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Alignment of glial cells stimulates directional neurite growth of CNS neurons *in vitro*

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Abstract

Olfactory ensheathing cells (OECs) together with olfactory nerve fibroblasts (ONFs) and neonatal astrocytes are potent stimulators of neurite growth in adulthood and during development, respectively. Since it is known that alignment of glial cells is important for the correct outgrowth of axon tracts, it was hypothesized that the alignment of glial cells stimulates directional and enhanced neurite outgrowth. Adult OEC/ONF and neonatal astrocytes were cultured either on biodegradable poly(D,L)-lactide matrices or in Petri dishes for 4 days. Thereafter neonatal cerebral cortical neurons were added. After a 2-days coculture period the cultures were fixed and processed for a combined MAP-2 and phosphorylated neurofilament (RT97) staining. The neurite growth (neurite elongation and neurite formation) and the neurite direction were assessed. We show that (1) OEC/ONF cultures are more potent in stimulating the length of the longest neurite of cocultured neurons, (2) alignment of glial is achieved *in vitro* on our biomatrices, (3) aligned glial/biomatrix complexes do not enhance neurite growth, and (4) aligned glial/biomatrix complexes direct neurite outgrowth. These data have significant implications for *in vivo* experiments focusing on glial transplantation. Transplanting glial/biomatrix complexes may stimulate the directional regrowth of severed axons across a lesion site.

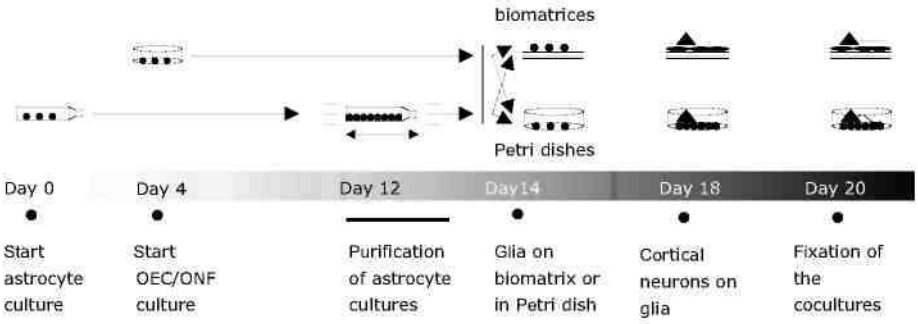
Key words: olfactory ensheathing cell, astrocytes, biomatrix, orientation, image analysis, spinal cord injury.

Abbreviations: DIV, days *in vitro*; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein-2; OB, olfactory bulb; OECs, olfactory ensheathing cells; OEC/ONF, non-pure OEC culture containing both OECs and ONFs; ONFs, olfactory nerve fibroblasts; PLA-*b*-PEO, poly(D,L-lactide-*b*-polyethylene oxide); PLL, poly-L-lysine; r.p.m., rotations per minute; RT97, phosphorylated neurofilament; SCI, spinal cord injury; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline with Triton X-100.

The interaction between glial cells and neurons is essential during the development of the CNS. Astroglial cells in general form a permissive substrate for neurite extension (Qian et al., 1992; Le Roux and Reh, 1994; Le Roux and Reh, 1995; Dijkstra et al., 1999). This stimulatory effect on neurite growth of both heterotypic and homotypic neurons may be mediated by a variety of diffusible factors, cell adhesion molecules, and extracellular matrix proteins, produced by astrocytes (McNaught and Jenner, 2000). In the adult olfactory system a population of axon-growth promoting cells, the olfactory ensheathing cells (OECs), is located (Doucette, 1990). These OECs are also known to stimulate neurite growth of both heterotypic and homotypic neuron populations (Denis-Donini and Estenoz, 1988; Goodman et al., 1993; Kafitz and Greer, 1998, 1999; Sonigra et al., 1999). OECs form a special type of glial cell, uniquely present within the olfactory system. The OECs are thought to be responsible for the continuous growth of olfactory axons from the peripheral epithelium into the central olfactory bulb (OB) that takes place throughout the whole lifespan of the organism. The ability of OECs to stimulate axon growth into the adult CNS makes them a very promising candidate for treatment of spinal cord injury (SCI). OECs have already been used in a variety of SCI models and this has resulted in regrowth of severed axons followed by partial functional recovery (Ramon-Cueto et al., 1998, 2000; Imaizumi et al., 2000; Verdu et al., 2003). It is suggested that OECs closely cooperate with olfactory nerve fibroblasts (ONFs), which then results in

a potent stimulation of axonal regrowth after injury (Li et al., 1997, 1998, 2003; Raisman, 2001; Keyvan-Fouladi et al., 2003). In contrast to the OECs, which *in vitro* can be selectively stained for the p^{75} low-affinity nerve growth factor receptor or S100β (Li et al., 1998; Ramon-Cueto and Nieto-Sampedro, 1992; Doucette and Devon, 1994; Barnett et al., 1993), the ONFs are not well described and may have characteristics of both fibroblasts and astrocytes (Li et al., 1998, 2003). After 14-17 days *in vitro* (DIV) an optimal mixture of OECs and ONFs is established (Raisman, 2001) which results in the stimulation of axonal regrowth after transplantation *in vivo* (Li et al., 1997, 1998). After transplantation of this optimal mixture of OECs and ONFs in a small spinal cord lesion, OECs and ONFs were observed in different locations in relation to the regrowing axons (Li et al., 1998), suggesting a different role of OECs and ONFs in the axon growth stimulation. Whereas OECs were found to be directly associated with the axons, the ONFs enclosed the OEC-enwrapped axons at some distance forming tubular sheaths (Li et al., 1998).

Table 1. Experimental time scheme for coculture experiments[a]



[a] *Note that culturing of the astrocytes starts 4 days prior to that of OEC/ONF. Circles in Petri dishes or flasks represent glial cells (OEC/ONF or astrocytes), triangles represent cocultured cortical neurons.*

In the olfactory system it is described that OECs ensheath the olfactory axons as they grow into the two outer glomerular layers of the OB (Raisman, 1985). The glomeruli of the OB are delineated by astrocytes (putative ONFs) that are thought to have an axon-growth inhibitory action (Gonzalez Mde et al., 1993). Hence, the interplay between OECs and ONFs is a prerequisite for a correct olfactory axon guidance. The respective locations of the OECs and ONFs around axons severed by a SCI, suggests they have a role in axon growth stimulation and guidance as well.

Alignment of glial cells may be another important factor in the appropriate guidance of axons. Alignment of astrocytes during development has been shown to be very important for the correct outgrowth of corticospinal tract fibers (Joosten and Gribnau, 1989). It has also been reported that the injection of an oriented population of OECs resulted in directional host axonal growth across natural anatomical barriers (Perez-Bouza et al., 1998). Hence, we suggested that glial alignment has a positive effect on the directionality of axon growth.

Alignment of glial cells may be achieved by culturing them on biodegradable poly(D,L)-lactide matrices. Many studies have cultured glial cells on biologically degradable matrices (Xu et al., 1995; Woerly et al., 1996; Oudega et al., 1997; Rabchevsky and Streit, 1997; Bamber et al., 2001). The majority of these studies reported a good viability of the glial cells on these artificial substrates, but there is no indication of any clear glial orientations. Poly(D,L)-lactide matrices consists of macroporous polylac-tide foams, which are highly oriented (Maquet et al., 2000). Transplantation of this type of biodegradable matrix in a spinal cord resulted in preferential axon growth along the main pore direction (Blacher et al., 2003). Hence, in the present study we investigated if glial cells cultured on this type of biodegradable matrix adopt a phenotype which is oriented along the main pore direction.

Although it is strongly suggesting that alignment has a positive effect on neurite growth, the hypothesis that aligned glia enhance neurite growth compared with non-aligned glia has not yet been tested. It has neither been quantitatively shown that glial cells can be grown oriented in an aligned way on a biomatrix. Here, adult OEC/ONF and neonatal astrocytes were cultured on biodegradable poly(D,L)-lactide matrices or in petridishes. The alignment of the glial cells was subsequently quantified using image analysis. Neonatal cerebral cortical neurons were cocultured on these glia/biomatrix and glia/Petri dish complexes. Neurite elongation, as the length of the longest neurite per neuron, and neurite formation, as the number of primary neurites per neuron, were measured for all conditions. In addition, it was tested by image analysis whether the cocultured neurons extended their neurites in the same direction as the underlying glial/biomatrix complexes. Besides studying the *in vitro* interactions and effects of aligned glial cells on neurite outgrowth these experiments can provide a foundation for the transplantation of glial/biomatrix complexes *in vivo* after SCI.

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EXPERIMENTAL PROCEDURES

OEC/ONF cultures were obtained from adult male Lewis rats aged 8–10 weeks. Neonatal cerebral cortical neurons and astrocyte cultures were obtained from postnatal day 1 male Lewis rat pups. All experimental procedures were performed according to the recommendations of the European Commission and protocols were approved by the Committee on Animal Research of the Maastricht University. Every attempt was made to minimize the number of animals and their suffering used in this study. Poly(D,L)-lactide matrices (Blacher et al., 2003) were used to obtain aligned growth of OECs and ONFs or astrocytes. Petri dishes were used to grow OEC/ONF cultures or astrocyte cultures without a clear growth direction preference. OEC/ONF cultures were maintained in culture for 10 days before transferring them either onto the biodegradable matrices (alignment-groups) or into Petri dishes (non-alignment-group). Astrocyte cultures were maintained in culture for 14 days before transfer either onto matrices or into Petri dishes. During the last two of these DIV14 a purification procedure was carried out to obtain over 95% pure astroglial cultures (Dijkstra et al., 1999; see below). The OEC/ONF and astrocyte cultures were allowed to grow onto poly(D,L)-lactide matrices or in Petri dishes for the next 4 days. Then, neonatal cerebral cortical neurons were added. These cocultures were maintained for 2 days before fixation. Importantly, the use of this protocol renders OEC/ ONF cultures which at the time of coculturing have the age of DIV14–16, at which they have been described as to have optimal reparative properties after transplantation *in vivo* (Raisman, 2001). The experimental time scheme is indicated in Table 1. For the purpose of this study the experiments were subdivided into several parts. It was examined if (1) OEC/ONF cultures enhanced neurite growth (length of longest neurite and number of primary neurites per neuron) compared with astrocyte cultures, (2) glial cultures showed alignment on the biomatrices versus Petri dishes, (3) alignment of glial cultures enhanced their neurite-growth stimulation, and (4) the aligned glial cells were able to direct neurite growth, i.e. if the alignment of the glial cells is similar to that of the neurites.

Coating of the Petri dishes

Plastic culture Petri dishes (35 mm diameter; Nunc, Roskilde, Denmark) were coated with poly-L-lysine (PLL; Sigma Chemical Co., St Louis, MO, USA; >300,000 molecular weight, 1% in purified water) for 15 min and then air-dried for at least 2 h.

Cell cultures

OEC/ONF cultures. The method of OEC and ONF culturing was based on the method described by others (Ramon-Cueto and Nieto-Sampedro, 1992), but without the purification step. In short, adult (8–9 weeks old) male Lewis rats, bred in the animal facilities of Maastricht University, were decapitated and the OBs were dissected and cleared of meninges. Then, the outer two glomerular layers were carefully dissected. The tissue was diced in small fragments and incubated with trypsin (Gibco; 0.1% in phosphate-buffered saline) at 37 °C for 15 min. Trypsinization was stopped by addition of culture medium, Dulbecco's Modified Eagle's medium (DMEM/NUT mix F12; Gibco) with glutamax-I supplemented with 10% inactivated fetal calf serum (Bodinco, Netherlands) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The tissue was washed twice with culture medium and collected in 1-ml culture medium. Then, single cell dissociation was achieved by three to five passes through a 5-ml pipette and 10-20 passes through a 1-ml pipette. The cells were plated in PLL-coated Petri dishes at a density of 200,000 cells per Petri dish. The cells were grown for 4-5 days before half of the medium was refreshed. Thereafter the medium was refreshed every 2 days. It is known that this protocol renders non-pure OEC cultures containing both OECs and ONFs. Therefore, these cultures will be named OEC/ ONF cultures.

Astrocyte cultures. The method of astrocyte culturing was similar to the method of others (Dijkstra et al., 1999), with minor modifications. In short, neonatal (postnatal day 1) Lewis rat pups, bred in the animal facilities of Maastricht University, were decapitated and the neocortex was dissected and cleared of meninges. The tissue was diced in small fragments and incubated in trypsin (0.05% in phosphate-buffered saline) at 37 °C for 15 min. Trypsinization was stopped by adding culture medium and the tissue was centrifuged at 500 rotations per minute (r.p.m.) for 1 min. The supernatant was discarded and the pellet was resuspended in 1-ml culture medium. Single cell dissociation was achieved by three to five passes through a 5-ml pipette (Greiner, Germany) and 10-20 passes through a 1-ml pipette (Greiner). Then, the tissue was centrifuged very briefly at 1000 r.p.m. to separate cells from tissue debris. The supernatant containing the cells was then plated into 25 cm^2 cell culture flasks (Corning, NY, USA) at a density of 10^6 cells per flask. Culture medium was refreshed after 4-5 days and every 2 days thereafter. At DIV12, the cultures reached confluence, and contaminating cells were shaken off on a rotary shaker (Rotofix 32; Hettich Zentrifugen) using a 2-days-lasting purification protocol. Briefly, this protocol involves shaking of the flasks at 135 r.p.m. and refreshment of the medium. At DIV14 the purification was

complete. This purification method renders >95% GFAP immunopositive astrocyte cultures (Dijkstra et al., 1999).

Neuron cultures. The protocol for isolation of neonatal cerebral cortical neurons was identical to that described by others (Dijkstra et al., 1999). After isolation of the cortical cells they were plated. In pilot studies we established that a plating density of approximately 40,000 cells per cm^2 of the glial cultures was appropriate for neurite-growth measurements after a coculture time of 2 days.

Poly(D,L)-lactide matrices

Poly(D,L)-lactide with inherent viscosity of 1.62 dl/g was purchased from Purac Biochem (The Netherlands). A diblock poly(D,L-lactide-*b*-polyethylene oxide) (PLA-*b*-PEO) copolymer with a PLA block of ca. approximately 4000 of molecular weight was synthesized by ring-opening polymerization of D,L-lactide from PEO-monomethylether (with molecular weight ca. approximately 5000, supplied by Sigma). Two different polymer foams were prepared, either from pure PLA or from a mixture of PLA containing 10 wt % of PLA-*b*-PEO amphiphilic copolymer (PLA/PLA-*b*-PEO). Polymer foams with an aligned pore structure were prepared by freeze-drying of polymer solutions, as previously described (Maquet et al., 2001). The polymer was dissolved in dimethylcarbonate to yield a 5 wt % polymer solution. The solution was rapidly frozen by immersion into liquid nitrogen in order to induce solvent crystallization. Then the frozen system was connected to a vacuum pump and the solvent was removed by sublimation at -10 °C and 10^{-2} Torr. After 48 h, the drying temperature was increased to 0 °C and the drying process was continued. The foams were finally dried at room temperature. Small polymer rods (1x1x10 mm) were cut from the freeze-dried foams using a razor blade and sterilized by UV exposure for 15 min.

Glia on poly(D,L)-lactide matrices or in Petri dishes

DIV10 OEC/ONF cultures and DIV14 astrocyte cultures were collected from the Petri dishes or flasks respectively by a 5-min-incubation in 0.1% trypsin/0.05 mM EDTA (Acros Organics, Belgium) solution at 37 °C. Then, OEC/ONF and astrocyte cultures were replated either onto the two types of biodegradable matrices (polylactide-matrix, PLA; or polylactide-matrix with the amphiphilic copolymer, PLA/PLA-*b*-PEO) or into PLL-coated Petri dishes to form a glial covering of the matrices and Petri dishes in 4 days. In pilot studies it was established that a 4-days culturing period was optimal for an appropriate covering of the matrix surface and of the Petri dish.

Fixation

For immunocytochemical stainings, the cultures were fixed with 4% paraformaldehyde (Merck, Germany) for 15 min. The fixed cultures were either stored in 0.5% paraformaldehyde or directly used for immunocytochemistry.

Immunocytochemistry

The following primary antibodies were used: mouse anti-microtubule-associated protein 2 (anti-MAP2, 1:200, Sigma), mouse anti-neurofilament RT97 (anti-RT97; 1:100; Hybridoma Bank, Iowa City, IA, USA), rabbit anti-S100β (1:1000; Swant, Bellinzona, Switzerland), mouse anti-low affinity nerve growth factor receptor p^{75} (anti-p^{75}; 1:10,000; Chemicon), mouse anti-glial fibrillary acidic protein (anti-GFAP; 1:1600; Sigma), rabbit anti-GFAP (1:1000; DAKO), rabbit anti-fibronectin (anti-fibronectin; 1:12,500; generous gift from S. Mollers, Universitätsklinikum, RWTH Aachen, Germany), and anti-vimentin (V9 clone; 1:12,500; Sigma). All primary antibodies were diluted in 0.3% Triton X-100 in Tris-buffered saline (TBS-T). All primary antibody incubations were overnight at room temperature. As secondary antibodies, Cy3-conjugated donkey anti-mouse (1:800), Cy3-conjugated donkey anti-rabbit (1:800), and Alexa488-conjugated goat anti-rabbit (1: 100; Molecular Probes) were used. For double immunostaining protocols Alexa 488-conjugated donkey anti-rabbit and Cy3-conjugated donkey anti-mouse were used. All secondary antibody incubations were 1.5 h at room temperature. When a double-staining was performed, the two primary antibodies were used in a mixture and then the incubation with Cy-3 labeled secondary antibody always preceded the incubation with Alexa-labeled secondary antibody. Before all antibody incubations, washing steps were carried out using subsequently TBS-T (10 min), Tris-buffered saline (TBS; 10 min) and TBS-T (10 min). All other washing steps involved three times 10 min with TBS. For visualization of nuclei in the cultures a 30-min incubation with Hoechst 33342 (1:500; Sigma) was used. Vimentin staining was performed to visualize all cells in the OEC/ONF cultures since both OECs and ONFs were found to express this intermediate filament protein (see also Li et al., 1998; Ramon-Cueto and Nieto-Sampedro, 1992). S100β staining was used to selectively visualize OECs in OEC/ONF cultures. GFAP staining

was used to visualize astrocytes. A combined neurofilament (RT97) and MAP2 staining was used to visualize neurons with their processes. Stained cultures were analyzed using an Olympus AX-70 microscope using epifluorescent illumination. The microscope was equipped with a x20 objective and a x10 projection lens. The signal for Alexa 488 (green) was detected using a narrowband MNIBA-type FITC filter, the signal for Cy3 (red) was detected using a MNG filter, and the signal for Hoechst (blue) was detected using a U-MNIBA filter (Chroma technology). Pictures of the stained cultures were made at a magnification of 20x using a Sony Power HAD 3CCD Color Video Camera (Olympus, The Netherlands). All pictures, used for neurite length measurements (see below), were analyzed with the image analyzing system analySIS Vers. 3.0. (analySIS, Germany).

Glial characterization

It is known that OEC/ONF cultures have optimal reparative properties at DIV14-17 (Raisman, 2001). This is probably due to the composition of the cell population at that time involving an optimal relative contribution of OECs and ONFs. In order to analyze the OEC contribution to the total cell population at subsequent DIVs, OEC+ONF cultures were fixed at DIV11, 14 and 16 and OECs were selectively stained. These DIVs were chosen for the following rationales: DIV11 is 1 day after transfer of the cultures onto matrix or into Petri dish, DIV14 is the first day of coculturing and DIV16 is the end-point. *In vitro,* the low-affinity nerve growth factor receptor p^{75} and the S100β protein are regarded as selective markers for OECs (Li et al., 1998; Ramon-Cueto and Nieto-Sampedro, 1992; Doucette and Devon, 1994; Barnett et al., 1993). All cells in the OEC/ONF cultures expressing either p^{75} or S100β were defined as OECs. The remaining cells in the OEC/ ONF cultures, the p^{75} or S100β negative cells, were defined as ONFs. The OEC contribution of the total OEC/ONF culture was quantitatively assessed using S100β immunostaining combined with a Hoechst nuclear staining. The composition of the ONF cell population in our OEC/ONF cultures was investigated by double stainings of the p^{75}-low affinity nerve growth factor receptor with either GFAP, fibronectin or vimentin, the latter two of which are known to be expressed by ONFs (Li et al., 1998). A combined Hoechst nuclear staining with these immunocytochemical double stainings was used to determine the number of ONFs with the phenotype p^{75}—/FN+ , p^{75}—/GFAP+, and p^{75}—/vimentin+. In order to assess the purity of the astrocyte cultures, a GFAP immunostaining was combined with a Hoechst nuclear staining. A total of 800-1200 cells were investigated for OEC/ONF and astrocyte cultures, respectively. All measurements were performed by investigators blinded for the conditions.

Alignment

OEC/ONF and astrocyte cultures were cultured either on the two types of biodegradable matrices or in Petri dishes for 4 days. The cultures were then fixed and processed for S100β or GFAP immunostaining. Pictures were taken from all experimental conditions (OEC/ONF or astrocyte on PLA matrix, on PLA/PLA-*b*-PEO matrix, or in Petri dish). Six to 12 pictures per group were quantified for alignment by image analysis.
Image treatment and statistic analysis were performed using two software programs, i.e. Visilog 5.2 from Neosis and Aphilion 3.6 from Adcis with a PC. Gray level image transformations, binary image processing and spectral analysis have been detailed elsewhere (Blacher et al., 2003). Spectral analysis measurements, which allow to evaluate the degree and direction of anisotropy of an image, are represented in rose plots. The intercept number, defined as the number of entries in an object along a given direction, have been calculated for each object of the binary images, in all directions between 0 and 360°, each 10°. Then, the degree of the object orientation was defined as the ratio y/x, where x is the maximum and y the minimum intercepts value. This implies that the higher the y/x ratio, the higher the degree of alignment.

Neurite growth

Neurite elongation and neurite formation. The neurite outgrowth stimulating potency of the aligned and non-aligned glia on neonatal cerebral cortical neurons was tested in coculture experiments. Cocultures were maintained for 2 days before fixation. Then the cocultures were stained for MAP-2 and RT97. Neurite elongation, as the length of the longest neurite, and neurite formation, as the total number of primary neurites per neuron, were measured using the analytical software AnalySIS (see Fig. 2). The investigator was blinded for the specific conditions. The longest neurite and the total number of primary neurites were investigated for approximately 50 neurons per group.

Direction of neurites in respect to the glial alignment. The ability of aligned glial cells to stimulate directional neurite growth was tested in coculture experiments. Cocultures of glial/biomatrix complexes and cortical neurons were again maintained for 2 days before fixation. Then, the cocultures were double stained for MAP-

2/RT97 (neurons with their processes) and GFAP or S100β (astrocytes and OECs respectively). Pictures were taken from immunostained aligned glial cells. Then, the neurons that were observed within this area were also photographed. The direction of the glial cells in this area was compared with that of the neurons in the corresponding area (see quantitative analysis of alignment). In the analyzed areas, the ratio between astroglial cells and neurons was about 10:1; between OECs and neurons this ratio was approximately 5:1.

Statistical analysis

The two-tailed *t*-test was used to compare the neurite-growth stimulating effects between OEC/ONF and astrocyte cultures. Also, the direction of the neurites was compared with that of the glia using the two-tailed *t*-test. The one-way analysis of variance was used to compare (1) the alignment of the glial cultures on PLA matrix, PLA/PLA-*b*-PEO matrix, and in Petri dish, (2) the neurite measurements (length of longest neurite and number of primary neurites per neuron) in alignment and non-alignment, and (3) the OEC contribution to the total OEC/ONF culture over subsequent DIVs. The Bonferroni was used as a post hoc test. A *P*-value below 0.05 was regarded as the level of significance.

RESULTS

Glial cultures

Our OEC/ONF cultures were found to contain two main cell types (see Fig. 1A). One cell type having a flattened fibroblast-like morphology in a naive (not-replated) primary OEC/ ONF culture and the other having an elongated, spindle-shaped morphology.

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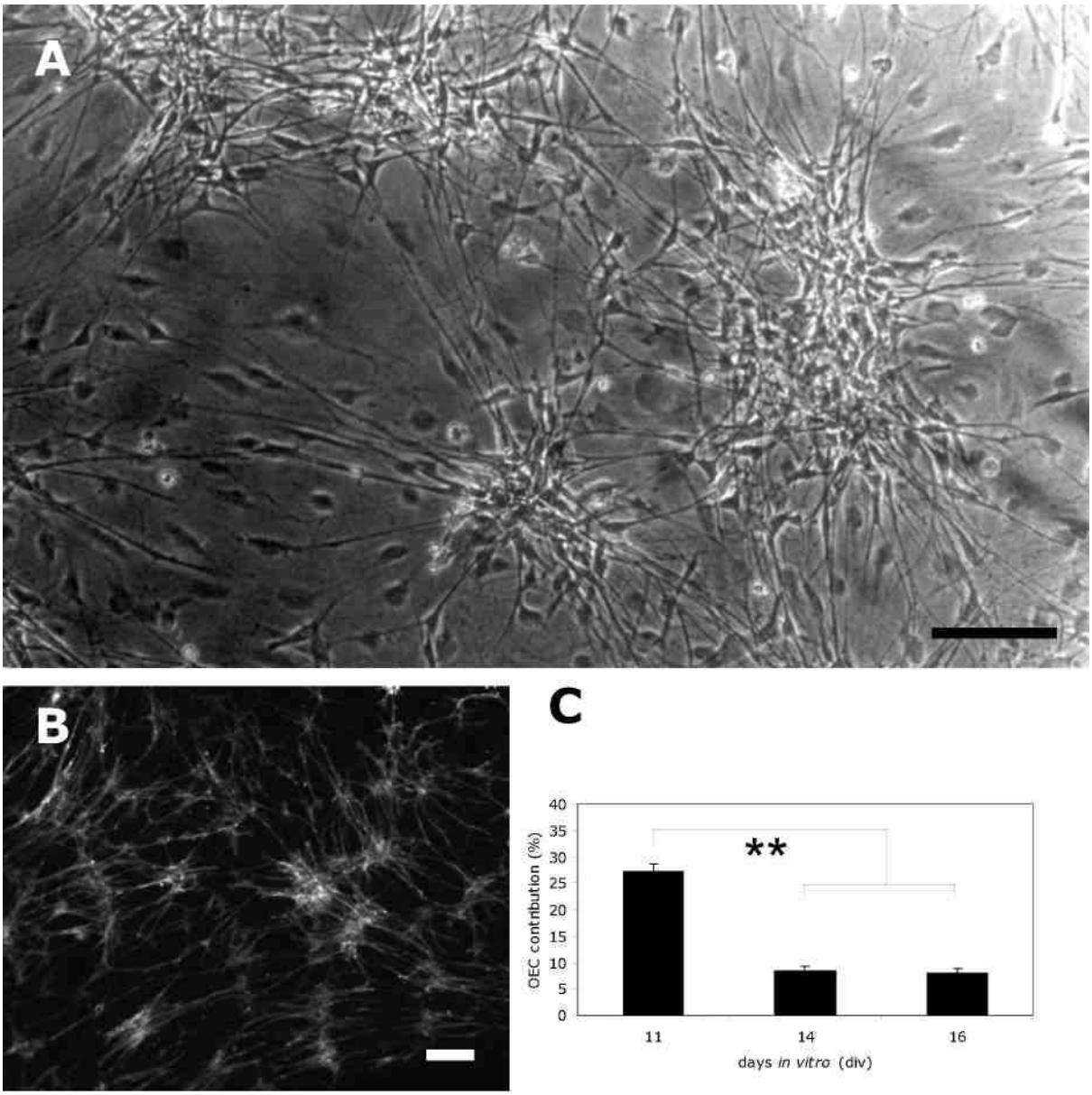

*Fig. 1. OEC/ONF cultures. (A) Phase-contrast photograph of a 14 days old OEC/ONF culture without replating (scale bar=200 µm); (B) a selective staining of OECs, using the marker p^{75}-low affinity nerve growth factor receptor (scale bar=200 µm); (C) the relative amount of OECs in our OEC/ONF cultures over time. ** P<0.01.*

The latter cell type was found to be p^{75} immunopositive (see Fig. 1B) and therefore defined as the OEC. The OECs in our OEC/ONF cultures were found to co-express S100β and GFAP. However, not all OECs expressed GFAP identically. The majority of OECs (86.9 ± 2.6%) expressed relatively low levels of diffuse GFAP; the remaining OECs (13.1 ± 2.6%) expressed high levels of filamentous GFAP. The expression of fibronectin was detected in a subpopulation of OECs (25.4 ± 2.5%). The cell type with a flattened morphology (see Fig. 1A) was regarded as an ONF, but due to the lack of a selective marker this is only suggestive. We defined all p^{75}-negative cells in our OEC/ONF cultures to be ONFs. This ONF cell population was found to be heterogeneous. Double-label experiments showed that 91.6 ± 1.6% of all p^{75}-negative cells expressed fibronectin. In contrast, the number of p^{75}-negative cells expressing GFAP was extremely low: 0.79 ± 0.0%. All cells in our OEC/ONF cultures were found to express vimentin.

The OEC contribution to total OEC/ONF cultures was determined at DIV11, 14, and 16. The OEC percentage was found to be highest at the earliest time point (DIV11: approximately 27%) and decrease significantly at subsequent DIVs (DIV14: approximately 8.5% and DIV16: approximately 8%; see Fig. 1C; $F_{2,11} = 105.9$, $P<0.01$).

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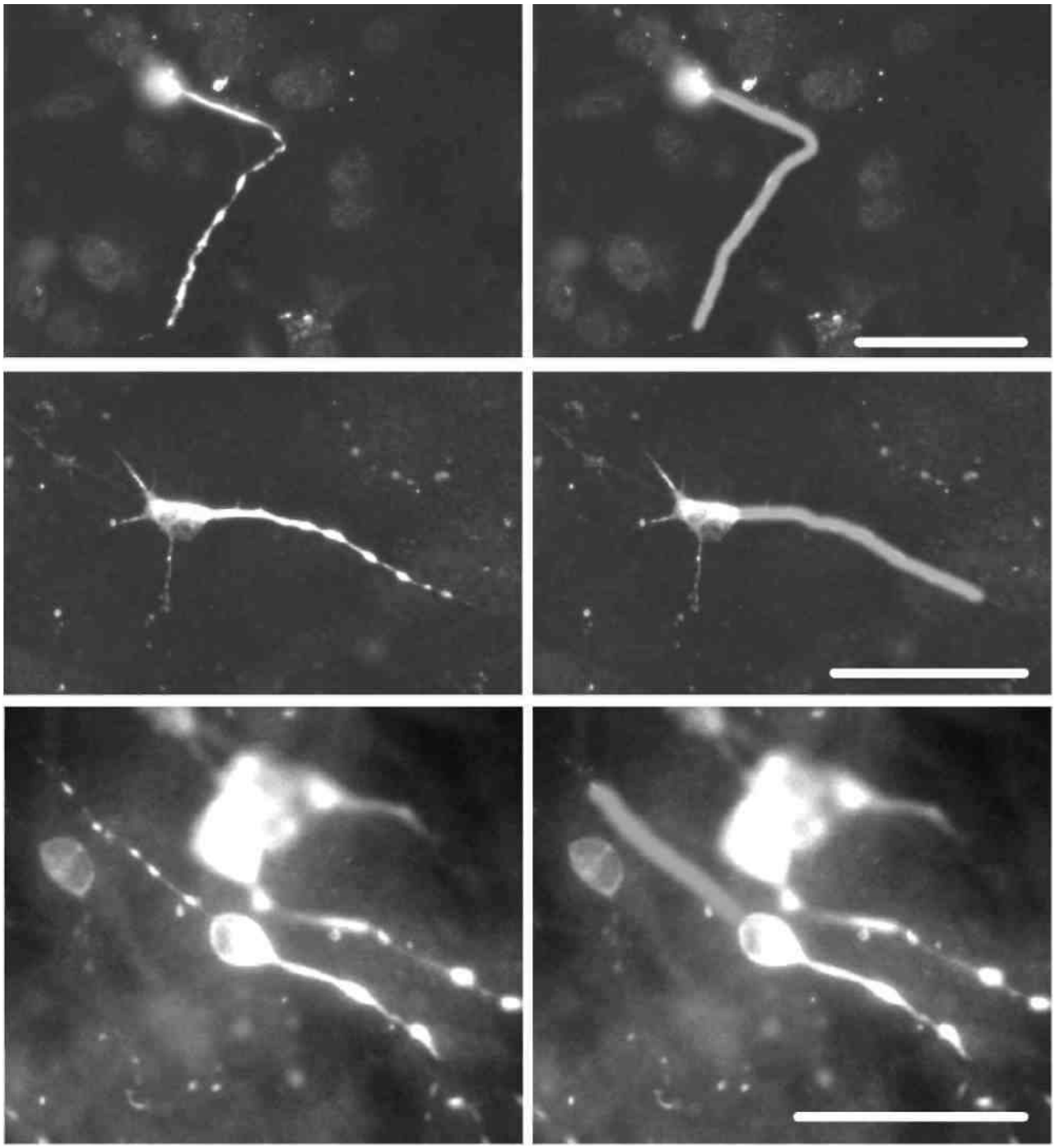

Fig. 2. Neonatal cerebral cortical neurons on glial cultures. Cortical neurons were plated on top of OEC/ONF or astrocyte cultures in a Petri dish or on biomatrices, fixed after 2 days, and the neurites were stained using a combined staining of RT-97 and MAP2. (A) Neuron on OEC/ONF in Petri dish; (C) neuron on astrocytes in Petri dish; (E) neuron on astrocytes on PLA/PLA-*b*-PEO; (B, D, and F) neurite length measurements of the same neurons as in A, C, and E are indicated. Scale bars=50 μm.

Although the OEC percentage was significantly lower between the earliest time point and the later two time points, there was no difference between the OEC percentage at DIV14 and DIV16.
The astrocyte cultures were found to be highly pure after the purification, having >99% GFAP immunoreactive cells.

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OEC/ONF cultures enhance the length of the longest neurite compared with astrocyte cultures

The neurite outgrowth stimulatory capacity of OEC/ONF cultures was compared with that of neonatal astrocyte cultures.
Neurite elongation and neurite formation were assessed as indicated in Fig. 2. As shown in Fig. 3 coculturing cortical neurons on OEC/ONF feeder layers resulted in an increased length of the longest neurite as compared with astrocyte feeders (P<0.05). In contrast, the number of primary neurites per neuron was lower on OEC/ONF feeders than on astrocyte feeders (P<0.05).

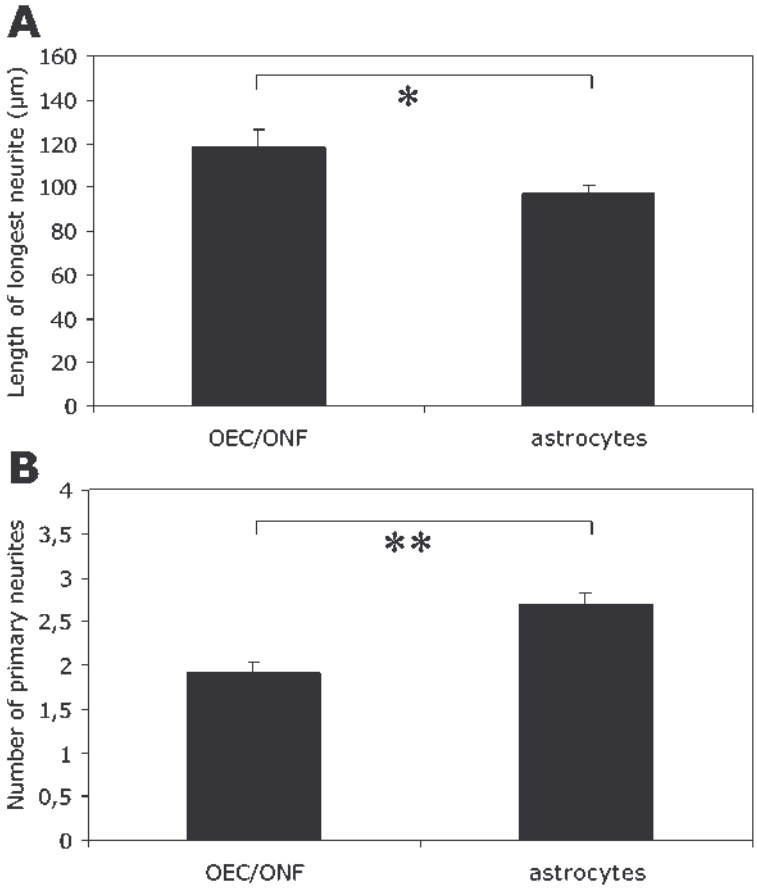

Fig. 3. Neurite-outgrowth stimulation by OEC/ONF and astrocyte cultures in Petri dish. OEC/ONF cultures enhance the length of the longest neurite, but lower the number of primary neurites per cocultured neuron compared with astrocyte cultures. * P<0.05; ** P<0.01.

Culturing glial cells on polylactide matrices induces aligned glial growth

Alignment of glial cells may be obtained by culturing them on poly(D,L)-lactide matrices (PLA and PLA/PLA-*b*-PEO). Culturing the glial cells in Petri dishes resulted in the absence of any orientation (see Fig. 4A, D). Already at low power magnification a clear orientation of glial cells plated on PLA or PLA/PLA-*b*-PEO matrices can be noted (see Fig. 4B, C, E, F). In order to substantiate the degrees of glial alignment under the three conditions (PLA, PLA/PLA-*b*-PEO, Petri dish), quantification of alignment using image analysis was carried out.
The quantitative data are shown in Fig. 5. Overall, there is a significant group difference for alignment of OEC/ONF conditions ($F_{2,24}$=33.90, P<0.01). Alignment of OEC/ONF cultures is best on PLA/PLA-*b*-PEO matrix (y/x=1.97) followed by PLA matrix (y/x=1.65) and Petri dish (y/x=1.42). Alignment of astrocytes differs significantly over all conditions as well ($F_{2,22}$=41.10, P<0.01). Alignment is best on both PLA and PLA/PLA-*b*-PEO matrices (y/x=1.99 and 1.87, respectively) and worst in Petri dish (y/x=1.39). There is only a significant difference between the matrices and Petri dish.

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Alignment of glial cells does not enhance neurite outgrowth stimulation

When glial cells were grown on the PLA or PLA/PLA-*b*-PEO matrix, they adopted an aligned phenotype (Figs. 4 and 5). The neurite outgrowth stimulating effect of the aligned glial feeder was compared with that of non-aligned glial feeder. Fig. 6 shows the results. There is an overall difference in the length of the longest neurite for OEC/ONF cultures on PLA matrix, PLA/PLA-*b*-PEO matrix, and in Petri dish ($F_{2,132}$=12.89, P<0.01). The length is longest on non-aligned OEC/ONF cultures (117.8 ± 8.2 μm) and is lower on OEC/ONF-PLA matrix (79.7 ± 9.1 μm) and OEC/ ONF-PLA/PLA-*b*-PEO matrix (66.2 ± 5.7 μm). There are only significant differences between the aligned and non-aligned groups (P<0.01). The number of primary neurites per neuron does not differ for the different OEC/ONF cultures ($F_{2,109}$=1.85, n.s.). The average number of primary neurites per neuron is between 1.6 and 1.9 for all OEC/ ONF cultures.

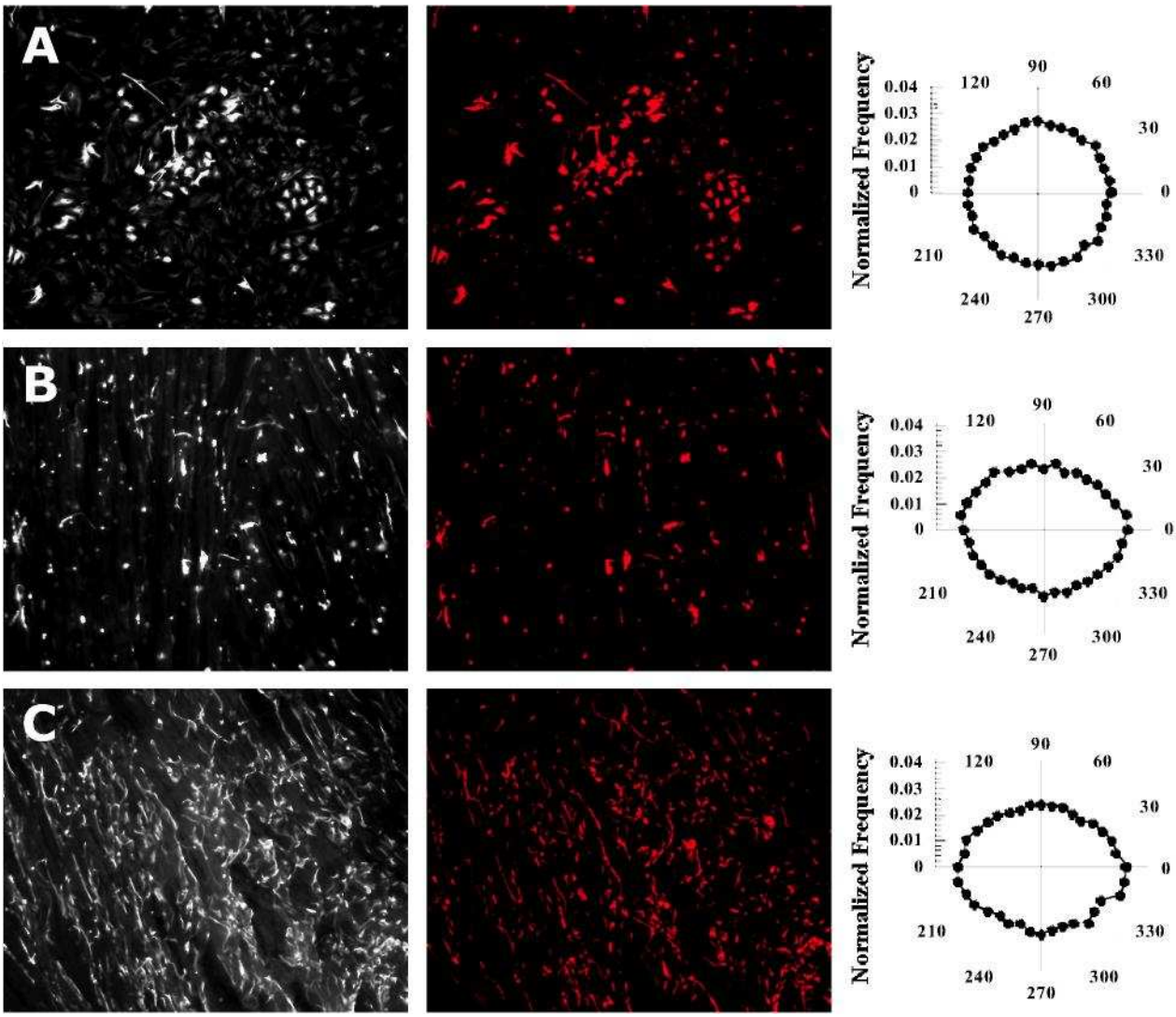

Fig. 4. Glial cultures in Petri dish, PLA and PLA/PLA-b-PEO matrix with binarized images and rose-plots. (A–C) DIV14 OEC/ONF cultures grown for 4 days in Petri dish, on PLA-matrix, or on PLA/PLA-b-PEO matrix; (D-F) DIV14 astrocyte cultures grown for 4 days in Petri dish, on PLA-matrix, or on PLA/PLA-b-PEO matrix.

In addition, the results show that there is an overall difference in the length of the longest neurite for the different astrocyte cultures ($F_{2,169}$=3.61, P<0.05). There is, however, only a significant reduction in the length of the longest neurite on PLA/PLA-*b*-PEO matrix (77.0 ± 5.3 μm) compared with Petri dish (97.1 ± 4.1 μm; P<0.05). Overall, the number of primary neurites per neuron differs for astrocyte groups ($F_{2,161}$ =29.28, P<0.01). This number is significantly reduced on both PLA (approximately 1.9) or PLA/PLA-*b*-PEO matrix (approximately 1.6) compared with Petri dish (approximately 2.7; P<0.01). There is no significant difference between PLA and PLA/PLA-*b*-PEO matrix.

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Directional growth of cortical neurites is achieved on aligned glial cells

Since OEC/ONF and astrocyte cultures adopt an aligned phenotype on the PLA and PLA/PLA-*b*-PEO matrices it is likely that neurons will also extend their processes in an aligned fashion. The results are shown in Fig. 7. All the neurites tested showed an identical orientation as the glial cells on PLA and PLA/PLA-*b*-PEO matrix. OEC/ONF cultures on PLA and PLA/PLA-*b*-PEO matrix had alignment values of 0.57 and 0.70 respectively, whereas these values were 0.58 and 0.66 for the cocultured neurons. Astrocyte cultures on PLA and PLA/PLA-*b*-PEO matrix had alignment values of 0.61 and 0.57, whereas these values were 0.58 and 0.58 for the cocultured neurons. There were no significant difference between the orientations of the different glia/biomatrix complexes and those of the neurites (Fig. 7).

DISCUSSION

The interactions between glial cells and neurons is crucial for the outgrowth of neurites. The astroglial cells and the OECs are known to be important for the correct outgrowth of axons during development and in adulthood (Joosten et al., 1995; Ramon-Cueto and Avila, 1998).

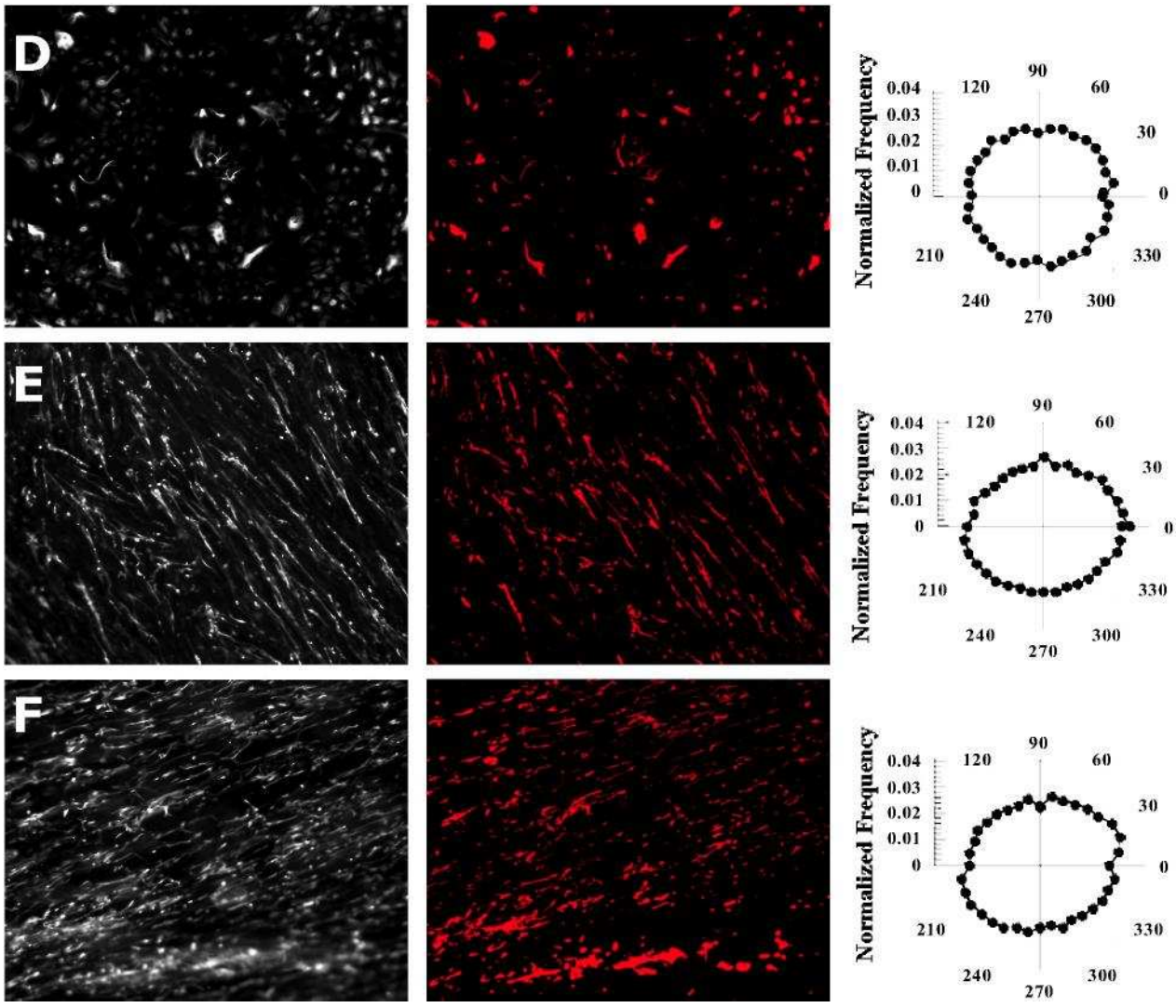

Fig. 4. (*Continued*).

Alignment of astroglial cells in the developing CNS has also been shown to be important for the correct outgrowth of several axon tracts, such as the corticospinal tract (Joosten, 1997). Furthermore, the injection of OECs in an aligned fashion highly supported directional host axonal growth (Perez-Bouza et al., 1998). We have shown that optimal OEC/ONF cultures from adult rats enhance the length of the longest neurite (neurite elongation) of cocultured neonatal cerebral cortical neurons compared with neonatal astrocyte cultures, whereas

the number of primary neurites (neurite formation) is increased on neonatal astrocyte cultures compared with OEC/ONF cultures. Furthermore, we have shown for the first time in a quantitative way that the glial cultures can be grown aligned *in vitro* on biologically degradable polylactide matrices. The alignment of glial cells results in a directed neurite outgrowth of cocultured cortical neurons along the glial/biomatrix complex. The alignment of the glial cells did not increase neurite growth of the cocultured neurons.
In our experiments, we used two types of glial cultures: neonatal astrocyte cultures and adult OEC/ONF cultures. We selectively stained OECs in our mixed OEC/ONF cultures using the markers p^{75} or S100β (Li et al., 1998; Ramon-Cueto and Nieto-Sampedro, 1992; Doucette and Devon, 1994; Barnett et al., 1993). Although OECs from different sources and grown under different conditions are known to express markers variably (Franceschini and Barnett, 1996; Ramon-Cueto and Avila, 1998; Barnett and Chang, 2004), the expression of p^{75} and S100β has been reported by OECs in mixed OEC/ONF cultures like ours (Li et al., 1998). Using these selective markers, we observed that all our OECs were GFAP positive. However, the GFAP expression pattern differed from high levels of filamentous GFAP to lower levels of diffuse GFAP as has also been found by others (Pixley, 1992; Franceschini and Barnett, 1996).

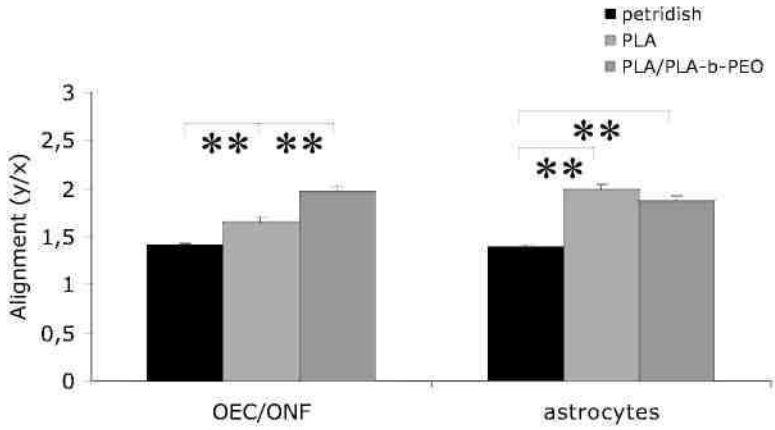

Fig. 5. Quantitative results on alignment of glia in Petri dish and on biomatrices. ** P<0.01.

The majority of OECs had a low level of diffuse GFAP expression. This may be explained by the serum in our culture medium, which is known to decrease the expression of GFAP by OECs (Franceschini and Barnett, 1996). Furthermore, we found a subpopulation of OECs to express fibronectin. This has also been found by others (Ramon-Cueto and Nieto-Sampedro, 1992) and again indicates the phenotypic heterogeneity of OECs. The ONF cell population, defined as the fraction of p^{75}-negative cells, in our mixed OEC/ONF cultures was found to contain more than one single cell type. The majority of ONFs (about 92%) was found to express fibronectin, suggesting these cells to be fibroblasts. This is in accordance with data of others (Ramon-Cueto and Nieto-Sampedro, 1992; Li et al., 1998). Only a very low proportion of ONFs was found to express GFAP (about 1%), suggesting an astrocytic phenotype of these cells. The identity of the remaining cells in the ONF population (7–8%) remains unknown.

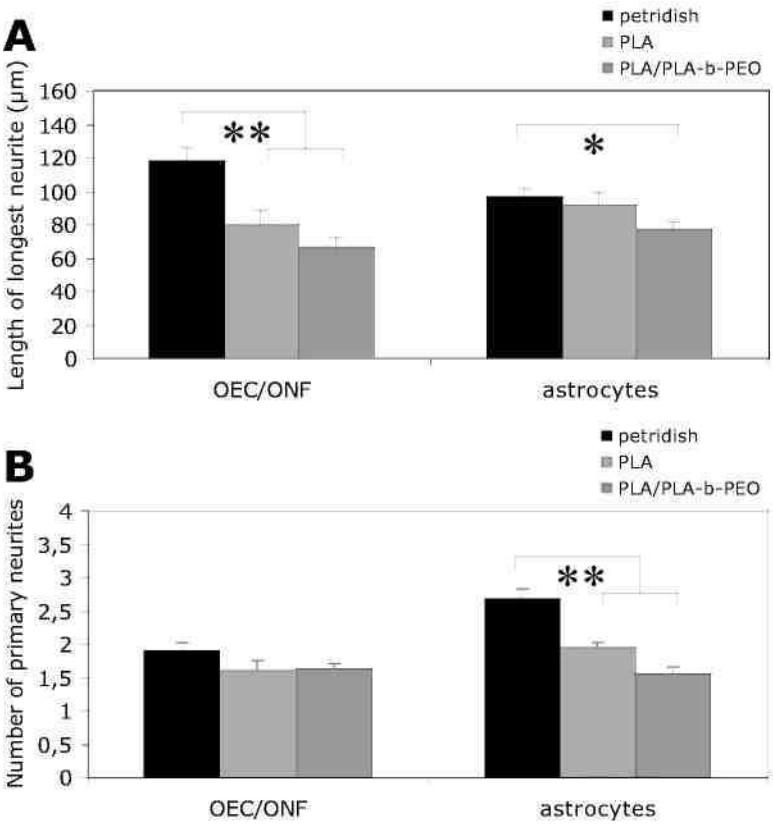

*Fig. 6. Neurite-outgrowth stimulation by aligned versus non-aligned OEC/ONF and astrocyte cultures. Alignment of the glial cultures results in a reduction of neurite-growth enhancement. * P<0.05; ** P<0.01.*

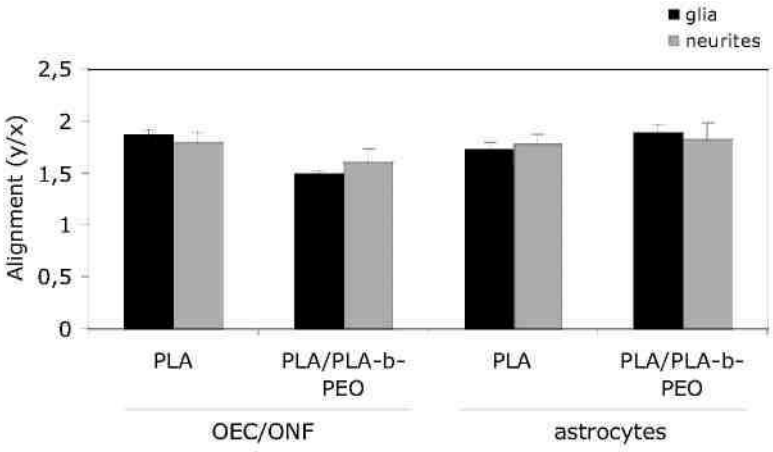

Fig. 7. The direction of the OEC/ONF or astrocyte cultures and the cocultured neurites. Quantitative results on the direction of the DIV16 glia and the cocultured neurons. Neurites obtain the same direction of outgrowth as the aligned glial/biomatrix complexes.

The ONF cell population may, thus, contain a proportion of fibroblasts, astrocytes and other glial or non-glial cell types. It is important to note here, that the use of serum in the medium can dramatically downregulate p^{75} expression by OECs. Hence, it cannot be ruled out that a proportion of ONFs consists of OECs which do no longer express the p^{75}-low affinity nerve growth factor receptor (Franceschini and Barnett, 1996).
The neuronal populations that we used were obtained from the cerebral cortex of neonatal rats. The cerebral cortex contains many differently sized neurons, suggesting highly variable neurite lengths in different cortical neuron populations. However, we encountered a relatively low variation in neurite lengths for these neuronal populations after 2 days of coculturing. The neurons and their processes were stained against phosphorylated neurofilament and microtubule-associated protein. We observed that the majority of neurons expressed these proteins, even at relatively short survival times of 6 h *in vitro* (unpublished results).

The neurite-growth promoting effects of astrocytes seems to be associated with the age of the donor-animal (Smith et al., 1990; Le Roux and Reh, 1995). This observation has been associated with a change in the expression of adhesion molecules on cortical astrocytes during maturation (Smith et al., 1993). We used neonatal astro-cyte cultures in our investigations to obtain strong neurite-growth promoting effects. The neurite-growth promoting effects of OECs have not been associated with donor age (Kafitz and Greer, 1999; Sonigra et al., 1999), but rather with time in culture (Raisman, 2001). Primary cultures containing both OECs and ONFs obtain growth-promoting characteristics only after at least a week *in vitro* (Raisman, 2001). This may be due to an optimal balance between the relative number of OECs and ONFs in the OEC/ONF cultures. This balance is known to change over time. We observed a decreased percentage of OECs in the OEC/ ONF cultures over subsequent DIVs. This is in accordance with the observed progressive increase in the proportion of ONFs over time *in vitro* (Raisman, 2001). The optimal OEC/ONF mixture has been reported to be obtained at DIV14 (Li et al., 1998). We observed an OEC proportion of 10% in these optimal OEC/ONF mixtures.
The neurite-growth promoting effect of our OEC/ONF cultures was reflected in an increased neurite elongation compared with neonatal astrocyte cultures. *In vitro*, OECs have been found to stimulate axonal elongation of olfactory receptor neurons, retinal ganglion cell neurons, and cortical neurons (Goodman et al., 1993; Kafitz and Greer, 1998, 1999). It was reported that adult OEC cell lines are more growth stimulatory than neonatal astrocyte cell lines (Goodman et al., 1993). It has been suggested that OECs enhance axonal growth by providing a favorable cellular substrate containing molecules that facilitate axonal binding and extension and that may release trophic factors that stimulate axonal growth (Kafitz and Greer, 1999). Adult OECs express the low-affinity nerve growth factor receptor p^{75} and adult ONFs express the cell adhesion molecule E-N-CAM (Sonigra et al., 1999), which are both known to be important for neurite outgrowth (Martini, 1994). PSA-N-CAM expressing cells, like adult ONFs, may be particularly involved in axonal elongation as it has been proposed that PSA is very important for regrowth of supraspinal axons (Aubert et al., 1998). Also, the cell adhesion molecules L1 and N-cadherin, expressed by adult OECs (Ramon-Cueto and Nieto-Sampedro, 1992; Sonigra et al., 1999), are involved in neurite-growth stimulation (Lagenaur and Lem-mon, 1987; Rathjen, 1991; Williams et al., 1994). In addition, laminin, which is known to promote extension of olfactory axons (Kafitz and Greer, 1997), is expressed by adult OECs (Ramon-Cueto and Nieto-Sampedro, 1992; Sonigra et al., 1999). Only few data are available on the neurotrophin-related factor profile of adult OECs. Although the expression of BDNF, NT-3, and NT4/5 by adult OECs has been proposed (Ramon-Cueto and Avila, 1998), it is only known that neonatal OECs express mRNA for NGF, BDNF, NT-4/5, neuregulin, CNTF, GDNF, and neurturin (Boruch et al., 2001; Wewetzer et al., 2001; Woodhall et al., 2001). Also, the release of BDNF, NGF, and the intracellular presence of NT4/5 has been reported for neonatal OECs (Boruch et al., 2001; Woodhall et al., 2001). Astrocytes express many of the same neurotrophic factors and cell adhesion molecules, like GDNF, BDNF, laminin, N-CAM, N-cadherin, and L1 (Tomaselli et al., 1988; Smith et al., 1990; McNaught and Jenner, 2000). Although both astrocytes and OECs express largely identical cell adhesion molecules involved in neurite growth, the relative contributions of these proteins to the total cellular protein profile may account for the observed differences in neurite-growth stimulation.
Neurite formation, reflected in the number of primary neurites per cocultured neonatal neuron, was found to be reduced on OEC/ONF cultures compared with the astrocyte cultures. This was somewhat surprising since the results of others suggest an increased neurite formation on adult OEC cultures compared with neonatal astrocytes (Sonigra et al., 1999). However, Sonigra et al. (1999) used the mean frequency of retinal ganglion cells with neurites as the readout parameter. Furthermore, our OEC/ONF cultures, in contrast to those of Sonigra et al. (1999), contain a relatively high abundance of ONFs. Within the ONF cell population there may be ONF-like cells associated with inhibition of neurite extensions in the glomeruli layers of the OB (Gonzalez Mde et al., 1993). In addition, it is known that ONFs form tubular perineurial sheaths after transplantation of OEC/ONF cultures into a SCI site (Li et al., 1998). The formation of this ONF-sheath may lead to an inhibition of side-branching. Hence, the ONFs in our OEC/ONF cultures may inhibit the formation of more neurites per neuron. Since we used mixed cultures of OECs and ONFs, it remains unclear from our experiments whether it is the mixture of these cell populations that accounts for the observed effects on neurite growth or it is the single cell populations within the OEC/ONF cultures that are responsible. It is comprehensive that the presence of the ONFs alters the function of the OECs. This is substantiated by the observation that OECs have increased myelination properties in the presence of a limited amount of meningeal fibroblasts (Lakatos et al., 2003). Hence, ONFs may affect the functional properties of OECs. Noteworthy is that the enhanced neurite elongation on OEC/ONF versus astrocytes is only moderate, i.e. about 20% increase. In this respect it has to be taken into account that the use of neonatal cortical neurons in our study renders heterotypic cocultures for OEC/ONF and homotypic cocultures for astrocytes. The use of homotypic versus heterotypic cultures can have beneficial effects on neurite growth (Qian et al., 1992; Kafitz and Greer, 1999), thereby potentially underestimating the effect of OEC/ONF on neurite elongation.
It was hypothesized that alignment of glial cells would further enhance their stimulatory effects on neurite growth and, in addition, stimulate the directionality of neurite growth. If cultured on a glial feeder layer, neurite

growth is enhanced, but since there is no clear glial orientation, neurite growth does not have a preferred direction (Qian et al., 1992; Le Roux and Reh, 1994; Dijkstra et al., 1999; Sonigra et al., 1999). Oriented matrices of various biomaterials, like collagen, poly(D,L)-lactide, and fibrin have been made thus far and this rendered enhanced neurite elongation and directionality of the neurites (Williams, 1987; Dubey et al., 1999; Miller et al., 2001). In this study, we were able to show that both OEC/ONF and neonatal astrocyte cultures obtain a clearly more aligned phenotype after 4 days on PLA or PLA/PLA-*b*-PEO matrices than after 4 days in a Petri dish. Although, there was no difference in the level of alignment of astrocytes on PLA or PLA/PLA-*b*-PEO matrix, OEC/ONF alignment was significantly better on PLA/PLA-*b*-PEO matrix as compared with PLA matrix. The PLA-*b*-PEO amphiphilic copolymer enhances the water absorption of the PLA matrices (Maquet et al., 2001). The resulting effect on the glial behavior is unknown, but it seems that the OEC/ONF cultures are clearly affected in their affinity for the matrix. Although the number of cells was not affected, the outgrowth of glial extensions on PLA/PLA-*b*-PEO matrix was clearly better than on PLA matrix. The exact chemical composition of the matrix seems, therefore, crucial for the cellular behavior on the matrix. A further chemical optimalization of the PLA and possibly of the PLA/PLA-*b*-PEO matrices may likely increase the affinity of the OEC/ONF cultures for the matrix.

We are the first to show that aligned glial/biomatrix complexes stimulate the directionality of neurite growth. Neurites of cocultured neurons were found to be oriented in the same direction as the glial/biomatrix complexes. Although there are studies indicating some alignment of glial cells on biomatrices and a subsequent neurite-orientation of cocultured neurons (Dubey et al., 1999; Miller et al., 2001), we show for the first time in a quantitative way that the neurites adopt the same direction as the underlying aligned glial cells. The guidance of neurites by the glial/biomatrices may be interpreted in two ways. First, guidance can be stimulated in a physical way. The neurites may prefer to grow over a glial surface. Since the glial surface is oriented along the underlying biomatrix, the neurites will adopt an identical orientation. Second, guidance may be mediated by chemical cues. Cell surface carbohydrates, such as cerebroglycan that is expressed on neuronal growth cones, have been suggested to mediate axon guidance (Kindt and Lander, 1995; Ivins et al., 1997). Interference with a signaling pathway involving cell surface carbohydrates was found to disrupt neurite guidance on oriented laminin substrata and on an OEC substrate (Kindt and Lander, 1995; Hayat et al., 2003). The stimulation of neurite-directionality by aligned glial/biomatrix complexes may be a very important finding for future investigations. In the field of experimental SCI our aligned glial/biomatrix complexes may be able to guide severed axons across a lesion site. It is known that severed axons are prevented from regeneration by a highly hostile CNS environment and the lack of a bridge to cross the lesion site. The use of aligned OEC/ONF-biomatrix complexes may be able to handle both aspects.

Although the directed neurite growth was stimulated by the aligned glial/biomatrix complexes, we found no enhanced neurite growth. In our cultures the alignment of OEC/ONF and astrocytes reduced the stimulation of neurite elongation and the formation of more neurites per neuron, respectively. This was surprising since enhanced axonal elongation has been found by the use of oriented polylactide or collagen matrices in the presence of glial cells (Dubey et al., 1999; Miller et al., 2001). However, polylactide matrices without glial cells have a very low neurite-growth promoting potency (R. Deumens, unpublished observations; V. Maquet, unpublished observations). Furthermore, the glial/biomatrix complexes do not have a continuous glial covering. Although the OEC/ONF and the astrocyte cultures have an aligned phenotype, the glial cells are not attached to each other and thus the glial cover may be interrupted. Hence, it may be argued that the culturing period of glial cells on the biomatrices has to be increased. However, in pilot studies we established that 4 days was optimal for covering the biomatrices. Longer culturing periods did not improve the glial connectivity on the biomatrices. Moreover, the glial pattern of cell adhesion molecule expression, important for neurite outgrowth stimulation, may be altered by the attachment to the matrices. This may subsequently lead to reduced neurite growth. In addition, a differential adhesion of OECs and ONFs to the biomatrices may modify the growth-promoting effects of the OEC/ONF cultures, which is suggested to be highly dependent on the balance between OECs and ONFs (Raisman, 2001). However, the balance of OECs and ONFs was not found to be different between Petri dish and any of the two polylactide matrices. Hence, it seems that a further optimalization of the matrix composition may increase the neurite outgrowth stimulation potency of the glial/biomatrix complexes.

In conclusion, our finding that aligned glial/biomatrix complexes can direct neurite growth *in vitro* suggests important benefits for the use of these complexes to stimulate the guidance of severed axons across a SCI site.

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