Autoimmune encephalomyelitis ameliorated by AMPA antagonists

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Multiple sclerosis is an immune-mediated disorder of the central nervous system leading to progressive decline of motor and sensory functions and permanent disability1,2. The therapy of multiple sclerosis is only partially effective, despite anti-inflammatory, immunosuppressive and immunomodulatory measures3. White matter inflammation and loss of myelin, the pathological hallmarks of multiple sclerosis, are thought to determine disease severity4,5. Experimental autoimmune encephalomyelitis reproduces the features of multiple sclerosis in rodents and in nonhuman primates6,7. The dominant early clinical symptom of acute autoimmune encephalomyelitis is progressive ascending muscle weakness6. The pathological hallmarks of multiple sclerosis in rodents and in nonhuman primates6,7. The dominant early clinical symptom of acute autoimmune encephalomyelitis is progressive ascending muscle weakness6. However, demyelination may not be profound and its extent may not correlate with severity of neurological decline8, indicating that targets unrelated to myelin or oligodendrocytes may contribute to the pathogenesis of acute autoimmune encephalomyelitis. Here we report that within the spinal cord in the course of autoimmune encephalomyelitis not only myelin but also neurons are subject to lymphocyte attack and may degenerate. Blockade of glutamate AMPA receptors ameliorated the neurological sequelae of autoimmune encephalomyelitis, indicating the potential for AMPA antagonists in the therapy of multiple sclerosis.

To induce experimental autoimmune encephalomyelitis (EAE), we immunized Lewis rats subcutaneously in both hind feet with inoculum containing guinea pig myelin basic protein emulsified with Freund’s complete adjuvant containing Mycobacterium tuberculosis. Neurological assessments demonstrated progressive development of tail and hindlimb paralysis, leading to paraplegia and loss of the righting reflex. The neurological decline started 11 days (11.13 ± 0.21; n = 40) after immunization, peaked after 13 days (13.08 ± 0.25), and waned after 16 days (15.75 ± 0.22) (Fig. 1). Rats showed a progressive loss of as much as 21% in body weight (maximal weight before onset, 207.74 ± 1.99 g compared with before, minimal weight after onset, 163.99 ± 2.22 g) starting 10 days (9.78 ± 0.21; n = 40) after immunization.

Morphological analysis by light and electron microscopy showed the presence of inflammation throughout lumbar spinal cord, with characteristic perivascular cuffs, lymphocytes and macrophages in white and gray matter. Demyelination was limited to root entry and exit zones. There was some edema in the white matter of dorsal columns at the peak of neurological symptoms. Ultrastructurally, lymphocytes initially approached motoneurons, attached to their membranes (Fig. 2a) and were subsequently internalized (Fig. 2b). Integrated lymphocytes within neurons appeared morphologically normal and were surrounded by a membrane forming a vacuolar structure (Fig. 2b). Nuclear chromatin in lymphocytes trapped in motoneurons formed clumps attached to the nuclear membrane. After the disintegration of the nuclear membranes, lymphocytic nucleoplasm and cytoplasm intermixed with apoptotic bodies were formed. At late stages of lymphocyte breakdown within intraneuronal vacuoles, cell masses and the apoptotic bodies were transformed into amorphous debris (Fig. 2c). As the process of sequestration of lymphocytes into motoneurons continued, the neuronal cytoplasm became overwhelmed, whereas the nucleus remained intact (Fig. 2c). At the end of this process, motoneurons decomposed and formed large circular vacuoles filled with apoptotic lymphocytes and amorphous cellular debris (Fig. 2d).

Analysis of the outer membranes of cells filled with lymphocytes showed that synaptic densities and presynaptic endings were preserved (Fig. 2b and c, insets), confirming their neuronal origin. The appearance in the spinal cord of neuronal vacuoles containing lymphocytes undergoing apoptosis correlated with clinical stages of the disease, with few vacuoles being detected at disease onset and the numbers increasing concomitant with highest disability scores and decreasing on recovery.

Morphometric analysis showed that the density of neurons in the ventral horns of the lumbar spinal cord decreased during the course of EAE by 30% (Table 1). There were no changes in the density of neurons in the intermediate and dorsal portions of the lumbar spinal cord (Table 1).

To determine whether non-neuronal cells were involved in the interaction with lymphocytes, we used immunohistochemistry for glial fibrillary acidic protein to identify astrocytes. In spinal cord sections from rats undergoing EAE at the peak of motor decline, cells positive for glial fibrillary acidic protein were distinct from those that contained lymphocytes (Fig. 3c). To determine the cell type entering neurons during acute EAE, we treated spinal cord sections with monoclonal antibodies specific...
for T, B, and natural killer lymphocytes, or macrophages/microglia. The cells entering neurons during the acute phase of EAE stained positive for CD2 but not for CD45RA, CD161 or ED1, showing that T lymphocytes were involved and not B or natural killer lymphocytes, or macrophages/microglia (Fig. 3a and b).

Spinal motoneurons have high densities of glutamate receptors and are sensitive to toxicity mediated by glutamate through non-NMDA (N-methyl-D-aspartate) receptors. Activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainate receptors mediates fast neurotransmission in the brain and spinal cord17. Administration of AMPA or kainate into the rat lumbar cord rapidly precipitates hindlimb weakness11.

To determine whether AMPA/kainate receptor antagonists affect the clinical and morphological outcome of acute EAE, we administered two competitive antagonists, NBQX (ref. 12) and MPQX (ref. 13), and two noncompetitive antagonists, GYKI52466 and GYKI53773 (ref. 14), intraperitoneally to immunized rats. Treatment with 30 mg/kg NBQX twice daily for 7 days starting on day 10 after immunization improved the neurological outcome, reducing the cumulative score from 11.06 ± 0.48 in vehicle-treated rats (n = 40) to 5.85 ± 0.98 in NBQX-treated rats (n = 30) (P < 0.001, Mann-Whitney U test) (Fig. 1a) and limiting neuronal death in the spinal cord (Table 1). NBQX at a dose of 30 mg/kg also decreased weight loss during the course of EAE by 19% (43.75 ± 1.19 g in vehicle-treated rats compared with 35.39 ± 1.84 g in NBQX-treated rats; P < 0.001, Student’s t-test), but had no effect on weight loss at doses of 10 mg/kg (n = 10) and 3 mg/kg (n = 10). Treatment with 10 mg/kg MPQX twice daily for 7 days starting on day 10 after immunization reduced the cumulative disease score from 10.95 ± 0.56 in vehicle-treated rats (n = 26) to 3.15 ± 1.29 in MPQX-treated rats (n = 16) (P < 0.001, Mann-Whitney U test). MPQX given twice daily at a dose of 5 mg/kg also reduced disease severity, decreasing the cumulative score to 8.35 ± 1.91 (n = 16), but it had no effect at a dose of 2.5 mg/kg (11.60 ± 1.43; n = 10) (Fig. 1b). MPQX at doses of 2.5–10 mg/kg had no effect on weight loss. GYKI52466 given to rats intraperitoneally at a dose of 40 mg/kg at 0, 6, 12, 18 and 24 hours on day 10 after immunization improved the clinical outcome, reducing the cumulative score from 13.81 ± 0.58 in vehicle-treated rats (n = 13) to 9.51 ± 0.55 in GYKI52466-treated rats (n = 12) (P < 0.001, Mann-Whitney U test). Treatment with 30 mg/kg GYKI52466 or GYKI53773, twice daily for 7 days starting on day 10 after immunization, improved the neurological outcome, reducing the cumulative score from 9.53 ± 1.71 (n = 9) in vehicle-treated rats to 6.90 ± 1.37 in GYKI52466-treated rats (n = 10) or 2.13 ± 0.70 in GYKI53773-treated rats (n = 10) (P < 0.001, Mann-Whitney U test). Treatment with 30 mg/kg GYKI52466 or GYKI53773 had no effect on weight loss.

To determine whether AMPA antagonists interact with the induction of immunization, we administered 30 mg/kg NBQX intraperitoneally to rats twice daily for 7 days starting 4 days after immunization. This treatment regimen did not affect the cumulative clinical score (11.40 ± 1.22, vehicle-treated (n = 118.59 ± 14.96 in NBQX-treated rats; not significant, Mann-Whitney U test) or decrease weight loss compared with that of vehicle-treated rats. We also used adoptive transfer of EAE in rats to determine whether AMPA antagonists interact with immunization. We transferred myelin basic protein-sensitized splenic lymphocytes from immunized rats to naive rats and began treatment with 30 mg/kg NBQX twice daily for 7 days on day 4 after transfer. Seven of eight rats given vehicle alone showed disability ranging from flaccid tail to hindlimb weakness, whereas one of eight rats treated with NBQX showed mild changes in tail tone and none showed motor disability (P < 0.005, compared with vehicle-treated rats, χ²-test).

To determine whether NBQX mitigates the CNS inflammation triggered by immunization, we analyzed sections of rat brainstem for the presence of perivascular cuffs. Treatment with 30 mg/kg NBQX twice daily for 7 days starting on day 10 after immunization improved the neurological outcome, reducing the cumulative scores from 10.33 ± 0.56 in vehicle-treated rats (n = 17) to 4.04 ± 1.04 in NBQX-treated rats (n = 17) (P < 0.001, Mann-Whitney U test). However, this treatment did not affect inflammation (histopathological scores: 166.94 ± 23.26 in vehicle-treated rats and 118.59 ± 14.96 in NBQX-treated rats; not significant, Mann-Whitney U test).

To assess whether AMPA antagonists improved disability in EAE because of immunosuppression, we studied the effects of NBQX, MPQX and of the glucocorticoid dexamethasone on immunogen-induced lymphocyte proliferation. Neither NBQX nor MPQX, at concentrations up to 100 µM, influenced incorpora-
Fig. 2 Electron micrographs of the ventral horn of the lumbar spinal cord from Lewis rats immunized with myelin basic protein, at the peak of the disease course, showing different stages of degeneration of motoneurons associated with lymphocyte entry. a, A lymphocyte (magnified in inset) is attached to the membrane of the motoneuron. b, A lymphocyte that has invaded the motoneuron is entirely engulfed by its cytoplasmic membrane and is beginning to undergo apoptosis. The host motoneuron seems intact. c, Several lymphocytes have entered the motoneuron, are filling up its cytoplasm and are in different stages of apoptosis. d, A decomposed motoneuron forms a vacuole filled with lymphocytes and amorphous cellular debris. The presence of synaptic densities on the cytoplasmic membranes (arrows; magnified in insets, a and d) identifies the cells as neurons. Original magnifications: × 3,500 (a), × 15,210 (a, inset), × 5,232 (b), × 35,900 (b, inset), × 3,486 (c), × 34,620 (c, inset), × 3,815 (d) and × 50,880 (d, inset).

Fig. 3 Light immunomicrographs showing that T lymphocytes but not macrophages/microglia or astrocytes invade motoneurons. a, A cell staining positive with OX34 specific for CD2 on T lymphocytes is present inside a motoneuron (arrow). b, Cells staining positive with ED1, which recognizes a lysosomal membrane-related antigen on macrophages/microglia, are present outside motoneurons and do not show propensity to invade them. c, Cells positive for glial fibrillary acidic protein (astrocytes) are present outside motoneurons and do not invade them. Similarly, cells positive for OX33 (specific for CD45RA on B lymphocytes) or clone 10/78 (specific for CD161 on natural killer lymphocytes) do not invade motoneurons. Original magnification, × 360; counterstained with hematoxylin.

Treatment with AMPA antagonists NBQX, MPQX, GYKI52466 and GYKI53773, begun at the onset of neurological decline, caused a profound reduction in the neurological deficits of acute EAE. The beneficial clinical effects of AMPA antagonists cannot be attributed to anti-inflammatory or immunomodulatory actions, as NBQX had no effect on neuroinflammation (perivascular cuffs), was effective in adoptive transfer EAE, and NBQX and MPQX did not inhibit the concanavalin A-induced proliferation of T lymphocytes. The reduction of neurological sequelae of acute EAE by AMPA antagonists and the beneficial effects of NBQX in chronic relapsing EAE substantiate the possibility of the involvement of glutamate in the disease pathogenesis.

Increased concentrations of glutamate in the cerebrospinal fluid of patients suffering from multiple sclerosis have been re-
null significantly improved neurological status in mice (F28-48(1,20)=2.76, or vehicle (n

nullmunization (stippled bar). Treatment with NBQX on days 10–16 after immunization significantly improved neurological outcome (hatched bar). Treatment with NBQX on days 26–42 after immunization also significantly improved neurological status in mice (F28-48(1,20)=2.76, P< 0.05). Data represent means ± s.e.m.

nullNeurological deficits were monitored daily during the course of EAE by a single observer ‘blinded’ to treatment status, before administration of vehicle or drugs, to ensure that the motor side effects of therapy with AMPA antagonists such as sedation or reduction of muscle tone did not interfere with disability scores. The following scoring system was used to grade neurologic impairment: 0, no detectable changes in muscle tone and motor behavior; 1, flaccid tail; 2, impairment of righting reflex and/or loss of muscle tone in hindlimbs; 3, complete hindlimb paralysis; 4, paraplegia; and 5, death. During the observation period, rats were housed in pairs in environmentally controlled conditions (6:00–18:00, 12-hour light/dark cycle; 24–25 °C) and were permitted free access to food and water. Body weight was monitored daily with a Sartorius model U 6100 balance.

nullMorphology. Rats were anesthetized with an overdose of pentobarbital and perfused with a fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (for combined light and electron microscopy). Coronal sections of the lumbar spinal cord were cut 10–15 μm thick, mounted on a glass slide and stained with hematoxylin and eosin or cresyl violet. For electron microscopy, the tissue was processed in osmium tetroxide and uranyl acetate, dehydrated in a series of increasing ethanol concentrations, ‘cleared’ in propylene oxide, embedded in araldite and viewed by transmission electron microscope. For light microscopy, ‘semi-thin’ coronal sections of the lumbar spinal cord 1 μm in thickness were cut and stained with toluidine blue.

nullQuantification of neuronal density in the spinal cord. To provide an estimate for possible neuronal loss in the spinal cord over the period of up to 16 d after immunization, numerical densities of neurons in the ventral and dorsal horns and in the intermediate zone of the lumbar cord were determined using a stereologic dissector13,14. An unbiased counting frame (0.1 mm × 0.1 mm; dissector height, 0.015 mm) and a high-aperture objective (x100) were used for the sampling13. Normal neurons were identified by the presence of the typical nuclei with clear nucleoplasm and distinct nucleolus surrounded by cytoplasm. An arbitrary horizontal line connecting the lateral ends of the spinal cord and crossing the ventral edge of the central commissure was considered the junction between the intermediate zone and the ventral horns. An arbitrary parallel horizontal line crossing the dorsal edge of the central commissure was considered the junction between the intermediate zone and the dorsal horns. The gray matter of the ventral horns contains motoneurons controlling function of the limbs and trunk, and interneurons. The intermediate zone contains sensory neurons projecting towards the cerebellum, pregangionic autonomic neurons and interneurons. The dorsal horns contain sensory projection neurons connecting supraspinal centers.

nullImmunohistochemistry. Immunohistochemical analysis of the lumbar spinal cord was done in rats with scores 3–4 at the peak of the disease course. The following murine monoclonal antibodies (all from Serotec, Raleigh, North Carolina, except 5.2E4: Institute of Neurology, London, UK) were used at the following dilutions to examine resident and infiltrating cells in the spinal cord: OX34 (1:500), specific for CD2 on T lymphocytes; OX33 (1:100), specific for CD45RA on B lymphocytes; clone 10/78 (1:100), specific for CD161 on natural killer lymphocytes; ED1 (1:500), which recognizes a lysosomal membrane-related antigen on rat macrophages/microglia; OX-42 (1:3,000), which binds the complement receptor 3 on microglia/macrophages; and 5.2E4 (1:1,000), for glial fibrillary acidic protein staining of astrocytes. Freshly frozen spinal tissue, cut into sections 10 μm in thickness, was fixed in ethanol, incubated with the primary antibody, and washed before a biotinylated rat-adsorbed antibody against mouse IgG (1:200 dilution; Vector Laboratories, Burlingame, California) was added. Peroxidase-labeled avidin–biotin complex (ABC; Vector Laboratories, Burlingame, California) was added to the sections, and peroxidase activity was detected in a 3,3′-diaminobenzidine (Sigma) solution in phosphate-buffered saline containing 0.01% hydrogen peroxide. Rinsed sections were counterstained in hematoxylin, dehydrated in a series of increasing alcohol concentrations, ‘cleared’ in xylene and mounted on glass slides. Sections with no primary antibody were included with each staining, as controls.

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Adaptive transfer of EAE. Ten days after being immunized with guinea pig myelin basic protein, Lewis rats were killed and their spleens were removed. Splenic lymphocytes were cultured for 72 h at 37 °C in an atmosphere of 5% CO2 and 95% O2; at a concentration of 2 × 107 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 µM 2-mercaptoethanol and 1 mg/ml indomethacin with 10 µg/ml myelin basic protein. Collected lymphocytes were washed in unsupplemented RPMI 1640, and 4 × 107 cells were transferred intraperitoneally to non-immunized recipients.

Quantification of neuroinflammation. Sections of rat brainstem (antero–posterior, −7.0 to −15.0; ventral, −8.60) (ref. 25) 10 mm in thickness were stained with hematoxylin and assessed for the presence of perivascular cuffs by an observer ‘blinded’ to sample identity. The following scoring system was used to grade histopathological changes induced by inflammation: 0, no detectable changes; 1, perivascular inflammation of up to 50% of the circumference of the vessel; 2, perivascular inflammation of up to 75% of the circumference of the vessel; 3, perivascular inflammation of more than 75% of the circumference of the vessel; and 4, parenchymal cell infiltrates. The histopathological score was calculated by adding all scores for lesions detected in a given section, and the mean of two sections was used for statistical analysis.

Quantification of immunosuppression in an in vitro stimulation assay. Splenic lymphocytes from Lewis rats were incubated at a concentration of 1 × 106 cells/ml in RPMI 1640 modified medium with 1 µg/ml concanavalin A and increasing concentrations of AMPA antagonists (0.01, 0.1, 1, and 100 µM) or dexamethasone (0.001, 0.01, 0.1 and 1 µM). Then, 96 h after the addition of the stimulating mitogen, 1 µCi [3H]-thymidine was added for an additional 6 h, then the cells were collected and incorporated radioactivity was measured. Lymphoproliferation in response to the lectins phytohemagglutinin or concanavalin A requires engagement of the T-lymphocyte receptor and synthesis of the T-lymphocyte growth factor interleukin-2. Agents that inhibit lymphoproliferation, such as dexamethasone, show immunosuppressive action.

Induction of chronic relapsing EAE. Spinal cords from Bizio mice (Ab/H, H-2ae), body weight 20–22 g; Harlan UK, Bicester, UK) were homogenized and freeze-dried. Lyophilized spinal cord homogenate was reconstituted in phosphate-buffered saline to a final concentration of 6.6 mg/ml. Incomplete Freund’s adjuvant was supplemented with M. tuberculosis (H37Ra; and M. butyricum (both from Difco, Detroit, Michigan) in a ratio of 8:1. Bizio mice were immunized subcutaneously in the flank at three sites on day 0 and day 7 with 0.3 ml of the emulsion (1 mg spinal cord homogenate and 60 µg of combined M. tuberculosis and M. butyricum). In addition, mice were treated intraperitoneally with 200 ng Bordetella pertussis toxin (2 µg/ml in phosphate-buffered saline; Calbiochem, La Jolla, California) immediately and 24 h after immunization with neurotoxins. Mice were treated intraperitoneally with 30 mg/kg NBQX twice daily on days 10–16 after immunization or once daily on days 26–42 after immunization. Neurological deficits were monitored daily by an observer ‘blinded’ to treatment status, before administration of vehicle or drugs. The following scoring system was used to grade neurological impairment: 0, no detectable changes; 1, flaccid tail; 2, impairment of righting reflex and/or mass weight loss; 3, complete hindlimb paralysis; 4, paraplegia; and 5, death. During the observation period, mice were housed in pairs in environmentally controlled conditions (6:00–18:00, 12-hour light/dark cycle; 24–25 °C) and were permitted free access to food and water.

Statistics. Data were analyzed statistically by means of analysis of variance, Student’s t-test, Mann-Whitney U test, χ2 test, and Fisher’s protected least-significant-difference test.

Acknowledgments

The assistance of M. Turmaine (University College London, UK) and L. Rivers is acknowledged. We thank C. Ikonomidou (Humboldt University, Berlin) for criticism and advice on electron microscopy and stereology. We also thank Y. Machida and Y. Yamanishi for advice and support.

RECEIVED 29 JUNE; ACCEPTED 21 OCTOBER 1999