Resistance of human adult oligodendrocytes to AMPA/kainate receptor-mediated glutamate injury

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Summary
Multiple sclerosis is an inflammatory disease of the CNS leading to the destruction of oligodendrocytes (OLs), myelin sheaths and axons. The mediators of tissue injury remain unknown. Glutamate, which can be released by activated immune cells or produced within the CNS, has been implicated as a potential mediator of tissue injury in multiple sclerosis. α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate are highly toxic when added to rodent OL cultures. Using OLs derived from human adult surgical specimens, we investigated AMPA/kainate receptor expression and the effects of receptor stimulation on the viability of human OLs. We find that human adult OLs in vitro express low levels of ionotropic glutamate receptors and are resistant to excitotoxicity mediated by high and sustained doses of AMPA or kainate, even when receptor desensitization is blocked. In contrast, rat OLs show strong AMPA receptor expression and are susceptible to excitotoxicity, as previously demonstrated. Furthermore, we show in human brain sections that OLs do not express AMPA receptors in situ and that glial expression of AMPA receptors is limited to astrocytes. The apparent lack of glutamate receptor expression on human OLs and their resistance to AMPA/kainate toxicity should be considered when postulating mechanisms of tissue injury in multiple sclerosis.

Keywords: glia; multiple sclerosis; astrocytes; oligodendroglia

Abbreviations: AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ConA = concanavalin A; CTZ: cyclothiazide; EAE = experimental autoimmune encephalomyelitis; GalC = galactocerebroside; GFAP = glial fibrillary acidic protein; GluR = glutamate receptor; MAG = myelin associated glycoprotein; MBP = myelin basic protein; NMDA = N-methyl-D-aspartate; OL = oligodendrocyte; PFA = paraformaldehyde.


Introduction
Multiple sclerosis is an inflammatory disease of the CNS characterized by the infiltration of activated immune cells, the destruction of oligodendrocytes (OLs) and their myelin sheaths, and axonal damage. The mediators of tissue injury remain largely unknown. Several studies have implicated the excitatory neurotransmitter glutamate as a contributor to the tissue injury. Glutamate is elevated in the CSF of multiple sclerosis patients (Stover et al., 1997a,b). Microglia and macrophages when activated in vitro can produce glutamate and upregulate glutaminase, the glutamate-producing enzyme (Piani et al., 1991). Werner et al. (2001) demonstrated altered glutamate homeostasis in multiple sclerosis lesions: high levels of glutaminase in microglia and macrophages and decreased expression of glutamine synthetase and glutamate dehydrogenase, the enzymes that break down glutamate, in OLs. Similar observations have been made previously in the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (Hardin-Pouzet et al., 1997). Glutamate levels in vivo could be increased further by altered glutamate transport within the multiple sclerosis brain, as has been demonstrated in EAE (Ohgoh et al., 2002).

Glutamate activates ionotropic receptors which gate membrane ion channels permeable to Na+ and Ca2+; these are
Oligodendrocytes are resistant to excitotoxicity

subdivided into AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate and NMDA (N-methyl-D-aspartate) receptors. Neurons are susceptible to excitotoxic injury mediated via highly Ca$^{2+}$-permeable NMDA receptors as well as Ca$^{2+}$-permeable AMPA and kainate receptors (Lipton and Rosenberg, 1994; Choi, 1995). Glutamate excitotoxicity has also been implicated as a pathological mechanism in both hypoxic–ischaemic (Fern and Moller, 2000; Kanellopoulos et al., 2000; Tekkok and Goldberg, 2001; Back et al., 2002) and traumatic (Wrathall et al., 1994, Agrawal and Fehlings, 1997; Li and Stys, 2000) white matter injury. In EAE, blocking of AMPA/kainate receptors with specific antagonists ameliorates disease outcome and promotes OL survival (Pitt et al., 2000; Smith et al., 2000), illustrating that glutamate excitotoxicity may also play an important role in autoimmune demyelination.

In vitro studies involving OL cell lines and rodent-derived OLs have addressed the issue of OL vulnerability to excitotoxicity. Rodent OLs express ionotropic receptors of the AMPA and kainate subclass and are vulnerable to injury mediated by their overactivation (Yoshioka et al., 1994; Brand-Schieber and Werner, 2003) and traumatic (Wraight et al., 1990; Agrawal and Fehlings, 1997; Li and Stys, 2000) white matter injury. In EAE, blocking of AMPA/kainate receptors with specific antagonists ameliorates disease outcome and promotes OL survival (Pitt et al., 2000; Smith et al., 2000), illustrating that glutamate excitotoxicity may also play an important role in autoimmune demyelination.

In this study, we investigated AMPA/kainate receptor expression and susceptibility to excitotoxicity in vitro, in primary cultures of human adult OLs. We further examined glial AMPA receptor expression in situ in sections of human brain. We find that human adult OLs express low levels of AMPA receptor subunits in vitro and are resistant to excitotoxicity even with prolonged activation of these receptors, in striking contrast to rat OLs. Our in situ results demonstrate that AMPA receptors are expressed by astrocytes but not mature OLs within the human brain.

**Materials and methods**

**Cell isolation and culture procedures**

These studies were carried out using tissue samples obtained from nine adults undergoing surgical treatment of epilepsy. Table 1 shows age, sex, type of surgery and age of onset of seizures for these patients, as well as findings from neuropathological reports of white matter adjacent to that used for cell culture (Table 1). Additional samples used were those obtained from peritumoral regions resected from two patients undergoing surgery for primary or secondary brain tumours. Table 1 also indicates the age, sex and diagnosis of these patients. The protocol was approved by an institutional review board according to the guidelines of the Canadian Institutes for Health Research. Unless specified, individual in vitro functional and immunohistochemical studies were conducted on OLs from tissue obtained from surgery for epilepsy.

OLs and microglial cells were isolated as previously described (Yong and Antel, 1992; D’Souza et al., 1996). Briefly, brain tissue was dissociated enzymatically with trypsin (Invitrogen, Burlington, Ontario) and Dnase 1 (Roche, Laval, Quebec), and mechanically by passage through a 132 μm nylon mesh (Industrial Fabrics, Minneapolis, MN). A mixed glial cell suspension consisting of ~70% OLs, 25% microglia and 5% astrocytes was obtained by separation on a 30% Percoll gradient (Amersham Pharmacia Biotech, Baie d’Urfe, Quebec). The mixed cell population was cultured for two subsequent overnight periods in uncoated tissue culture flasks (VWR Scientific Products, Montreal, Quebec) in minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS), penicillin, streptomycin, 2 mM glutamine and 0.1% glucose (all

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<th>Table 1. Characterization of tissue used for the isolation of human adult OLs</th>
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<td><strong>Age</strong>&lt;br&gt;(years)</td>
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<td><strong>Craniotomy for the treatment of seizures</strong></td>
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<td><strong>Craniotomy for tumour resection: peritumoral tissue</strong></td>
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The table indicates age and sex of operated patients who donated tissue for this study. For patients operated for the treatment of seizures, the tissue source, the patient’s age at seizure onset as well as neuropathological findings for white matter of cortical tissue adjacent to that used for cells isolation is indicated. For patients operated for tumour resection, the diagnosis is specified.
from Invitrogen). To obtain enriched OL cultures, the less adherent cells were washed off (adherent cells consist primarily of microglia) and plated at $1 \times 10^5$ cells per well onto poly-L-lysine (Sigma, Oakville, Ontario)-coated glass tissue culture chamber slides (Invitrogen) for cell death assays or at $1.5 \times 10^6$ cells per well onto poly-L-lysine-coated plastic 24 well plates for protein or RNA isolation and $45\text{Ca}^{2+}$ uptake experiments. OLs were left to adhere and extend processes for 1–2 weeks post-plating. As previously reported, cultures were found to consist of >90% OLs, with <10% contaminating microglia and <2% astrocytes (D’Souza et al., 1996) for seizure cases, and ~85% OLs with <5% contaminating microglia and ~15% astrocytes for peritumoral tissue.

Rat OL progenitor primary cultures were prepared as described by Almazan et al. (1993) and modified from McCarthy and de Vellis (1980). Cultures were prepared from the brains of newborn Sprague–Dawley rats. Cerebral hemispheres and brainstems were removed, placed in Ham’s F12 medium (Invitrogen) and successively passed through a 230 and a 150 μm nylon mesh. The resulting cell suspension was centrifuged and resuspended in a 1 : 1 mixture of Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) and Ham’s F12 medium with 12.5% FCS. Cells were plated on poly-L-ornithine (Sigma)-coated flasks and grown for 9–11 days. On day 10, flasks were placed on a rotary shaker at 225 r.p.m. at 37°C for 3 h to remove loosely adherent macrophages. OL progenitors were removed following shaking for 18 h at 260 r.p.m. The cell suspension was filtered through a 30 μm nylon mesh and plated on bacteriological-grade Petri dishes (VWR) for 3 h. Under these conditions, astrocytes and microglia attach to the plastic surface while OL progenitors remain in suspension. The final cell suspension was plated onto poly-D-lysine (Sigma)-coated glass coverslips (VWR). The cultures were first maintained in serum-free medium containing 2.5 ng/ml platelet-derived growth factor AA (Roche) and 2.5 ng/ml basic fibroblast growth factor (Sigma), to promote self-renewal and prevent differentiation. Serum-free medium consists of 1 : 1 DMEM–F12, 10 mM HEPES, 0.1% bovine serum albumin (BSA), 25 g/ml insulin, 16 g/ml putrescine, 30 mM sodium, 50 U/ml penicillin and 50 g/ml streptomycin (all from Sigma). After 4 days, cultures were differentiated to yield mature rat OLs by removing growth factors, and 24 h later were maintained in a 1 : 1 mixture of DMEM and Ham’s F12 medium supplemented with 3% FCS. The morphological and biochemical properties of 6–12 day differentiated rat OL cultures have been described previously (Khordoch et al., 2002).

**Western blot and RT–PCR**

For western blotting, cells were lysed in NP-40 buffer [10 mM Tris–HCl, 10 mM NaCl, 3 mM MgCl₂, and 0.5% NP-40 (Sigma)] and 30 μg per lane was electrophoresed on an 8% SDS–polyacrylamide gel under reducing conditions. Proteins were transferred to a PVDF membrane (Biorad, Mississauga, Ontario) and blocked for 1 h at room temperature in 5% milk. Membranes were incubated further overnight with rabbit anti-rat glutamate receptor (GluR) 1, 2/3 or 4 (a generous gift from Dr. Wenhong, National Institutes of Health, Bethesda, MD) (Wenhold et al., 1992) antibody diluted in milk, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Dako, Carpiniteria, CA). For antibodies against GluR1, GluR2 and GluR4, peptides used for rabbit immunizations are 100% conserved in the human proteins. For the anti-GluR2/3 antibody, the peptide used for immunization is 100% conserved for human GluR2 and has one amino acid difference (out of 13, i.e. 92.3% conserved: I → T) for human GluR3. Membranes were stripped subsequently of antibodies and reprobed with a monoclonal anti-β-actin antibody (ICN Biomedicals, Aurora, OH) followed by a rabbit anti-mouse–HRP (Dako). In all cases, specific binding was visualized using the ECL (enhanced chemiluminescence) system (Amersham Pharmacia Biotech).

For RT–PCR (reverse transcription–polymerase chain reaction), total RNA from $3 \times 10^6$ cells was isolated using the TRizol reagent (Invitrogen) and 3 μg were transcribed into cDNA using 3.3 mM hexamer primers (Roche), 3 mM dNTPs, 0.6 μl of RNA guard (Amersham Pharmacia Biotech), 3 mM dithiothreitol (DTT), reverse RT buffer (50 mM Tris–HCl pH 8.3, 7.5 mM KCl, 3 mM MgCl₂, 10 mM DTT) and 400 μM of Moloney murine leukemia virus (MMLV) RT (Invitrogen). The reaction mixture was incubated at 42°C for 1 h followed by 10 min at 75°C. Subsequently, 300 ng of cDNA was added to a reaction mixture containing PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl; Invitrogen), 0.4 mM dNTPs, 1.5 mM MgCl₂, 50 pM forward (F) and reverse (R) primers and 2.5 U of Taq polymerase (Invitrogen). Primers were designed as follows (size of amplification product, in bp, is shown in parentheses; Sigma): GluR1F, GGATACCGGATGCTCTTTCA; R, GGCCGG-AAATGTGCTGTTGT(243); GluR2F, AACGCCTGTAATTCTTGGAAC; R, CTTGTGCTTCTTAGGTGGCG (343); GluR3F, GTTG-GTTGCAAGGGAATATGG; R, CATTGTGGAGTACGGAG-GA(382); GluR4F, TACTATTGGTGTCGCGTGTT; R, GCCAG-GCTTTCTGACCTTTC(323); GluR5F, TAAATCAAACTCCGC-CAGC; R, ATGACACGTGAGGTTGTC(281) GluR6F, TTAGCGCTTTTGCAGT; R, GGTGACAGTCCGGTGGTA(292); GluR7F, CACCAAGATGACCGTGAC; R, CTCTTCTAGAGGTGAC(398); KA1F, TAATGCTGAAAGGGAAC-CAC; R, TCAATACCTTCTCCCGTTC (250); KA2F, AGATC-CAACCGGATCATCGAG; R, CGAAGCGAAGGTTGTAAGG (252); β-actin F, GAGGCCATACCCCTCGTAGAT; R, CAGAAG-GATCCCTTATGGC (378). The reaction mixture was placed in a PTC-100 thermal cycler (MJ Research, Watertown, MA) for 30 cycles of 94°C for 1 min, 62°C for 45 s and 72°C for 1 min. After amplification, 20 μl of each sample was resolved on a 1.5% agarose gel with ethidium bromide.

**Cell survival/cell death assays**

To assay for toxicity of glutamate receptor agonists, cells were incubated in serum-free medium. When applicable, cells were pre-treated with cyclothiazide (CTZ; 25 μM for rat and 50 μM for human; Tocris, Ellisville, MO) or concanavalin A (ConA; 10 μM; Sigma) for 10 min prior to the addition of agonists AMPA or kainate (Tocris, Ellisville, MO) or concanavalin A (ConA; 10 μM; Sigma) for 10 min prior to the addition of agonists AMPA or kainate (Tocris). Apoptotic cell death was assessed using the Cell Death Detection ELISAPLUS (Roche), which detects cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) that are generated upon enzymatic cleavage of DNA in apoptotic cell death. For human cells, each experiment was conducted on cells derived from at least two different brain preparations and each treatment was performed in duplicate or triplicate wells. For rat cells, experiments were conducted in duplicate wells. Enzyme-linked immunoassay (ELISA) results represent pooled data from individual experiments and are plotted as the mean of the sample absorbance minus the mean untreated sample absorbance (relative absorbance), and expressed as the mean of replicate wells ± SEM. Cell survival
was assayed using the XTT assay [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium hydroxide; Sigma]. In this assay, mitochondrial dehydrogenase activity is assessed by reduction of the tetrazolium salt XTT to orange-coloured compounds of formazan and provides an index of cell viability. Cells were incubated in Opti-MEM (Invitrogen) containing XTT for 4–6 h at 37°C and the absorbance of cell supernatants was measured at 450 nm (reference 690 nm) using a spectrophotometer. Untreated sister cultures were taken to represent 100% survival. Each experiment was conducted on cells derived from at least two different brain preparations. Treatments were performed in duplicate or triplicate wells for human, and duplicate for rat cells. Results represent pooled data from the indicated number of individual experiments and are expressed as mean survival percentage of replicates ± SEM.

45Ca2+ uptake

For 45Ca2+ uptake (Perkin Elmer, Guelph, Ontario), cells were washed and incubated for 30 min in pre-warmed Mg2+-free Locke’s solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 1.3 mM CaCl2, 5.6 mM d-glucose, 5 mM HEPES, pH 7.4). Drugs blocking receptor desensitization were added for 10 min. The solution was replaced subsequently with Locke’s containing 10 μCi/ml 45Ca2+ (2.5 μCi/well) for human OLs and microglia or 1 μCi/ml 45Ca2+ (0.5 μCi/well) (Liu et al., 1997) for rat OLs, for 10 min at 37°C, as well as AMPA, kainate or ionomycin (10 μg/ml, Sigma). Cells were washed on ice three times with ice-cold choline buffer (154 mM choline chloride, 2 mM EGTA and 10 mM HEPES, pH 7.4) and lysed with 0.1 M NaOH/0.1% Triton X-100 (Sigma). Radioactivity was measured by liquid scintillation counting.

Immunocytochemistry

For staining of OLs in culture, cells were fixed in 2% paraformaldehyde (PFA) and permeabilized with ice-cold methanol for the myelin-associated glycoprotein (MAG) stain. The primary antibody was diluted in HHG (1 mM HEPES, 2% horse serum, 10% goat serum in Hank’s buffered salt solution): 1 : 200 for anti-MAG (monoclonal; Immunologicals, Camarillo, CA; or FITC-linked goat anti-rabbit for GFAP, 1 : 200 Caltag Laboratories, Burlingame, CA) following the manufacturer’s instructions. Sections subsequently were washed and incubated for 30 min with Cy3-conjugated streptavidin (1 : 1000 in blocking buffer, Jackson ImmunoResearch). Sections were then double-stained using mouse monoclonal anti-MAG (1 : 100 dilution) as a marker for OLs, rabbit polyclonal anti-γ-aminobutyric acid (GABA) (GFAP 1 : 200, Dako) for astrocytes, or mouse monoclonal anti-β-tubulin III (1 : 400, Sigma) for neurons. Sections were permeabilized for 15 min with ice-cold 5% acetic acid in 95% ethanol (GFAP), ice-cold methanol (MAG) or 0.3% Triton X-100 (β-tubulin III) and blocked for 30 min with HHG. Primary antibodies were incubated for 2 h at 37°C and appropriate secondary antibodies were incubated for 30 min at room temperature [fluorescein isothiocyanate (FITC)-linked goat anti-mouse for MAG and β-tubulin III, 1 : 50 Tago Immunomarkers, Camarillo, CA; or FITC-linked goat anti-rabbit for GFAP, 1 : 200 Cedarlane, Hornby, Ontario]. Hoechst was used as a nuclear stain. In order to quench autofluorescence of the tissue, for stainings presented in Figure 7, sections were treated with 1% Sudan black (Sigma) in 70% ethanol for 3 min following all antibody stainings presented in Figure 7, sections were treated with 1% Sudan black (Sigma) in 70% ethanol for 3 min following all antibody incubations, and washed thoroughly. Control stainings were done in the absence of each one of the primary antibodies (either anti-GluR, anti-GFAP or anti-MAG). Staining was visualized using a Leica microscope.

Statistical analyses

Results of in vitro cell cytotoxicity and survival assays are presented as the mean ± SEM for the number of experiments indicated. Differences between treatments were evaluated by two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test to compare differences between treatments at specific time points. Results of 45Ca2+ uptake per 106 cells are expressed as mean of duplicate wells ± SEM. Statistical differences between samples were calculated using one-way ANOVA followed by Bonferroni’s multiple comparison test. Only probability values p < 0.05 were considered to be statistically significant.

Results

Characterization of human adult oligodendrocyte cultures

An example of our dissociated human OL cultures is presented in Fig 1. Phase contrast is shown in Fig. 1A, with processes being best demonstrated by MAG staining (Fig. 1B). We previously have determined that our OL cultures are ≥90% pure with ≤10% contaminating microglia and ≤2% astrocytes (D’Souza et al., 1996). The human adult OLs express all mature myelin protein markers, including MAG (Fig. 1B), galactocerebroside (GalC) and myelin basic protein (MBP), as determined by immunocytochemistry (data not shown). The spontaneous rate of cell death in these cultures as measured by propidium iodide, annexin V
or TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling) staining is <5% for up to 2 weeks in culture following the isolation protocol (data not shown).

Mature rat OLs have been described previously to express the major glycolipids and proteins of myelin, including GalC, MBP, proteolipid protein and MAG, as well as the enzyme 2',3'-cyclic-nucleotide 3'-phosphodiesterase (Khorchid et al., 2002).

AMPA/kainate receptor subunit expression in human adult oligodendrocytes in vitro

To assess non-NMDA glutamate receptor expression in human OLs from adult brain, cell homogenates were analysed by western blotting using antibodies specific for AMPA receptor subunits. Two preparations of human adult OLs were used with each antibody. As shown in Fig. 2A, human adult OLs show low levels of expression of the GluR2 protein; a stronger band is evident with the antibody recognizing both GluR2 and GluR3 (GluR2/3), probably indicating that OLs also express the GluR3 subunit protein. No expression is evident for either GluR1 or GluR4. Rat OL lysates were used as positive controls and human cortical tissue was used to assess antibody cross-reactivity to the human proteins. Membranes were reprobed with β-actin to ensure equal protein loading. (B and C) PCR for AMPA (B) and kainate (C) receptor subunits. Human OLs express GluR2, 3 and 4 mRNAs as well as low levels of GluR6 and KA1 mRNAs. GluR1, 5, 7 and KA2 are undetectable. Fetal neuron (N) mRNA was used as positive control. β-Actin was amplified to control for equal RNA loading.
used to control for antibody reactivity to the human protein, as antibodies were raised against rat peptides. Because human adult brain shows very modest reactivity, lack of GluR4 expression by human OLs could be due to weak antibody cross-reactivity with the human protein, due to differences in protein folding between species. All membranes were stripped and reprobed with an antibody recognizing β-actin, to ensure that protein loading was equal in all wells.

To assess AMPA/kainate receptor mRNA expression further in human OLs, we isolated total RNA and performed RT–PCR for AMPA receptor subunits GluR1–4 and kainate receptor subunits GluR5–7 and KA1–2. We amplified β-actin mRNA to control for equal RNA loading. RNA was also isolated from human fetal neurons as a positive control for expression of all AMPA and kainate glutamate receptor subunits. As shown in Fig. 2B, human adult OLs express AMPA receptor subunits GluR2, GluR3 and GluR4 mRNAs, while GluR1 is undetectable. Human adult OLs also express lower levels of kainate receptor subunit GluR6 and KA1 mRNAs, but have no detectable GluR5, GluR7 or KA2 (Fig. 2C). To validate that the amplified reaction products were indeed correspondent and specific for the cDNAs of the different AMPA receptor subunits, we sequenced isolated PCR products. Sequences obtained were shown to be specific for the corresponding genes using the NCBI BLAST program/database.

In summary, human adult OLs express low levels of protein for GluR2 and GluR3. This is in striking contrast to rat OLs which show high protein levels for GluR2, GluR2/3 and GluR4.

**Sustained activation of AMPA receptors does not induce apoptosis in human adult oligodendrocytes**

Recent pathological findings demonstrate that glutamate homeostasis is altered in the brain of multiple sclerosis patients (Werner et al., 2001). Numerous studies demonstrate that rodent OLs are highly vulnerable to glutamate-mediated excitotoxicity through AMPA receptors (Yoshioka et al., 1996; Matute et al., 1997; Matute, 1998, McDonald et al., 1998; Sanchez-Gomez and Matute, 1999; Kavanaugh et al., 2000; Alberdi et al., 2002). Excitotoxic cell death is thought to occur through the activation of apoptotic pathways.

To assess the susceptibility of human adult OLs to excitotoxicity via prolonged activation of AMPA receptors, we treated human adult OLs with high concentrations of AMPA (500 µM) (Fig. 3A and C) or kainate (500 µM) (Fig. 3B and D) for periods ranging from 2 to 48 h and assessed apoptotic cell death using the cell death detection ELISA (nucleosome ELISA), which detects histone-associated mono- and oligonucleosomes that are generated upon enzymatic cleavage of DNA. To determine whether human OL AMPA/kainate receptors underwent rapid desensitization as reported in rodent systems (see references above), we also examined the viability of human OLs treated with agonist in the presence of the benzothiadiazine CTZ, an allosteric modulator which selectively blocks desensitization of AMPA receptors. CTZ is tested in the presence of both agonists, as AMPA receptors bind both AMPA and kainate (four
brain preparations were tested, \( n = 4 \). In addition, to verify that responses to receptor agonists were not due to the fact that our human OL cultures are isolated from epileptic tissue and may have altered properties with regards to receptor expression and susceptibility to sustained activation of AMPA receptors, we also performed experiments on OLs isolated from peritumoral tissue (Fig. 3C and D) \( (n = 2) \).

We find that human adult OLs do not undergo apoptosis with up to 48 h of AMPA or kainate treatment, regardless of receptor desensitization with CTZ, as no significant differences between treatments were found. This is true for OLs isolated from epileptic tissue as well as peritumoral OLs. Lower doses of agonist were also tested (ranging from 1 to 500 \( \mu M \)) and were found to have no effect. Human OL morphology was also not affected by either treatment (data not shown).

To confirm previous findings regarding the susceptibility of rodent OLs, we used primary cultures of mature rat OLs. We treated rat OLs with AMPA (500 \( \mu M \)) (Fig. 3E) or kainate (500 \( \mu M \)) (Fig. 3F) with and without CTZ for periods ranging from 6 to 24 h and assessed apoptotic cell death using the nucleosome ELISA. Exposure of rat OLs to AMPA (Fig. 3E) or kainate (Fig. 3F) alone induces a moderate increase in cell death within 6 h which decreases by 18 h. Concomitant receptor desensitization with CTZ strongly potentiates apoptotic cell death of rat OLs through both AMPA and kainate \( (P < 0.01 \) kainate plus CTZ compared with kainate alone at 6 and 24 h, \( P < 0.001 \) at 18 h; \( P < 0.001 \) comparing AMPA plus CTZ and AMPA alone at 6, 18 and 24 h; \( P < 0.001 \) agonist and CTZ compared with CTZ alone at 6, 18 and 24 h). Maximal levels of apoptosis are reached by 18 h of treatment.

In both rat and human OL preparations, CTZ alone did not induce apoptosis. Also, treatment with glutamate itself (up to 500 \( \mu M \)) for up to 48 h did not induce apoptotic cell death of human OLs (data not shown), excluding the possibility that glutamate at these concentrations could be toxic to OLs through a non-receptor-mediated mechanism. We cannot exclude, however, that higher concentrations could be toxic.

In summary, human adult OLs are resistant to apoptotic cell death mediated via AMPA receptors, regardless of blocking receptor desensitization. This is in sharp contrast to rat OLs, where, in accordance with previous reports, either agonist alone induces measurable apoptosis which is greatly increased when AMPA receptor desensitization is blocked.

**Sustained activation of AMPA receptors does not affect the viability of human adult oligodendrocytes**

Because human OLs are resistant to AMPA/kainate-induced apoptosis, we wanted to assess whether cell viability could be affected by AMPA/kainate through pathways other than apoptotic ones. We treated human OLs with AMPA (500 \( \mu M \)) (Fig. 4A and C) or kainate (500 \( \mu M \)) (Fig. 4B and D) in the presence or absence of CTZ, for periods ranging from 2 to 48 h. We subsequently assessed overall cell viability using the XTT assay. Untreated sister OL cultures were used to represent 100% survival. As previously,
we tested both human adult OLs isolated from epileptic tissue (Fig. 4A and B) (n = 5) and peritumoral OLs (Fig. 4C and D) (n = 2), and we used mature rat OLs (treated for periods ranging from 6 to 72 h) as a positive control (Fig. 4E and F).

Exposure of human OLs to AMPA or kainate alone, for up to 48 h, is not toxic as determined by XTT (Fig. 4A–D). At no time point did we see a statistical difference between agonist plus CTZ and CTZ alone, thereby differences in cell viability seen between agonist alone and agonist plus CTZ are attributable to the toxicity of CTZ. These changes in cell viability are not promoted further by the addition of either AMPA or kainate, demonstrating that sustained activation of AMPA receptors does not affect the viability of human adult OLs.

In control rat OL cultures, exposure of rat OLs to AMPA alone (Fig. 4E) does not result in a decrease in cell survival over 72 h. However, by 18 h of treatment with AMPA plus CTZ, cell survival decreases below 50% and is maintained throughout the rest of the time course (P < 0.001 for AMPA plus CTZ when compared with AMPA or CTZ alone at 6, 18, 24, and 72 h). Exposure of rat OLs to kainate alone (Fig. 4F) results in an ~50% decrease in cell survival by 6 h and does not decrease further over 72 h. When CTZ is added, cell survival per h falls to 25% and remains ≤25% up to 72 h (P < 0.001 for kainate plus CTZ when compared with kainate or CTZ alone at 18, 24, 48, and 72 h). Rat OLs treated with CTZ alone throughout the time course did not show decreased viability. Cell viability data are in accordance with apoptosis data: for both agonists, cell viability reaches its minimum between 18 and 24 h, while maximum apoptosis was observed at 18 h of treatment.

In summary, human adult OLs in vitro, in contrast to rat OLs, are resistant to excitotoxicity mediated by sustained activation of AMPA receptors.

Human adult oligodendrocytes are resistant to sustained activation of kainate receptors

Because the PCR data indicate that human OLs also express kainate receptor subunits GluR6 and KA1 mRNA, albeit weakly, we tested whether kainate receptor overactivation is toxic to human adult OLs. To this end, we treated OLs for time periods ranging from 6 to 24 h with kainate (500 µM) (Fig. 5) with or without the lectin ConA, which selectively blocks kainate receptor desensitization. No sustained decrease in cell viability was observed upon the addition of kainate alone or in combination with ConA, indicating that prolonged activation of kainate receptors is not toxic to human adult OLs. Lower agonist concentrations were also tested and were found to have no effect. Furthermore, OL morphology was not altered under these conditions (data not shown).

In summary, human adult OLs in vitro are resistant to excitotoxic injury mediated through prolonged activation of kainate receptors.

Sustained activation of AMPA or kainate receptors does not induce calcium influx into human adult oligodendrocytes

AMPA receptors in rat OLs are permeable to Na+ and Ca2+, and Ca2+ overload is necessary to initiate excitotoxicity in cultured rodent OLs (Alberdi et al., 2002). We therefore measured 45Ca2+ influx into human OLs upon activation of AMPA or kainate receptors in the presence of CTZ or ConA, respectively. Rat OLs were used as a positive control. Treatment with ionomycin, a Ca2+ ionophore, was used as a control.

Human adult OLs (Fig. 6A) showed no significant 45Ca2+ uptake in response to kainate or AMPA alone or in conjunction with CTZ or ConA when compared with the relevant controls (non-treated cells or cells treated with agonist or drug alone, P > 0.05). Maximal Ca2+ influx was obtained with ionomycin and was found to be 4.1-fold over baseline (P < 0.001). Although the presence of the GluR2 subunit in AMPA receptors confers resistance to Ca2+ permeability and could explain the lack of 45Ca2+ uptake in response to agonist alone, the presence of CTZ should force Ca2+ into the receptor channel and inside the cell, regardless of GluR2 expression. These results are hence indicative of low Ca2+ permeability of human OLs because of low AMPA/kainate receptor expression or the expression of these molecules by only a small subset of cells in the culture.

Treatment of rat OLs (Fig. 6B) with AMPA (500 µM) alone did not induce a Ca2+ influx above baseline, while kainate alone elicited only a weak uptake, 3.2-fold over baseline (P > 0.05). Significant responses were obtained with kainate or AMPA in the presence of CTZ: P < 0.01 and P < 0.001 for kainate plus CTZ when compared with agonist alone or CTZ alone, respectively, and P < 0.001 for AMPA in conjunction with CTZ when compared with either AMPA or CTZ alone. These results indicate that AMPA receptors are permeable to Ca2+ in rat OLs, especially when receptor desensitization is blocked.
Human OLs

A.

counts (x10^3)

per 10^6 cells

CTZ
ConA
CTZ

Rat OLs

B.

counts (x10^3)

per 10^6 cells

CTZ
CTZ

Human Microglia

C.

counts (x10^3)

per 10^6 cells

CTZ
ConA
CTZ

Human OLs

A.

without ConA for human cells. 45Ca^{2+} uptake was measured by black bars), with or without CTZ for all cell types and with or in response to AMPA (500 µM, grey bars) or kainate (500 µM, black bars), with or without CTZ for all cell types and with or without ConA for human cells. 45Ca^{2+} uptake was measured by scintillation counting. Treatment with ionomycin (hatched bars) was used as positive control. (A) Human OLs show background 45Ca^{2+} influx for either agonist alone and no significant 45Ca^{2+} uptake for agonist plus CTZ or ConA. (B) Rat OLs show none (for AMPA) to low 45Ca^{2+} influx with agonist alone (3.2-fold over background for kainate, P > 0.05). Kainate in conjunction with CTZ induced robust 45Ca^{2+} uptake when compared with kainate (**P < 0.001) or CTZ (*P < 0.001) alone, as did AMPA plus CTZ in comparison with CTZ or agonist alone (*P < 0.001). (C) Human microglia display background levels of 45Ca^{2+} uptake under all conditions tested.

To test whether the weak (although not significant) 45Ca^{2+} uptake seen with human OLs could be attributed to microglial contamination of the cultures (~5% of total cells), we assessed 45Ca^{2+} uptake of pure human microglial cultures (Fig. 6C) under the same conditions. Microglia showed no significant 45Ca^{2+} influx in response to either agonist regardless of the presence of CTZ or ConA.

In summary, stimulation of AMPA or kainate receptors induces only a minimal Ca^{2+} influx in human adult OLs, as compared with rat OLs.

In situ AMPA receptor expression in the human brain

Neuronal expression of AMPA receptors has been demonstrated in the mammalian brain (Petraila and Wenthold, 1992; Martin et al., 1993; Ozawa et al., 1998). We sought to determine whether AMPA receptors were expressed on OLs in the human CNS. To this end, we immunostained tissue sections from the surgical and post-mortem specimens described previously for AMPA glutamate receptors GluR1 (Fig. 7A–F), GluR2/3 (Fig. 7G–L) and GluR4 (Fig. 7 M–R) (revealed in red), followed by double staining for MAG (a mature OL marker) (Fig. 7) (shown in green), and looked at both white matter (areas with dense MAG staining: Wm, Fig. 7A–C, G–I and M–O) and grey matter areas (sparse MAG staining: Gm, Fig. 7D–F, J–L and P–R).

In white matter, we found dense MAG staining (Fig. 7A, G and M) as expected, but only very rare immunolabelling for the selected glutamate receptor (Fig. 7B, H and N), and no overlay of GluR and MAG staining (Fig. 7C, I and O), indicating that OLs in white matter do not express AMPA glutamate receptors. Abundant cell bodies ensheathed by MAG immunoreactivity can be seen in the overlay pictures. It should be noted that in each of the MAG-rich areas, there are abundant nuclei, identified by Hoechst nuclear staining (in blue), most of which are OLs (data not shown).

In grey matter, MAG staining was scarce (Fig. 7D, J and P), in contrast to robust GluR expression (Fig. 7E, K and Q). We found no evidence for co-localization of MAG and GluR (Fig. 7F, L and R), indicating that OLs in grey matter also do not express AMPA receptors.

Additionally, to determine GluR expression by other cell types, we immunostained sections for GluR1 (Fig. 8A–C), GluR2/3 (Fig. 8G–L) and GluR4 (Fig. 8D–F) (revealed in red) followed by double staining for GFAP (a marker for astrocytes) (Fig. 8A–I) or β-tubulin III (a marker for neurons) (Fig. 8 J–L). We found that selected astrocytes in the human CNS were positive for AMPA receptor expression as GFAP staining coincided with GluR1 (Fig. 8A–C), 2/3 (Fig. 8G–I) and 4 (Fig. 8D–F), as evidenced in the merged pictures (Fig. 8C, F and I). Only occasional GFAP-positive astrocytes in the white matter have detectable GluR expression (data not shown); the majority of GluR-positive astrocytes we have encountered, and that we illustrate in Fig. 8, are in the grey matter. Consistent results were obtained using sections from each of these tissue sources. This is in agreement with previous data reporting that subpopulations of astrocytes express AMPA receptors in situ (Petraila and Wenthold, 1992; Martin et al., 1993; Conti et al., 1994; Matute et al.,
Fig. 7 Oligodendrocytes do not express AMPA receptors in situ in adult human brain. Human brain sections were immunostained for GluR1 (A–F), GluR2/3 (G–L) and GluR4 (M–R) (in red) followed by MAG staining for OLs (in green), and both white matter (Wm; MAG dense areas, A–C, G–I and M–O) and grey matter (Gm; areas of scarce MAG staining, D–F, J–L and P–R) from the same sections were analysed. The left-hand column shows MAG staining of sections (green), the middle displays immunolabelling with GluR subtype-specific antibody (red), and an overlay of the two, along with Hoechst nuclear stain is shown in the right-hand column. In white matter (Wm), dense MAG staining is present (A, G and M), but immunolabelling for the selected glutamate receptor (B, H and N) is rare. White matter OLs do not express AMPA receptors as there is no overlay of MAG and GluR stains (C, I and O). In grey matter (Gm), MAG staining is less abundant (D, J and P) and immunolabelling for specific GluR subunits is robust (E, K and Q). There is no overlay of GluR and MAG stains (F, L and R), indicating that OLs do not express AMPA receptors in the grey matter. Scale bars 50 μm.
As expected, selective neuron populations were also positive for distinct glutamate receptor subunits. For example, Fig. 8 J–L shows a β-tubulin III-positive neuron (Fig. 8 J, in green) which expresses GluR2/3 (Fig. 8K, in red), as evidenced by the merged picture (Fig. 8L). Thus neurons account for the GluR2/3-positive and GFAP-negative cells seen in Fig. 8I. Moreover, vascular brain endothelial cells also expressed GluR4 (a GluR4-positive vessel can be seen in Fig. 7 M–O), in accordance with previous in vitro findings (Krizbai et al., 1998; Parfenova et al., 2003). Control immunostainings performed in the absence of each one of the primary antibodies (either anti-GluR or anti-GFAP, anti-MAG or anti-β-tubulin III) did not reveal any positive signal (data not shown).

**Discussion**

In this report, we investigated the properties of mature human OLs with regards to glutamate receptor expression and susceptibility to excitotoxicity mediated through AMPA/kainate receptors. We demonstrate that human adult OLs express very low levels of AMPA and kainate receptors in vitro and are resistant to excitotoxicity mediated through sustained activation of these receptors. In addition, we show in situ in human brain that OLs do not express AMPA receptors and that glial expression of AMPA receptors is limited to astrocytes. We believe our study is of importance to the field of excitotoxic injury to OLs as it addresses, for the first time, the effects of glutamate on OLs from the human brain.

Our in vitro findings with human OLs are in contrast to rat OLs, which express high levels of AMPA receptor protein and which undergo apoptotic cell death upon sustained activation of these receptors, as previously reported (Yoshioka et al., 1996; Matute et al., 1997; Matute, 1998; McDonald et al., 1998; Sanchez-Gomez and Matute, 1999; Kavanaugh et al., 2000; Alberdi et al., 2002). Besides species differences, several potential distinctions between human and rodent cell preparations could contribute to our results. The rat OLs used, just as in other rodent studies using cerebral tissue (McDonald et al., 1998), were isolated as progenitors early postnatally (P1–P3) and subsequently induced to mature in vitro.
Oligodendrocytes are resistant to excitotoxicity

OLs, on the other hand, are isolated from adult human brain and hence are cells that have matured in vivo. Itoh et al. (2002) have shown recently that AMPA glutamate receptor-mediated signalling is transiently enhanced during development of OLs and downregulated as OLs mature. Rosenberg et al. (2003) demonstrated that mature MBP-expressing rat brain OLs do not express several kainate receptors or the AMPA receptor GluR2 and are resistant to kainate, in contrast to progenitor and less mature cells which express these receptors and are susceptible. The fact that rodent OLs matured in vitro acquire markers of mature cells such as myelin protein, need not indicate that they are fully physiologically mature; down-regulation of receptors could be a subsequent step and different in vitro means of maturing cells could or could not provide the appropriate cues.

Several studies also demonstrate that optic nerve OLs isolated from up to 12-day-old animals are susceptible to AMPA/kainate-mediated excitotoxicity (Matute et al., 1997; Matute, 1998; Sanchez-Gomez and Matute, 1999; Alberdi et al., 2002). We cannot address the question of regional differences in OL phenotype, as our surgical samples are all derived from the cortical region.

Just as in human tissue, in situ studies on adult rodent brain have found little evidence for AMPA/kainate receptor expression on mature OLs in the brain. Numerous reports, however, do show receptor expression on astrocytes (Petralia and Wenthold, 1992; Martin et al., 1993; Conti et al., 1994; Matute et al., 1994; Tachibana et al., 1994; Matthias et al., 2003), a finding we confirm in our study, and on developing OLs or OL progenitor cells in situ (Barres et al., 1990; Fulton et al., 1992; Bergles et al., 2000; Itoh et al., 2002). McDonald et al. (1998) described co-localization of GluR2/3 staining with the OL marker Rip in rat subcortical white matter. Recent studies describe kainate receptor KA2-expressing OLs in mouse spinal cord and a subset of CNPase-positive cells expressing GluR2/3 and GluR4 in the rat spinal cord (Brand-Schieder and Werner, 2003a,b). However, in these studies, comparisons between mouse and rat CNS were done systematically and the authors caution that important inter-species differences exist.

Our data demonstrate that healthy human OLs are not killed by glutamate through the action of AMPA/kainate receptors, due to the absence of the receptors on these cells. In the context of multiple sclerosis, we did consider in our study whether pro-inflammatory cytokines would induce receptor expression and sensitize OLs to excitotoxic cell death. Precedent for this is provided by reports that tumour necrosis factor-α (TNF-α) enhances synaptic plasticity in rodent hippocampal neurons by increasing surface expression of AMPA receptors (Beattie et al., 2002), allows for glutamate toxicity in human fetal neurons (Chao and Hu, 1994), and potentiates glutamate-mediated neuronal cell death in rat spinal cord (Hermann et al., 2001). Yong and colleagues show in mixed mouse glial cell cultures that interleukin-1β (IL-1β) and TNF-α promote killing of OLs (up to 15%) through a mechanism involving glutamate excitotoxicity (Takahashi et al., 2003). In our hands, the pro-inflammatory cytokines TNF-α, interferon-γ and IL-1β did not induce/upregulate AMPA receptor expression in OLs in vitro nor did they potentiate killing (data not shown).

Our results do not preclude an important role for excitotoxicity in multiple sclerosis. It is possible that other factors present in the multiple sclerosis milieu or a combination of such factors could induce glutamate receptor expression and susceptibility of OLs to excitotoxicity. Another possibility is that glutamate could be toxic to immature or developing OLs which are increasingly thought to be involved in tissue repair in multiple sclerosis. Furthermore, the capacity for neuronal directed injury by glutamate is well established and thus this mediator may be particularly relevant for this component of the injury process (Chang et al., 2002). It remains to be established whether neuronal injury can indirectly impact on OL/myelin properties either by release of intermediaries that can induce injury or by interruption of trophic support needed for continued OL/myelin integrity.

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