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System $x_c^{-}$ and Glutamate Transporter Inhibition Mediates Microglial Toxicity to Oligodendrocytes

María Domercq,* María Victoria Sánchez-Gómez,* Catherine Sherwin,† Estibaliz Etxebarria,* Robert Fern,† and Carlos Matute2*

Elevated levels of extracellular glutamate cause excitotoxic oligodendrocyte cell death and contribute to progressive oligodendrocyte loss and demyelination in white matter disorders such as multiple sclerosis and periventricular leukomalacia. However, the mechanism by which glutamate homeostasis is altered in such conditions remains elusive. We show here that microglial cells, in their activated state, compromise glutamate homeostasis in cultured oligodendrocytes. Both activated and resting microglial cells release glutamate by the cystine-glutamate antiporter system $x_c^{-}$. In addition, activated microglial cells act to block glutamate transporters in oligodendrocytes, leading to a net increase in extracellular glutamate and subsequent oligodendrocyte death. The blocking of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors or the system $x_c^{-}$ antiporter prevented the oligodendrocyte injury produced by exposure to LPS-activated microglial cells in mixed glial cultures. In a whole-mount rat optic nerve, LPS exposure produced wide-spread oligodendrocyte injury that was prevented by AMPA/kainate receptor block and greatly reduced by a system $x_c^{-}$ antiporter block. The cell death was typified by swelling and disruption of mitochondria, a feature that was not found in closely associated axonal mitochondria. Our results reveal a novel mechanism by which reactive microglia can contribute to altering glutamate homeostasis and to the pathogenesis of white matter disorders. The Journal of Immunology, 2007, 178: 6549–6556.

In addition, the paralysis of microglia and/or macrophages arrest the progression of experimental autoimmune encephalitis (EAE) (14), an animal model of MS showing oligodendrocyte death, myelin loss, and axonal damage. Thus, understanding how microglia contributes to oligodendrocyte cell death is crucial for the development of therapeutic approaches to white matter disorders.

A mechanism of cellular injury unique to the CNS is excitotoxicity, which is caused by excessive extracellular glutamate and the subsequent overstimulation of glutamate receptors in neurons and oligodendrocytes (15–18). Glutamate homeostasis is altered in EAE because glutamate-metabolizing enzymes and glutamate transporters are down-regulated (19, 20) and treatment with an antagonist of the glutamate receptor ameliorates both oligodendrocyte cell death and neurological deficits associated with this experimental condition, although it does not prevent inflammation (21, 22). These findings suggest that autoimmunity to myelin and the subsequent microglial activation induce an alteration in glutamate homeostasis leading to oligodendrocyte excitotoxicity, which contributes to tissue damage in MS (23). Indeed, active MS lesions are characterized by the presence of activated microglia and/or macrophage/microcyte infiltrates and by the loss of oligodendrocytes concomitant with an increase in glutamate levels (24). A possible source of glutamate is from the cystine-glutamate antiporter, called system xc$^{-}$, which exchanges extracellular cystine for intracellular glutamate (25). The system $x_c^{-}$ is a heterodimeric protein complex consisting of a catalytic L chain (xCT) and a regulatory H chain (xCT$^+$), which is essential for membrane localization of the transporter (26). The system $x_c^{-}$ appears to be especially abundant in macrophage/microglia and immune cells (27, 28).

In this study we analyze the effects of resting and LPS-activated microglia in glutamate homeostasis and excitotoxicity in cocultured oligodendrocytes. We show that microglia release glutamate by system $x_c^{-}$ and alter glutamate homeostasis by

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3 Abbreviations used in this paper: MS, multiple sclerosis; AAA, aminoadipic acid; AM, acetoxyethyl ester; AMPA, N-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; aCSF, artificial cerebrospinal fluid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EAE, experimental autoimmune encephalitis; IC$_1^{-}$, 5,5',6,6'-tetrahydro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(1)quinoxaline; RGN, rat optic nerve; ROS, reactive oxygen species; TBOA, ti-fluoreyl-benzyloxyxyparitate.

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inhibiting glutamate transporters in oligodendrocytes. In addition, we provide evidence indicating that extracellular glutamate accumulation after microglia activation is toxic to oligodendrocytes in vitro and that a similar mechanism operates in isolated optic nerves.

Materials and Methods

Oligodendrocyte and microglia cultures

Oligodendrocyte cultures were obtained from optic nerves of 12-day-old Sprague-Dawley rats using a previously described procedure (29) with modifications (16). Cells were seeded onto 24-well plates bearing 10 µg/ml poly-t-lysine-coated coverslips at a density of 5,000 or 25,000 cells per well for toxicity and uptake experiments, respectively. Cell culture medium (see Refs. 16 and 29) was supplemented with N-acetyl-cysteine to avoid glutathione depletion and oxidative glutamate toxicity (30). Cultures were used after 3–5 days in vitro when almost all cells (>98%) were O1+/galactocerebroside C+ differentiated oligodendrocytes. Microglial cells were isolated from confluent monolayers of cultured astrocytes derived from the cerebral cortex of newborn rats as previously described (31). Astrocytes flask were mechanical shackled and free-floating microglia were collected and purified by preplating on plastic dishes for 1 h. For oligodendrocyte-microglia coculture, microglial cells were seeded on oligodendrocyte cultures at a density 1,000 or 5,000 cells per well for toxicity and uptake experiments, respectively. To activate microglia, cocultures were treated with LPS (100 ng/ml; Escherichia coli O11:B4; Sigma-Aldrich) 1 day after plating and used for experiments after 48 h. Media were conditioned by microglial cultures for 48 h and added immediately to oligodendrocyte cultures. Cocultures were characterized by immunocytochemistry to the oligodendrocyte marker galactocerebroside C (5 µg/ml; Boehringer-Mannheim) and by labeling with Griffonia simplicifolia isocerin B4 (10 µg/ml; Vector Laboratories), a selective marker of both resting and activated microglia.

Electrophysiology

Standard whole-cell recordings were performed at room temperature on an inverted microscope (CK-40; Olympus) using the EPC-7 patch-clamp amplifier (HEKA) as described (32). Recordings were low-pass filtered at 2 kHz, digitized at 5 kHz, and stored as data files on a computer using the pClamp 8.2 program (Axon Instruments) for later analysis. The extracellular bath solution contained 140 mM NaCl, 5.4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES (pH 7.3). Glutamate transporter currents were recorded in the presence of intracellular thiocyanate to potentiate the transporter anionic conductance. Patch clamp pipettes (3–5 MΩms) were filled with a solution containing 140 mM KSCN, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 11 mM EGTA, and 2 mM sodium ATP (pH 7.3). Currents were recorded at a holding membrane potential of ~70 mV. Local micropipetron of agonist was performed with a multibarreled apparatus connected to an electronically driven rotatory motor (RSC-100; Bio-Logic).

l-[^3H]Glutamate uptake and glutamate release

l-[^3H]Glutamate uptake was assessed at 37°C in 300 µl of saline solution containing 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, and 4.5 g/L glucose (pH 7.4). Cells were equilibrated for 5 min in the absence or presence of uptake inhibitors and then incubated for 10 min with 100 nM l-[^3H]Glutamate and unlabeled glutamate (1 µM) as previously described (31). Uptake was stopped with two washes in ice-cold PBS, followed immediately by cell lysis in 0.1 N NaOH and 0.1% Triton X-100. Sodium-dependent uptake was calculated to be the difference between the amount of radioactivity in the presence of sodium and the amount observed in the choline-containing buffer. Sodium-dependent glutamate uptake was normalized to the number of viable cells analyzed by the dye O1 (100 nM; Calbiochem), a selective marker of both resting and activated microglia. Glutaraldehyde (36 U/ml; Sigma-Aldrich) as previously described (3, 32). Glutamate was oxidized by L-glutamate dehydrogenase (36 U/ml; Sigma-Aldrich) 1 day after plating and used for experiments after 48 h. Media were conditioned by microglial cultures for 48 h and added immediately to oligodendrocyte cultures. Cocultures were characterized by immunocytochemistry to the oligodendrocyte marker galactocerebroside C (5 µg/ml; Boehringer-Mannheim) and by labeling with Griffonia simplicifolia isocerin B4 (10 µg/ml; Vector Laboratories), a selective marker of both resting and activated microglia.

Western blotting

Western blot analysis of cystine-glutamate antiporter expression was done in microglial cultures by conventional SDS-PAGE polyacrylamide electrophoresis. The system X-100 was visualized with Abs against the cystine-glutamate antiporter xCT (1/250) generously provided by Dr. P. Kalivas (Medical University of South Carolina, Charleston, SC; Ref. 33). Densitometric analysis was performed using the NIH Image program (n = 3).

LPS toxicity assays

LPS (E. coli O11:B4; Sigma-Aldrich) was added to mixed cultures or oligodendrocyte cultures 24 h after plating and cell death was determined after 48 h. To determine oligodendrocyte viability, we counted cells stained with the oligodendrocyte O1 Ab marker and the vital dye calcein-AM, as follows. Cells were loaded with calcein-AM (1 µM) for 30 min and fixed with 4% paraformaldehyde for 20 min. Cells were then processed with an mAb to the O1 oligodendroglial marker (10 µg/ml; Chemicon) and labeling was visualized with Alexa 594-conjugated goat anti-mouse IgG (1/200; Molecular Probes). The number of living oligodendrocytes (O1+ and calcein+ cells) in each well was counted blind to the treatments done and results were expressed as the percentage of cell death vs nontreated oligodendrocyte pure cultures considered as control. The changes in mitochondrial membrane potential were monitored with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1; Molecular Probes). In the cytosol the monomeric form of this dye fluoresces green (emission read at 527 nm), whereas within the mitochondrial matrix highly concentrated JC-1 forms aggregates that fluoresce red. After drug treatment, cells were loaded with JC-1 (3 µM) at 37°C for 30 min. JC-1 aggregates were detectable in the propidium iodide channel (emission at 590), JC-1 detectable in the propidium iodide channel (emission at 520), and the changes in mitochondrial potential were calculated as the red/green ratio in each condition.

Isolated rat optic nerve (RON) experiments

RONs were dissected from 12-day-old litter hooded rats and placed in artificial cerebrospinal fluid (aCSF) composed of 124 mM NaCl, 3 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 26 mM NaHCO3, 2 mM NaH2PO4, and 10 mM dextrose. LPS was dissolved in aCSF immediately before the experiment to give a final concentration of 1 µg/ml. RONs were incubated at 37°C in 1 ml of aCSF with or without LPS for 3 h, the period during which maximal cell death was previously observed (12). Death cells were determined using ethidium bromide staining, a lipophilic nuclear dye that does not stain intact living cells. RONs were incubated for 10 min at room temperature with 0.5 mM ethidium bromide and then washed in aCSF and live mounted on a Nikon LABOPHOT-2 epifluorescence microscope equipped with a ×20 fluorescence objective and appropriate filter. Whole RON images (excluding the cut ends) were captured with a charge-coupled device camera aging 16 frames. Cell counting was performed blind using NIH ImageJ. The mean number of dead cells found following exposure to any given condition was compared with the number seen in control RONs (180 min aCSF only) and is expressed as a percentage change relative to this level of control cell death.

Electron microscopy

RONs were exposed to 1 µg/ml LPS in aCSF for 180 min before washing in Sorenson’s buffer and, postfixation, in 3% glutaraldehyde and Sorenson’s buffer for 90 min at room temperature. The RONs were subsequently postfixed with 2% osmium tetroxide and dehydrated in ethanol and propylene oxide. The nerves were infiltrated in epoxy resin and the ends were cut back before ultrathin sections were taken. Sections were counterstained with uranyl acetate and lead citrate and examined with a JEOL 100CX electron microscope. To avoid bias in the data, electron micrographs were collected blind (R.F.K.) and the entire procedure used to produce each sample. Mitochondrial areas were drawn by hand using ImageJ. Apoptosis was examined by immunoelectron microscopy of annexin-V (1:50; Santa Cruz Biotechnology), an early marker.
Statistical analysis
Data are presented as mean ± SEM. The significance between two data sets was tested with unpaired t test or ANOVA as appropriate.

Results
Microglia alters glutamate homeostasis in cultured oligodendrocytes
Oligodendrocytes express functional glutamate transporters and contribute to maintaining glutamate homeostasis in white matter tracts (18, 31–32, 34). We therefore studied whether the function of glutamate transporters in these cells is altered by an activated microglia such as that found in inflammation. To this end, we added microglial cells to oligodendrocytes in vitro and cells were subsequently incubated with LPS (100 ng/ml) to activate microglial cells (Fig. 1a). LPS alone had no significant effect in glutamate uptake (n = 4; Fig. 1b). However, we found that LPS-activated microglia, but not microglia in its resting state, blocked glutamate uptake in oligodendrocyte-microglia cocultures (n = 6; Fig. 1b). This effect was mainly due to impaired glutamate transport in oligodendrocytes because control and LPS-treated microglia cultured alone lack significant Na⁺-dependent glutamate uptake (data not shown). The blockade of glutamate transport by activated microglia was not secondary to an effect on cell viability, because glutamate uptake was normalized to the number of oligodendrocytes in each condition. In addition, medium conditioned by LPS-activated microglia also induced a partial inhibition of glutamate uptake in oligodendrocytes (n = 3; Fig. 1b). However, to further exclude any effect on cell viability, we analyzed the effect of microglia on glutamate transporter function in single oligodendrocytes by electrophysiology. The application of D-aspartate (1 mM) elicited an inward current in oligodendrocytes clamped at −70 mV

FIGURE 1. Activated microglia inhibits Na⁺-dependent glutamate transporter in oligodendrocytes. a, Control (left) and LPS-treated (right) cocultures of oligodendrocytes (galactocerebroside C¹ cells (GalC) in red) and microglia (IB4⁺ cells (IB4) in green). Note the reactive appearance of microglia in the presence of LPS. Scale bar, 50 μm. b, Na⁺-dependent glutamate uptake in pure oligodendrocyte cultures (OL), oligodendrocyte-microglia cocultures (OL+MG) with or without LPS (100 ng/ml), or OL cultures treated with microglia-conditioned medium with or without LPS (100 ng/ml). Glutamate uptake was normalized to the number of oligodendrocytes in each condition and expressed as a percentage of that measured in pure OL cultures. Data are mean ± SEM from at least three independent experiments performed in triplicate. **, p < 0.001. c, Membrane currents (top) in oligodendrocytes (OL) clamped at −70 mV in response to 1 mM D-aspartate applied for the duration indicated by the bars in the same culture conditions described before. The addition of microglia (OL+MG) did not significantly affect glutamate transporter currents in oligodendrocytes, whereas LPS-activated microglia (OL+MG+LPS) greatly diminished D-aspartate currents in oligodendrocytes. Bottom, Histogram depicts mean amplitudes ± SEM of steady-state currents induced by D-aspartate application to 10–20 oligodendrocytes. *, p < 0.01. d, Na⁺-dependent glutamate uptake in pure oligodendrocyte (OL) cultures and in LPS-treated oligodendrocyte plus microglia (OL+MG) cultures in the absence or presence of TNF-α (100 ng/ml), IL-1β (10 ng/ml), paraquat (15 μM), anti-TNF Ab (100 μg/ml) and Trolox (10 μM). Data are mean ± SEM from at least three independent experiments performed in triplicate. Symbols denote statistical significance vs OL cultures or vs LPS-treated OL+MG mixed cultures ( * and #, respectively). **, p < 0.01; #, p < 0.05.
glutamate levels measured in the medium after the addition of the L-glutamate dehydrogenase (GDH) enzyme to an oligodendrocyte-microglia coculture (OL+MG) with or without LPS (100 ng/ml) and microglia alone (MG). Bottom, Histogram illustrates extracellular glutamate levels in the culture conditions referred to above and also in oligodendrocyte alone or after addition of LPS (OL and OL+LPS, respectively), or the cystine-glutamate antiporter inhibitor AAA (2.5 mM). Note that glutamate levels are similar in oligodendrocyte plus microglia (OL+MG) cultures incubated with LPS or the glutamate transporter inhibitor TBOA (1 mM) and in microglia alone. a, Extracellular glutamate levels in microglia in the absence (Control) or in the presence of TBOA (1 mM), the cystine-glutamate antiporter inhibitor AAA (2.5 mM) and LPS (100 ng/ml). Data are mean ± SEM from three determinations in sister wells from at least three independent experiments. **, p < 0.001. b, Up-regulation of the cystine-glutamate antiporter xCT in microglia after LPS (100 ng/ml) treatment for 48 h. Data were normalized to actin and expressed as the percentage of xCT expression vs control (n = 3). *, p < 0.05.

(mean amplitude = 26.5 ± 7.3, n = 20; Fig. 1c) that is dependent on extracellular sodium and inhibited by the glutamate transporter inhibitor d-threo-β-benzoyloxyaspartate (TBOA) (data not shown) as shown previously (32). Similarly as in uptake assays, currents elicited by D-aspartate in oligodendrocytes cultured alone were not significantly affected by the presence of resting microglia (n = 10, Fig. 1c) but were greatly reduced by LPS-activated microglia (n = 15; Fig. 1c).

We tested the effect of different factors released by microglia upon activation of glutamate transporter activity in oligodendrocytes. Neither TNF-α (100 ng/ml) nor IL-1β (10 ng/ml) have any effect on glutamate uptake in oligodendrocytes (n = 3; Fig. 1d). However, paraquat (15 μM), a redox-cycling agent capable of generating reactive oxygen species (ROS) intracellularly (35), blocked glutamate transporters in oligodendrocytes (n = 3; Fig. 1d), and Trolox (10 μM), a ROS scavenger, prevented glutamate transporter inhibition by LPS-activated microglia (n = 3; Fig. 1d).

We next checked whether the inhibition of glutamate transporters by LPS-activated microglia increased glutamate levels in the culture medium. The concentration of glutamate in control and LPS-treated oligodendrocyte cultures was 0.7 μM (Fig. 2a, OL; n = 9) and 0.65 μM, respectively (Fig. 2a, OL+LPS; n = 4). Coculture of microglial cells with oligodendrocytes during a 48-h period raised glutamate concentration to 12 μM (n = 9; Fig. 2a). Notably, the addition of LPS to activate microglia in these co-cultures further elevated glutamate levels to 34 μM (n = 10; Fig. 2a). To investigate the cellular source of glutamate, we analyzed extracellular glutamate levels in pure microglia cultures at the same density. Interestingly, we detected the same levels of extracellular glutamate in microglia alone as in mixed cultures treated with LPS, suggesting that microglia is a major source of glutamate under these conditions (n = 10; Fig. 2a). In addition, the levels of glutamate in mixed cultures treated with LPS were similar to those after incubation with the glutamate transporter inhibitor TBOA (1 mM; n = 5; Fig. 2a). Together, these data suggest that glutamate released by microglial cells is normally taken up by oligodendrocytes in control conditions, whereas in the presence of LPS glutamate accumulates in the medium following an inhibition of oligodendrocyte glutamate uptake.

We next investigated the mechanisms by which microglia releases glutamate. We found that glutamate released by microglial cells is not inhibited by TBOA and thus, it is not due to a reversal function of transporters (1 mM; n = 3; Fig. 2b). Another possible glutamate release mechanism by microglia is the cystine-glutamate antiporter, which exchanges extracellular cystine for intracellular glutamate. Indeed, aminoacidic acid (AAA; 2.5 mM), an inhibitor of cystine-glutamate antiporter xCT (2, 28), induced a significant reduction in glutamate release by microglia (n = 3; Fig. 2b). In addition, AAA also inhibited glutamate release in mixed oligodendrocyte-microglia cultures treated with LPS (n = 3; Fig. 2a). Finally, a moderate increase in glutamate levels was detected after the exposure of microglial cells to LPS (100 ng/ml, n = 5; Fig. 2b), indicating that glutamate release by the system xCT− is enhanced in activated microglia. In agreement with this finding, we observed that the expression of the cystine-glutamate antiporter xCT is increased in microglia after LPS treatment as revealed by Western blot analysis (n = 3; Fig. 2c).

Activated microglia induced oligodendroglial excitotoxicity

We next checked whether activated microglia contributes to LPS toxicity in oligodendrocyte-microglia cocultures. The addition of
resting microglia or LPS did not alter oligodendrocyte viability (n = 3 or 4, respectively; Fig. 3, a and b), in agreement with previous data showing no alteration in glutamate homeostasis (see Figs. 1b and 2a). However, LPS-activated microglia induced oligodendrocyte cell death, which was prevented by the AMPA and kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (30 μM; n = 4; Fig. 3, a and b) and by the ROS scavenger Trolox (10 μM; n = 3; Fig. 3b). Oligodendroglial excitotoxicity is associated with calcium overload, alteration of the mitochondrial membrane potential and a rise in ROS (36). Because of that, we next analyzed whether LPS-activated microglia alters mitochondrial membrane potential in oligodendrocytes. After treatment with LPS (100 ng/ml), we detected an early increase in the JC-1 ratio that is indicative of mitochondria membrane hyperpolarization in mixed oligodendrocyte-microglia cultures (3h; n = 3; Fig. 3c). The same treatment did not induce any change in mitochondrial membrane potential in pure microglial cell cultures (Fig. 3c).

Because a major source of extracellular glutamate in mixed cultures is the microglial system x_{c}^{-}, we next examined whether the inhibition of the cystine-glutamate antiporter prevented LPS toxicity to oligodendrocytes. The inhibition of system x_{c}^{-} with 2.5 mM AAA had no significant effect on cell viability in pure oligodendrocyte cultures (n = 3; Fig. 3d). However, AAA abolished LPS-activated microglial toxicity to oligodendrocytes (n = 3; Fig. 3d). Taken together, these results suggest that glutamate excitotoxicity contributes to LPS-activated, microglia-induced oligodendrocyte cell death.

Oligodendrocytes in white matter tracts express glutamate transporters and contribute to glutamate homeostasis (31, 32, 34). In addition, acute exposure to LPS produces oligodendrocyte cell death in isolated neonatal RONs (12). Oligodendrocytes were easily identified by the presence of myelinating axons imbedded within the cells in addition to features such as the presence of numerous mitochondria, a light cytoplasm, and a thin endoplasmic reticulum (Fig. 4a). Oligodendrocytes in nerves exposed to LPS (1 μg/ml for 3 h) did not exhibit the kind of frank necrotic changes produced by acute ischemia (37–39) and were typified by the swelling and disruption of mitochondria and, to a lesser degree, of the endoplasmic reticulum (Fig. 4, b–g). LPS treatment produced a significant increase in the mitochondrial area in oligodendrocytes, whereas a similar change was not found in the mitochondria of neighboring axons (Fig. 4h). To detect early changes related to apoptosis, we analyzed annexin V expression in LPS treated nerves. Annexin V translocation to the cell membrane was detected in naturally apoptotic cells in control nerves, whereas no annexin V translocation was apparent in damaged oligodendrocytes in LPS-treated nerves (Fig. 5).

Oligodendrocyte death induced by LPS was completely abolished by the non-N-methyl-d-aspartate (non-NMDA) glutamate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof(l)quinoxaline (NBQX) (10 μM; Fig. 4i). In addition, the x_{c}^{-} antiporter blocker AAA (2.5 mM) significantly reduced the number of dead cells found in LPS-treated optic nerves, implicating the antiporter in the glutamate release cascade in the whole mount optic nerve.

FIGURE 3. LPS-activated microglia induces oligodendroglial excitotoxicity. Pure oligodendrocyte cultures or mixed oligodendrocyte-microglia cultures were treated with LPS (100 ng/ml; 48 h) in the presence or absence of the AMPA/kainate receptor antagonist CNQX (30 μM) and the system x_{c}^{-} inhibitor AAA (2.5 mM; a). Double fluorescence staining with Abs to oligodendroglial marker O1 (red) and with the vital-dye calcine-AM (green) in untreated oligodendrocyte cultures (OL) and in mixed oligodendrocyte-microglia cultures (OL + MG) with or without LPS (100 ng/ml; 48 h). Only LPS-activated microglia is toxic to oligodendrocytes. Scale bar, 50 μm. b, Histogram shows LPS-activated microglia toxicity to oligodendrocytes, which is blocked in the presence of CNQX (30 μM) and Trolox (10 μM) (n = 3–4). c, Effect of LPS on mitochondrial membrane potential. Mixed oligodendrocyte-microglia cultures (OL + MG) or microglia alone cultures (MG) were treated with LPS (100 ng/ml) during a 3-h period and mitochondrial membrane potential was analyzed by fluorometry after loading cells with the fluorescent dye JC-1 (3 μM). Data was expressed as a JC-1 red/green ratio in percentage vs nontreated control cells. d, Blockade of the system x_{c}^{-} by AAA in LPS-activated microglia (MG) prevents oligodendrocyte (OL) excitotoxicity. Data are mean ± SEM of at least three independent experiments performed in triplicate. Symbols denote statistical significance vs control or LPS-treated cultures (+ and #, respectively). **, p < 0.01; #, p < 0.05; ##, p < 0.01.
Discussion

In this study we describe how activated microglia can kill oligodendrocytes by a dual mechanism leading to excitotoxicity. First, activated, but not resting, microglia release ROS and inhibit glutamate uptake in oligodendrocytes, which results in an increase in extracellular glutamate levels. Then we provide evidence that glutamate release by the cystine-glutamate antiporter expressed in microglia contributes substantially to the observed increase in extracellular glutamate. Finally, we show that elevated glutamate levels resulting from microglial activation causes oligodendrocyte excitotoxicity by the activation of AMPA and kainate receptors both in mixed cultures and in isolated optic nerves and that blocking this increase in the number of dead cells following LPS treatment that is prevented by NBQX (10 μM). Cell death in optic nerve in LPS and LPS with AAA (AAA + LPS). Note the significantly lower degree of cell death in the presence of AAA (2.5 mM). Scale bars = 1 μm; *** p < 0.001.

FIGURE 4. LPS-induced toxicity in optic nerve oligodendrocytes is glutamate receptor mediated. a, Oligodendrocytes (OL) in control optic nerve showing numerous mitochondria (arrows) and endoplasmic reticulum (arrowheads). Note the healthy appearance of the organelles within the cytoplasm and the numerous myelinated and remyelinated axons surrounding and embedded within the cells. b-d, Structural alterations of an oligodendrocyte (OC) within an LPS-treated optic nerve (1 μg/ml; 3 h). The boxed areas are shown at a higher gain in c and d. Note the swollen and disrupted mitochondria (arrows) and the swollen endoplasmic reticulum (arrowheads). Vacuoles (asterisks) are also apparent in the processes while axons (AX) embedded within the cells appear healthy. e-g, Appearance of another oligodendrocyte (OL) in an LPS-treated optic nerve. Boxed areas are shown at a higher gain in f and g for viewing the alterations in the mitochondria and endoplasmic reticulum. h, The mean mitochondrial area measured in oligodendrocytes (OL) and neighboring axons (AX) in control and LPS-treated (+LPS) optic nerves. Note the significant increase in the mitochondrial area in oligodendrocytes but not in axons after LPS treatment. i, Cell death in optic nerve treated with LPS and LPS with NBQX (NBQX + KOS) as compared with nontreated nerves (Control). Note the significant

FIGURE 5. LPS-mediated oligodendrocyte injury in an isolated P10 RON is not apoptotic. a, Natural cell death in the control optic nerve showing a cell with highly condensed chromatin that appears to be in the process of forming apoptotic bodies. Annexin V staining is localized by immunogold electron microscopy to the cell membrane (gold particles are indicated by the arrow) with no staining apparent in the cytoplasm. b and c, Oligodendrocytes in an LPS-treated nerve showing significant areas of mitochondrial vacuolization (asterisks) and only cytoplasmic annexin V staining (e.g., arrows). Scale bars = 400 nm (a) and 1 μm (b and c).
pathway alleviates oligodendrocyte death in culture and in the isolated optic nerve.

The immediate or innate immune response is the first line of defense against diverse microbial pathogens and requires the expression of recently discovered TLRs. TLR4 serves as a specific receptor for LPS, which in culture binds exclusively to microglia (11). Consistent with this idea, we found that LPS toxicity to oligodendrocytes requires the presence of microglial cells, as previously demonstrated (11). NO and the cytokines TNF-α and IL-1β are the main mediators of activated microglia-induced oligodendrocyte death (1, 10–12). A recent study (13), moreover, suggests that peroxynitrite, produced by the reaction between NO and superoxide anion, also mediates oligodendrocyte demise by LPS-stimulated microglia. We show here that excitotoxicity is an additional mechanism contributing to activated microglia-induced oligodendrocyte death.

Our findings indicate that activated microglia inhibit Na+-dependent glutamate transporters in cultured oligodendrocytes, resulting in extracellular glutamate accumulation and the subsequent overactivation of ionotropic AMPA/kainate glutamate receptors. These results are consistent with those reporting that LPS-activated microglia can also cause neuronal excitotoxicity (2). In addition, previous evidence indicates that TNF-α or IL-1β renders oligodendrocytes vulnerable to excitotoxicity in mixed glial cultures by inhibition of the function of glutamate transporters in astrocytes (40, 41). In contrast, we did not detect any change in glutamate uptake in oligodendrocytes after treatment with either cytokine, TNF-α or IL-1β. The different sensitivity between astrocytes and oligodendrocytes could be explained by the expression of different subtypes of transporters in each cellular type. Thus, whereas astrocytes express mainly the GLT-1 subtype, oligodendrocytes also express GLAST and EAAC1 (31, 32, 34), the latter being up-regulated after treatment with TNF-α (42). Notably, we observed that ROS inhibited glutamate uptake in oligodendrocytes and that the antioxidant Trolox prevented activated microglia-induced glutamate transporter inhibition and toxicity to oligodendrocytes. In addition to ROS, peroxynitrite, generated by the reaction between the superoxide anion and NO, has been identified as a major oxidant released by LPS-activated microglia (13). Indeed, peroxynitrite may indirectly induce excitotoxicity because it is a potent and fast inhibitor of the glutamate transporters GLAST, GLUT-1, and EAAC1 (43).

A link between inflammation and excitotoxic neurodegeneration due to glutamate transporter inhibition has been proposed as a component in the etiology of MS. In MS white matter active lesions, which are characterized by the presence of activated microglia and/or macrophage/microcyte infiltrates and the loss of oligodendrocytes, the expression of glutamate transporters is severely down-regulated (44) and glutamate levels are increased (24). Notably, glutamate levels are also altered in serum, suggesting that monocyte/macrophage lineage contributes to glutamate homeostasis alteration in this disease (45). In this study we demonstrate for the first time a causative link between inflammation and the loss of glutamate transporter function in oligodendrocytes and white matter. In addition to microglia, a recent study has demonstrated that autoantigen-activated myelin basic protein-specific T cells are also able to inhibit glutamate transporter expression in glial cells (46). Altogether, these data could explain glutamate homeostasis alterations in EAE as well as in MS plasma and cerebrospinal fluid.

Under conditions in which Na⁺-dependent glutamate transporters are not functional, tonic glutamate release by the microglial system x⁻ can be increased in LPS-treated microglia. This feature is consistent with the up-regulation of cystine-glutamate antiporter xCT transcripts in macrophages and microglia in response to bacterial LPS or β-amyloid that others and we have detected (27, 28). Together, these results suggest that the microglial system x⁻ is relevant to the maintenance of glutamate homeostasis in axonal tracts. Indeed, the tonic release of glutamate that contributes to maintaining extracellular glutamate in the micromolar range outside synapse is mainly dependent on system x⁻ (47). The system x⁻ has been implicated in neurological conditions such as β-amyloid toxicity (28, 48), virally induced encephalopathy (49), and periventricular leukomalacia (30). The present findings bring about the possibility that blocking microglial system x⁻ during inflammation could contribute to the prevention of oligodendrocyte damage in white matter disorders.

In summary, this study provides evidence that the concomitant glutamate release from activated microglia by the cystine-glutamate antiporter and the inhibition of Na⁺-dependent glutamate uptake by activated microglia can induce a local increase in extracellular glutamate that leads to excitotoxic oligodendrocyte death. In addition, these results suggest that counteracting these deleterious mechanisms of microglia could be exploited to treat white matter disorders.

Disclosures

The authors have no financial conflict of interest.

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