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ORIGINAL ARTICLE

ATP and glutamate released via astroglial connexin 43 hemichannels mediate neuronal death through activation of pannexin 1 hemichannels

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Abstract

Inflammation contributes to neurodegeneration in post-ischemic brain, diabetes, and Alzheimer's disease. Participants in this inflammatory response include activation of microglia and astrocytes. We studied the role of microglia treated with amyloid-β peptide (Aβ) on hemichannel activity of astrocytes subjected to hypoxia in high glucose. Reoxygenation after 3 h hypoxia in high glucose induced transient astroglial permeabilization via Cx43 hemichannels and reduction in intercellular communication via Cx43 cell-cell channels. Both responses were greater and longer lasting in astrocytes previously exposed for 24 h to conditioned medium from Aβ-treated microglia (CM-Aβ). The effects of CM-Aβ were mimicked by TNF-α and IL-1β and were abrogated by neutralizing TNF-α with soluble receptor and IL-1β with a receptor antagonist. Astrocytes under basal conditions protected neurons against hypoxia, but exposure to CM-Aβ made them toxic to neurons subjected to a sub-lethal hypoxia/reoxygenation episode, revealing the additive nature of the insults. Astrocytes exposed to CM-Aβ induced permeabilization of cortical neurons through activation of neuronal pannexin 1 (Panx1) hemichannels by ATP and glutamate released through astroglial Cx43 hemichannels. In agreement, inhibition of NMDA or P2X receptors only partially reduced the activation of neuronal Panx1 hemichannels and neuronal mortality, but simultaneous inhibition of both receptors completely prevented the neurotoxic response. Therefore, we suggest that responses to ATP and glutamate converge in activation of neuronal Panx1 hemichannels. Thus, we propose that blocking hemichannels expressed by astrocytes and/or neurons in the inflamed nervous system could represent a novel and alternative strategy to reduce neuronal loss in various pathological states including Alzheimer's disease, diabetes and ischemia.

Keywords: Alzheimer's disease, amyloid β-peptide, connexin, cytokines, diabetes mellitus, gap junctions, pannexin, stroke.

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The most common acute brain insult is ischemic stroke, where transient or permanent reduction in cerebral blood flow deprives the tissue of oxygen and glucose and permits build-up of potentially toxic substances, effects that together lead to rapid or delayed cell death (Dirnagl *et al.* 1999). An association between Alzheimer's disease (AD) and ischemic stroke has been established. Indeed, patients that on autopsy show cerebral infarcts and AD pathology are more cognitively impaired than patients with AD pathology alone (Snowdon *et al.* 1997). Moreover, the presence of high levels

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Abbreviations used: AD, Alzheimer's disease; AU, arbitrary units; BBG, brillian blue G; CM, conditioned medium; CM-Aβ, conditioned medium from Aβ-treated microglia; DM, diabetes mellitus; DMEM, Dulbecco's modified Eagle's medium; FCS, Fetal Calf Serum; F-Jade, Fluoro-Jade C; GFAP, glial fibrillary acidic protein; LY, Lucifer yellow; Panx1, pannexin 1; PBS, phosphate-buffered saline.

of a neurotoxic fragment of amyloid β-peptide (Aβ_{25-35}) in a model of focal cerebral ischemia is associated with increased infarct size, greater inflammation, and more pronounced cognitive deficits (Whitehead *et al.* 2007).

It has long been known that hyperglycemia worsens the outcome of acute brain ischemia by increasing the extent of tissue injury in animals and in humans (Kagansky *et al.* 2001). Interestingly, both AD and hyperglycemic conditions developed during diabetes mellitus (DM) produce persistent inflammation that can cause neuronal death (Pasquier *et al.* 2006; LaFerla *et al.* 2007). More relevant to this point is that DM accelerates memory dysfunction via cerebrovascular inflammation and Aβ deposition in an AD transgenic mouse (Takeda *et al.* 2010). Thus, inflammation seems to be a common factor in the neuronal damage induced by stroke, AD and DM. However, the molecular and/or cellular targets involved in these processes remain to be elucidated.

Microglia are the most sensitive CNS detectors of adverse conditions, including fibrillar Aβ depositions (Block *et al.* 2007). Interestingly, upon stimulation with lipopolysaccharide, microglial cells release TNF-α and IL-1β which reduce intercellular communication via gap junctions and increase hemichannel activity in astrocytes (Retamal *et al.* 2007). More prominent changes are observed in astrocytes exposed to hypoxia in high glucose (Orellana *et al.* 2010). Gap junctions are membrane specializations that provide a direct cytoplasmic pathway between contacting cells by aggregates that contain a few tens to thousands of cell–cell channels, termed gap junction channels (Sáez *et al.* 2003). They are formed by the docking of two hemichannels, one contributed by each contacting cell (Sáez *et al.* 2003). Each hemichannel is formed by oligomerization of six protein subunits termed connexins (Cxs), which are expressed by astrocytes, microglia and neurons (Orellana *et al.* 2009). A more recently described three-member protein family, termed pannexins (Panxs), can also form hemichannels at the cell surface of diverse mammalian cells, including astrocytes and neurons (Thompson *et al.* 2008; Iglesias *et al.* 2009). They are permeable to ATP and are activated by intracellular Ca^{2+} and extracellular ATP acting on P2 receptors (Locovei *et al.* 2006; Pelegrin and Surprenant 2006).

It has been proposed that under pathological conditions the increased hemichannel opening and reduced gap junctional communication in astrocytes deprive neurons of glial protective functions, which could increase neuronal vulnerability and the incidence of neuronal death (Orellana *et al.* 2009). In agreement with this notion, it was recently demonstrated that astroglial hemichannel opening induced by pro-inflammatory cytokines potentiates glutamate-induced neurotoxicity (Froger *et al.* 2010). However, soluble factors released by activated astrocytes that enhance neuronal vulnerability to injuries remain to be identified. The aim of this study was to evaluate if changes in hemichannels and/or gap junction channels of cultured cortical astrocytes exposed to sub-threshold pro-inflammatory conditions *in vitro* are potentiated. In addition, the impact of changes in astroglial hemichannels/gap junction channels and neuronal hemichannels on the viability of cortical neurons was evaluated, while both glutamate and ATP released via astroglial hemichannels were identified as relevant mediators of the observed neuronal death.

Materials and methods

Reagents and antibodies

SuperSignal kit for enhanced chemiluminescence detection, Sulfo-NHS-SS-biotin, and immobilized NeutrAvidin were purchased from Pierce. Gap26 (VCYDKSFPISHVR, first extracellular loop domain of Cx43, Cx32 and Cx26), Gap27 (SRPTEKTIFII, second extracellular loop domain of Cx43, Cx37, Cx32 and Cx26), ^{10}panx1 (WRQAAFVDSY, extracellular loop domain of Panx1), and E1b (SSFSWRQAAFVDS, extracellular loop domain of Panx1) peptides were obtained from NeoMPS, SA. (Straousburg, France). Aβ_{25-35} and Aβ_{35-25} peptide were purchased from Bachem (King of Prussia, PA, USA). HEPES, Dulbecco's modified Eagle's medium (DMEM), H_2O, $LaCl_3$ (La^{3+}), ethidium (Etd) bromide, Lucifer yellow (LY), cytosine arabinoside (Ara-C), glutamate, 3-[(*R*)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid, oATP, suramin, brillian blue G (BBG), apyrase, ATP, anti-MAP-2 monoclonal antibody H-M2 and probenecid were purchased from Sigma-Aldrich (St Louis, MO, USA). Penicillin, streptomycin, isolectin GS-IB4 and goat anti-mouse Alexa Fluor 488 were obtained from Invitrogen (Carlsbad, CA, USA). TNF-α and IL-1β were obtained from Roche Diagnostics (Indianapolis, MI, USA). Proteinase K was purchased from Promega (Madison, WI, USA). Anti glial fibrillary acidic protein (GFAP) monoclonal antibody was purchased from ICN Chemicals, (Irvine, CA, USA). Anti-Cx43 monoclonal antibody was obtained from BD Biosciences (Franklin Lakes, NJ, USA). A soluble form of the TNF-α receptor (sTNF-aR1) and a recombinant receptor antagonist for IL-1β (IL-1ra) were from R&D Systems (Minneapolis, MN, USA). $Cx43^{E2}$ antibody specifically for blocking hemichannels was generated and affinity purified as previously described (Siller-Jackson *et al.* 2008).

Animals

Microglia, neuron and astrocyte cultures were prepared from OF1 mice (Charles River, L'Arbresle, France). In addition, Cx43-deficient astrocytes were obtained from neonatal mice born to $Cx43^{+/-}$ female mice (Reaume *et al.* 1995). Homozygous mutant ($Cx43^{-/-}$) and their wild-type control ($Cx43^{+/+}$) neonatal mice were the product of mating between heterozygous $Cx43^{+/-}$ mice. Genotyping was performed from a tissue sample, using PCR analysis, as previously described (Naus *et al.* 1997). All experiments were carried out in accordance with the European Community Council Directives of November 24, 1986 (86/609/EEC) and all efforts were made to minimize the number of animals used and their suffering.

Cell cultures

Astrocyte cultures

Primary astrocyte cultures were prepared from the cortex of newborn OF1 mice. Briefly, the brains were removed, and the cortices were dissected. Meninges were carefully peeled off and tissue was mechanically dissociated. Cells were seeded into 100-mm diameter plastic dishes (Nunc, Roskilde, Denmark) at a density of 3×10^6 cells/dish or into 60 mm diameter plastic dishes at a density of 2×10^6 cells/dish in DMEM, supplemented with penicillin (5 U/mL), streptomycin (5 μg/mL), and 10% Fetal Calf Serum (FCS). Alternatively, cells were seeded on glass coverslips (Gassalem, Limeil-Brevannes, France) placed inside 16-mm diameter 24-well plastic plates (NunClon) at the density of 1×10^5 cells/well in the same culture conditions. After 8–10 days, when cells had reached confluence, 1 μM of cytosine-arabinoside was added to the culture medium for 3 days to eliminate proliferating microglial cells. At that stage, these cultures contained > 95% GFAP+ cells and > 95% S100β+ cells. No neurons were detected as judged by MAP-2 staining. At the end of these experiments, cell cultures were stained with DAPI to quantify the total number of astrocytes per culture.

$Cx43^{-/-}$ and $Cx43^{+/+}$ astrocyte cultures. $Cx43^{-/-}$ and $Cx43^{+/+}$ astrocyte cultures were prepared from the cortex of $Cx43^{-/-}$ and wild-type mice, as described for OF1 mice. The mouse genotype was determined by PCR analysis as described previously (Naus *et al.* 1997).

Microglial cultures, astrocyte-microglia co-cultures, and conditioned media

After dissociation, astroglial cells were seeded into 100-mm diameter culture dishes (NunClon) at 3×10^6 cells/10 mL/dish in DMEM, containing 10% heat-inactivated FCS (Abcys, Paris, France). The medium was changed at 1 and 3 DIV, and microglia were collected at 10 DIV by shaking the culture dishes to detach cells adherent to the astrocyte monolayer. The collected population resulted in > 98% of cells bearing the Mac-1 antigen, a specific marker of macrophage cells. Freshly collected microglia were either seeded on confluent astrocytes (astrocyte-microglia co-cultures, 3×10^4 cells/16 mm wells) or cultured to generate conditioned medium (CM). Co-cultures were maintained for 24 h in DMEM containing 5% FCS and then treated (or not for control) for another 24 h. Immunostaining with astrocyte and microglia markers (GFAP and isolectin B4, respectively) indicated that astroglial cultures contained 98.9 ± 0.2 astrocytes (GFAP-positive) and 1.1 ± 0.1% microglia (isolectin B4-positive) ($n = 3$), whereas astrocyte-microglia co-cultures contained 81.5 ± 0.1% astrocytes and 18.5 ± 0.5% microglia ($n = 3$).

To obtain CM from microglia, freshly collected microglia were seeded in DMEM containing 5% FCS (1.7×10^6 cells/mL/dish in 35 mm dishes) and treated with 10 μM $A\beta_{25-35}$ (CM-$A\beta_{25-35}$) for 24 h. CM of non-activated microglia was obtained from sister cultures, and effects of activated and non-activated CM on astrocyte cultures were compared. In addition, to obtain CM from astrocytes (CM-Ast), astrocytes were treated for 24 h with CM-$A\beta_{25-35}$ and then exposed to 3 h of hypoxia in fresh medium containing 5 mM glucose followed by 1 h of reoxygenation in fresh medium. The final supernatants from treated microglia and astrocytes were collected, filtered (0.22 μm), and stored at −20°C before use.

Neurons and astrocyte-neuron co-cultures

Neuron-astrocyte co-cultures were obtained by plating cell suspensions dissociated from E16 mouse cerebral cortex (5×10^4 cells/coverslip) on 3-week-old astrocyte monolayer in MEM containing 5% horse serum and 5% FCS. After 24 h, the medium was replaced by one containing 2×10^{-5} M 5′-fluoro-2-deoxyuridine + uridine (10^{-5} M), insulin (5 μg/mL), pyruvate (110 μg/mL), 5% horse serum and 1% Ultroser-G, a serum substitute (Pall-Biosepra). Partial medium changes (1/4) were performed twice a week. In this medium, astrocytes are healthy, neuronal cells differentiate, and potentially dividing cells (neural progenitors and/or microglial cells) are killed because of the continuous presence of an anti-mitotic agent. Enriched neuronal cultures were switched to the co-culture medium and submitted to the same medium changes as their sister co-cultures.

Cell treatments

Some astrocyte-microglia co-cultures were treated for 24 h with 10 μM $A\beta_{25-35}$ and then used for experiments. Astrocyte or neuron cultures and astrocyte-neuron co-cultures were treated with either CM-$A\beta_{25-35}$ (diluted four times at the final concentration) or the mixture of cytokines TNF-α plus IL-1β (10 pg/mL of each) for 24 h and then exposed to an *in vitro* hypoxia model in the presence of normal or high glucose. Briefly, astrocyte-microglia and astrocyte-neuron co-cultures or highly enriched astrocytes and neuron cultures were subjected to 3 h of hypoxia in ischemic brain solution (in mM: 51 NaCl, 65 K-gluconate, 0.13 $CaCl_2$, 1.5 $MgCl_2$, 10 HEPES, and pH 6.8) (Orellana *et al.* 2010), containing normal (5 mM) or high (27 mM) glucose concentrations. Hypoxia was induced as described before (Orellana *et al.* 2010). In brief, cell cultures were kept inside a chamber with the air removed by a CO_2/N_2 flow for 7 min and maintaining the chamber closed for 3 h. Then, oxygenation was restored, and the medium replaced with normal medium. In hypoxic protocols in 5 mM glucose, 22 mM sucrose was added to achieve the same osmolarity as the high, 27 mM glucose medium. Connexin hemichannel blockers, La^{3+} (200 μM) and synthetic peptides, Gap26 and Gap27 (200 μM), were co-applied with Etd for uptake measurements. In other experiments, Panx1 hemichannel blockers, ^{10}panx1 (200 μM), E1b (200 μM) and probenecid (200 μM), were applied similarly. In some experiments, CM-Aβ was preincubated (2 h) with 100 ng/mL sTNF-aR1, a soluble form of the receptor that binds TNF-α, and/or 100 ng/mL IL-1ra, an IL-1β receptor blocker were applied before the addition of CM to astrocyte cultures.

Scrape loading/dye transfer technique

Gap junction permeability was evaluated at 24°C using the scrape-loading/dye transfer technique, on either astrocyte cultures or microglia-astrocyte co-cultures. Briefly, cultures were washed for 10 min in HEPES-buffered salt solution containing the following (in mM): 140 NaCl, 5.5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 10 HEPES, pH 7.4 followed by washing in a Ca^{2+}-free HEPES solution for 1 min. Then, a razor blade cut was made in the monolayer in a HEPES-buffered salt solution with normal Ca^{2+} concentration containing the fluorescent dye LY. After 1 min, LY (100 μM) was washed out several times with HEPES-buffered salt solution. Eight minutes after scraping, fluorescent images were captured using an inverted fluorescent microscope equipped for epifluorescence (Diaphot-Nikon, Tokyo, Japan). For each trial, data were quantified by measuring fluorescence areas in five representative fields using an image analyzer system (Lucia-Nikon, Tokyo, Japan). Quantification of changes in gap junctional communication induced by different treatments was performed by measuring the fluorescence area, expressed as arbitrary units (AU).

Dye uptake

For single image visualization of dye uptake, astrocytes were exposed to 5 μM Etd for 10 min at 37°C with Hank's Buffered Salt Solution (HBSS in mM: 137 NaCl, 5.4 KCl, 0.34 Na_2HPO_4, 0.44 KH_2PO_4, pH 7.4) with 1.2 mM $CaCl_2$ (HBSS-Ca^{2+}), mounted in Fluoromount, and examined by epifluorescence (518 nm excitation and 605 nm emission) using an inverted microscope (Diaphot-Nikon) equipped with a CCD camera (Nikon). Captured images were analyzed with image analyzer software (Lucia-Nikon) and the NIH ImageJ program.

For time lapse fluorescence imaging, fluorescence of cells bathed with HBSS-Ca^{2+} containing 5 μM Etd was recorded every 30 s using a Olympus BX 51W1I microscope. To test for changes in slope, regression lines were fitted to points before and after various treatments using Microsoft (Seattle, WA, USA) Excel, and mean values of slopes were compared using Graphpad Software (San Diego, CA, USA).

Biotinylization

Confluent cultures in 100 mm diameter dishes were washed three times with HBSS-Ca^{2+}. Three milliliters of Sulfo-NHS-SS-biotin (0.5 mg/mL dissolved in HBSS-$Ca^{2+)}$ was added to cultures, which were then incubated for 30 min at 4°C. Cells were washed three times with HBSS-Ca^{2+} solution plus 15 mM glycine (pH 8.0), to quench unreacted biotin, and harvested by scraping with a rubber policeman in the presence of protease inhibitors (200 μg/mL soybean trypsin inhibitor, 1 mg/mL benzamidine, 1 mg/mL ε-aminocaproic acid, and 2 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (see below, western blot analysis). Then, cells were pelleted and lysed by sonication in 50 μL of ice cold solution containing proteases and phosphatases inhibitors. NeutrAvidin was added to the samples (1 μL of NeutrAvidin solution per 3 μg of biotinylated protein, based on the assumption that 40% of total membrane protein was biotinylated), and the mixture was maintained for 1 h at 4°C. One milliliter of binding buffer (HBSS, pH 7.2, plus 0.1% sodium dodecyl sulfate and1% NP-40) was added, mixed by vortexing, and centrifuged for 2 min at 1 957 × g at 4°C, and the supernatant was removed. This wash procedure was repeated three times. After the final wash, 40 μL of HBSS, pH 2.8 (to release the protein from the avidin) plus 0.1 M glycine was added to the pellet, which was resuspended and centrifuged at 1 957 × g for 2 min at 4°C. The supernatant was removed and placed in a 1.5 mL Eppendorf (Westbury, NY, USA) tube, and pH was adjusted to 7.4 immediately by adding 10 μl of 1 M Tris, pH 7.4. Relative Cx43 levels present in each sample were measured by western blot analysis (see below).

Western blot analysis

Cultures were rinsed twice with phosphate-buffered saline (PBS), pH 7.4, and harvested by scraping with a rubber policeman in ice solution containing protease and phosphatase inhibitors (1 mM orthovanadate, 10 mM α-glycerophosphate) and complete miniprotease inhibitor (Roche Diagnostics). Pelleted cells were resuspended in 40 μL of the protease and phosphatase inhibitor solution, placed on ice, and lysed by sonication (Ultrasonic cell disrupter, Microson, Ultrasons, Annemasse, France). Proteins were measured in aliquots of cell lysates with the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA). Samples were stored at −80°C or analyzed by immunoblotting. Aliquots of cell lysates (50 μg of protein) or biotinylated surface membrane proteins were resuspended in a final concentration of 1× Laemli's sample buffer, boiled for 5 min, separated on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose sheets as described previously (Orellana *et al.* 2010). Non-specific protein binding was blocked by incubation of nitrocellulose sheets in PBS-BLOTTO (5% non-fat milk in PBS) for 30 min, and then blots were incubated with primary monoclonal antibody for 1 h at 24°C or overnight at 4°C, followed by four 15 min PBS washes. Blots were incubated with goat anti-mouse antibody conjugated to horseradish peroxidase. Immunoreactivity was detected by enhanced chemiluminescence detection using the SuperSignal kit (Pierce, Rockford, IL, USA) according to the provider instructions.

Immunofluorescence and confocal microscopy

For all immunostaining experiments, cells grown on coverslips were fixed at 24°C with 2% paraformaldehyde for 30 min and then washed three times with PBS. Then, they were sequentially incubated in 0.1 M PBS-glycine, three times for 5 min each, and then in PBS-0.1% Triton X-100 containing 10% Normal Goat Serum for 30 min. To identify astrocytes and microglia, we used a specific molecular marker for each one (GFAP antibody and isolectin B4, respectively). We first incubated cells for 2 h at 24°C with anti-GFAP monoclonal antibody (IgG1, 1 : 500) diluted in 0.1% PBS-Triton X-100 with 2% Normal Goat Serum. After three rinses in 0.1% PBS-Triton X-100, cells were then incubated for 50 min at room temperature with both goat anti-mouse Alexa Fluor 355 (1 : 1500) and isolectin GS-IB4 (1 : 100), diluted in the same solution as the first antibody. To identify neurons an anti-MAP-2 monoclonal antibody (1/500) was used following the same protocols mentioned above. After several washes, coverslips were mounted in Fluoromount and examined with an upright microscope equipped with epifluorescence (Eclipse E800, Nikon). To visualize double immunostaining, a confocal laser-scanning microscope (TBCS SP2; Leica, Wetzlar, Germany) was used. Stacks of consecutive confocal images taken with a 63 × objective at 500 nm intervals were acquired sequentially with two lasers (argon 488 nm and helium/neon 543 nm), and Z projections were reconstructed using the Leica confocal software.

Measurement of ATP and glutamate release

Astrocytes were plated in multi well culture trays (10^6 cells/well/0.5 mL) and 48 h after later were used for experiments. Extracellular ATP was measured by luciferin/luciferase bioluminescence assay kit (Sigma-Aldrich). Levels of extracellular glutamate were determined using an enzyme-linked fluorimetric assay as described by Genever *et al.* (Genever and Skerry 2001). In the presence of glutamate dehydrogenase and $NADP^+$, glutamate is oxidized to α-ketoglutarate, yielding NADPH, which can be determined fluorimetrically (excitation and emission wavelengths of 355 and 460 nm) to provide an indirect quantification of glutamate concentration.

For each assay, standard curves were constructed by using known concentrations of ATP or glutamate. The concentration of ATP and glutamate in samples of extracellular medium were calculated from standard curves and referred to 10^6 cells. The fraction of ATP or glutamate released by cells to the extracellular milieu was estimated by the difference between the concentration detected in the medium of cells under resting conditions and the concentration measured after stimulation in the presence or absence of hemichannel inhibitors.

Neuronal death quantification

Neuronal death was measured as the fraction of Fluoro-Jade C (F-Jade) positive cells as described previously (Noraberg *et al.* 1999; Schmuck and Kahl 2009). For F-Jade measurements the cell culture were fixed in cold ethanol (4°C) for 10 min. Cells were then treated with detergent (0.3% Triton X-100 in PBS, Sigma, Deisenhofen, Germany) for 10 min and washed twice with distilled water. F-Jade is stable in a stock solution in distilled water (0.01%), which can be stored at 4°C. The final concentration for staining was 0.001% in distilled water. The cells were covered with the dye and gently shaken for 30 min in the dark. The dye was then removed from the cell cultures, the cells were washed, and fluorescence was determined with an upright microscope equipped with epifluorescence (Eclipse E800, Nikon).

Data analysis and statistics

For each data group, results were expressed as mean ± SE, and n refers to the number of independent experiments. For statistical analysis, each treatment was compared with its respective control, and significance was determined using a one-way ANOVA followed by a Tukey *post hoc* test. For multiple group treatments, significance was determined using a two-way ANOVA followed by a Bonferroni *post hoc* test.

Results

Aβ-treated microglia potentiate the changes in connexin-based channels induced by hypoxia/reoxygenation in cortical astrocytes

Recently, it was reported that 3 h of hypoxia in high glucose induces a transient increase in Cx43 hemichannel activity and decrease in gap junctional communication in astrocytes during reoxygenation (Orellana *et al.* 2010). To evaluate if microglia and other pro-inflammatory agents could potentiate these effects on Cx based channels, astrocytes alone or in co-culture with microglia were treated with a fragment of Aβ peptide (Aβ$_{25\text{-}35}$) previously shown to be toxic for neurons and astrocytes (Pike *et al.* 1995; Assis-Nascimento *et al.* 2007); this fragment and retains most of neurotoxic and pro-inflammatory effects of Aβ [1–42] peptide found *in vivo* (Yankner *et al.* 1990; Meda *et al.* 1995). To examine hemichannel and gap junction channel activities Etd uptake and scrape loading/LY transfer technique were employed, respectively. As reported previously (Orellana *et al.* 2010), control astrocytes exhibited a low Etd uptake (Figure S1a) and high LY intercellular diffusion (Figure S2a). However, astrocytes subjected to 3 h hypoxia in high glucose (27 mM) showed at 1 h reoxygenation a large increase in Etd uptake (743.5 ± 57.5% normalized to control; $n = 4$) (Fig. 1a and Figure S1b) and decrease in LY diffusion (53.7 ± 9.4% normalized to control; $n = 4$) (Fig. 2a and Figure S2b). In contrast, normal glucose (5 mM) during hypoxia did not induce the effects mentioned above (Fig. 1a and 2a). The changes in Etd uptake and LY diffusion induced by hypoxia in high glucose were abolished in astrocytes co-cultured for 24 h with resting microglia (Fig. 1b and 2b and Figures S1e and 2e). However, astrocytes co-cultured with microglia for 24 h in the presence of 10 μM Aβ$_{25\text{-}35}$ showed a prominent increase in Etd uptake (Fig. 1c and Figure S1h) and decrease

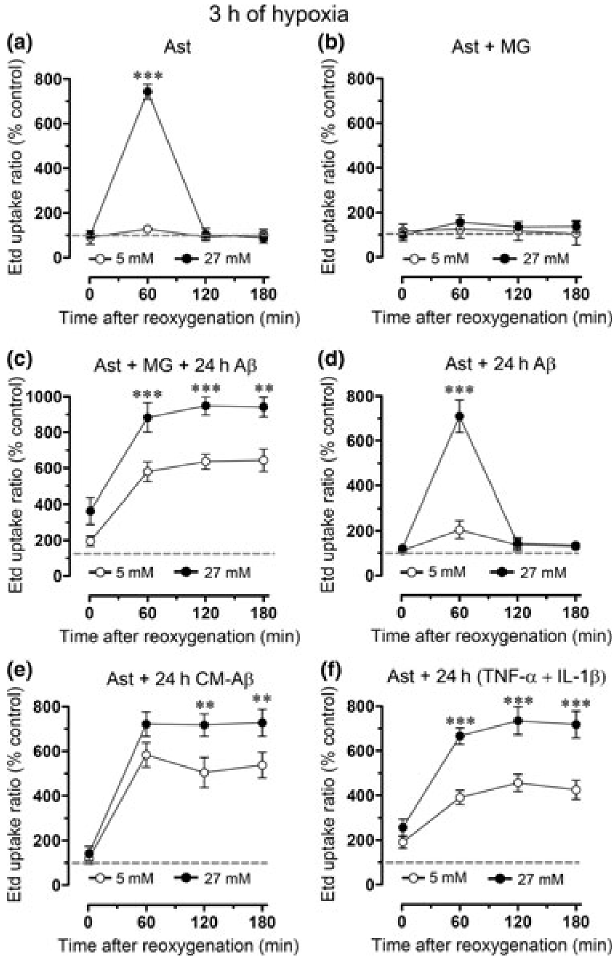

Fig. 1 Aβ$_{25\text{-}35}$-treated microglia potentiate astroglial Etd uptake induced by hypoxia in high glucose. (a–f) Averaged data normalized to control (dashed line) of Etd uptake by astrocytes alone (Ast) or co-cultured for 24 h with microglia (MG) in presence or absence of 10 μM Aβ$_{25\text{-}35}$ (Aβ) and then exposed to 3 h hypoxia in 5 mM (○) or 27 mM (●) glucose followed by several periods of reoxygenation. Shown is the Etd uptake of astrocyte pre-treated for 24 h with 10 μM Aβ$_{25\text{-}35}$ or with conditioned media from microglia exposed for 24 h to 10 μM Aβ$_{25\text{-}35}$ (CM-Aβ) and then subjected to hypoxia/reoxygenation. Also shown is the Etd uptake of astrocytes pre-treated for 24 h with TNF-α and IL-1β (10 pg/mL of each). ** $P < 0.005$, *** $P < 0.001$, (●) versus (○) at each time point. Each value corresponds to mean ± SE of four independent experiments.

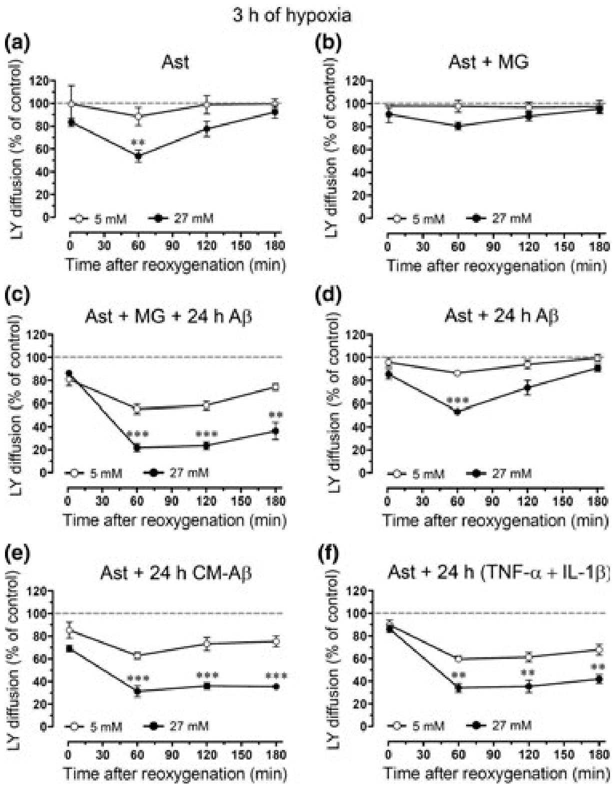

Fig. 2 $A\beta_{25\text{-}35}$-treated microglia potentiate the reduction of astroglial coupling induced by hypoxia in high glucose. (a–f) Averaged data normalized to control (dashed line) of area of LY diffusion in astrocytes alone (Ast) or astrocytes co-cultured for 24 h with microglia (MG) in presence or absence of 10 μM $A\beta_{25\text{-}35}$ (Aβ) and then exposed to 3 h hypoxia in 5 mM (○) or 27 mM (●) glucose followed by several periods of reoxygenation. Shown is the LY diffusion between astrocytes pre-treated for 24 h with 10 μM $A\beta_{25\text{-}35}$ or with conditioned media from microglia exposed for 24 h to 10 μM $A\beta_{25\text{-}35}$ (CM-Aβ) and then subjected to hypoxia/reoxygenation. Also shown is the LY diffusion between astrocytes pre-treated for 24 h with TNF-α and IL-1β (10 pg/mL of each).***P* < 0.005, ****P* < 0.001, (●)versus (○) at each time point. Each value corresponds to mean ± SE of four independent experiments.

in LY diffusion (Fig. 2c and Figure S2h) approaching a plateau at ~1 h reoxygenation that persisted for at least 3 h (Figures S1i and S2i). These responses were substantially greater with higher than normal glucose for Etd uptake (at 1 h of reoxygenation 882.8 ± 142.1% and 579.8 ± 95.1%, respectively normalized to control; *n* = 4) and LY diffusion (55.3 ± 7.4% and 21.8 ± 6.4%, respectively, normalized to control; *n* = 4). It is noteworthy that the above-mentioned responses in Etd uptake and LY diffusion were not produced by the inverted sequence of toxic Aβ ($A\beta_{35\text{-}25}$, not shown).

These actions of $A\beta_{25\text{-}35}$ required the presence of microglia because they were not observed in astrocytes treated with $A\beta_{25\text{-}35}$ alone (Figs 1d and 2d). Supporting this view, astrocytes incubated for 24 h with conditioned medium from

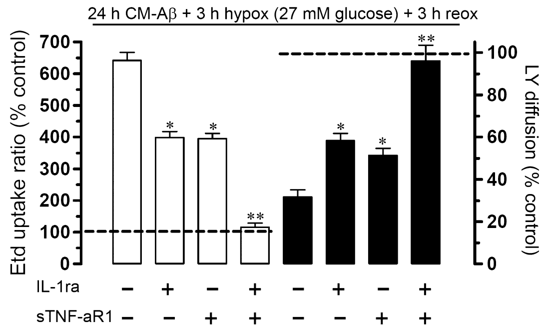

Fig. 3 TNF-α and IL-1β account entirely for the changes in astroglial hemichannels and gap junctions induced by CM-Aβ. Astrocyte cultures were treated for 24 h with CM-Aβ followed by 3 h hypoxia in 27 mM glucose and 3 h reoxygenation. In some experiments, the CM-Aβ treatment was in the presence of 100 ng/mL of IL-1ra, a recombinant antagonist of the IL-1β receptor, or of 100 ng/mL of sTNF-aR1, a soluble form of the TNF-α receptor that binds TNF-α. Graphs of Etd uptake (left, white bars) and the area of LY diffusion after scrape loading (right, black bars) normalized to their respective controls (dashed lines). **P* < 0.05, ***P* < 0.005, effect of the respective antagonist compared to treatment effect (CM-Aβ + hypoxia). Each value corresponds to mean ± SE of four independent experiments.

$A\beta_{25\text{-}35}$-treated microglia (CM-Aβ) and then exposed to hypoxia in high glucose exhibited elevated Etd uptake (at 1 h of reoxygenation 722.4 ± 94.1%, normalized to control; *n* = 4) (Fig. 1e) and decreased LY diffusion (at 1 h of reoxygenation 31.2 ± 8.5% normalized to control; *n* = 4) (Fig. 2e). Interestingly, similar results were observed at 1 h reoxygenation on Etd uptake (666.1 ± 63.8% normalized to control; *n* = 4) (Fig. 1f) and LY diffusion (34.1 ± 6.8% normalized to control; *n* = 4) (Fig. 2f) in astrocytes pre-treated for 24 h with TNF-α and IL-1β (10 pg/mL). These data suggest TNF-alpha and IL-1beta as candidates to mediate the effect of $A\beta_{25\text{-}35}$-treated microglia on astroglial hemichannels and gap junction channels during reoxygenation. To address this hypothesis, we evaluated whether sTNF-aR1 (soluble form of the TNF-α receptor that binds TNF-α) and IL-1ra (recombinant receptor antagonist for IL-1β) affect the above-mentioned responses. Treatment with sTNF-aR1 and IL-1ra for 24 h prior to hypoxia in high glucose completely prevented the increase in Etd uptake (from 641.7 ± 44.7% to 115.9 ± 21.9% normalized to control; *n* = 3) and the reduction in LY diffusion (from 31.7 ± 5.8% to 95.6 ± 16.8% normalized to control, *n* = 3) induced by CM-Aβ during reoxygenation (Fig. 3). When these agents were applied alone (each at 100 ng/mL), partial prevention was observed (Fig. 3).

Notably, protocols that produced persistent (CM-Aβ or TNF-α/IL-1β) increase in Etd uptake (Fig. 4a) and reduction in intercellular LY diffusion (not shown) during reoxygenation did not generate similar responses when applied alone (24 h before hypoxia). Thus, treatments for 24 h with CM-Aβ or lower TNF-α/IL-1β concentrations were unable to

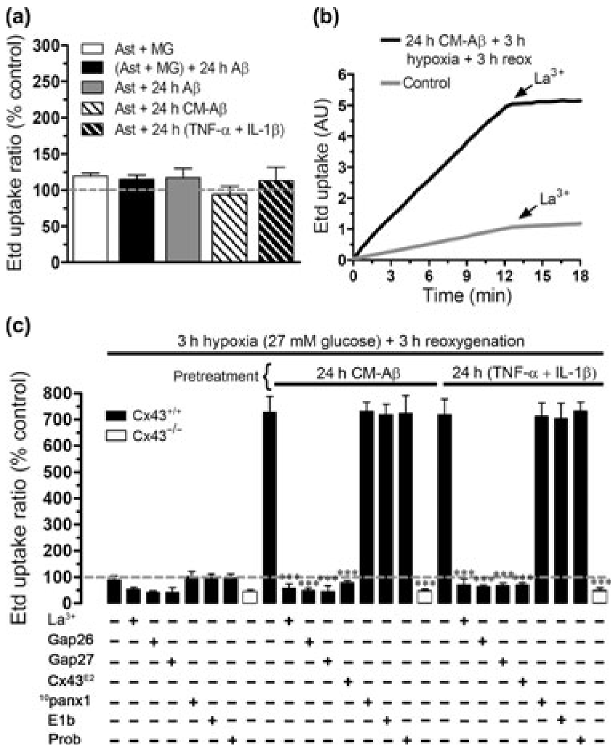

Fig. 4 Increase in astroglial uptake induced by $A\beta_{25-35}$-treated microglia is mediated through Cx43 hemichannels. (a) Averaged data normalized to control (dashed line) of Etd uptake by astrocytes (Ast) co-cultured for 24 h with microglia (MG) without (white bar) or with 10 μM $A\beta_{25-35}$ (black bar). Also shown is the Etd uptake by astrocytes treated for 24 h with 10 μM $A\beta_{25-35}$ (gray bar), with CM-Aβ (lighter cross-hatched bar) or 10 pg/mL each of TNF-α + IL-1β (darker cross-hatched bar). (b) Time-lapse measurements of Etd uptake in astrocytes under control conditions (gray line) or exposed for 24 h to CM-Aβ (black line) and then subjected to 3 h of hypoxia in 27 mM glucose followed by 3 h of reoxygenation. (c) Graphs representing Etd uptake normalized to control (dashed line) by astrocytes pretreated or not (left) for 24 h with CM-Aβ (middle) or 10 pg/mL of TNF-α + IL-1β (right) and then subjected to 3 h of hypoxia in 27 mM glucose followed by 3 h reoxygenation. For each group is shown the effect on dye uptake of the connexin hemichannel blockers La^{3+} (200 μM), Gap26 (200 μM), Gap27 (200 μM), or $Cx43^{E2}$ (1 : 500 dilution); or pannexin hemichannel blockers ^{10}panx1 (200 μM), Eb1 (200 μM) or probenecid (1 mM). All blockers were used co-incubated with Etd. Moreover, $Cx43^{-/-}$ astrocytes (open bars) subjected to the above-mentioned protocols exhibited low uptake like that of wild type cells treated with connexin blockers. ***$P < 0.001$, compared with the respective treatment. Each value corresponds to mean ± SE of four independent experiments.

change hemichannel activity by themselves, indicating that hypoxia/reoxygenation is necessary to observe an increase in hemichannel activity. Finally, the decrease in intercellular LY diffusion was not due to LY leakage through astroglial hemichannels, since similar results were observed in experiments performed in the absence and presence of La^{3+} (200 μM), a connexin hemichannel blocker (not shown).

To investigate the identity of the astroglial pathway mediating the CM-Aβ-induced Etd uptake during reoxygenation, we examined the effect of several connexin hemichannel blockers. In 'time-lapse' experiments, the CM-Aβ-induced Etd uptake during reoxygenation was rapidly blocked by 200 μM La^{3+} (from 0.62 ± 0.02 to 0.05 ± 0.008 AU/min, $n = 4$) (Fig. 4b) and by 200 μM Gap26 or 200 μM Gap27 in 'snapshot' experiments (from 727.7 ± 104.1% to 48.9 ± 15.6% or 44.5 ± 38.8%, respectively; $n = 5$) (Fig. 4c); Gap26 and Gap27 are mimetic peptides of the first and second extracellular loop of Cx43 hemichannels, respectively (Evans and Leybaert 2007). Moreover, to elucidate more specifically the contribution of Cx43 hemichannels in this response, we employed an antibody directed to the second extracellular loop of Cx43 ($Cx43^{E2}$), which block specifically Cx43 hemichannels, but not Cx43 gap junction channels (Siller-Jackson *et al.* 2008). We found that this antibody completely inhibited the CM-Aβ-induced Etd uptake (from 727.7 ± 104.1% to 78.9 ± 12.1%; $n = 3$) (Fig. 4c). As expected, the Etd uptake induced by low concentrations of TNF-α and IL-1β during reoxygenation was blocked as well by the above-mentioned blockers (Fig. 4c). As it has been shown that astrocytes express functional Panx1 hemichannels *in vitro* (Iglesias *et al.* 2009), we studied their possible contribution on the CM-Aβ-induced Etd uptake observed during reoxygenation. For this purpose, we used two mimetic peptides of the second extracellular loop of Panx1 (^{10}panx1 and E1b) and probenecid, which blocks Panx1 hemichannels (Pelegrin and Surprenant 2006). ^{10}panx1 (200 μM), E1b (200 μM), or probenecid (1 mM), failed to reduce the Etd uptake elicited during reoxygenation in astrocytes pre-treated with CM-Aβ (from 727.7 ± 104.1% to 731.4 ± 62.3%, 718.1 ± 71.3% and 723.2 ± 117.9%, respectively; $n = 5$) or TNF-α and IL-1β (from 718.2 ± 103.9% to 712.1 ± 90.3%, 703.8 ± 101.4% and 732.1 ± 60.1%, respectively; $n = 5$) (Fig. 4c**).** Furthermore, protocols that induced Etd uptake in wild-type astrocytes did not induce these responses in astrocytes cultured from $Cx43^{-/-}$ mice (44.2 ± 12.9% normalized to control, $n = 3$) (Fig. 4c), indicating that astroglial Etd uptake occurred largely if not exclusively through Cx43 hemichannels.

We have previously demonstrated that 3 h hypoxia in high glucose followed by reoxygenation causes a transient increase in surface astroglial Cx43 hemichannels that could explain the transient increase in Etd uptake observed under these conditions (Orellana *et al.* 2010). Both changes were transient and showed maximal values at 1 h of reoxygenation, but were back to normal at 3 h of reoxygenation and were not modified by reoxygenation in cells exposed to 3 h hypoxia in normal glucose (Orellana *et al.* 2010). To study if a similar association might occur in the present study, we determined the effect of CM-Aβ on total and surface levels of Cx43 during reoxygenation. Comparable levels of total Cx43 were detected in astrocytes under control conditions or after treatment for 24 h with CM-Aβ and then exposed to 3 h of hypoxia in normal or high glucose followed by 3 h reoxygenation (113.2 ± 14.8% or 95.4 ± 13.2%, respectively, normalized to control; *n* = 3) (Fig. 5a and c). Moreover, surface levels of Cx43 were comparable to control levels at 3 h reoxygenation after the hypoxia period in high or normal glucose at (117.4 ± 8.4% and 105.4 ± 10.8%, respectively, normalized to control; *n* = 3) (Fig. 5b and c). However, pre-treatment with CM-Aβ for 24 h followed by hypoxia in normal or in high glucose induced a prominent increase in surface levels of Cx43 at 3 h reoxygenation (319.2 ± 33.9% and 306.5 ± 36.7%, respectively, normalized to control; *n* = 3) (Fig. 5b and c), suggesting summation of stimuli (hypoxia in low glucose and CM-Aβ).

Astroglial Cx43 hemichannel activity induced by Aβ-treated microglia promotes neuronal death by opening neuronal Panx1 hemichannels

To explore whether astrocytes can potentiate neuronal vulnerability via hemichannels, astrocytes alone or co-cultured with neurons were treated with CM-Aβ and then subjected to hypoxia in normal glucose. Firstly, we evaluated if 3 h hypoxia induce cell death in neuronal cultures. Under control conditions a small amount of neuronal death was observed by F-Jade staining (5.9 ± 2.9% normalized to total cells, *n* = 4) (Fig. 6a), whereas after hypoxia in normal glucose neuronal death was prominently increased only after 24 h reoxygenation (49.9 ± 10.6% normalized to total cells, *n* = 4) (Fig. 6a). Importantly, this death was associated with beading of neuronal processes, but not with Etd uptake (Figure S3c), suggesting that this was a process independent of hemichannel activity. In contrast, the above-mentioned protocol did not decrease neuronal viability and change morphology at 24 h reoxygenation when neurons were co-cultured with astrocytes (6.9 ± 1.9% normalized to total cells, *n* = 5) (Fig. 6a and Figure S3i), indicating that under these conditions astrocytes were neuroprotective. Interestingly, when neurons were co-cultured with astrocytes in the presence of CM-Aβ for 24 h a significant increase in neuronal death (66.8 ± 6.2% normalized to total cells, *n* = 4) (Fig. 6a) and neuronal Etd uptake (Figure S3k) was observed at 1 h reoxygenation. Nevertheless, neurons treated for 24 h with CM-Aβ presented similar Etd uptake (Figure S3f) and neuronal death (51.1 ± 12.7% normalized to total cells, *n* = 4) (Fig. 6a and Figure S3f) than neurons not treated with CM-Aβ (Fig. 6a and Figure S3c). Importantly, Gap26 decreased the CM-Aβ-induced neuronal death (11.9 ± 8.2% normalized to total cells, *n* = 4) (Fig. 6a) and Etd uptake in neurons and astrocytes at 1 h reoxygenation (Figure S3n and o). The neuroprotective effect of Gap26 is probably caused by its blocking effect on astroglial hemichannels because it did not affect the reduction in astroglial dye coupling induced by hypoxia/reoxygenation in high glucose (62 ± 4.2% and 61.2 ± 5.1%, respectively, normalized to control, *n* = 3) (Fig. 6b). Further indication of the role of astroglial Cx43 hemichannels in neuronal death was obtained by blocking these channels with a specify antibody.

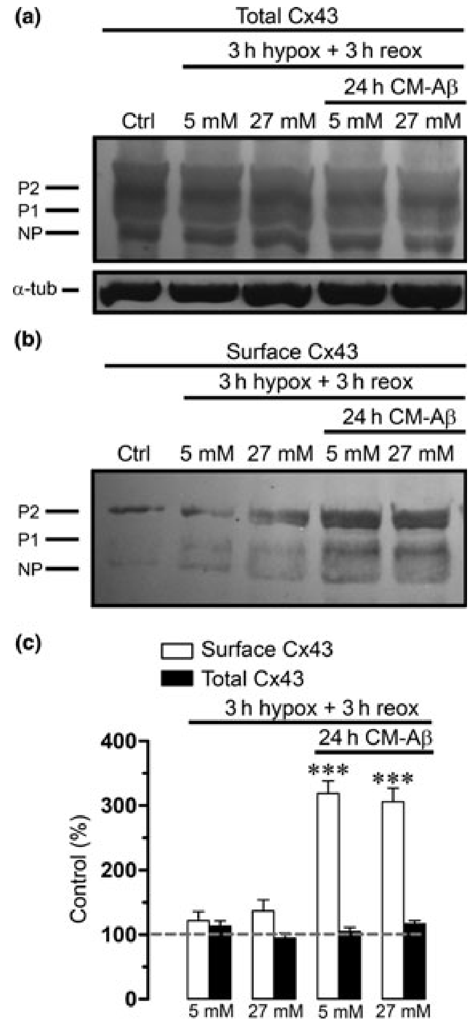

Fig. 5 $A\beta_{25\text{-}35}$-treated microglia induces increase in surface levels of Cx43 after hypoxia. (a, b) Astrocytes cultures were controls or were subjected to 3 h hypoxia in 5 or 27 mM glucose followed by 3 h reoxygenation. Other cultures were pre-incubated for 24 h in CM-Aβ and then subjected to 3 h hypoxia in 5 or 27 mM glucose followed by 3 h reoxygenation. Levels of total Cx43 and surface Cx43 isolated by biotinylation were measured by western blot analysis. (a) Western blot of total Cx43 present in homogenates. None of the treatments affected quantities of the phosphorylated (P1-P2) and non-phosphorylated (NP) forms of Cx43 (markers on the left). (b) Western blot of surface Cx43 from astrocytes under the same conditions. (c) Quantification of surface and total Cx43 normalized to the control in the treatments mentioned above. ***$P < 0.001$, compared with control. Each value corresponds to mean ± SE of at least three independent experiments.

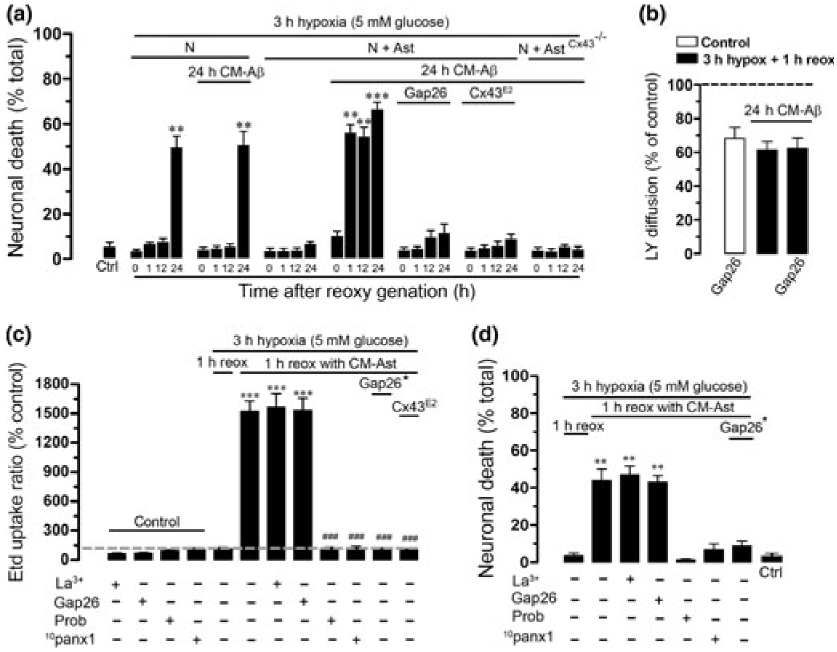

Fig. 6 Astroglial Cx43 hemichannel activity induced by $A\beta_{25\text{-}35}$-treated microglia followed by hypoxia/reoxygenation accelerates neuronal death caused by opening of neuronal Panx1 hemichannels. (a) Cell death was monitored as percent of neurons positive to F-Jade staining. Neurons alone (N), or co-cultured with astrocytes (N + A) were treated or untreated with CM-Aβ for 24 h and then subjected to 3 h hypoxia in 5 mM glucose followed by several periods of reoxygenation (0, 1, 12 or 24 h). In some experiments, 200 μM Gap26 or $Cx43^{E2}$ (1 : 500 dilution) was applied during the reoxygenation period. Also it is shown data from neurons co-cultured with astrocytes from $Cx43^{-/-}$ mice. (b) LY diffusion (normalized to control) by astrocytes incubated for 1 h with 200 μM Gap26 (white bar) or by astrocytes exposed to CM-Aβ for 24 h and then subjected to 3 h hypoxia in 5 mM glucose (black bars) followed by 1 h reoxygenation without (middle bar) or with 200 μM Gap26 (right bar). (c) Etd uptake in neurons normalized to control conditions (dashed line) or subjected to 3 h hypoxia in 5 mM glucose followed by 1 h reoxygenation in CM-Ast or not. Also shown is the effect of La^{3+}, Gap26, probenecid (Prob) or ^{10}panx1 (200 μM each) on Etd uptake of control or treated neuronal cultures. Gap26 (*); effect on neuronal Etd uptake of CM-Ast made in presence of Gap26 during reoxygenation. In some experiments, the effect on neuronal Etd uptake of CM-Ast made in the presence of $Cx43^{E2}$ (1 : 500 dilution) during reoxygenation was studied. (d) Cell death in cultures of neurons subjected to 3 h hypoxia in 5 mM glucose followed by 1 h reoxygenation with the same treatments as in panel c. $^{***}P < 0.001$, $^{**}P < 0.005$, compared with control; $^{\#\#\#}P < 0.001$, compared with hypoxia plus Ast-CM effect. Each value corresponds to mean ± SE of at least four independent experiments.

In co-cultures treated with the $Cx43^{E2}$ antibody during reoxygenation neuronal death was drastically reduced (Fig. 6a). Moreover, a similar reduction in neuronal death was observed in co-cultures of $Cx43^{-/-}$ astrocytes and wild-type neurons. In the latter cultures, treatment for 24 h with CM-Aβ followed by hypoxia and 1 h reoxygenation did not significantly affect neuronal survival compared with control (4.5 ± 2.2% normalized to control, n = 3) (Fig. 6a).

As the above data suggested that astrocytes release neurotoxins via Cx43 hemichannels, conditioned medium was prepared from astrocytes treated for 24 h with CM-Aβ and then exposed to 3 h hypoxia followed by 3 h reoxygenation. Thus, neuronal cultures were subjected to hypoxia in normal glucose and then reoxygenated in the above-mentioned conditioned medium (CM-Ast). After 3 h hypoxia followed by 1 h of reoxygenation in CM-Ast, prominent neuronal death was observed (44.1 ± 12.1% normalized to total cells, n = 4) and neurons that remained alive exhibited a greater Etd uptake (1521 ± 218.6% normalized to control, n = 5) (Fig. 6c and d). Interestingly, CM-Ast made from astrocytes exposed to Gap26 or $Cx43^{E2}$ antibody during reoxygenation did not increase neuronal Etd uptake either in normal or high glucose (102.8 ± 34.8% or 98.2 ± 24.2%, respectively, normalized to control, n = 5). Moreover, Gap26 also reduced neuronal death at 1 h reoxygenation under the same protocol (8.8 ± 12.3% normalized to total cells, n = 4) (Fig. 6c and d). These observations suggested that soluble factors released from astroglial Cx43 hemichannels affect hemichannel activity and viability of neurons. In agreement with the previous interpretation, ^{10}panx1 or probenecid, but not 200 μM La^{3+} or Gap26, prevented the neuronal Etd uptake (114.9 ± 29.9%, 119.8 ± 34.7%, 1565.6 ± 278.8% and 1531.8 ± 255.2%, respectively, normalized to control, n = 4) and death in neurons (3.2 ± 1.7%, 6.9 ± 2.8%, 46.9 ± 8.1% and 43.1 ± 6.1%, respectively, normalized to total cells, n = 4) induced during reoxygenation in CM-Ast (Fig. 6c and d).

Death of cortical neurons is mediated by opening of Panx1 hemichannels activated via P2 and NMDA receptors

As it has been proposed that glutamate (Thompson *et al.* 2008) and ATP (Pelegrin and Surprenant 2006), acting through NMDA and P2 receptors, respectively, activate Panx1 hemichannels, we investigated if CM-Ast alone could induce neuronal dye uptake and death and if so, whether this effect could be prevented by glutamate and/or P2 receptor blockers.

In neuronal cultures treatment with CM-Ast alone for 1 h, we observed increased Etd uptake (1127.1 ± 74.1% normalized to control, $n = 4$) and cell death (50.3 ± 9.8% normalized total cells, $n = 4$) evaluated with F-Jade (Fig. 7 and Figure S4b). The increase in Etd uptake and neuronal death induced by CM-Ast was reduced partially by degradation of extracellular ATP with apyrase (376.7 ± 69.9% and 39.2 ± 8.2%, respectively, $n = 4$) or treatment with blockers of P2X receptors: oATP (445.9 ± 64.3% and 40.1 ± 5.2%, respectively, $n = 4$), suramin (389.8 ± 74.8% and 41.8 ± 7.2%, respectively, $n = 4$), and BBG (421.3 ± 73.2% and 42.7 ± 6.3%, respectively, $n = 4$) or a blocker of the NMDA receptor: 3-[(*R*)-2-Carboxypiperazin-4-yl]-propyl-1-phosphonic acid (523.8 ± 96.3% and 30.8 ± 10.7%, respectively, $n = 4$) and was almost completely blocked when activation of both glutamate and P2 receptors was inhibited (Fig. 7 and Figure S4c). Similarly, both the Etd uptake and neuronal death were abolished by probenecid (115.1 ± 24.6% and 8.1 ± 2.6%, respectively, $n = 4$) or ^{10}panx1 (114.8 ± 16.3% and 5.6 ± 2.1%, respectively, $n = 4$) (Fig. 7).

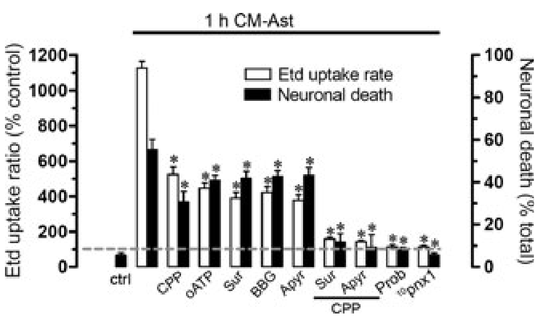

Fig. 7 Neuronal death induced by astrocytes is prevented by Panx1 hemichannel blockers but not by connexin hemichannel blockers; protection by P2X and NMDA receptor blockers. Averaged Etd uptake (white bars) and death (black bars) normalized to control of neurons treated for 1 h with CM-Ast alone or after 20 min pretreatment and continued application of 20 μM 3-[(*R*)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP) (NMDA receptor blocker), 300 μM oATP (P2X receptor blocker), 200 μM suramin (P2 receptor blocker), 10 μM BBG ($P2X_7$ receptor blocker) or 10 U/mL apyrase (ATPase). Shown is the effect of probenecid (Prob) and ^{10}panx1 in the above-mentioned responses. Prob and ^{10}panx1 were applied at 200 μM during reoxygenation. $^*P < 0.001$, compared with Ast-CM effect. Each value corresponds to mean ± SE of at least four independent experiments.

To elucidate the possible role of glutamate and ATP in this response, we measured the concentration of these molecules in CM-Ast generated by wild type astrocytes under control conditions or treated with Cx or Panx1 hemichannel blockers during reoxygenation as well as in the CM-Ast generated by $Cx43^{-/-}$ astrocytes. In the extracellular medium of astrocytes maintained under control conditions, levels of glutamate and ATP were 23.4 ± 5.3 nmol/10^6 cells ($n = 3$) and 11.3 ± 1 nmol/10^6 cells ($n = 3$), respectively (Fig. 8). Conditioned medium obtained during reoxygenation from astrocytes not treated with CM-Aβ presented similar levels of glutamate and ATP than control astrocytes (not shown). However, levels of glutamate and ATP were much higher in CM-Ast compared with control (82.2 ± 7.8 nmol/10^6 cells and 62.5 ± 5.6 nmol/10^6, respectively, $n = 4$) (Fig. 8). Interestingly, in CM-Ast made from astrocytes exposed to Gap26 during reoxygenation the levels of glutamate and ATP were similar to control conditions (18.1 ± 2.3 nmol/10^6 cells and 10.2 ± 1.4 nmol/10^6, respectively, $n = 4$) (Fig. 8). But, neither ^{10}panx1 nor probenecid applied during reoxygenation decreased the levels of glutamate (77.1 ± 7.9 nmol/10^6 cells and 73.5 ± 4.9 nmol/10^6, respectively, $n = 3$) or ATP (60.7 ± 9.2 nmol/10^6 cells and 58.1 ± 7.2 nmol/10^6, respectively, $n = 3$) (Fig. 8).

The above data suggest that glutamate and ATP released by astrocytes occurred via Cx43 hemichannels. In support of this notion, low levels of extracellular glutamate and ATP were detected in the extracellular mediun of $Cx43^{-/-}$ astrocytes (16.9 ± 1.6 nmol/10^6 cells and 9.3 ± 2.5 nmol/10^6, respectively, $n = 3$) (Fig. 8). To study the possible role of vesicular ATP release, the effect of 10 μM brefeldin A, an inhibitor of vesicular transport, was studies and it was found that this compound did not affect the levels of glutamate and

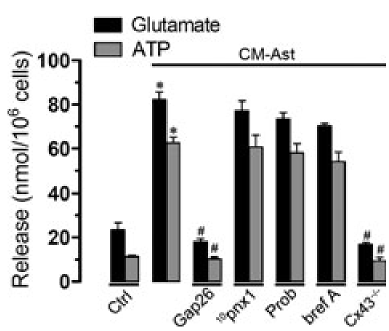

Fig. 8 $A\beta_{25-35}$-treated microglia increase the release of glutamate and ATP from astroglial Cx43 hemichannels during reoxygenation. Release of glutamate (black bars) and ATP (gray bars) by astrocytes under control conditions or treated with CM-Aβ for 24 h and then subjected to 3 h hypoxia in 5 mM glucose followed by 1 h reoxygenation. In some experiments, 200 μM Gap26, 500 μM probenecid (Prob), 200 μM ^{10}panx1 and 10 μM brefeldin A (Bref A) were applied during the reoxygenation period. Also shown is data from astrocytes from $Cx43^{-/-}$ mice subjected to the same above-mentioned protocols.

ATP in CM-Ast (70.1 ± 2.1 nmol/10^6 cells and 54.2 ± 7.2 nmol/10^6 cells, respectively, $n = 3$) (Fig. 8), suggesting that under these conditions the main pathway of ATP and glutamate release occurs via hemichannels.

ATP and glutamate open pannexin hemichannels in neurons

Because ATP and glutamate probably mediated the CM-Ast-induced Etd uptake and death in neurons, we also investigated whether both molecules could affect the activity of neuronal hemichannels. ATP or glutamate alone increased neuronal hemichannel activity after 1 h incubation but with different concentration/response relation (Fig. 9a). Glutamate increased the Etd uptake in a concentration dependent manner with two ascending steps, whereas ATP increased Etd uptake with the maximal effect at 100 μM (373.1 ± 87.9% normalized to control, $n = 4$) and progressively decline at higher concentrations (Fig. 9a). To exclude the possibility that the effects were mediated by breakdown products of ATP (e.g. ADP and adenosine), we treated neurons for 1 h with several concentrations of ADP or adenosine. Under these conditions, no changes in Etd uptake were observed (Figure S5a). Additionally, the ATP-induced Etd uptake was inhibited after inhibiting $P2X_7$ receptors with oATP and BBG (Iglesias *et al.* 2009; Orellana *et al.* 2010) but not with 8-cyclopentyl-1,3-dipropylxanthine, an A1 adenosine receptor blocker (Cechova *et al.* 2010) (Figure S5b). When glutamate and ATP were co-applied to neurons, Etd uptake was increased synergistically up to 100 μM, but for higher concentrations, the effect of both molecules became progressively weaker (Fig. 9a). The increase in neuronal Panx1 hemichannel activity induced by 100 μM ATP/glutamate (1012.7 ± 143.7% normalized to control, $n = 3$) was prevented by 1 mM probenecid (123.1 ± 9.1% normalized to control, $n = 3$), but was not affected by 200 μM La^{3+} (886.9 ± 83.1% normalized to control, $n = 3$) (Fig. 9b). Similarly, the neuronal death induced by ATP or glutamate alone or in combination was prevented by Panx1 but not Cx43 hemichannel blockers (Fig. 9c).

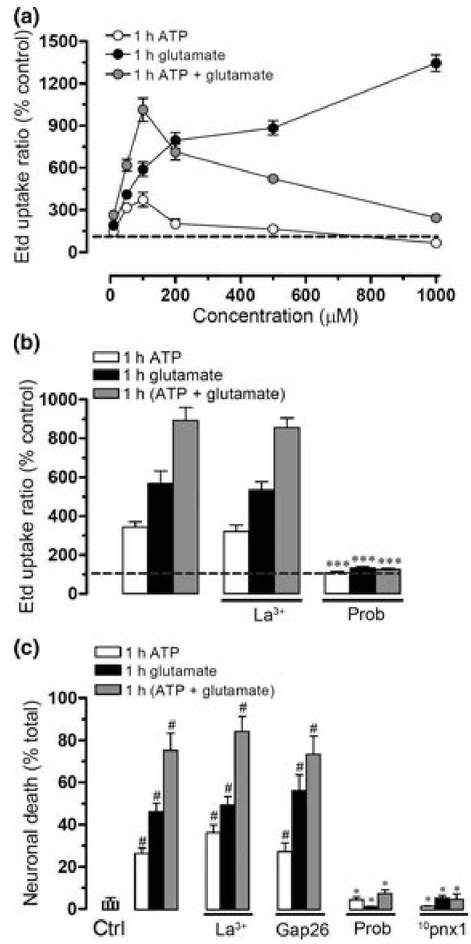

Fig. 9 ATP and glutamate act together to permeabilize and kill neurons in neuronal cultures. (a) Etd uptake ratio normalized to control (dashed line) in neuronal cultures exposed for 1 h to various concentrations of ATP (white circles), glutamate (black circles) or ATP plus glutamate (gray circles). (b) Etd uptake normalized to control (dashed line) in neuronal cultures exposed for 1 h to 100 μM ATP (white bars), 100 μM glutamate (black bars) or 100 μM ATP plus 100 μM glutamate (gray bars). Etd uptake was unaffected by La^{3+} (200 μM, middle bars) but blocked by probenecid (200 μM, right bars). (c) Cell death measured as percent of F-Jade-positive cells in neuronal cultures treated with glutamate and/or ATP as in panel b. Probenecid and ^{10}panx1 provided complete protection, but not La^{3+} and Gap26. $^{\#}P < 0.001$; compared with control; $^{*}P < 0.001$, compared with the respective treatment. Each value corresponds to mean ± SE of four independent experiments.

Discussion

In this study, we have demonstrated that Aβ-treated microglia potentiate the increase in astroglial hemichannel activity and reduction in gap junctional communication induced by hypoxia in high glucose. In addition, the extracellular medium of inflamed astrocytes was neurotoxic because of its glutamate and ATP content, molecules which activated neuronal Panx1 hemichannels via NMDA/P2X receptors leading to neuronal death. Therefore, neurons could be efficiently protected from ischemia and neurotoxicity by blocking NMDA and P2X receptors as already proposed, but also by targeting either glial or neuronal hemichannels composed by Cx43 and Panx1, respectively.

When microglia are activated with $A\beta_{25\text{-}35}$ they release pro-inflammatory cytokines (Block *et al.* 2007). Accordingly, medium conditioned by microglia treated with $A\beta_{25\text{-}35}$ (CM-Aβ) induced a persistent change in Cx based channels of astrocytes subjected to a 3-h hypoxia in high glucose, suggesting the action of soluble factors present in the CM-Aβ. In agreement with this interpretation, simultaneous neutralization of TNF-α and IL-1β with IL-1ra and sTNFaR1 completely prevented the changes induced by CM-Aβ. Persistent and opposite responses of astroglial Cx43 hemichannels and gap junction channels was recently demonstrated during reoxygenation after 6 h of hypoxia (Orellana *et al.* 2010) and after lipopolysaccharide treatment of co-cultured microglia (Froger *et al.* 2009). Here, a similar persistent response was observed in astrocytes pre-treated with CM-Aβ or low concentrations of TNF-α and IL-1β followed by only 3 h of hypoxia. None of these conditions alone caused detectable or persistent changes, but CM-Aβ-treated astrocytes presented similar responses during reoxygenation to that induced by 6 h of hypoxia (Orellana *et al.* 2010), indicating convergence and addition of their effects at the level of astroglial Cx based channels. Supporting this interpretation, the additive effect was also evident in the increase of Cx43 levels at the cell membrane, which has been shown to account for the increase in hemichannel activity evoked by pro-inflammatory conditions (Orellana *et al.* 2010). In addition, astroglial Etd uptake induced by CM-Aβ or TNF-α/IL-1β after 3 h hypoxia was exclusively dependent on Cx43 hemichannels, because: 1) Etd uptake was not observed in $Cx43^{-/-}$ astrocytes, 2) pharmacological treatments known to block Cx43, but not Panx1 hemichannels (e.g. La^{3+} and Gap26), inhibited this response and 3) two Panx1 mimetic peptides, ^{10}panx1 and E1b, as well probenecid, a Panx1 hemichannel blocker, failed to block the increase in Etd uptake.

Microglia not stimulated with $A\beta_{25-35}$ prevented the increase in astroglial hemichannel activity observed at 3 h reoxygenation after 3 h hypoxia in high glucose. The latter is in agreement with the known neuroprotective effect of resting microglia (Block *et al.* 2007). In fact, microglia suppress both the effect of H_2O_2 on astroglial gap junctional communication and its toxicity (Rouach *et al.* 2004). In contrast, activated microglia and pro-inflammatory cytokines induce astroglial uncoupling (Faustmann *et al.* 2003), astroglial hemichannel opening and neuronal death (Block *et al.* 2007; Retamal *et al.* 2007).

It is well established that astrocytes under resting conditions can prevent neuronal damage induced by hypoxia during reoxygenation (Trendelenburg and Dirnagl 2005). In contrast, astrocytes treated with pro-inflammatory conditions promote neuronal damage (Thornton *et al.* 2006). We here show that this process, in addition to involving increased opening of astroglial Cx43 hemichannels, is also associated with the activation of neuronal Panx1 hemichannels. Indeed, Gap26 directly prevented astroglial hemichannel activity but also had a secondary preventative action on Panx1 hemichannel activity in neurons. The functional role of gap junctional communication in animal models of stroke remains controversial (Orellana *et al.* 2009). By selectively blocking astroglial hemichannels without interfering with their gap junctions, we have shown that inhibition of hemichannels is neuroprotective. However, as gap junctional communication is reduced in our conditions it remains to be demonstrated if recovery of intercellular communication, independently of changes in hemichannel activity, could increases neuronal resistance to pro-inflammatory conditions.

The concentration of both glutamate and ATP was much higher in CM-Ast generated by astrocytes with functional Cx43 hemichannels. Due to the enhanced activity of astroglial Cx43 hemichannels, more glutamate and ATP could have been released through this signaling pathway (Ye *et al.* 2003; Kang *et al.* 2008). Accordingly, the presence of Cx43 hemichannel blockers during conditioning of the culture media by astrocytes treated with CM-Aβ or astrocytes without Cx43 expression (astrocytes from $Cx43^{-/-}$ mice) prevented the increase in glutamate and ATP concentration observed in the extracellular media harvested from untreated wild type astrocytes. These findings and the inability of brefeldin A to inhibit the glutamate/ATP release rule out the possible participation of vesicular release of these molecules (Rossi and Volterra 2009).

A striking finding of the present work was that in the presence of $A\beta_{25-35}$, astroglial hemichannels are implicated in neuronal death induced by pro-inflammatory conditions. Accordingly, the CM-Ast was deleterious only when it was harvested from astrocytes not treated with Cx43 hemichannel blockers applied at time zero of reoxygenation. These findings suggested that during reoxygenation soluble effectors were released via astroglial Cx43 hemichannels. This interpretation is supported by the following findings: (i) the increase in neuronal Etd uptake and death were partially prevented with inhibition of NMDA or P2X receptors, but was completely prevented by inhibition of both receptor types, (ii) exogenous ATP and glutamate mimicked the Etd uptake and neuronal death induced by CM-Ast and (iii) higher ATP and glutamate concentrations were detected in CM-Ast generated by astroglial cells expressing functional Cx43 hemichannels. These findings provide an explanation of a recent report in which we observed that Etd uptake occurred in neurons cocultured with astrocytes treated with proinflammatory cytokines (TNF-α and IL-1β) and we showed that hemichannels of inflamed astrocytes worsen the NMDA excitotoxicity in neurons (Froger *et al.* 2010).

The *in vitro* neurotoxic effect of glutamate has been well documented (Lau and Tymianski 2010). In addition, the extracellular ATP concentration is known to increase in ischemic brain (Melani *et al.* 2005) and ATP could be neurotoxic acting directly on neurons (Amadio *et al.* 2005) or indirectly by inducing astroglial release of glutamate (Fellin *et al.* 2006). In the neurodegeneration model used in the present work, excessive extracellular ATP and glutamate enhance neuronal mortality via Panx1 hemichannels. This observation implies that persistent activation of Panx1 hemichannels is more deleterious to neurons than prolonged activation of P2 and/or NMDA receptors. In support of this possibility, studies in knock out animals for $P2X_7$ receptors

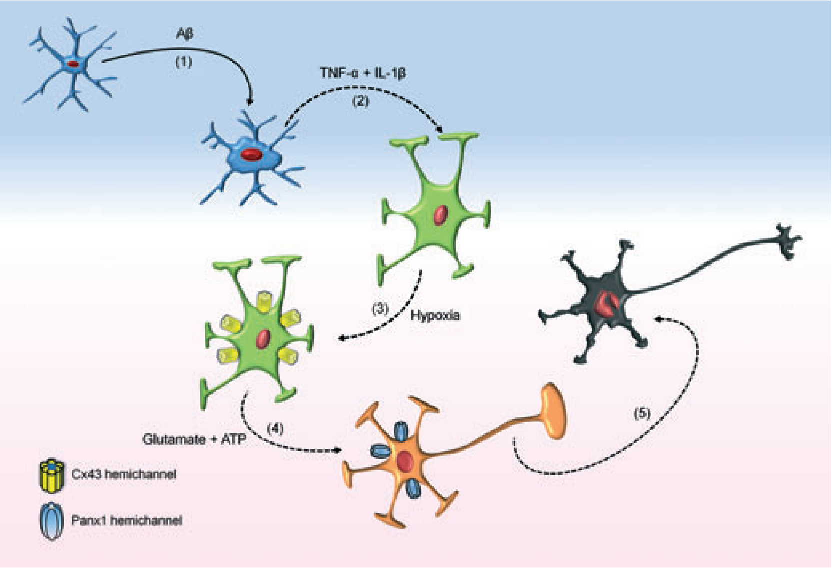

Fig. 10 Signaling that leads to neuronal death through the contribution of inflamed glial cells. Initially, microglia (in blue) upon activation with Aβ (1) release pro-inflammatory cytokines (TNF-α/IL-1β) (2), which increase astroglial hemichannel activity when it is followed by hypoxia in high glucose (3). Then, astrocytes (in green) release glutamate and ATP via Cx43 hemichannels, which activate opening of Panx1 hemichannels in neurons (4). ATP released as a result of Panx1 hemichannel opening could contribute in the progression of neuronal death (orange to black) by a vicious cycle because it will activate more P2X receptors leading to more Ca^{2+} entry and activation of intracellular neurotoxic cascades (5).

show similar neuronal sensitivity in ischemic and excitotoxic brain (Le Feuvre *et al.* 2003) and knock-out animals for the NMDA subunits NR1 and NR2 show only reduction in the affected brain area (Morikawa *et al.* 1998). Previous evidence indicates that Panx1 hemichannels can be activated by extracellular ATP through $P2X_7$ receptors (Locovei *et al.* 2006; Pelegrin and Surprenant 2006) as well as through glutamate receptors (Thompson *et al.* 2008). However, in hippocampal pyramidal cells inhibitors of glutamate receptors, but not inhibitors of Panx1 hemichannels, prevent the anoxic depolarization (Madry *et al.* 2010), suggesting that opening of Panx1 hemichannel might depend on experimental conditions.

We propose that microglia activated by $A\beta_{25\text{-}35}$ release pro-inflammatory cytokines for which an increase in astroglial hemichannel activity has been demonstrated (Retamal *et al.* 2007) (Fig. 10). The activation of such hemichannels allows the release of neurotoxic molecules including glutamate and ATP, which can act on microglia inducing further cytokine release (Block *et al.* 2007) (Fig. 10). Then, opening of neuronal Panx1 hemichannels could be triggered as a result of the rise in $[Ca^{2+}]_i$ via activation of NMDA and P2X receptors by glutamate and ATP, respectively (Fig. 10). Panx1 hemichannels are likely to contribute to the intracellular Ca^{2+} overload that activates neurotoxic intracellular cascades during brain ischemia and excitotoxicity (Szydlowska and Tymianski 2010). The complete neuronal death inhibition elicited by Panx1 hemichannel blockade suggests that other mechanisms known to contribute to the Ca^{2+} overload, including ionotropic receptors and channels (e.g. NMDA, 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid and kainate receptors, TRPM, and P2X receptors and CaV1.2 channels) and membrane transporters (Szydlowska and Tymianski 2010), may also act as activators of Panx1 hemichannels or participate downstream of Panx1 hemichannels.

Neurodegenerative processes, as in DM and AD, which are accompanied by neuro-inflammation, including micro strokes (Snowdon *et al.* 1997; Pasquier *et al.* 2006), might cause enhanced astroglial and neuronal hemichannel activity, leading to cell death and impairment of CNS function. AD and DM are two of the most common and devastating health problems in the elderly. Several studies have shown associations between DM and moderate cognitive impairment of both memory and executive functions (Pasquier *et al.* 2006; Takeda *et al.* 2010). Moreover, the risk of both vascular dementia and AD is greater in patients with type 2 DM (Pasquier *et al.* 2006). Interestingly, increase in Cx43 expression has been observed in reactive astrocytes from AD patients (Nagy *et al.* 1996) and a double transgenic mouse developing Aβ plaques (Mei *et al.* 2010). Thus, dysregulation of Cx43 and Panx1 based channels may contribute to the development of CNS pathologies and Cx as well as Panx1 hemichannels might represent potential and alternative targets for therapeutic intervention in neuro-inflammatory diseases.

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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. (a–c) Fluorescence micrographs of Etd uptake for 10 min in astrocytes under control conditions or after 3 h hypoxia in 27 mM glucose followed by 1 or 3 h reoxygenation. (d–f) Astrocytes and microglia co-cultured under control conditions or after 3 h hypoxia in 27 mM glucose followed by 1 or 3 h reoxygenation. (g–i) Astrocytes and microglia co-cultured only with $A\beta_{25\text{-}35}$ for 24 h and then exposed to 3 h hypoxia in 27 mM glucose followed by 1 or 3 h reoxygenation. Scale bar, 150 μm.

Figure S2. (a–i) Fluorescence micrographs of scrape loading/dye transfer with LY in astrocytes under control conditions or after 3 h hypoxia in 27 mM glucose followed by 1 or 3 h reoxygenation. (d–f) Astrocytes and microglia co-cultured under control conditions or after 3 h hypoxia in 27 mM glucose followed by 1 or 3 h reoxygenation. (g–i) Astrocytes and microglia co-cultured only with $A\beta_{25\text{-}35}$ for 24 h and then exposed for 3 h hypoxia in 27 mM glucose followed by 1 or 3 h reoxygenation. Scale bar, 100 μm.

Figure S3. CM-Aβ pretreatment followed by hypoxia-reoxygenation increases neuritic beading and death of neurons in co-culture with astrocytes.

Figure S4. Inhibition of NMDA and P2X receptors reduce the increase in F-Jade-positive neurons induced by activated astrocytes.

Figure S5. Breakdown products of ATP does not affect neuronal hemichannel activity.

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