Oligodendrocytes, the myelinating cells of the CNS, are highly vulnerable to glutamate excitotoxicity, a mechanism involved in tissue damage in multiple sclerosis. Thus, understanding oligodendrocyte death at the molecular level is important to develop new therapeutic approaches to treat the disease. Here, using microarray analysis and quantitative PCR, we observed that dual-specific phosphatase-6 (Dusp6), an extracellular regulated kinase-specific phosphatase, is up-regulated in oligodendrocyte cultures as well as in optic nerves after AMPA receptor activation. In turn, Dusp6 is overexpressed in optic nerves from multiple sclerosis patients before the appearance of evident damage in this structure. We further analyzed the role of Dusp6 and ERK signaling in excitotoxic oligodendrocyte death and observed that AMPA receptor activation induces a rapid increase in ERK1/2 phosphorylation. Blocking Dusp6 expression, which enhances ERK1/2 phosphorylation, significantly diminished AMPA receptor-induced oligodendrocyte death. In contrast, MAPK/ERK pathway inhibition with U0126 significantly potentiates excitotoxic oligodendrocyte death and increases cytochrome c release, mitochondrial depolarization, and mitochondrial calcium overload produced by AMPA receptor stimulation. Upstream analysis demonstrated that MAPK/ERK signaling alters AMPA receptor properties. Indeed, Dusp6 overexpression as well as incubation with U0126 produced an increase in AMPA receptor-induced inward currents and cytosolic calcium overload. Together, these data suggest that levels of phosphorylated ERK, controlled by Dusp6 phosphatase, regulate glutamate receptor permeability and oligodendroglial excitotoxicity. Therefore, targeting Dusp6 may be a useful strategy to prevent oligodendrocyte death in multiple sclerosis and other diseases involving CNS white matter.

Multiple sclerosis (MS) is a chronic, degenerative disease of the CNS, which is characterized by focal lesions with inflammatory, demyelination, infiltration of immune cells, oligodendroglial death, and axonal damage (1, 2). These cellular alterations are accompanied by neurological deficits such as sensory disturbances, lack of motor coordination, and visual impairment (3). MS usually begins with an autoimmune inflammatory reaction to myelin components and progresses later to a chronic phase in which oligodendrocytes, myelin, and axons degenerate (4). Although the precise cause of MS remains unclear, several lines of evidence support the hypothesis that glutamate excitotoxicity may be involved in this pathology (5–8).

Glutamate, the main excitatory transmitter of the CNS, contributes to oligodendrocyte excitotoxicity and axonal dysfunction by acting on ionotropic AMPA (5, 6), kainate (9, 10), and NMDA receptors (11–13). Activation of AMPA/kainate receptors in vivo can induce inflammation, demyelination, and other pathological features that are typical of MS lesions (9). A central event to glutamate excitotoxicity is Ca2+ influx upon receptor activation and the ensuing accumulation of this cation within mitochondria, which leads to depolarization of this organelle and increased production of radical oxygen species (14, 15). Dysfunction of mitochondria leads to the translocation of cytochrome c to the cytosol, where it can activate caspase-9 and downstream caspase-3 and trigger apoptosis (16). However, the molecular cascades initiated by AMPA and kainate receptors upstream to mitochondrial dysfunction are not identical, indicating that different intracellular domains are involved in executing the death program triggered by these receptors (15). Cell death induced by mild excitotoxic insults can be prevented by the overexpression of the anti-apoptotic Bcl-2 (15, 17) or by preventing bax translocation and cytochrome c release after IGF-1 exposure (18, 19).

To understand the mechanism by which glutamate receptor activation induces apoptosis in oligodendrocytes and select possible therapeutic targets, we have analyzed the expression of genes in models of oligodendroglial cell death using gene array technology. We have detected increases in dual-specific phosphatase-6 (Dusp6) or MKP-3 from different putative proapoptotic genes. Dusp6 is an intracellular enzyme, which catalyzes the removal of phosphate groups from both threonine and tyrosine residues on its substrate, ERK1/2 (20). ERKs act at the end of a kinase cascade that is activated in response to a variety of signals from hormones, growth factors, cytokines, neurotransmitters, ethylbenzimidazolocarbocyanine iodide; EGF, enhanced GFP; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CTZ, cyclothiazide; FCCP, p-trifluoromethoxyphenylhydrazine.
Mitters, and stresses. Upon activation, ERK phosphorylates and activates membrane-associated and cytoplasmic proteins and translocates into the nucleus and phosphorylates transcription factors such as c-Jun, c-Fos, elk-1, and c-Myc (21). In this study, we have therefore analyzed the role of Dusp6 and ERK signaling in oligodendroglial cell death.

**MATERIALS AND METHODS**

**Oligodendrocyte Cultures and Toxicity Assays**—Primary cultures of oligodendrocytes derived from optic nerves of 12-day-old Sprague-Dawley rats were obtained as described previously (15). Cells were maintained at 37 °C and 5% CO2 in a chemically defined medium (15). After 2–4 days in vitro, cultures were composed of at least 98% O4/GalC+ cells. No microglial cells were detected in these cultures. For RNA expression analysis, cultures were treated with AMPA (10 μM) in the presence of cyclothiazide (100 μM) during 2, 10, and 15 min. RNA was collected immediately after stimulation in the first two cases and 1 h later for the 15-min treatment. For toxicity assays, cell death after AMPA receptor stimulation (15 min) was determined 24 h later using calcein-AM (Invitrogen) as described previously (22). The total calcein fluorescence on each coverslip was measured using a Synergy-HT fluorimeter (BioTek Instruments), and data were expressed as percentage of cell death versus the respective control.

Dusp6 expression was blocked with antisense oligonucleotides (ODNs). The sequences of Dusp6 antisense ODNs are as follows: Dusp6-1 (antisense), GCCCGGGATGGCCACGT-TGA; Dusp6-2 (antisense), GATTGCAGGGAGTCCACTTG; and Dusp6 (scrambled), CATAGTGGTCGTCGACGTGA. The 5' end of ODNs were conjugated to Texas Red to monitor the transfection efficiency, and the ODNs were delivered into oligodendrocytes using Lipofectin (Invitrogen) following the manufacturer’s instructions. When using antisense, cell viability was determined by counting calcein-positive cells in the transfected population (Texas Red+ cells). Cell counts were performed using a 20× objective in at least 10 fields per coverslip. Experiments were done in triplicate, and counts were carried out by two independent observers. Results were expressed as mean ± S.E. of at least three independent experiments performed in triplicate.

**Human Tissue Samples**—Postmortem optic nerve samples from 13 longstanding MS patients and 12 control subjects (who died from non-neurological diseases) were obtained at autopsy under the management of the Netherlands Brain Bank. All patients and controls had previously given written approval for the use of their tissue, according to the guidelines of the Netherlands Brain Bank. Clinical characteristics for control and patient groups have been described previously (23; see supplemental Table 1). We used clinical data together with macroscopic tissue analysis to classify MS samples as normal appearing or damaged optic nerves, when showing macroscopic plaques, atrophy, and/or optic neuritis. For comparisons, MS samples were matched with control samples for age, sex, and postmortem delay.

**Preparation of Rat Optic Nerves and Drug Perfusion**—Adult male Sprague-Dawley rats were deeply anesthetized with iso-flurane and then decapitated. Optic nerves were freed from their meninges in artificial cerebrospinal fluid (126 mM NaCl, 3 mM KCl, 2 mM MgSO4, 26 mM NaHCO3, 1.25 mM Na2HPO4, and 2 mM CaCl2·2H2O) supplemented with 10 mM glucose. Subsequently, the nerves were placed into a chamber and perfused for 3 h with oxygen-saturated artificial cerebrospinal fluid, to which glutamate (1 mM) alone or in the presence of CNQX (30 μM, Sigma), was applied. After perfusion, total RNA was extracted, and synthesis of cDNA was carried out as described below.

**Microarray Analysis**—The probes for gene expression analysis were performed according to Affymetrix protocol. Briefly, total RNA from oligodendrocyte cultures were isolated using a TRIzol reagent kit (Invitrogen), and RNA was reverse-transcribed to double-stranded cDNA. The double-stranded cDNA was purified and transcribed in vitro to biotin-labeled cRNA and subsequently fragmented. 15 μg of labeled and fragmented cRNA was hybridized to Rat Genome U34 and 230 Gene Chips (Affymetrix, Santa Clara, CA). The chips were stained, washed (Affymetrix Fluidics Station 400), and scanned (Affymetrix GeneChip Scanner 3000) according to the manufacturer’s protocol. Microarray data were extracted from the scanned images by using MAS software (version 5.0; Affymetrix). Microarray data were processed using Genespring software (Agilent Technologies, Santa Clara, CA). The data were normalized using Genespring’s per chip normalization and per gene normalization. A 3-fold difference in normalized expression was used to identify gene transcript that are differentially expressed. The data set consisted of a logarithm of fold-change generated by Genespring normalization and comparison analysis. p values were calculated using t test. Real time quantitative RT-PCR was used to confirm the data obtained.

**RNA Isolation and Quantitative Real Time PCR**—Total RNA was extracted from oligodendrocytes, HEK cells, and rat and human optic nerves using the TRIsol reagent kit (Invitrogen) and purified using the RNeasy Mini kit (Qiagen). The RNA from cultured cells was treated with DNase I (Ambion) to eliminate contaminating DNA. No DNase I treatment was performed in RNA obtained from rat and human optic nerves. The integrity of RNA was analyzed by ethidium bromide-agarose gel or using a Bioanalyzer (Agilent). cDNA synthesis, 2 μg of total RNA was used using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen). Mock reverse transcription (without enzyme) for each sample served as negative controls.

The relative expression level of genes were measured according to the manufacturer’s protocol with quantitative real time PCR (ABI Prism 7700 sequence detector, Applied Biosystems) as described previously (23). The expression level was normalized with a normalization factor obtained using the Genorm software (24) based on the expression of three to six housekeeping genes (see supplemental Table 1). Primers (supplemental Table 2) were designed with PrimerExpress software (Applied Biosystems). We verified that generated fluorescence was not overestimated by contamination resulting from residual genomic DNA amplification (using RT negative controls) and from primer dimer formation or external DNA contamination (no template controls). Quantitative PCR products were also subjected to a dissociation protocol to ensure that a single amplicon of the expected melting temperature was indeed obtained.
Analysis of Mitochondrial Membrane Potential—The changes in mitochondrial membrane potential were monitored with 5,5′,6′,6″-tetrachloro-1,1′,3,3″-tetracehylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes) according to the manufacturer’s protocol. Immediately after AMPA stimulation, cells were loaded with JC-1 (3 μM) at 37 °C for 15 min. In the cytosol the monomeric form of this dye fluoresces green (excitation at 485 nm and emission at 527 nm), whereas within the mitochondrial matrix, highly concentrated JC-1 forms aggregates that fluoresce red (excitation at 485 nm and emission at 590 nm). Both JC-1 monomers and aggregates were detectable using a Synergy-HT fluorimeter (Bio-Tek Instruments, Inc., Beverly, MA), and the changes in mitochondrial potential were calculated as the red/green emission ratio in each condition.

Immunocytochemistry and Western Blot—Oligodendrocyte cultures were fixed in 4% paraformaldehyde in PBS and processed for immunocytochemistry as described previously (15). Polyclonal primary antibodies were as follows: Dusp6 (N terminus; 1:100; Santa Cruz Biotechnology), phospho-p44/42 MAPK (1:250; Cell Signaling), and cytochrome c (1:100; Santa Cruz Biotechnology). Oligodendroglial expression of Dusp6 phosphatase was examined by double immunofluorescence using a goat polyclonal antibody to Dusp6 and a monoclonal antibody to galactocerebroside C (3 μg/ml; Chemicon) as described previously (10). Primary antibodies were visualized with the corresponding Alexa Fluor 488 or 594 secondary antibodies (1:200; Invitrogen). Controls by omitting the primary antibody gave no labeling. Dusp6 immunostaining after ODN transfection was analyzed using the ImageJ program in at least five different fields from three different experiments. For phospho-ERKs and cytochrome c immunostaining, cells were counterstained with Hoechst 33258 to simultaneous visualize nucleus and nuclear condensation. Cells presenting diffuse cytochrome c labeling were counted, and data were plotted as a percentage of positive cells versus control coverslips.

Total protein from oligodendrocytes cultures were extracted by scraping the cells in SDS/sample buffer. Samples were boiled for 5 min and subjected to SDS-PAGE in 12.5% polyacrylamide gels. After electrophoretically on nitrocellulose membranes, proteins were visualized using primary antibodies to phospho-ERK1/2 (1:500; Cell Signaling) and total ERK (1:500; Cell Signaling) and secondary peroxidase-coupled goat anti-rabbit antibody (1:5000; Sigma).

Electrophysiology and Ca2+ Imaging—Whole cell recordings were performed at room temperature and at a holding membrane potential of −70 mV. Extracellular bath solution with a pH of 7.3 contained the following: 140 mM NaCl, 5.4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES. Patch clamp pipettes (3–5 meghoms) were filled with internal solution at a pH of 7.3 containing the following: 140 mM CsCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 11 mM EGTA, and 2 mM Na-ATP. To analyze the role of Dusp6 overexpression, oligodendrocytes were cotransfected with Dusp6 plasmid (1 μg; RZPD-German Centre for Genome Research) and enhanced GFP (EGFP, 1 μg) using a Nucleofector kit following the manufacturer’s instructions (Amaxa Boisystems). Responses were analyzed 24–48 h after transfection.

For Ca2+ recording, cells were loaded with fura-2 AM (5 μM; Molecular Probes, Eugene, OR) in culture medium for 30 min at 37 °C. Experiments were carried out in a coverslip chamber continuously perfused with a buffer containing 20 mM HEPES, 2 mM CaCl2, and 10 mM glucose in HBSS at 1 ml/min. The perfusion chamber was mounted on the stage of a Zeiss inverted epifluorescence microscope (Axiovert 35), equipped with a 150-watt xenon lamp Polychrom I (TILL Photonics GMBH) and a Plan Neofluor 40× oil immersion objective (Zeiss, Germany). Cells were visualized with a high resolution digital black/white CCD camera (ORCA; Hamamatsu Photonics Iberica, Barcelona, Spain), and image acquisition and data analysis were carried out using the Aquacosmos software program (Hamamatsu Photonics Iberica). Images were acquired every 5 ms at 37 °C. [Ca2+]i was estimated by the 340/380 ratio method, using a Kd value of 224 nm. At the end of the assay, in situ calibration was carried out with the successive addition of 10 mM ionomycin and 2 M Tris/50 mM EGTA, pH 8.5. Data were analyzed with Excel and Prism software.

Rhod-2 AM (Molecular Probes, Invitrogen) was used to measure mitochondrial Ca2+ according to the previous procedure with modifications (25). Rhod-2 AM has a net positive charge, which facilitates its sequestration into mitochondria due to membrane potential-driven uptake. Cells were loaded with 1.25 μM Rhod-2 AM for 40 min. The residual cytosolic fraction of the dye was eliminated when the cells were kept in culture for an additional 18 h after loading, whereas the mitochondrial dye fluorescence was maintained. Fluorescence images of Rhod-2 were acquired using 550 nm for excitation and 590 nm for emission. The fluorescence of Rhod-2 was not calibrated in terms of [Ca2+]mit as it is not a ratiometric dye.

Statistical Analysis—Data are presented as mean ± S.E. p values are from Student’s two-tailed t test except for multiple comparisons, which were performed with one-way analysis of variance and Bonferroni post hoc tests.

RESULTS

Gene Expression Profile in Oligodendrocyte Culture—We performed a genome-wide transcription profiling to identify the molecular signaling of oligodendrocyte cell death. Specifically, we compared RNA from control cultures with RNA obtained from AMPA (10 μM)-stimulated cultures at different times: 2 min (n = 3), 10 min (n = 3), and 15 min (n = 3). This is a mild stimulus that induces oligodendroglial apoptosis by activation of the mitochondrial caspase pathway (15). The complexity of the data were reduced removing the nonsignificant probe sets (i.e. those not expressed and those not changing). After data scaling, normalizing and filtering, 8680 probe sets remained for further statistical analysis. Hierarchical clustering showed differences between control and AMPA-treated cultures. Specifically, 219 probe sets were up-regulated, and 143 probe sets were down-regulated. Most genes that were differentially expressed after AMPA treatment are related to gene transcription and metabolism (supplemental Tables 3–5). However, among genes that were up-regulated at all different times, we identified the Dusp6 or MAPK phosphatase-3, a highly specific ERK-phosphatase (Table 1).
To independently test the validity of the differential expression of Dusp6 determined by microarray analysis, we measured the expression patterns of some of the putative biomarkers. We assessed gene expression of four representative genes in all culture samples using quantitative real time polymerase chain reaction. In all cases, the quantitative RT-PCR results of the analyses confirmed the differences in our microarray analysis and showed that AMPA treatment induced a rapid up-regulation of Dups6 phosphatase (Table 1).

Dusp6 Phosphatase Controls Oligodendroglial Excitotoxicity

TABLE 1
Quantitative PCR of changes in gene expression in oligodendrocytes after AMPA receptor activation

Data are derived from quantitative PCR experiments (qRT-PCR) and from Affymetrix chip analysis (arrays) and are depicted as fold change of expression in stimulated versus control oligodendrocytes at the post-activation time points indicated below. Values for four up-regulated or down-regulated representative genes are illustrated. iNOS, inducible NO synthase; NGF, nerve growth factor; FSH, follicle-stimulating hormone.

| Gene                          | GenBank™ accession no. | AMPA (t = 2 min) | AMPA (t = 10 min) | AMPA (t = 15 min) |
|-------------------------------|------------------------|------------------|-------------------|------------------|
|                               |                        | qRT-PCR          | Arrays            | qRT-PCR          | Arrays            | qRT-PCR          | Arrays            |
| NGF-induced protein A         | M18416                 | 5.25             | 11.50             | 8.41             | 22.10             | 10.87             | 42.90             |
| c-Fos                        | X06769                 | 1.20             | 6.40              | 1.49             | 3.10              | 3.91              | 15.70             |
| FSH-regulated protein         | L26292                 | 1.20             | 1.90              | 1.50             | 1.10              | 8.10              | 13.10             |
| Dusp6                         | U42627                 | 1.24             | 2.60              | 1.05             | 3.50              | 2.26              | 5.90              |
| α-Globin                      | X56325                 | −2.63            | −10.20            | −9.81            | −7.40             | −67.05            | −3.10             |
| β-Globin                      | M94918                 | −46.04           | −9.20             | −5.43            | −10.00            | −9.63             | −3.40             |
| iNOS                          | U03699                 | −6.73            | −4.70             | −0.72            | −4.50             | −1.20             | −5.30             |
| Interleukin-1α                | D00403                 | −3.78            | −3.00             | −3.32            | −2.90             | −10.47            | −6.50             |

FIGURE 1. Quantitative PCR analysis of Dusp6 phosphatase RNA expression in glutamate treated optic nerves and in human optic nerves from MS patients. A, dose-response study of the effect of glutamate (3 h) on Dups6 RNA expression in rat optic nerves. B, Change in the expression of Dusp6 RNA levels in rat optic nerves after addition of 1 mM glutamate (3 h) with or without CNQX (30 μM). Statistical analysis showed a significant increase in Dusp6 in rat optic nerves treated with glutamate, which was abolished in the presence of the AMPA/kainate antagonist CNQX. Symbols denote statistical significance versus control (*) or AMPA-treated optic nerves (#). *, p < 0.05; ##, p < 0.01. C, expression of Dusp6 in control optic nerves and in optic nerves from MS patients. Expression in MS samples have been normalized to controls matched by age and sex (n = 13). a.u., arbitrary units. D, summary of Dusp6 expression changes in human MS optic nerves with or without apparent damage. Dusp6 was up-regulated exclusively in optic nerves without damage (n = 6). *, p < 0.05. NAON, normal appearing optic nerve; DON, damage optic nerves (as described under "Materials and Methods"). Error bars show the S.E.

Dusp6 Expression Is Increased in Glutamate-treated Optic Nerves and in MS Optic Nerves—We next analyzed whether elevated concentrations of glutamate are sufficient to induce an up-regulation of Dusp6 in an axonal tract such as the optic nerve. To this end, isolated adult rat optic nerves were perfused with oxygen-saturated artificial cerebrospinal fluid and expression of Dusp6 was assayed by quantitative PCR. Addition of glutamate (1 mM for 3 h) to the perfusate resulted in a significant increase of −1.5-fold in the expression of Dusp6 compared
with controls ($n = 4; p < 0.05; EC_{50} = 13 \mu M$; Fig. 1, A and B). This effect was partially blocked by adding to the perfusate the AMPA/kainate receptor antagonist CNQX (30 \mu M; $p < 0.01$; Fig. 1F). This result indicates that glutamate receptor activation up-regulates Dusp6 in rat optic nerves.

Levels of glutamate are altered in demyelinating diseases, in particular in MS (26). Because of that, we next studied the expression of Dusp6 in post-mortem optic nerves samples of MS patients. This cohort of samples has been characterized in terms of ERK1/2 phosphorylation. Indeed, following AMPA (10 \mu M) stimulation, a rapid increase in ERK1/2 phosphorylation was detected. The kinetics of activation of the two kinases was similar (Fig. 2B). Maximal phosphorylation of ERKs was detected at 10 min following AMPA receptor stimulation, decreased after 15 min, and reached basal levels thereafter (Fig. 2B). The activation of ERK1/2 was significantly inhibited in the presence of the AMPA/kainate antagonist CNQX (30 \mu M) and in the presence of UO126 (10 \mu M), an inhibitor of the MEK, the upstream kinase of ERK ($p < 0.05$; Fig. 2C). Immunocytochemical analysis revealed that active ERKs translocate to the nucleus after AMPA receptor stimulation (data not shown), where they can phosphorylate certain transcription factors, regulating the expression of genes involved in cell survival. ERK translocation to the nucleus was inhibited in the presence of CNQX antagonist (30 \mu M; data not shown).

Because there is no pharmacological agent to selectively block Dusp6 phosphatase, we used a molecular approach to analyze the role of Dusp6 in oligodendrogial cell death. Oligodendrocytes were transfected with Texas Red-labeled antisense ODNs against Dusp6 (see “Materials and Methods”). The efficacy of antisense ODNs to reduce Dusp6 expression was assessed on HEK 293 cells transfected with Dusp6 plasmid by quantitative PCR. Treatment with two antisense ODNs, named A and B, induced a 56 and 65% reduction in the corresponding mRNA, respectively (Fig. 3A). In addition, the two antisense ODNs induced a significant reduction in Dusp6 protein expression, as revealed by immunocytochemistry (Fig. 3B), whereas no change was detected with the sense ODNs. In turn, 10 \mu M AMPA (plus 100 \mu M CTZ)-induced cell death was significantly blocked in the presence of both antisense probes (Fig. 3C), demonstrating that Dusp6 expression is necessary for oligodendroglial excitotoxicity. In contrast, no effect on AMPA-induced cell death was observed with sense or scrambled ODNs (Fig. 3C).

If the prosurvival effect of Dusp6 antisense was due to an overactivation of ERK signaling, blocking this pathway should have the opposite effect. Indeed, oligodendrocytes pretreated with UO126 (10 \mu M; 10 min) showed an increase in AMPA (10 \mu M plus 100 \mu M CTZ)-induced oligodendroglial cell death (Fig. 3D), indicating that levels of phosphorylated ERK are critical to determine the survival or death of the cell after glutamate receptor activation. UO126 had no effect on cell viability in the absence of stimulus (data not shown). The effect of UO126 was observed when AMPA was applied at low concentrations, which are known to induce apoptotic oligodendroglial cell death (15). In contrast, no effect was detected when AMPA was applied at higher concentrations (100 \mu M; Fig. 3D), an insult which results in necrotic oligodendrocyte death (15).

**ERK Regulation of Mitochondrial Function—**Mild excitotoxic insults mediated by AMPA receptors in oligodendrocytes trigger apoptosis through the mitochondrial or intrinsic pathway characterized by cytochrome c release into the cytosol, which subsequently activates caspase-9 and -3 (15). To determine the signaling cascades triggered by ERK phosphorylation in oligodendrocytes, we studied mitochondrial dysfunction induced by AMPA in the absence or in the presence of UO126. Cytochrome c release into the cytoplasm can be detected immunocytochemically as a transition from a localized punctate-type labeling to a more diffuse-type labeling with antibodies against cytochrome c. As described previously (15), we detected an increase in cytochrome c release into the cytosol 1 h after AMPA receptor stimulation (10 \mu M AMPA plus 100 \mu M CTZ; 15 min; Fig. 4A), which was further elevated by blocking ERK signaling with UO126 (n = 3; p < 0.05; Fig. 4A). Because cytochrome c release is secondary to mitochondrial depolarization (16), we analyzed the role of UO126 in mitochondrial potential after AMPA receptor stimulation. AMPA (10 \mu M plus 100 \mu M CTZ; 15 min) induced a reduction of JC-1 ratio, indicative of mitochondrial depolarization, an effect that was exacerbated with UO126 (n = 4 in triplicate; p < 0.05; Fig. 4B).

We next studied mitochondrial Ca$^{2+}$ uptake using Rhod-2 AM, a cationic indicator which loads selectively into respiring mitochondria. To assess that feature, we used 25 \mu M FCCP, a protonophore that collapses the mitochondria membrane potential and releases Ca$^{2+}$ from mitochondria. Only cells where FCCP induced a reduction of Rhod-2 fluorescence at the beginning of the experiment were selected for further analysis (Fig. 4C). Under those conditions, we assessed the effects of AMPA ± UO126 on [Ca$^{2+}$]$_{mit}$. Acute application of AMPA (10 \mu M in the presence of CTZ 100 \mu M; 30 s) induced a transient increase in mitochondrial calcium, that returned to basal levels after washing (Fig. 4D). Preincubation with UO126 did not alter
the increase on $[\text{Ca}^{2+}]_{\text{mit}}$ induced by acute application of AMPA ($n = 63$ and 96 cells for control and UO126 treated cultures; Fig. 4D). In contrast, sustained activation of AMPA receptors (AMPA 10 $\mu$M plus CTZ 100 $\mu$M; 5 min) induced a higher increase on $[\text{Ca}^{2+}]_{\text{mit}}$ ($n = 84$ cells; Fig. 4E) that was potentiated in the presence of UO126 (10 $\mu$M; $n = 87$ cells; $p < 0.05$; Fig. 4, E and F). Together, these results demonstrated that mitochondrial function and $\text{Ca}^{2+}$ buffering were altered by blocking ERK1/2 signaling.

**AMPA Receptor Inward Currents Are Regulated by ERK**—In oligodendrocytes, cell death induced by low stimulation of AMPA receptors is secondary to a massive increase in cytoplasmic calcium (15). To further characterize the signaling cascades triggered by ERK phosphorylation in oligodendrocytes, we checked whether upstream mechanisms such as inward currents and cytosolic calcium overload after AMPA receptor stimulation was altered by blocking ERK1/2 signaling. To that end, we cotransfected oligodendrocytes with Dusp6 and EGFP...
expression plasmids. Because the cotransfected plasmids enter the cells with equal probability, EGFP-positive cells were likely to contain Dusp6 DNA. Indeed, EGFP-positive oligodendrocytes showed an increased expression of Dusp6 as revealed by immunocytochemical analysis (data not shown). In addition, phosphorylation of ERK1/2 after AMPA receptor stimulation was significantly reduced in oligodendrocytes transiently transfected with Dusp6 plasmid (Fig. 5A). Then, we analyzed AMPA responses in EGFP+/H11001 and EGFP+/H11001/Dusp6+/H11001 cells by means of electrophysiology recording and Ca$^{2+}$/H11001 imaging. Using whole-cell patch clamp recording, we showed that 1 mM AMPA-evoked membrane inward currents were significantly enhanced in cells overexpressing Dusp6 ($I_{\text{peak}}$ in control cells = $-337 \pm 35$ pA, $n = 70$ cells; $I_{\text{peak}}$ in Dusp6 overexpressing cells = $-538 \pm 53$ pA, $n = 78$ cells; $p < 0.01$; Fig. 5B). Similarly, cytosolic Ca$^{2+}$/H11001 accumulation after AMPA receptor stimulation (10 $\mu$M AMPA and 100 $\mu$M CTZ) is potentiated in Dusp6-overexpressing cells ($n = 18$ and 21 control and Dusp6+ cells, respectively; $p < 0.01$; Fig. 5C).

Long term changes in AMPA receptor peak current amplitude associated to Dusp6 overexpression could be due to changes in glutamate receptor expression. However, acute pharmacological inhibition of MEK with UO126 induced similar effects. AMPA-induced inward currents were recorded in oligodendrocytes both before and after a 10-min treatment with UO126 (10 $\mu$M). UO126 induced an acute increase in AMPA-induced membrane inward currents ($I_{\text{peak}}$ in UO126-treated cells = $538 \pm 53$ pA, $n = 78$ cells; $p < 0.01$; Fig. 6A). In accordance, cells pretreated with UO126 (10 $\mu$M, 10 min) showed a higher increase in cytosolic [Ca$^{2+}$/H11001] after AMPA receptor stimulation ($n = 29$ and 34 cells in control and UO126-treated cultures respectively; $p < 0.01$; Fig. 6B). Altogether, these data indicate that Dusp6 and ERK signaling control glutamate receptor response.

DISCUSSION

Cells recognize danger signals and activate death-inducing pathways but also several prosurvival mechanisms in an attempt to cope with stress. We reported here that glutamate receptor overactivation in oligodendrocytes induced a rapidly activation of ERK1/2 signaling and a rapid increase in Dusp6 phosphatase and that levels of phosphorylated ERK1 and -2, controlled by the levels of Dusp6 phosphatase, are pivotal in determining cell death or survival. We demonstrated that
**FIGURE 4. Effects of ERK signaling on mitochondrial function and calcium overload after AMPA receptor stimulation.**

**A**, release of cytochrome c after AMPA receptor activation. Cultures were treated with AMPA (10 μM plus 100 μM CTZ) for 15 min and immunostained 1 h later with an antibody to cytochrome c (cyt c) and with Hoechst 33258. UO126 (10 μM), added 10 min prior to AMPA receptor stimulation, induced a significant increase in the number of cells showing diffuse cytochrome c labeling. The histogram represents mean ± S.E. of three different experiments. *, p < 0.05.

**B**, effects of ERK signaling on AMPA-induced changes on mitochondrial membrane potential. Oligodendrocytes were treated with AMPA (10 μM plus 100 μM CTZ; 15 min) with or without UO126 (10 μM) and mitochondrial membrane potential was analyzed by fluorimetry after loading cells with the fluorescent dye JC-1 (3 μM). Data were expressed as a JC-1 red/green ratio in percentage versus nontreated control cells (n = 4 independent experiments performed in triplicate). *, p < 0.05.

**C**, effects of FCCP on mitochondrial calcium in oligodendrocytes loaded with Rhod-2 AM. FCCP (25 μM) was used as an internal control to release Ca^{2+} from mitochondria. Calcium was normalized to the corresponding baseline levels of each cell.

**D**, effects of acute application of AMPA (10 μM plus 100 μM CTZ, 30 s) with or without UO126 (10 μM) on [Ca^{2+}]_{mit}. No significant change was detected on [Ca^{2+}]_{mit} peak amplitude. Recordings illustrate average ± S.E. responses of 63 and 96 cells, respectively, for control and UO126 treated cultures.

**E** and **F**, effects of continuous application of AMPA (10 μM plus 100 μM CTZ, 5 min) with or without UO126 (10 μM) on [Ca^{2+}]_{mit}. The histogram in **F** represents average ± S.E. of [Ca^{2+}]_{mit} peak amplitude for each condition (n = 84 and 87 cells for AMPA and AMPA + UO126 treated cultures, respectively. UO126 induced a significant increase in calcium accumulation in mitochondria. *, p < 0.05.
ERK1/2 acts as a prosurvival agent in oligodendrocytes by acting at different steps of the mitochondrial apoptotic pathway. Furthermore, the elevated expression of Dups6 in normal appearing axon tracts in MS suggests that this feature may be a risk factor associated with early lesion formation in this disease.

Dusp6 phosphatase expression is increased in oligodendrocytes in vitro after AMPA receptor stimulation as well as in optic nerves after glutamate stimulation. In accordance with our data, overexpression of the Dusp6 gene has also been detected in brain after kainic acid injection (in conditions that also activate AMPA receptors) (28). Moreover, cells overexpressing Dusp6 mRNA showed clear signs of cell death, including condensed nuclei, dendritic retraction, and cytoskeletal collapse (28), suggesting a role of Dups6 in neuronal cell death.

**FIGURE 5.** Dusp6 overexpression increases AMPA-evoked inward currents and cytosolic Ca$^{2+}$ increases in oligodendrocytes. A, Western blot analysis of ERK1/2 phosphorylation in oligodendrocytes transfected with EGFP plasmid alone or in conjunction with Dusp6 plasmid. Dusp6-overexpressing cells showed a significant reduction on AMPA receptor-induced ERK1/2 phosphorylation (10 μM AMPA plus 100 μM CTZ, 15 min). The histogram represents the mean ± S.E. of three experiments. *, p < 0.05. B, left, representative traces showing AMPA (1 mM)-evoked inward currents in control and in EGFP-Dusp6” oligodendrocytes whole-cell clamped at −70 mV. Control cultures were transiently transfected with an EGFP plasmid. Right, bar graph summarizing the currents in control and in EGFP-Dusp6” oligodendrocytes (n = 70 and 78 cells, respectively, from four independent experiments). **, p < 0.01. C, cytosolic [Ca$^{2+}$], increase induced by AMPA (10 μM) in the presence of CTZ (100 μM) in control and in EGFP-Dusp6” oligodendrocytes. Cells overexpressing Dusp6 showed a clear increase in [Ca$^{2+}$]. **, p < 0.01.
addition to glutamate receptor stimulation, an increased expression of Dusp6 has also been detected in peri-infarcted cortex after focal cerebral ischemia in rats, which are not accompanied by neuronal injury (29, 30) and in the hippocampal CA1 region following transient global ischemia induction in rats (31).

The induction of Dusp6 gene expression provides a sophisticated transcriptional mechanism for targeted inactivation of selected ERK1 and ERK2 kinase activities (21, 27). Notably, Dusp6 gene induction and ERK1 and ERK2 phosphorylation in oligodendrocytes have similar kinetics and are detected as early as 2 min following AMPA receptor stimulation, a time course which comparable to that observed for Dusp6 in CA1 neurons following transient global ischemia induction (31). In turn, activation of both presynaptic and postsynaptic AMPA receptors results also in activation of MAPK or to inhibition of adenylate cyclase (32–34), which is in line with our observation illustrating that oligodendroglial AMPA receptors are coupled to the MAPK signaling pathway. However, it is not known whether Dusp6 induction in oligodendrocytes is secondary to ERK1/2 phosphorylation or directly activated by AMPA receptor.

The role of ERK1/2 in cell death remains controversial (35). ERK1/2 has been commonly viewed as a promoter of cell survival induced in response to stimuli deleterious to neurons (36, 37). However, other studies have suggested a death-promoting role for ERK1/2 in several models of neuronal death such as traumatic brain injury, ischemia, and Alzheimer disease (reviewed in Ref. 38). In addition, glutamate oxidative toxicity and neuronal death induced by glutathione depletion was shown to be abolished when reactive oxygen species-dependent activation of ERK1/2 was inhibited by UO126 (39). Indeed, the kinetics and duration of ERK1/2 activation may direct ERK1/2 toward downstream targets that will either promote or limit neuronal survival (38, 40). The dual roles of ERK1/2 as a pro-survival and death-promoting kinase can be observed within a single neuronal cell type (i.e. HT22 cells) exposed to a single toxin (i.e. glutamate) (40). Thus, whereas early receptor-mediated ERK1/2 activation is linked to a prosurvival response, more sustained and retarded activation of ERK1/2 secondary to glutamate oxidative toxicity after prolonged exposure to glutamate is required to activate prodeath signals (39–42). In oligodendrocytes, we observed that AMPA induces a rapid and transient activation of ERK1/2 and, accordingly, blocking ERK1/2 signaling with UO126 exacerbates oligodendroglial excitotoxicity. In contrast, blocking Dusp6 phosphatase, and thus increasing ERK1/2 phosphorylation, nearly abolished AMPA-induced oligodendrocyte cell death.

**FIGURE 6.** Blocking ERK signaling with UO126 increases AMPA responses. A, left, representative traces showing two pulses of AMPA (1 mM) at t = 0 and 10 min and a pulse of AMPA (1 mM) followed by second pulse of AMPA (1 mM) after 10 min incubation with UO126 (10 μM; 10 min). Right, bar graph summarizing the currents normalized to the first pulse of AMPA to avoid current run down. *, p < 0.05. B, AMPA receptor-induced increase in cytosolic calcium in control oligodendrocytes cultures (black line) and in cultures pretreated with UO126 (10 μM; 10 min prior to AMPA application; gray line). Right, histogram represents average ± S.E. of [Ca²⁺] peak amplitude. UO126-treated oligodendrocytes showed a higher increase in cytosolic calcium after AMPA application. **, p < 0.01.
Activation of the ERK pathway inhibits apoptosis in various cellular models by different mechanisms, including inactivation of the proapoptotic factor Bad (43), interference with caspase activation at a step downstream of cytochrome c release (44), phosphorylation of Bcl-2 (45, 46), and inhibition caspase-9 through phosphorylation (47). In contrast, Dusp6 induces apoptosis via dephosphorylation of ERK1/2, leading to Bcl-2 proteolysis (48). In oligodendrocytes, ERK1/2 activation acts at different pre- and postmitochondrial apoptotic phases. Mitochondrion is a critical organelle for Ca\(^{2+}\) buffering in neurons as well as in oligodendrocytes (15). Excessive mitochondrial Ca\(^{2+}\) uptake is detrimental to cell viability because it causes mitochondrial membrane depolarization, which can lead to the release of cytochrome c and other proapoptotic molecules into the cytosol (reviewed in Ref. 16). In the current study, we observed that blocking ERK1/2 after AMPA receptor activation in oligodendrocytes potentiates cytochrome c release, mitochondrial depolarization, and mitochondrial Ca\(^{2+}\) overload. These effects are probably due to the altered Ca\(^{2+}\) buffering observed after blocking ERK signaling. However, other mechanisms such as a direct dephosphorylation of other targets, including Bcl-2 and Bax, should not be excluded. Indeed, mitochondrial depolarization can occur as a consequence of translocation of Bax from the cytosol to the mitochondria in the presence of U0126 (49). Overall, these data suggested that ERK1/2 signaling secondary to AMPA receptor activation constitutes a prosurvival mechanism in an attempt to prevent oligodendrogial excitotoxicity.

In addition to induce mitochondrial damage, blocking ERK1/2 signaling with U0126 or overexpression of Dusp6 phosphatase potentiates the responses to AMPA, including Ca\(^{2+}\) influx and the amplitude of the electrophysiological responses. This potentiation could be due to changes in the phosphorylation state and/or number of AMPA receptors, as well as changes in AMPA receptor subunit composition and Ca\(^{2+}\) permeability (50). The latter is rapidly modified by the self activation of AMPA receptors (51) and is induced by activation ERK/MAPK signaling pathway (52). Interestingly, AMPA receptor subunit composition, controlled by the transcriptional factor cAMP-response element-binding protein, is essential to determine glutamate receptor permeability and sensitivity to excitotoxicity in oligodendrocytes (53). However, further experiments will be required to characterize the mechanisms involved in ERK1/2-induced potentiation of AMPA mediated inward currents.

In conclusion, we have shown that AMPA receptor activation in oligodendrocytes induces the expression of Dusp6, which potentiates the activity of the receptors, inhibits the MAPK signaling pathway and promotes mitochondrial alterations leading to apoptotic death. Pharmacological inhibition of the MAPK pathway induces similar effects. Collectively, these data point to a protective role of the MAPK signaling in oligodendrocyte death and to Dusp6 as relevant negative regulator of this survival pathway.

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REFERENCES

1. Trapp, B. D., Peterson, J., Ransohoff, R. M., Rudick, R., Mörk, S., and Bö, L. (1998) *Neur. Eng. J. Med.* 338, 278–285
2. Primeas, J. W., McDonald, W. I., and Franklin, R. J. (2002) in *Greenfield’s Neuropathology* (Graham, D. I., and Lantos, P. L., eds.) 8th Ed., pp. 471–510, Edward Arnold Publisher, London
3. Steinman, L. (2001) *Nat. Immunol.* 2, 762–764
4. Steinman, L., Martin, R., Bernard, C., Conlon, P., and Oksenberg, J. R. (2002) *Arnu. Rev. Neurosci.* 25, 491–505
5. Matute, C., Sánchez-Gómez, M. V., Martínez-Millán, L., and Miledi, R. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 8830–8835
6. McDonald, J. W., Althomsons, S. P., Hyrc, K. L., Choi, D. W., and Goldberg, M. P. (1998) *Nat. Med.* 4, 291–297
7. Pitt, D., Werner, P., and Raine, C. S. (2000) *Nat. Med.* 6, 67–70
8. Smith, T., Groom, A., Zhu, B., and Turski, L. (2000) *Nat. Med.* 6, 62–66
9. Matute, C. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 10229–10234
10. Sánchez-Gómez, M. V., and Matute, C. (1999) *Neurobiol. Dis.* 6, 475–485
11. Káradóttir, R., Cavelier, P., Bergeersen, L. H., and Attwell, D. (2005) *Nature* 438, 1162–1166
12. Salter, M. G., and Fern, R. (2005) *Nature* 438, 1167–1171
13. Micu, I., Jiang, Q., Codere, E., Ridsdale, A., Zhang, L., Woulfe, J., Yin, X., Trapp, B. D., McRory, J. E., Rehak, R., Zamponi, G. W., Wang, W., and Stys, P. K. (2006) *Nature* 439, 988–992
14. Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003) *Nat. Rev. Mol. Cell Biol.* 4, 552–565
15. Sánchez-Gómez, M. V., Alberdi, E., Ibarretxe, G., Torre, I., and Matute, C. (2003) *J. Neurosci.* 23, 9519–9528
16. Galluzzi, L., Blomgren, K., and Kroemer, G. (2009) *Nat. Rev. Neurosci.* 10, 481–494
17. Lawrence, M. S., Ho, D. Y., Sun, G. H., Steinberg, G. K., and Saposky, R. M. (1996) *J. Neurosci.* 16, 486–496
18. Ness, J. K., and Wood, T. L. (2002) *Mol. Cell Neurosci.* 20, 476–488
19. Ness, J. K., Scaduto, R. C., Jr., and Wood, T. L. (2004) *Glia.* 46, 183–194
20. Muda, M., Theodosiou, A., Rodrigues, N., Boschert, U., Camps, M., Gillieron, C., Davies, K., Ashworth, A., and Arkingstall, S. (1996) *J. Biol. Chem.* 271, 2705–2708
21. Camps, M., Nichols, A., and Arkingstall, S. (2000) *FASEB J.* 14, 6–16
22. Domercq, M., Perez-Samartin, A., Aparicio, D., Alberdi, E., Pampoleo, O., and Matute, C. (2010) *Glia.* 58, 730–740
23. Vallejo-Illarramendi, A., Domercq, M., Pérez-Cerdá, F., Ravid, R., and Matute, C. (2006) *Neurobiol. Dis.* 21, 154–164
24. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002) *Genome Biol.* 3, RESEARCH0034
25. Hajnóczky, G., Robb-Gaspers, L. D., Seitz, M. B., and Thomas, A. P. (1995) *Cell* 82, 415–424
26. Sarchielli, L., Greco, L., Floridi, A., Floridi, A., and Gallai, V. (2003) *Arch. Neurol.* 60, 1082–1088
27. Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U., and Arkingstall, S. (1998) *Science* 280, 1262–1265
28. Boschert, U., Dickenson, R., Muda, M., Camps, M., and Arkingstall, S. (1998) *Neuroreport* 9, 4081–4086
29. Soriano, M. A., Tessier, H., Certa, U., and Gill, R. (2000) *J. Cereb. Blood Flow Metab.* 20, 1045–1055
30. Lu, A., Tang, Y., Ran, R., Clark, J. F., Arnow, B. J., and Sharp, F. R. (2003) *J. Cereb. Blood Flow Metab.* 23, 786–810
31. Kawahara, N., Wang, Y., Mukasa, A., Furuya, K., Shimizu, T., Hamakubo, T., Aburatani, H., Kodama, T., and Kirino, T. (2004) *J. Cereb. Blood Flow Metab.* 24, 212–223
32. Hayashi, T., Umemori, H., Mishina, M., and Yamamoto, T. (1999) *Nature* 397, 72–76
33. Perkinton, M. S., Siha, T. S., and Williams, R. J. (1999) *J. Neurosci.* 19, 5861–5874
34. Shenk, U., and Matteoli, M. (2004) *Biol. Cell.* 96, 257–260
35. Cheung, E. C., and Slack, R. S. (2004) *Sci. STKE* 251, PE45
36. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) *Science* 270, 1326–1331
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37. Grewal, S. S., York, R. D., and Stork, P. J. (1999) Curr. Opin. Neurobiol. 9, 544–553
38. Subramaniam, S., and Unsicker, K. (2010) FEBS J. 277, 22–29
39. Stanciu, M., Wang, Y., Kentor, R., Burke, N., Watkins, S., Kress, G., Reynolds, I., Klann, E., Angioli, M. R., Johnson, J. W., and DeFranco, D. B. (2000) J. Biol. Chem. 275, 12200–12206
40. Luo, Y., and DeFranco, D. B. (2006) J. Biol. Chem. 281, 16436–16442
41. de Bernardo, S., Canals, S., Casarejos, M. J., Solano, R. M., Menendez, J., and Mena, M. A. (2004) J. Neurochem. 91, 667–682
42. Levinthal, D. J., and Defranco, D. B. (2005) J. Biol. Chem. 280, 5875–5883
43. Jin, K., Mao, X. O., Zhu, Y., and Greenberg, D. A. (2002) J. Neurochem. 80, 119–125
44. Tashker, J. S., Olson, M., and Kornbluth, S. (2002) Mol. Biol. Cell 13, 393–401
45. Breitschopf, K., Haedeler, J., Malchow, P., Zeiher, A. M., and Dimmeler, S. (2000) Mol. Cell. Biol. 20, 1886–1896
46. Wang, C. X., Song, J. H., Song, D. K., Song, V. W., Shuai, A., and Hao, C. (2006) Cell Death Differ. 13, 1203–1212
47. Allan, L. A., Morrice, N., Brady, S., Magee, G., Pathak, S., and Clarke, P. R. (2003) Nat. Cell Biol. 5, 647–654
48. Rössig, L., Haendeler, J., Hermann, C., Malchow, P., Urbich, C., Zeiher, A. M., and Dimmeler, S. (2000) J. Biol. Chem. 275, 25502–25507
49. Zhang, X. D., Borrow, J. M., Zhang, X. Y., Nguyen, T., and Hersey, P. (2003) Oncogene. 22, 2869–2881
50. Liu, S. J., and Zukin, R. S. (2007) Trends Neurosci. 30, 126–134
51. Liu, S. Q., and Cull-Candy, S. G. (2000) Nature 405, 454–458
52. Liu, Y., Formisano, L., Savtchouk, I., Takayasu, Y., Szabó, G., Zukin, R. S., and Liu, S. J. (2010) Nat. Neurosci. 13, 223–231
53. Deng, W., Neve, R. L., Rosenberg, P. A., Volpe, J. J., and Jensen, F. E. (2006) J. Biol. Chem. 281, 36004–36011