N-Methyl-D-Aspartate Receptor Blockade Is Neuroprotective in Experimental Autoimmune Optic Neuritis

Kurt-Wolfram Sühs, MD, Richard Fairless, PhD, Sarah K. Williams, PhD, Katrin Heine, Adolfo Cavalié, PhD, and Ricarda Diem, MD

Abstract

Optic neuritis is a common clinical manifestation of the chronic inflammatory CNS disease multiple sclerosis that can result in persistent visual impairment caused by degeneration of optic nerve axons and apoptosis of retinal ganglion cells (RGCs). Using a model of experimental autoimmune encephalomyelitis with optic neuritis (Brown Norway rats), we show that administration of the N-methyl-D-aspartate (NMDA) receptor antagonists memantine or MK801 results in RGC protection, axon protection, and reduced demyelination of optic nerves. Calcium imaging revealed that RGC responses to glutamate stimulation predominantly occurred via NMDA receptors and were inhibited by memantine in a dose-dependent manner. In contrast, oligodendrocytes were mainly responsive through the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor. This suggests that NMDA receptor blockade protected RGCs directly and that the protection was independent of effects on oligodendrocytes. Moreover, increased RGC survival was observed before the onset of optic nerve demyelination—when RGC degeneration had already started. These results indicate an important pathophysiologic role for NMDA receptor–mediated glutamate toxicity during the induction phase of this disease model and highlight a potential target for therapeutic neuroprotection in human optic neuritis.

Key Words: Glutamate, Memantine, Multiple sclerosis, Neurodegeneration, Neuroprotection, NMDA receptor, Optic neuritis, Retinal ganglion cells.

INTRODUCTION

Optic neuritis is a frequent and early event in patients with multiple sclerosis (MS) that can result in clinically significant decreases in visual acuity in 30% to 50% of patients (1). Persistent visual impairment after optic neuritis is caused by neurodegenerative changes within optic nerves that can be detected by magnetic resonance imaging after only a single episode of optic nerve inflammation (2, 3). Optical coherence tomography in patients with optic neuritis further suggests degeneration of retinal ganglion cells (RGCs), the axons of which form the optic nerve (4).

Experimental autoimmune encephalomyelitis (EAE) induced by immunization with myelin oligodendrocyte glycoprotein (MOG) leads to optic neuritis in 80% to 90% of female Brown Norway (BN) rats. In previous work, we have shown that this model closely resembles the optic nerve axon and RGC degeneration present in patients with optic neuritis (5–8).

A major cause of neurodegeneration in many disease models (e.g. brain trauma and cerebral ischemia) is glutamate-mediated excitotoxicity; both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors have been implicated in these conditions (9). Support for a similar role of excitotoxicity in MS comes from elevated levels of glutamate found in the cerebrospinal fluid of patients with MS (10, 11) and also in brain lesions, as assessed by magnetic resonance spectroscopy (12). In addition, we found elevated concentrations of glutamate in the retina of rats around the time point of maximal inflammatory cell infiltration within the optic nerves in our autoimmune optic neuritis model (13).

The receptor subtypes potentially responsible for glutamate-mediated excitotoxicity in EAE are, at present, unclear. Previous studies have shown that inhibiting AMPA/kainate glutamate receptors leads to myelin protection and neuroprotection in autoimmune inflammatory disease models, again demonstrating the pathogenetic role of excitotoxicity (14, 15). In contrast, the role of NMDA receptors is not well established; however, a study using the noncompetitive NMDA receptor antagonist memantine suggested the involvement of NMDA receptors in blood-brain barrier dysfunction in EAE (16). In contrast to these findings, memantine did not influence inflammatory cell infiltration in another study, although it did ameliorate the clinical course of EAE (17). Because NMDA receptors are present in RGCs (18, 19), we wished to assess the contribution of NMDA receptor activation...
to neurodegeneration in autoimmune optic neuritis and whether this activation could be targeted.

In the present study, we show that blockade of NMDA receptors in experimental optic neuritis protects both neurons and myelin, resulting in sparing of RGCs and oligodendrocytes. Using calcium imaging, we demonstrate that NMDA receptor blockade acts specifically on RGCs and has almost no effect on oligodendrocytes. Furthermore, by analyzing early time points in our disease model, we demonstrate that this protective effect on RGCs can be detected before inflammatory changes and demyelination are observed in the optic nerves. This suggests that blockade of NMDA receptors directly protects RGCs from MOG-induced neurodegeneration, independently of protective effects on demyelination. Targeting of NMDA receptors may therefore prove to be an effective strategy for protecting neurons against neurodegeneration in patients with MS.

MATERIALS AND METHODS

Rats

Female BN rats (8–10 weeks old) were used in the experiments. They were obtained from Charles River (Sulzfeld, Germany) and kept under environmentally controlled conditions without pathogens. All experiments involving animal use were performed in compliance with relevant laws and institutional guidelines. These experiments have been approved by the local authorities of Saarpfalz-Kreis and Karlsruhe, Germany.

Immunogen, Induction, and Evaluation of EAE

Recombinant rat MOG was synthesized as previously described (20) and injected subcutaneously at the base of the tail. It was administered at a dose of 100 μg in saline emulsified (1:1) with complete Freund adjuvant (Sigma, St Louis, MO) containing 200 μg of heat-inactivated Mycobacterium tuberculosis (strain H 37 RA; Difco Laboratories, Detroit, MI) to a total volume of inoculum of 200 μL. Rats were scored daily for clinical signs of EAE using a scale from 0 to 4, as previously described (5): Grade 0, no signs; Grade 0.5, distal paresis of the tail; Grade 1, complete tail paralysis; Grade 1.5, paresis of the tail and mild hindleg paresis; Grade 2.0, unilateral severe hindleg paresis; Grade 2.5, bilateral severe hindlimb paresis; Grade 3.0, complete bilateral hindlimb paralysis; Grade 3.5, complete bilateral hindlimb paralysis and paresis of a front limb; Grade 4, complete paralysis (tetraplegia), moribund state, or death. Experiments were repeated at least in duplicate for each group.

Treatments

Animals were randomly assigned to different treatment groups to receive intraperitoneal injections of 250 μL of physiologic saline with or without additional drug every other day. Treatments consisted of vehicle (saline alone), memantine hydrochloride (Fagron GmbH and Co KG, Barsbüttel, Germany) 20 or 60 mg/kg body weight, or MK801 (Sigma Aldrich, Taufkirchen, Germany) 0.15 mg/kg body weight. Drug doses were adapted from previous studies (16, 17, 21, 22). Treatments were started on the day of immunization and continued until animals were killed at the following time points: Day 7 postimmunization (n = 7 animals or n = 14 eyes per treatment group), Day 1 after the onset of clinical EAE (n = 6 animals or n = 12 eyes per treatment group), or Day 8 of clinical EAE (n = 24 for vehicle/memantine 20 mg/kg; n = 8 for memantine 60 mg/kg).

Retrograde Labeling of RGCs and Quantification of RGC Density

A week before immunization, RGCs were retrogradely labeled following a previously described procedure (6). The 1-week period between Fluorogold injection into both superior colliculi and immunization of the rats ensures full labeling of RGCs and excludes underestimation of RGC density caused by disturbances in axonal transport (6). At the end of the in vivo part of the study, the rats were given an overdose of ketamine and xylazine and perfused, via the aorta, with 4% paraformaldehyde in PBS. Brain, optic nerves, and both eyes were removed and postfixed overnight in 4% paraformaldehyde, and the retinas were dissected, flat-mounted on glass slides, and examined by fluorescence microscopy (Axiohot 2; Zeiss, Göttingen, Germany) using an ultraviolet filter (365/397 nm). Retinal ganglion cell densities were determined as previously described (5). Briefly, Fluorogold-positive cells were counted within a graticule placed in each quadrant of the retina at the inner, middle, and outer positions to avoid localized variations in retinal densities (12 positions per retina).

Optic Nerve Histopathology

Animals were perfused with 4% paraformaldehyde and postfixed overnight. Optic nerves were removed, processed routinely, and embedded in paraffin. Histologic evaluation was performed on 0.5-μm-thick slices stained with Luxol fast blue and Bielschowsky silver impregnation to assess demyelination and axonal pathology, respectively. Demyelinated areas were determined as a percentage of the whole optic nerve cross section. The surface area of the optic nerve was measured using AxioVision 4.5 software (Zeiss). Photographs were made with a CCD camera (Color View II; Soft Imaging System 1) using an Axioplan 2 microscope (Zeiss). Relative axonal densities were determined in cross sections of the optic nerves prepared with Bielschowsky silver impregnation by point sampling using a 25-point Olympus eyepiece. Point sampling overcomes the density artifacts that might arise when the optic nerve area is increased because of edema (23). Random points (n = 25) were superimposed on the entire section of the optic nerve cross section. The number of points crossing axons was measured as the fraction of the total number of points of the stereologic grid. The degree of axon reduction is given as the percentage of axon density compared with the average axon density in healthy optic nerves. Inflammatory infiltration was assessed through immunohistochemistry performed on optic nerve cross sections using anti-CD3 antibody (1:500, BZL03543; Biozol, Eching, Germany) to detect T cells and anti-ED1 antibody (1:500, MCA341R; Serotec, Oxford, United Kingdom) to detect activated microglia/macrophages. Visualization was subsequently performed using avidin-biotin detection (ABC kit; Vector Laboratories, Peterborough, United Kingdom). In addition, hematoxylin and eosin staining was also performed on 0.5-μm-thick sections to assess overall inflammatory infiltration.
Neurofilament staining was achieved by incubation of optic nerve sections with anti–pan-neurofilament antibody (1:2000, SMI-312; Covance Research Products Inc, Berkeley, CA), and visualization was achieved either by avidin-biotin detection or by fluorescent labeling with an anti-mouse Cy3-conjugated secondary antibody (1:400; Jackson Immunoresearch, West Grove, PA). To assess β-amyloid precursor protein (βAPP) accumulation, we performed immunohistochemistry on optic nerve cross sections using an antibody against βAPP (1:1000, MAB348; Millipore, Billerica, MA) and visualized them by avidin-biotin detection. Quantification of ED1-positive cells, CD3-positive cells, and βAPP-positive axons was achieved by overlaying a morphometric grid on optic nerve sections and counting total positive cells per axon (expressed as density [per square millimeter]). The investigators who performed neuropathologic examinations were blinded to the applied treatments, and a minimum of 10 sections was taken throughout the length of each optic nerve.

**Cell Cultures**

Primary RGCs were obtained from 6- to 8-day-old BN rats according to a previously described 2-step immunopanning protocol (24). Cells were seeded onto poly-D-lysine (0.01 mg/mL)—coated 26-mm dishes and cultured in serum-free medium (Neurobasal; Invitrogen, Karlsruhe, Germany) containing glutamine, N-acetyl cysteine (both from Sigma Aldrich), B27 supplement, sodium pyruvate, and triiodothyronine (both from Invitrogen) and in Sato medium (transferrin, bovine serum albumin, progesterone, putrescine, and sodium selenite; all from Sigma Aldrich) and in Sato medium (transferrin, bovine serum albumin, progesterone, putrescine, and sodium selenite; all from Sigma Aldrich) supplemented with forskolin (4.1 μg/mL), brain-derived neurotrophic factor, triiodothyronine, and N-acetyl cysteine (both from Sigma Aldrich) at 37°C in 5% CO₂.

Oligodendrocytes were purified from 6- to 8-day-old BN rats. Optic nerves were dissected, meninges were removed, and tissue was digested in collagenase and DNase (both from Sigma Aldrich) at 37°C for 30 minutes to isolate oligodendrocyte precursor cells. After trituration and washing, cells were plated onto poly-D-lysine–coated 26-mm dishes and maintained in serum-free medium containing glutamine, sodium pyruvate, insulin, and Sato medium supplemented with platelet-derived growth factor-α (10 ng/mL), brain-derived neurotrophic factor (50 μg/mL); both from Peprotech Germany, Hamburg, Germany). From morphologic assessment, cultures appeared to contain more than 90% oligodendrocyte precursor cells. After 7 days in culture, the medium was removed and replaced with one lacking platelet-derived growth factor-α and basic fibroblast growth factor but containing ciliary neurotrophic factor, triiodothyronine, and N-acetyl cysteine to stimulate the differentiation of oligodendrocyte precursor cells into oligodendrocytes. After a further 5 to 7 days, oligodendrocytes were identified morphologically by their elaborate processes, with more than 95% differentiation achieved.

**Cytokine Evaluation**

Serum samples were collected on Day 8 of EAE. Collected blood was allowed to clot overnight at 4°C, centrifuged for 10 minutes at 12,000 rpm, and stored at −80°C. Serum was analyzed using ELISA kits specific for tumor necrosis factor alpha (TNFα; Gen-Probe, San Diego, CA) and transforming growth factor β1 (TGFβ1; eBioscience, Frankfurt, Germany) according to the manufacturers’ instructions.

**Calcium Imaging of RGCs and Oligodendrocytes**

Purified RGCs and oligodendrocytes were labeled with Fura-2 AM by 30-minute incubation in growth medium supplemented with 5 μmol/L Fura-2 AM (Invitrogen). After wash, coverslips were mounted on a microscope chamber, and an experimental solution (10 mmol/L hydroxyethyl piperazine ethanesulfonic acid buffer, 5 mmol/L CaCl₂, 2.8 mmol/L KC1, 120 mmol/L NaCl, 10 mmol/L glucose, and 10 μmol/L glycine, but no MgCl₂; all from Sigma Aldrich) was added to encourage maximal NMDA responses (25). Loaded cells were then imaged on a fluorescent microscope by excitation alternatively at 340 and 380 nm with a Polychrome V (TILL Photonics, Gräfelfing, Germany) for 10 milliseconds each (emission, 510 nm). Repeated image pairs containing approximately 20 cells per frame were taken every 2 seconds for the duration of the experiment and analyzed using TILLvisION software (TILL Photonics). Ratiometric values (F340/380) were established after background subtraction, and ΔF340/380 was calculated by subtracting F340/380 at Time 0. All treatments were made by dissolving neurotransmitters and drugs in the experimental solution. Experiments were performed at least in triplicate, using different cell preparations.

**Statistical Analysis**

Statistical comparisons were made using SPSS SigmaPlot version 12. Nonparametric 2-tailed Mann-Whitney U test was used for clinical score comparisons because of the ordinal nature of the score used. Student t-test was used to evaluate statistical significance in histopathologic parameters (following Shapiro-Wilk normality test). A value of p < 0.05 was considered significant.

**RESULTS**

**Clinical Disease**

To ascertain the effect of systemic NMDA receptor blockade on rats with optic neuritis, we randomly assigned the rats into 3 groups and induced optic neuritis by immunization with MOG. This protocol reliably results in optic neuritis in BN rats (5). One group received in traperitoneal delivery of vehicle (n = 24); the other groups received either memantine 20 mg/kg (n = 24) or memantine 60 mg/kg (n = 8). Doses were chosen based on previous studies, where 1.5 mg/kg failed to elicit any significant effect on clinical disease severity in mice (26) whereas 20 mg/kg and 40 to 80 mg/kg elicited significant effects on clinical disease severity in rats (16, 17). Animals were observed until 8 days after the manifestation of neurologic signs associated with EAE. After immunization, the day of disease manifestation was significantly delayed in the memantine 60 mg/kg–treated group in comparison with the placebo group (Fig. 1C; p < 0.05). Vehicle-treated animals developed clinical signs on Day 15.6 ± 0.7 (mean ± SE) after immunization, and memantine 20 mg/kg–treated animals developed signs on Day 17.0 ± 0.8, whereas the
FIGURE 1. Effects of memantine on the clinical parameters of EAE. (A) Mean clinical score for each treatment group shown as days postimmunization. Memantine 60 mg/kg (Mem 60) treatment resulted in the most significantly reduced disease course; there were time points at which there were significant differences between the memantine 20 mg/kg (Mem 20) group and the vehicle-treated group. The preclinical time point of Day 7 postimmunization is marked with a black arrow. (B) Mean clinical score for each treatment group with animals aligned for the day of onset of clinical signs. Time points of the histopathologic analysis of animals (i.e. Day 1 of EAE, the first day of clinical signs; Day 8 of EAE, 8 days after disease onset) are marked with black arrows. (C) The average day of onset of clinical disease (days postimmunization). Onset of clinical disease was significantly delayed in the Mem 60-treated group. (D) Disease incidence was similarly reduced in the Mem 60–treated group. (E, F) Serum levels of TNFα (E) and TGFβ1 (F) were assessed by ELISA, with no significant differences observed between the treatment groups. * p < 0.05, ** p < 0.01, *** p < 0.001 (n = 24 for vehicle/Mem 20; n = 8 for Mem 60). N.S., not significant.
first neurologic signs appeared in memantine 60 mg/kg–treated animals on Day 23.5 ± 5.5. Clinical severity (Fig. 1A), as well as disease incidence (Fig. 1D), was significantly lower in both memantine treatment groups. None of the rats in the low-dose or vehicle group showed apparent adverse effects of the treatment. Animals treated with memantine 60 mg/kg showed mild gait abnormality with reduced coordination.

Sera taken on Day 8 of EAE were measured for circulating cytokine levels and compared with sera collected from healthy unimmunized animals to assess whether memantine treatment had any systemic effects on the immune response that caused the reduced EAE incidence and severity. Tumor necrosis factor alpha (TNFα) was assessed by ELISA as a proinflammatory cytokine, and TGFβ1 was assessed by ELISA as an anti-inflammatory cytokine (Figs. 1E, F). Although both cytokines were elevated in the 3 EAE groups, compared with healthy unimmunized controls, no significant differences in either serum TNFα or serum TGFβ1 were seen among the treatment groups—TNFα: vehicle, 19.0 ± 2.9 pg/mL; memantine 20 mg/kg, 27.4 ± 4.7 pg/mL (p = 0.16); memantine 60 mg/kg, 20.5 ± 3.2 pg/mL (p = 0.75); TGFβ1: vehicle, 393.7 ± 68.4 pg/mL; memantine 20 mg/kg, 352.3 ± 64.9 pg/mL (p = 0.68); memantine 60 mg/kg, 302.9 ± 38.9 pg/mL (p = 0.25).

Optic Nerve Histopathology

To compare the severity of optic neuritis on Day 8 of EAE after treatment with memantine or after treatment with vehicle, we analyzed demyelination, inflammation, and axonal damage. The incidence of optic neuritis was assessed by histopathology and correlated with disease incidence (Fig. 1D). First, the extent of demyelination (expressed as the percentage of demyelinated area with respect to the whole optic nerve cross section) was found to be significantly reduced in the memantine-treated groups compared with the vehicle-treated...
FIGURE 3. Axonal pathology of optic nerves on Day 8 of EAE. (A–C) Bielschowsky silver impregnation of optic nerve cross sections from rats treated with vehicle (A), memantine 20 mg/kg (B), or memantine 60 mg/kg (C). Boxes indicate further magnified regions. (D) Quantification of axon density in treated groups (percentages of healthy animals) showing significant preservation of axons in both memantine-treated groups. Degree of axon reduction is expressed as the percentage of axon density compared with the average axon density in healthy optic nerves. (E–G) Representative optic nerve cross sections stained with anti-βAPP antibody from vehicle-treated (E), memantine 20 mg/kg-treated (F), or memantine 60 mg/kg–treated (G) animals. Boxes indicate further magnified regions, with arrows indicating βAPP-positive axons. (H) Quantification of the density of βAPP-positive axons. Although reduced numbers were observed in the memantine 60 mg/kg group, they did not reach the significance threshold. (I–L) Representative immunohistochemical staining for neurofilament in healthy (I), vehicle-treated (J), memantine 20 mg/kg–treated (K), and memantine 60 mg/kg–treated (L) optic nerve sections. (M–P) Representative confocal fluorescent images of anti-neurofilament–stained optic nerve sections from healthy (M), vehicle-treated (N), memantine 20 mg/kg–treated (O), and memantine 60 mg/kg–treated (P) groups. White arrows indicate damaged axonal swellings. *p < 0.001 (n = 20 for vehicle/memantine 20 mg/kg; n = 8 for memantine 60 mg/kg). Scale bars = (C) 50 μm; (G, L, P) 25 μm.
FIGURE 4. Determination of RGC density. (A–C) Representative retinal whole mounts showing Fluorogold-labeled RGCs in vehicle-treated (A), memantine 20 mg/kg–treated (B), and MK801 0.15 mg/kg–treated (C) rats taken on Day 8 after the onset of clinical EAE (Day 8 of EAE). Open arrows, microglia; bold arrows, RGCs. (D) Quantification of surviving RGCs per square millimeter of retina. Greater numbers of RGCs survive in memantine- and MK801-treated retinas than in vehicle-treated retinas on both Day 1 and Day 8 of EAE (n ≥ 12). (E–G) Representative whole mounts from vehicle-treated (E), memantine-treated (F), and MK801-treated (G) rats taken on Day 7 postimmunization. (H, I) Representative Luxol fast blue (H) and hematoxylin and eosin (I) stains of an optic nerve from an untreated rat on Day 7 postimmunization showing no demyelination or infiltrating immune cells, respectively. (J) Quantification of surviving RGCs on Day 7 postimmunization (n = 14). * p < 0.05, ** p < 0.01. Scale bars = (G) 10 μm; (I) 100 μm.
group (vehicle, 84.7% ± 3.7%; memantine 20 mg/kg, 41.4% ± 8.1%; memantine 60 mg/kg, 4.9% ± 1.9%; p < 0.001; Figs. 2A–D).

In addition, the degree of inflammatory infiltration after treatment with memantine was significantly reduced in both treatment groups. The number of CD3-positive cells per square millimeter was lower with memantine than with vehicle (vehicle, 41.9 ± 4.7; memantine 20 mg/kg, 21.1 ± 6.7; p < 0.05; memantine 60 mg/kg, 5.5 ± 1.8; p < 0.001; Figs. 2E–H). Similarly, fewer ED1-positive cells per square millimeter were found in memantine-treated animals (vehicle, 168.2 ± 27.5; memantine 20 mg/kg, 71.8 ± 18.9, p < 0.05; memantine 60 mg/kg, 4.55 ± 2.8; p < 0.01; Figs. 2I–L).

Axonal loss was then assessed by Bielschowsky silver impregnation (expressed as the percentage of axon density compared with the average axon density in healthy optic nerves; n = 20). This was significantly lower in both memantine-treated groups compared with vehicle-treated group (vehicle, 70.4% ± 4.6%; memantine 20 mg/kg, 33.9% ± 5.8%; memantine 60 mg/kg, 6.7% ± 2.9%; p < 0.001; Figs. 3A–D). In axons remaining at this time point, there was a trend toward reduced βAPP deposition in the high-dose memantine treatment group (but not in the low-dose group) compared with vehicle-treated optic nerve sections (74.98 ± 19.5 vs 228.4 ± 49.3 positive axons/mm², p < 0.126; Figs. 3E–H), indicating that axonal damage resulting in breakdown of fast axonal transport was reduced.

To further investigate differences in surviving axons between the treatment groups, we used an anti–pan-neurofilament antibody. Using light microscopy, increased axonal densities were observed in the memantine-treated groups, particularly in the 60 mg/kg treatment group (Figs. 3I–L). This was more clearly seen on confocal imaging of fluorescently labeled optic nerve sections, where the presence of acutely damaged axons (resulting in axonal swellings) was also reduced in the high-dose treatment group (Figs. 3M–P).

In conclusion, memantine treatment, particularly in the high-dose group (60 mg/kg), significantly reduced demyelination and inflammatory cell infiltration and protected against axonal loss and damage.

**RGC Survival**

To compare RGC survival, we prelabeled RGCs by injecting Fluorogold into the superio colliculi 1 week before immunization. On Day 8 of EAE, we counted the number of remaining Fluorogold-positive RGCs after treatment with either vehicle or memantine 20 mg/kg (Figs. 4A, B, D). In control retinas of sham-immunized rats, the mean RGC density was 2,046 ± 120.2 cells/mm² (n = 5). Retinal ganglion cell counts in vehicle-treated animals dropped to 683.5 ± 42.6 cells/mm² by Day 8 of EAE, whereas the rats treated with memantine 20 mg/kg showed significantly higher numbers of surviving RGCs at this time point (929.1 ± 60.9 cells/mm²; p < 0.05 vs vehicle).

To control for the specificity of memantine action on neuroprotection, we administered an alternative inhibitor of the NMDA receptor, MK801, intraperitoneally into another group of immunized rats. Again, similar to memantine administration, MK801 given at a dose of 0.15 mg/kg (maximal tolerated dose) did not cause any long-lasting neurologic adverse effects. MK801 treatment, similar to memantine 20 mg/kg treatment, did not affect the timing of disease onset after immunization (data not shown). In a comparison of the numbers of RGCs present in retinal whole mounts of animals on Day 8 of EAE, MK801 reduced the number of RGCs lost similarly to memantine (906.7 ± 31.2 cells/mm²; Figs. 4C, D). On the day of onset (Day 1 of EAE), retinal whole mounts were also taken from animals that had received vehicle, memantine 20 mg/kg, or MK801 treatment. Both memantine-treated and MK801-treated groups had higher RGC densities in the retina (1,152.1 ± 133.6 and 1,154.6 ± 115.2 cells/mm², respectively) compared with vehicle-treated rats (865.7 ± 43.8 cells/mm², p < 0.05; Fig. 4D).

In our model of MOG-induced optic neuritis, degeneration of RGCs starts during the induction of the disease, preceding histopathologic changes in the optic nerve (6, 8). To determine whether NMDA receptor blockade exerted a direct protective effect on RGCs or whether this was secondary to the effects of NMDA receptor blockade on inflammatory infiltration and concomitant reduction in demyelination on Day 8 of EAE, we next investigated RGC survival on Day 7 postimmunization, before demyelination of the optic nerve had started. We confirmed that, at this time point, before any visible clinical disease, there was no significant demyelination or inflammatory infiltration of the optic nerves (Figs. 4H, I), similar to previously reported data (8). This was also true of animals that had received either memantine or MK801 at this time point (data not shown). Retinal ganglion cell density in all groups (treated with memantine, MK801, or vehicle; n = 7 animals or n = 14 eyes per treatment group) was significantly reduced compared with sham-immunized healthy controls. However, there were more RGCs in memantine-treated animals (1,415 ± 74.3 cells/mm²) and MK801-treated animals (1,363.6 ± 129.1 cells/mm²) than in vehicle controls (1,064 ± 61.9 cells/mm²; p < 0.01; Fig. 4J). Thus, NMDA receptor blockade was protective of...
RGCs even before demyelination occurred, suggesting that this was not secondary to protective effects on oligodendrocytes.

**Calcium Imaging of RGCs and Oligodendrocytes**

Because both RGCs and oligodendrocytes were protected during systemic memantine treatment, as assessed on Day 8 of EAE, we next wished to determine whether memantine acted directly on either RGCs or oligodendrocytes. Calcium imaging with Fura-2 AM was used to study changes in intracellular calcium levels. Upon adding 200 μmol/L glutamate to both isolated RGCs and oligodendrocytes, intracellular calcium rose rapidly in both cell types (Figs. 5A, D), although a greater calcium response was observed in oligodendrocytes. Preincubation with 12 μmol/L memantine (concentration chosen based on concentration dependence experiments; Fig. 5B) significantly reduced calcium response only in RGCs (not in oligodendrocytes), suggesting that calcium response in oligodendrocytes was mainly independent of NMDA receptors. This was confirmed by performing dose-response experiments with memantine, in which memantine was found to reduce calcium response in RGCs when given at a concentration of 1 μmol/L or more. Memantine, even when given at high doses, had no effect on oligodendrocytes. In addition, direct addition of 100 μmol/L NMDA to the cells elicited a high calcium response only in RGCs, but not in oligodendrocytes (Figs. 5I, L). Conversely, oligodendrocytes were more responsive than RGCs to the blockade of AMPA and kainate receptors with the addition of the AMPA/kainate receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (Figs. 5J, G). Response to the direct addition of 100 μmol/L AMPA was also larger in oligodendrocytes (Fig. 5K), although a response was still observable in RGCs. The action of NBQX on AMPA receptors was confirmed by its reduction of the AMPA response back to baseline calcium levels (Figs. 5H, K).

Together, these data demonstrate that RGCs and oligodendrocytes have different glutamate receptor profiles whereby only RGCs are significantly responsive to memantine. This also provides further evidence that RGC survival might be directly elicited by NMDA receptor inhibition rather than being secondary to the protective effects of NMDA receptor inhibition on oligodendrocytes.

**DISCUSSION**

In this study, we show that NMDA receptor blockade with memantine, a noncompetitive antagonist, is neuroprotective and myelin-protective in experimental optic neuritis. This was confirmed by the neuroprotective effect of MK801 treatment, which controlled for the specificity of memantine action on NMDA receptors. Calcium imaging of RGCs revealed significant inhibition of glutamate-induced calcium influx by NMDA receptor blockade. In contrast, calcium response to glutamate in oligodendrocytes was primarily mediated via AMPA/kainate receptors. Because NMDA receptor–mediated protection of RGCs was already observed on Day 7 postimmunization, a time point when demyelination in the optic nerve is minimal, we conclude that blockade of NMDA receptors may directly protect RGCs and optic nerve axons. Reduced infiltration of immune cells at later disease stages under NMDA blockade may in turn cause partial preservation of the myelin sheath, allowing for further axonal protection.

Earlier studies in MS models have shown the involvement of voltage- and ligand-gated cation channels in inflammation-induced myelin degeneration and neurodegeneration, stressing their importance in the development of MS and making them a potential therapeutic target (14, 15, 27–29). In the context of neurodegeneration, NMDA receptors have attracted attention because their overactivation triggers excessive calcium influx, which can lead to neuronal cell death (9). Because memantine is a noncompetitive NMDA receptor antagonist, it is an interesting candidate for addressing the role of NMDA receptor overactivation (with limited effects on normal physiologic glutamate signaling) in patients with MS (30, 31). In contrast, MK801 has been reported to cause cognitive disruption, likely through inhibition of long-term potentiation (32), and to induce psychosis (33). The neuroprotective properties of memantine have been shown in a number of in vitro studies and in animal models of noninflammatory neurodegeneration (34, 35) (e.g. it acts on cortical, cerebellar, and retinal neurons) (21, 36). With respect to its clinical use, it is an already approved drug for the treatment of patients and has been tested in clinical trials for Alzheimer disease, Parkinson disease–related dementias, and vascular dementia (37–39).

**Memantine Acts Directly on RGCs**

Here, we confirm previous EAE studies showing that memantine significantly reduced the severity of the disease course (16, 17) perhaps by reducing the number of inflammatory infiltrates able to penetrate the blood-brain barrier (16, 40). However, the effects of memantine on neurodegeneration in MS models have not been investigated. We show that memantine protected both RGC bodies and axons. This could be expected because RGCs express NMDA receptors, which remain present during the progression of the disease (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A582 demonstrates NMDA receptor expression in RGCs throughout the disease course). Because neurodegeneration in this model has been shown to precede inflammatory infiltration and blood-brain barrier disturbances in the optic nerve (8), preclinical time points were analyzed to determine whether memantine was able to exert neuroprotective effects independent of its reduction of immune cell infiltration. In accordance with this hypothesis, there was a significant reduction in RGC loss that was independent of both inflammation and demyelination. This time frame correlated with microglial activation in the retina and disturbances in the blood-retina barrier (8), which may provide a source of glutamate via microglial release (41), and extravasation of glutamate from high plasma concentrations to the retina (42).

Calium imaging of isolated cells supports a direct action of memantine on RGCs. Although glutamate-induced calcium influx in RGCs occurs mainly via the NMDA receptor, oligodendrocyte calcium responses were mainly independent of this receptor. Instead, they were more dependent on AMPA/kainate receptor signaling, as previously described (43). Although some studies have suggested a possible role for NMDA receptors in oligodendrocytes because of their reported (and previously overlooked) expression (44, 45), a more recent study demonstrated...
that this expression had no influence on their susceptibility to immune-mediated degeneration because selective ablation of NR1 in oligodendrocytes had no influence on the clinical severity of EAE or on axonal survival (46). Thus, memantine is unlikely to protect RGCs through its action on oligodendrocytes but may instead exert a direct influence on RGC glutamatergic responses.

Clinical Perspective

Together with previous findings showing beneficial effects of AMPA/kainate receptor blockade on EAE (14, 15), our present results corroborate the involvement of glutamate in the pathophysiology of the animal model. However, the efficacy of glutamate receptor antagonism in MS remains largely uninvestigated. Coapplication of ionotropic receptor antagonists with different specificities might positively influence several disease aspects and augment neuroprotection. However, it might be wise to choose drugs that regulate abnormal receptor function without totally blocking physiologic activity to minimize unwanted adverse effects during therapeutic application. Such an approach targeting nonimmunologic sites should be regarded as a potential additional therapy to established immunomodulatory treatments.

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REFERENCES

4. Trip SA, Schlottmann PG, Jones SJ, et al. Optic nerve atrophy and retinal nerve fibre layer thinning following optic neuritis: Evidence that axonal loss is a substrate of MRI-detected atrophy. Neuroimage 2006;31:286–89
19. Lipton SA. Paradigm shift in neuroprotection by NMDA receptor antagonists with different specificities might positively influence several disease aspects and augment neuroprotection. However, it might be wise to choose drugs that regulate abnormal receptor function without totally blocking physiologic activity to minimize unwanted adverse effects during therapeutic application. Such an approach targeting nonimmunologic sites should be regarded as a potential additional therapy to established immunomodulatory treatments.


44. Salter MG, Fern R. NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. Nature 2005;438:1167–71


46. Guo F, Maeda Y, Ko EM, et al. Disruption of NMDA receptors in oligodendroglial lineage cells does not alter their susceptibility to experimental autoimmune encephalomyelitis or their normal development. J Neurosci 2011;32:639–45