<td>Decrease</td>
<td>0.30</td>
<td>X03639</td>
<td>Impulse conduction</td>
</tr>
</tbody>
</table>

cDNA microarray analysis identified differentially expressed genes in the spinal cord during EAE, including those listed above which showed robust changes in transcript levels (at least 2-fold difference between EAE and control) and which were highly expressed (at least 30- to 40-fold over background). The genes are classified according to their best characterized function. The results obtained with all the listed genes were corroborated subsequently by RT–PCR in 2–3 independent experiments. The ratio of spot intensity reflects the relative transcript levels in EAE over control.

were then washed four times in 2× SSC (standard saline citrate), 1% SDS, and once in 0.1× SSC, 0.5% SDS for 30 min, each wash at 68°C, and exposed to phosphorimager screens for 5 days. A Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA) and ImageQuant software were used to scan images. Gene expression was custom analysed using Atlas Image 2.0 software by Clontech. The intensity of each spot, quantified after background correction, reflected the level of expression of each gene. Experimental variation in the overall intensity of the signal was corrected by normalization of the gene signal with respect to the housekeeping genes in the same array. For each gene, a ratio was generated by dividing the intensity of the spot by the average of the sum of housekeeping genes. Only those housekeeping genes with signal intensity below saturation were considered for normalization. Reproducibility of this technique (80% concordance of signals) was verified by hybridizing probe mixtures generated from two aliquots of the same RNA source to identical arrays. The signal levels of genes selected for further analysis in our study were 30- to 40-fold over background. The genes are classified according to their best characterized function. The results obtained with all the listed genes were corroborated subsequently by RT–PCR in 2–3 independent experiments. The ratio of spot intensity reflects the relative transcript levels in EAE over control (Table 1).

**Preparation of riboprobes for in situ hybridization**

RT–PCR products generated by use of specific primers for PMCA2 or α-tubulin were cloned into pCR II-TOPO vector containing SP6 and T7 promoters (Invitrogen, Carlsbad, CA, USA) and sequenced for further confirmation. Antisense and sense riboprobes were generated using the Digoxigenin (DIG) RNA Labelling Kit according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN, USA). A 1 μg aliquot of linearized plasmid was transcribed at 37°C for 2 h in the presence of 1 mM ATP, CTP and GTP, 0.65 mM UTP, 0.35 mM DIG-UTP, 20 U of RNASin, 40 U of SP6 or T7 RNA polymerase in 40 mM Tris buffer pH 8.0, 10 mM NaCl, 10 mM dithiothreitol and 6 mM MgCl₂. The DNA template was removed by treatment with DNase I and the probe was precipitated with LiCl and ethanol. The concentration of DIG-labelled riboprobe was determined by utilizing DIG quantification test strips according to the manufacturer’s instructions (Boehringer Mannheim).

**RT–PCR**

This was performed utilizing the Retroscript reverse transcription kit and SuperTaq Polymerase (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. A 1–2 μg aliquot of DNase-treated total RNA was reverse transcribed in a total volume of 20 μl. A 2–5 μl aliquot of RT mix was then used for PCR, which was performed in a total volume of 50 μl by denaturation at 94°C for 30 s, annealing at 55°C for 45 s and polymerization at 68°C for 1 min. PCR was completed by 10 min extension at 72°C. PCRs were performed for 30–35 cycles, within the linear range. Products were separated on a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and the image was captured using an Alpha digital imaging system. The optical density in each band was then quantified utilizing the Un-Scan-It software (Silk Scientific, Orem, UT, USA). Some PCR products were analysed further by sequencing.
In situ hybridization
Controls and EAE (E3) rats (six animals/group) were sacrificed by cardiac perfusion of saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.5. Lumbar spinal cords were dissected out, post-fixed in phosphate buffer for 4 h, cryoprotected in 10 and 15% sucrose and cryosectioned at 10 μm. Sections obtained from control and EAE lumbar spinal cords were mounted on the same slides to minimize experimental variations, fixed in 4% paraformaldehyde/phosphate buffer for 15 min at room temperature, and treated with 6% H2O2 in phosphate-buffered saline (PBS) containing 0.1% Tween (PBST). Sections were then digested with proteinase K (1 μg/ml) for 15 min at 37°C, followed by incubations in glycine (2 mg/ml, 10 min) and 4% paraformaldehyde/0.2% gluteraldehyde/PBST for 15 min. Prehybridization was performed in buffer containing 50% deionized formamide, 5× SSC pH 4.5, 1% SDS, 50 μg/ml yeast tRNA and 50 μg/ml heparin at 65°C for 1 h. Hybridization was performed overnight at 65°C in the same buffer containing 1 μg/ml DIG-labelled sense or antisense probe. The sections were then washed in 5× SSC/50% formamide/1% SDS at 70°C, followed by 4× SSC/50% formamide at 65°C, blocked in 10% heat-inactivated goat serum/PBS for 1 h at room temperature and incubated in anti-DIG–alkaline phosphatase-conjugated antibody (1 : 1000) in PBS at 4°C overnight. Endogenous alkaline phosphatase activity was blocked by pre-treatment with 10 mM levamisole. The colour reaction was developed overnight in the presence of 250 μg/ml NBT (4-nitro blue tetrazolium chloride) and 130 μg/ml BCIP (5-bromo-4-chloro-3-indolyl-phosphate) as substrate.

Immunocytochemistry
Lumbar spinal cord sections, prepared as described above, were first treated with 10% goat serum/0.1% Triton X-100/PBS for 1 h at room temperature. They were then incubated in ED-1 (1 : 500; Serotec, Oxford, UK)/0.1% Triton X-100/PBS overnight at 4°C. The rest of the procedure was performed at room temperature. The sections were washed in PBS, incubated in anti-mouse IgG [1/500 (v/v)] followed by avidin–biotin complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Immunopositive cells were visualized by 3,3¢-diaminobenzidine reaction product.

Western blot analysis
Lumbar spinal cords of controls or EAE (E1–E3) rats (three animals/group) were homogenized on ice with a motorized pestle in 1 ml of buffer A (10 mM HEPES pH 7.0, 10% sucrose, 0.4 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin). The homogenate was then passed five times through a syringe with a 22-gauge needle and centrifuged at 800 g for 5 min. The supernatant was removed and centrifuged at 20 000 g for 45 min. The crude plasma membrane preparation was suspended in buffer A and aliquots were kept at −70°C until use. Total protein concentrations were determined utilizing the CBQCA protein quantification kit according to the manufacturer’s instructions (Molecular Probes, Eugene, OR, USA). A 5 μg aliquot of total protein was loaded on each lane of an 8% SDS–polyacrylamide Novex, Tris-glycine gel (Invitrogen). Electrophoresis was performed for 90 min at 125 V. The protein was then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane for 45 min at 50 V, and stained with BLOT-FastStain (Chemicon, Temecula, CA, USA) according to the manufacturer’s instructions. Immunodetection was performed employing a Western Breeze kit (Invitrogen). Primary antibodies were utilized as follows: anti-PyCA2 (1 : 5000, polyclonal; Research Diagnostic, Flanders, NJ, USA), anti-synapsin IIa (1 : 5000, monoclonal; BD Sciences, Palo Alto, CA, USA) and anti-α-tubulin (1 : 20 000, monoclonal; Sigma). Signal was visualized by exposure of blots to Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, NJ) for 30–120 s. Between each immunoblot, membranes were stripped with Re-Blot recycling kit (Chemicon). Bands were quantified using the Un-scant-it software (Silk Scientific).

Spinal cord slice cultures
Adult rats were sacrificed by exposure to CO2/O2. Lumbar spinal cords were excised, sliced into 1 mm sections and immediately placed in 1 ml of defined medium consisting of L-15: neurobasal medium (2 : 1; Gibco/BRL, Rockville, MD, USA) containing insulin (5 μg/ml), transferrin (100 μg/ml), selenium (40 ng/ml), putrescine (16 ng/ml), progesterone (60 ng/ml) and 1 U of penicillin/streptomycin. Kainic acid (60 ng/ml) and 1 U of penicillin/streptomycin. Kainic acid was then introduced at the concentrations indicated in the Results. Controls were maintained in medium containing vehicle. The slices were incubated for 6 h at 37°C. At the end of the incubation period, the slices were homogenized in Trizol reagent and RNA was prepared as described above. Some slices were fixed in 4% paraformaldehyde, cryoprotected in 10 and 20% sucrose and frozen on dry ice. Cryostat sections (10 μm) were mounted on poly-L-lysine-coated slides and utilized for assessment of DNA fragmentation by TUNEL assay, a hallmark of cell death. TUNEL assay was performed utilizing the ApopTag kit (Intergen, Purchase, NY, USA) according to the manufacturer’s instructions. Eight control and eight kainic acid-treated slices were analysed. At least five sections from each slice were used for TUNEL assay. Control and experimental samples were mounted on the same slide, side by side. The experiment was repeated twice and yielded similar results.

Results
To identify differentially expressed genes in the EAE lumbar spinal cord, we first performed cDNA microarray analysis at
disease stage E3. We selected a number of differentially expressed genes for further studies because they are expressed exclusively or mainly by neurons, they play critical roles in important cellular functions including ion homeostasis, neurotransmitter exocytosis, energy metabolism or impulse conduction, and their transcript level was altered greatly. The genes were classified according to the best characterized function of the protein which they encode (Table 1). Thus, cDNA microarray profiling provided an initial and global insight into potential cellular mechanisms that may be affected during a defined stage of the disease. We further corroborated these findings by semi-quantitative RT–PCR utilizing the same source of RNA as that employed for cDNA microarray analysis (stage E3). Subsequently, we further validated our findings by performing at least 2–3 additional, independent experiments employing distinct groups of rats (2–3 rats/group) during the course of the disease (stages E1–E3), as reported below.

**PMCA2 mRNA and protein levels are decreased at onset of symptoms**

One of our observations by cDNA microarray analysis was a pronounced change in the expression of several pumps which modulate ion, and in particular Ca\(^{2+}\), homeostasis including plasma membrane Ca\(^{2+}\) ATPase 2 (PMCA2), sarcomplasmic and endoplasmic reticulum Ca\(^{2+}\)-activated ATPase 2 (SERCA2) and Na\(^{+}/K^{+}\) transporting ATPase \(\beta 2\) subunit (Table 1). As Ca\(^{2+}\) balance plays a pivotal role in the function and survival of cells, including neurons, we further analysed these genes and corroborated our microarray findings by semi-quantitative RT–PCR utilizing specific primers for each pump (Table 2). We examined the expression of the aforementioned ion pumps during the course of the disease starting at E1 (tail weakness only; clinical score 1) in order to determine the clinical stage when the first significant changes in expression occur. Transcript levels of PMCA2, a major pump mediating Ca\(^{2+}\) extrusion from cells (Carafoli, 1987; Miller, 1991; Garcia and Strehler, 1999), were significantly decreased at E1 [Fig. 1, 88% decrease, \(P < 0.001\) by analysis of variance (ANOVA); Scheffe’s post hoc test] and consistently remained low until E3, the latest clinical stage examined. Transcript levels of SERCA2, which mediates the sequestration of intracellular Ca\(^{2+}\) into the endoplasmic reticulum, and Na\(^{+}/K^{+}\) transporting ATPase \(\beta 2\) subunit, which regulates Na\(^{+}\) homeostasis, were not significantly altered at E1 or E2 but dramatically decreased at E3 (Fig. 1; 100 and 90% decrease for SERCA2 and Na\(^{+}/K^{+}\) ATPase, respectively, \(P < 0.001\) by ANOVA, Scheffe’s post hoc test).

To ascertain that the changes in PMCA2 mRNA were also reflected at the protein level, we performed western blot analysis utilizing an antibody which previously has been reported to be specific for the PMCA isoform 2 (Stauffer et al., 1995). Indeed, we did not detect any signal when crude membranes from kidney, a tissue which does not express PMCA2, were tested, indicating specificity of the antibody (data not shown). In contrast, in spinal cord, one large band of mol. wt ~135–140 kDa and another one of 130 kDa were visualized (Fig. 2), in accordance with the 130, 135 and 138 kDa PMCA2 isoforms previously described (Stauffer et al., 1995). The intensity of both bands decreased by 50% at E1, and by 80% at subsequent stages (Fig. 2). The results by western analysis confirm that the reduction in PMCA2 mRNA is accompanied by a decrease in protein level. Our findings, taken together, raise the possibility of ion dyshomeostasis as one of the mechanisms leading to neural dysfunction or injury during EAE.

### Table 2: List of primers utilized in RT–PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’ primer (bp)</th>
<th>3’ primer (bp)</th>
<th>Predicted size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane Ca(^{2+}) ATPase 2</td>
<td>667–690</td>
<td>1659–1681</td>
<td>1014</td>
</tr>
<tr>
<td>Na(^{+}/K^{+}) transporting ATPase (\beta 2) subunit</td>
<td>499–522</td>
<td>1039–1062</td>
<td>563</td>
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<tr>
<td>SERCA2</td>
<td>548–570</td>
<td>1357–1379</td>
<td>823</td>
</tr>
<tr>
<td>Synapsin IIa</td>
<td>1884–1909</td>
<td>2531–2556</td>
<td>672</td>
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<td>Syntaxin 1B</td>
<td>211–235</td>
<td>918–942</td>
<td>731</td>
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<tr>
<td>SNAP-25</td>
<td>1–22</td>
<td>588–609</td>
<td>609</td>
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<td>Rab16</td>
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<td>Cytoplasmic (c) oxidase subunit Vb</td>
<td>17–38</td>
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<td>481</td>
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<td>333–356</td>
<td>858–834</td>
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<tr>
<td>Na(^{+}) channel (\beta 2) subunit</td>
<td>319–339</td>
<td>735–755</td>
<td>436</td>
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<tr>
<td>Na(^{+}) channel II</td>
<td>213–233</td>
<td>772–793</td>
<td>580</td>
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<tr>
<td>(\alpha)-Tubulin</td>
<td>68–90</td>
<td>687–708</td>
<td>640</td>
</tr>
</tbody>
</table>

The region selected for the design of primers and the predicted size for each RT–PCR product are presented. GenBank accession Nos are given in Table 1.
PMCA2 expression is restricted to grey matter

To determine the cell type-specific expression and modulation of PMCA2, we performed in situ hybridization on lumbar spinal cord sections utilizing DIG-labelled riboprobes specific for this isoform. PMCA2 mRNA was localized exclusively to grey matter (Fig. 3A–D). We did not detect any signal in white matter even when the substrate reaction was continued for 48 h. Many cells in the grey matter, including those in the ventral horn exhibiting motor neuron-like morphology, expressed PMCA2 (Fig. 3C and D). The exclusive expression of PMCA2 mRNA in spinal cord grey matter is consistent with the findings of Stauffer et al. (1997) reporting PMCA2 immunoreactivity primarily associated with neurons in the brain.

In situ hybridization studies further confirmed the results obtained by microarray analysis or RT–PCR, and indicated a
inflammatory reaction in the lumbar spinal cord is quite extensive, as indicated by the distribution of ED-1-immunopositive activated microglia/macrophages in the grey and white matter (Fig. 3L–N).

**PMCA2 expression is modulated by kainic acid**
Glutamate excitotoxicity has been implicated recently in EAE as a trigger that induces neuronal and oligodendrocyte loss and axonal injury (Pitt et al., 2000; Smith et al., 2000). It has also been reported that kainic acid rapidly suppresses hippocampal PMCA expression, *in vivo*. Reductions in PMCA2 levels in the dentate gyrus were already observed 4 h following the administration of kainic acid, *in vivo* (Garcia et al., 1997). Taken together, these findings suggested that glutamate might be a possible modulator of PMCA2 expression in the spinal cord during EAE. To begin assessing this possibility, we exposed lumbar spinal cord slices to defined medium containing kainic acid (4–100 μM) or medium alone for 6 h at 37°C. Kainic acid (4 μM) decreased PMCA2 transcript levels by 86% as compared with controls (Fig. 4A and B; *P < 0.001 by t test*). Exposure to 20 or 100 μM kainic acid completely suppressed PMCA2 expression (not shown). In contrast, α-tubulin mRNA levels were unaltered, indicating specificity in the changes observed (Fig. 4A).

Previous reports have shown that prolonged exposure of neurons to kainic acid induces cell death, as indicated by DNA fragmentation and TUNEL immunoreactivity (Simonian et al., 1996; Cheung et al., 1998; Venero et al., 1999; Fujikawa et al., 2000; Giardina and Beart, 2001). Although our cultures were treated with kainic acid only for 6 h, we ascertained that the decrease in PMCA2 expression was not due to death of grey matter cells, by performing TUNEL assay on sections obtained from sister cultures maintained under the same experimental conditions. In controls, TUNEL-positive cells were infrequent (Fig. 4C) and were found only occasionally in the white matter and in the immediate vicinity of the dorsal root entry zone. Following exposure to kainate, TUNEL-positive cells were sparse in the ventral horn or intermediate zone grey matter, regions rich in PMCA2-expressing cells (Fig. 4D), whereas they increased in the dorsal root entry zone and white matter (Fig. 4E). We conclude that the decrease in PMCA2 mRNA levels in response to kainic acid is due to alterations in expression rather than to cell death.

**Synaptic protein expression is decreased at onset of symptoms**
cDNA microarray analysis also indicated changes in the expression of several neuronal proteins which mediate exocytosis. The most prominent decrease occurred in synapsin IIa, syntaxin 1B and SNAP-25 (synaptosome-associated protein of 25 kDa) transcript levels, whereas the expression of Rab12, 14 and 16 was increased (Table 1). Some members of the Rab family GTPases previously have

**Fig. 2** Modulation of PMCA2 and synapsin IIa protein levels at different phases of EAE (E1, E2, E3) and control (C) as assessed by western blot analysis. The figure shows a representative western blot. α-Tubulin was used as control for experimental variations. Quantification of PMCA2 (135/138 and 130 kDa) band intensities revealed a 50% decrease at E1, and 80% at subsequent stages. As expected, a single band of 78 or 50 kDa was visualized with the synapsin IIa and α-tubulin antibodies, respectively. Quantification of synapsin IIa band intensities indicated a 30, 60 and 80% decrease at E1, E2 and E3, respectively. The experiment was repeated twice and yielded similar results.
Cellular localization and modulation of PMCA2 mRNA assessed by in situ hybridization. Bright field photomicrographs of lumbar spinal cord (L4-L5) sections obtained from controls (A–D) and diseased rats (E and F) hybridized to DIG-labelled antisense riboprobe. (G) A control section hybridized to a sense riboprobe. Labelled cells were found exclusively in grey matter of control sections (A). (B) A higher magnification photomicrograph of the section shown in A, demonstrating labelling in cells exhibiting motor neuron-like morphology in the ventral horn (examples shown within the square) and in smaller cells of the intermediate zone (arrows). (C and D) Higher magnification photomicrographs of the cells in B (indicated by arrows and enclosed within the square, respectively). Expression of PMCA2 dramatically decreased in the spinal cord during EAE (E), confirming results obtained by cDNA microarray analysis and RT-PCR. Note that the diseased spinal cord is usually enlarged as compared with controls. (F) A higher magnification picture of the section shown in E. Arrows point at some weakly labelled cells. To ensure that the reduction in PMCA2 expression was not due to a generic decrease in mRNA levels, adjacent sections obtained from control (H) and experimental (I) spinal cord were hybridized to an antisense α-tubulin riboprobe. (H and I) A group of motor neuron-like cells in the ventral horn expressing the housekeeping gene at comparable levels. The presence of cresyl violet-stained cells in sections obtained from spinal cord of control (J) and experimental (K) rats indicated that the decrease in PMCA2 was not due to non-specific cell loss. To confirm the inflammatory reaction in the lumbar spinal cord, consecutive sections were stained with haematoxylin–eosin (not shown) and immunolabelled with ED-1, a marker for activated microglia/macrophages. ED-1-immunopositive cells are rare in control (L), and very abundant in EAE (M) spinal cord grey and white matter. The grey matter in the control section is delineated by dashed lines. (N) A higher magnification photomicrograph of a region in the section shown in M. Arrows point at examples of immunopositive cells. Sections obtained from six controls and six EAE rats (clinical score 3, E3) were analysed. The reduction in PMCA2 expression was observed in all diseased rats, although the magnitude of the change was variable and ranged from dramatic decreases as illustrated in this figure to more moderate but distinctly discernable reductions. Scale bar = 400 μm (A, E, G, L and M); 200 μm (B and F); 100 μm (C and D); 40 μm (N).
been implicated in vesicular function (for a review see Geppert and Sudhof, 1998), although the exact role of the aforementioned Rab isoforms has yet to be determined.

As synapsins, syntaxins and SNAP-25 are essential for vesicular function and neurotransmitter exocytosis, we further analysed the temporal pattern of their expression during the course of EAE. At E1, transcript levels of synapsin IIA and syntaxin 1B were decreased by 93 and 84%, respectively (P < 0.001 by ANOVA, Scheffe’s post hoc test) and remained low thereafter (Fig. 5). In addition, western blot analysis indicated 30, 60 and 80% reductions in synapsin IIA protein levels at E1, E2 and E3, respectively (Fig. 2). SNAP-25 expression showed a tendency to decrease at E1 and E2, but the results were more variable and did not reach statistical significance (Fig. 5). By E3, however, SNAP-25 levels had decreased by 96% (P < 0.001 by ANOVA, Scheffe’s post hoc test), in agreement with a recent study on chronic EAE in mice (Ibrahim et al., 2001). In contrast to synapsins, syntaxin and SNAP-25, the transcript levels of Rab12, Rab14 and Rab16 were 2.9-, 4.3- and 2.6-fold increased, respectively (Fig. 6; P < 0.001 for Rab12 and 14, and P < 0.01 for Rab16, by t test). These results raise the possibility of disturbances in neurotransmission at onset of EAE symptoms.

Increased cytochrome c oxidase expression suggests deregulation of mitochondrial metabolic activity at E2
cDNA microarray analysis indicated a strong increase in expression of cytochrome c oxidase subunits IV and Vb, suggesting altered mitochondrial metabolic activity (Table 1). We corroborated this finding by RT-PCR and found that the most robust increases in cytochrome c oxidase IV and Vb subunit expression (~11- and 15-fold, respectively) occur at E2 and E3 (Fig. 7). Mitochondrial dysfunction has often been associated with neuronal injury in CNS diseases and especially in response to intracellular Ca²⁺ overload or oxidative stress (Fiskum, 2000; Hirai et al., 2001). It is conceivable that changes in mitochondrial activity contribute to EAE pathology in a fashion similar to that observed in other CNS disorders such as ischaemia.

Reduced sodium channel expression occurs at advanced clinical phases
Sodium channels are critical mediators of impulse conduction along the axon. Therefore, the reduced expression of Na⁺ channel β1 and 2 subunits and brain sodium channel II, as indicated by cDNA microarray analysis, was analysed further by RT-PCR. We found that the expression of all the aforementioned channels substantially decreased at E3 (Fig. 8; 80–90% decrease), confirming the results obtained by microarray analysis. In contrast, no changes were observed at E1 and E2. Thus, potential abnormalities in Na⁺ channel repertoires in nerve cells leading to impairment in impulse activity may contribute to the progression of symptoms at more advanced disease phases.

Discussion
The present study reports alterations in the expression of specific genes in the lumbar spinal cord during the clinical course of EAE, with special emphasis on the initial phase of symptomatic disease. In particular, we analysed the temporal expression pattern of genes which encode proteins playing critical roles primarily in neuronal function. Our results, taken together, indicate that changes in neuronal gene expression coincide with onset of symptoms. Thus, our findings provide novel insights into the potential molecular mechanisms that may underlie the recently described neuronal dysfunction or injury during EAE (Ferguson et al., 1997; Trapp et al., 1998, 1999; De Stefano et al., 1999; Bitsch et al., 2000; Smith et al., 2000; Bjartmar and Trapp, 2001; Bjartmar et al., 2001; Peterson et al., 2001). We propose that ion dyshomeostasis due to aberrant Ca²⁺ extrusion and abnormal neurotransmitter release resulting from anomalies in the expression of vesicular proteins may promote the onset of neural deficits. As the disease progresses, perturbed mitochondrial function and reduced impulse conduction due to decreases in Na⁺ channel expression may exacerbate clinical symptoms.

Glutamate-mediated changes in PMCA2 expression may induce neuronal dysfunction at onset of EAE symptoms
Increases in intracellular Ca²⁺ often have been associated with cell dysfunction, injury and death in many pathological conditions of the CNS, including ischaemia, Alzheimer’s and Parkinson’s disease (for reviews see Paschen, 2000; O’Neill et al., 2001; Paschen and Frandsen, 2001). Yet, the contribution of Ca²⁺ dyshomeostasis to EAE and the affected mechanisms have not been defined. A loss in Ca²⁺ balance may result from defects in mechanisms that regulate Ca²⁺ entry, extrusion or intracellular sequestration. A role for voltage-dependent channel-mediated Ca²⁺ influx in axonal and neuronal degeneration during multiple sclerosis and EAE has been reported (Kornek et al., 2000, 2001). To our knowledge, our study is the first report implicating a pivotal role for distinct ion pumps involved in calcium extrusion and sequestration in EAE pathogenesis.

Whereas PMCA’s are major pumps modulating Ca²⁺ extrusion, SERCA isoforms play critical roles in sequestration of Ca²⁺ into the endoplasmic reticulum. Our results indicate a profound decrease in PMCA2 expression at the earliest manifestation of symptoms, which is then followed by a significant reduction in SERCA2 transcript levels at subsequent clinical phases. Such temporal changes in ion pump expression may promote accumulation of Ca²⁺ in the cytoplasm, increasing the vulnerability of neurons to cal-
calcium-mediated injury. This may be one possible candidate mechanism underlying neuronal decline, and implicates PMCA2 and SERCA2 as potential targets for therapeutic interventions. Importantly, our results may be relevant to the human disease, as a recent study found a decrease in PMCA2 transcript levels by microarray analysis of three multiple sclerosis brains (Lock et al., 2002, online supplementary table B).

The PMCA gene family encodes four isoforms which initially were considered to be necessary only for the maintenance of basal Ca$^{2+}$ levels. However, emerging evidence indicates that they may play important roles in pathological conditions (Garcia and Strehler, 1999) especially after overstimulation of glutamate receptors which causes intracellular Ca$^{2+}$ overload (Choi, 1992; Mody and MacDonald, 1995). PMCA2 is expressed primarily in brain and heart, in a region- and cell-type specific manner (Garcia and Strehler, 1999). The highly restricted tissue distribution of PMCA2 suggests that this isoform plays unique, specialized and non-redundant roles in the CNS. Indeed, PMCA2-null mice exhibit deafness, and unsteady gait and balance, indicating that the presence of other isoforms does not compensate for the absence of PMCA2 (Kozel et al., 1998).

The triggers that induce changes in the expression of Ca$^{2+}$ pumps during EAE have not been defined. Glutamate is an appealing candidate because of its well-known effects on Ca$^{2+}$ homeostasis (Choi, 1992) and because of the recently reported contribution of excitotoxicity to EAE pathogenesis (Pitt et al., 2000; Smith et al., 2000). Administration of AMPA/kainate antagonists ameliorates EAE symptoms in mice and rats, and prevents neuronal and oligodendrocyte loss and axonal damage. Moreover, kainate-induced decreases in PMCA1 and PMCA2 expression in hippocampal pyramidal cells are followed by neuronal death, in vivo (Garcia et al., 1997). These findings raise the possibility of kainate receptor-mediated alterations in PMCA2 expression in the spinal cord during EAE. In fact, we found that expression of spinal cord slice cultures to relatively low concentrations of kainate (4 μM) for 6 h dramatically decreased PMCA2 transcript levels. Thus, even low glutamate levels may be sufficient to affect PMCA2 expression when cells are exposed to the neurotransmitter continuously. We propose that, at onset of EAE, persistent stimulation of kainate receptors following moderate but sustained elevations in glutamate concentrations may lead to downregulation of Ca$^{2+}$ pump expression, promote Ca$^{2+}$ overload and initiate the previously described injury cascade (Shields et al., 1998).
Aberrant neurotransmitter exocytosis may affect neuronal communication during the early course of the disease

In addition to PMCA2, the expression of synapsin IIa and syntaxin 1B, vesicular proteins that are crucial for exocytosis in presynaptic terminals, was decreased dramatically at onset of clinical symptoms. Synapsins are a family of synaptic vesicle-associated phosphoproteins that are required for the tethering of vesicles to each other and to cytoskeletal proteins such as actin. Phosphorylation of synapsins liberates vesicles from the cytoskeleton, enabling their trafficking in the presynaptic terminal. Synapsins are essential for the formation, maintenance and regulation of the reserve synaptic pool in the vicinity of the active zone. Decreases in synapsin
expression reduce the number of synaptic vesicles distal to the active zone, leading to synaptic fatigue (Pieribone et al., 1995; Rosahl et al., 1995). Moreover, perturbations in synapsin function inhibit or slow down neurotransmitter release.

Syntaxins belong to a class of proteins called SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) hypothesized to be essential for the docking and fusion of synaptic vesicles. Vesicle-associated SNAREs, such as synaptobrevin, and target membrane-associated SNAREs, such as syntaxins and SNAP-25, form a high affinity complex. Formation of the SNARE complex draws vesicles to the target membrane and induces membrane fusion. In our experimental paradigm, we observed a decrease in both syntaxin 1B and SNAP-25. However, the dramatic reduction in syntaxin 1B occurred at onset of symptoms, whereas SNAP-25 showed a tendency to decrease, which was statistically significant only at a later clinical stage (E3). Hence, changes in the expression of some synapsin and SNARE proteins can interfere with normal neuronal communication during EAE.

The notion of synapse pathology is suggested further by changes observed in Rab12, 14 and 16 expression. Rab isoforms belong to the Ras superfamily of small GTP-binding proteins, and have been implicated in the attachment of vesicles to target membranes. A member of this family, Rab3, is localized to synaptic vesicles and limits the amount of neurotransmitter released in response to Ca$^{2+}$ signal. However, a similar function for Rab12, 14 and 16 has not yet been defined. Rab12 and 14 are also expressed in oligodendrocytes and may play additional roles (Burcelin et al., 1997). Therefore, the exact contribution of the aforementioned Rab isoforms to EAE pathogenesis remains to be determined.

**Perturbations in mitochondrial function may contribute to cellular injury at advanced EAE stages**

Cellular injury often has been associated with disturbances in mitochondrial function. Interestingly, our microarray analysis indicated a pronounced increase in the expression of mitochondrial cytochrome c oxidase subunits IV and Vb, a finding which was validated further by RT–PCR. Cytochrome c oxidase has been used frequently as a marker for neuronal metabolic activity, especially in pathological conditions involving oxidative stress. Neurons subject to oxidative...
damage show abnormalities in mitochondrial dynamics even in the absence of any apparent indication of degeneration (Sayre et al., 1997; Smith et al., 1997). Elevations in cytochrome c oxidase expression or function in vulnerable neuronal subpopulations in Alzheimer’s disease, following traumatic brain injury and preceding apoptotic death of spinal cord motor neurons after sciatic nerve avulsion, have been reported (Martin et al., 1999; Harris et al., 2001; Hirai et al., 2001). Thus, an increase in cytochrome c oxidase expression may reflect aberrant energy metabolism and oxidative damage in the spinal cord during EAE.

Alterations in sodium channel expression may exacerbate symptoms by affecting impulse conduction

Neurological symptoms in multiple sclerosis and EAE models associated with extensive myelin loss have been attributed largely to a failure in impulse conduction in demyelinated axons. However, our results indicating a dramatic decrease in the expression of important Na⁺ channels at advanced EAE stages (E3) suggest that impaired nerve conduction may also be due to alterations in Na⁺ channel levels. Indeed, the expression and function of various other sodium channels are affected after nerve injury, inflammation and demyelination (Dib-Hajj et al., 1996; Cummins and Waxman, 1997; Schild et al., 1997; Gould et al., 1998; Black et al., 2000). A change in the expression of sensory neuron-specific sodium channels in Purkinje cells during EAE has been reported (Black et al., 2000).

In conclusion, our results suggest that the onset of EAE symptoms may be due partially to disruption of neuronal function and communication as a consequence of changes in mechanisms regulating calcium extrusion and neurotransmitter exocytosis. Exacerbation of symptoms at later stages may result from abnormalities in additional processes including impulse conduction and energy metabolism. Future studies will determine the role of the differentially expressed genes in the onset and progression of EAE and their precise contribution to neuronal dysfunction during the different stages of the disease.

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References


Bjartmar C, Trapp BD. Axonal damage and neuronal degeneration


Garcia ML, Streher EE. Plasma membrane calcium ATPases as critical regulators of calcium homeostasis during neuronal cell function. [Review]. Front Biosci 1999; 4: D869±82.


Giardina SF, Beart PM. Excitotoxic profiles of novel, low-affinity kainate receptor agonists in primary cultures of murine cerebellar granule cells. Neuropharmacology 2001; 41: 421±32.


