Cell. Mol. Life Sci. 64 (2007) 1303 – 1309
1420-682X/07/101303-7
DOI 10.1007/s00018-007-7106-y

Cellular and Molecular Life Sciences

Research Article

Effect of olfactory ensheathing cells on reactive astrocytes *in vitro*

D. A. O'Toole, A. K. West and M. I. Chuah*

NeuroRepair Group, University of Tasmania, Hobart, Tasmania 7001 (Australia), Fax: +61 3 62262679, e-mail: Inn.Chuah@utas.edu.au

Received 27 February 2007; received after revision 30 March 2007; accepted 3 April 2007
Online First 20 April 2007

Abstract. Olfactory ensheathing cells have been used in several studies to promote repair in the injured spinal cord. However, cellular interaction between olfactory ensheathing cells and glial cells induced to be reactive in the aftermath of injury site has not been investigated. Using an *in vitro* model of astrogliosis, we show that reactive astrocytes expressed significantly less glial fibrillary acidic protein (GFAP) when cultured both in direct contact with olfactory ensheathing cells and when the two cell types were separated by a porous membrane. Immunofluorescence staining also suggested that reactive astrocytes showed decreased chondroitin sulfate proteoglycans in the presence of olfactory ensheathing cells, although the reduction was not statistically significant. No down-regulation of GFAP was observed when reactive astrocytes were similarly cultured with Schwann cells. Cell viability assay and bromodeoxyuridine uptake showed that proliferation of reactive astrocytes was significantly increased in the presence of olfactory ensheathing cells and Schwann cells.

Keywords. Olfactory ensheathing cells, reactive astrocytes, glial fibrillary acidic protein, proliferation, astrogliosis.

Introduction

The lack of repair in the injured central nervous system (CNS) is widely attributed to the inhibitory environment of the lesion site, most notably the formation of a glial scar that forms a physical and physiological barrier to axon regeneration. A number of experimental strategies have been used to induce repair in the injured CNS, including transplantation of olfactory ensheathing cells, whose normal physiological role is to support the regeneration and growth of olfactory neurons. Previous studies have shown that olfactory ensheathing cells secrete a number of growth factors in addition to expressing neurite-promoting adhesion molecules [1, 2]. It is not surprising then that over the last decade olfactory ensheathing cells have been used in trials as a possible agent for promoting repair of injured axons [3–5]. A previous *in vivo* study from our laboratory demonstrated that olfactory ensheathing cells were able not only to promote regeneration of injured axons, but they also induced the growth of collateral branches from intact nerve tracts [5]. We showed that this process was mediated by the secretion of soluble factors from olfactory ensheathing cells as well as direct contact with injured axons [6]. Whether soluble factors derived from olfactory ensheathing cells also influence expression of glial fibrillary acidic protein (GFAP) in reactive astrocytes in the injury site remains to be elucidated.

* Corresponding author.

Such an effect appears likely given that, *in vitro*, olfactory ensheathing cells migrate and intermingle with astrocytes, whereas Schwann cells and astrocytes remain segregated [7]. Furthermore, the ability of olfactory ensheathing cells to migrate within the injured spinal cord is greater than that of Schwann cells [8]. It has also been noted that injured spinal cords that were transplanted with olfactory ensheathing cells had comparatively less astrogliosis, as suggested by less intense immunostaining for chondroitin sulfate proteoglycan (CSPG) and GFAP [9, 10]. Whether this outcome is a direct influence of olfactory ensheathing cells on astrocytes remains to be determined.

In this study, we utilized an *in vitro* model of astrogliosis to compare the effects of olfactory ensheathing cells and Schwann cells on GFAP and CSPG expression in reactive and non-reactive astrocytes. We investigated whether olfactory ensheathing cells were able to influence directly GFAP expression in astrocytes through secretion of soluble factors and/or physical contact, and whether olfactory ensheathing cells affect astrocyte proliferation.

Materials and methods

Olfactory ensheathing cell, astrocyte and Schwann cell cultures. All procedures conducted on animals were approved by the Animal Experimentation Ethics Committee of the University of Tasmania and are consistent with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Tissues for cell culture were harvested from 2-day old Hooded Wistar rats and cells were cultured in DMEM + 10% fetal calf serum (JRH Biosciences, Brooklyn, VIC, Australia) + 1% penicillin-streptomycin-amphotericin B solution (DMEM-10S; Sigma, St Louis, MO). For olfactory ensheathing cell, astrocyte and Schwann cell cultures, previously established techniques were used [11, 12] with minor modifications as previously described [13]. Briefly, olfactory ensheathing cells were cultured from the olfactory nerve layer and olfactory mucosa, purified for 3 days in 100 μM cytosine-β-D-arabinofuranoside (AraC; Sigma) and expanded for 4 days in 100 μg/ml bovine pituitary extract (BPE; Sigma). With this method of culture, about 95% of the cells were identified as olfactory ensheathing cells by their positive staining for $p75^{NTR}$. Schwann cells were cultured from the brachial plexus and sciatic nerve, purified for 4 days in 100 μM AraC and expanded for 4 days in 20 μg/ml BPE + 2 μM forskolin (Sigma). Schwann cell cultures were ~95% $p75^{NTR}$-positive. Cortical astrocytes were grown to confluence in a flask coated with 0.01% poly-L-lysine (PLL; Sigma), purified by shaking at 200 rpm for 18 h, followed by 4 days in 20 μM AraC, then passaged and grown to confluence. Astrocyte cultures were >98% GFAP positive.

Types of co-cultures. Two types of co-cultures were investigated: (1) reactive astrocytes with olfactory ensheathing cells or Schwann cells on the same coverslip, and (2) reactive astrocytes on the coverslip, and olfactory ensheathing cells or Schwann cells in inserts.

Astrocyte, olfactory ensheathing cell and Schwann cell cultures were synchronized to allow for the establishment of appropriate co-cultures. Cells were harvested from flasks by treatment with 0.25% trypsin and centrifugation for 10 min at 500 *g*. Astrocytes were replated onto PLL-coated 13-mm-diameter round glass coverslips (Nunc, Rochester, NY) at a density of 1×10^4 cells/well. Once these cells reached confluence, some cultures were scratched with a sterile plastic pipette tip and TGF-β1 (10 ng/ml) was added to the medium, to induce a reactive astrocyte phenotype in an *in vitro* scratch wound [14]. Olfactory ensheathing cells or Schwann cells were added either directly onto confluent astrocytes (non-reactive or reactive) monolayers, or onto Falcon cell culture inserts incorporating polyethylene terephthalate (PET) track-etched membranes of 1-μm pore size (Becton Dickinson Labware, Franklin Lakes, NJ), at the same plating density as the astrocytes. Co-cultures were maintained in serum-free DMEM for 24 h before the cells were prepared for immunostaining, Western blot analysis, or luminescent proliferation assay.

Immunocytochemistry and analysis. Cultures on coverslips were fixed in 4% paraformaldehyde for 20 min after 24 h of co-culture. They were then washed in PBS and post-fixed in ethanol:acetic acid (95:5) solution for 20 min at –20°C. Next, nonspecific binding was blocked with DAKO serum-free protein block (DakoCytomation, Carpinteria, CA) for 15 min. Primary antibodies against GFAP (1:400, DakoCytomation) and CSPG (1:100, Sigma) were applied for 1 h at room temperature. Following washing with PBS, secondary antibodies goat anti-rabbit Alexa Fluor 594 or goat anti-mouse Alexa Fluor 488 (both 1:1000; Molecular Probes) were applied for 1 h in the dark. Coverslips were washed in PBS and mounted using aqueous Permafluor mounting medium (Immunotech, Marseille, France). Specimens were viewed on a fluorescence microscope (Olympus BX50) and images were captured at the same exposure time and magnification (400×) with an Olympus DP50 digital camera, plus Viewfinder Lite and Studio Lite 1.0 software (Pixera). For each antigen and culture condition, 15 digital frames representing 15 coverslips obtained from more than three separate experiments were analyzed. Changes in GFAP and CSPG were qualitatively determined by mean intensity values, similar to that used previously [15, 16]. Images were converted to gray scale, and the intensity value of each image measured (Adobe Photoshop 7.0). The mean intensity value of the reactive astrocytes group in each experiment (as represented by R in the figures) was normalized to 100 and the values of the other groups were expressed in terms of this standard. The data were analyzed by ANOVA with Tukey's post-hoc tests for pair-wise comparisons, using Graph Pad Prism v. 4.03 (Graph Pad Software, Inc., San Diego, CA).

Western blotting. Depending on the type of co-culture, at the conclusion of the culture period astrocytes or astrocytes + olfactory ensheathing cells were scraped from coverslips, and spun for 10 min at 500 *g*. A standard lysis buffer solution (50 mM Tris, 2 mM $CaCl_2$, 80 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail, pH 8.0) was added to the resulting cell pellets and incubated for 20 min at 4°C [17] before being spun at 3500 *g* for 30 min. The resulting supernatant was used to establish samples for SDS-PAGE. Protein concentrations of supernatants were determined by Bradford Assay (Bio-Rad Laboratories, Regents Park, NSW, Australia). For gel electrophoresis, 1 μg protein was loaded into each lane for three conditions of astrocyte cultures (*i.e.*, unreactive astrocytes; reactive astrocytes; reactive astrocytes separated from olfactory ensheathing cells in inserts). Twice the amount of protein was loaded into the lane that corresponded to co-cultures of reactive astrocytes in direct contact olfactory ensheathing cells because at the beginning of each experiment, these cultures already contained twice the number of cells. Proteins were resolved by SDS–PAGE through 10% polyacrylamide, and were transferred to a nitrocellulose membrane under 20 V overnight at 4°C. The membrane was probed using a polyclonal rabbit anti-GFAP antibody (1:400, DakoCytomation), followed by goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase (DakoCytomation) diluted in 2.5% blocking solution (1:1000). Proteins were visualized using the SuperSignal West Pico Chemiluminescent kit (Pierce Biotechnology, Rockford, IL), according to the manufacturer's instructions.

Cell number and proliferation assay. The number of proliferating cells was assessed using two methods: bromodeoxyuridine (BrdU) uptake and CellTiter-Glo® Luminescent Cell Viability Assay

(Promega, Madison, WI). Astrocyte cultures were scratched, and 1 mM BrdU and TGF-β1 (10 ng/ml) were added. Following overnight incubation, alone or with olfactory ensheathing cells or Schwann cells, astrocytes were fixed in 4% paraformaldehyde, incubated with 2 M HCl for 30 min at room temperature, and then washed three times in 0.2 M borate buffer. Next, they were treated with DAKO serum-free protein block, and anti-BrdU primary antibody (1:200, Sigma), using the immunostaining protocol as outlined above. Micrographs of astrocytes were captured at 400× magnification and the number of BrdU-positive cells was counted from a total of 12 frames for each treatment group. The 12 frames were obtained from six coverslips from two separate experiments. The procedure for the cell viability assay was performed according to the manufacturer's instructions. Briefly, the media in wells containing coverslips of astrocytes was reduced to 100 µl and mixed with 100 µl of CellTiter-Glo® Reagent. The plate was then shaken for 2 min to induce cell lysis of the astrocytes. The plate was left to incubate for 10 min to stabilize the luminescence signal. Then 100 µl of the solution from each well was transferred to a 96-well plate for recording using a GENios microplate reader (Tecan, Qld, Australia). A standard curve conforming to the equation $y = mx + c$ was created using coverslips containing known numbers of cells. This was used to determine the number of viable cells per coverslip in separate experiments. For each treatment group, the mean value was obtained from a total of six coverslips derived from two separate experiments.

Results

Reactive astrocytes co-cultured with olfactory ensheathing cells express less GFAP. Confluent astrocyte monolayers were induced to become reactive by physically scratching the cultures and treating them with10 ng/ml TGF-β1. Under these conditions, we observed no distinct difference in the morphology of non-reactive (Fig. 1A, B) and reactive (Fig. 1C, D) astrocytes; the astrocytes were largely polygonal and remained confluent. The scratch wound produced a narrow cell-free zone into which astrocytes extended broad processes in the ensuing 24 h, as shown in left part of Figure 1E. Immunofluorescence staining (Fig. 1A, C) and densitometry (Fig. 2A) showed that physically scratching the cultures and treating them with 10 ng/ml TGF-β1 resulted in significantly increased GFAP expression ($p<0.001$), similar to that described previously [18, 19]. The expression of CSPG in reactive astrocytes was also increased significantly, but to a lesser extent ($p<0.01$; Fig. 2A). Therefore, the combined treatment of 10 ng/ml TGF-β1 and scratch wounding is effective in increasing astrocytic expression of GFAP and CSPG *in vitro* (Figs 1A–D, 2A), thus representing a valid model of astrogliosis.

When olfactory ensheathing cells were added directly to reactive astrocytes, the intensity of immunostaining for GFAP was significantly decreased (Figs 1E, 2A; $p<0.001$). Similarly, when olfactory ensheathing cells were co-cultured with reactive astrocytes in a non-contact manner, a decrease in GFAP expression was observed (Figs 1G, 2A; $p<0.01$). Under these two conditions, CSPG was observed also to decrease

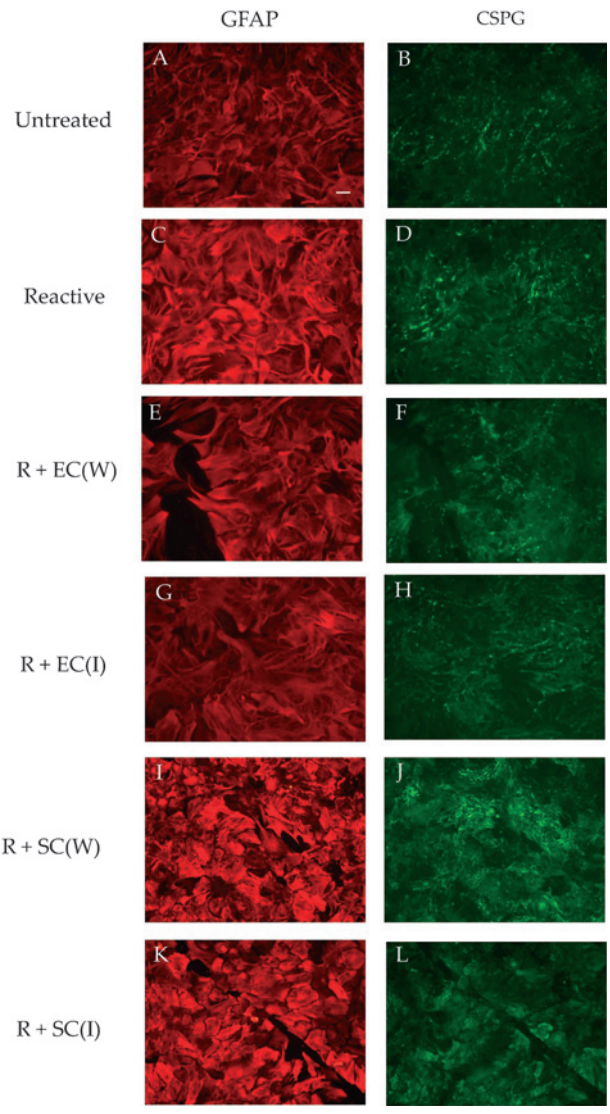


Figure 1. Immunofluorescence staining of glial fibrillary acidic protein (GFAP; A, C, E, G, I, K) and chondroitin sulfate proteoglycans (CSPG; B, D, F, H, J, L) in astrocytes under various conditions. Compared to the untreated condition (A, B), astrocytes induced to become reactive (C, D) expressed more intense staining for GFAP and CSPG. The staining for GFAP (E, G) and CSPG (F, H) was decreased when olfactory ensheathing cells were seeded directly into the wells containing reactive astrocytes [R + EC(W)] or when olfactory ensheathing cells were cultured in inserts [R + EC(I)]. No reduction in GFAP (I, K) and CSPG (J, L) were observed when Schwann cells were seeded directly into the wells containing reactive astrocytes [R + SC(W)] or when Schwann cells were cultured in inserts [R + SC(I)]. Scale bar = 20 µm.

(Fig. 1F, H) but the quantitative intensity analyses revealed that the decrease was not statistically significant (Fig. 2). The decrease in GFAP was supported by Western blot data, which showed that smaller bands corresponding to GFAP were detected when astrocytes were co-cultured with olfactory ensheathing cells in direct contact, or separated from each other by inserts (Fig. 2B).

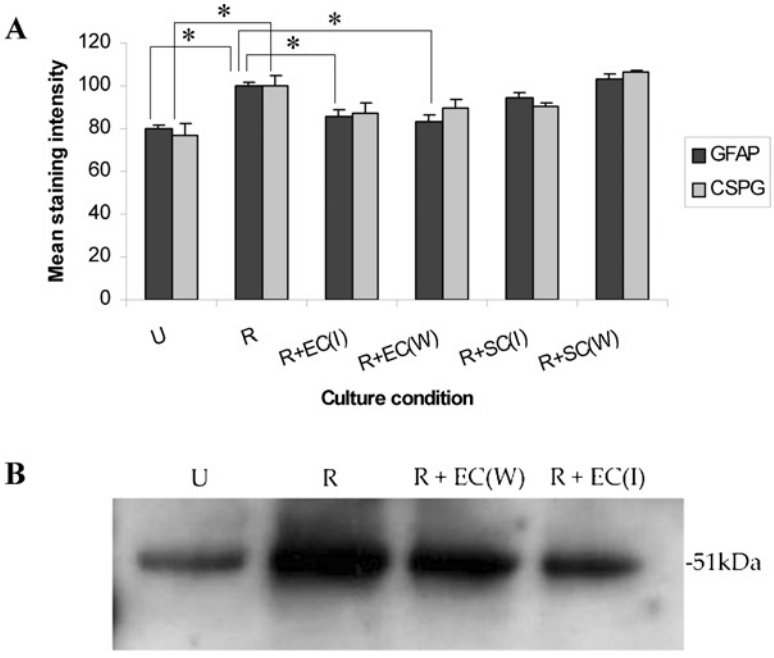


Figure 2. (*A*) Mean intensity values of GFAP and CSPG in astrocytes under various conditions. GFAP and CSPG were significantly increased in reactive astrocytes (R) compared to untreated astrocytes (U) ($p<0.001$ and $p<0.01$, respectively). When reactive astrocytes were co-cultured with olfactory ensheathing cells in inserts [R+EC(I)] or in the same well [R+EC(W)], the means intensity of value of GFAP was significantly reduced ($p<0.01$ and $p<0.001$, respectively). No reduction in GFAP and CSPG was obtained when reactive astrocytes were cultured with Schwann cells in inserts [R+SC(I)] or in the same well [R+SC(W)]. Error bars represent standard error of means. (*B*) Western blots of GFAP expressed in astrocytes under various conditions. The band corresponding to reactive astrocytes (R) is the most prominent, while smaller bands are observed when reactive astrocytes are co-cultured with olfactory ensheathing cells in the same well [R + EC(W)] or when olfactory ensheathing cells are present in inserts [R + EC(I)]. Untreated or non-reactive (U) astrocytes express low levels of GFAP.

When Schwann cells were cultured either directly in contact with reactive astrocytes (Fig. 1I) or separated by inserts (Fig. 1K), no down-regulation of GFAP and CSPG expression was observed (Fig. 2A). This indicates a distinct difference in the action of olfactory ensheathing cells and Schwann cells in respect to their interaction with reactive astrocytes *in vitro*.

Olfactory ensheathing cells and Schwann cells induce proliferation of reactive astrocytes *in vitro*. The treatment of scratch wounding and TGF-β1 addition did not induce any significant change in astrocyte cell number as shown by both BrdU uptake and the results of the cell viability assay (Fig. 3A, B). Immunostaining for BrdU showed that there was no obvious increased aggregation of dividing astrocytes at the scratch wound site (Fig. 3C, D). However, when reactive astrocytes were cultured with olfactory ensheathing cells in inserts, the number of astrocytes per coverslip was found to increase significantly over 24 h (Fig. 3A, B), as indicated by the viability assay ($p<0.05$) and BrdU uptake ($p<0.001$). This increase was also observed in the percentage of BrdU-positive astrocytes following co-culture with Schwann cells in inserts (Fig. 3B, $p<0.01$). The cell viability assay also indicated an increase in astrocyte number when they were co-cultured with Schwann cells in inserts, although the increase was not significant; this may be due to the fact that a smaller sample size ($n=6$) was used in the cell viability assay as compared to the sample size ($n=12$) in the BrdU-uptake experiments. These quantitative results suggest that olfactory ensheathing cells and Schwann cells release some soluble factor or factors, which can induce reactive astrocyte proliferation *in vitro*.

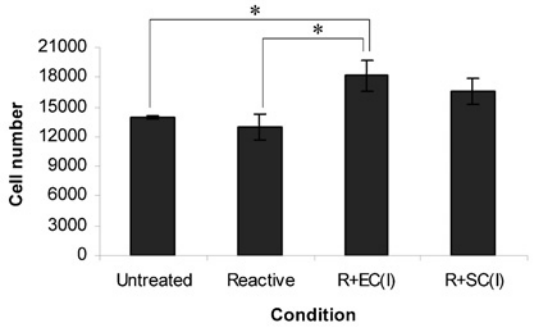
Discussion

We have used an *in vitro* model to investigate the effects of olfactory ensheathing cells and Schwann cells on reactive astrocytes. The results show that olfactory ensheathing cells are able to decrease GFAP expression in reactive astrocytes by the secretion of soluble factors and possibly also by direct contact between cells. Schwann cells failed to induce down-regulation of GFAP expression, although both olfactory ensheathing cells and Schwann cells increased proliferation of reactive astrocytes.

A major reason why regeneration of injured axons in the CNS fails to occur under normal circumstances is because astrocytes and oligodendrocytes contribute to an overall prohibitive environment [20]. Reactive astrocytes induced by injury are thought to have both beneficial and undesirable effects on neuronal well-being. They release growth factors that are beneficial to neurons but at the same time also express extracellular matrix molecules that are non-permissive to axonal regeneration [21, 22].

Results from some studies indicate that olfactory ensheathing cells transplanted into the injured CNS exert various effects on astrocytes. In photochemically damaged spinal cords, astrocytes appeared phenotypically different from those of injured spinal cords that were not implanted with olfactory ensheathing cells. The astrocytes appeared to show less hypertrophy, and

A



B

Condition	BrdU positive	Total cells	% BrdU positive
Untreated	121	1034	11.70
Reactive	92	1050	8.76
R + EC (I)	284	1045	27.18 *
R + SC (I)	233	1036	22.49 *

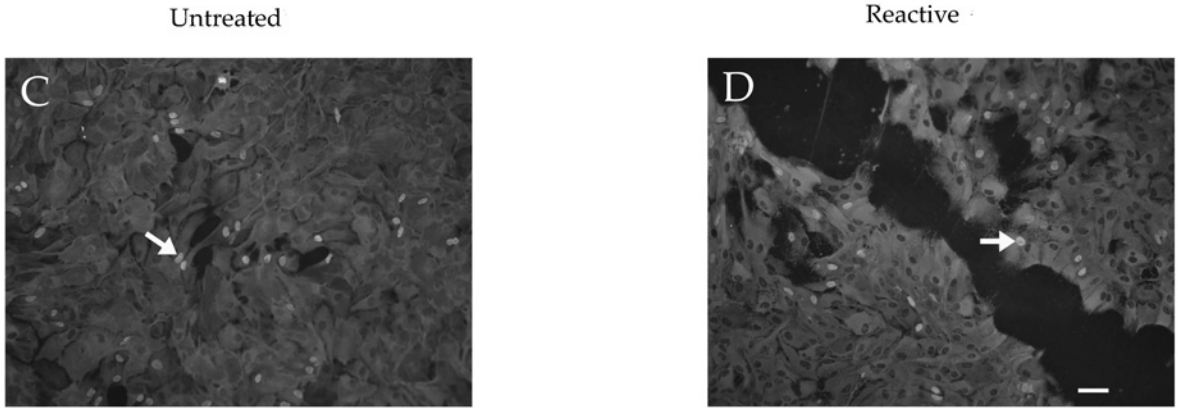


Figure 3. (*A*) Cell viability assay. Mean number of untreated or reactive astrocytes per well was determined following 24 h of co-culture with olfactory ensheathing cells [R + EC(I)] or Schwann cells in inserts [R + SC(I)]. The number of reactive astrocytes in the [R + EC(I)] group was significantly increased compared to reactive astrocytes alone or untreated non-reactive astrocytes (both $p<0.05$). The cell counts for each group was based on a total of six coverslips derived from two separate experiments. Error bars represent standard error of means. (*B*) Bromodeoxyuridine (BrdU) uptake by astrocytes. The percentage of BrdU-positive cells was significantly increased when reactive astrocytes were cultured either with olfactory ensheathing cells or Schwann cells in inserts ($p<0.001$ and $p<0.01$, respectively). For each group a total of 12 random frames derived from two separate experiments were counted. (*C, D*) Immunofluorescence staining for BrdU-positive nuclei (arrows) in untreated (*C*) and reactive (*D*) astrocyte cultures. Cell-free region in (*D*) indicates the width of the scratch wound. Scale bar = 20 μm.

possessed short thin processes [23]. However, because the spinal cord injury site is a complex environment containing reactive astrocytes, oligodendrocytes, infiltrating fibroblasts, immune cells and a mix of growth factors, inhibitory factors, cytokines and other agents, it is not possible to determine whether such observed changes in astrocytes is a direct response to transplanted olfactory ensheathing cells. The *in vitro* model in this study induces astrogliosis and has permitted us to observe interaction between two specific populations. We did not find a marked change in the morphology of the reactive astrocytes when they were cultured in contact with olfactory ensheathing cells or separated by a porous membrane. One reason for this discrepancy may be because changes in astrocytic morphology *in vivo* was a secondary effect of transplanted olfactory ensheathing cells or resulted from olfactory ensheathing cells acting in concert with other immune cells such as leukocytes and microglia. This is a distinct possibility given that in our recent microarray analysis of the transcriptome of olfactory ensheathing cells [13], we discovered that olfactory ensheathing cells expressed a number of innate immune factors, including lysozyme, the transcription factor Cebpb (which regulates the production of nitric oxide and some cytokines), and the cytokines CXCL1 (Gro1) and CCL2 (MCP-1). CXCL1 and CCL2 are signaling factors for recruiting neutrophils and macrophages, respectively. Another reason for the difference in findings may lie in the astrocytes. The astrocytes in our study are cortically derived and hence are composed of type 1 (protoplasmic) astrocytes, whereas astrocytes in the spinal cord as referred to in *in vivo* studies are composed mainly of type 2

(fibrous) and to a lesser extent, type 1 astrocytes [24, 25]. These two types of astrocytes have been shown to be developmentally and antigenically different [25, 26].

The immunofluorescence staining and Western blotting suggest that olfactory ensheathing cells are capable of regulating astrocytic GFAP expression, and that this is in part due to the release of soluble factors by olfactory ensheathing cells. The smaller band corresponding to decreased GFAP in co-cultures of reactive astrocytes and olfactory ensheathing cells [R+EC(W) in Fig. 2B] is particularly striking given that olfactory ensheathing cells themselves express GFAP, albeit at a lower level than astrocytes [27] and that twice as much protein was loaded into this lane. This indicates strongly that the amount of GFAP expressed per reactive astrocyte, including newly generated ones, is significantly reduced in the presence of olfactory ensheathing cells.

Although astrocytes have been reported to hypertrophy, increase expression of GFAP and other molecules, and proliferate in response to injury [28–30], several studies have found that few reactive astrocytes divided [31–33]. The *in vitro* model of reactive astrocytes used in this study similarly did not show increased cell division compared to the non-reactive astrocytes. Perhaps more interesting is the finding that olfactory ensheathing cells significantly increased astrocyte division through the secretion of soluble factors. The next logical step is to question which molecules expressed by olfactory ensheathing cells may be mitogenic for astrocytes. Candidate molecules include fibroblast growth factor 2 (FGF2) and platelet-derived growth factor-B known to be present in olfactory ensheathing cells [34, 35], and which have been shown to be mitogenic to rat astrocytes [36, 37]. Another possible candidate is myelin basic protein (MBP), which can be induced in olfactory ensheathing cells *in vitro* even though they do not myelinate olfactory axons *in vivo* [38, 39]. Previous studies show that MBP interacts with both fibroblast growth factor receptor (FGFR) and ganglioside GM1 on astrocytes to trigger proliferation [40]. Because olfactory ensheathing cells are also known to express FGFR [41], it is possible that both FGF2 and MBP may act in an autocrine manner to stimulate olfactory ensheathing cells proliferation as well.

The issue of whether olfactory ensheathing cells promote repair of the injured CNS by enhancing the beneficial effects of astrogliosis warrants further investigation. Our major finding that olfactory ensheathing cells directly down-regulate GFAP expression in reactive astrocytes and yet stimulate their proliferation, underscores the possibility that olfactory ensheathing cells could exert apparently conflicting effects on astrogliosis. It should also be noted that microglia conditioned medium in combination with MBP are a more potent mitogen for astrocytes than each condition individually [40]. Hence additional studies are needed to investigate the contribution of other cell types and their interaction with olfactory ensheathing cells to regulate the reactive astrocyte phenotype. Astrogliosis is a central phenomenon of CNS injury and understanding the intercellular effects and signaling mechanisms involved in this condition will provide new clues for the development of therapeutic treatments to promote repair.

Acknowledgements. This work was supported by a grant from the National Health and Medical Research Council (No. 253907).

1 Chuah, M. I. and Au,, C. (1993) Cultures of ensheathing cells from neonatal rat olfactory bulbs. Brain Res. 601, 213 – 220.
2 Woodhall, E., West, A. K. and Chuah, M. I. (2001) Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors. Brain Res. Mol. Brain Res. 88, 203 – 213.
3 Li, Y., Field, P. M. and Raisman, G. (1997) Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells. Science 277, 2000 – 2002.
4 Lu, J., Feron, F., Mackay-Sim, A. and Waite, P. M. (2002) Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord. Brain 125, 14 – 21.
5 Chuah, M. I., Choi-Lundberg, D., Weston, S., Vincent, A. J., Chung, R. S., Vickers, J. C. and West, A. K. (2004) Olfactory ensheathing cells promote collateral axonal branching in the injured adult rat spinal cord. Exp. Neurol. 185, 15 – 25.
6 Chung, R. S., Woodhouse, A., Fung, S., Dickson, T. C., West, A. K., Vickers, J. C. and Chuah, M. I. (2004) Olfactory ensheathing cells promote neurite sprouting of injured axons *in vitro* by direct cellular contact and secretion of soluble factors. Cell. Mol. Life Sci. 61, 1238 – 1245.
7 Lakatos, A., Franklin, R. J. and Barnett, S. C. (2000) Olfactory ensheathing cells and Schwann cells differ in their *in vitro* interactions with astrocytes. Glia 32, 214 – 225.
8 Li, Y., Field, P. M. and Raisman, G. (1998) Regeneration of adult rat corticospinal axons induced by transplanted olfactory ensheathing cells. J. Neurosci.18, 10514 – 10524.
9 Garcia-Alias, G., Lopez-Vales, R., Fores, J., Navarro, X. and Verdu, E. (2004) Acute transplantation of olfactory ensheathing cells or Schwann cells promotes recovery after spinal cord injury in the rat. J. Neurosci. Res. 75, 632 – 641.
10 Lakatos, A., Barnett, S. C. and Franklin, R. J. (2003) Olfactory ensheathing cells induce less host astrocyte response and chondroitin sulphate proteoglycan expression than Schwann cells following transplantation into adult CNS white matter. Exp. Neurol. 184, 237 – 246.
11 Brockes, J. P., Fields, K. L. and Raff, M. C. (1979) Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. Brain Res. 165, 105 – 118.
12 McCarthy, K. D. and De Vellis, J. (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J. Cell Biol. 85, 890 – 902.
13 Vincent, A. J., Taylor, J. M., Choi-Lundberg, D. L., West, A. K. and Chuah, M. I. (2005) Genetic expression profile of olfactory ensheathing cells is distinct from that of Schwann cells and astrocytes. Glia 51, 132 – 147.

14 Yu, A. C., Lee, Y. L. and Eng, L. F. (1993) Astrogliosis in culture: I. The model and the effect of antisense oligonucleotides on glial fibrillary acidic protein synthesis. J. Neurosci. Res. 34, 295 – 303.
15 Chung, R. S., Vickers, J. C., Chuah, M. I. and West, A. K. (2003) Metallothionein-IIA promotes initial neurite elongation and postinjury reactive neurite growth and facilitates healing after focal cortical brain injury. J. Neurosci. 23, 3336 – 3342.
16 Levadoux, M., Mahon, C., Beattie, J.H., Wallace, H.M. and Hesketh, J.E (1999) Nuclear import of metallothionein requires its mRNA to be associated with the perinuclear cytoskeleton. J. Biol. Chem. 274, 34961 – 34966.
17 Safavi-Abbasi, S., Wolff, J. R. and Missler, M. (2001) Rapid morphological changes in astrocytes are accompanied by redistribution but not by quantitative changes of cytoskeletal proteins. Glia 36, 102 – 115.
18 Wu, V. W. and Schwartz, J. P. (1998) Cell culture models for reactive gliosis: new perspectives. J. Neurosci. Res. 51, 675 – 681.
19 Morgan, L., Jessen, K. R. and Mirsky, R. (1991) The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04+) to a myelin phenotype (P0+, GFAP-, N-CAM-, NGF-receptor-) depends on growth inhibition. J. Cell Biol. 112, 457 – 467.
20 Silver, J. and Miller, J. H. (2004) Regeneration beyond the glial scar. Nat. Rev. Neurosci. 5, 146 – 156.
21 Giulian, D. (1993) Reactive glia as rivals in regulating neuronal survival. Glia 7, 102 – 110.
22 McKeon, R. J., Hoke, A. and Silver, J. (1995) Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Exp. Neurol. 136, 32 – 43.
23 Verdu, E., Garcia-Alias, G., Fores, J., Gudino-Cabrera, G., Muneton, V. C., Nieto-Sampedro, M. and Navarro, X. (2001) Effects of ensheathing cells transplanted into photochemically damaged spinal cord. Neuroreport 12, 2303 – 2309.
24 Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R. and Noble, M. (1983) Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides, and growth characteristics. J. Neurosci. 3, 1289 – 1300.
25 Miller, R. H. and Raff, M. C. (1984) Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. J. Neurosci. 4, 585 – 592.
26 Navarro, A., Del Valle, E. and Tolivia, J. (2004) Differential expression of apolipoprotein d in human astroglial and oligodendroglial cells. J. Histochem. Cytochem. 52, 1031 – 1036.
27 Chuah, M.I. and Au, C. (1991) Olfactory Schwann cells are derived from precursor cells in the olfactory epithelium. J. Neurosci. Res. 29, 172 – 180.
28 Eng, L. F., Reier, P. J. and Houle, J. D. (1987) Astrocyte activation and fibrous gliosis: glial fibrillary acidic protein immunostaining of astrocytes following intraspinal cord grafting of fetal CNS tissue. Prog. Brain Res. 71, 439 – 455.
29 Janeczko, K. (1989) Spatiotemporal patterns of the astroglial proliferation in rat brain injured at the postmitotic stage of postnatal development: a combined immunocytochemical and autoradiographic study. Brain Res. 485, 236 – 243.
30 Raivich, G., Bohatschek, M., Kloss, C. U., Werner, A., Jones, L. L. and Kreutzberg, G. W. (1999) Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain Res. Brain Res. Rev. 30, 77 – 105.
31 Miyake, T., Hattori, T., Fukuda, M., Kitamura, T. and Fujita, S. (1988) Quantitative studies on proliferative changes of reactive astrocytes in mouse cerebral cortex. Brain Res. 451, 133 – 138.
32 Di Prospero, N. A., Zhou, X. R., Meiners, S., McAuliffe, W. G., Ho, S. Y. and Geller, H. M. (1998) Suramin disrupts the gliotic response following a stab wound injury to the adult rat brain. J. Neurocytol. 27, 491 – 506.
33 Hampton, D. W., Rhodes, K. E., Zhao, C., Franklin, R. J. and Fawcett, J. W. (2004) The responses of oligodendrocyte precursor cells, astrocytes and microglia to a cortical stab injury, in the brain. Neuroscience 127, 813 – 820.
34 Chuah, M. I. and Teague, R. (1999) Basic fibroblast growth factor in the primary olfactory pathway: mitogenic effect on ensheathing cells. Neuroscience 88, 1043 – 1050.
35 Kott, J. N., Westrum, L. E., Raines, E. W., Sasahara, M. and Ross, R. (1994) Olfactory ensheathing glia and platelet-derived growth factor B-chain reactivity in the transplanted rat olfactory bulb. Int. J. Dev. Neurosci. 12, 315 – 323.
36 Biesiada, E., Razandi, M. and Levin, E. R. (1996) Egr-1 activates basic fibroblast growth factor transcription. Mechanistic implications for astrocyte proliferation. J. Biol. Chem. 271, 18576 – 18581.
37 Luo, J. and Miller, M. W. (1999) Platelet-derived growth factor-mediated signal transduction underlying astrocyte proliferation: site of ethanol action. J. Neurosci. 19, 10014 – 10025.
38 Devon, R. and Doucette, R. (1992) Olfactory ensheathing cells myelinate dorsal root ganglion neurites. Brain Res. 589, 175 – 179.
39 Devon, R. and Doucette, R. (1995) Olfactory ensheathing cells do not require L-ascorbic acid *in vitro* to assemble a basal lamina or to myelinate dorsal root ganglion neurites. Brain Res. 688, 223 – 229.
40 South, S. A., Deibler, G. E., Tzeng, S. F., Badache, A., Kirchner, M. G., Muja, N. and De Vries, G. H. (2000) Myelin basic protein (MBP) and MBP peptides are mitogens for cultured astrocytes. Glia 29, 81 – 90.
41 Hsu, P., Yu, F., Feron, F., Pickles, J. O., Sneesby, K. and Mackay-Sim, A. (2001) Basic fibroblast growth factor and fibroblast growth factor receptors in adult olfactory epithelium. Brain Res. 896, 188 – 197.