System x_c^- and Apolipoprotein E Expressed by Microglia Have Opposite Effects on the Neurotoxicity of Amyloid- β Peptide 1–40

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Because senile plaques in Alzheimer's disease (AD) contain reactive microglia in addition to potentially neurotoxic aggregates of amyloid- β (A β), we examined the influence of microglia on the viability of rodent neurons in culture exposed to aggregated A β 1–40. Microglia enhanced the toxicity of A β by releasing glutamate through the cystine-glutamate antiporter system x_c^- . This may be relevant to A β toxicity in AD, because the system x_c^- -specific xCT gene is expressed not only in cultured microglia but also in reactive microglia within or surrounding amyloid plaques in transgenic mice expressing mutant human amyloid precursor protein or in wild-type mice injected with A β . Inhibition of NMDA receptors or system x_c^- prevented the microglia-enhanced neurotoxicity of A β but also unmasked a neuroprotective effect of microglia mediated by microglial secretion of apolipoprotein E (apoE) in the culture medium. Immunodepletion of apoE or targeted inactivation of the apoE gene in microglia abrogated neuroprotection by microglial conditioned medium, whereas supplementation by human apoE isoforms restored protection, which was potentiated by the presence of microglia-derived cofactors. These results suggest that inhibition of microglial system x_c^- might be of therapeutic value in the treatment of AD. Its inhibition not only prevents glutamate excitotoxicity but also facilitates neuroprotection by apoE.

Key words: Alzheimer's disease; microglia; NMDA receptor; apolipoprotein E; system x_c^- ; neuronal death

Introduction

Amyloid β -peptides (A β) 1–40 and 1–42 play a major role in the pathogenesis of Alzheimer's disease (AD) (Hardy and Selkoe, 2002). In AD and mouse models of the disease, neuronal death colocalizes with congophilic plaques containing aggregated A β and activated microglia (Haga et al., 1989; Itagaki et al., 1989; Calhoun et al., 1998; Stalder et al., 1999; Urbanc et al., 2002), and aggregated A β injected in the CNS or added to cell cultures can trigger neuronal death (Pike et al., 1993; Geula et al., 1998). Microglia stimulated by A β may promote the death of neurons by producing toxins, such as free radicals, cytokines, or glutamate receptor agonists (Giulian et al., 1995; Meda et al., 1995; McDonald et al., 1997; Weldon et al., 1998; Ishii et al., 2000). However, microglia may clear A β through phagocytosis (Frautschy et al., 1992; Wyss-Coray et al., 2001; Rogers et al., 2002). Understanding how microglia enhance or limit A β neurotoxicity is therefore crucial for the development of therapeutic approaches to AD.

A potential therapeutic target is the microglial system x_c^- antiporter composed of the widely expressed 4F2hc chain common to several amino acid transporters and a specific xCT protein (Sato et al., 1999). The release of cytotoxic concentrations of glutamate through the antiporter is coupled to the uptake of extracellular cystine and is promoted by oxidative stress, tumor necrosis factor- α (TNF- α), or secreted amyloid precursor protein (APP) (Piani and Fontana, 1994; Barger and Basile, 2001; Sato et al., 2001).

Because microglial system x_c^- might be involved in A β neurotoxicity, we hypothesized that preventing microglial glutamate release might be neuroprotective in itself but might also permit neuroprotection by microglial factors such as lipid-associated apolipoprotein E (apoE) (Mahley and Rall, 2000), which accumulates in amyloid plaques and is recognized by several neuronal receptors (Namba et al., 1991; Dickson et al., 1997; Herz and Beffert, 2000). The human apoE gene encodes three major isoforms, apoE2 (Cys112, Cys158), apoE4 (Arg112, Arg158), and apoE3 (Cys112, Arg158), the most abundant. Whereas the E2 allele is associated with a decreased risk of AD, the E4 allele increases the risk (Corder et al., 1993, 1994). Furthermore, apoE has isoform-specific effects on A β aggregation (Dodart et al., 2005). In neuronal cultures, recombinant apoE2 or apoE3 protects against A β toxicity, whereas apoE4 can be toxic in itself or by promoting A β cytotoxicity (Jordan et al., 1998; Pedersen et al., 2000; Ji et al., 2002). ApoE expression has been detected in astroglial, neuronal, and microglial cells (Pitas et al., 1987a; Poirier et

Received Aug. 11, 2005; revised Feb. 2, 2006; accepted Feb. 5, 2006.

This work was supported by the Institut National de la Santé et de la Recherche Médicale, Université Pierre et Marie Curie, and Sanofi-Aventis. We thank Drs. Merle Ruberg, Bernard Zalc, and Anna Williams for critical reading of this manuscript.

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DOI:10.1523/JNEUROSCI.5186-05.2006

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al., 1991; Stone et al., 1997; Xu et al., 1999; Saura et al., 2003; Mori et al., 2004), but the role of microglial apoE has not been assessed previously.

We analyzed the effects of microglia on cultured neurons exposed to A β 1–40. We show that the microglial system x_c^- potentiates A β -induced neuronal death and prevents neuroprotection by microglia-derived apoE. These observations may be relevant to AD, because the system x_c^- -specific xCT gene is expressed by microglia in animal models in which amyloid deposits are formed.

Materials and Methods

Animals

Three-month-old male C57BL/6 mice and pregnant Wistar rats were obtained from Elevage Janvier (Le-genest-St-isle, France). Transgenic Thy1-APP₇₅₁ mice (TgAPP) expressing the human APP bearing the Swedish (S: KM595/596NL) and London (L: V6421) mutations (Blanchard et al., 2003) and apoE knock-out (KO) mice (Piedrahita et al., 1992) were bred and maintained on a C57BL/6 genetic background and obtained from Charles River (St. Aubin les Elbeuf, France). All animal procedures were performed in accordance with the guidelines for care and use of experimental animals of the European Economic Community (ref 86/609/EEC) and approved by the ethics committee for animal experimentation in Ile-de-France.

In vitro experiments

Reagents for cell cultures. The amyloid peptides A β 1–40 and A β 40–1 (Bachem, Voisins-le-Bretonneux, France) were dissolved in sterile distilled water at 5 mg/ml, incubated at 37°C for 1 week to form aggregated A β 1–40, and stored at –80°C. Recombinant human apoE2, apoE3, and apoE4 were obtained from Calbiochem (La Jolla, CA). 2-Amino-5-phosphonopentanoic acid (APV), (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK801), amino adipate (AAA), aminopimelate (APA), cysteine, cystine, insulin, iron-free transferrin, progesterone, putrescine, and selenium salt were obtained from Sigma (Saint Quentin Fallavier, France). Mouse monoclonal anti-basic FGF (bFGF) IgG1 neutralizing rat bFGF (clone bFM-1) was obtained from Upstate Biotechnology (Euromedex, Mundolsheim, France). All other components of the culture media including fetal calf serum (FCS) with low endotoxin content (<0.05 ng/ml) were obtained from Invitrogen (Cergy Pontoise, France). Cells were cultured in plastic dishes (TPP, Trasadingen, Switzerland).

Cell cultures. Cultured neurons and microglial cells were derived from the cerebral cortex of 17-d-old Wistar rat embryos [embryonic day 17 (E17)], according to procedures described previously (Thery et al., 1991; Dobberty et al., 1997) with a slight modification of the neuronal culture medium. Embryos were taken from pregnant rats killed by prolonged exposure to increasing concentrations of carbon dioxide. Microglia were also prepared from the cerebral cortex of newborn C57BL/6 wild-type (WT) or apoE-KO mice.

Primary neuronal cultures. E17 cells dissociated mechanically were seeded in 96-well culture plates (2×10^4 cells per well) precoated with 15 μ g/ml polyornithine and cultured in a Sato-based chemically defined medium (CDM; 50 μ l per well) consisting of DMEM supplemented with 100 μ g/ml transferrin, 25 μ g/ml insulin, 20 nM progesterone, 60 μ M putrescine, 30 nM Na₂SeO₃, 5 U/ml penicillin, and 5 μ g/ml streptomycin. After 4 d *in vitro* (DIV), 50 μ l of CDM was added to each well with or without other reagents. Cells were further cultured for up to 2 d (6 DIV) before analysis of neuronal morphology and survival. For biochemical analyses, neurons were cultured in 35 mm culture dishes (1.5×10^6 cells per dish), and the purity (>95%) was checked before and after treatment (4–6 DIV) by immunocytochemical detection of neuronal and glial markers (Dobberty et al., 1997). When indicated, neurons were cultured in CDM with 10 μ M instead of 0.2 mM cystine supplemented with 100 μ M cysteine.

Microglial cultures. Pure amoeboid microglial cells (>99%) were isolated from 2-week-old primary glial cultures grown in DMEM supplemented with 10% FCS. The cells were washed three times in DMEM and

plated in CDM (4×10^4 cells per well of 96-well plates or $1\text{--}1.5 \times 10^6$ cells per 35 mm dish).

Neuron–microglia cocultures. Freshly isolated microglial cells and reagents were added to 4 DIV primary neuronal cultures grown in 96-well plates at a microglia/neuron seeding ratio of 2/1 (4×10^4 microglial cells added together with 50 μ l of CDM per well). Control neuronal cultures were supplemented with an equal amount of cell-free CDM.

Microglia conditioned medium. Purified microglia (10^6) plated in 35 mm dishes were cultured for 2 d in 900 μ l of CDM, which was then recovered, cleared of contaminating cells by centrifugation ($10,000 \times g$; 5 min), and stored at –80°C. Conditioned medium (CM) was added with or without other reagents to 4 DIV neuronal cultures grown in 96-well plates (50 μ l per well; final dilution of CM was 1:2). Control neuronal cultures were supplemented with an equal amount of unconditioned CDM or reagents.

Immunodepletion of apoE in CM. CM was incubated for 1.5 h at 37°C with protein G-Sepharose fast flow beads coated with goat IgG recovered either from goat anti-human apoE antiserum (Chemicon, Hampshire, UK) or from nonimmune goat serum (700 μ g antibody per 1 ml of CM; Invitrogen). The beads were then sedimented by centrifugation ($10,000 \times g$; 15 min), and the immunodepleted CM (CM^{apoE–} and CM^{apoE+}) was recovered and filtered (0.2 μ m filter pores; Sartorius, Palaiseau, France) before the incorporation into 4 DIV neuronal cultures.

Delipidation of immunodepleted CM. CM^{apoE–} was mixed at room temperature with an equal volume of either n-hexane or diethyl ether. The mixture was centrifuged ($10,000 \times g$; 3 min) to separate the aqueous and organic phases. The aqueous phase was recovered and further extracted twice with the same organic solvent before the incorporation into neuronal cultures. Unconditioned CDM submitted to the same extraction procedure was used as a control.

Immunocytochemical and lectin fluorescent staining. Neurons and microglia were immunostained as reported previously (Dobberty et al., 1997; Lima et al., 2001) with slight modifications. Briefly, cell cultures were fixed with 4% paraformaldehyde for 20 min and incubated at 4°C overnight with 0.5 μ g/ml Alexa Fluor 594 (red)-conjugated isolectin B4 from *Griffonia simplicifolia* (Invitrogen). To detect the neuronal microtubule associated protein-2 (MAP2), fixed cultures, stained or not with isolectin B4, were blocked with PBS containing 0.1% Triton X-100 and 10% goat serum for 1 h at room temperature (RT) and incubated with 20 μ g/ml mouse monoclonal anti-MAP2 IgG1 (Sigma) diluted in blocking solution at 4°C overnight. Bound antibodies were revealed with Alexa Fluor 488 (green)-conjugated goat anti-mouse. Nuclei of fixed cultures were labeled by incubation with 2 μ g/ml Hoechst 33342 (Sigma) in PBS at RT for 1 min. Stained cultures were analyzed with an inverted Leica (Nussloch, Germany) microscope.

Quantification of cell survival. Neuronal survival was quantified with an ELISA of MAP2 levels as described previously (Thery et al., 1991). Briefly, cultures in 96-well plates were fixed by adding paraformaldehyde (4% final) to the medium at 4°C for 1 h. Fixed cells were saturated at 37°C for 30 min in PBS containing 0.1% Tween 20 and 10% FCS (buffer A) before sequential incubations (1 h at 37°C) with 2 μ g/ml mouse monoclonal anti-MAP2 IgG1 and 1:1000 peroxidase-conjugated anti-mouse IgG (Amersham Biosciences, Arlington Heights, IL) diluted in buffer A. Bound peroxidase was revealed with 0.04% o-phenylenediamine (Sigma) and 0.02% H₂O₂ in citrate buffer, pH 5, for 20 min at 37°C. Quantification was performed by measuring optical density (O.D.) as absorbance at 490 nm using an automated plate reader (Dynatech, Plaisir, France). The specific MAP2 O.D. was determined by subtracting absorbance resulting from nonspecific IgG binding that was measured in sister wells by replacing anti-MAP2 IgG1 with standard mouse unrelated IgG1 (BD Biosciences, Le Pont de Claix, France). MAP2 O.D. was shown to increase linearly with the number of neurons in cultures of E17 cells seeded in 96-well plates at a cell density ranging between 1×10^3 and 4×10^4 cells per well (Thery et al., 1991). In the present study, we verified in representative experiments that the variations in MAP2 O.D. were related to changes in the number of neurons immunostained with anti-MAP2 antibody in sister wells. For each well, MAP2-positive cell bodies were counted in four microscopic fields covering 3% of the total well surface.

Cell survival in pure microglia cultures was estimated by the enzymatic conversion of nonfluorescent cell-permeant calcein *O,O'*-diacetate tetraakis (acetoxymethyl) ester (calcein-AM; Sigma) to fluorescent calcein (Papadopoulos et al., 1994). Culture medium was removed, and the cells were incubated with 1 μ M calcein-AM diluted in PBS for 45 min at 37°C. Calcein fluorescence was measured at 530 nm after excitation at 495 nm using the SpectraMAX Gemini microplate spectrofluorometer (Molecular Devices, St. Grégoire, France).

Measurement of glutamate released by cultured microglia. Glutamate released from microglial cultures plated in CDM without phenol red (4×10^4 cells in 100 μ l of CDM per well of 96-well plate) was quantified with the glutamate dehydrogenase-based colorimetric assay described by Beutler (1985). Briefly, the medium of microglia cultures was recovered and heated at 95°C for 10 min, before dilution (1:2, v/v) and incubation at RT for 15 min in an assay mixture containing 72 mM triethanolamine, 10 mM PBS, 0.6% Triton X-100, 0.4 mM nicotinamide-adenine dinucleotide, 80 μ M iodonitrotetrazolium chloride, 0.2 U/ml diaphorase, and 10 U/ml glutamate dehydrogenase (all from Sigma). Glutamate concentrations were determined by reading O.D.₄₉₂. O.D. standard curves were generated with graded concentrations of L-glutamate (Sigma) diluted in CDM.

Reverse transcription-PCR analysis. Total RNA was extracted from neuronal or microglial cultures (1.5×10^6 cells per 35 mm dish) after the lysis of cells with TRIZOL reagent according to the instructions of the manufacturer (Invitrogen). RNA was quantified spectrophotometrically, and 3 μ g samples were reverse-transcribed using SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) and pd(N)6 random hexamers (Amersham Biosciences) as described previously (Lima et al., 2001). Primers were designed to amplify both rat and mouse products. Primer sequences product sizes and numbers of the related cDNA sequences were as follows: 5'-CTC GTG ACA GCT GTG GGC AT-3' and 5'-GGC ACT AGA CTC AAG AAC TGT G-3' for xCT (1244 bp; accession number NM_011990 and XM_227120); 5'-CTC CCA GGA AGA TTT TAA AG-3' and 5'-TTC ATT TTG GTG GCT ACA AT-3' for 4F2hc (144 bp; accession number AB015433); 5'-CCT GAA CCG CTT CTG GGA TTA C-3' and 5'-AGC ATG GTG TTT ACC TCG TTG C-3' for apoE (291 bp; accession number X04979); 5'-CAT TGA GAG CAA TGC CAG CC-3' and 5'-TAT CGG ACG CCT GGT TAC CA-3' for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (875 bp; accession number X02231 and M32599). cDNA were amplified by 20 cycles (apoE), 27 cycles (GAPDH or 4F2hc), or 30 cycles (xCT) with the following conditions: denaturation at 94°C for 30 s (xCT, 4F2hc, or GAPDH) or 1 min (apoE) annealing at 58°C (xCT), 60°C (4F2hc), or 54°C (GAPDH) for 30 s or at 57°C for 1 min (apoE), extension at 72°C for 1 min (xCT), 45 s (4F2hc or GAPDH), or 90 s (apoE) and final elongation at 72°C for 8 min. Amplified products were analyzed by electrophoresis on 1% agarose gels. No products were amplified from RNA samples that were not reverse-transcribed. The identity of PCR products was verified by subcloning into the pGEM-T easy vector (Promega, Charbonnières, France) and sequencing.

Western blot analyses

Assessment of A β degradation. Aggregated A β was diluted (0.4 μ g/ml final) and incubated at 37°C for 16 h in CM or CDM with or without proteinase K (1 μ g/ml). Before heat denaturation at 95°C for 10 min, 13 μ l samples of each medium were separated on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) using reducing reagents provided by the manufacturer and transferred to a nitrocellulose membrane (Millipore, Saint-Quentin en Yvelines, France). Blots were blocked for 30 min at RT in Tris-buffered saline [TBS; 50 mM Tris-(hydroxymethyl) aminomethane and 150 mM NaCl, pH 7.5] containing 5% nonfat skin milk and incubated sequentially at RT for 1 h with mouse monoclonal WO2 anti-A β antibody (1:20,000; ABETA, Heidelberg, Germany) and horseradish peroxidase-linked sheep anti-mouse antibody (1:3000; Amersham Biosciences). Bound antibodies were detected by enhanced chemiluminescence (Perbio, Brebières, France) using BioMax Light-2 films (Amersham Biosciences).

Cell production of apoE. For total extracts, cultured microglial cells (10^6 cells in 35 mm dishes) were lysed after three washes with PBS in 400 μ l of 1% SDS (VWR, Fontenay-sous-Bois, France) preheated to 95°C.

The lysate was then homogenized by pipetting and further heated to 95°C for 1 min. Protein concentrations were quantified by the bicinchoninic acid assay (Sigma). Microglial protein extracts (5 μ g per sample) or culture media (4 μ l per sample) recovered from cell cultures and cleared by centrifugation (10,000 $\times$ g; 10 min) were resolved by 10% SDS-PAGE, and the resulting Western blots were processed for immunodetection as above using goat anti-apoE antiserum (1:1000) and revealed with a horseradish peroxidase-linked rabbit anti-goat antibody (1:5000; DAKO, Trappes, France).

In vivo experiments

A β injection. Three-month-old (25 g) male C57BL/6 mice were deeply anesthetized by injection of a mixture of 0.42% ketamine (Merial, Lyon, France) and 0.33% xylazine (Bayer, Puteaux, France). A β (1.8 μ g) in 400 nl of PBS or PBS alone (control) was injected unilaterally into the brain, using stereotaxic coordinates for the hippocampus, relative to bregma: –2 mm anteroposterior, 2 mm lateral, and 2 mm depth. Mice were killed 1 week after injections for histological analyses.

Tissue preparation for in situ hybridization and immunocytochemistry. Three-month-old wild-type C57BL/6 mice injected or not injected with A β or PBS, 15- or 19-month-old TgAPP and age-matched WT mice were deeply anesthetized and perfused with 4% (v/v) paraformaldehyde. Brains were postfixed overnight in the same fixative, cryoprotected by overnight immersion in PBS containing 15% (w/v) sucrose, and frozen in melting isopentane. Sections (10 μ m thick) cut on a cryostat (Microm, Heidelberg, Germany) were mounted on Superfrost Plus glass slides (Menzel-Glaser, Freiburg, Germany). Four animals were used for each treatment, genotype, and age.

In situ hybridization. A cDNA (1.2 kb) corresponding to nucleotides 818–2061 of the mouse xCT mRNA (NM_011990) was obtained by reverse transcription-PCR as described above from mouse microglial cultures derived from E18 Swiss mice embryos. The cDNA was cloned into the pGEM-T Easy vector (Promega) and verified by sequencing. Digoxigenin-UTP (DIG)-labeled antisense and sense cRNA probes were transcribed using the SP6 and T3 RNA polymerases, respectively (Promega). *In situ* hybridization (ISH) was performed according to the protocol of Strahle et al. (1994) modified by Myat et al. (1996). In brief, cryostat sections were hybridized overnight at 65°C with 0.15 μ g/ml DIG-labeled cRNA in hybridization buffer containing 50% formamide, 10 mM Tris-HCl, 1 mM Tris-base, 5 mM NaH₂PO₄·2H₂O, 5 mM Na₂HPO₄, 5 mM EDTA, 10% dextran sulfate, and 1 mg/ml yeast RNA and then washed twice at 65°C for 30 min in 1 $\times$ SSC (0.15 M NaCl and 15 mM Na₂CO₃, pH 7.0) with 50% formamide and 0.1% Triton X-100, and once at RT for 30 min in 100 mM maleic acid, 150 mM NaCl and 1% Tween 20, pH 7.5. Hybridized probes were detected with an alkaline phosphatase-conjugated sheep anti-DIG antibody, using 5-bromo-4-indoyl phosphate/nitroblue tetrazolium chloride (Roche, Meylan, France) as the substrate.

Immunocytochemistry, lectin peroxidase, and Congo red staining in combination with ISH. Microglial cells were detected using the rat monoclonal anti-mouse CD11b IgG1 (Serotec, Oxford, UK) or peroxidase-coupled isolectin B4 (Sigma). For staining with anti-CD11b, cryostat sections were blocked at RT for 1 h in PBS containing 0.1% Triton X-100 and 10% goat serum and then incubated overnight at 4°C with 3 μ g/ml rat anti-CD11b diluted in blocking buffer. Bound anti-CD11b antibody was detected with a biotinylated goat anti-rat IgG (Amersham Biosciences; antibodies diluted to 1/300; 2 h at RT) followed by incubation with streptavidin biotinylated horseradish peroxidase (diluted to 1/400, 1.5 h at RT; Amersham Biosciences). Peroxidase activity was revealed with 3,3'-diaminobenzidine (DAB chromogen; DAKO) and H₂O₂ in TBS, pH 7.6. To detect both CD11b and xCT mRNA, immunocytochemistry was performed as above in sterile buffer containing 1 $\times$ protectRNA RNase inhibitor (Sigma) and then ISH was performed as above after rinsing in PBS. The specificity of the immunostaining was verified by replacing anti-CD11b with unrelated rat IgG1 (BD Biosciences). Isolectin B4 staining was performed alone or in combination with ISH. In the latter case, ISH was performed first, and then the sections were washed in PBS, and isolectin B4 staining was performed as described previously (Lima et al., 2001) using DAB to reveal bound lectin. The specificity of

the staining was verified by saturating the lectin binding sites with D-(+)-galactose (300 μ g/ml). Congo red staining was performed alone or after ISH by incubating sections for 30 min at RT with 0.2% Congo red (Sigma) diluted in 80% ethanol containing 2.5 mM NaOH saturated with 0.5% NaCl. The stained sections were mounted in Eukitt (O. Kindler, Freiburg, Germany) for examination under a Leica DMRB microscope.

Results

Effect of A β and microglial cells on neuronal survival

In the present study, the neurotoxic effect of A β on neurons was assessed by treating 4 DIV pure neuronal cultures with increasing concentrations of aggregated A β 1–40, a protein that accumulates in AD plaques containing microglia (Fukumoto et al., 1996). After a 2 d exposure to A β (20 μ g/ml), the neurons developed tortuous neurites and showed signs of apoptotic-like cell death characterized by chromatin condensation, nuclear fragmentation, and complete loss of MAP2 immunoreactivity (Fig. 1*A,B*). Neuronal death quantified by ELISA determination of MAP2 levels shown to correlate with cell loss (see Materials and Methods) was dose dependent beginning at a concentration between 5 and 10 μ g/ml (Fig. 1*D*). To determine whether microglia modulate the toxicity of A β , amoeboid microglial cells were added to 4 DIV neuronal cultures together with increasing concentrations of A β . In the absence of A β , microglia had no significant effect on neuronal survival (data not shown), but microglia potentiated the neurotoxicity of A β , which became toxic at 2.5 μ g/ml (Fig. 1*C,E*). Neuronal degeneration was not observed when cultures were treated with the reverse sequence A β 40–1 (Fig. 1*D,E*).

Microglial system x_c^- is involved in the microglia-enhanced death of neurons exposed to a low dose of A β

To investigate the role of system x_c^- -dependent glutamate release in the microglia-enhanced neurotoxicity of A β , we quantified glutamate levels in the medium of pure microglial cultures treated with increasing doses of A β and seeded at the same density as in cocultures (4×10^4 cells per well of 96-well plate). In the absence of A β , purified microglia readily released glutamate that reached an average of 70 μ M after 2 d in culture. The level increased significantly in cultures exposed to A β from 5 μ g/ml (Fig. 2*A*).

To determine whether system x_c^- was implicated in glutamate release by microglial cells exposed to A β at 2.5 μ g/ml, a dose that triggered neuronal death in neuron–microglia cocultures, the microglial cultures were treated with 2.5 mM AAA and APA that inhibit the antiporter (Watanabe and Bannai, 1987; Piani and Fontana, 1994). As shown in Figure 2*B*, the levels of extracellular glutamate were reduced by 60–90%, but neither AAA treatment nor APA treatment altered microglial survival. The NMDA receptor antagonist MK801 used as a negative control had no effect (Fig. 2*B*).

To confirm the presence of the x_c^- antiporter in the microglial cells, expression of the xCT gene encoding the specific short chain and the 4F2hc gene encoding the nonspecific chain of the

Figure 1. Effects of A β on the survival of neurons cultured with or without microglial cells. Neurons derived from E17 rat cerebral cortex were cultured for 6 d with or without A β or microglia that were present during the last 2 d in the cultures. *A–C*, Triple fluorescent staining with mouse anti-MAP2 antibody (Alexa green), isolectin B4 (Alexa red), and Hoechst 33342 (blue) in untreated neuronal cultures (*A*) or cultures treated with 20 μ g/ml A β 1–40 (*B*) or 2.5 μ g/ml A β 1–40 in the presence of microglia (*C*). Hoechst staining shows nuclei in cell corpses that display no MAP2 immunoreactivity. Arrowheads in *C* point to cell corpses engulfed by microglial cells. Scale bar, 20 μ m. *D, E*, Pure neuronal cultures (*D*) and neuron–microglia cocultures (*E*) were treated with the indicated concentrations of A β 1–40 or A β 40–1 before quantification of neuron survival by ELISA determination of MAP2 levels (MAP2 O.D.). The MAP2 O.D. is expressed as a percentage of the mean control value determined in the cultures without A β and defined as 100%. Data are means $\pm$ SD from four to five determinations in sister wells from three independent experiments. The asterisk indicates significant difference between untreated and A β -treated cultures (* p < 0.01; one-way ANOVA followed by Dunnett's test).

antiporter was examined by reverse transcription-PCR. Both xCT and 4F2hc transcripts were presented in microglial cells that were exposed to 2.5 μ g/ml A β for 1 d (Fig. 2*C*). xCT transcripts were not detected in 5 DIV pure neuronal cultures treated for the last 24 h of culture with A β . 4F2hc transcripts were expressed at low levels in the neuronal cultures, probably related to the presence of other amino acid transporters (Stevens and Vo, 1998; Heckel et al., 2003). Both the 4F2hc and xCT genes were also expressed in purified microglial cultures in the absence of A β (data not shown).

To determine how the microglial system x_c^- contributes to the death of neurons in cocultures exposed to 2.5 μ g/ml A β , neuronal survival was compared in cultures treated with or without 2.5 mM AAA. As shown in Figure 2*D*, the neuronal death resulting from the presence of microglial cells was totally suppressed in the presence of AAA and was restored by supplementing the medium of AAA-treated cocultures with glutamate at a dose of 70 μ M, which is equivalent to the amount of glutamate released by untreated microglial cultures (Fig. 2*B,D*). In the absence of microglial cells, 70 μ M glutamate was not sufficient to kill neurons exposed or not to sublethal doses of A β (2.5–5 μ g/ml). At 200 μ M, however, slight (<30%) but significant toxicity was observed in pure neuronal cultures, whether or not the cells were treated with a sublethal dose of A β (Fig. 2*E*). Although A β used at doses <5 μ g/ml did not significantly stimulate the microglial production of glutamate (Fig. 2*A*), our results show that glutamate released through the microglial system x_c^- is required to trigger neuronal death in cocultures treated with 2.5 μ g/ml A β and that the neurotoxicity of glutamate is enhanced when neurons are exposed to microglia together with A β .

Figure 2. System x_c^- expression and release of neurotoxic glutamate by microglia in the presence of Aβ. **A**, Aβ stimulates a dose-dependent release of glutamate by microglia. Purified microglial cells (4×10^4 cells per 100 μl of medium) were incubated for 2 d with increasing concentrations of Aβ before measuring glutamate concentrations in the culture medium. Data are means $\pm$ SD from six determinations in sister wells from three independent experiments. The asterisk indicates significant difference between untreated and Aβ-treated cultures ($*p < 0.01$; one-way ANOVA followed by Dunnett's test.). **B**, Effect of system x_c^- blockers on glutamate release and cell survival in pure microglial cultures exposed to Aβ. Cells were incubated for 2 d with 2.5 μg/ml Aβ in the absence (Ctrl.) or the presence of 2.5 mM AAA, 2.5 mM APA, or 2.5 μM MK801 before assessment of glutamate release or cell survival measured by calcein fluorescence. Results are expressed as the percentage of the mean value in control cultures. Data are means $\pm$ SD from three or six determinations in sister wells from three independent experiments. The asterisk indicates significant difference between control (Ctrl.) and cultures treated with AAA or APA ($*p < 0.001$; one-way ANOVA followed by Tukey–Kramer multiple comparisons test). **C**, Ethidium bromide-stained agarose gels showing reverse transcription-PCR products generated from xCT, 4F2hc, and GAPDH mRNA in 5 DIV neuronal cultures or in microglial cells cultured for 24 h after purification. Cultures were treated with 2.5 μg/ml Aβ for the last 24 h. **D**, **E**, Role of microglial system x_c^- and glutamate in neuronal death in the presence of Aβ. **D**, Cultured neurons were grown for 4 d and then treated for 2 d with 2.5 μg/ml in the absence (pure neuronal cultures) or presence of microglia (neuron–microglia cocultures) before quantification of neuron survival by ELISA determination of MAP2 levels. When indicated, AAA (2.5 mM) and glutamate (Glu 70 μM) were added to the medium together with Aβ. The MAP2 O.D. is expressed as the percentage of the mean control value determined in pure neuronal cultures without AAA defined as 100%. **E**, Four-day-old pure neuronal cultures were treated for 2 d with or without indicated doses of glutamate and Aβ. MAP2 O.D. is expressed as the percentage of untreated neuronal cultures. Data are means $\pm$ SD from five determinations in sister wells from two independent experiments. The asterisk indicates significant difference ($*p < 0.01$; one-way ANOVA followed by Tukey–Kramer multiple comparisons test).

When NMDA receptors or system x_c^- are blocked, microglia become neuroprotective

Under standard conditions, in the presence of microglia, a normally sublethal concentration (2.5 μg/ml) of Aβ became toxic to neurons (Fig. 3A). This toxicity implicated glutamate release through the system x_c^- , because two agents that inhibit the antiporter, AAA and APA, prevented microglial potentiation of Aβ-induced cell death (Fig. 3B,C). We then investigated whether

Figure 3. Inhibition of system x_c^- or NMDA receptors in cultures exposed to Aβ 1–40 prevents the neurotoxic effect of microglia and unmask their neuroprotective potential. Cultured neurons were grown for 4 d and then treated for 2 d with 2.5 μg/ml or 20 μg/ml Aβ (Aβ 2.5 or Aβ 20) in the absence or the presence of microglia before quantification of neuron survival by ELISA determination of MAP2 levels (MAP2 O.D.). Cultures were performed in standard CDM (containing 0.2 mM cystine) (**A**), standard CDM supplemented for the last 2 d with AAA (2.5 mM) (**B**), APA (2.5 mM) (**C**), MK801 (2.5 μM) (**E**), or APV (50 μM) (**F**), or CDM with 10 μM cystine and 100 μM cystine (low cystine) (**D**). In each graph, the MAP2 O.D. in pure neuronal cultures (open bars) or in neuron–microglia cocultures (filled bars) is expressed as the percentage of the mean value determined in pure neuronal cultures treated with 2.5 μg/ml Aβ defined as 100%. Data are mean $\pm$ SD from five determinations in sister wells from five (**A**, **B**), three (**C**, **D**), four (**E**), or two (**F**) independent experiments. The asterisk indicates significant difference between pure neuronal and neuron–microglia cocultures ($*p < 0.01$; one-way ANOVA followed by Tukey–Kramer multiple comparison test).

inhibition of system x_c^- permitted microglial protection against the neurotoxicity of Aβ. A high concentration (20 μg/ml) of Aβ was toxic to neurons in pure cultures (Fig. 3A–F), but microglia were neuroprotective at this Aβ concentration in cocultures if the deleterious release of glutamate through the antiporter was inhibited by AAA or APA (Fig. 3B,C). The role of system x_c^- was confirmed by reducing the extracellular concentration of cystine to a level that prevents system x_c^- -mediated microglial release of glutamate (Piani and Fontana, 1994) (Fig. 3D). Similar effects were observed with the noncompetitive and competitive NMDA receptor antagonists MK801 and APV, respectively, which prevented the deleterious effect of system x_c^- -dependent glutamate release and enhanced neuronal survival in the presence of microglia and high concentrations of Aβ (Fig. 3E,F). In the absence of microglia, increasing Aβ concentration from sublethal (2.5 μg/ml) to high levels (20 μg/ml) triggered neuronal death that was not caused by excitotoxicity, because it was not prevented by NMDA receptor antagonists (Fig. 3E,F).

Microglia-derived soluble factors rescue neurons from A β toxicity when system x_c^- -mediated excitotoxicity is inhibited

To determine whether neuroprotection by microglia requires the phagocytic elimination of A β or the release of factors into the culture medium, 4 DIV pure neuronal cultures were supplemented with CM recovered from 2 DIV pure microglia cultures treated or not with AAA and then exposed for 2 d (4–6 DIV) to 20 μ g/ml A β . MK801 was added to neuronal cultures to prevent excitotoxicity when assessing the effect of CM prepared in the absence of system x_c^- blockers. As illustrated in Figure 4*A–H*, CM obtained from microglia cultured with or without AAA prevented A β -induced neuronal death, indicating that microglia released one or more neuroprotective factors that prevented neuronal death induced by a high concentration of A β .

To determine whether these factors included proteases that degraded preaggregated A β , the aggregated peptide (<2 μ g/ml) was incubated overnight in CM or in CDM and analyzed by PAGE electrophoresis and Western blotting. A β incubated in CDM appeared as a smear of high molecular weight (MW) species (>250 kDa) and two polymers with apparent MWs between 50 and 75 kDa. No monomers (4 kDa) or small oligomers of A β were detected (Fig. 4*I*, lane 1). A similar pattern was observed when A β was incubated in CM (Fig. 4*I*, lane 2). As a positive control for A β proteolysis, aggregated A β was clearly degraded by proteinase K (Fig. 4*I*, lane 3). These results strongly suggested that the neuroprotective activity of CM did not result from A β degradation by secreted proteases.

Microglia-derived apoE is required for protection of neurons against direct A β -cytotoxicity

Preliminary physicochemical characterization of the neuroprotective activity in CM indicated that it was not lost after ultracentrifugation at 30,000 $\times g$ for 1 h or filtration (0.2 μ m filter pore diameters), ruling out the involvement of membrane particles that were possibly shed by cultured microglia. Dialysis experiments showed that the MWs of factors required for the protective activity exceeded 10 kDa (data not shown). Because human isoforms of apoE were previously shown to protect cultured neurons against A β cytotoxicity (Puttfarcken et al., 1997; Pedersen et al., 2000), we tested whether the neuroprotective factor released from microglia was apoE. We first performed reverse transcription-PCR to detect apoE transcripts. ApoE mRNA were clearly detected in purified microglia exposed or not to 20 μ g/ml A β for 2 d but also in 5 DIV pure neuronal cultures, although at much lower levels than in microglia (Fig. 5*A*). Western blot analyses detected an apoE immunoreactive band with an apparent MW of 34 kDa in microglial cell extracts and CM but not in the

Figure 4. Effects of microglial conditioned medium on neuronal cultures exposed to A β . *A–H*, Four DIV neuronal cultures were treated for 2 d with or without 20 μ g/ml A β and/or CM recovered from pure microglia cultures incubated for 2 d without (*A–G*) or with (*H*) 2.5 mM AAA. MK801 (2.5 μ M) was also added to 4 DIV neuronal cultures when assessing the effect of CM prepared without AAA (*A–G*). *A–F*, Optical fields of 6 DIV pure neuronal cultures treated for the last 2 d with MK801 and in the absence (*A*, *D*) or the presence (*B*, *E*) of 20 μ g/ml A β or 20 μ g/ml A β and CM (*C*, *F*). *A–C*, Phase contrast views of living cultures. Arrowheads in *B* and *C* point to A β deposits. *D–F*, Fluorescent staining with anti-MAP 2 antibody (Alexa green). Scale bar, 20 μ m. *G*, Quantification of neuronal survival by determination of MAP2 levels in 6 DIV cultures that were treated with MK801 and incubated with or without 20 μ g/ml A β , CM, or A β plus CM. Cultures without CM were supplemented with the same volume of unconditioned CDM (see Materials and Methods). The MAP2 O.D. is expressed as the percentage of the mean value in pure neuronal cultures without A β and CM set at 100%. Filled bars correspond to CM-treated cultures. Data are means $\pm$ SD from five determinations in sister wells from six independent experiments. *H*, Assessment of neuronal survival in cultures treated as in *G* but without MK801 and using CM derived from microglia treated with 2.5 mM AAA (means $\pm$ SD from five determinations from two independent experiments). The asterisk indicates significant difference (* p < 0.001; one-way ANOVA followed by Tukey–Kramer multiple comparisons test). *I*, Western blot analysis of A β incubated in culture media. A β (0.4 μ g/ml) was incubated overnight at 37°C in CDM (lane 1), CM (lane 2), or with 1 μ g/ml proteinase K (lane 3). Samples (13 μ l) of each medium were separated by SDS-PAGE and probed with polyclonal anti-A β antibodies that were visualized by enhanced chemiluminescence.

medium of pure neuronal cultures (Fig. 5*B*). Released apoE was also clearly detected in pure microglial cultures and in neuron–microglia cocultures treated with AAA or A β (Fig. 5*B*).

To show that apoE was responsible for neuroprotection by microglia, CM was depleted of apoE content by immunoprecipitation with anti-apoE antibodies (CM^{apoE−}), before it was added to neuronal cultures treated with A β in the presence of MK801. Because the neurotoxicity of A β tends to be reduced at concentrations <20 μ g/ml (Fig. 1*A*), neuronal cultures were treated with A β in excess (40 μ g/ml). As shown in Figure 6*A*, CM^{apoE−} did not prevent the death of neurons exposed to a toxic dose of A β , whereas control CM treated with unrelated antibodies (CM^{apoE+}) was fully protective.

To determine whether a specific isoform of apoE was responsible for neuroprotection in the absence of system x_c^- -mediated

Figure 5. Microglial expression of the apoE gene. **A**, Ethidium bromide-stained agarose gel of reverse transcription-PCR products generated from apoE and GAPDH mRNA in 5 DIV pure neurons (neu.) or in purified microglial cells cultured for 24 h in the absence (mic.) or the presence (mic. + A β) of 20 μ g/ml A β . **B**, Western blot detection of apoE in microglia cell extracts (5 μ g of total protein, extract mic.) or in medium (4 μ l per lane) recovered from 2 DIV pure microglia cultures (mic.), neuron–microglia cocultures (coc.), or 6 DIV pure neuronal cultures (neu.). Cultures were treated for 2 d with or without 2.5 mM AAA or 20 μ g/ml A β .

Figure 6. Effects of apoE immunodepletion and supplementation on the survival of neurons exposed to A β . Four DIV neuronal cultures were treated for 2 d with MK801 (2.5 μ M) and with or without A β (40 μ g/ml), CM, or purified recombinant human apoE isoforms (80 nM). **A**, Effects of CM after immunodepletion of apoE (CM^{ApoE-}) compared with CM after immunoprecipitation with unrelated antibodies (CM^{ApoE+}). The effectiveness of the depletion is illustrated by Western detection of apoE in the conditioned medium before immunoprecipitation (CM), after depletion (CM^{ApoE-}), and after treatment with nonrelevant antibodies (CM^{ApoE+}). **B, C**, Effects of CM^{ApoE-} supplemented with recombinant human apoE2 (E2), apoE3 (E3), or apoE4 (E4). **C**, Illustration of a control experiment verifying that variations in MAP2 O.D. in **B** correspond to changes in the number of MAP2-positive cells (see Materials and Methods). **D**, Effects of recombinant human apoE (E2, E3, or E4) added to unconditioned CDM. The MAP2 O.D. is expressed as the percentage of the mean value determined in untreated control (Ctrl.) neuronal cultures defined as 100%. Data are means $\pm$ SD from 5 or 10 determinations in sister wells from three (**A, B**), one (**C**), or four (**D**) independent experiments. The asterisk indicates significant difference between CM^{ApoE-}-treated and CM^{ApoE+}-treated neurons (**A**) or between A β -treated neurons and A β plus recombinant human apoE-treated neurons (**B–D**) (* p < 0.001 in **A, B**, and **D**; p < 0.05 in **C**; one-way ANOVA followed by Tukey–Kramer multiple comparisons test).

excitotoxicity, CM^{ApoE-} was supplemented with recombinant human apoE2, E3, or E4 at a final concentration of 80 nM that is physiologically relevant (Pitas et al., 1987b). All three isoforms significantly increased neuronal survival in the presence of a toxic concentration of A β (Fig. 6B). However, apoE2 was significantly more effective than apoE3 and apoE4 (p < 0.01; one-way

ANOVA followed by Tukey–Kramer multiple comparisons test), and apoE3 and apoE4 were similar in efficacy (p > 0.05) (Fig. 6B). Estimations of neuronal survival by counting the number of MAP2-positive cells (Fig. 6C) confirmed that CM^{ApoE-} supplemented with each of the apoE isoforms afforded significant protection against A β , with the same order of potencies (apoE2 > apoE3 = apoE4). Thus, apoE was necessary for neuroprotection by CM.

To determine whether purified apoE alone in the absence of CM or microglial cells could protect neurons, cultures exposed to a toxic dose of A β were treated with each of the apoE isoforms alone at 80 nM. Figure 6D shows that in the absence of CM^{ApoE-}, apoE4 and apoE3 did not increase the survival of cultured neurons in the presence of a toxic concentration of A β , whereas human apoE2 had a slight but significant protective effect (+22%). These results indicated that CM^{ApoE-} contained additional cofactors that strongly potentiated neuroprotection by human apoE.

Although the function of apoE is modulated by its binding to lipid components (Mahley and Rall, 2000), extraction of CM^{ApoE-} with organic solvents such as diethyl ether or n-hexane before incorporation of apoE2 did not reduce the capacity of apoE2-supplemented CM^{ApoE-} to effectively protect neurons against A β cytotoxicity (Fig. 7A,B). Basic FGF (FGF-2) was previously shown to reduce the *in vitro* neurotoxicity of A β (Mark et al., 1997) and can be produced in small amounts by purified microglia (Shimojo et al., 1991). However, addition of a blocking antibody against bFGF (up to 50 μ g/ml) to CM^{ApoE-} did not decrease neuroprotection by the medium (Fig. 7C). Cofactors other than lipids or bFGF are therefore likely to be implicated in the neuroprotective activity of human apoE.

To further evaluate the importance of apoE in the neuroprotective activity of microglia, we compared mouse microglia derived from WT and homozygous apoE-KO mice. Inactivation of the apoE gene had no effect on the survival of purified mouse microglia cultured in CDM. Purified WT mouse microglia secreted apoE (data not shown), and CM recovered from pure WT mouse microglial cultures protected rat neurons from A β cytotoxicity in the presence of MK801 (Fig. 8). Medium conditioned by apoE-deficient microglia had no protective activity. Supplementation of mouse apoE-deficient medium with human apoE2 (80 nM) was sufficient to obtain a neuroprotection similar to that observed with WT microglial CM (Fig. 8).

Altogether our results indicate that microglial secretion of apoE plays a major role in the capacity of microglia to protect neurons from A β neurotoxicity in the absence of system x_c^- excitotoxicity.

A β promotes microglial expression of the xCT gene *in vivo*

The preceding experiments show that the activity of microglial system x_c^- is critical in determining whether microglial cells are neurotoxic or neuroprotective in the presence of A β . To investigate whether system x_c^- is expressed in microglial cells associated with amyloid deposits *in vivo*, we injected A β into the hippocampus of adult mice and examined the expression of xCT transcripts in the brains fixed 1 week later. Consistent with the observation of Sato et al. (2002), using a DIG-labeled xCT mRNA probe, xCT transcripts were not detected in noninjected hippocampus or cerebral cortex. However, as illustrated in Figure 9, A and B, the xCT gene was strongly expressed in the hippocampus injected with A β at sites remote from the needle track but not in noninjected or PBS-injected hippocampus. Controls performed with an xCT sense probe were negative. xCT transcripts were most

Figure 7. Anti-bFGF antibodies and lipid extractions with organic solvents do not prevent neuroprotection by CM^{ApoE-} supplemented with human apoE2. Four DIV rat neuronal cultures were treated for 2 d with MK801 (2.5 μ M) with or without A β (40 μ g/ml) or CM^{ApoE-} supplemented with apoE2 (80 nM). **A, B**, CDM and CM^{ApoE-} were extracted three times with diethyl ether (**A**) or with n-hexane (**B**) before supplementation of CM^{ApoE-} with human apoE2 and incorporation into 4 DIV cultures (see Materials and Methods). The MAP2 O.D. is expressed as the percentage of the mean value determined in untreated neuronal cultures defined as 100%. **C**, Anti-bFGF IgG (50 μ g/ml) was added to 4 DIV cultures as indicated, and neuronal survival was quantified by counts of the number of MAP2-positive cells. Data are means $\pm$ SD from five determinations in sister wells from one (**C**) or two (**A, B**) independent experiments.

prominent in the region of A β deposits stained with Congo red (Fig. 9E) and were expressed by microglia as shown by colocalization with isolectin-B4 staining (Fig. 9F). Although A β deposits clearly promoted the recruitment of xCT mRNA-expressing microglia, such cells also responded to mechanical lesions, as indicated by the presence of xCT transcripts in the reactive microglia surrounding the needle track in mice brain injected with PBS (Fig. 9G,H).

Expression of xCT gene by microglia within or around senile plaques

To further evaluate the microglial expression of system x_c^- in association with pathological accumulations of A β , we examined xCT expression in the cerebral cortex of 15- or 19-month-old mice with a mutated human APP transgene. ISH detected xCT transcripts (Fig. 10A) that were codistributed with Congo red-stained amyloid deposits in the cerebral cortex (Fig. 10B). xCT transcripts were expressed by anti-CD11b-stained reactive microglial cells in the vicinity of or within the plaques (Fig. 10C). xCT mRNA was not detected in the cerebral cortex of age-matched wild-type mice (Fig. 10D).

Figure 8. Effects of medium conditioned by apoE-KO mouse microglia on the survival of neurons exposed to A β . Four DIV rat neuronal cultures were treated for 2 d with MK801 (2.5 μ M) with or without A β (40 μ g/ml), CM recovered from wild-type (CM-wt) or apoE-KO (CM-apoE KO) mouse microglial cultures, or CM-apoE KO supplemented with human apoE isoforms (80 nM final). The MAP2 O.D. is expressed as the percentage of the mean value in control neuronal cultures. Data are means $\pm$ SD from five determinations in sister wells from two independent experiments. The asterisk indicates significant difference (* p < 0.001; one-way ANOVA followed by Tukey–Kramer multiple comparisons test).

Discussion

Deposits of A β in rodent or human brain form in association with microglial activation and neuronal death. The present study demonstrates the dual capacity of microglia to either promote or prevent the death of neurons exposed to A β . Using cocultures of microglia and neurons, we show that system x_c^- -mediated release of glutamate from A β -stimulated microglia is neurotoxic, whereas apoE released from microglia protects against A β cytotoxicity. We also provide evidence for the *in vivo* upregulation of the system x_c^- -specific gene xCT in activated microglia recruited in the vicinity of A β deposits.

Our study raises the question whether the neutralization of a specific neurotoxic signal from microglia might unmask their neuroprotective potential. Previously, microglial production of NO, superoxide ion, peroxynitrite, or TNF- α were shown to be implicated in the death of cultured neurons exposed to A β -stimulated microglial cells (Combs et al., 2001; Qin et al., 2002; Xie et al., 2002). However, other studies suggested that excitotoxicity also played a role in the *in vivo* or *in vitro* death of neurons exposed to TNF- α or free radical-producing microglia activated with A β or lipopolysaccharide, because neuron survival increased after treatments with NMDA receptor antagonists (Willard et al., 2000; Golde et al., 2002; Miguel-Hidalgo et al., 2002; Floden et al., 2005). Enhanced release of glutamate has been observed in cultures of purified peritoneal macrophages or microglia treated with A β 1–40, A β 1–42, fibrillogenic A β 25–35 fragment, or secreted derivatives of APP (Klegeris and McGeer, 1997; Noda et al., 1999; Barger and Basile, 2001; Ikezu et al., 2003), but it was not known whether system x_c^- was involved in A β neurotoxicity.

We now show in neuron–microglia cocultures, that microglial

Figure 9. Expression of xCT gene and microglial activation in the adult mouse hippocampus 7 d after injection of A β . **A–D**, Sagittal sections of the hippocampus of two animals injected with A β diluted in PBS (**A**, **C**) or PBS alone (**B**, **D**). Scale bar, 100 μ m. **A**, **B**, ISH detection of xCT mRNA. CA3 and CA1 fields and the fimbria (fi) are indicated. **C**, **D**, Staining of microglia with peroxidase-conjugated isolectin B4. The microglial reaction and xCT mRNA are most prominent in the ventral part of CA3 after A β injection. **E**, Colocalization of injected A β (Congo red staining) and xCT mRNA (ISH, blue) in the ventral part of CA3. Scale bar, 100 μ m. **F**, ISH detection of xCT mRNA (blue) in reactive microglial cells stained with peroxidase-conjugated-isolectin B4. Scale bar, 20 μ m. **G**, **H**, Region lining the needle track in an animal injected with PBS. **G**, Staining with peroxidase-conjugated-isolectin B4. **H**, ISH detection of xCT mRNA. Scale bars, 50 μ m.

system x_c^- increases the vulnerability of neurons to A β 1–40 by releasing glutamate that acts on neuronal NMDA receptors. However, the concentration of A β necessary to enhance glutamate release by purified microglia was higher than the concentration required to promote the death of neurons in contact with

Figure 10. Detection of xCT mRNA, amyloid plaques, and activated microglia in the cerebral cortex of a 15-month-old TgAPP mouse. **A–C**, Representative fields of the cerebral cortex from a TgAPP mouse. **A**, ISH detection of xCT mRNA. **B**, Congo red staining of amyloid plaques. **C**, Microglia stained with an anti-CD11b antibody (brown peroxidase staining). Inset, Double detection of xCT mRNA (ISH, blue) and reactive microglial cells (anti-CD11b, brown). **D**, Representative field of the cerebral cortex from an age-matched wild-type mouse: ISH does not detect xCT mRNA. Scale bars: **A–D**, 100 μ m; inset, 10 μ m.

microglia. Previous studies have shown that exposure of neurons to A β enhances neuronal responses to glutamate, including the rise of intracellular calcium and subsequent cell death (Koh et al., 1990; Mattson et al., 1992). In our study, sublethal doses of A β did not clearly increase the vulnerability of neurons to exogenous glutamate in the absence of microglia, whereas the neurotoxic effect of glutamate was enhanced when microglia were present in A β -treated cultures. This suggests that other cytotoxic compounds produced by A β -stimulated microglia contribute, together with glutamate, to the death of neurons, in agreement with previous demonstrations of a role for microglia-derived free radicals or cytokines. Our data, however, indicate that the effect of microglia on neurons challenged with A β is strongly determined by system x_c^- -dependent excitotoxicity. Indeed, blocking of either NMDA receptors or system x_c^- not only prevented microglial enhancement of A β cytotoxicity but also permitted efficient microglial protection of neurons against other cytotoxic activities of A β unrelated to excitotoxicity.

The capacity of microglial cells to clear A β deposits by phagocytosis was not required for microglial protection of neurons against A β 1–40, and extracellular degradation of aggregated A β by microglia-derived proteases was not detected. In contrast, microglial secretion of apoE played a major role. Immunodepletion of apoE from the culture medium abrogated the protective activity of microglia, which was restored by the addition of recombinant human apoE2, E3, or E4 to the medium. These observations confirm that human apoE can reduce the neurotoxicity of aggregated A β (Puttfarcken et al., 1997; Pedersen et al., 2000) and provide evidence that microglia potentiate the neuroprotective activity of the three major apoE isoforms.

In cell cultures exposed to soluble forms of A β and treated with purified human apoE, isoform-specific modulations of cell death have been attributed to the formation and receptor-mediated clearance of stable apoE/A β complexes, inhibition of A β binding to cell membranes, or changes in the polymerization of soluble A β into neurotoxic aggregates (Ma et al., 1996; Jordan et al., 1998; Drouet et al., 2001; Lee et al., 2002). How apoE

rescues neurons exposed to aggregated A β remains unclear. The neurotoxicity of aggregated A β has been reported to implicate alterations in calcium homeostasis, expression of apoptosis-related genes, generation of proapoptotic lipid metabolites, and reactive oxygen species (Mattson et al., 1993; Estus et al., 1997; Morishima et al., 2001; Cutler et al., 2004). The protective activities of purified apoE isoforms against A β have been correlated with their antioxidant properties or capacity to bind lipid peroxidation products (Miyata and Smith, 1996; Pedersen et al., 2000). However, the effects of apoE isoforms, and most notably apoE4, are strongly modulated by posttranslational modifications of apoE or by the presence of cofactors that bind to apoE, including lipids, lipoproteins, or neuronal growth factor. ApoE4, in association with purified very light density lipoproteins, increases lysosomal leakage and apoptosis induced by aggregated A β in neuro-2A cell (Ji et al., 2002). Even in the absence of A β , N-terminal apoE4 fragments generated by thrombin cleavage or C terminal-truncated apoE4 secreted by neurons are neurotoxic in themselves (Marques et al., 1996; Huang et al., 2001), whereas native apoE4 bound to CNTF favors the survival of cultured neurons (Gutman et al., 1997).

In our study, culture medium conditioned by microglia (CM) potentiated neuroprotection by the three apoE isoforms, although apoE2 was more protective than apoE3 and apoE4 against A β -induced neuronal death. The microglial factors responsible for enhancement of neuroprotection by apoE remain to be elucidated. However, the persistence of such cofactors in CM that was depleted of apoE content and then extracted with organic solvents argues against a significant contribution of lipid components. A protective role of bFGF (Mark et al., 1997) also seems unlikely, because addition of blocking antibodies against bFGF to apoE-depleted CM had no effect. However, our observations clearly show that microglial production of apoE plays an essential role in the mechanism by which microglia rescue neurons challenged with aggregated A β from death. Indeed, microglia genetically deficient for apoE failed to develop any compensatory mechanisms that even partly restored their neuroprotective capacity.

Altogether, our analyses in cell cultures showed that microglial expression of apoE and system x_c^- plays an important role in the control of A β neurotoxicity. The physiopathological relevance of these observations is supported in part by previous neuropathological investigations in AD or mouse models of the disease in which the accumulation of apoE in senile plaques has been observed (Dickson et al., 1997; Bales et al., 1999; Navarro et al., 2003; Motoi et al., 2004). Although astrocytes and neurons can also produce apoE, microglia appear to be a major source of apoE in senile plaques (Uchihara et al., 1995). However, the expression of system x_c^- in microglia *in vivo* has not been investigated previously. In the normal adult mouse CNS, xCT transcripts are scarce except in diencephalic regions facing the ventricles (Sato et al., 2002). We have shown here that injecting A β into the hippocampus favors the local recruitment of activated microglia expressing the xCT gene and that the AD-like amyloid plaques in TgAPP mice are associated with xCT-expressing microglia. These observations are consistent with a role for the microglial x_c^- antiporter in the neurodegenerative processes triggered by A β . In addition to releasing glutamate, system x_c^- takes up cystine that fuels the intracellular production of the anti-oxidant glutathione, and increases in xCT mRNA expression and system x_c^- activity have been observed in macrophage or fibroblast cultures undergoing oxidative stress (Bannai et al., 1989; Sato et al., 2001; Sasaki et al., 2002). It is therefore possible that the marked xCT expression by microglia associated with amyloid plaques resulted from

the pro-oxidant effects of amyloid deposit previously documented by *in situ* detection of reactive oxygen species in rodent models of AD and in human AD tissue (Weldon et al., 1998; Ishii et al., 2000; McLellan et al., 2003).

Conclusions

This study provides evidence of two molecular mechanisms that determine whether microglial cells enhance or limit the cytotoxicity of aggregated A β . The activity of the glutamate transporter system x_c^- supports the hypothesis that microglia enhance the cytotoxic effect of A β through an excitotoxic mechanism, which has been implicated in the pathogenesis of AD (Hynd et al., 2004). Blocking system x_c^- -mediated glutamate efflux or neuronal NMDA receptors unmasked the microglial secretion of apoE as a new mechanism by which microglia can protect neurons from A β cytotoxicity provided that excitotoxicity is kept under control. This mechanism may partly explain the reported improvement in clinical outcome observed in patients treated with the NMDA receptor antagonist memantine (Reisberg et al., 2003; Farlow, 2004). Our study therefore suggests that inhibition of system x_c^- may promote neuronal survival in patients with AD by two complementary mechanisms. It prevents excitotoxicity without the unwanted side effects of NMDA receptor blockade and at the same time unmasks the neuroprotective potential of microglial apoE and its cofactors. The role of apoE and system x_c^- therefore deserves additional investigation in AD but also other neurodegenerative diseases and pathologies such as stroke or trauma, in which microglia may be friends or foes.

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