Glutamate excitotoxicity in a model of multiple sclerosis

DAVID PITT[2], PETER WERNER[1,3] & CEDRIC S. RAINE[1,2,4]

Departments of [1]Neurology, [2]Pathology and [4]Neuroscience, F-121N, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York, 10461, USA
[3]Dept. of Neurology, Beth Israel Medical Center, 10 Union Square East, New York, New York 10003, USA
D.P and P.W. contributed equally to this study.
Correspondence should be addressed to P.W.; email: pwerner@aecom.yu.edu.

Glutamate excitotoxicity mediated by the AMPA/kainate type of glutamate receptors damages not only neurons but also the myelin-producing cell of the central nervous system, the oligodendrocyte[1]. In multiple sclerosis, myelin, oligodendrocytes and some axons are lost as a result of an inflammatory attack on the central nervous system[2]. Because glutamate is released in large quantities by activated immune cells[3], we expected that during inflammation in MS, glutamate excitotoxicity might contribute to the lesion. We addressed this by using the AMPA/kainate antagonist NBQX to treat mice sensitized for experimental autoimmune encephalomyelitis, a demyelinating model that mimics many of the clinical and pathologic features of multiple sclerosis. Treatment resulted in substantial amelioration of disease, increased oligodendrocyte survival and reduced dephosphorylation of neurofilament H, an indicator of axonal damage[4]. Despite the clinical differences, treatment with NBQX had no effect on lesion size and did not reduce the degree of central nervous system inflammation. In addition, NBQX did not alter the proliferative activity of antigen-primed T cells *in vitro*, further indicating a lack of effect on the immune system. Thus, glutamate excitotoxicity seems to be an important mechanism in autoimmune demyelination, and its prevention with AMPA/kainate antagonists may prove to be an effective therapy for multiple sclerosis.

For this study, we determined whether glutamate excitotoxicity due to increased extracellular glutamate is important in damage to oligodendrocytes and axons in experimental autoimmune encephalomyelitis (EAE). As oligodendrocytes have only the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)/kainate type of excitatory glutamate receptor and are exquisitely vulnerable to glutamate excitotoxicity[1,5], we treated mice subjected to EAE with NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)-quinoxaline-2,3-dione), an AMPA/kainate antagonist. We induced EAE by adoptive transfer of myelin basic protein (MBP)-specific lymph node cells from previously immunized donor SJL/J mice into naive recipients. In two independent experiments, adoptively sensitized mice ($n = 19$) received three subcutaneous injections per day of 300 µg NBQX in 200 µl phosphate-buffered saline (PBS) beginning day 5 after transfer and continuing until the end of the experiment (day 15). Control mice with EAE ($n = 18$) received vehicle (PBS) only. A clinical score from 0 (healthy) to 5 (moribund or dead) was assigned to assess neurologic impairment[6]. Treatment with NBQX led to a highly significant reduction ($P < 0.01$) in clinical impairment compared with treatment with vehicle alone (Fig. 1). The difference in clinical score was significant ($P < 0.05$) by day 3 after disease onset (day 7 of treatment), and continued to increase until the time of sampling ($P < 0.01$), attaining a final mean difference between experimental and vehicle-treated groups of 1.8 clinical score points. Histologic examination of the neuraxes of two representative mice in each group showed they had similar degrees of inflammation, indicating that NBQX did not function by modulating immune responsiveness. Thus, we assessed the effect of NBQX on the immune response by testing the proliferation of lymph node cells *in vitro* in the presence of NBQX. We stimulated cultured lymph node cells from MBP-sensitized mice with 50 µg/ml MBP and determined their proliferation rates in the presence or absence of NBQX. In three independent experiments (six samples each), there was no significant difference in ^{3}H-thymidine incorporation by NBQX-treated cells (counts per minute: unstimulated, 352 ± 63; MBP, 2,200 ± 441) and control cells (counts per minute: unstimulated, 261 ± 19; MBP, 2,610 ± 567). In addition, pretreatment of MBP-activated lymph node cells with NBQX for 72 hours did not reduce their efficacy in inducing EAE when injected into naive mice but resulted in the usual form of EAE (ref. 6). Thus, NBQX substantially ameliorated the clinical course of EAE, indicating that glutamate excitotoxicity was responsible for a substantial portion of the clinical impairment in vehicle-treated mice with EAE. This effect was not due to a change in T-cell function or central nervous system (CNS) inflammation.

The principal cellular target in multiple sclerosis (MS) is the oligodendrocyte. To evaluate the role of glutamate excitotoxicity in the loss of oligodendrocytes during EAE, we examined oligodendrocytes in detail by immunohistochemistry of lumbar spinal cord tissue from representative mice from the NBQX- and vehicle-treated groups. We immunolabeled frozen sections with antibody against CNPase and counted the total number of oligodendrocytes within the dorsal columns in 20–30 transverse sections (Fig. 2*a*). In mice with EAE, dorsal columns commonly have inflammatory lesions centered on midline vessels, and this feature allowed us to reproducibly evaluate the effect of NBQX on the lesion area. Within this well-defined region, the number of oligodendrocytes was significantly less ($P < 0.001$) in vehicle-treated EAE than in normal, age-matched mice. However, the number of oligodendrocytes in mice with EAE treated with NBQX was only slightly less than that in normal mice (Fig. 2*b*). Expressed as percentage of cell loss in comparison to normal mice, 23% of oligodendrocytes ($P < 0.001$) were lost in mice with vehicle-treated EAE, compared with only 9% in NBQX-treated mice ($P > 0.05$) (Fig. 2*c*). Overall, more than 60% of the total oligodendrocyte loss in vehicle-treated mice was prevented with treatment with NBQX. Thus, our results indicate that AMPA/kainate receptor-mediated glutamate excitotoxicity is a chief factor in oligodendrocyte death in EAE.

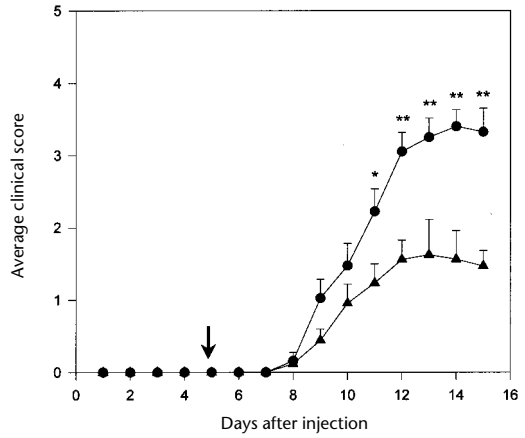

Fig. 1 Clinical course of adoptive transfer EAE: Effect of treatment with NBQX. SJL mice were injected with MBP-activated cells. Starting on day 5 after immunization (downward arrow), mice were treated with NBQX or vehicle (PBS) until day 15 (day 10 of treatment). Data represent means ± s.e.m. from two independent experiments, with a total of 18 mice until day 12, and 9 mice for days 13–15. *, $P < 0.05$ and **, $P < 0.01$, vehicle-treated compared with NBQX-treated, Student's unpaired, two-tailed *t*-test.

Axonal damage is another important feature of MS lesions and has been shown to increase with lesion activity[4,7]. A recently exploited immunohistochemical marker of demyelinated and dystrophic axons in MS, the presence of abnormal dephosphorylation of heavy chain neurofilament H (NF-H), has been used to assess axonal damage[4]. Using the same technology, with western blot analysis, we showed that as in MS, the central nervous systems of mice with EAE had a large increase of abnormally dephosphorylated NF-H, demonstrable both structurally (Fig. 3*a*) and semi-quantitatively (Fig. 3*b* and *c*). 'Terminal' EAE mice (that is, grade 5) had large amounts of abnormally dephosphorylated NF-H, whereas normal mice had much smaller amounts (Fig. 3*b* and *c*). A background level of reactivity in the spinal cords of normal mice was consistent with the presence of dephosphorylated NF-H in neuronal cell bodies and some large-diameter axons[8]. Treatment of EAE with NBQX resulted in a substantial reduction of abnormally dephosphorylated NF-H compared with that in vehicle-treated mice (Fig. 3*b* and *c*), indicating involvement of glutamate excitotoxicity in the axonal changes. This is in agreement with the well-known vulnerability of neurons to glutamate excitotoxicity and the reported presence of ionotropic glutamate receptors on myelinated axons[9]. Thus, treatment of mice with EAE with the AMPA/kainate antagonist NBQX led to a considerable amelioration of clinical outcome, which corresponded pathologically to a reduced loss of oligodendrocytes as well as diminished axonal damage.

These observations relate directly to oligodendrocyte death and axonal damage in MS, the underlying mechanisms of which are believed to be immune-associated. Activated immune cells present in the inflammatory infiltrates in CNS lesions produce cytotoxic factors such as tumor necrosis factor α (ref. 10), matrix metalloproteinases[11], active oxygen species[12] and autoantibodies[13], and may also kill by direct cell-to-cell contact. During inflammation, glutamate is produced and released into the extracellular space[3] by activated leukocytes and microglia. However, this topic has received little attention. Within the CNS, increase in extracellular glutamate during inflammation may be further enhanced by reduction of the glial glutamate-metabolizing enzymes, glutamate dehydrogenase and glutamine synthetase, as shown in EAE (ref. 14). Accordingly, increased glutamate levels have been found in the cerebrospinal fluid of patients with CNS inflammatory conditions, such as acute encephalitis, meningitis and MS (refs. 15,16). An increase in extracellular glutamate, the main excitatory neurotransmitter in the CNS, can have potentially serious consequences, as it is capable of precipitating excitotoxic cell death by overstimulation of ionotropic glutamate receptors[1,17], two types of which are recognized: NMDA (N-methyl D-aspartate) and AMPA/kainate. Both types are found on neurons, whereas glial cells have only AMPA/kainate receptors[1,5].

In the context of EAE, given the plethora of other cytotoxic factors known to be present in EAE lesions, it was unexpected that more than 60% of the oligodendrocyte loss in our experiments could be attributed to glutamate excitotoxicity. It is unlikely that

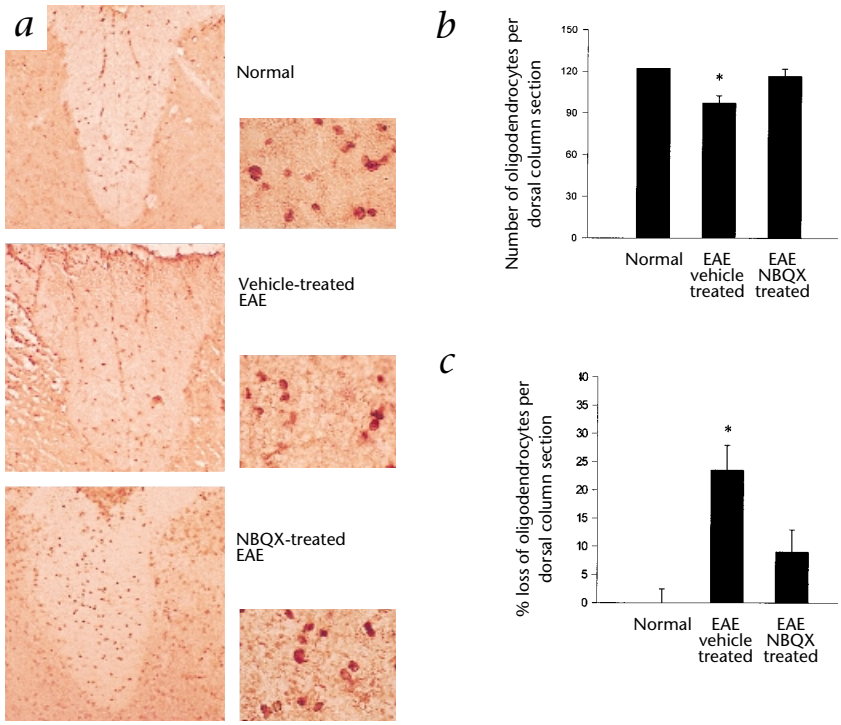

Fig. 2 CNPase-immunoreactive oligodendrocytes in lumbar spinal cord sections. ***a***, Transverse sections of dorsal columns immunolabeled with antibody against CNPase (amplified with ABC and visualized with HRP/DAB; hematoxylin counterstaining). Original magnifications, × 30 (left) and × 100 (right). ***b***, Average number of oligodendrocytes per transverse section of dorsal columns. Each bar represents two representative mice from the normal, vehicle-treated and NBQX-treated groups, with 20–30 sections analyzed per mouse. ***c***, Oligodendrocyte loss in treated and untreated EAE mice and normal mice. Data are expressed as percentage of cells lost per dorsal column and represent means ± s.e.m. *, $P < 0.001$, vehicle-treated compared with normal, and *P*, not significant, vehicle-treated compared with NBQX-treated, ANOVA followed by post-hoc Tukey.

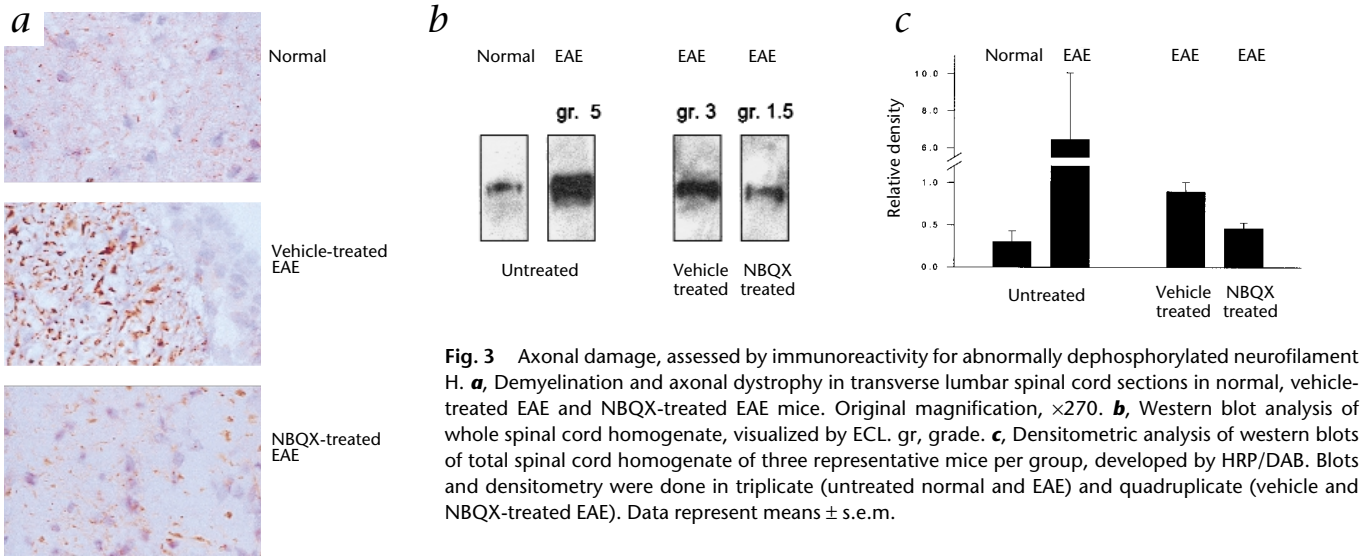

Fig. 3 Axonal damage, assessed by immunoreactivity for abnormally dephosphorylated neurofilament H. ***a***, Demyelination and axonal dystrophy in transverse lumbar spinal cord sections in normal, vehicle-treated EAE and NBQX-treated EAE mice. Original magnification, ×270. ***b***, Western blot analysis of whole spinal cord homogenate, visualized by ECL. gr, grade. ***c***, Densitometric analysis of western blots of total spinal cord homogenate of three representative mice per group, developed by HRP/DAB. Blots and densitometry were done in triplicate (untreated normal and EAE) and quadruplicate (vehicle and NBQX-treated EAE). Data represent means ± s.e.m.

blockade of glutamate receptors in the lesion center would prevent cell death; however, the degree of protection in the surrounding parenchyma indicated a substantial bystander effect, akin to the spreading excitotoxic damage described in stroke[18], in which AMPA/kainate receptor antagonists have received considerable attention[19]. Although the loss of oligodendrocytes in chronic EAE parallels that in MS (ref. 7), we have now quantified the phenomenon in evolving lesions and shown that depletion of oligodendrocytes can be reduced, even during the acute phase. The extent of protection against oligodendrocyte loss demonstrated here indicates glutamate excitotoxicity may be one of the main mechanisms of oligodendrocyte demise in EAE, and perhaps in MS. Our results are supported by a recent report demonstrating that NBQX protects against white matter damage in spinal cord crush injury, a situation in which large amounts of glutamate are released into the extracellular space[20]. Whether changes in glutamate production can be detected in the cerebrospinal fluid of EAE-affected mice is now being investigated using a published method[21], and this forms part of an ongoing study on glutamate metabolism in these conditions (P.W. *et al.*, manuscript in preparation).

Dephosphorylation of NF-H has provided a useful quantitative molecular marker for the severity of EAE, emphasizing the association between axonal involvement and neurologic dysfunction and confirming recent immunohistochemical results with MS lesions[4]. This raises the possibility that retrograde degeneration of neurons in demyelinating diseases[22] may be related to excitotoxic events. The beneficial effects of NBQX on the course of EAE seemed to be due to axonal sparing and oligodendrocyte protection in the absence of a detectable effect upon inflammation, a new concept in this MS paradigm.

In conclusion, we have shown glutamate excitotoxicity mediated by AMPA/kainate receptors to be important in CNS damage in EAE and by extrapolation, possibly in MS. Thus, AMPA/kainate antagonists, now being tested in stroke patients, may afford promising avenues in the treatment of this devastating human disease.

Methods

Induction of EAE. Female adult SJL/J mice 4–6 weeks old (Jackson Laboratories, Bar Harbor, Maine), were housed in a light- and temperature-controlled environment in accordance with National Institutes of Health and Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Myelin basic protein (MBP; Sigma) was dissolved in sterile PBS at a concentration of 8 mg/ml and emulsified with an equal volume of incomplete Freund's adjuvant supplemented with 6 mg/ml *Mycobacterium tuberculosis* (Difco, Detroit, Michigan). Ten days after antigen was injected into the flanks of SJL/J mice, lymph node cells were obtained from draining lymph nodes, cultured and grown for 4 d in the presence of 50 μg/ml MBP, and were subsequently injected intravenously into syngeneic mice at a dose of 3×10^7 cells/mouse. Onset of disease occurred usually after 7–9 d, and mice were graded daily according to a standard clinical index[15] of 0–5 by an individual 'blinded' to mouse identity.

Treatment of EAE. NBQX (Tocris, Ballwin, Missouri) was administered in three daily subcutaneous injections of 300 μg in 200 μl PBS for the duration of the experiment (7 and 10 d). Control mice were treated with PBS alone.

Neuropathology. After 7 and 10 d of treatment, respectively, mice from the vehicle- and NBQX-treated groups were perfused with ice-cold PBS or glutaraldehyde and the CNS was prepared for frozen or epoxy sections, respectively. Epoxy sections 1 μm in thickness from nine levels of the neuraxis (optic nerve, cerebrum, brainstem and spinal cord at C7, Th2, L2, L5, L6 and S1) were stained with toluidine blue and examined by light microscopy, by an individual 'blinded' to the coded sample identity. Frozen sections from lumbar spinal cord (10μm) were acetone-fixed and immunolabeled using the avidin:biotinylated enzyme complex technique (ABC; Vector Laboratories, Burlingame, California). The following antibodies were used: antibody against CNPase (Sigma) at a dilution of 1:80; and antibody against non-phosphorylated neurofilament-H (SMI 32; Sternberger Monoclonal, Lutherville, Maryland) at a dilution of 1:10,000. Sections were viewed with a Zeiss Photomicroscope III, and cells positive for antibody against CNPase were counted using an ocular graticule.

T-cell proliferation assay. Lymph node cells from MBP-immunized mice (described above), were grown in 96-well plates at a concentration of 0.5×10^6 cells per well in 200 μl medium containing 50 μg/ml MBP in the presence or absence of 5 μM NBQX (Tocris, Ballwin, Missouri). After 3 d, cells were 'pulsed' with 1 μCi/ml ^{3}H-thymidine for 12 h before being collected onto glass fiber mats. The incorporated radioactivity was measured in a scintillation counter.

Western blot analysis. PBS-perfused spinal cords were homogenized in 8.5 M urea containing 2 mM PMSF. Samples were denatured in sampling buffer for 2 min at 100 °C, then separated by 10% SDS–PAGE and blotted onto a PVDF membrane. After this transfer, the membrane was blocked for 10 min in 5% fat-free instant milk. Immunodetection was accomplished by incuba-

tion overnight at 4 °C with primary monoclonal antibody SMI 32 (1:10,000 dilution) and, as a standard, antibody against tubulin (1:1,000 dilution; Sigma). After being washed, the blots were incubated with horseradish peroxidase (HRP)-labeled goat antibody against mouse IgG1 (1:500 dilution; Southern Biotechnology, Birmingham, Alabama), for 1 h and then washed. Blots were developed with an ECL kit (Amersham, Piscataway, New Jersey). Alternatively, biotinylated secondary antibody was used and the avidin:biotinylated enzyme complex (ABC) technique was used, with diaminobenzidine (DAB) as substrate.

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