

# Copper-Zinc Superoxide Dismutase (SOD1) Is Released by Microglial Cells and Confers Neuroprotection against 6-OHDA Neurotoxicity

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## Key Words

Microglia · Conditioned medium · SOD1 · Neuroprotection · Cerebellar granule neurons · 6-Hydroxydopamine

## Abstract

Microglial-neuronal interactions are essential for brain physiopathology. In this framework, recent data have changed the concept of microglia from essentially macrophagic cells to crucial elements in maintaining neuronal homeostasis and function through the release of neuroprotective molecules. Using proteomic analysis, here we identify copper-zinc superoxide dismutase (SOD1) as a protein produced and released by cultured rat primary microglia. Evidence for a neuroprotective role of microglia-derived SOD1 resulted from experiments in which primary cerebellar granule neurons (CGNs) were exposed to the dopaminergic toxin 6-hydroxydopamine (6-OHDA). Microglial conditioned medium, in which SOD1 had accumulated, protected CGNs from degeneration, and neuroprotection was abrogated by SOD1 inhibitors. These effects were replicated when exogenous SOD1 was added to a nonconditioned medium. SOD1 neuroprotective action was mediated by increased cell calcium from an external source. Further experiments demonstrated the specificity of SOD1 neuroprotection against 6-OHDA com-

pared to other types of neurotoxic challenges. SOD1, constitutively produced and released by microglia through a lysosomal secretory pathway, is identified here for the first time as an essential component of neuroprotection mediated by microglia. This novel information is relevant to stimulating further studies of microglia-mediated neuroprotection in *in vivo* models of neurodegenerative diseases.

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## Introduction

Microglia, the resident immune cells of the central nervous system, possess distinctive physiological and pathological properties, the understanding of which has considerably progressed in recent years [1–7]. Some of this research has led to the development of novel concepts in microglial function. In particular, attention has been focused on microglia-derived neuroprotection and on immunomodulation to boost the physiologic properties of these cells, thus counteracting the pathologic consequences of their excessive activation [8].

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The neuroprotective role of microglia is exerted not only as a response to a neuropathologic threat but also in physiologic conditions. 'Quiescent' or 'dormant' microglia actually play a role in continuous surveillance of the brain microenvironment, participate in synapse remodeling and function, influence neurogenesis, and provide trophic support for the survival of neurons and maintenance of neural circuits [9–13]. This led to the idea that physiological microglial functions are exerted through the production and release of substances with neuroprotective, survival-favoring properties. Moreover, several experimental approaches have been developed to demonstrate this kind of neuroprotection, as well as to identify microglia-derived neuroprotective molecules. In vitro models based on neuron-microglia co-cultures or on neuronal exposure to microglial conditioned media (MCM) have been particularly useful. In co-culture conditions, microglia has been shown to protect neurons from nitrosative stress, metabolic impairment, and maintenance in nontrophic conditions [14–16]. A similar microglia-mediated neuroprotection has also been granted to organotypic hippocampal cultures exposed to oxygen-glucose deprivation [17]. Furthermore, MCM has been shown to promote the survival of rat mesencephalic neurons [18] and protect cerebellar granule neurons (CGNs) from nontrophic conditions, including staurosporine-induced apoptosis and excitotoxicity [16, 19], and cortical neurons from  $\beta$ -amyloid toxicity [20]. Some of the molecules released by microglia have already been identified and, in some cases, a neuroprotective activity has been characterized. In the above mentioned experiments on cortical neurons [20], the MCM neuroprotective effect was attributed to the release of apolipoprotein E from microglia. In cultured dopaminergic mesencephalic neurons, microglia-derived plasminogen is neuroprotective through dopamine uptake [21]. In CGNs exposed to the dopaminergic toxin 6-hydroxydopamine (6-OHDA), we recently identified transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2) as a neuroprotective molecule released by microglia [22]. In addition to constitutively released neuroprotective factors, exposure of microglia to media conditioned by apoptotic neurons may increase their neuroprotective action [16] as demonstrated by the release of immunoregulatory and pro-survival factors, such as prostaglandin E<sub>2</sub>, NGF, and TGF- $\beta$  [23].

Here we identify for the first time Cu-Zn superoxide dismutase (SOD1) as an effector of MCM-mediated neuroprotection. SOD1 is a particularly intriguing protein for brain pathophysiology, as mutations in its codifying gene are responsible for the familial forms of the neuro-

degenerative disorder amyotrophic lateral sclerosis (ALS) [24]. We demonstrate that SOD1 is released by microglia and accumulates in culture medium. Released SOD1 contributes to the neuroprotective properties of the conditioned medium itself, with neuroprotection being reproduced by the addition of exogenous SOD1 to a nonconditioned medium. Evidence points to increased calcium levels from extracellular sources as the mediator of SOD1 action. This novel knowledge may improve our understanding of the complex relationships between neurons and microglial cells with particular reference to the mechanisms of microglia-mediated neuroprotection and to their molecular effectors.

## Materials and Methods

### *Microglial Cell Culture and MCM*

Primary cultures of microglial cells were prepared from newborn Wistar rat cerebral cortices as previously described [16]. All animal experiments were authorized by a local bioethical committee and performed in accordance with the Italian and European Community law on the use of animals for experimental purposes. Briefly, brain tissue was cleaned from meninges and trypsinized for 15 min at 37°C; after mechanical dissociation, the cell suspension was washed and plated on poly-L-lysine (10  $\mu$ g/ml; Sigma-Aldrich, St. Louis, Mo., USA)-coated flasks (75 cm<sup>2</sup>). Mixed glial cells were cultured for 7–8 days in Basal Medium Eagle (BME; Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM glutamine, and 100  $\mu$ M gentamicin sulfate (Sigma-Aldrich). Microglial cells were harvested from mixed glial cell cultures by mechanical shaking, resuspended in serum-free BME, and plated on uncoated 40-mm  $\emptyset$  dishes at a density of  $1.5 \times 10^6$  cells/1.5 ml medium/well. Cells were allowed to adhere for 30 min and were then washed to remove nonadhering cells. After 2, 24, and 48 h, MCM was collected, filtered through 0.22- $\mu$ m filters, and stored at -20°C until use. For the evaluation of SOD1 release, microglial cells were plated on uncoated 12-well plates at a density of  $7.5 \times 10^5$  cells/well and stimulated with lipopolysaccharide (LPS), rat recombinant interferon- $\gamma$  (IFN- $\gamma$ ), or both, as well as with adenosine triphosphate (ATP) at different concentrations (all chemicals were from Sigma-Aldrich). After 24 h (1 h for ATP treatment) the media were collected and then lyophilized using Microcon-YM-3 (Millipore, Billerica, Mass., USA), and the expression of SOD1 was analyzed via Western blot analysis.

### *Microglial Transfection*

Microglial cells were transfected with the mammalian expression vector pcDNA3 (Invitrogen) containing full-length cDNA encoding wild-type or mutated (G93A) human SOD1 (pcDNA3-SOD1wt/G93A), a kind gift from Prof. Angelo Poletti [25], or two different commercially available siRNA (Sigma) against SOD1, previously tested for their efficacy by the Rosetta bioinformatics system. Transfection was performed using LipofectAMINE 2000 Reagent (Invitrogen), following the manufacturer's protocol, in OptiMEM/serum-free BME (Invitrogen) without antibiotics for

4 h; the medium with lipid-DNA complex was then replaced with serum-free BME. Twenty-four hours after plasmid transfection or 48 h following siRNA transfection, MCM were collected, cleared of contaminating cells by filtration (through 0.22- $\mu$ m filters) or by centrifugation (10,000 g for 5 min), and stored at  $-20^{\circ}\text{C}$  until use for Western blot and neuroprotection analysis on CGNs. The vector pmaxGFP<sup>®</sup> (Amaxa, Cologne, Germany) was used both as a plasmid control and as a test for transfection efficiency.

#### CGN Cultures

Primary cultures of CGNs were prepared from 7-day-old Wistar rats, as previously described [26]. Briefly, cells were dissociated from cerebella and plated on 96-well plates or in 40-mm  $\emptyset$  dishes, previously coated with 10  $\mu\text{g}/\text{ml}$  poly-L-lysine, at a density of  $2 \times 10^5$  cells/ $\text{cm}^2$  in BME supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM glutamine, 100  $\mu\text{M}$  gentamicin sulfate, and 25 mM KCl (all from Sigma-Aldrich). Sixteen hours later, 10  $\mu\text{M}$  cytosine arabino-furanoside (Sigma-Aldrich) was added to avoid glial proliferation. After 7 days in vitro, differentiated neurons were shifted to serum-free BME medium containing 25 mM KCl and treated with 20  $\mu\text{M}$  6-OHDA (Sigma-Aldrich). The neuroprotective effect of MCM, as well as the one of exogenous SOD1 (Sigma-Aldrich), was tested on this in vitro model of neurotoxicity. Specific SOD inhibitors, ammonium tetrathio-molybdate and disulfiram, or the extracellular calcium-chelating agent ethylenediaminetetraacetic acid (EDTA) were examined to block the neuroprotective effect of exogenous SOD1 as well as that of microglia-secreted SOD1 present in MCM (all chemicals from Sigma-Aldrich). The possible SOD1 neuroprotection was also studied in other classical models of CGN neurotoxicity, i.e. the shift of differentiated neurons to a low potassium concentration (5 mM KCl) for 24 h or the chronic (24 h) exposure of neurons to 100  $\mu\text{M}$  glutamate (all from Sigma-Aldrich). Neuronal survival was analyzed using the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) test. To measure the neuroprotection exerted by MCM conditioned for 2, 24, and 48 h, nucleus counting following Hoechst staining was performed as described below.

#### MTT Assay

The viability of CGN cultures was evaluated by thiazolyl blue (MTT) assay [27]. This method is based on conversion of the tetrazolium salt to a colored compound, a reaction that only occurs in viable cells since the chemical reaction is carried out by mitochondrial dehydrogenases. MTT (Sigma-Aldrich) was added to the culture medium to reach a final concentration of 0.1 mg/ml. Following 15 min of incubation at  $37^{\circ}\text{C}$  in the dark, formed crystals were dissolved in 0.1 M Tris-HCl buffer containing 5% Triton X-100 (all from Sigma-Aldrich) and the absorbance was read at 570 nm in a Multiplate Spectrophotometric Reader (Bio-Rad Laboratories, Hercules, Calif., USA).

#### Proteomic Analysis of MCM

MCM were concentrated using Amicon ultra-15 (3-kDa centrifugal filter; Millipore), and albumin was removed using a SwellGel<sup>®</sup> Blue Albumin Removal Kit (Pierce, Rockford, Ill., USA) according to the manufacturer's instructions. Starting from 60 ml (containing 700  $\mu\text{g}$  of total proteins) of 48-hour MCM (from  $60 \times 10^6$  microglial cells) to reach 400  $\mu\text{g}$  of total proteins, samples were loaded on the gel after de-albuminization. The con-

ditioned medium was concentrated and then brought to a final volume of 350  $\mu\text{l}$  with rehydration buffer made of 8 M urea, 0.5% CHAPS, 18 mM DTT, 0.002% bromophenol blue (all from Sigma-Aldrich), and 0.8% pH 3–10 Amersham Pharmalytes (GE Healthcare, Chalfont St. Giles, UK). The samples were loaded into a rehydration strip tray, and 18-cm pH 3–10 NL IPG strips (GE Healthcare) were placed facing the samples and left to rehydrate for at least 12 h (overnight). The first dimension was run using a Multiphor III (GE Healthcare) at 50  $\mu\text{A}$  per strip for 9 steps until a total of 80 kV was reached. Once the first dimension was finished, the strips were stored at  $-80^{\circ}\text{C}$  until needed for the second dimension. Before the second dimension, the strips were equilibrated by incubation in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, and 0.002% bromophenol blue) first in 1% DTT for 15 min and then in 2.5% iodoacetamide for 15 min. Following the equilibration, the strips were briefly washed in Laemli SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) (all chemicals were from Sigma-Aldrich). The equilibrated strips were loaded onto 15% polyacrylamide gels (Bio-Rad Laboratories) together with Bio-Rad wide-range protein standard markers. The IPG strips were overlaid with agarose sealing solution (0.5% agarose and 0.002% bromophenol blue in Laemli SDS electrophoresis buffer, all from Sigma-Aldrich). The second dimension was performed keeping the buffer tank temperature constant at  $20^{\circ}\text{C}$  throughout the entire second dimension for 30 min at 2 W per gel and 5–6 h at 15 W per gel until the blue dye front reached the bottom of the gel.

After the second dimension was finished, gels were immediately stained in Coomassie Brilliant Blue G-250 solution (10% acetic acid, 40% methanol, x% CBB) with constant shaking for 48 h. Afterwards, the gels were left in deionized water with constant shaking until the protein spots were clearly visible. Gel images were captured using a Pharos FX (Bio-Rad) scanner at a resolution of 50  $\mu\text{m}$ . Raw images (each sample was analyzed in three different gels) were analyzed using Proteomweaver 4.0 software (Bio-Rad). Differentially expressed spots, with intensity significantly increased on the average map, were collected. Peptide identification was performed by the proteomics laboratory at CRBA, Center for Applied Biomedical Research at S. Orsola-Malpighi University Hospital in Bologna, Italy, by trypsin digestion and MALDI-ToF.

#### SOD Activity Determination

SOD1 activity in 20  $\mu\text{l}$  of 48-hour MCM concentrated 2- to 4-fold was determined using the SOD determination kit from Fluka (Sigma-Aldrich). This method is based on the ability of SOD1 to catalyze the dismutation of the anion superoxide, previously produced by xanthine oxidase starting from xanthine and  $\text{O}_2$ , into molecular oxygen and hydrogen peroxide, which in turn can reduce a highly soluble tetrazolium salt (WST-1) to a formazan dye that can be easily revealed through a spectrophotometer. Since the absorbance of formazan dye is proportional to the amount of anion superoxide, SOD activity is quantified as an inhibition activity by measuring the decrease in color development. For quantification, a standard inhibition curve was prepared with a known concentration of exogenous SOD1 (Sigma-Aldrich) from 1.125 to 10 U/ml and the reaction was followed for at least 20 min.

#### Immunocytochemistry and Nuclear Staining

CGNs were fixed for 20 min with 4% paraformaldehyde in phosphate buffer at  $25^{\circ}\text{C}$  and then washed in phosphate-buffered

saline (PBS). Aspecific sites were blocked with normal goat serum (Sigma-Aldrich) for 1 h at 25°C. After several washes, cells were incubated overnight at 4°C with both rabbit anti-SOD1 and mouse anti-lysosome-associated membrane protein-1 (LAMP-1; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif, USA) and further incubated with the secondary antibodies for 1 h and 30 min at 25°C (anti-rabbit fluorescein isothiocyanate for SOD1 and anti-mouse tetramethyl rhodamine isothiocyanate for LAMP-1; Sigma-Aldrich). At the end, nuclei were stained with Hoechst 33258 (0.1 mg/ml; Sigma) for 5 min at 25°C. Stained cultures were photographed with a fluorescence microscope (Eclipse Hoechst staining TE 2000-S microscope; Nikon, Tokyo, Japan) equipped with an AxioCam MRm (Zeiss, Oberkochen, Germany) digital camera.

#### Western Blot Analysis

To analyze the level of SOD1 released into the media or expressed by microglial cell cultures after different times of conditioning and/or after treatments with different stimuli, MCM and cells were collected. Five hundred microliters of MCM for each condition were lyophilized using Amicon YM-3 (Millipore) and resuspended in 15 µl of loading buffer 2× (0.05 M Tris-HCl pH 6.8; 40 g/l sodium dodecyl sulfate; 20 ml/l glycerol; 2 g/l bromophenol blue, and 0.02 M dithiothreitol; all chemicals were from Sigma-Aldrich). In parallel, 1.5/10<sup>6</sup> microglial cells from each well were collected directly in 50 µl of loading buffer 2×. Fifteen microliters per lane of concentrated MCM samples or of microglial cell samples were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE; Bio-Rad Laboratories). After electrophoresis and transfer to a nitrocellulose membrane (GE Healthcare), the membranes were blocked for 1 h with a blocking solution made of 5% nonfat dried milk (Bio-Rad)/0.1% Tween-20 in phosphate buffer solution (Sigma-Aldrich), pH 7.4, and incubated overnight at 4°C with primary antibodies (rabbit polyclonal anti-SOD1, 1:1,000; Santa Cruz Biotechnology) in 0.1% Tween-20/PBS. Then, nitrocellulose membranes were incubated with a secondary antibody, a goat anti-rabbit antibody conjugated to horseradish peroxidase (1:2,000; Santa Cruz Biotechnology), for 90 min at 24°C in 0.1% Tween-20/PBS. The labeled bands were visualized using the enhanced chemiluminescence method (ECL; Santa Cruz Biotechnology).

#### Calcium Microfluorometry

Variations in intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) were monitored through ratiometric microfluorometry using the fluorescent Ca<sup>2+</sup> detector fura-2 AM (Molecular Probes; Invitrogen, Milano, Italy). For microfluorometric experiments the control bath saline was 140 mM NaCl, 4 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM TES, and 5 mM glucose, pH 7.4, with NaOH and osmolarity adjusted to ~320 mOsm with mannitol. The Ca<sup>2+</sup>-free extracellular saline was prepared by removing CaCl<sub>2</sub> salt and adding 0.5 mM EGTA. When using high (50 mM) K<sup>+</sup> external solutions, salts were replaced equimolarly. Stock solution of SOD1 was diluted in control and Ca<sup>2+</sup>-free media at the final concentrations of 0.75, 1.5, 3.125, 6.25, and 12.5 U/ml. The 48-hour MCM solution was obtained by dissolving the lyophilized powder in 10 ml of the control bath saline with and without calcium. Before measurements, low-density granular cells seeded in coverslips were loaded with 10 µM fura-2 AM dissolved in standard bath solution for 45 min at 25°C. For microfluorometric analysis cell coverslips

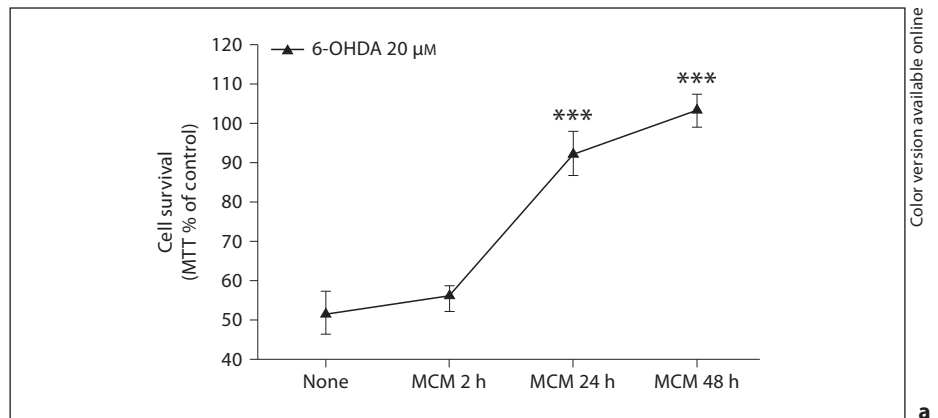
were mounted on a perfusion chamber containing 100 µl bath saline. Cells were continuously perfused at a rate of 0.5 ml/min with different salines at room temperature (22–24°C) as previously described [28]. Measurements of [Ca<sup>2+</sup>]<sub>i</sub> were performed using an inverted fluorescence microscope (Nikon Eclipse TE2000U; Nikon, Italy) equipped with a long-distance dry objective (×40) and appropriate filters. The emission fluorescence of selected astrocytes was passed through a 510-nm narrow-band filter and acquired with a digital charge-coupled device camera (VTi; Visi-Tech International Ltd., Sunderland, UK). Monochromator settings, chopper frequency, and complete data acquisition were controlled by QuantiCell 2000 (VisiTech). The excitation wavelength was alternated between 340 and 380 nm with a sampling rate of 0.25 or 0.5 Hz. The fluorescence ratio measured at 340 and 380 nm (F340/F380) was used as an indicator of [Ca<sup>2+</sup>]<sub>i</sub> changes. The calibration of the 340/380 ratio in terms of the free Ca<sup>2+</sup> concentration was based on the procedure previously described [29].

#### Statistical Analysis

All results were subjected to statistical analysis with Student's *t* test or with one-way ANOVA followed by Bonferroni's post hoc comparisons test in order to evaluate the significance of the differences.

## Results

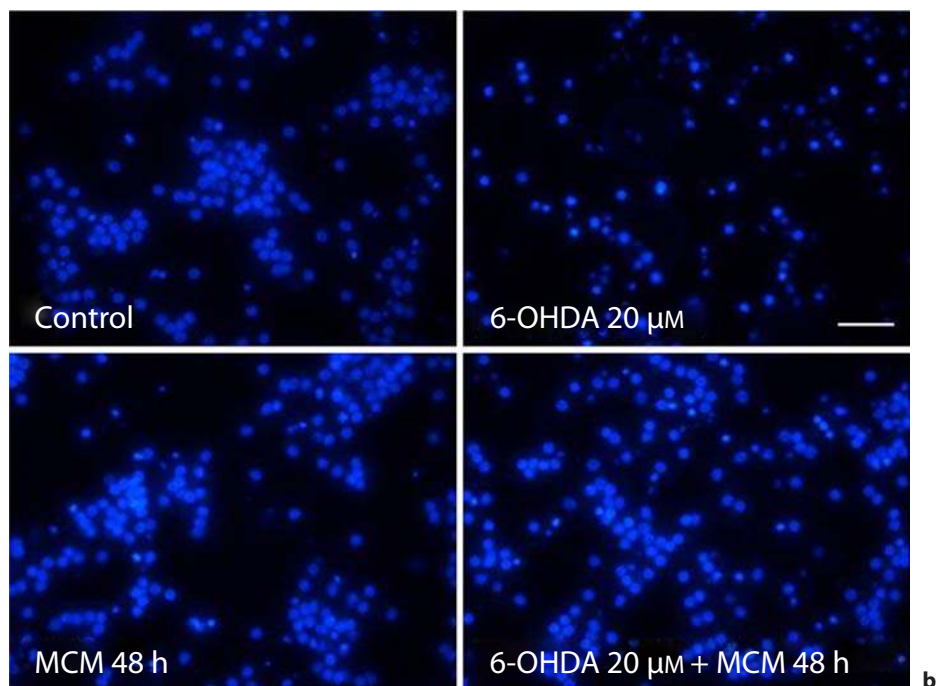
As previously demonstrated [22, 30], exposure of differentiated rat CGNs to the dopaminergic toxin 6-OHDA (20 µM) resulted in approximately 50% cell death after 24 h (fig. 1a). While a medium conditioned by microglia for 2 h (2-hour MCM) did not protect neurons from toxicity, media conditioned for 24 and 48 h (24-hour and 48-hour MCM) showed neuroprotection (fig. 1a, b), thus suggesting that neuroprotective substances released by microglia progressively accumulated in the medium. We previously demonstrated [22] that heat inactivation of the neuroprotective MCM, as well as its pre-treatment with peptidases/proteases, results in a significant decrease in MCM-mediated protection against 6-OHDA neurotoxicity in CGNs, leading to the idea that peptidic factor(s) contribute to neuroprotection. In order to identify the neuroprotective microglia-released proteic factors, we performed a proteomic analysis of 48-hour MCM. This analysis demonstrated the presence of several spots, revealing the accumulation of various peptides in the conditioned medium. Among them, one spot was identified through mass spectroscopy analysis as the Cu-Zn superoxide dismutase SOD1 (fig. 2a). Western blot analysis confirmed that SOD1 was actually present and accumulated in 24-hour and 48-hour MCM, while it was much less evident after 2 h of conditioning (fig. 2b), thus suggesting that SOD1 could contribute to MCM neuroprotection. In order to estimate the amount of this



Color version available online

a

**Fig. 1.** Neuroprotective effect of MCM on 6-OHDA-induced neurotoxicity in CGNs. CGNs were treated for 24 h with 20  $\mu$ M 6-OHDA in the presence or absence of medium conditioned for 2, 24, and 48 h by unstimulated microglial cell cultures (100% of the culture media). Cell viability was determined through MTT assay (**a**) or visualized via Hoechst staining (**b**). **a** Each point is the mean  $\pm$  SE of at least four different experiments run in triplicate. \*\*\*  $p < 0.001$  compared to the conditions of treatment with 6-OHDA in nonconditioned medium. **b** Representative Hoechst staining of CGNs cultured for 24 h in the presence of 20  $\mu$ M 6-OHDA. Note the decrease in cell density caused by exposure to the neurotoxin and the protection of the medium conditioned by microglia for 48 h. Scale bar = 30  $\mu$ M.

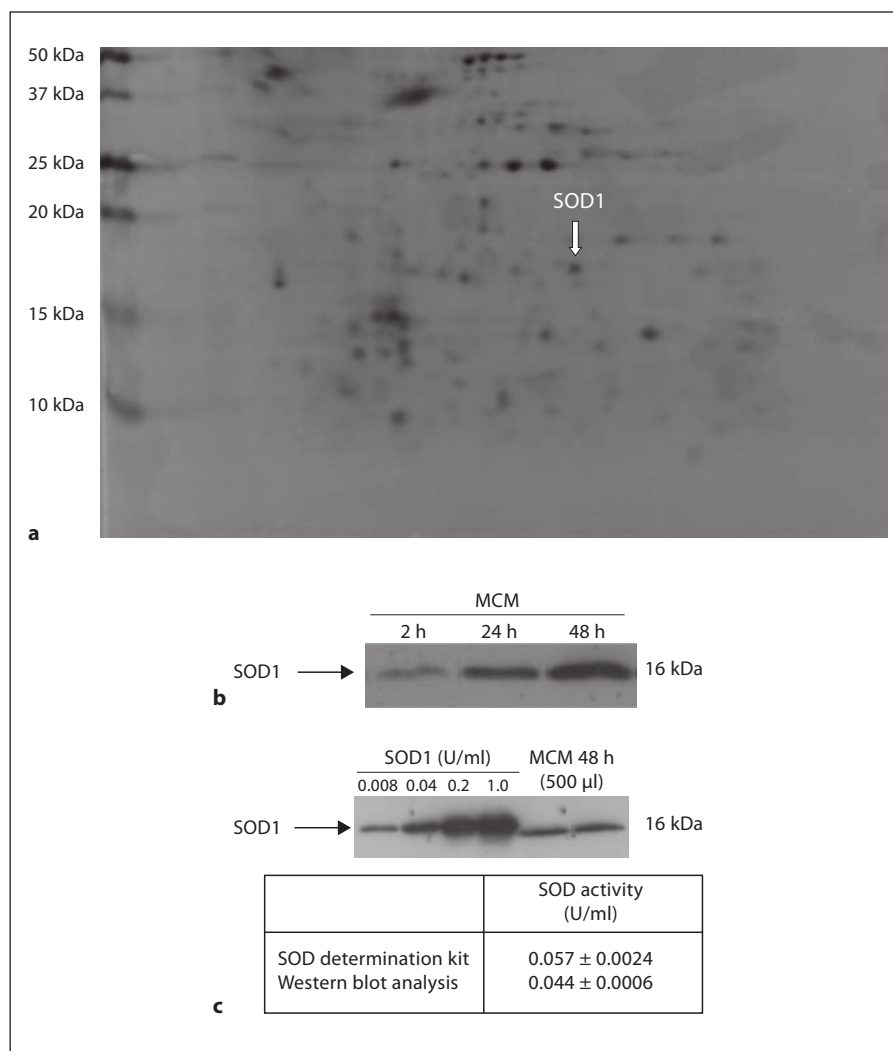


b

enzyme in the neuroprotective medium (48-hour MCM), we measured its catalytic activity using a commercially available SOD-assay kit (fig. 2c). In parallel, we compared the intensity of Western blot bands derived from a known volume of the 48-hour MCM (i.e. 500  $\mu$ l) with the intensity of bands obtained by loading known amounts of recombinant SOD1 (fig. 2c). Both methods yielded similar values, corresponding to 0.04/0.05 U/ml in the 48-hour MCM which is around 10 ng/ml (according to the activity/concentration relationship given by the producer of the recombinant SOD1). As the release and accumulation of SOD1 in the conditioned medium appeared to be constitutive, i.e. related to the normal partially activated in vitro microglial conditions, we tested

whether a more activated state could increase SOD1 microglial secretion. Activation of microglia with LPS, IFN- $\gamma$ , or both did not increase the SOD1 level in a medium conditioned for 24 h (fig. 3a) and it did not change the intracellular level of the enzyme, while, as expected, it did induce a strong increase in iNOS expression, thus demonstrating the actual activated state of microglia under these conditions (fig. 3a). SOD1 secretion should be atypical since this protein lacks the secretion signal sequence at its amino terminal polypeptide chain. However, its extracellular release has been well documented in several cell types through a mechanism involving a vesicle-related and, in some cases, ATP-dependent pathway [30–36]. Since ATP has been shown to stimulate the

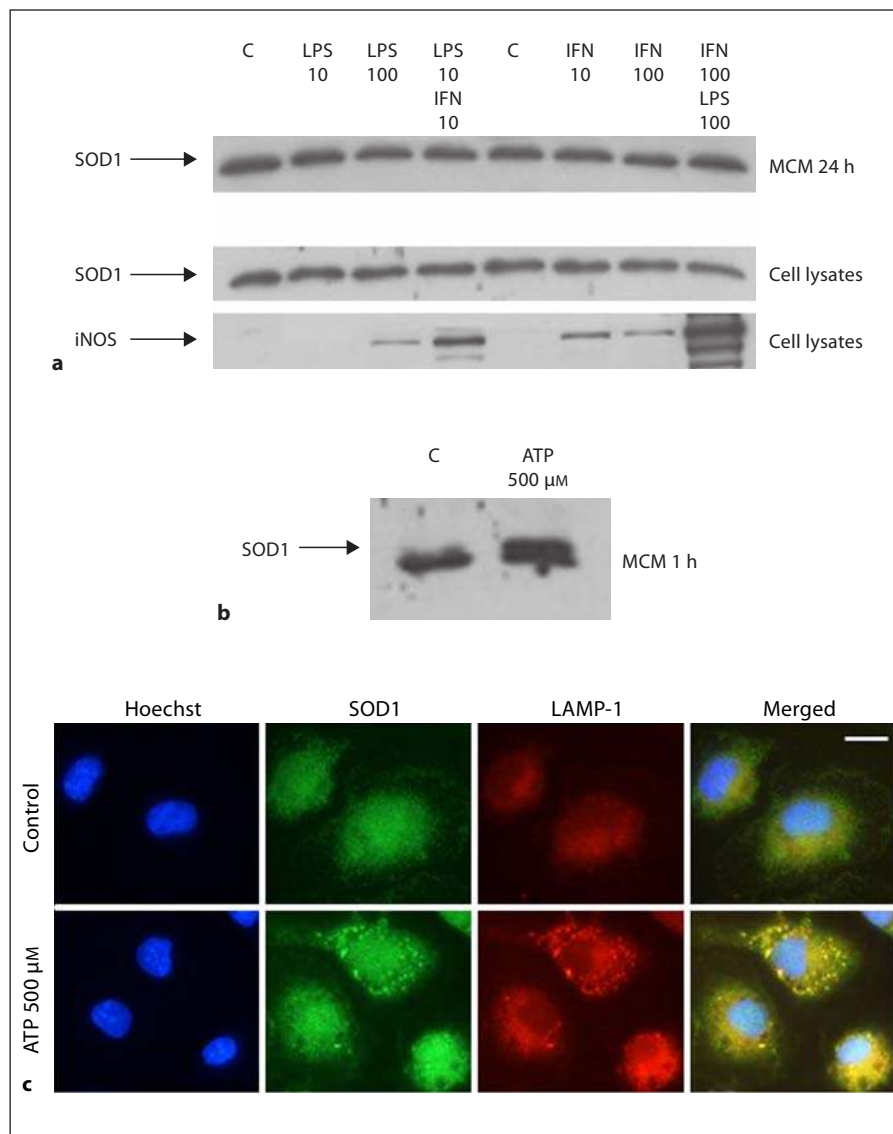
**Fig. 2.** Identification of SOD1 release and accumulation in the MGM. **a** Microglial cell cultures were maintained for 48 h in serum-free medium. Then, 60 ml of the medium (from  $60 \times 10^6$  microglial cells) were purified from BSA and 400  $\mu$ g of total proteins were separated according to their isoelectric point followed by standard SDS-PAGE, as described in Materials and Methods. A representative Coomassie Blue-stained 2-D gel is illustrated and the arrow indicates the position of the spot identified as SOD1. **b** Representative Western blot analysis of SOD1 protein expression in different media conditioned by microglia for 2, 24, and 48 h. **c** Determination of SOD1 units in 48-hour MCM through two different methods: comparative analysis of SOD1 protein expression (Western blot) in 48-hour MCM, compared to different known units of SOD1 from human erythrocytes, and determination of SOD activity using a specific kit.



extracellular release of the lysosomal compartment content from microglia [37, 38], we investigated the effect of this purine on microglial SOD1 secretion. As shown in figure 3b, 1 h of stimulation with 500  $\mu$ M ATP increased the level of SOD1 in culture medium, thus suggesting that SOD1 could be released by microglial cells through a lysosomal secretory pathway. To further support this hypothesis, we studied SOD1 co-localization with LAMP-1, a vesicular marker of the lysosomal secretory pathway, using an immunocytochemical approach [39]. Therefore, we performed double immunofluorescent staining with an antibody against SOD1 and one against LAMP-1. This analysis demonstrated that the finely granular and partially overlapping cytoplasmic distribution of the two proteins in unstimulated microglia (fig. 3c, upper panel) shifted towards a clear co-localiza-

tion in secretory granules after 1 h of ATP stimulation (fig. 3c, lower panel).

In order to demonstrate a relationship between SOD1 accumulated in MCM and neuroprotection, we added different amounts of exogenous SOD1 to a nonconditioned medium, thus testing it for neuroprotection in 6-OHDA-challenged CGNs. Exogenously added SOD1 was significantly neuroprotective under these conditions, although at concentrations  $\sim$ 40 times higher than those estimated in MCM (fig. 4a). Contradictory previously published results [40, 41] suggested that SOD1 could inhibit the spontaneous process of 6-OHDA auto-oxidation, leading to generation of superoxide radicals and quinones putatively responsible for neurotoxicity. In order to ensure that, in our conditions, SOD1 neuroprotection was a true biological effect and not simply the

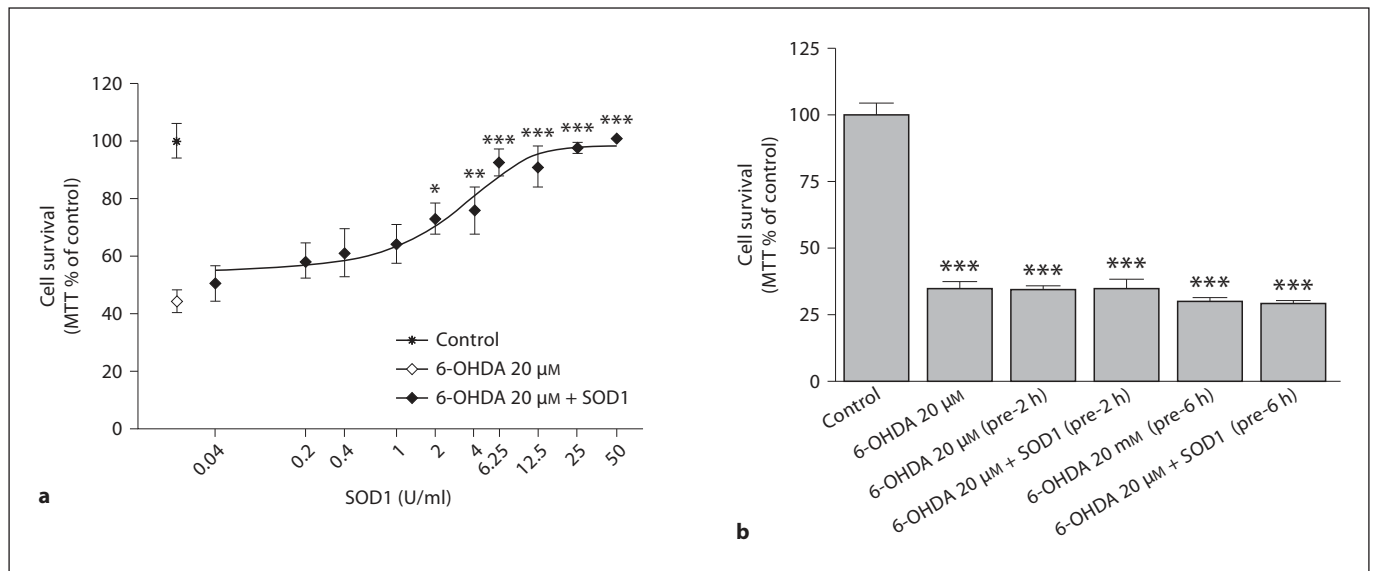


**Fig. 3.** Constitutive release of SOD1 by microglial cells is not modulated by pro-inflammatory agents but is increased by ATP. **a** SOD1 protein expression was analyzed using Western blot in different conditioned media and in cell lysates of microglia cell cultures treated or co-treated for 24 h with different concentrations of LPS and IFN- $\gamma$ . The increase in the intracellular expression of iNOS was also analyzed to confirm microglial activation. **b** The microglial release of SOD1 was detected via Western blot in the medium of microglial cell cultures stimulated for 1 h with 500  $\mu$ M ATP. **c** Immunocytochemical analysis of SOD1 and LAMP-1 in microglial cell cultures in basal conditions or after stimulation with 500  $\mu$ M ATP for 1 h. Note how treatment induces a much more evident co-localization of SOD1 with LAMP-1 in vesicles of the lysosomal secretory pathway. Scale bar = 10  $\mu$ M. C = Control.

result of a toxin concentration decrease in the medium due to a purely chemical effect, we performed a specific control experiment. Nonconditioned medium containing 20  $\mu$ M 6-OHDA was pre-incubated for 2 or 6 h with a strongly neuroprotective concentration of SOD1 (12.5 U/ml), and then this medium was passed through a filter with a molecular cutting of 10 kDa in order to trap SOD1, but not 6-OHDA, thus leaving the toxin in the medium. The resulting medium maintained the same toxic potency of a medium to which 20  $\mu$ M 6-OHDA has been freshly added (fig. 4b). These data showed that pre-incubation with SOD1 did not result in dampened neurotoxicity of 6-OHDA attributable to an SOD1-depend

ent chemical modification, thus demonstrating that SOD1-mediated neuroprotection was not due to a non-specific decrease in 6-OHDA neurotoxicity but was the result of an unambiguous neuroprotective effect of this protein.

In an attempt to demonstrate the specificity of the SOD1 neuroprotective effect in the MCM, we tried to deplete the medium by knocking down the SOD1 expression in microglial cells through an siRNA approach. With Western blot analysis we observed a partial decrease, but not complete abrogation, of SOD1 in the medium conditioned for 48 h by the siRNA-transfected microglial cells, as MCM maintained its neuroprotective



**Fig. 4.** Effect of exogenous SOD1 on neurotoxicity induced in CGNs by 6-OHDA. **a** Cell survival (MTT assay) of CGN cultures treated for 24 h with 20  $\mu$ M 6-OHDA in the presence or absence of different amounts of exogenous SOD1 (0.04–50 U/ml). Each point is the mean  $\pm$  SE of eight different experiments run in triplicate. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  compared to 6-OHDA

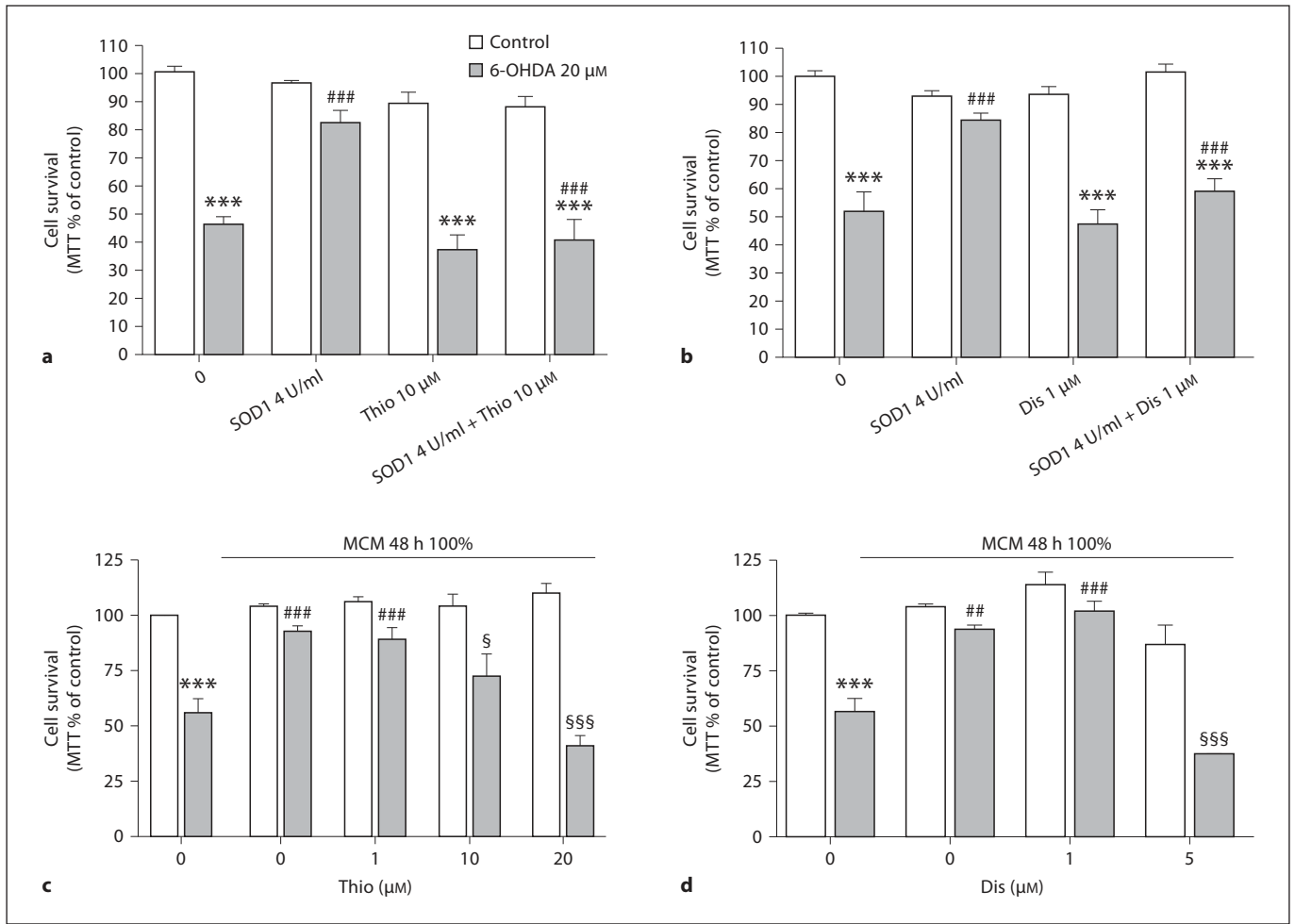
treatment. **b** Cell survival (MTT assay) of CGN cultures treated for 24 h with 20  $\mu$ M 6-OHDA freshly prepared or 20  $\mu$ M 6-OHDA pre-incubated for 2 h at 37°C with or without SOD1 (12.5 U/ml). Bars are the means  $\pm$  SE of four different experiments run in triplicate. \*\*\*  $p < 0.001$  compared to control conditions. Bonferroni's test after ANOVA.

activity (online suppl. fig. 1; for all online suppl. material, see [www.karger.com/doi/10.1159/000337115](http://www.karger.com/doi/10.1159/000337115)). Considering that silencing its expression was not an adequate method to block SOD1 in our experimental model, we decided to use a pharmacologic approach to interfere both with the catalytic activity of SOD1 and with the ability of this protein to regulate target cells through receptor interactions. To this aim, we used two SOD1 inhibitors, ammonium tetrathiomolibdate and disulfiram [42–44], and we showed that both of them were able to fully reverse the effect of neuroprotective concentrations of exogenous SOD1 (fig. 5a, b). Moreover, these SOD1 inhibitors substantially decreased the neuroprotective potency of 48-hour MCM against 6-OHDA neurotoxicity (fig. 5c, d), thus confirming the neuroprotective role of SOD1 in the MCM.

In view of previous evidence that released SOD1 could regulate cellular calcium levels in a paracrine way through receptor-mediated interactions in neuroblastoma cells [45–47], we sought to analyze the involvement of a similar mechanism in the neuroprotective action of the protein and of the conditioned medium containing it. To this end, a microfluorometric analysis of intracellular calcium dynamics was performed in 2 mM  $[Ca^{2+}]_o$  by measur-

ing changes in the fluorescence emission ratio of fura-2-loaded CGNs. Extracellular challenge with SOD1 (6.25 U/ml) promoted a significant rise in  $[Ca^{2+}]_i$  (fig. 6). Notably, 90% of all of the granular cells tested responded to SOD1, and the viability of responding and nonresponding cells was checked in every experiment by the application of high  $K^+$  (50 mM) external solution (not shown). The  $[Ca^{2+}]_i$  responses were variable both in amplitudes and dynamics depending on the presence of the extracellular calcium. The most representative behavior in calcium dynamics in response to SOD1 was a significant increase in  $[Ca^{2+}]_i$  signals occurring with a time lag of  $\sim 2$  min (fig. 6a). The  $[Ca^{2+}]_i$  responses were always dependent on  $[Ca^{2+}]_o$  presence because omission of extracellular  $Ca^{2+}$  caused a rapid reduction of  $[Ca^{2+}]_i$  signals as a result of administration of SOD1 (fig. 6a). This result was corroborated by the fact that when perfusion of neurons started in SOD1-containing,  $Ca^{2+}$ -free medium there was no response until  $Ca^{2+}$  was added to the medium (fig. 6b). Responses similar to those described for the addition of exogenous SOD1 were elicited by SOD1-containing medium conditioned for 48 h by microglia (fig. 6c, d). Conversion of fluorimetric data into direct evaluation of intracellular calcium levels and statistical



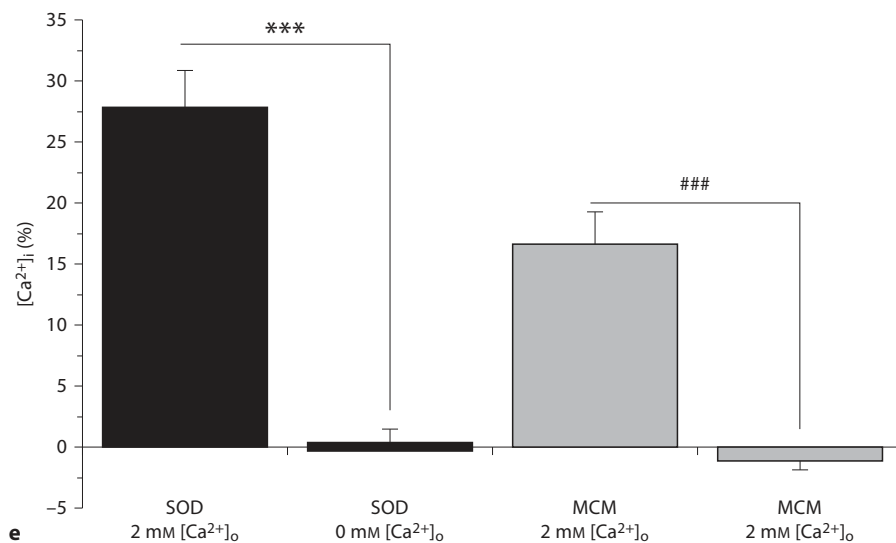
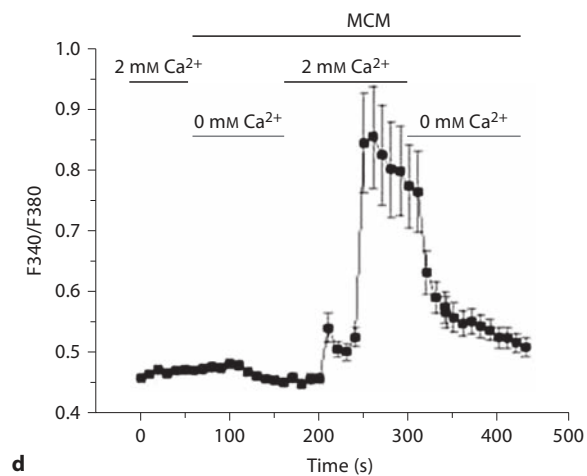
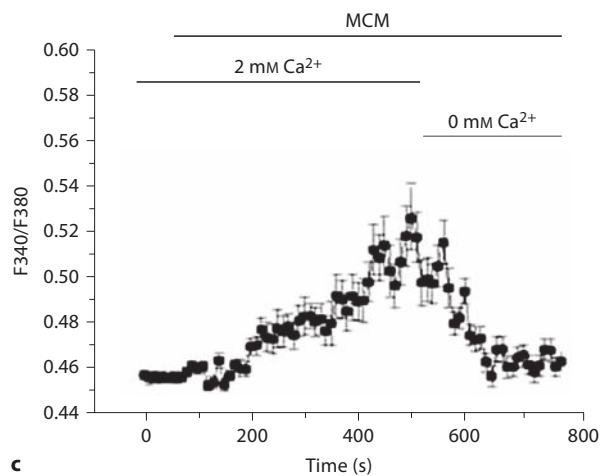
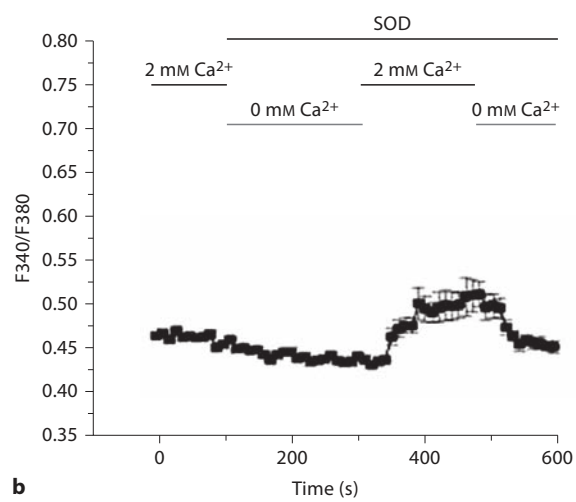
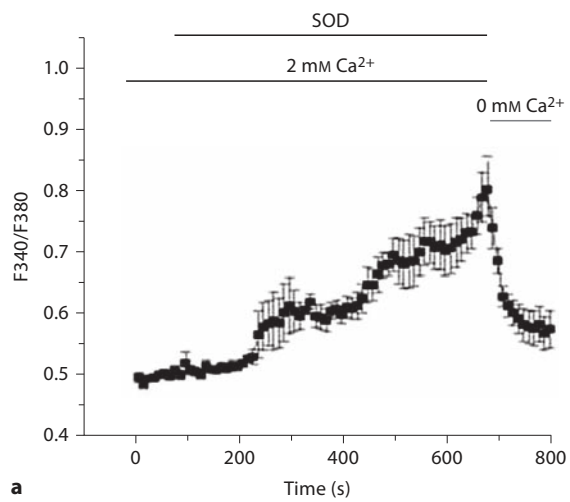


**Fig. 5.** Two inhibitors of superoxide dismutase, ammonium tetrathio-molybdate (Thio) and disulfiram (Dis), block the neuroprotective effect of exogenous SOD1 and MCM. Thio 10 μM (a) or Dis 1 μM (b) was added to CGN cultures treated for 24 h with 20 μM 6-OHDA in the presence or absence of exogenous SOD1 (4 U/ml). Cell survival was analyzed via MTT assay. Bars are the means ± SE of four different experiments run in triplicate. \*\*\* p < 0.001 compared to its own control condition; # p < 0.01 compared to the 6-OHDA condition (with neither SOD1 nor the SOD inhibitor); ### p < 0.001 compared to the parallel SOD1 condition (with 6-OHDA and SOD1 at the same concentration but without the

SOD inhibitor). Thio (10 or 20 μM) (c) or Dis (1 or 5 μM) (d) was added to the cultures of CGNs treated for 24 h with 20 μM 6-OHDA in presence or absence of medium conditioned for 48 h by unstimulated microglial cell cultures (100% of the culture media). Cell survival was analyzed through MTT assay. Bars are the means ± SE of four different experiments run in triplicate. \*\*\* p < 0.001 compared to control conditions; # p < 0.01, ### p < 0.001 compared to 6-OHDA conditions (with neither 48-hour MCM nor the SOD inhibitor); § p < 0.05, §§§ p < 0.001 compared to the condition with 6-OHDA and 48-hour MCM (without the SOD inhibitor). Bonferroni's test after ANOVA.

analyses confirmed that SOD1-induced calcium signals were significantly higher than at baseline and dependent upon  $[Ca^{2+}]_o$  (fig. 6e). Altogether, these data suggest that perfusion with SOD1 promotes complex  $[Ca^{2+}]_i$  signals that are dependent on the presence of  $[Ca^{2+}]_o$ . Interestingly, administration of SOD1 at different concentrations, from 0.75 to 12.5 U/ml, was able to induce a dose-dependent increase in  $[Ca^{2+}]_i$  (fig. 7a). In order to verify

whether the effect of SOD1 on calcium dynamics depended on its catalytic activity, we performed a control experiment in which an SOD1 mimetic substance, MnTMPyP, possessing similar catalytic activity failed to affect the calcium dynamics (data not shown), similarly to what previously shown in neuroblastoma cells [45]. On the basis of these results, we supposed that if the calcium flux was important for neuroprotection, interfering with it



6

could abrogate the neuroprotection provided by SOD1 itself, either exogenously added or endogenously present in the conditioned medium. This was actually the case since the extracellular  $\text{Ca}^{2+}$  chelator EDTA, which has no effect on the Cu-Zn site of SOD1 [48], significantly reverted neuroprotection mediated by exogenous SOD1 and by 48-hour MCM (fig. 7b).

To investigate the specificity of SOD1 protection against different forms of neurotoxicity, we added the recombinant enzyme at high concentrations to CGNs exposed to two well-known and widely used models of neurotoxicity, i.e. a shift from high to low potassium medium or chronic exposure to glutamate. While, in agreement with our previous results [16, 19], 48-hour MCM significantly protected CGNs in both conditions, addition of exogenous SOD1 to a nonconditioned medium had no significant neuroprotective effects (fig. 8a, b). These results confirmed that the neuroprotective effect of SOD1 was specific towards 6-OHDA-induced neurodegeneration and suggested that microglia-mediated neuroprotection could be exerted by multiple released factors with diverse effects on different types of neurodegeneration, further demonstrating the complexity of microglia-mediated neuroprotection.

## Discussion

In the present work, we started to characterize neuroprotective molecules produced and released by microglia by means of their identification through proteomic analysis of media conditioned by rat primary microglial cultures. Using this approach, we present here novel evi-

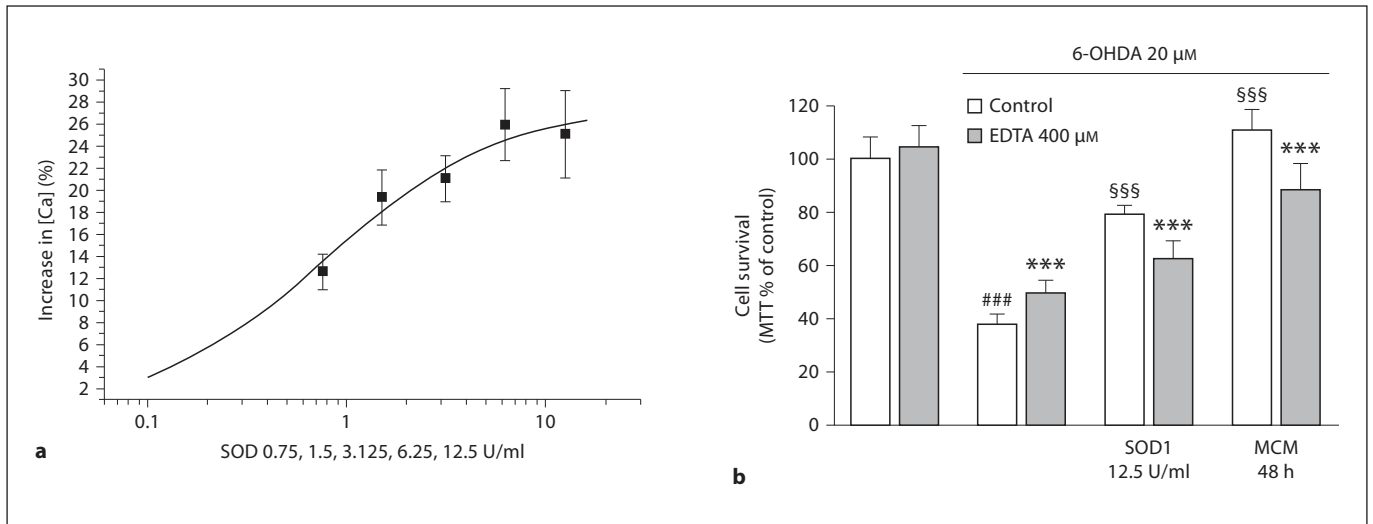
**Fig. 6.** SOD1 and MCM promote  $[\text{Ca}^{2+}]_i$  elevation in cultured CGNs. Representative variations in  $[\text{Ca}^{2+}]_i$  measured in fura-2-loaded cultured CGNs stimulated with 6.25 U/ml SOD1 in the presence (a) and absence of 2 mM  $[\text{Ca}^{2+}]_o$  (b). Representative  $[\text{Ca}^{2+}]_i$  traces measured upon MCM challenge in the presence (c) and absence of 2 mM  $[\text{Ca}^{2+}]_o$  (d). The traces are representative of 10–15 cells of each experimental group studied in at least three different experimental sessions. e Histogram of the mean increase in maximal  $[\text{Ca}^{2+}]_i$  rise with respect to the basal level upon exposure to 6.25 U/ml SOD1 (d) and MCM with and without  $[\text{Ca}^{2+}]_o$ . The results are reported as intracellular calcium increases (% peak/basal). Data are expressed as the means  $\pm$  SE of several cells in the various conditions. Triple marks indicate the statistical analysis performed with Student's t test. \*\*\*  $p < 0.001$  compared the effect of SOD1 on  $[\text{Ca}^{2+}]_i$  to  $[\text{Ca}^{2+}]_o$  conditions; ###  $p < 0.001$  compared the effect of MCM on  $[\text{Ca}^{2+}]_i$  to  $[\text{Ca}^{2+}]_o$  conditions.

dence that a neuroprotective protein accumulated in the MCM is the enzyme SOD1, which we show protected CGNs from death caused by the neurotoxin 6-OHDA. We confirmed the neuroprotective action of SOD1 by adding the exogenous protein to a nonconditioned medium and by inhibiting it through SOD1 antagonists. SOD1 neuroprotective action was mediated by increased cell calcium from an external source, and full neuroprotection depended on the maintenance of extracellular free calcium levels.

The identification of microglia-released molecules through the proteomic dissection of 'microglia secretoma' in culture conditions, which are close to the physiologic situation, is essential to better understanding the neuroprotective properties of these cells and may lead to discovery of new neuroprotective microglia-secreted molecules [49–52]. In recent years, several studies have focused on strategies of immunomodulation to specifically increase neuroprotective phenotypes of microglial cells [53–56], mainly through the release of neuroprotective molecules, whose identification and characterization could be very important for developing novel therapies [8, 57]. Previous proteomic studies [49, 50] have detected SOD1 among the proteins released by microglia, even following activation. While superoxide dismutase is usually released by cells in its SOD3 isoform [58, 59], also SOD1 is released by several cell lines [31–34]. Moreover, high concentrations of SOD1 may protect neurons from some toxic insults [60–66]. Here we show that SOD1 is produced and released by microglia and is a neuroprotective factor against 6-OHDA neurotoxicity in CGNs.

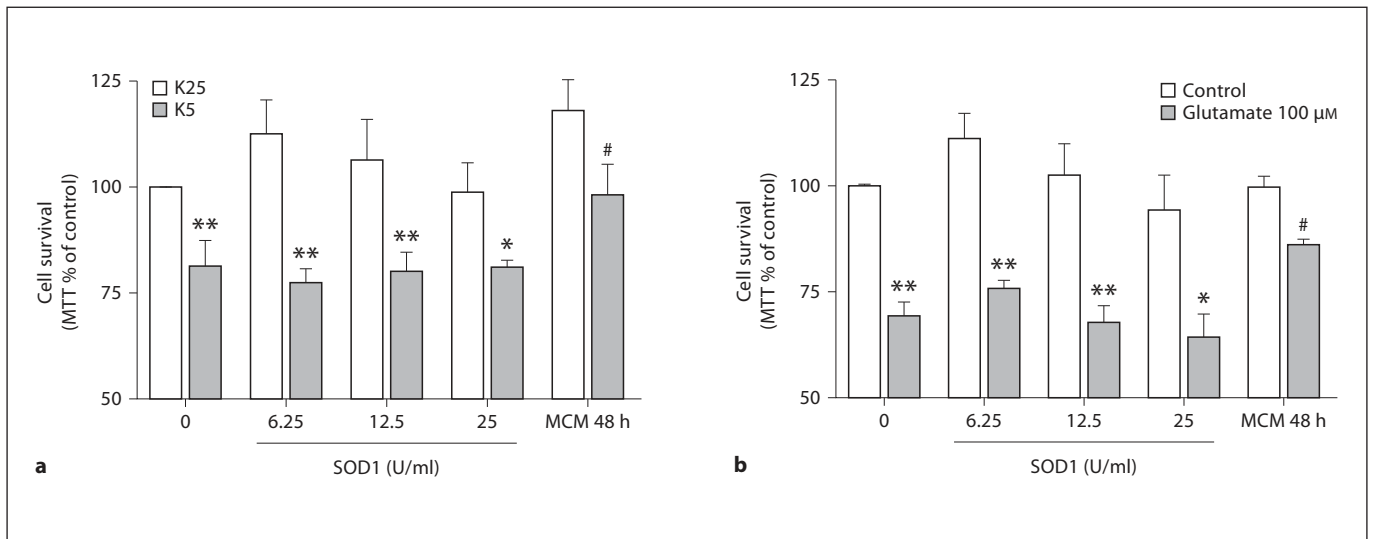
We have recently succeeded in identifying TGF- $\beta$ 2, a known regulator of neuronal survival [67], as an active neuroprotective molecule released by microglia, and other groups have found additional factors [20, 23, 68, 69].

From our data, SOD1 appears to be constitutively released by cultured microglia, as it accumulates in the medium in the absence of experimentally induced activation. Stimulation for 24 h with known pro-inflammatory activators of microglia did not affect SOD1 production and release. This lack of effect on SOD1 agrees with previous data showing that pro-inflammatory cytokines stimulate expression of SOD2 and SOD3, but not SOD1, in microglia [70, 71]. Interestingly, we demonstrate here that ATP increases the constitutive release of SOD1 in the medium, in agreement with a general modulatory response of microglia to this nucleotide [72, 73]. Release of SOD1 has been demonstrated to be ATP dependent through a microvesicle pathway in neuronal cell lines [34], and secretion of several proteins occurs



**Fig. 7.** SOD1 promotes [Ca<sup>2+</sup>]<sub>i</sub> elevation in cultured CGNs and the extracellular Ca<sup>2+</sup> chelator EDTA decreases the neuroprotective effect of both MCM and SOD1 on 6-OHDA neurotoxicity. **a** Dose-dependent effect curve of SOD1 on [Ca<sup>2+</sup>]<sub>i</sub> increase at different concentrations (0.75, 1.5, 3.125, 6.25, and 12.5 U/ml). The results are reported as intracellular calcium increases (% peak/basal) and are representative of 25–30 cells of each experimental group studied in at least three different experimental sessions.

The plot was fitted to the equation  $y = \max \cdot x / (x + k)$ . **b** MTT assay on CGN cultures treated for 24 h with 20 μM 6-OHDA in the presence or absence of exogenous SOD1 12.5 U/ml, 48 h MCM, and 400 μM EDTA. Bars are the means ± SE of 6–8 experiments; ### p < 0.001 compared to the control without 6-OHDA; SSS p < 0.001 compared to the control with 6-OHDA; \*\*\* p < 0.001 compared to its own control with 6-OHDA.



**Fig. 8.** Effect of exogenous SOD1 on different models of neurotoxicity induced in CGCs by a shift to low potassium and glutamate excitotoxicity. **a** Cell survival (MTT assay) of CGN cultures shifted to low potassium for 24 h in the presence or absence of different amounts of exogenous SOD1 (6.25, 12.5, and 25 U/ml) or of medium conditioned for 48 h by unstimulated microglial cell cultures (100% of the culture media). Bars are the means ± SE of four different experiments run in triplicate. \* p < 0.05; \*\* p < 0.01 compared to its own control condition; # p < 0.05 compared to low

potassium conditions (without SOD1). **b** Cell survival (MTT assay) of CGNs cultures treated for 24 h with 100 μM glutamate in the presence or absence of different amounts of exogenous SOD1 (6.25, 12.5, and 25 U/ml) and of medium conditioned for 48 h by unstimulated microglial cell cultures (100% of the culture media). Bars are the means ± SE of four different experiments run in triplicate. \* p < 0.05; \*\* p < 0.01 compared to its own control condition; # p < 0.05 compared to glutamate conditions (without SOD1). Bonferroni's test after ANOVA.

through a lysosomal secretory pathway in microglial cells [37, 38]. The evidence here presented that microglial ATP exposure results in a rapid increase in SOD1 localization in secretory vesicles and in the parallel increase in SOD1 released in the medium strongly argues in favor of this noncanonical secretion of the enzyme by microglia cells.

By adding exogenous SOD1 to a nonconditioned medium, even at concentrations remarkably higher than those present in the conditioned medium, we have reproduced the neuroprotective effect on 6-OHDA insult to CGNs. This raises the obvious question of the actual contribution of the low SOD1 concentration present in the conditioned medium to its neuroprotective action. Explanation of this apparent contradiction likely resides in the fact that the low SOD1 concentration present in the conditioned medium could cooperate with other neuroprotective factors, such as the ones mentioned in the introduction (TGF- $\beta$ 2, apolipoprotein E, and plasminogen), to protect neurons [21, 22, 27]. Consequently, it is not surprising that when SOD1 is added as the only neuroprotective molecule to a nonconditioned medium higher concentrations are required. In addition, the SOD1 increase in the medium conditioned for 24 h by primary microglia transfected with plasmids expressing human wild-type SOD1 (see online suppl. fig. 2) does not show any improvement in neuroprotection compared to the 24-hour standard conditioned medium (fig. 1a), thus confirming that the amount of protein normally released by microglia is enough to grant protection. Conceivably, once proteomic studies have definitely disclosed the pattern of neuroprotective molecules released by microglia in the medium, it could be possible to design specific mixtures of molecules granting neuroprotection at individually low concentrations through an additive/synergic effect. In this respect, it is relevant to consider that exogenous SOD1, even at very high concentrations, is not able to protect CGNs from neurotoxicity caused by other neurotoxic agents besides 6-OHDA. Therefore, the identification of other microglia-released factors involved in neuroprotection will likely contribute to explaining why other types of neurotoxicity (a shift of CGNs to nondepolarizing conditions or excitotoxicity) are significantly reverted by MCM, but not by exogenous SOD1. Differences in SOD1 neuroprotection against different toxic stimuli are in line with previous works that have or have not found neuroprotective effects of SOD1 in nonconditioned media, sometimes using very high amounts of exogenously added protein [60–66]. To further complicate this situ-

ation, it has to be considered that SOD1 may act on target cells both through its catalytic activity and through receptor interaction [46, 47]. In the present study, we started to characterize the mechanism of neuroprotection mediated through SOD1. We found that SOD1-mediated neuroprotection depends on increased cellular calcium from an external source. Furthermore, we obtained preliminary evidence that this mechanism does not depend on the protein catalytic activity but it could be attributed to a receptor-mediated mechanism, as previously observed in neuroblastoma cells [45]. This mechanism, together with the fact that there is an SOD1 concentration-dependent increase in  $\text{Ca}^{2+}$  entry into CGNs and a concomitant decrease in neuroprotection in the presence of the  $\text{Ca}^{2+}$  chelator EDTA in the medium, strongly suggests that SOD1 neuroprotection is related to a  $\text{Ca}^{2+}$  increase from an extracellular source. This knowledge could pave the way for future steps aimed to fully characterize the cellular neuroprotective pathway elicited by SOD1. Moreover, identification of the intracellular  $\text{Ca}^{2+}$  increase as a mechanism involved in SOD1-mediated neuroprotection could also explain the lack of an SOD1 neuroprotective effect against other types of neurotoxic stimuli for CGNs, i.e. the shift to nondepolarizing conditions and the excitotoxicity of glutamate, as shown here. In fact,  $\text{Ca}^{2+}$  entry from the extracellular compartment is limited if the neuronal membrane is not depolarized, as occurs in the first type of neuronal death mentioned above, since in this condition both voltage-sensitive  $\text{Ca}^{2+}$  channels and NMDA receptors, which are the main ways of entrance for  $\text{Ca}^{2+}$  in these cells, are inactive [74]. Instead, it could be supposed that SOD1 is not neuroprotective against glutamate excitotoxicity, as this type of neurodegeneration is largely attributable to an excessive increase in the intracellular calcium levels leading to a condition of calcium overload that may explain why the  $\text{Ca}^{2+}$ -mediated neuroprotection of SOD1 cannot be effective in this situation [75].

In order to further demonstrate the SOD1 neuroprotective function in MCM, we tried to perform an siRNA knockdown of its expression, but we only obtained a decrease that was not sufficient to reduce the MCM neuroprotection. This is not surprising as cultured microglia are obtained from mixed glial primary cultures and, once detached, microglial cells are able to maintain their phenotypic features and to be healthy for not more than 48 h in a serum-free medium like the one we use for MCM. In contrast, it has been previously demonstrated that siRNA knockdown needs more time to completely

silence the expression of the target gene, i.e. at least 4/5 days after transfection [76–78], a timing that is not compatible with our experimental system. Thus, either a slight decrease in SOD1 expression through partial silencing or an increase through transfection with plasmids expressing human wild-type SOD1 (see above) does not show any change in MCM neuroprotection. Therefore, we used SOD1 inhibitors interfering either directly with Cu<sup>2+</sup> bound to the enzyme [43] or indirectly through the action on the Cu<sup>2+</sup> chaperon protein [44] to abrogate both the neuroprotective effect of SOD1 exogenously added to a nonconditioned medium or endogenously present in the conditioned medium. By decreasing the Cu<sup>2+</sup> loading on SOD1, these inhibitors not only interfere with its enzymatic activity but also affect SOD1 structure and dimerization [79]. As a result, in agreement with the lack of an SOD1 mimetic neuroprotective effect, the inhibitor-mediated reduction of both exogenous SOD1 and MCM neuroprotection could be determined by a possible change in SOD1 structure due to Cu<sup>2+</sup> chelation and by the consequent alteration of SOD1 interactions with receptors [45–47].

In conclusion, the present results identify SOD1 as a potential neuroprotective molecule produced and secreted by microglia. Relevant to the present model, in which toxicity was delivered to CGNs through the dopaminergic toxin 6-OHDA, are previous results demonstrating that SOD1 gene transfer protects cultured nigral dopaminergic neurons from the same toxin [80]. Experiments could thus be devised in which microglia are forced to overproduce SOD1 to protect nigro-striatal dopaminergic neurons in models of experimental Parkinson-like neurodegeneration. This could be obtained through specific infection of microglia with viral vectors [8, 81] and may be translated to therapy by finding ways to stimulate the neuroprotective microglia phenotype in order to increase the constitutive production and release of neuroprotective factors by microglia, including SOD1 [53–57].

Mutations of SOD1 are found in familial cases of ALS and it has been demonstrated that protein mutations in microglia/macrophage cells are directly implicated in the disease [82–85]. Our novel evidence that normal SOD1 is constitutively released by noninflammatory microglia and protects neurons may be relevant to better understanding the pathogenic/protective role of this protein, as well as of its release by microglia in ALS. A recent study showed that, in SOD1-related ALS mice, repopulation of the brain with microglia not expressing the pathogenic form of mutated SOD1 slowed down motor neuron de-

generation and prolonged lifespan [82]. It is equally interesting that intraspinal infusion of wild-type SOD1 in a mouse model of ALS was able to reduce the damage in affected segments of the spinal cord, slow down disease progression, and prolong the lifespan of animals [35]. These data strongly support the hypothesis that, in this neuropathology, there is not only a gain in microglial neurotoxic potential but also a loss of microglia physiological neuroprotective function [86]. In this framework, our preliminary results (see online suppl. fig. 2) obtained by transfecting primary microglia with plasmids expressing either human SOD1 wild-type or the pathogenic G93A mutation which is linked to inherited ALS [24] demonstrate that mutated SOD1 is released by microglia and that the medium conditioned by microglia expressing mutated SOD1 is not neurotoxic by itself, but its neuroprotective activity seems to be partially reduced, at least in this model of neurotoxicity. This is not surprising as it has already been suggested that SOD1 transgenic microglia have a reduced neuroprotective function [87]. However, further experiments must be performed using media conditioned by microglia for different times and by testing these media on different types of neurotoxicity, even in different neuronal types. Therefore, on the basis of our in vitro results and in view of possible therapeutic translations, novel approaches could be devised to test neuroprotection related to microglia-secreted molecules, including SOD1, in animal models of neurodegenerative diseases.

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