Autoimmune encephalomyelitis ameliorated by AMPA antagonists

TERENCE SMITH, ANTHONY GROOM, BIN ZHU & LECHOSLAW TURSKI

Eisai London Research Laboratories, Bernard Katz Building, University College London, Gower Street, London WC1E 6BT, UK

Correspondence should be addressed to L.T. or T.S.; email: lechoslaw.turski@solvay.com or terence_smith@eisai.net

Multiple sclerosis is an immune-mediated disorder of the central nervous system leading to progressive decline of motor and sensory functions and permanent disability[1,2]. The therapy of multiple sclerosis is only partially effective, despite anti-inflammatory, immunosuppresive and immunomodulatory measures[3]. White matter inflammation and loss of myelin, the pathological hallmarks of multiple sclerosis, are thought to determine disease severity[4,5]. Experimental autoimmune encephalomyelitis reproduces the features of multiple sclerosis in rodents and in nonhuman primates[6,7]. The dominant early clinical symptom of acute autoimmune encephalomyelitis is progressive ascending muscle weakness[6]. However, demyelination may not be profound and its extent may not correlate with severity of neurological decline[8], 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 in 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. 2*a*) and were subsequently internalized (Fig. 2*b*). Integrated lymphocytes within neurons appeared morphologically normal and were surrounded by a membrane forming a vacuolar structure (Fig. 2*b*). 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 and 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. 2*c*). As the process of sequestration of lymphocytes into motoneurons continued, the neuronal cytoplasm became overwhelmed, whereas the nucleus remained intact (Fig. 2*c*). At the end of this process, motoneurons decomposed and formed large circular vacuoles filled with apoptotic lymphocytes and amorphous cellular debris (Fig. 2*d*). Analysis of the outer membranes of cells filled with lymphocytes showed that synaptic densities and presynaptic endings were preserved (Fig. 2*b* 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. 3*c*). To determine the cell type entering neurons during acute EAE, we treated spinal cord sections with monoclonal antibodies specific

Table 1 Density of neurons in the lumbar spinal cord of rats subjected to EAE, and the effect of NBQX on cell loss in the ventral horns

	Density of neurons (N_V)						
Treatment	Ventral horns	%	intermediate zone	%	Dorsal horns	%	n
Sham + Vehicle	11,746 ± 408	100	38,952 ± 820	100	77,747 ± 1,353	100	7
EAE + Vehicle	8,264 ± 386*	70	36,934 ± 2,056	95	72,098 ± 2,701	93	8
EAE + NBQX	10,250 ± 715**	87	39,040 ± 1,685	100	76,983 ± 2,661	99	8

Neuronal loss was estimated 13–16 days after immunization with myelin basic protein. Data represent mean ± s.e.m. cells/mm^3. N_V, numerical density. *$P < 0.001$, compared with sham-immunized, vehicle-treated rats; **, $P < 0.05$, compared with immunized, vehicle-treated rats; Student's *t*-test.

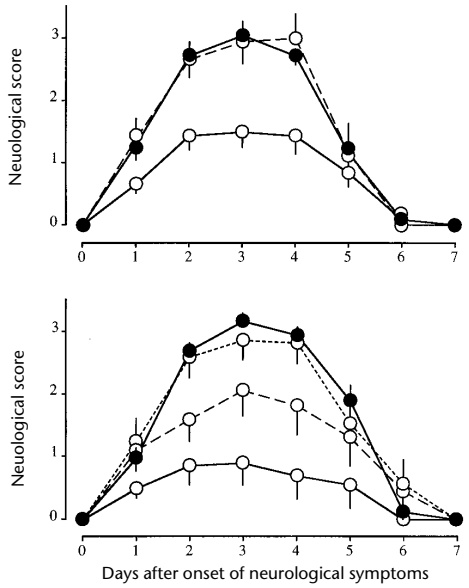

Fig. 1 Dose–response relationship of AMPA/kainate antagonists on motor disability induced by autoimmune encephalomyelitis in rats. NBQX, MPQX or vehicle were administered intraperitoneally twice daily for 7 d starting on day 10 after immunization. ***a***, ○, NBQX at a dose of 3 mg/kg (n = 10; dashed line) or 30 mg/kg (n = 30; solid line); ●, vehicle (n = 40). NBQX reduced disability in rats subjected to EAE ($F(3,76) = 14.86$, $P < 0.001$, analysis of variance with multiple comparisons). NBQX at a dose of 30 mg/kg reduced disability compared with vehicle ($F(1,62) = 36.24$, $P < 0.001$) and either 3 mg/kg NBQX ($F(1,31) = 32.54$, $P < 0.01$) or 10 mg/kg NBQX ($F(1,29) = 32.94$, $P < 0.01$) (n = 10), whereas 3 and 10 mg/kg NBQX did not differ from each other or vehicle. Onset of neurological symptoms was not affected by 3–30 mg/kg NBQX, whereas 30 mg/kg NBQX shortened duration of the disease ($P < 0.001$, Fisher's protected least-significant-difference test). ***b***, ○, MPQX at a dose of 2.5 mg/kg (n = 10; dotted line), 5 mg/kg (n = 10; dashed line), 10 mg/kg (n = 12; solid line); ●, vehicle (n = 26). MPQX reduced disability in rats subjected to EAE in a dose-dependent manner ($F(3,42)=13.40$, $P < 0.001$). MPQX at a dose of 10 mg/kg reduced disability compared with vehicle ($F(1,27) = 55.83$, $P < 0.001$) and 2.5 mg/kg MPQX ($F(1,15) = 54.97$, $P < 0.001$), whereas 2.5 and 5 mg/kg MPQX did not differ from each other or vehicle. Onset of neurological symptoms was not affected by 2.5–10 mg/kg MPQX, whereas 10 mg/kg MPQX shortened duration of the disease ($P < 0.001$, Fisher's protected least-significant-difference test). Data represent means ± s.e.m.

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. 3*a* 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[9]. Activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainate receptors mediates fast neurotransmission in the brain and spinal cord[10]. Administration of AMPA or kainate into the rat lumbar cord rapidly precipitates hindlimb weakness[11].

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 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. 1*a*) 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. 1*b*). 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 = 10); 9.38 ± 0.82, NBQX-treated (n = 10); 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[6] 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, χ^2-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-

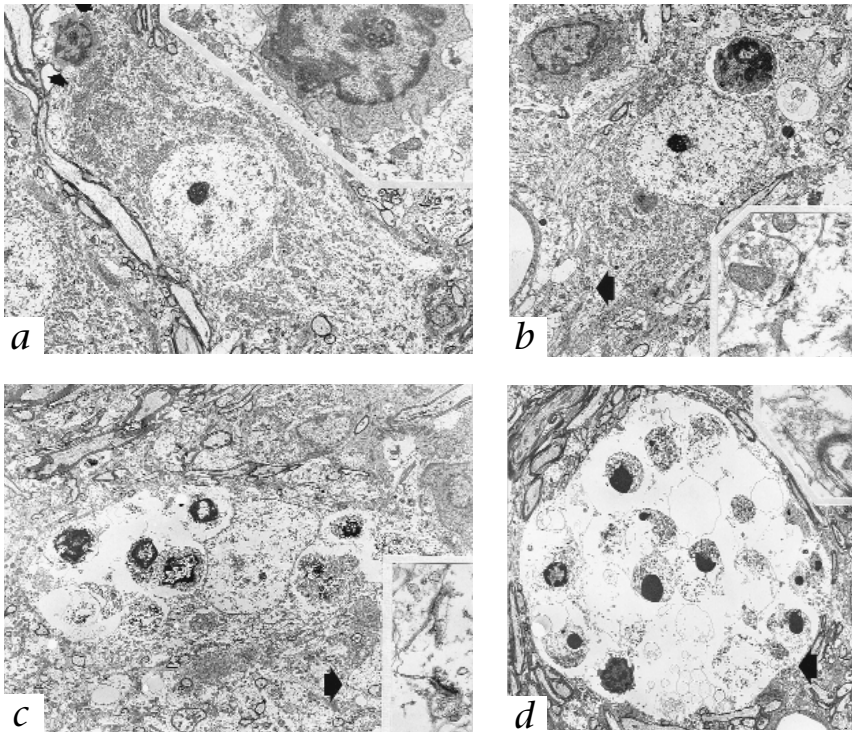

Fig. 2 Electron micrographs of the ventral horn of the lumbar spinal cord from Lewis rats immunized with myelin basic protein, at the peak of the disease course, showing different stages of degeneration of motoneurons associated with lymphocyte entry. ***a***, A lymphocyte (magnified in inset) is attached 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, *b*, *c* 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).

tion of thymidine into T lymphocytes, whereas dexamethasone was effective, with an ED_{50} (dose required ti suppress thymidine incorporation by 50%) of 0.43 μM (range, 0.32–0.59 μM.

To determine whether AMPA/kainate antagonists affect clinical outcome of chronic relapsing EAE (ref. 6), we administered NBQX intraperitoneally to immunized mice. Treatment with 30 mg/kg NBQX twice daily for 7 days starting on day 10 after immunization improved the neurological outcome, reducing disease severity between days 10 and 48 after immunization ($F(1,38)=9.21$; $P < 0.001$) (Fig. 4*a*). Treatment with 30 mg/kg NBQX once daily for 17 days beginning on day 26 after immunization also reduced disease severity between days 28 and 48 after immunization ($F(1,20)=2.76$; $P < 0.05$) (Fig. 4*b*).

Ultrastructural analysis of the spinal cord in rats has indicated involvement of neurons as well as non-neuronal cell populations (lymphocytes, macrophages and oligodendrocytes) in the pathogenesis of EAE. Lymphocytes initiate inflammation during EAE, and the degree of infiltration of spinal cord by T lymphocytes correlates with the time course of the disease[8]. T lymphocytes may approach and enter neurons during acute EAE. Temporary internalization of a morphologically normal cell by another cell without morphological or functional harm to both host and guest cells is known as emperipolesis[15]. Emperipolesis is different from phagocytosis, during which morphologically altered cells or cell debris are internalized and disposed of[16]. The interaction between lymphocytes and motoneurons in the course of acute EAE shares features with both emperipolesis and phagocytosis.

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-

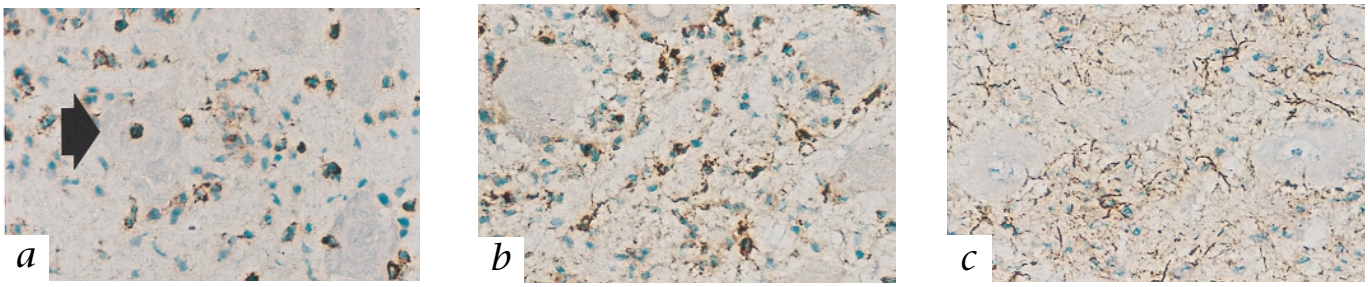

Fig. 3 Light immunomicrographs showing that T lymphocytes but not macrophages/microglia or astrocytes invade motoneurons. ***a***, A cell staining positive with OX34 specific for CD2 on T lymphocytes is present inside a motoneuron (arrow). ***b***, Cells staining positive with ED1, which recognizes a 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.

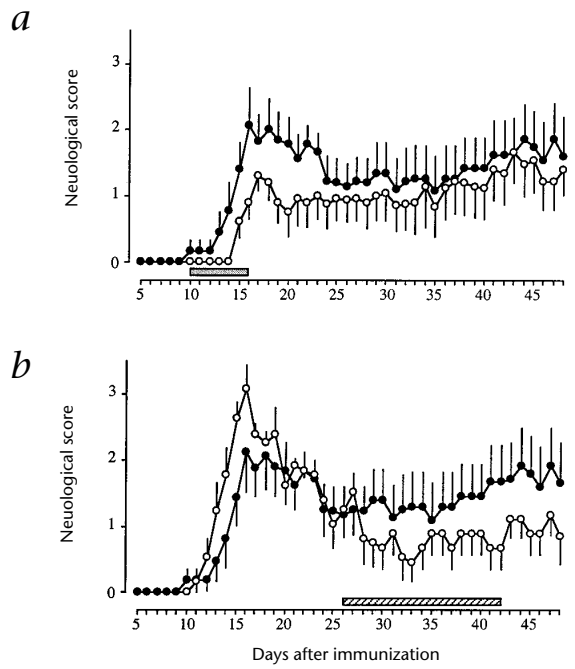

Fig. 4 Effect of AMPA/kainate antagonist NBQX on chronic relapsing EAE in mice. ***a***, Mice were given 30 mg/kg NBQX (n = 10; open circles) or vehicle (n = 9; ●) intraperitoneally twice daily on days 10–16 after immunization (stippled bar). ***b***, Mice were given 30 mg/kg NBQX (n = 7; ○) and vehicle (n = 10; ●) intraperitoneally once daily on days 26–42 after immunization (hatched bar). Treatment with NBQX on days 10–16 after immunization significantly improved neurological outcome ($F_{10\text{-}48}(1,38)=9.21$, $P < 0.001$, repeated measures analysis of variance); delayed treatment with NBQX on days 26–42 after immunization also significantly improved neurological status in mice ($F_{28\text{-}48}(1,20)=2.76$, $P < 0.05$). Data represent means ± s.e.m.

ported[17]. The activity of glutamine synthetase and glutamate dehydrogenase is compromised in astrocytes during EAE, affecting the reuptake of glutamate[18]. AMPA receptors mediate the death of oligodendrocytes *in vitro*[19], whereas microinfusions of kainate into the optic tract in rodents produce demyelination[20]. These observations indicate that glutamate may target both non-neuronal and neuronal elements in the spinal cord to induce neurological decline during EAE. Thus, the clinical and morphological changes in EAE may be attributed to the 'unleashed' toxic action of glutamate or related agents on various cellular populations in the spinal cord.

These conclusions may be relevant for the therapy of multiple sclerosis and perhaps other demyelinating disorders. The treatment of multiple sclerosis relies exclusively on immunosuppressive agents[3]. Interferon-β has proven efficacious in the relapsing/remitting form of multiple sclerosis[3]. Our results indicate that therapeutic strategies for multiple sclerosis should be complemented by neuroprotective measures to reduce neurological disability and neuronal injury.

Methods

Induction of EAE. Lewis rats (200–220 g in body weight; Charles River, Manson, UK) were immunized subcutaneously in the dorsal surface of each hind foot with 50 μl inoculum containing 50 μg guinea pig myelin basic protein, prepared according to a published method[21,22] and emulsified in Freund's complete adjuvant containing 5.5 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, Michigan). 'Sham-immunized' rats received Freund's complete adjuvant containing *M. tuberculosis* alone "subcutaneously".

Neurological assessment. Neurological 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.

Morphology. 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, 'semithin' coronal sections of the lumbar spinal cord 1 μm in thickness were cut and stained with toluidine blue.

Quantification 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 dissector[23,24]. An unbiased counting frame (0.1 mm x 0.1 mm; dissector height, 0.015 mm) and a high-aperture objective (x100) were used for the sampling[24]. 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, preganglionic autonomic neurons and interneurons. The dorsal horns contain sensory projection neurons connecting supraspinal centers.

Immunohistochemistry. 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 (1:100); 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-absorbed 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.

Treatment regimen. Rats were treated with the following competitive AMPA/kainate antagonists intraperitoneally twice daily for 7 days starting on day 10 after immunization: 3–30 mg/kg NBQX (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline) or 2.5–10 mg/kg MPQX ([1,2,3,4-tetrahydro-7-morpholin-yl-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methylphosphonate). This treatment regimen was chosen to ensure that sufficient concentrations were achieved in the brain to interact with AMPA/kainate receptors[12,13]. Rats received 40 mg/kg GYKI52466 (non-competitive AMPA antagonist 1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-5H-2,3-benzodiazepine) intraperitoneally at 0, 6, 12, 18 and 24 h on day 10 after immunization. This treatment regimen allows for maintenance of relevant concentrations of GYKI52466 in the brain for several days[14]. Rats were treated with 30 mg/kg of the non-competitive AMPA antagonists GYKI52466 and GYKI53773 ((-)1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-4,5-dihydro-3-methylcarbamoyl-2,3-benzodiazepine) intraperitoneally twice daily for 7 days starting on day 10 after immunization. This treatment regimen was used to ensure that relevant concentrations in the brain to interact with AMPA dependent ion channels were reached[14]. NBQX, MPQX, GYKI52466, GYKI53773 and vehicle were administered in a volume of 0.5 ml/100 g body weight.

Adoptive 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% CO_2 and 95% O_2 at a concentration of 2×10^6/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 4×10^7 cells were transferred intraperitoneally to non-immunized recipients.

Quantification of neuroinflammation. Sections of rat brainstem (anterior–posterior, –7.0 to –15.0; ventral, –8.60) (ref. 25) 10 μm 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 three cell layers; 2, perivascular inflammation of more than three cell layers; and 3, 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 immunosuppresion in an *in vitro* stimulation assay. Splenic lymphocytes from Lewis rats were incubated at a concentration of 1×10^6 cells/ml in RPMI 1640 modified medium with 1 μg/ml concanavalin A and increasing concentrations of AMPA antagonists (0.01, 1, 10 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 ^{3}H-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 immunosuppresive action.

Induction of chronic relapsing EAE. Spinal cords from Biozzi mice (Ab/H, H-2^{dq1}, 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. Biozzi 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 injected 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 neuroantigens. Mice were treated intraperitoneally with 30 mn/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 loss of muscle tone; 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, χ^2 test, and Fisher's protected least-significant-difference test.

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