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# Neuroprotection of microglia conditioned media from apoptotic death induced by staurosporine and glutamate in cultures of rat cerebellar granule cells

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ABSTRACT

Microglia, the immune cells of the mammalian CNS, have often been indicated as dangerous effector cells for their activation in response to traumatic CNS injuries or immunological stimuli and for their involvement in many chronic neurodegenerative diseases. Recently, several in vitro and in vivo studies have emphasized that microglial activity is essential in promoting neuronal survival. We have tested the efficacy of media directly conditioned by microglia or conditioned by microglia after having been exposed to apoptotic neurons, towards neuroprotection of rat cerebellar granule cells (CGCs) challenged with staurosporine or glutamate. Apoptotic death of CGC caused by staurosporine, as well as by a mild excitotoxic stimulus delivered through sub-chronic glutamate treatment, was significantly counteracted by microglia conditioned media. On the other hand, an acute excitotoxic insult delivered through a short pulse of glutamate exposure in the absence of magnesium and resulting in a mix of apoptotic and necrotic death was only marginally counteracted by microglia conditioned media. The present results extend the available information regarding the neuroprotective role of microglia and support the usefulness of employing the culture approach for perspective identification of neuroprotective factors released by these cells. Furthermore, the use of media previously exposed to apoptotic neurons to elicit the neuroprotective response of microglia, indicate the feasibility to re-create also in the isolated culture conditions, at least some of the elements at the basis of neuron/microglia cross-talk.



Functioning of the CNS depends on a continuous and reciprocal exchange of molecular signals between neurons and microglia [1,29,33,35]. This interactive "cross-talk" starts during development, continues in the adult brain and is enhanced in conditions of brain injury or infection. Neurons influence microglia functions through direct cell-to-cell interactions and release of diffusible mediators, able to control proliferation, migration and, in a more general way, the activation state of microglia [25,30–32]. Conversely, during development microglia interact with neurons from early stages of differentiation through secretion of many neurotrophic factors and regulatory molecules [5,11,23]. In adult healthy brain microglial cells are in a resting ramified state controlled by neurons [4,12] while in response to traumatic CNS injuries or immunological stimuli they are readily activated [2,14,16,32]. For many years, microglial activation has been considered a harmful mechanism that may enhance neuronal injury [10]. Chronic microglial activation has been involved in many neurodegenerative diseases such as Alzheimer's disease [38], Parkinson's disease [15,39], Huntington's disease [34] and prion disease [7]. Recent studies have highlighted a neuroprotective function of microglia, based on its involvement in axonal regeneration in PNS, axonal regrowth in SNC [18] and for the release of anti-inflammatory cytokines (TGF-β1) in transient middle cerebral artery occlusion [19]. In addition to in vivo data, some studies in culture have demonstrated that microglia produce substances that protect neurons from damage and death both in conditions of co-culture and upon exposure of neurons to media previously conditioned by microglia [26–28]. In a former study, we demonstrated that a medium previously conditioned by microglia for 48 h protects cerebellar neurons from apoptotic death caused by the shift of the medium from depolarizing to non-depolarizing conditions [28]. Furthermore, when the medium to be conditioned by microglia had been previously exposed to apoptotic neurons, a shorter (24-h) exposure to microglia was sufficient for neuroprotection, thus suggesting that substances produced by apoptotic neurons enhanced the neuroprotective function of microglia [28].

With the present report we extend information on the neuroprotective role of microglia conditioned media by demonstrating neuroprotection of cerebellar neurons challenged with different apoptotic stimuli and absence of significant protection when neurons are challenged with a largely non-apoptotic death stimulus.

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Microglial cells were prepared from cerebral cortex of newborn Wistar rats as previously described [21]. Briefly, brain tissue was cleaned from meninges, trypsinized for 15 min, and after mechanical dissociation cell suspension was washed and plated on poly-L-lysine (Sigma, 10 μg/ml) coated flasks (75 $cm^2$). Mixed glial cells were cultured for 10–13 days in Basal Medium Eagle (BME, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Sigma) and 100 μM gentamicin sulphate (Sigma). Microglial cells were harvested from mixed glial cells cultures by mechanical shacking, resuspended in serum-free BME and plated on uncoated 40 mm dishes at a density of $1 \times 10^6$ cells/ml medium. Cells were allowed to adhere for 30 min, then washed to remove non-adhering cells and cultured in serum-free BME. After 48 h microglial conditioned medium (MCM) was collected, filtered through 0.22 μM filters, aliquoted and stored at −20 °C until used.

Primary cultures of rat cerebellar granule cells (CGCs) were prepared from 7-day-old Wistar pups as previously described [9]. Briefly, cells were dissociated from cerebella and plated on 40 mm dishes or in 24-well plates coated with 10 μg/ml poly-L-lysine at a density of $2 \times 10^5$ cells/$cm^2$ in BME supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 μM gentamicin sulphate and 25 mM $K^+$. After 16 h, 10 μM cytosine arabino-furanoside (Sigma) was added to avoid glial proliferation. Part of the cultures was used to prepare double conditioned medium (DCM), i.e. a medium exposed for 24 h to apoptotic neurons and subsequently conditioned for 24 h by microglia [28]. To this aim, CGC cultures were shifted after 7 DIV (days in vitro) for 24 h to serum-free BME at low potassium concentration (5 mM KCl) and the same medium was used as culture medium of microglial cells for additional 24 h.

After 8 DIV differentiated neurons were shifted to serum-free BME medium or to MCM or to DCM, all of them added with KCl to obtain a final $K^+$ concentration of 25 mM, and exposed to staurosporine (100 nM) or glutamate (100 μM) for 24 h. Using a different excitotoxic paradigm of acute exposure to glutamate, cultures were exposed for 15 min at room temperature to 100 μM glutamate in $Mg^{++}$-free Locke buffer to maximize the neurotoxic effect mediated by the NMDA receptor [6], before to be incubated for 24 h in the aforementioned media. After 24 h neuronal viability, death or survival was evaluated by different methods. The viability of CGC culture was evaluated by thiazolyl blue (MTT) assay [13]. This method is based on the conversion of the tetrazolium salt to a colored compound, a reaction that only occurs in viable cells since the chemical reaction is carried on by mitochondrial dehydrogenases. MTT was added to the culture medium to reach a final concentration of 0.1 mg/ml. Following 15 min of incubation at 37 °C the dark crystals formed were dissolved in 0.1 M Tris–HCl buffer containing 5% Triton X-100 and the absorbance was read at 570 nm in a Multiplate Spectophotometric Reader (Biorad). To quantify neuronal cell death, normal and condensed nuclei were counted after Hoechst stain. CGCs were fixed for 20 min with 4% paraformaldehyde in phosphate buffer, washed in PBS and incubated for 5 min at room temperature with 0.1 μg/ml Hoechst 33258. Cultures were observed and photographed with a fluorescence microscope using a 20× objective and count was performed on five randomly selected fields per dish. Quantitative evaluation of cell death was determined by calculating the percent ratio: condensed nuclei/condensed + normal nuclei [24]. In experiments based on acute excitotoxic pulse of glutamate, living cells were stained with fluorescein diacetate (FDA) and nuclei of dead cells with propidium iodide (PI). Cells were washed with Locke's solution, stained for 3 min at room temperature with the same solution containing 15 μg/ml FDA and 5 μg/ml PI, washed, observed and photographed with the fluorescence microscope. Experiments were approved by a local bioethical committee and were conducted in accordance with the Italian and European Community law on the use of experimental animals for research purposes. Statistical significance between different treatments was calculated through one-way analysis of variance (ANOVA) followed by post hoc comparison through Newman–Keuls test. A value of $P < 0.05$ was considered statistically significant.

Exposure to staurosporine resulted in apoptotic death of CGC, as revealed by the appearance of condensed nuclei in Hoechst-stained cultures (Fig. 1A). Through cell counting, it was determined that about 30% of cultured neurons were apoptotic by the end of the 24-h treatment and that MCM, and to a greater extent DCM, significantly protected CGC from the neurotoxic insult (Fig. 1B). A similar degree of neuroprotection of the media conditioned by microglia was assessed by using the MTT assay to evaluate cell viability (Fig. 1C).

Sub-chronic glutamate excitotoxicity obtained through continuous exposure of the cultures to 100 μM glutamate added to the

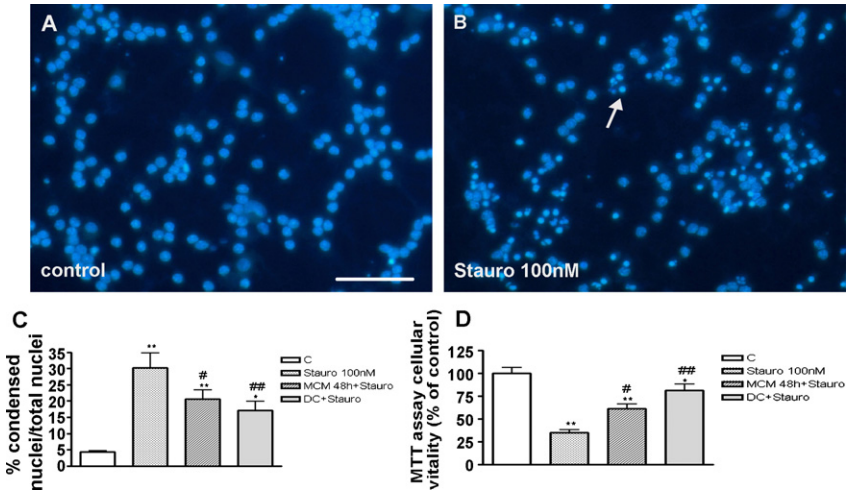


**Fig. 1.** Hoechst stain of control CGC at 8 DIV (A) or corresponding cultures treated for 24 h with staurosporine (B). Arrows point at condensed nuclei; calibration bar, 30 μm. (C) Cell count of condensed and total nuclei after Hoechst stain. Data were expressed as percentage of condensed nuclei over total stained nuclei. Bars are means ± S.E. from four to eight cultures for each condition. $^{**}P < 0.001$, $^{*}P < 0.01$ compared to control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to staurosporine in non-conditioned medium. Newman–Keuls multiple comparison test after ANOVA. (D) MTT assay on corresponding CGC cultures. Bars are means ± S.E. from four to eight cultures for each condition. $^{*}P < 0.05$, $^{**}P < 0.001$ compared to control; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ compared to staurosporine in non-conditioned medium. Newman–Keuls multiple comparison test after ANOVA.