

Culture of neural cells of the eyestalk of a mangrove crab is optimized on poly-L-ornithine substrate

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Abstract Although there is a considerable demand for cell culture protocols from invertebrates for both basic and applied research, few attempts have been made to culture neural cells of crustaceans. We describe an *in vitro* method that permits the proliferation, growth and characterization of neural cells from the visual system of an adult decapod crustacean. We explain the coating of the culture plates with different adhesive substrates, and the adaptation of the medium to maintain viable neural cells for up to 7 days.

Scanning electron microscopy allowed us to monitor the conditioned culture medium to assess cell morphology and cell damage. We quantified cells in the different substrates and performed statistical analyses. Of the most commonly used substrates, poly-L-ornithine was found to be the best for maintaining neural cells for 7 days. We characterized glial cells and neurons, and observed cell proliferation using immunocytochemical reactions with specific markers.

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This protocol was designed to aid in conducting investigations of adult crustacean neural cells in culture. We believe that an advantage of this method is the potential for adaptation to neural cells from other arthropods and even other groups of invertebrates.

Keywords Glia · Neurons · Cell culture · Immunocytochemistry · Visual system · Invertebrates

Introduction

The study of invertebrate nervous systems, including crustaceans, previously focused on neuronal cytoarchitecture and circuitry, however little attention has been given to characterizing functional roles or understanding the development of the neurons and glia (Hu et al. 2015; Miguel et al. 2002; Stowe 1977; Strausfeld and Nässel 1981). Further, the characterization of invertebrate neurons and glia either in vivo or in vitro has not been explored as thoroughly as it has in vertebrates (Barres 2008). Culturing invertebrate nervous system cells may allow experimental manipulations under controlled conditions, facilitating studies on their morphology (Xu et al. 2010) and functions (Stepanyan et al. 2004).

In general, invertebrates have a rather small number of neurons and glial cells as compared to vertebrates, although essential functional features, such as patterns of synaptic connections and neurotransmitter contents, are conserved (Matheson 2002). As in vertebrates, invertebrate glial cells are responsible for many important nervous system functions: recycling the neurotransmitter glutamate at synapses (Tsacopoulos et al. 1997), interacting metabolically with neurons, guiding the axons to their targets during development (Abbott 1995; Allen and Barres 2009; Duffy et al. 2014; Hartline 2011; Pentreath 1987; Radojicic and Pentreath 1979), and acting as stem-like cells during and after the development of the nervous system (Corty and Freeman 2013; Sullivan et al. 2007). Since we are interested in the roles of glia, including in adult crustacean neurogenesis (Chaves da Silva et al. 2012; Zhang et al. 2009), we were motivated to obtain a primary culture, in order to characterize glial cells, in addition to neurons, in vitro.

For this purpose, we used the visual system of the adult mangrove crab *Ucides cordatus* (Crustacea,

Decapoda), because not only we have been using the visual system of this species as a model for studies of neuronal and glial cells (Allodi et al. 1999; Chaves da Silva et al. 2010, 2013; Corrêa et al. 2008; da Silva and Allodi 2000, 2001, 2004; Fusco et al. 2014; Hollmann et al. 2015; Miguel et al. 2002, 2005, 2007), but because in adult decapods neurogenesis occurs in the optic lobe (Schmidt 1997). The visual system of *U. cordatus* consists of a retina with photoreceptors projecting to the optic lobe, which is composed of neurons and glial cells constituting the *lamina ganglionaris* (La), the external medulla (EM) and the internal medulla (IM) (Corrêa et al. 2004; da Silva et al. 2003). Decapod crustaceans are excellent models because of their well-organized nervous system, attention-grabbing behavior patterns ranging from reflexes to complex social interactions (Sandeman et al. 1992) and ease of handling. To our knowledge, few protocols for nervous-tissue culture have been reported for adult or developing decapod crustaceans (Chun-Lei et al. 2003; Mitsuhashi 2002; Stepanyan et al. 2004; Toullec 1999; Xu et al. 2010).

In this study we developed a protocol that provides a basis for culturing neurons and glial cells from the visual system of the adult mangrove crab *U. cordatus* in order to facilitate experiments to analyze the contribution of extrinsic and intrinsic factors to the control of cell proliferation and differentiation. We believe that the method we describe here for maintaining neurons and glial cells from an adult decapod in culture can be adapted to other arthropods and even to other groups of invertebrates.

The difference between our culture protocol and other cell culture descriptions is that ours includes the isolation of neuronal elements from visual system of the crab and the characterization of cell populations by cell biology methods. Using conventional light microscopy, immunofluorescence with specific molecular markers and scanning electron microscopy (SEM), we described the different cell types. The markers used were: glial fibrillary acidic protein (GFAP), glutamine synthetase (GS), and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) for characterizing glial cells. For neuronal progenitors we used nestin, for young neurons, β tubulin isotype III (β III-tub) and for characterizing mature neurons, we used neuronal nuclei (NeuN), S-100 α -subunit (S-100A) and medium neurofilament chain (NF-160). Additionally, since we developed a protocol for culturing

nervous system cells and therefore needed to determine whether the cells were dividing properly, we used a marker for proliferating cells (Ki-67).

Materials and methods

Animals

Healthy adult male intermolt specimens of *U. cordatus* ($n = 20$; with carapace lengths between 7.5 and 8.5 cm and weight between 143 and 163 g) were obtained from mangroves in Itambi, Niterói, Rio de Janeiro State, Brazil (S-22°43'59.99", W-42°58'00.00"). All procedures adopted in this study, including access to the location where the animals were caught, were conducted under license from the “Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis” (IBAMA, Certificate #14689-1/IBAMA/2008, animal-use permit #2440408) and by the Ethics Commission on Research Animals of the Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro (protocol DHEICB 005).

The crabs of the species *U. cordatus* were maintained in aquaria, in constant conditions (water salinity 20, temperatures from 25 to 28 °C, 12/12-h light/dark cycle, and fed with small pieces of the mangrove species *Avicennia schaueriana*). Then, they were cryoanesthetized at -4 °C for 20 min. The whole optic stalks with the carapace were washed with 1 % sodium hypochlorite, and rinsed with 70 % ethanol (Sashikumar and Desai 2008; Jose et al. 2011). The optic stalks were cut and, under a stereoscopic microscope, the eye and the carapace surrounding the internal structures (muscle, connective tissue, nervous tissue and vessels) of the optic stalk were removed, and only the three ganglia (La, EM and IM) were left intact. The ganglia were placed on Petri dishes containing the medium for dissection.

Adult primary cell culture

The ganglia were dissected and pooled in a Falcon tube where 500 µL of TrypLE™ Express (Gibco, Life Technologies, Sao Paulo, Brazil), was added for enzymatic dissociation. After gentle agitation, the ganglia were placed in a water bath for 4 min at 37 °C, and the TrypLE™ was removed. Then, 1000 µL of culture medium (see below) was gently added to the

Falcon tube to wash the remaining TrypLE™, which was then centrifuged at 1800×g for 5 min. The pellet was resuspended in 2000 µL of culture medium in the Falcon tube and mixed gently to completely dissociate the cells. Next, 10 mL of culture medium was added and the cells were plated on culture dishes (approximately 10⁵ cells/mL per culture dish), in the presence or absence of the above-mentioned substrates (500 µL each). The cells were grown in the culture medium for 7 days, at 28 °C and the cultures were observed on the first and on the seventh day after the former procedure. We note that CO₂ is not needed for crustacean cell cultures (Chun-Lei et al. 2003; Mitsunashi 2002). Cell viability was determined using the Trypan blue (0.4 %) dye exclusion test (data not shown).

Culture dishes and medium

Cells were cultured with Leibovitz's L-15 medium with L-glutamine (L-15 medium, Sigma Aldrich, St. Louis, MO, USA). The L-15 medium typically supplies essential nutrients for cellular growth, including salts, amino acids, vitamins, D-galactose, phenol red, and L-glutamine. The medium was reconstituted at a double concentration (2×) with ddH₂O, and filtered on 0.22 µm cellulose membranes. Then, for dissection we added to the L-15 medium 1.5 % of antibiotics (10,000 units/mL penicillin and streptomycin, Sigma Aldrich) and 2.5 µg/mL of the sterile antifungal drug Anforicin B® (Cristália, São Paulo, SP, Brazil). Cultures were maintained until 7 days with L-15 medium, 1.5 % antibiotics, 2.5 µg/mL Anforicin and 10 % fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil). FBS is a complex supplement that acts as a source of minerals, lipids, and hormones, and contains growth and adhesion factors to promote cells attachment and proliferation (Freshney 2005).

Glass coverslips (15 mm, Glasscyto, Meclab, Jacarei, Brazil) were coated with various substrates and arranged on 24-well plastic culture dishes (TPP®, Trasadingen, Switzerland). In the first horizontal row of the culture plate, the substrate was collagen type I (Sigma Aldrich); the second horizontal row contained poly-D-lysine (Sigma Aldrich); the third row contained poly-L-ornithine (Sigma Aldrich); and the fourth horizontal row had no substrate on the glass coverslips. This row served as the control for the cell culture. The culture medium in the left three columns was changed every 2 days (changed medium, MC).

The culture medium in the right three columns was not changed during the 7 days of culture (not changed medium, MNC). During this period, 100 μL of L-15 culture medium with FBS, antibiotics and Anforicin B was added to prevent the normal evaporation from the wells (Fig. 1).

Immunocytochemistry

Our goal was to compare the pattern of cell adhesion on the selected substrates, but we also wanted to test whether replacing the culture medium (100 μL L-15 culture medium with FBS, antibiotics and Anforicin B) every 2 days (MC) or not changing (NMC) it had an effect. To test this, the cultures were grown for 1 and 7 days and fixed with 4 % paraformaldehyde (PFA) for 10 min at room temperature on a shaker with gentle agitation, washed 3 times for 5 min in PBS-crustacean (PBS-C) containing crustacean saline (31.0 g NaCl, 0.99 g KCl, 2.35 g MgCl_2 , 0.22 g NaHCO_3) and phosphate buffer, pH 7.4. The cultures were then incubated with 200 μL fluorescent probe DAPI and left for 2 min, rinsed with sterile distilled water for another 2 min, and, because the cells were plated directly on the coverslips, the coverslips were mounted with glass microscope slides, using Fluoromount/PlusTM, in order to be observed under the

Fig. 2 To evaluate the patterns of cell behavior in the selected substrates and culture medium conditions, neurons and glial cells grown for 1 and 7 days are incubated with DAPI. **a, d, g, j** Cells cultured for 1 days show no difference when the substrates are compared. Cell morphology always shows a round or oval shape. **b, e, h, k** A 7 days culture in which the medium was changed (MC) reveals a significant difference in the number of cells between substrates. **c, f, i, l** In addition, when cultures in which the medium was changed (MC) are compared with cultures in which the medium was not changed (MNC), the number of cells differs significantly. **m** Quantification of neurons and glial cells cultured for 7 days with MC. Significant differences in the total number of cells were observed between the poly-L-ornithine substrate and the substrates collagen type I ($p < 0.001$) and poly-D-lysine ($p < 0.001$), and the uncoated coverslips ($p < 0.001$). **n** Culture with MNC. Significant differences in the total number of cells were observed between the poly-L-ornithine substrate and the substrates collagen type I ($p < 0.05$) and poly-D-lysine ($p < 0.05$), and uncoated coverslips ($p < 0.001$). **o** Significant differences were observed between the culture with MC and the culture with MNC (*poly-D-lysine vs. poly-D-lysine [$p < 0.001$], **poly-L-ornithine vs. poly-L-ornithine [$p < 0.001$], and ***coverslips vs. coverslips [$p < 0.05$]). Scale bars (**a–l**), 5 μm

microscope. As stated above (Adult primary cell culture) approximately 10^5 cells was the total number of cells plated on each culture dish.

Next, in order to compare the pattern of cell proliferation on the different substrates or no substrate, and with different culture-medium conditions, cells

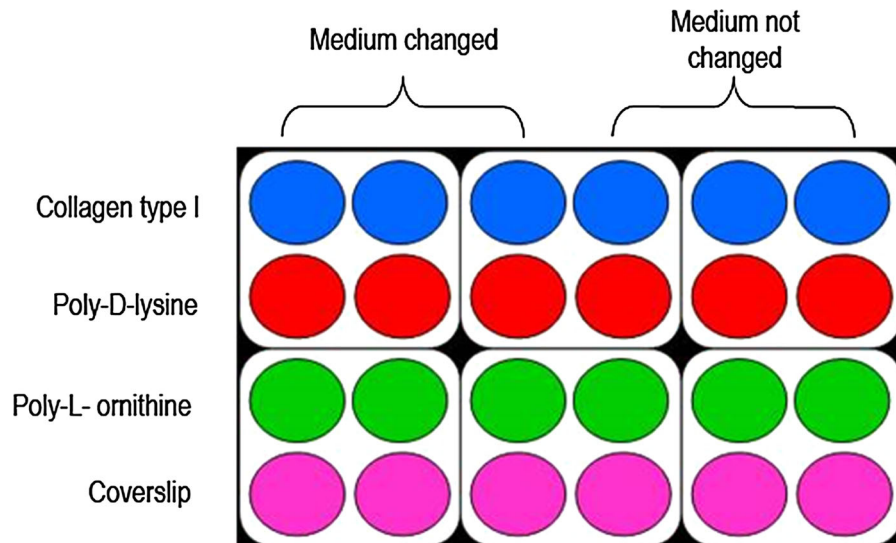
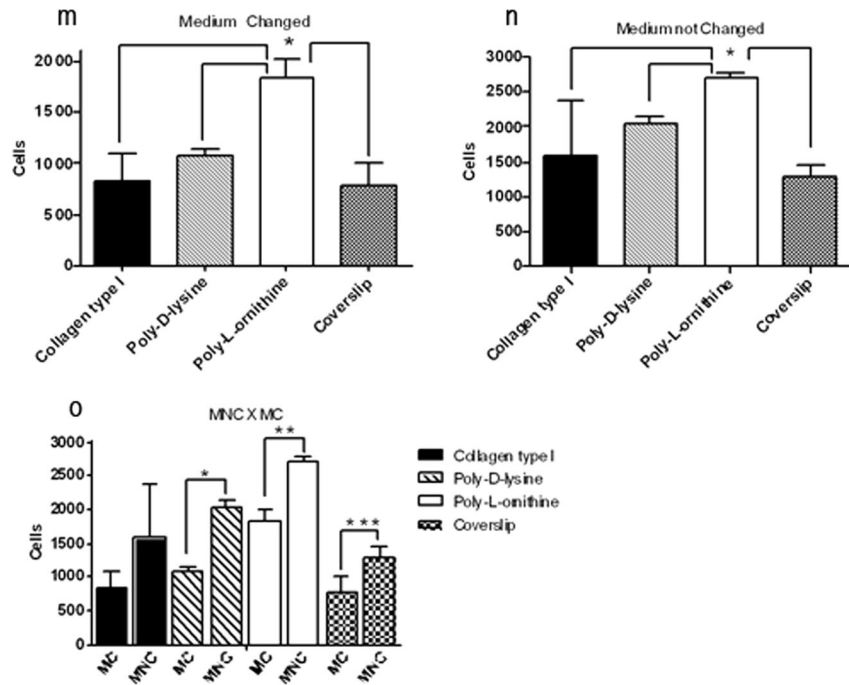
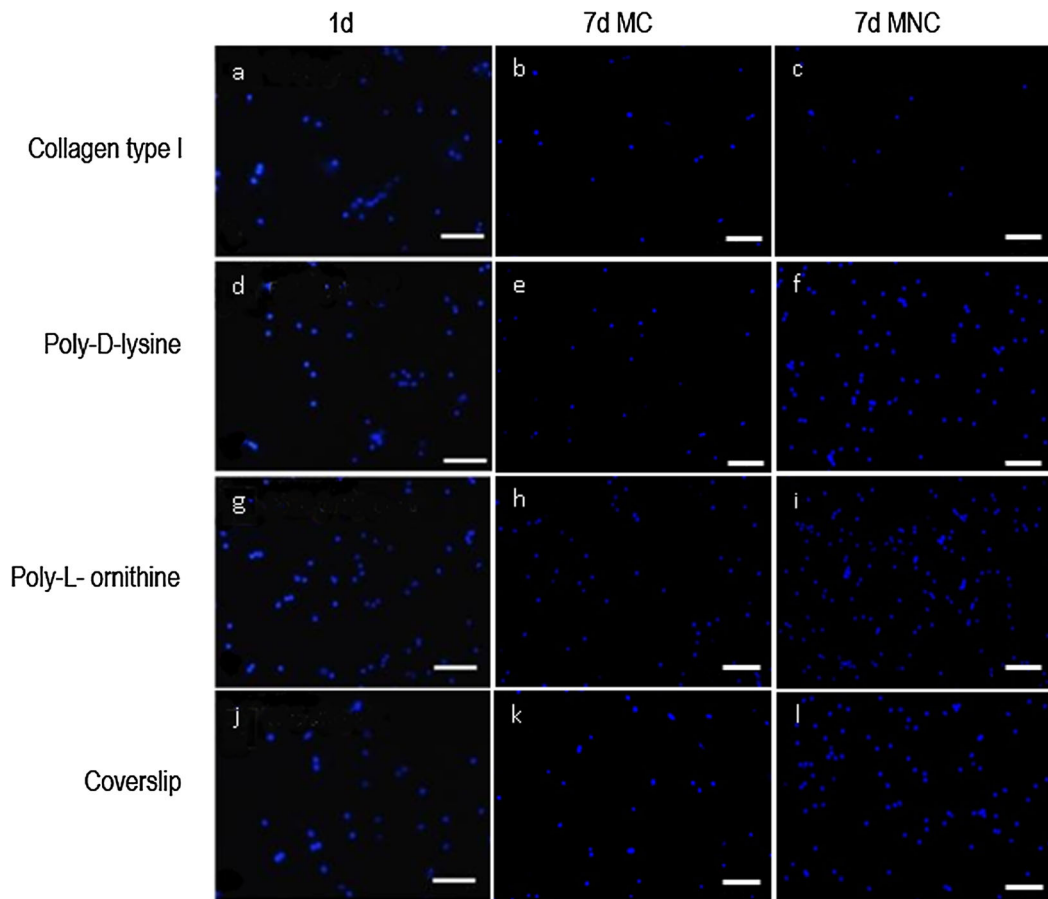


Fig. 1 Illustration of the provision of coverslips with different substrates, and the best procedure to maintain the L-15 medium. Glass coverslips are placed in a culture plate with 24 wells. The first six coverslips are coated with collagen type I (blue). In the next rows, the coverslips are coated with poly-D-lysine (red) and

poly-L-ornithine (green). The coverslips in the last row have no adhesive substrates (pink). The same plate is divided into two parts. The culture medium in the left three columns is changed every 2 days. The culture medium in the right three columns is not changed during the 7 days of culture. (Color figure online)



cultured for 7 days were fixed with 4 % PFA and immunostained with the anti-Ki-67 (rabbit polyclonal, Abcam, UK). For this procedure, after fixation the cultures were washed 3 times for 5 min in PBS-C and the cells were permeabilized with 300 μ L 0.3 % Triton X-100 in PBS-C twice, for 5 min each. Next, nonspecific sites were blocked by adding 300 μ L 10 % PBS-C BSA for 1 h at room temperature and the primary antibody Ki-67, diluted in PBS-C (1:100), was applied and left for 2 days in the dark at 4 °C. The antibody was revealed by incubation with goat anti-rabbit-Alexa 456 secondary antibody (Molecular Probes, Eugene, OR, USA) for 1 h in the dark at room temperature. The cells were then washed and the cultures were incubated with 200 μ L fluorescent probe DAPI and left for 2 min, rinsed with sterile distilled water for another 2 min, and mounted with glass microscope slides upside down using Fluoromount/PlusTM.

In order to identify glial cells we applied the antibodies against GFAP (Sigma Aldrich, St. Louis, MO, USA, rabbit polyclonal, diluted 1:100), GS (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, mouse monoclonal, diluted 1:50) and CNPase (Sigma Aldrich, mouse monoclonal, diluted 1:100). To characterize neuronal progenitors we used the antibody against nestin (clone Rat-401, Millipore, Billerica, MA, USA, mouse monoclonal, diluted 1:40), and for young neurons, the antibody against β III-tub (clone SDL3D10, Sigma Aldrich, mouse monoclonal, diluted 1:50). For neurons, the antibodies used were: anti-neuronal nuclei (NeuN, clone A60, Millipore, mouse monoclonal, diluted 1:50), anti-medium neurofilament chain (NF-160, clone BF10, Novocastra, Newcastle, UK, mouse monoclonal, diluted 1:50), and anti-S-100A (clone SH-A1, Sigma Aldrich, mouse monoclonal, diluted 1:100). The procedures for the other immunocytochemical reactions were the same, except for the anti-NeuN. In this case, the cells fixed for 10 days were permeabilized with 300 μ L 0.5 % Triton X-100 in PBS-C for 5 min. Afterwards, primary antibodies diluted in PBS-C were applied individually overnight in the dark at 4 °C, except for the anti-NeuN, which remained for 3 days in the dark at 4 °C. Antibodies were detected by incubation with goat anti-mouse-Alexa 488 (diluted 1:200) or goat anti-rabbit-Alexa 456 (diluted 1:200) secondary antibodies (Molecular Probes) applied for 1 h in the dark at room temperature. The cells were subsequently washed and

the cultures were incubated with 200 μ L fluorescent probe DAPI and left for 2 min, rinsed with sterile distilled water for another 2 min, and mounted with coverslips with Fluoromount/PlusTM. They were analyzed (and counted) under a Zeiss AxioImager.Z1/ApoTome microscope. The images obtained were recorded using the Axiocam MRm Rev. 3 and the software Axiovision Rel 4.8Cell MTM. The cell count was performed after capturing 49 images using the microscope mentioned above, with 20 \times magnification objective lenses. Certain criteria were taken into account: (1) the nucleus had to be clearly visible; (2) only the cells that were within each quadrant were considered; (3) the grouped cells were counted only when they were easily distinguishable.

Negative controls for the reaction were prepared by omitting the primary antibodies.

Scanning electron microscopy

Cells in culture plates were fixed with 4 % PFA and 2.5 % glutaraldehyde solution for 1 day. The wells of the plates were rinsed with phosphate buffer (0.1 M) 3 times for 5 min, rinsed with 0.1 M cacodylate buffer for 5 min, and then with 0.1 M cacodylate buffer 3 times for 5 min each. Next, the cells were post-fixed for 2 h with 1 % osmium tetroxide, 0.8 % potassium ferrocyanide and 5 mM calcium chloride, in 0.1 M cacodylate buffer (pH 7.4) for 2 h, maintaining the samples protected from light. The cells were dehydrated in graded ethanol concentrations from 50 to 100 % for 1 min in each step, and the samples were submitted to the critical-point reactions. The samples were mounted on conductive tabs, vacuum-dried in a CPD 030 BAL TEC Critical Point Dryer, and sputter-coated with gold for 2 min. The images were obtained using the JSM 5310 scanning electron microscope belonging to the Rudolf Barth Electron Microscopy Platform of the Oswaldo Cruz Institute/Fiocruz (Rio de Janeiro, Brazil).

Statistical analyses

The statistical analyses were performed using the SPSS Statistical Package for Windows, release 17.0 (IBM SPSS, IBM Inc., New York, NY, USA). The level of statistical significance was set at $p < 0.05$, and values are reported as the mean \pm SD.

Table 1 Means and standard deviations of the total number of cells and the number of cells labeled with Ki-67 in changed culture medium

Substrate	Total number of cells	Number of Ki-67-labeled cells
Collagen type I	830.8 ± 261.3	92 ± 51.8
Poly-D-lysine	1077.4 ± 72.3	97.4 ± 21.3
Poly-L-ornithine	1846.6 ± 167.2	187 ± 32.1
Coverslip	785.2 ± 216.4	80.4 ± 66.2

Table 2 Means and standard deviations of the total number of cells and the number of cells labeled with Ki-67 in unchanged culture medium

Substrate	Total number of cells	Number of Ki-67-labeled cells
Collagen type I	1585.6 ± 777.4	185.2 ± 103.1
Poly-D-lysine	2048.2 ± 97.3	243.6 ± 75.3
Poly-L-ornithine	2709 ± 71.4	697.6 ± 75.6
Coverslip	1275.8 ± 192.5	138.4 ± 63.4

Results

Analyses of the primary cultures (1 and 7 days) plated with different conditions

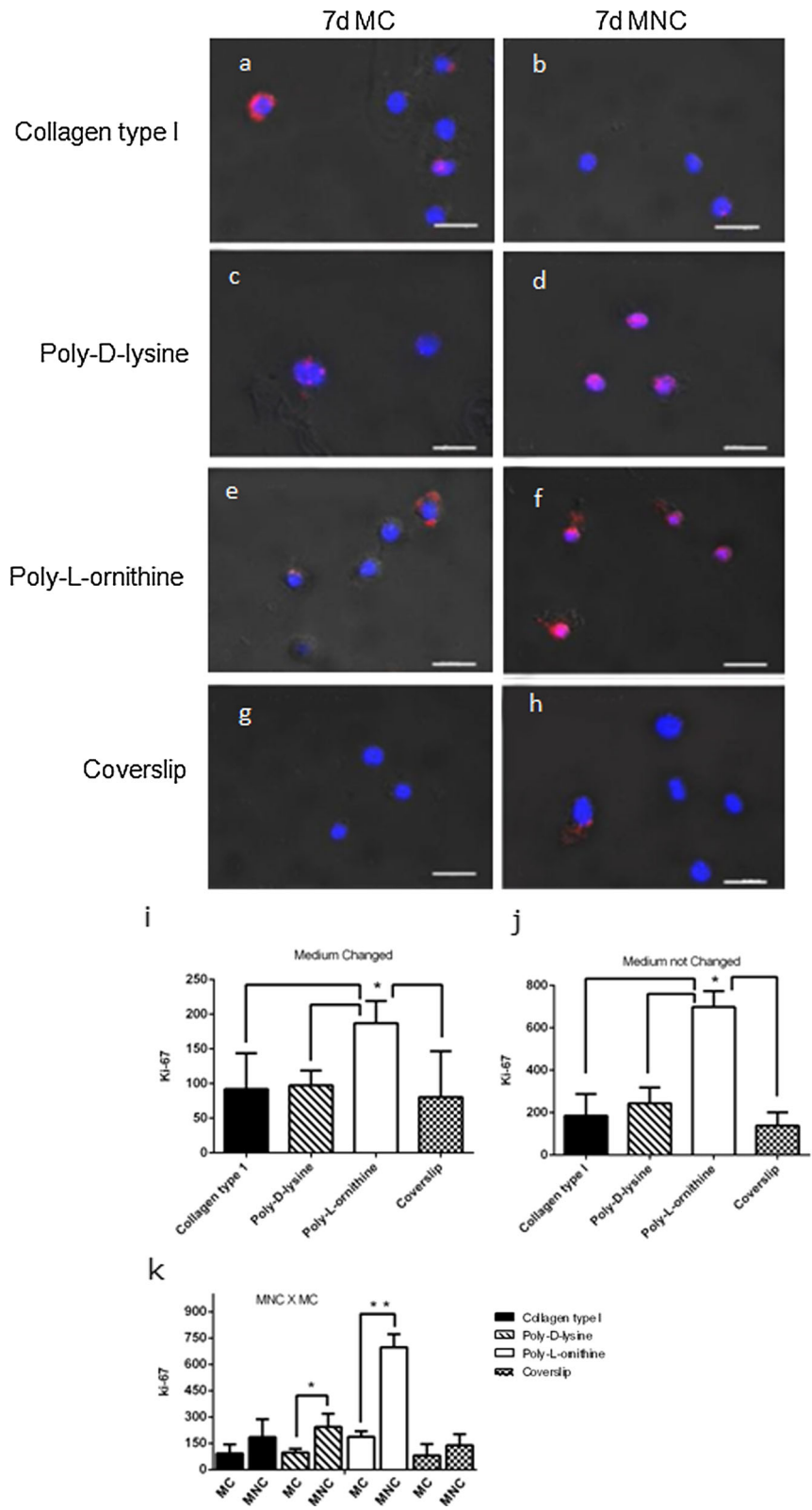
Nuclei of cells grown for 1 and 7 days and labeled with DAPI are shown in Fig. 2a–o. Cells cultured for 1 day did not display any difference in adhesion when the substrates were compared (collagen type I, Fig. 2a; poly-D-lysine, Fig. 2d; poly-L-ornithine, Fig. 2g; and coverslips with no adhesive substrate, Fig. 2j). Cells cultured for 7 days were tested for adhesion on the same substrates as mentioned above for 1-d cultures and for different conditions of the culture medium: the culture medium was either changed every 2 days (MC) or not changed (MNC). Also, we tested the MNC with the addition of 100 µL of fresh culture medium after 4 days of culture to adjust to the initial volume of the medium. Cells cultured on collagen type I with both MC (Fig. 2b) and MNC (Fig. 2c) showed fewer cells compared with the other substrates (MC, Fig. 2b, e, h, k and MNC, Fig. 2c, f, i, l). No significant differences were found in cell numbers when cells were grown on both poly-D-lysine (Fig. 2f) and with glass coverslips alone (Fig. 2l) in the MNC condition. However, the cell number increased when the cells were grown on poly-L-ornithine in the MNC condition (Fig. 2i,n). The cell counts revealed that in all cultures, not changing the culture medium was better for culturing cells (Fig. 2o).

For 7 days cultures with MC, the total number of cells differed significantly among substrates ($p < 0.001$; Fig. 2m), i.e., between the poly-L-ornithine substrate and the substrates collagen type I and poly-D-lysine ($p < 0.05$) and uncoated coverslips ($p < 0.001$; Fig. 2n). Significant differences were also observed between the culture with MC and the culture with MNC [poly-D-lysine vs. poly-D-lysine ($p < 0.001$), poly-L-ornithine vs. poly-L-ornithine ($p < 0.001$), and coverslips vs. coverslips ($p < 0.05$)] (Tables 1, 2).

Influence of the substrates and culture medium on cell proliferation

Additional 7 days MC cultured cells confirmed the experimental results shown in Fig. 2. The antibody against Ki-67 was used to observe cell proliferation in cultures (Fig. 3a–k) with collagen type I, poly-D-lysine, poly-L-ornithine and coverslips with no adhesive substrate, combined with MC (Fig. 3a, c, e, g) or MNC (Fig. 3b, d, f, h). Few labeled cells were seen with MC (Fig. 3i), while cells grown in MNC (Fig. 3j) were strongly labeled if grown on poly-D-lysine (Fig. 3d) or poly-L-ornithine (Fig. 3f). Poly-L-ornithine proved to be the best substrate to maintain proliferating cells. As shown in Fig. 3k, significant differences were seen between the cultures with MC and MNC and the different substrates ($p < 0.001$) (Tables 1, 2).

Fig. 3 Ki-67 antibody labeling. This antibody was used to assess cell proliferation (cells labeled in red) in 7 days cultures with different substrates and cell maintenance in the different environments. **a, c, e, g** In cultures in which the medium was changed (MC), only some cells are dividing. **b, d, f, h** Cultures in which the medium was not changed (MNC) cells are labeled. **i** Quantification of neurons and glial cells labeled with the Ki-67 antibody after culturing for 7 days with MC. Significant differences in the total number of Ki-67+ cells were observed between the poly-L-ornithine substrate and the substrates collagen type I ($p < 0.05$) and poly-D-lysine ($p < 0.05$), and uncoated coverslips ($p < 0.05$). **j** Culture with MNC. Significant differences in the total number of Ki-67 cells were observed between the poly-L-ornithine substrate and the substrates collagen type I ($p < 0.05$) and poly-D-lysine ($p < 0.05$), and uncoated coverslips ($p < 0.001$). **k** Significant differences between the culture with MC and the culture with MNC. Significant differences between MC and MNC (*poly-D-lysine vs. poly-D-lysine [$p < 0.001$], and ** poly-D-ornithine vs. poly-D-ornithine [$p < 0.001$]). Scale bars **a–h** 5 μm . (Color figure online)



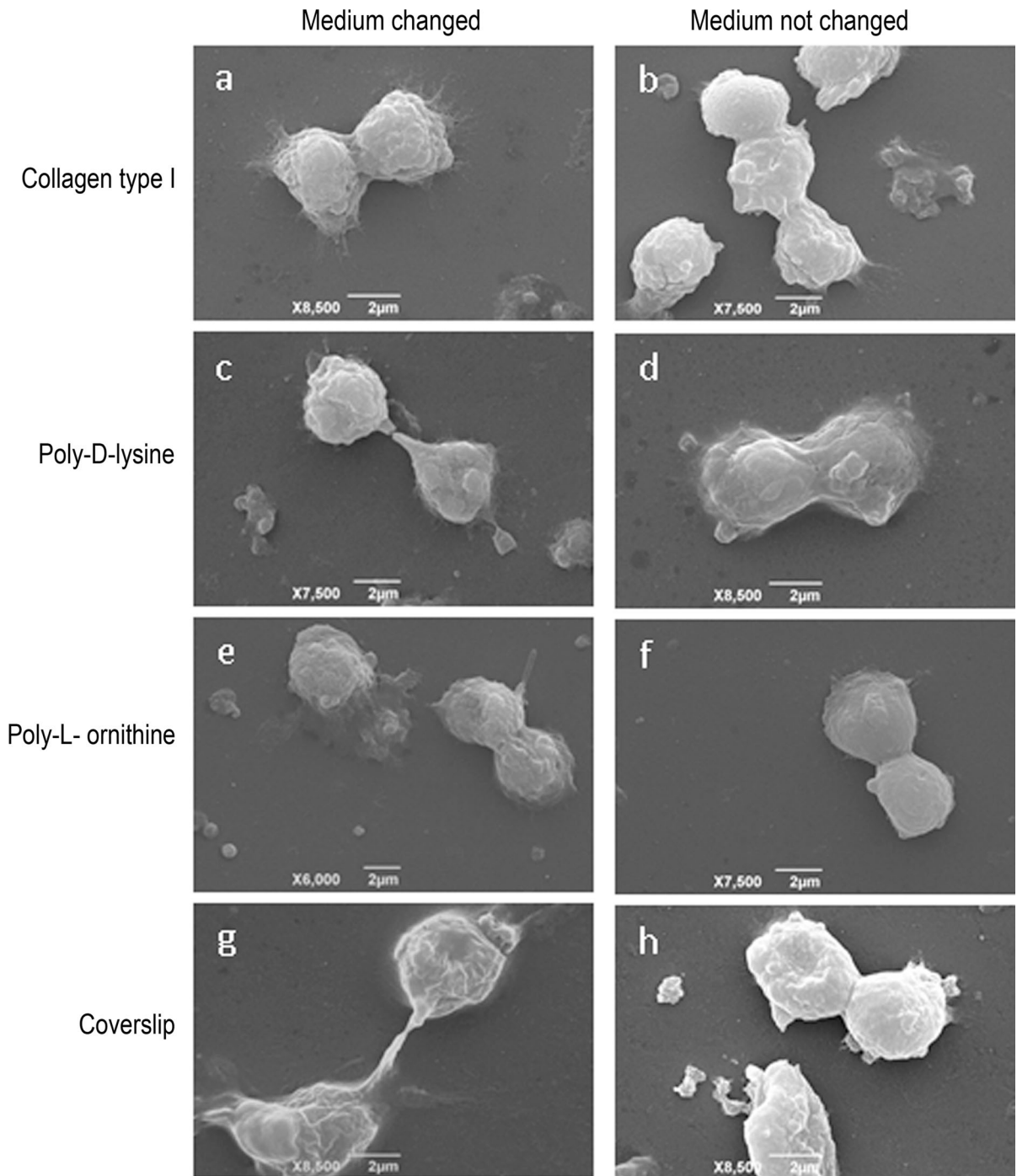


Fig. 4 Scanning electron micrograph of a 7 days culture of neurons and glial cells. **a, c, e, g** The culture medium was changed. **b, d, f, h** A culture in which the medium was not changed shows round cells with protrusions from the surface,

and cells that appear to be dividing. The basal outline appears to be expanded as filamentous edges, suggesting cell adhesion. **g, h** The cells appear dehydrated and also to be dividing

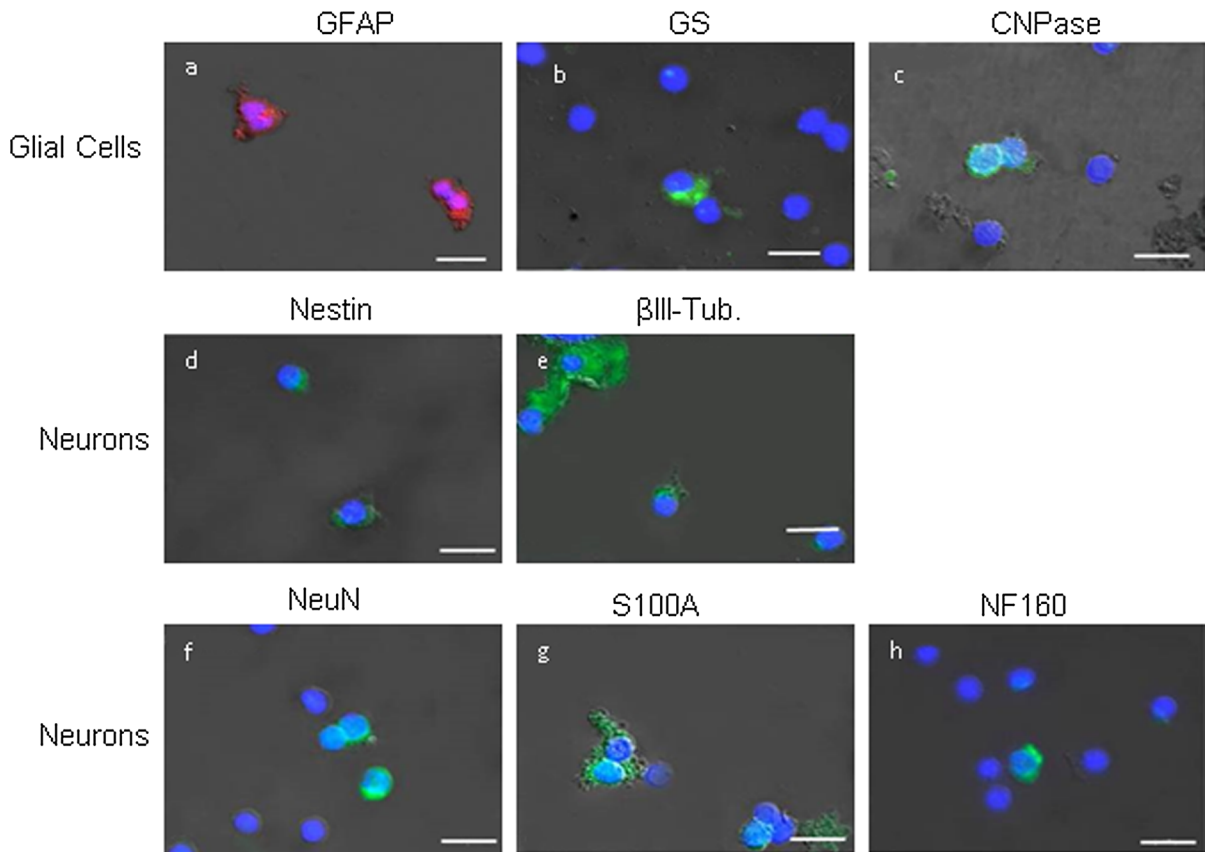


Fig. 5 Characterization of a 7 days culture of neurons and glial cells. The medium is not changed, and the substrate is poly-L-ornithine. **a** The cells are immunoreactive for anti-GFAP (a glial cell marker). **b** Some neural cells show intense staining for glutamine synthetase (GS; a glial cell marker) (green). **c** Some neural cells show cytoplasm labeled with anti-CNPase (a glial cell marker). **d** Cells labeled with anti-nestin (a neuronal

progenitor marker). **e** The cells are immunoreactive with anti- β III-tub, suggesting that the culture contains young neurons. **f** Cells labeled for NeuN (which indicates neurons). **g** Cells labeled with anti-S100A (a marker for neurons). **h** Neural cells labeled with an antibody against the medium neurofilament subunit (anti-NF160). Scale bars 5 μ m. (Color figure online)

Scanning electron microscopy of a 7 days culture of nervous system cells

Scanning electron micrographs were taken to evaluate in detail the external morphology of cells grown on the different substrates (Fig. 4) either with MC (Fig. 4a, c, e, g) or with MNC (Fig. 4b, d, f, h). Cultures with MNC showed round cells with small protrusions on the surface and cells that appeared to be dividing. The basal outline appeared to be expanded as filamentous edges, suggesting cell adhesion. The cells grown only on coverslips appeared dehydrated, and only some of them seemed to be dividing (Fig. 4g, h).

Characterization of cells grown on poly-L-ornithine

The above data suggest that the substrate that gave the best results in terms of cell number and cell adhesion was poly-L-ornithine, and the best condition was not changing the medium in the 7 d culture. The next step was to identify the different cell types. Therefore we used antibodies against glial cells and neurons (Fig. 5). GFAP (Fig. 5a), glutamine synthetase (GS) (Fig. 5b) and CNPase (Fig. 5c) revealed different glia phenotypes. When labeled with anti-nestin (Fig. 5d) and anti- β III-tub (Fig. 5e), this suggests that the culture

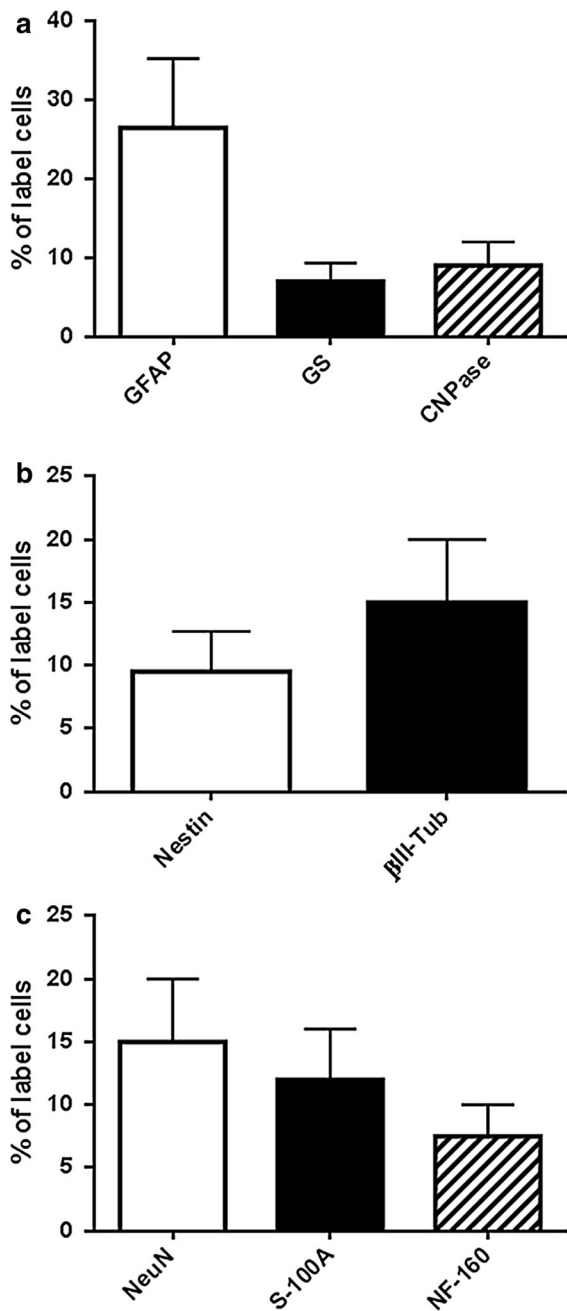


Fig. 6 Percentage of the total number of cells in the 7 days cultures. **a** Of the total number of cells 42.5 % are glial cells (26.5 % are labeled with GFAP, 7 %, with GS and 9 %, with CNPase). **b** 9.5 % of the cells are labeled with nestin, a marker for neuronal progenitors, and 15 % are labeled with β III-tub cells, a marker for young neurons. **c** Mature neurons were 34.5 % of the total number of cells (15 % NeuN-positive cells, 12 %, S-100A-positive cells and 7.5 %, NF-160-positive cells)

contained neuron progenitors and young neurons. Additionally, neurons were identified with antibodies against NeuN (Fig. 5f), S100A (Fig. 5g) and anti-NF160 (Fig. 5h). We counted how many (% of the total number of cells in the cultures) were labeled with each of the antibodies used (Fig. 6). Future studies are planned to complete experiments to understand the different phenotypes of the cultured cells.

Discussion

The novel aspect of this study is the description and implementation of a protocol designed to maintain the glial cells and neurons of crustaceans, since we are aware of no description in the literature of this experimental approach used for this group of animals.

Protocols have been developed for nervous system cells of invertebrates, particularly adult and developing insects and adult worms (Beadle 2006; Srivatsan and Peretz 1997). However, for adult decapod crustaceans, we are aware of only a few reports of nerve cell culture: Chun-Lei et al. (2003) described the morphology of cultured neurons from the terminal medulla of the Chinese white shrimp *Fenneropenaeus chinensis*; Stepanyan et al. (2004) described a protocol for olfactory neurons from the American lobster *Homarus americanus*, focusing on olfactory physiology; and Xu et al. (2010) studied the morphology of the cells of the cerebral ganglion of the mud crab *Scylla paramamosain*. None of these reports described a cell-culture protocol that aimed to characterize cell types from the adult decapod crustacean nervous system in detail, as we describe here. Our study provides useful information concerning suitable culture conditions for the survival and proliferation of neurons and glial cells of adult crustaceans. This is important because crustaceans have been used as models for studies on nervous control mechanisms, on sensory organization, and on the role of neurotransmitters or neuropeptides, to mention some (Wiese et al. 1990; Wiese 2002).

Regarding substrates, we chose to test: (1) collagen type I, an abundant protein in the extracellular matrix, and the collagen type most used for invertebrate cells (Odintsova et al. 2010); (2) poly-D-lysine, a polymer of positively charged amino acids that promotes cell adhesion, and therefore increases the interaction with

the plasma membrane of the cells (Stepanyan et al. 2004; Odintsova et al. 2010); and (3) poly-L-ornithine, described as an appropriate substrate for vertebrate neural-cell growth and differentiation (Smith 1994; Perígolo-Vicente et al. 2013). Additionally, we tested these substrates with changed (MC) and unchanged media (MNC).

The culture medium for maintaining the cultures of neurons and glial cells was chosen from literature sources: L-15 medium with L-glutamine has been the most widely used for maintaining invertebrate neurons and glial cells and/or brain ganglia in culture (Pearce et al. 2003; Sashikumar and Desai 2008; Weigel et al. 2012; Zanetti et al. 2007). However, the base medium, comprising inorganic salts, amino acids and vitamins, was not sufficient to maintain cell viability; it had to be supplemented with other factors (Valk et al. 2010). Because, like other aquatic animals, crustaceans are susceptible to a wide variety of pathogens, including viruses, bacteria, fungi, and protozoa (Lin 1995), we added antibiotics and Anforicin B. FBS is an effective supplement for almost all cell cultures, providing basic nutrients and hormonal factors that stimulate cell growth and proliferation, among other benefits (Maurer 1992). In order to determine the optimal concentration of FBS in our cultures, we conducted tests using 5, 10 and 20 % FBS. Cells in cultures with 5 % FBS did not survive (data not shown) and since cells grown with 10 and 20 % FBS showed no differences in morphology and cell survival, we chose to use 10 % FBS in all our cultures.

We also analyzed the length of time that the cells remained in the culture medium. In this protocol, we maintained the same culture medium for 7 days at 28 °C in a dry atmosphere. As a result, the medium was reduced during the culture period due to evaporation, which could lead to cell distress or death. Therefore, in order to restore the initial medium volume, 100 µL of fresh culture medium was added to 4 days cell cultures.

Taken together, our results with different substrates and changed/not changed medium demonstrated that poly-L-ornithine was the best substrate for growing nervous system cells from this crab. The results of the analyses of variance revealed significant differences, both among the substrates in the culture medium and between changed and unchanged media, as shown in Fig. 2 and Tables 1 and 2. The Bonferroni post hoc test showed that poly-L-ornithine supported better growth of neurons and glial cells in comparison to collagen type I, poly-D-lysine and coverslips.

Some protocols reported for other tissues and for developing organs in adult invertebrates (George and Dhar 2010; Jiang et al. 2006; Noonin et al. 2012) were taken into consideration in order to develop our own method. Acetylcholinesterase (Srivatsan and Peretz 1997; Stepanyan et al. 2004), insulin (Odintsova et al. 2010), hemolymph and muscle extracts (George and Dhar 2010) have been used in nervous tissue cultures. We initially tested insulin in our cultures, but did not obtain satisfactory results. To our surprise, when we added only FBS to the culture medium, with no additional growth factors, we obtained the best results.

Immunofluorescence labeling allowed the phenotypic characterization of neurons and glial cells cultured for 7 days without changing the culture medium. We were unable to locate any article that characterized the types of crustacean cells cultured in vitro, as we did using our approach. Here, the in vitro nervous system cells were labeled with antibodies that evidence glial cells and neurons in vivo, in both crustaceans (Allodi et al. 2006; Beltz et al. 2011; Corrêa et al. 2004; da Silva et al. 2003, 2004; Fusco et al. 2014) and mammals (Alexander et al. 1991; Lee et al. 1990). Additionally, because we used an antibody for cell proliferation, we can assume that the cells were proliferating in these culture conditions. SEM revealed the morphology of the cells on different substrates and showed in detail that the cells had a healthy appearance; it was also possible to observe protrusions on their surface and expansions on their basal surface with filamentous edge contours, suggesting cell adhesion. Therefore, our protocol can be used in studies to determine the potential for the in vitro differentiation of glial cells and the close relationship between neurons and glia. It can also be used in studies that aim to characterize crustacean nervous system cells and their differentiation potential.

The methodology reported here is important because the approach necessary to grow crustacean cells is different from the known protocols needed for vertebrate cell cultures; moreover, such a protocol has never been described for invertebrates. To conclude, this protocol provides a basis for studying neurons and glial cells for a range of applications, including culture activation, coculture, and functional interactions with other cell types, analyses of gene expression and epigenetic regulatory mechanisms, and proteomic and single-cell analyses.

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