Acta Biologica Hungarica 55 (1–4), pp. 273–285 (2004)

NEURON-MICROGLIA COMMUNICATION IN THE CNS OF THE FRESHWATER SNAIL *PLANORBARIUS CORNEUS**

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(Received: August 31, 2003; accepted: December 1, 2003)

The aim of the present study was to identify molecules that may be involved in neuron-microglia communication in the CNS of freshwater snail *Planorbarius corneus.* Messenger molecules are exchanged in normal and pathological conditions and we tried to identify some of them by immunocytochemistry on whole ganglia and cell cultures. In particular, we examined neurons and microglia for the expression of some cytokines, IL-1α, IL-1β, IL-6 and TNF-α and the neurotransmitter glutamate. These substances may be released by suffering or injured neurons and communicate to microglia the damaging event. Even microglia, on own turn, once activated, express and released the same or other substances in order to re-restablish the system homeostasis, depending on modalities and times of activation. We discuss the possibility that hyperactivated microglia can shift from neuroprotective to neurodegenerative. Moreover, we examined in neuron-microglia co-coltures the direct interaction effects in terms of neuronal survival and improved neurite regeneration.

Keywords: Neuron – microglia – cell communication – cytokines – glutamate – *Planorbarius corneus*

INTRODUCTION

In many invertebrates, glial cells constitute quantitatively the major component of the central nervous system (CNS) and different types of glia with specific functions have been characterized [23]. In *Planorbarius corneus,* a Gastropod Pulmonate, we found a type of glia we identified for the morpho-functional properties as "invertebrate microglia" [30, 31]. According to their role as primary immune effector cells of the nervous system, microglia respond in a rather stereotypic pattern to any type of event endangering the CNS, i.e. traumatic injuries or the presence of pathogens [10]. Once activated, these cells rapidly change in morphology by retracting processes and enlarging, start to proliferate and part of them become motile and migrate towards the site of damage. In the presence of extensive neuronal degeneration or

* Presented at the 10th ISIN Symposium on Invertebrate Neurobiology, July 5–9, 2003, Tihany, Hungary.

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infections agents, as a last step of activation, microglial cells transform into fully differentiated macrophages [13]. A further important aspect concerns the expression and secretion by activated microglia of both survival-promoting and toxic molecules, including cytokines, prostanoids, nitric oxide and growth factors [13, 20, 32] the repertoire and quantities of them depending on whether and how the cells are activated [38]. At the basis of microglia activation and subsequent response in terms of neuroprotection or neurotoxicity, it is thus evident that, in order to re-establish tissue homeostasis, there are reciprocal microglia-neuron interactions and a signalling system subserving them, either via diffusible molecules or via direct contact.

The aim of our research was to investigate by immunocytochemistry in a "simple" invertebrate model, the freshwater snail *P. corneus*, for the presence of putative communication molecules exchanged between neurons and microglia in conditions of normality or expressed following neuronal damage and chemical activation. For this study we used both whole ganglia and cell culture preparations. We put a special emphasis on signal molecules that might turn microglia from neuroprotective to neurodegenerative following hyperactivation like cytokines and glutamate.

MATERIALS AND METHODS

Animals

Adult specimens of the freshwater snail *Planorbarius corneus* (L.) (shell size 20–25 mm) were collected from a ditch near Modena (Italy) or sent from the Balaton Limnological Research Institute, kindness of Dr. L. Hernádi. The snails were kept under standard conditions in our laboratory aquatic facility (photoperiod: 8.00 a.m.–8.00 p.m., water temperature 20 ± 2 °C) and fed with boiled lettuce and marrows.

Activation experiments on whole ganglia and immunocytochemistry on sections

As a control, ganglionic rings from *P. corneus* adult specimens were dissected and immediately fixed in 4% paraformaldehyde in 0.1 M sodium phosphate-buffer (PB; pH 7.4) at 4 °C for 6 h. For experimental activation we used two graded modalities: the first was the neurological trauma represented by ganglia dissection and subsequent maintenance of them in culture conditions for at least 72 hours. The second modality (hyperactivation) was to proceed with a biochemical activation of the cultured ganglia. In brief, dissected ganglia were washed in physiological solution with antibiotics, passed in a modified L-15 culture medium with antibiotics [15, 30] for 72 h in a incubator at 20 °C and then fixed. Half of the ganglionic cultures (hyperactivation experiments) were charged with lipopolysaccharide (LPS) 10 μg/ml followed by ATP 10^{-3} M, respectively for 2 and 1 h. These substances are recognized as immune stimulants leading to glia activation [19]. The ganglia were then fixed

immediately or left after biochemical activation for a further period (24 h) in culture conditions in order to give the possibility to the stimulated cells to express newly synthetized molecules. After fixation, all the tissues were sequentially rinsed in PB (3 × 10 min) and incubated in 25% sucrose in PB at 4 °C overnight. The ganglia were embedded in Tissue Tek OCT Compound (Gurr, BDH, Laboratory Supplies, UK), frozen and cut at –25 °C on a cryostat (Microm, Heidelberg, Germany). Consecutive sections (14 μm thick) were mounted on gelatin-coated glass slides. For the immunocytochemical detection, a standard procedure was performed using the following polyclonal antibodies supplied from Santa Cruz Biotechnology (CA, U.S.A.): anti-IL-1α (113-271) raised in rabbit (1 : 500); anti-IL-1β (117-269) raised in rabbit (1 : 200); anti-IL-6 raised in goat (1 : 200); anti-TNFα (77-233) raised in rabbit (1 : 500). Polyclonal anti-glutamate raised in rabbit (1 : 200) was bought from Biogenesis (U.K.). To visualise the immunoreaction we performed the technique proposed by Hsu and Raine [12]. Control slides were made replacing the primary antiserum with normal serum. Each section image was acquired with a Polaroid DMC Ie video camera connected to a Leiz DMRB microscope.

In order to compare the results obtained with different techniques, in some cases the slides were stained by immunofluorescence technique using a secondary FITC labelled antiserum (1 : 20) (DAKO A/S, Denmark) in PBS-Triton for 1 h at 20 °C and counterstained for nuclei with 0.005% propidium iodide. The ganglionic sections were mounted in a solution of glycerine with an antifading (DABCO, Sigma) and examined under a Zeiss Axioplan fluorescent microscope equipped with a CCD Exwave HAD SONY videocamera or under a scanning confocal microscope Leica IRBE equipped with a laser beam Ar-Kr at "Centro Interdipartimentale Grandi Strumenti Universitŕ di Modena e Reggio Emilia" (C.I.G.S.).

Activation experiments on microglia cell cultures and immunocytochemistry

Microglial cells were obtained by culturing for 72 h ganglionic rings in Petri dishes (3 cm in diameter) containing a modified L-15 medium. After dissection, a subpopulation of glial cells (microglia), moves towards the site of lesion (cut nerves) and passes into the dishes adhering to the bottom surface. For chemical activation experiments we used the same procedures described for ganglia. At the end of experiments, microglial cell cultures were washed 3 × 5 min in PB, fixed at 37 °C in 4% paraformaldehyde vapours for 5 min and then dried. For immunocytochemistry, the dishes were incubated with the previously described antibodies, and the same procedures performed. The cultured cells were examined under an ECLIPSE TE 300 Nikon inverted microscope equipped with a SONY SSC-DC 50 AP digital camera or under confocal microscope (C.I.G.S.) as previously described.

Neuron-microglia co-cultures

Pure neuronal cell cultures were prepared on Petri dishes in which part of the plastic bottom was substituted with a 13 mm diameter glass coverslip previously coated with 0.05% poly-L-lysine (Sigma) in 0.1 M tetraborate buffer, pH 8.2 for 24 h and then conditioned for the successive 24 h with *Aplysia* filtered hemolymph (generous gift from Prof. P. G. Montarolo, Univ. of Torino, Italy). In brief, ganglia from young animals (5 mm shell diameter) were quickly removed and passed for 2 h in physiological solution with antibiotics for washing and sterilizing the tissues. The ganglionic rings were then incubated in 1% neutral protease (Dispase II, Boehringer, Manneim, Germany) in L-15 medium for 2h 30 min at 34 °C, then transferred into fresh L-15 medium to block the enzyme activity. The periganglionic sheaths were removed by microneedles and the ventral prominence of the left cerebral ganglion, named "E cluster", was isolated and transferred with a flame-polished Pasteur pipette in the pre-conditioned culture dishes. The neurons were dispersed by gentle microneedle vibrations and left to adhere to the coverslips overnight. The day after, when neurons had firmly adhered and some of them started to sprout new neuritic processes, the medium was changed. For co-culture experiments, isolated neurons were seeded directly into the microglial cells cultures obtained 24 h in advance as previously described and left to adhere onto the conditioned coverslips. Cells were examined every 12 h under ECLIPSE TE 300 Nikon inverted microscope equipped with a SONY SSC-DC 50 AP digital camera.

RESULTS

Immunoreactivity related to cytokines

We did not find appreciable differences in the immunocytochemical results using the different detection methods. Immunofluorescence was useful for studying the preparations in the confocal microscope.

IL-1α

Immunoreactivity for this cytokine was found only in some neurons on cytoplasmic level but only very rarely and showing a weak signal in microglia both in ganglionic sections and in cell cultures. Even after the different activation modalities, the distribution and amount of immunoreactivity related to IL-1α did not change (Figs 1 and 2).

IL-1β

The presence of immunoreactivity to this cytokine is variable depending on the state of activation. On sections from control ganglia and from cultured ganglia (simple

trauma) it was found expressed only at the cytoplasmic level in neurons localized in different ganglia (Fig. 3). After combined LPS/ATP ganglia exposure (2 + 1 h) followed by immediate fixation, the immunoreactivity was still limited to neurons, but leaving the ganglia for further 24 h in culture, an additional immunopositivity in the microglial cells of all ganglia appeared (Fig. 4). A similar response was obtained on the cultured microglial cells: non-activated cells were negative and the same result was found exposing microglia to LPS alone (2 h) or even followed by ATP exposure (1 h) and then immediately fixed. Only leaving the microglia cultures alive for additional 24 h after combined LPS/ATP activation treatment, many microglial cells expressed IL-1β immunoreactivity (Fig. 5).

IL-6

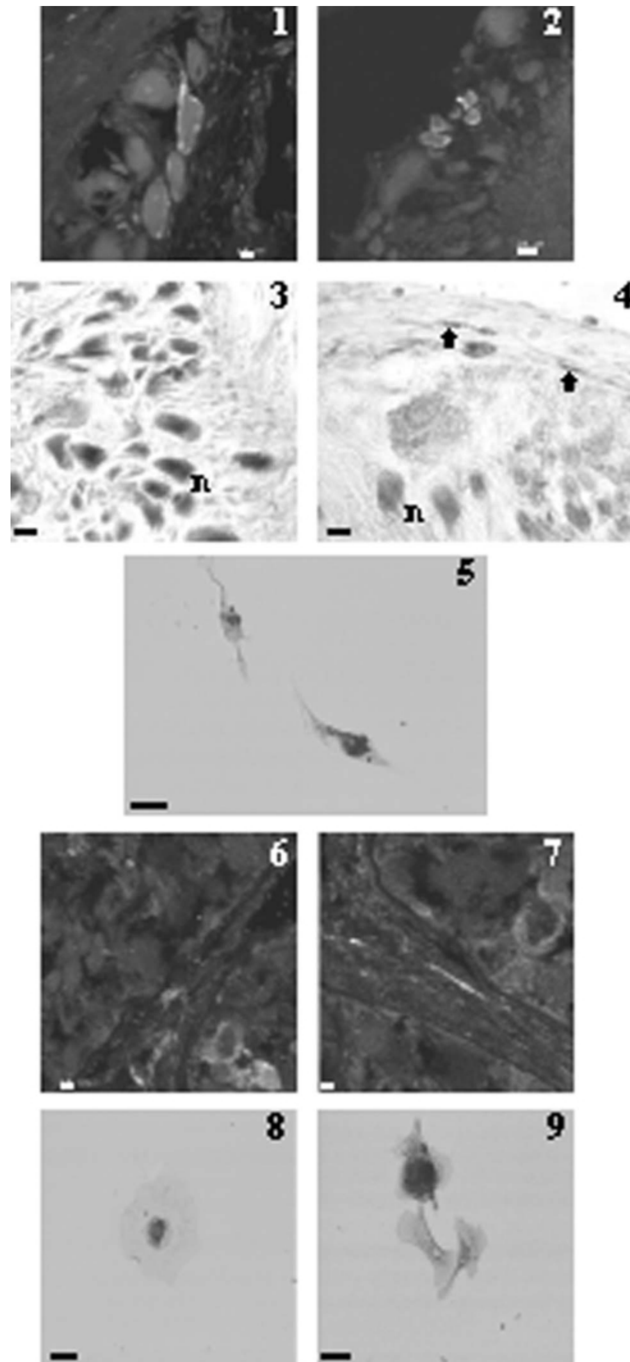
In sections from control ganglia we found the presence of immunoreactivity related to IL-6 in several neurons in different locations and in a part of glial cells corresponding presumably to microglia (Fig. 6). Both activation procedures did not change appreciably, at immunocytochemical level, the amount and distribution of the signal (Fig. 7). All microglial cultured cells showed always an high level anti-IL-6 immunopositivity and a further increase after LPS/ATP exposure was not visualised (Figs 8 and 9).

TNF-α

In sections from control ganglia, an immunopositivity to this cytokine was found at neuronal level in several cells wherever distributed (Fig. 10) but not in microglia. Leaving the dissected ganglia for at least 72 h in culture medium (simple trauma modality), microglial cells became hypertrophic and motile and appeared to have a granular content very rich in TNF-α immunoreactivity (Fig. 11). Even in cells cultured for at least 72 h we found the same type of immunocytochemical distribution (Fig. 12) and a further activation treatment did not change substantially the amount of visualised immunoreactivity.

Immunoreactivity related to glutamate

In control ganglia and in ganglia cultured for 72 h we found immunopositivity anti-glutamate without appreciable differences in some neurons and in microglial cells localized in all the ganglia (Figs 13 and 14). Also cultured microglia cells showed an intense immunoreactivity to anti-glutamate antibody (Fig. 15). We did not visualise a further increase in immunoreactivity after the activation procedures (Fig. 16).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Neuron-microglia co-cultures

In order to standardised the results, for our *in vitro* experiments, we selected the "E cluster" neurons because they belong to an homogeneous population (Fig. 17). Indeed, these neurons have the same morpho-functional and biochemical characteristics in respect to the majority of ganglionic neurons that constitute a very heterogeneous populations. The "E cluster" is formed by about two hundred monopolar neurons whose axons, through the small asymmetrical cerebral nerve, innervate the lateral penis complex. All these cells showed immunoreactivity for both FMRFamide and SCP_B (unpublished data). The isolated neurons adhered to poly-L-lysine substrate and then they survived for some days also in absence of growth factors. When the substrate was conditioned with *Aplysia* haemolymph, the neurons showed a good percentage (about 25% of the cells) of neurite regeneration, usually sprouting from the axonal stump (Fig. 18). In our co-culture, the fact that neurons were seeded directly in dishes in which microglia were already present did not compromise the neuronal adhesion. Preliminary comparative data showed that neuronal survival was improved by the presence of microglia. Moreover, we found that when adhered neurons started to sprout new neurites, microglial cells moved to contact them and their presence increased elongation and branching (Figs 19 and 20).

DISCUSSION

In this study we demonstrated by immunocytochemistry the presence of constitutive and induced expression of different cytokines (IL-1α, IL-1β, IL-6, TNF-α) and the presence of the neurotransmitter glutamate in neurons and glial cells of the nervous

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Figs 1–2. Ganglionic sections immunostained with anti-IL-1α. Immunoreactivity was found only in some neurons at cytoplasmic level. Fig. 1: control ganglia. Bar = 10 µm. Fig. 2: ganglia activated with LPS/ATP. Bar = 20 µm. Even after the different activation modalities, the distribution and amount of immunoreactivity related to IL-1α did not change

Fig. 3. Section from control ganglia immunostained with anti-IL-1β. IL-1b-like immunoreactivity is expressed only at cytoplasmic level in different neurons. Bar = 20 µm

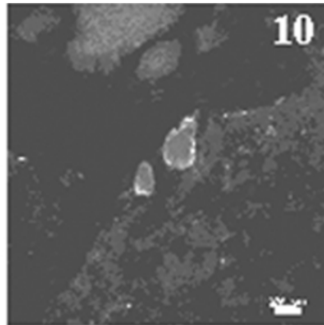
Fig. 4. Section from ganglia activated with LPS/ATP and cultured for further 24 h. Microglial cells appeared immunopositive to the reaction. Bar = 20 µm

Fig. 5. Microglial cells cultured for 72 h, activated with LPS/ATP and then cultured for additional 24 h after activation treatment. Many microglial cells showed to express IL-1β immunoreactivity. Bar = 10 µm

Fig. 6. Section from control ganglia immunostained with anti-IL-6. Several neurons and microglia cells show immunopositivity to the reaction. Bar = 10 µm

Fig. 7. Section from hyperactivated ganglia immunostained with anti-IL-6. The activation procedures did not change appreciably the amount and distribution of the signal. Bar = 10 µm

Figs 8–9. Cultured microglial cells immunostained with anti-IL-6. Fig. 8: control culture. Fig. 9: chemically activated culture. All microglial cultured cells show always an high level in anti-IL-6 immunopositivity. Bar = 10 µm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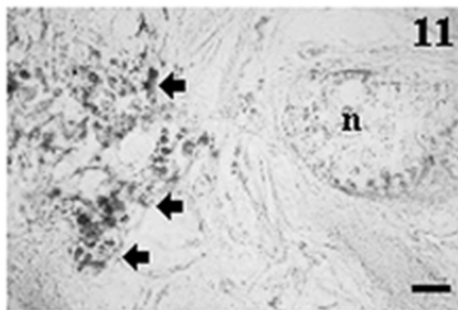
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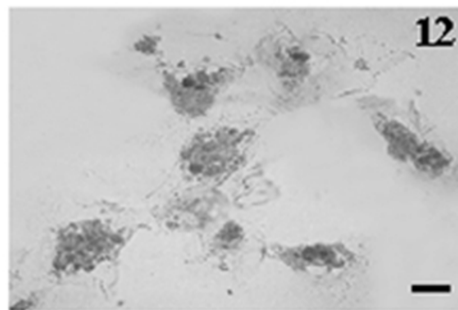
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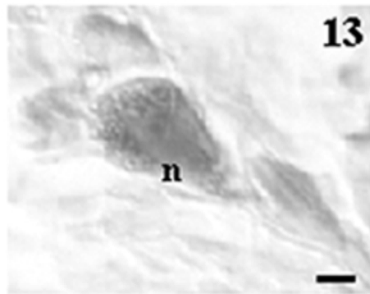
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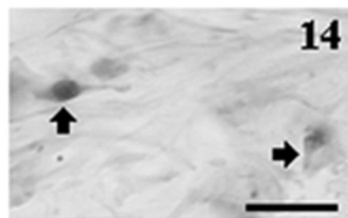
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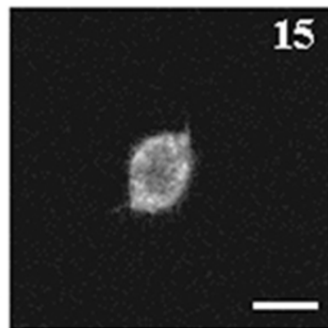
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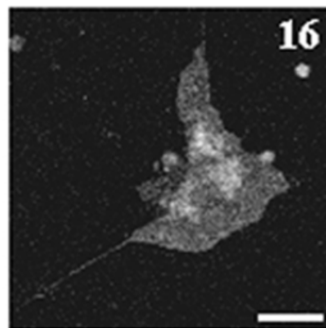
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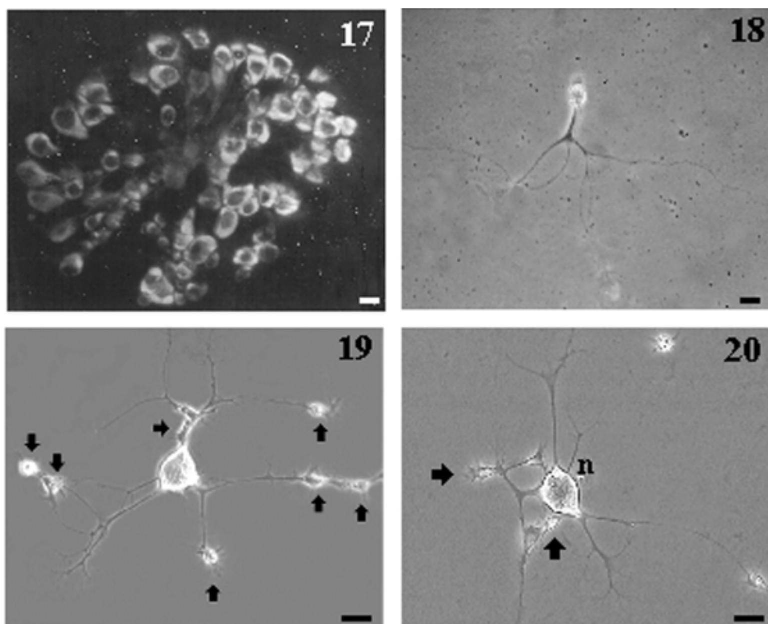

Fig. 17. "E cluster" neurons. Bar = 10 μm

Fig. 18. "E cluster" neuron cultured on conditioned substrate for 24 h; regenerated neurites usually are present. Bar = 20 μm

Fig. 19. Co-culture experiment: "E cluster" neuron cultured for 24 h beside microglial cells. Microglial cells contact the regenerated neurites. Bar = 10 μm

Fig. 20. Co-culture experiment: "E cluster" neuron cultured for 48 h beside microglial cells. Microglial cells contact the regenerated neurites. Bar = 10 μm

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Fig. 10. Section from control ganglia immunostained with anti-TNF-α. Immunopositivity is present at neuronal level in several cells but not in microglia. Bar = 10 μm

Fig. 11. Section from ganglia cultured for 72 h immunostained with anti-TNF-α. Microglial cells show TNF-α immunoreactivity. Bar = 20 μm

Fig. 12. Microglial cells cultured for 72 h and immunostained with anti-TNF-α: immunoreactivity to this cytokines is found. Bar = 10 μm

Figs 13–14. Sections from ganglia cultured for 72 h and immunostained with anti-glutamate. Fig. 13: neurons are immunopositve to the reaction. Fig. 14: also in microglia cells we found anti-glutamate-immunoreactivity. Bar = 20 μm

Figs 15–16. Cultured microglia cells immunostained with anti-glutamate. Fig. 15: control cultured cells show an intense immunoreactivity to anti-glutamate antibody. Fig. 16: activated cultured cells did not show a further increase in immunoreactivity. Bar = 10 μm

system of the freshwater snail *P. corneus.* Furthermore, we obtained some evidence about the possible role of these mediators in the neuron-microglia communication through a series of *in vitro* activation experiments. We have to notice that our results were obtained using polyclonal antibodies to human antigens: the positive reactions does not mean that the studied molecule is present by sure, but merely indicates that molecules from distant species groups at least share the epitope recognized by the antibodies used. Additional immunotechniques and molecular biology investigations are necessary to confirm the presence and chemical sequence of a postulated substance. Nevertheless, a vaste literature on invertebrate studies confirms the presence of cytokines [9, 14, 25] and glutamate [4, 8, 16, 33] in different phyla and consequently the old filogeny of the investigated molecules. The novelty of our research consists in considering these substances as relevant molecular correlates of the cross-talk between neurons and microglia even in the invertebrate nervous system. In the vertebrate healthy brain, microglia are described as cells in a state of quiescence or alertness [3] and this condition is mantained by the existence of neuronal imputs that, through both humoral and cell-cell contact mechanisms, are able to suppress the potentially neurotoxic properties of microglia. Nevertheless, there is still a controversy evidence about whether, once activated, microglia aid neuronal survival or exacerbate the neuropathological state. Indeed, it has been postulated that microglia response is graded and in a first phase primarily reflects a neuroprotective effort through a limited and specific production of factors that defend the metabolically impaired neurons and preserve them from induced apoptosis. Successively, the definitive loss of specific communication from severely injured neurons or the increased release of the same factors or new molecules from them (as well as the reception of specific signals from invading pathogenic microrganisms) may account for the turning of microglia towards hyperactivation, leading to an acclaimed neurotoxicity and ending in a circumscribed neuronal death, justified to preserve the remaining neurons [26]. Among the substances released by impaired neurons and known to elicit microglial activation there are multifunctional cytokines and some of them were examined in our study. For instance, it has been reported that in vertebrates, traumatic lesions induce the expression of IL-l and TNF-α by local and distant neuronal populations at the site of injury [34] and IL-6 is released by axotomised neurons following peripheral nerve injury [2]. The expression of these molecules in *P. corneus* neurons may account for their informative role, especially following the trauma caused by ganglia dissection. Other molecular signals reported to be released by injured neurons are ATP [35], and extracellular potassium ions [5]. In our activation experiments, we used LPS and ATP in a subsequent administration procedure [19]. Both these substances are considered chemo-stimulants leading to glia activation and release of various mediators, including cytokines and nitric oxide, and in high doses cause neurodegeneration [6, 11, 18, 28, 29]. Concerning the communication molecules expressed by microglia in our invertebrate model we found the most interesting results. Once activated, microglia produce and release biologically active substances possessing a wide range of functions influencing neuronal survival or death. Among the molecules we found in *P. corneus* microglia, TNF-α, IL-1 and IL-6 are

the best known vertebrate pro-inflammatory cytokines and it is of great importance that their expression in the snail appears only after activation procedures: apart from IL-6 we never found in our controls immunopositive microglia for the studied cytokines both on sections and *in vitro* preparations. In vertebrates, the temporal expression of these cytokines is related to the stimuli and in particular, TNF level increases in the first hours after the trauma or before neural death, while IL-1β is expressed in the following 24 h [17, 36]. The activation experiments we made leaving dissected ganglia and microglia cells in culture medium for 72 h or adding to the medium LPS/ATP gave similar results. In particular IL1-β is expressed by microglia only in experiments in which the activated cells have been cultured for the following 24 h. Furthermore, when the cells were activated only with LPS for two hours, they never revealed immunopositivity to the antibody but the addition of ATP to LPS stimulated microglia causes the expression of great amount of IL-1β as in vertebrates [28]. These data suggest that IL-1β is not expressed in the mature form in normal conditions, but only after a specific activation, and the presence of ATP permits the cleavage from a precursor procytokine. IL-6 immunoreactivity was found in both neurons and microglia cells in each experimental condition. The role of IL-6 in the neuroprotection mecchanisms is well documented, indeed its overexpression in pathological condition acts as an activating stimulus for microglia [24]. Several study show that IL-6 production by both neurons and microglia is stimulated by other pro-inflammatory cytokines like IL-1β and TNF-α. The presence of glutamate in both *P. corneus* neurons and microglia suggests the occurrence of neurotransmiter-mediated neuron-microglia communication, which may appear in pathological states [7]. We have to take into account that various subtypes of glutamate receptors have been found in microglial cell in culture and it is also interesting to recall the presence of glutamate-transporters on microglial cells membranes [22] which suggests a potential neuroprotective role of microglia against glutamate excytotoxicity [37]. Furthermore, different studies show that microglial cells produce neuroprotective factor under their stimulation [21].

Recently, we set up neuron-microglia co-coltures in order to test *in vitro* the effects of microglia in terms of neuronal survival and neurite regeneration through humoral and cell-cell contact communication. In preliminary comparative experiments, we have seen that neuronal survival is improved by the direct presence of microglia that, probably attracted by chemotaxis, tend to contact closely the neurons as we can observe *in vivo*. Neurites regeneration itself is improved by the beneficial interaction with microglia. On the other hand, several studies on vertebrates demonstrated the ability of microglial cells to promote axonal sprouting and regeneration in both central and peripheral nervous systems and a contact mechanism involving adhesion molecules has been hypotesized [1, 27].

In conclusion, the potential role of microglial cells in rescuing injured neurons and in promoting neuro-regeneration depends on the efficacy of a balanced cross-talk between damaged neurons and microglial cells.

We surmize that a better understanding of the nature and the mechanisms of action of the neuronal signals exchanged with microglia, even with studies carried out in

invertebrates, is possible and will be essential to improve to our knowledge on many neuropathological states affecting man as well as to devise therapeutic tools against them.

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