Cellular and Molecular Biology of Ensheathing Cells

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ABSTRACT Ensheathing cells are the glial cells that envelop olfactory axons as they course from the olfactory epithelium to the bulb. They are derived from the olfactory placode and differ from the typical glia in terms of sharing the phenotypes of both astrocytes and Schwann cells. The aims of this study are to review (1) cellular characterisation of ensheathing cells in vivo and in vitro, (2) molecular insight into their growth promoting properties, and (3) their role in olfactory development and potential function as a therapeutic agent for nerve repair. Much of the characterisation of ensheathing cell property has developed from immunohistochemical studies that have been supplemented with new molecular methodologies in recent years. Many pieces of evidence clearly indicate that ensheathing cells actively produce growth-promoting molecules, which act in a paracrine and, in some cases, autocrine manner. However, a review of the available literature also suggests that there is a great deal that remains to be elucidated regarding the cell biology of ensheathing cells, for example, their rate of formation and turnover. In addition, the apparent antigenic heterogeneity as revealed by numerous in vitro studies warrants further analysis, particularly in view of the fact that in recent years these cells have been touted as a possible agent for central nerve repair. New molecular methodologies such as the microarray techniques will prove to be crucial for defining the unique characteristics of ensheathing cells. *Microsc. Res. Tech. 58: 216–227, 2002.*

INTRODUCTION

It is well recognized that the olfactory epithelium is one of the few sites in the body that exhibits continuous neurogenesis and axonal growth during an organism's life. Studies using ^{3}[H] thymidine labeling and quantitative analysis suggested that olfactory neurons are short-lived, being present in the epithelium for approximately 28 days (Graziadei and Monti Graziadei, 1979; Moulton, 1975). When the mature neurons die, they are replaced by new ones that are generated from the division of globose basal cells (Calof and Chikaraishi, 1989). Regeneration studies in which olfactory nerves are axotomized, leading to neuron death and replacement, have also indicated a similar limited life span (Samanen and Forbes, 1984). Subsequent studies have shown, however, that some olfactory neurons have a much longer life span, capable of surviving up to one year (Hinds et al., 1984). Regardless of the causal events preceding cell death and reconstitution, newly generated olfactory neurons have to grow an axon that can successfully fasciculate with others, elongate in the right direction, penetrate the cribriform plate, and eventually synapse with the dendrites of secondary neurons in the olfactory bulb. No doubt, the growing axon, particularly its growth cone, encounters numerous molecular signals as it maneuvers through the lamina propria and into the target bulb in the central nervous system. There is evidence to indicate that guidance of olfactory axons operates via a hierarchical arrangement with various molecules playing a crucial role at different points along the pathway (Lin and Ngai, 1999). An investigation into these regulatory molecules necessarily implicates the ensheathing cells, since they are closely associated with olfactory axons throughout their course.

The presence of a group of cells that follows and ensheathes olfactory axons as they grow had been observed almost a century ago (Bedford, 1904). They were originally termed "Schwann cells" in accordance with the terminology commonly applied to peripheral nerve glia (Gasser, 1956; De Lorenzo, 1957). Subsequent studies revealed, however, that they were developmentally and structurally different from typical Schwann cells (Chuah and Au, 1991; Cuschieri and Bannister, 1975a,b). This was followed by numerous studies that characterised the phenotypic expression of these cells both in vivo and in vitro (e.g., Doucette, 1993a ; Pixley, 1992; Ramon Cueto and Nieto-Sampedro, 1992). In part, the rationale for conducting these studies lies in the belief that an understanding of these cells will shed new light on the mechanisms of olfactory axonal growth. This review summarizes the existing data on the cellular and molecular features of this unique group of cells as obtained from different laboratories, some of which point to the existence of a heterogeneous population of ensheathing cells (Astic et al., 1998; Franceschini and Barnett, 1996; Pixley, 1992). In addition to discussions on how the molecular properties of

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ensheathing cells could conceivably play a role in olfactory axon guidance, the latter part of this review focuses on recent studies in which properties of ensheathing cells have been advantageously utilized in stimulating repair in injured central nervous tissue.

ENSHEATHING CELLS IN THE PRIMARY OLFACTORY PATHWAY

The notion that the olfactory epithelium is the source of several types of extraepithelial cells, including ensheathing cells, was first suggested by the observation that during development clusters of cells were present beneath the basal lamina of the epithelium (Doucette, 1990; Farbman and Squinto, 1985; Mendoza et al., 1982). In mouse and rat, the presence of these cells coincides with the initiation of olfactory axon outgrowth at E10.5 and E13, respectively, and their subsequent penetration of the basal lamina the following day (Doucette, 1990; Farbman and Squinto, 1985). The placodal origin of ensheathing cells has been established by a study from our laboratory that showed that when the embryonic olfactory epithelium devoid of lamina propria was cultured, ensheathing cells expressing glial fibrillary acidic protein, differentiated from the explant (Chuah and Au, 1991).

Prior to their emergence from the epithelium, olfactory axons are already enveloped by ensheathing cell processes that form a cradle-like structure immediately above the basal lamina (Tennent and Chuah, 1996). Further development is marked by continuous downgrowth resulting in an evagination in which ensheathing cells are sandwiched between the fascicle of axons and basal lamina. Soon after, the basal lamina breaks down and the bundle of axons exits from the epithelium.

Once in the lamina propria, ensheathing cells extend cytoplasmic processes around groups of axons, separating the bundle into smaller fascicles (Barber and Lindsay, 1982; De Lorenzo, 1957). It has been observed that unlike peripheral nerves, there is a progressive increase in the number of axons per fascicle as development progresses (Fraher, 1982). Ultrastructurally, ensheathing cells are described as having cytoplasm that is of medium electron density and containing scattered intermediate filaments (Doucette, 1984, 1993a). As the olfactory axons elongate, ensheathing cell processes are always observed ahead of the axon terminals, suggesting that they may be somehow involved in providing directional guidance (Tennent and Chuah, 1996). However, it is likely that ensheathing cells are not the sole candidate that is crucial to axon guidance, particularly during the adult stage. Unlike the scenario during ontogeny, an elongating olfactory axon is confronted with a large array of cells and molecules as represented by preexisting bundles of axons and their accompanying ensheathing cells, not to mention the connective tissue through which the nerve bundles course. It is not known how a growing axon is able to recognize and fasciculate with others in the appropriate bundle en route to its target, the olfactory bulb.

During embryonic development, the ensheathing cells that migrate together with the growing olfactory fascicles accumulate as a superficial mass on reaching the telencephalic vesicle, forming the presumptive olfactory nerve layer (Valverde et al., 1992). It has been found through our in vitro studies that the ensheathing cells migrate along a concentration gradient of soluble factor(s) released by the olfactory bulb (Liu et al., 1995). Soon after the growing olfactory axons establish contact with the telencephalic vesicle, the physical barrier of glia limitans between the two components breaks down. This process paves the way for the formation of the definitive olfactory nerve layer as the ensheathing cells begin to form a new glia limitans along the external surface (Doucette, 1993a). It is with the development of this new glia limitans that the ensheathing cells come to reside eventually in a part of the central nervous system.

Many proliferating cells are present in the olfactory nerve layer of the bulb during the last one third of gestation and the first 10 days of postnatal life (Hinds, 1968). Presumably, these mitotic figures include ensheathing cells and astrocytes that arise from glioblasts originally derived from the subventricular germinal layer (Doucette, 1993b). Although developmental studies are able to account for the spatial distribution of ensheathing cells, little is known about their cell dynamics. It is conceivable that when olfactory axons are undergoing robust growth during ontogeny, there is a net increase in the number of ensheathing cells both in the lamina propria and in the maturing olfactory nerve layer of the bulb. However, once maturation is reached and growth of the olfactory epithelium has tapered off, it is expected that the ensheathing cell number will also be in equilibrium. There is no data on the time course of maturation of these cells subsequent to their migration into the lamina propria. Similarly it is also not known whether in the adult animal, they continue to exit from the epithelium or whether growing axons utilize pre-existing ensheathing cells in the lamina propria as signposts to guide them to their target.

STRUCTURAL AND MOLECULAR HETEROGENEITY OF ENSHEATHING CELLS IN VIVO AND IN VITRO

Ultrastructural observations of olfactory nerves of developing mice were the first to suggest that ensheathing cells may be composed of different populations (Cuschieri and Bannister, 1975b). It was noted that during the perinatal period, ensheathing cells of different cytoplasmic electron densities were present. Presumably the darker ones found within the nerve bundles represented a less mature population. Subsequent immunohistochemical studies have confirmed some degree of heterogeneity both in terms of development and expression of antigenic markers (Astic et al., 1998; Franceschini and Barnett, 1996) (Table 1).

During development, there appears to be a proximodistal gradient of antigenic expression along the primary olfactory pathway, with cells in the olfactory nerve layer demonstrating delayed expression of S-100 and glial fibrillary acidic protein (GFAP). Generally, the expression of S-100 protein precedes that of GFAP by one or two days with the earliest detection of the former being demonstrated at E14 in the embryonic rat (Astic et al., 1998). More recently, another developmental study found that in perinatal and neonatal rats, ensheathing cells in the olfactory nerve layer of the bulb and its vicinity show positive immunoreactivity

TABLE 1. Intracellular and receptor molecules expressed by ensheathing cells[1]

Antigen molecule	In vivo	In vitro	Reference
S-100	NLB, ON	+	Astic et al. (1998); Franceschini and Barnett (1996); this paper
GFAP	dNLB, ON	+	Astic et al. (1998); Barber and Lindsay (1982)
Ephrin-B2[2]	NLB	n.d.	St. John and Key (2001)
O4	NLB	+	Barnett et al. (1993); Franceschini and Barnett (1996)
NGFr	dNLB, ON	+	Gong et al. (1994); Franceschini and Barnett (1996); Sonigra et al. (1999)
NPY	NLB, ON	n.d.	Ubink et al. (1994)
Nestin	n.d.	+	Sonigra et al. (1999)

[1]NLB = olfactory nerve layer of bulb; dNLB = deep region of NLB; ON = olfactory nerves; n.d. = not determined; + = present in most ensheathing cells.
[2]Level is greatly reduced during and after the second postnatal week.

for Ephrin-B2, a ligand for the Eph family of receptor tyrosine kinases (St. John and Key, 2001). Interestingly those ensheathing cells associated with nerve bundles closer to the olfactory epithelium fail to express Ephrin-B2 and, within 2 weeks of birth, Ephrin-B2 in ensheathing cells is greatly reduced. This change in expression is interpreted by the authors as supporting evidence for a regulatory action of Ephrin-B2 in desfasciculating growing olfactory nerves rather than a reflection of distinct populations of ensheathing cells distributed along the olfactory pathway.

Using a panel of antibodies, Franceschini and Barnett (1996) showed that antibodies against O4 antigen, N-CAM, and vimentin label the entire thickness of the olfactory nerve layer while S-100 and GAP-43 are similarly extensive except for a thin layer on the surface of the bulb. Interestingly, the low affinity NGF receptor (NGFr), which is commonly used as a marker for ensheathing cells in culture (see below), show variable expression, being most extensive and associated with ensheathing cells of the olfactory nerves during development (Gong et al., 1994). It is also present in the olfactory nerve layer during the perinatal period and decreases progressively as the animal matures (Franceschini and Barnett, 1996). In the 1-month-old rat, only a faint and variable presence in the deepest and outermost regions of the olfactory nerve layer remains. GFAP is found to be restricted to the deepest region of the olfactory nerve layer (Franceschini and Barnett, 1996). However, immunopositivity in any or all regions of the olfactory nerve layer does not necessarily confer positive presence of the antigen in ensheathing cells particularly when the antigen is also localised heavily in olfactory axons and, consequently, immunoreaction in ensheathing cells may not be discerned clearly. A more convincing alternative is the use of immunoelectron microscopy, which has been employed to demonstrate the presence of neuropeptide tyrosine (NPY) in the Golgi compartment of ensheathing cells (Ubink et al., 1994).

Heterogeneity of ensheathing cells in terms of morphology and antigenic profile has also been observed in tissue culture studies with each research group applying different antigenic expression as a criterion for determining the identity of ensheathing cells (Barber and Lindsay, 1982; Barnett et al., 1993; Franceschini and Barnett, 1996; Pixley, 1992; Ramon-Cueto and Nieto-Sampedro, 1992). The observed heterogeneity can be attributed to different methods of isolation (e.g., Barnett et al., 1993; Chuah and Au, 1993) culture conditions (e.g., Franceschini and Barnett, 1996; Pixley, 1992), and possibly the age of the animal from which the ensheathing cells are obtained (Ramon-Cueto and Avila, 1998). Barber and Lindsay (1982), who were the first to report that ensheathing cells resembled astrocytes in their expression of GFAP, described the presence of two morphologically distinct types of ensheathing cells in cultures of cells dissociated from the olfactory mucosa. The first type was "spindly-bipolar" while the other was "flat" and angular-looking, bearing the characteristic filamentous staining for GFAP. However, the dichotomy between these two types may be less distinct than originally suggested by this paper. In a later study, it was shown that in contrast to serum-supplemented medium, serum-free medium supplemented with 4 μg/ml laminin induced the ensheathing cells to grow longer processes with a resultant decrease in the volume of cytoplasm in the cell body (Pixley, 1992). As a result, there appeared to be increased numbers of bipolar ensheathing cells. This transition from a "flat" to a more spindly morphology in serum-free medium has been shown to occur with ensheathing cells isolated from both embryonic and neonatal rats (Doucette, 1993b; Pixley, 1992, 1996).

In our laboratory, we have confirmed that the specific type of chemically-defined serum free medium can also exert an influence on ensheathing cell morphology. We have grown confluent cultures of ensheathing cells in a chemically-defined medium (CM) whose formula was modified from that originally developed by Bottenstein and Sato (1979) (Chuah and Teague, 1999) and also in Neurobasal medium with B-27 supplement (NB) (Fig. 1). There are subtle differences between cells maintained in these two media. In CM, ensheathing cells tend to elaborate longer and straighter processes that often align parallel to each other. Ensheathing cells in NB also elaborated processes but they frequently radiate out from the cell body and are not as straight. The respective phenotypes are malleable as medium change from one to the other results in a reversible morphological change that is observable within 24 hours.

In a recent in vitro study, it was reported that different morphologies of ensheathing cells could be correlated with heterogeneity in antigenic expression (Sonigra et al., 1999). For example, E-N-CAM was expressed by ensheathing cells with a flattened morphology while NGFr, over an increasing length of time in culture, became mainly associated with spindly-looking cells, although a small number of flat ensheathing cells also expressed NGFr (Franceschini and Barnett, 1996). Those ensheathing cells with a flattened morphology expressed high levels of fibrous GFAP while the spindly population only contained diffuse amounts of GFAP. It is not known whether the spindly-shaped NGFr-positive cells in culture were those that had orig-

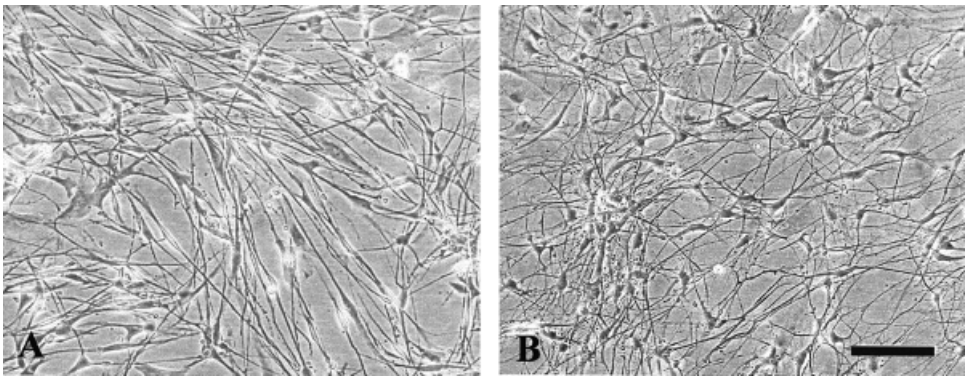

Fig. 1. Ensheathing cells cultured in two different serum-free chemically defined media, (**A**) medium whose chemical composition has been established in an earlier study (Chuah and Teague, 1999) (CM) and (**B**) Neurobasal medium with B-27 supplement (NB). In CM, ensheathing cells appear more spindly, bipolar with many of the processes oriented parallel to each other, while in NB, two or more processes tend to radiate out from the cell body. Bar = 100 μm.

inally resided in the deepest and outermost regions of the olfactory nerve layer, as indicated by immunohistochemical studies (Franceschini and Barnett, 1996). Interestingly, S-100, N-CAM, vimentin, and nestin have been reported to be expressed by the majority of cultured ensheathing cells, regardless of their morphology in culture (Barnett and Franceschini, 1996; Sonigra et al., 1999; Fig. 2). The presence of vimentin in ensheathing cells is particularly intriguing because it is generally associated with immature glia in the CNS and is gradually replaced by GFAP as astrocytes mature (Bignami et al., 1982). Its presence in ensheathing cells suggests that these cells do not completely evolve to a "mature" phenotype.

In summary, the evidence demonstrates some degree of antigenic heterogeneity among ensheathing cells in vivo that may be complicated further by culture conditions. The current status of identifying ensheathing cells primarily on the basis of morphology, and expression of relatively non-specific markers such as NGFr and S-100 protein, which are also present in other glial cells, and that could vary during culture, is not ideal. It is conceivable that with the development of microarray techniques, it will be possible to allow selection of a new definitive marker (or set of markers) to unequivocally characterise ensheathing cells, particularly during establishment and expansion of cultures.

Notwithstanding the complex antigenic phenotype of ensheathing cells in culture, a few research groups have produced clonal ensheathing cell lines (Goodman et al., 1993; Sonigra et al., 1996). Goodman and coworkers have generated an ensheathing cell line after transduction of cells with DNA encoding SV40 large T antigen and classified them as such based on the in vitro morphology and immunogenicity for NGFr. In another study, a spontaneously immortalised variant of a subcultured olfactory nerve cell line was isolated and was shown to express GFAP, S-100, and NGFr (Sonigra et al., 1996). Although both these cell lines have been shown to support neurite growth from retinal ganglion neurons, their facilitatory role to axonal growth from olfactory neurons in culture has not been reported.

In contrast, the behavior of ensheathing cells derived from primary and subsequent sub-cultures, in response to olfactory neurons, has been characterised extensively. Ensheathing cells obtained from the olfactory epithelium and bulb were shown to extend processes to enfold olfactory axons (Chuah and Au, 1991, 1994; Ramon-Cueto et al., 1993). Interestingly, it was noted that soon after the ensheathing cell process enveloped an olfactory axon, immunoreactivity for NGFr disappeared at the site of membrane contact between these two cells, indicating that expression of NGFr in ensheathing cells is influenced in part by interaction with axonal membrane (Ramon-Cueto et al., 1993). In contrast, expression of GFAP does not appear to be affected by contact with neuronal membrane (Chuah and Au, 1991) but instead is regulated by intracellular levels of cAMP (Doucette and Devon, 1995).

Although ensheathing cells are non-myelinating glia cells, it has been established in vitro that they have the capacity to myelinate dorsal root ganglion neurites, being induced to resemble the phenotype of myelinating Schwann cells (Devon and Doucette, 1992). The capacity to myelinate also extends into the central nervous system. Ensheathing cells, derived both from the rat and human, when implanted into the spinal cord are able to myelinate demyelinated central axons, assuming a typical peripheral one-to-one relationship with each axonal internode segment (Barnett et al., 2000; Imaizumi et al., 1998; Li et al., 1998; Kato et al., 2000). It appears then that when induced to myelinate in vivo, ensheathing cells preferentially express a Schwann cell phenotype.

However, there have been some conflicting data from in vitro investigations. One of the first studies reporting the isolation of ensheathing cells from adult rat olfactory bulb showed that some of the cells stain positively for myelin basic protein (MBP), which is a molecule normally associated with oligodendrocytes (Ramon-Cueto and Nieto-Sampedro, 1992). In contrast, in a later study it was reported that only about 10% of ensheathing cells cultured in medium that would normally support the differentiation of oligodendrocytes, express galactocerebroside while none express MBP (Doucette and Devon, 1995). More recently, immunohistochemical staining showed the absence of immunoreactivity for MBP in both the olfactory nerve layer of the bulb and ensheathing cells in culture (Santas-Silva and Cavalcante, 2001). Interestingly, the ensheathing cells showed weak reactivity for the non-compact myelin protein 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), which is an oligodendroglial/Schwann marker, known to be involved in the ensheathment process preceding myelin compaction (Amur-Umarjee

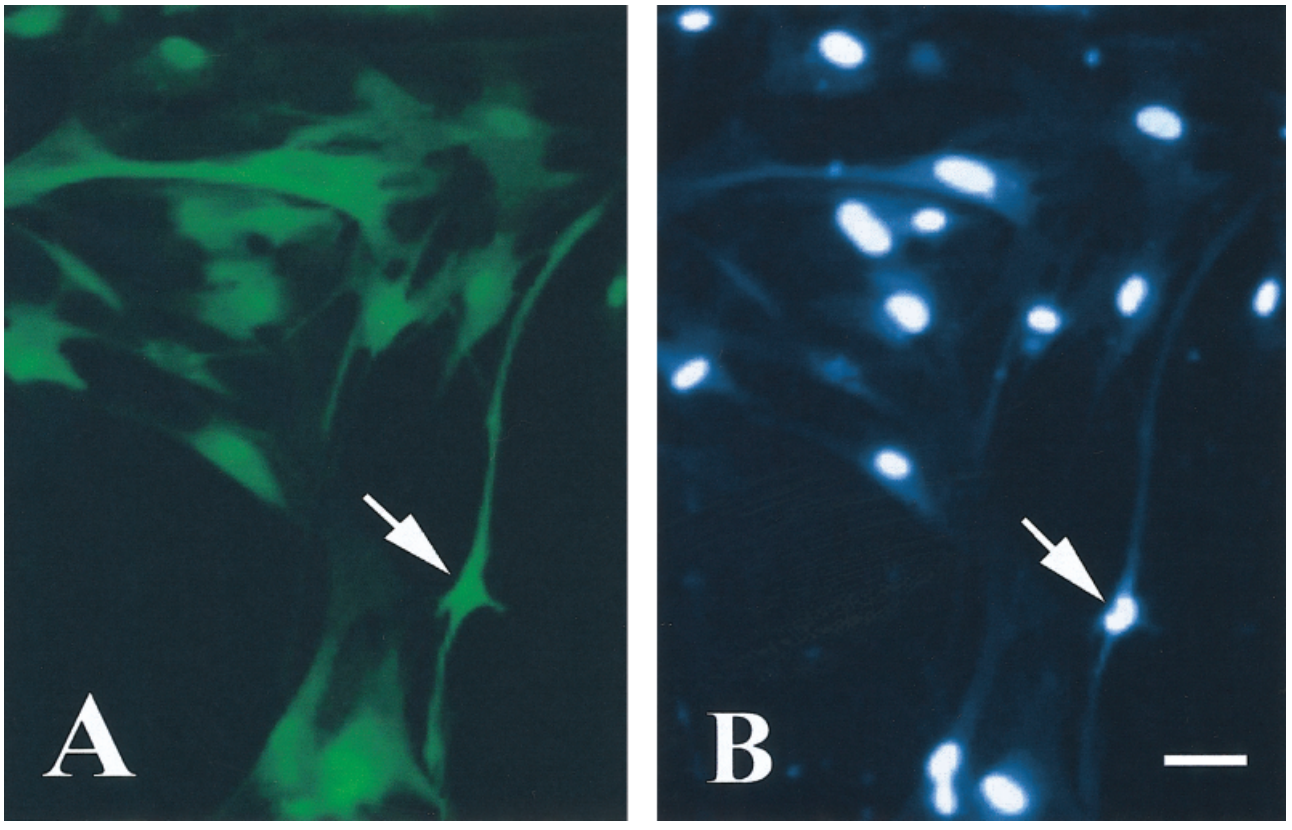

Fig. 2. Ensheathing cells of morphology stained with anti-S-100 (**A**) and Nuclear Yellow (**B**). Arrow indicates a spindly bipolar cell amongst several other cells that appear polygonal and flatter. Bar = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 1990; Braun et al., 1988; Yoshino et al., 1985). In spite of these uncertainties, it should be noted that there are distinct differences between Schwann cells and ensheathing cells with regard to their in vitro behaviour in the presence of astrocytes (Lakatos et al., 2000). Ensheathing cells interact more freely with astrocytes, migrating frequently to areas containing astrocytes, and with no indication of causing the astrocytes to hypertrophy. This lack of aversion to astrocytes probably explains why ensheathing cells are shown to be increasingly more effective than Schwann cells as an experimental agent for central nerve repair (Ramon-Cueto et al., 1998).

EXPRESSION OF MEMBRANE SURFACE MOLECULES

The successful growth of olfactory axons to the olfactory bulb is probably mediated by a combination of extracellular matrix molecules, cell adhesion molecules, and soluble chemotropic factors, in much the same manner as these extrinsic agents are thought to mediate axonal elongation in other parts of the nervous system. Many of these growth-associated molecules were first identified in immunohistochemical studies that reported their spatiotemporal presence either in the lamina propria along the route taken by olfactory nerves or in the olfactory nerve layer of the bulb. The distribution of these molecules is consistent with the notion that they play a role in supporting olfactory axon growth, fasciculation, and sorting (Table 2).

Laminin was first localised in the olfactory nerve layer of the bulb (Liesi, 1985) and is produced by ensheathing cells and olfactory axons as early as E13 in the rat (Treloar et al., 1996). When dissociated olfactory neurons were plated at a density of 1.5×10^5 /cm^2 they were able to adhere to laminin and subsequently differentiate into bipolar cells (Pixley and Pun, 1990). However a lower plating density of about 10^4 cells/cm^2 failed to induce significant axon growth (Chuah et al., 1991). Interestingly, when olfactory epithelial explants were grown on laminin, extensions of single neurites and fascicles were clearly visible within a few days (Whitesides and LaMantia, 1996). Taken together, the data from these studies show that increasing the density and proximity of cells to each other enhances axonal extension on laminin. Significantly, the fact that low density cultures on laminin failed to induce neurite growth indicates that other neurotrophic agents are probably required for this process.

It is possible that laminin has multiple roles in the olfactory system. In explant cultures, laminin has been shown to promote cellular migration including that of ensheathing cells from the epithelium (Calof and Lander, 1991; Tisay and Key, 1999). This action has been mapped to the distal long arm domain E8 of the molecule and is mediated by $\alpha_6\beta_1$ integrin (Calof et al.,

TABLE 2. Membrane-associated molecules expressed by ensheathing cells

Antigen molecule	Presence in axons (a) or Lamina propria (lp)	Reference
Laminin	a	Liesi (1985); Treloar et al. (1996)
N-CAM	a, lp	Miragall et al. (1989); Whitesides and LaMantia (1996)
L1	a, lp	Miragall et al. (1989); Whitesides and LaMantia (1996)
Galectin-1	lp	St. John and Key (1999)
HSPG	lp	Treloar et al. (1996)
Semaphorin 3A	lp	Schwarting et al. (2000)

1994). The enhanced exit of ensheathing cells from the epithelium in response to laminin and bulb-derived soluble factors (Liu et al., 1995) acts as a stimulus for promoting olfactory nerve growth.

In addition to laminin, ensheathing cells are known to express N-CAM and L1, which are also present in olfactory axons and the surrounding mesenchyme (Calof and Chikaraishi, 1989; Gong and Shipley, 1996; Miragall et al., 1989; Whitesides and LaMantia, 1996). The use of antibodies against these adhesion molecules on cultures of dissociated olfactory neurons growing on astrocyte monolayers has shown that neurite outgrowth from olfactory neurons is inhibited by antibodies to N-CAM and L1 (Chuah et al., 1991). Interestingly the trajectory of the olfactory nerves in the N-CAM-180 null mutant mice appears largely normal other than the olfactory bulb having a relatively thicker olfactory nerve layer because some of the axons fail to exit and contribute towards glomeruli formation (Treloar et al., 1997). Perhaps the absence of N-CAM can be compensated for by the presence of other adhesion molecules such as L1. The Src-family tyrosine kinases $p59^{fyn}$ and $pp60^{c\text{-}src}$, which are associated with the transduction of adhesive signals from cell adhesion molecules, are present on olfactory axons (Morse et al., 1998). Homozygous null mutant mice, which lack both the *src* and *fyn* genes, show extensive defasciculation among the olfactory nerve bundles (Morse et al., 1998). This finding points to the possibility that olfactory axonal growth is mediated by a combination of several molecules.

The expression of proteoglycans and carbohydrate binding proteins by ensheathing cells has also been implicated in the sorting out and fasciculation of olfactory axons during development (Treloar et al., 1996). An example is galectin-1, which is first apparent in the mesenchyme surrounding the nasal cavity at E15 and can be localised distinctly to ensheathing cells at E17 (St. John and Key, 1999). Expression of galectin-1 is maintained throughout development and adulthood where it can be observed in the ensheathing cells surrounding the axon bundles in the lamina propria as well as those residing in the olfactory nerve layer. Heparan sulfate proteoglycans (HSPG) are expressed early during development in the rat at about E13 in the mesenchyme between the olfactory pit and presumptive olfactory bulb (Treloar et al., 1996). They are subsequently localised to ensheathing cells as well.

As the aforementioned studies indicate, much of the data is consistent with the notion of ensheathing cells expressing growth-promoting molecules that facilitate olfactory axonal growth. In recent investigations, it has been shown that ensheathing cells are also involved in providing directional cues to olfactory axons by expressing semaphorin 3A, which bears chemorepulsive properties for neuropilin, which is expressed on olfactory axons (Rohm et al., 2000; Schwarting et al., 2000). Mutant mice that lack semaphorin 3A have aberrant axon trajectories, particularly in the olfactory nerve layer and, consequently, the axons terminate in inappropriate glomerular targets (Schwarting et al., 2000).

The path of the olfactory nerves has been described as consisting of three morphological domains: the interface area between the olfactory epithelium and underlying lamina propria, the extra- and intracranial connective tissue region, and the interface area between the connective tissue and olfactory bulb (WhitesidesI and LaMantia, 1996). It is thought that different combinations and levels of adhesion and extracellular matrix molecules in these domains act together to mediate axonal growth. They probably also regulate the direction of nerve growth and the degree of fasciculation. Although the ensheathing cells are an active agent in producing these growth-promoting molecules, it should be noted that many of them are also expressed by the olfactory axons and surrounding connective tissue.

EXPRESSION OF GROWTH FACTORS AND THEIR RECEPTORS

Growth factors are commonly regarded as a target-derived, long-range cue crucial to the normal development of neurons. In recent years, the number of growth factors that have been identified in the olfactory system has increased dramatically although many of their respective functions still remain to be elucidated (Mackay-Sim and Chuah, 2000). In earlier studies, the axonal-promoting properties of ensheathing cells have largely been attributed to their expression of membrane surface associated molecules; few investigations have speculated on the possibility of ensheathing cells synthesising and secreting soluble growth factors. Perhaps part of the problem is the fact that even with the localisation of growth factors, there have been some inconsistencies with regard to their spatial distribution in the primary olfactory pathway (Mackay-Sim and Chuah, 2000). Whilst immunohistochemical evidence is often conveniently cited as a first indicator of growth factor synthesis, confirmation using other techniques is required because the protein-containing cells may have taken up the growth factor from surrounding tissues.

In the rat primary olfactory pathway, both acidic and basic fibroblast growth factors (FGF1 and FGF2, respectively) have been localised to ensheathing cells and olfactory nerves (Chuah and Teague, 1999; Gall et al., 1994; Key et al., 1996). In addition, ensheathing cells

TABLE 3. Growth Factors and Receptors Expressed by Ensheathing Cells[1]

Growth factor	Receptor	Reference
FGF1, FGF2	FGFr1, perlecan	Gall et al. (1994); Key et al. (1996); Chuah and Teague (1999); Hsu et al. (2001)
PDGF-B	n.d.	Kott et al. (1994)
NGF	NGFr	Gong et al. (1994); Franceschini and Barnett (1996); Woodhall et al. (2001)
BDNF	Trk B	Woodhall et al. (2001)
NT-4/5	Trk B	Boruch et al. (2001); Woodhall et al. (2001)
GDNF, NTN	GFRα-1, GFRα-2	Woodhall et al. (2001)
CNTF	n.d.	Guthrie et al. (1997)
Neuregulin family	ErbB-2, ErbB-3 (?) ErbB-4 (?)	Salehi-Ashtiani and Farbman (1996); Perroteau et al. (1998); Pollock et al. (1999); Chuah et al. (2000)

[1]n.d. = not determined; (?) = discrepancy between in vitro and in vivo studies.

have been shown to express the high affinity receptor FGFr1 and perlecan, which is required for the binding of FGF to their receptors (Chuah and Teague, 1999; Hsu et al., 2001). Immunohistochemical staining has also revealed the presence of platelet-derived growth factor-B (PDGF-B) (Kott et al., 1994). Investigation into the qualitative and quantitative production of growth factors by ensheathing cells has been facilitated by their successful isolation and culture in a number of laboratories (e.g., Chuah and Au, 1993; Franceschini and Barnett, 1996; Ramon-Cueto and Nieto-Sampedro, 1992). Increasingly, the evidence indicates that ensheathing cells are a rich source of growth factors (Table 3).

Recent immunohistochemical and reverse transcriptase-polymerase chain reaction (RT-PCR) experiments from our laboratory show that ensheathing cells synthesise nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurturin, and glial growth factor 2 (GGF2) (Chuah et al., 2000; Woodhall et al., 2001). Interestingly, neurotrophin-3 (NT-3) was not detected in ensheathing cells. Enzyme linked immunosorbent assay demonstrated that ensheathing cells are active secretors of NGF, producing seven times as much NGF as BDNF (Woodhall et al., 2001). Similar results confirming the production of NGF, BDNF, but not NT-3 have also been obtained with the ensheathing cell line that was previously established by Goodman and co-workers (1993). This cell line was shown also to synthesise neurotrophin 4/5 (NT-4/5) but not ciliary neurotrophic factor (CNTF) (Boruch et al., 2001). The absence of CNTF is surprising given that in an earlier study, it was found that CNTF mRNA was present in the olfactory nerve ensheathing cells (Guthrie et al., 1997). Analysis of RT-PCR results show that it is likely that certain isoforms of growth factors synthesised by ensheathing cells may be a resultant effect of culture conditions. For example, ensheathing cells in vitro express a 500 base pair fragment of NGF and a BDNF transcript containing exon 3 that are not normally present in the olfactory bulb (Woodhall et al., 2001).

Whilst the expression of the low affinity neurotrophin receptor NGFr has been widely accepted as one of the markers for ensheathing cells, of the three high affinity receptors Trk A, B, and C, only Trk B is present. In addition, ensheathing cells expressed mRNA for GFRα-1 and GFRα-2 the receptors for GDNF and neurturin (NTN), respectively, but not RET (Woodhall et al., 2001). Although GFRα confers ligand specificity, RET provides the capacity for signal transduction (Airaksinen et al., 1999). The absence of TrkA and RET suggest that it is unlikely that NGF, GDNF, and NTN could elicit intracellular cytoplasmic signals within these cells. Instead, the receptors could function to bind and present NGF, GDNF, or NTN to surrounding olfactory axons.

In addition to neurotrophins, members of the neuregulin family are also synthesised by ensheathing cells. The multiple isoforms of the neuregulin gene (NRG-1), which is located on the short arm of chromosome 8, are encoded through alternative splicing (Orr-Urtreger et al., 1993). Up to 15 isoforms of neuregulin have been identified although it is possible that more exist. The protein products of the neuregulin gene are composed of several distinct structural domains including a signal peptide at the N-terminus, an immunoglobulin-like domain, a glycosylation rich spacer domain, an epidermal growth factor (EGF)-like domain, a juxtamembrane domain, a transmembrane domain, and a highly conserved cytoplasmic domain (Wen et al., 1992). Some of them, Neu differentiation factors (NDF), have been demonstrated immunohistochemically in vivo (Salehi-Ashtiani and Farbman, 1996) and in the ensheathing cell line (Boruch et al., 2001).

In a recent study (Woodhall et al., 2000), we performed RT-PCR analysis on ensheathing cells using primers previously designed by Rosenbaum and coworkers (1997). The results showed that ensheathing cells expressed the α-EGF, β-EGF, and transmembrane transcripts of sensory and motorneuron-derived factor (SMDF) and NDF (Table 4).In addition, a single transcript of GGF/β-EGF was also detected. The results presented here are consistent with a recently published study (Thompson et al., 2000), which suggested that several isoforms of neuregulins are present in ensheathing cells.

In terms of their receptors, immunohistochemical staining showed that ErbB-2 and -3 are present in ensheathing cells (Perroteau et al., 1998; Salehi-Ashtiani and Farbman, 1996). However, perhaps due to the culture conditions, a recent in vitro study showed that ensheathing cells expressed ErbB-2 and -4 but not ErbB-3 (Pollock et al., 1999).

The finding that ensheathing cells express receptors for some of the factors that they produce raises implications regarding the possible roles of these soluble agents. It is probable that these growth factors exert both an autocrine and paracrine action. With regard to paracrine activity from ensheathing cells and other olfactory sources, most studies have investigated growth factor influence on olfactory neuron survival

TABLE 4. Neuregulin Isoforms Expressed by Ensheathing Cells

Neuregulin	Sub-type	Predicted PCR product (bp)	Ensheathing cells	Fibroblasts
SMDF	α	540	●	●
	β	530	●	-
	S	657	-	-
	T	711	●	-
GGF	α	625	-	-
	β	610	●	-
	S	637	-	-
	T	790	-	-
NDF	α	580	●	●
	β	570	●	●
	S	603	?	-
	T	657	●	●

[1]α = α-EGF; β = β-EGF; S = secreted; T = transmembrane; ? = inconclusive.

and proliferation (e.g., NGF: Ronnett et al., 1991; FGF2: Ensoli et al., 1998). In contrast, it is perhaps surprising that few studies have investigated the effect of soluble growth factors on axonal growth, given that the ensheathing cells are so intimately associated with olfactory axons.

Scarcity of these studies may be a consequence of earlier reports that revealed that medium conditioned by ensheathing cells failed to promote neurite growth from olfactory neurons isolated from 4–5-week old rats (Chuah and Au, 1994). However, a recent study by Kafitz and Greer (1999) demonstrated that if embryonic olfactory neurons and ensheathing cells were spatially separated but sharing the same culture medium, the length of olfactory neurites was similar to that obtained when they were cultured in direct contact with each other. The discrepancy in these two studies could be due to the possibility that diffusible factors secreted by the ensheathing cells are rapidly inactivated in culture or that that they are required in high levels by the differentiating neurons. Alternatively, it is possible that embryonic olfactory neurons are more dependent on soluble growth factors for neurite extension than are postnatal neurons. We have initiated studies into the possible role of NGF in promoting neurite extension from embryonic olfactory neurons and results indicate that it enhances neurite growth but is probably one of a number of agents that are able to perform this function (Martin et al., 2002).

EFFECT OF GROWTH FACTORS ON ENSHEATHING CELLS

Despite the emerging importance of ensheathing cells in the olfactory system and their use in the promotion of repair in the central nervous system (see below), there is still a dearth of information concerning the cell dynamics of this unique glia. For example, although it is well established that they originate from the olfactory placode (Chuah and Au, 1991), little is known about the mechanisms regulating their proliferation or lifespan once they emerge into the lamina propria or enter the olfactory nerve layer of the bulb. It is conceivable that during development when increasing numbers of olfactory axons are growing towards the bulb, there is a net increase in the number of ensheathing cells in the primary olfactory pathway. However, once a mature pattern is established, it is logical to assume that the number of newly formed ensheathing cells is balanced by a dying population. How this balance is achieved is not known. In addition, it is also unclear what happens to ensheathing cells that are associated with neurons that die during the normal course of life. Experiments that utilise intranasal irrigation with zinc sulphate solution, which kills most of the olfactory epithelial cells, show that, devoid of olfactory axons, many of the ensheathing cells migrate to the olfactory bulb while a small number gather in a narrow zone beneath the olfactory epithelium (Chuah et al., 1995). However, this procedure causes massive degeneration of olfactory axons and the resultant migratory behaviour of ensheathing cells may not be representative of the normal condition in which degenerating axons constitute a very small proportion of the olfactory nerves.

In view of the finding that ensheathing cells can assume different phenotypes, it is possible that some type of regulatory mechanism may be mediated by growth factor signalling. In an attempt to understand the dynamics of ensheathing cells, several laboratories have undertaken experiments to examine the effect of growth factors on ensheathing cells. Such experiments are also partly fueled by a renewed interest in discovering growth factors that are able to effectively expand ensheathing cells in order that sufficient quantities can be achieved for CNS transplantation studies (see Use of Ensheathing Cells in Nervous Tissue Repair). In vitro studies from our laboratory show that bFGF, at 10 ng/ml, is able to elicit a threefold increase in the number of ensheathing cells (Chuah and Teague, 1999). However, the increased proliferation rate is not maintained with higher doses of bFGF (Chuah and Teague, 1999; Yan et al., 2001). In our study, NGF was found not to be effective in eliciting a significant change in the proliferation.

Following an earlier report that medium conditioned by astrocytes is mitogenic for ensheathing cells (Franceschini and Barnett, 1996), subsequent analysis has revealed that products of the neuregulin gene, such as NDFβ1, -2, -3 contained in the medium are responsible for the effect (Pollock et al., 1999). More recently, other neuregulins, GGF2 and heregulin β1 (HG), have also been shown to promote ensheathing cell proliferation (Chuah et al., 2000; Yan et al., 2001). Whilst NDFβ3 and NDFα2 enhance ensheathing cell survival by inhibiting apoptosis, it was also noted that they induce a flattened and process-bearing morphology in the ensheathing cells (Pollock et al., 1999). Consistent with this finding, it was found that ensheathing cells exposed to GGF2 express more extracellular matrix on their membrane and contain more cytoskeletal filaments, thus making the cellular processes more robust (Chuah et al., 2000). Unlike Schwann cells, which show increased motility in response to GGF2 (Mahanthappa et al., 1996), the growth factor does not appear to exert any chemotactic effect on ensheathing cells (Chuah et al., 2000).

The study conducted by Yan and co-workers (2001) employed four different growth factors either separately or in various combinations. It was found that the combination of HG and bFGF is most potent and it promotes ensheathing cell proliferation in an additive manner, suggesting that they may be acting indepen-

dently. When present separately in the culture medium, the proliferative effect of HG and bFGF can be enhanced further by the addition of forskolin, which is known to increase intracellular cAMP levels (Weinmaster and Lemke, 1990). The link between cAMP levels and the proliferative effect of HG and bFGF has not been elucidated although it has been shown that cAMP induces the expression of receptors for PDGF (Weinmaster and Lemke, 1990). Interestingly, PDGF-B and insulin-like growth factor 1 fail to stimulate ensheathing cell proliferation in serum-free medium (Yan et al., 2001).

Some of the growth factors that have been shown to influence proliferation and phenotype (e.g., GGF2 and NDF) are produced by the ensheathing cells themselves, indicating that this is an autocrine activity. These studies pave the way for future investigations into the intracellular signalling pathways that ultimately control the kinetics of ensheathing cell biology. Understanding the mechanisms that control ensheathing cell's proliferation will emerge as an important area of research in view of increasing interest in using them in nerve regeneration studies.

USE OF ENSHEATHING CELLS IN NERVOUS TISSUE REPAIR

The last five years have witnessed a dramatic explosion in the application of the unique features of the olfactory system to promote repair of injured nervous tissue. Two features of the olfactory system stand out: (1) the easy accessibility of a stem cell population that is capable of generating new neurons throughout life and (2) the presence of a unique glia, the ensheathing cells, that is capable of existing both in the peripheral and central nervous system. This section will assess the results that have been obtained so far in regeneration studies using ensheathing cells, including those currently conducted in our laboratory, and discuss how these studies could shed further light on ensheathing cell biology.

One of the first studies to suggest the feasibility of using ensheathing cells to repair traumatically damaged neural pathways was conducted by Kott and coworkers (1994). In their study, they transplanted fetal olfactory bulbs into the space created as a result of bulbectomy in neonatal rats. It was found that donor ensheathing cells not only survived but also ensheathed both olfactory and non-olfactory axons deep within the host tissue. Since this study, numerous reports have surfaced in which ensheathing cells have been implanted into various sites of the nervous system, e.g., spinal cord (Li et al., 1997, 1998; Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto et al., 2000), thalamus (Perez-Bouza et al., 1998), brainstem (Gudino-Cabrera et al., 2000), fimbria-fornix (Smale et al., 1996), and the sciatic nerve (Verdu et al., 1999). The different models of spinal cord injury that have been treated with transplants of ensheathing cells have been summarised in a recent review by Bartolomei and Greer (2000).

All of the above studies report varying degrees of success in the regrowth of injured tracts except for the study in which ensheathing cells were implanted into the brainstem (Gudino-Cabrera et al., 2000). In this latter study, it was found that ensheathing cells migrated in a direction opposite to the intended axonal target and, consequently, failed to provide directional guidance to new axons. Interestingly, in a recent review, it was reported that when ensheathing cells are obtained from the olfactory bulb and transplanted immediately without prior culturing, they fail to exhibit reparative properties (Raisman, 2001). Hence, it appears that the growth promoting activity of ensheathing cells may in part be attributed to culture conditions.

Once transplanted into the central nervous system, the precise location and orientation of ensheathing cells appear to be crucial in influencing axonal regeneration. In those studies where new axons had elongated in the correct direction, ensheathing cells adopted a spindle-shaped morphology forming a "glial bridge" or scaffold in addition to being concentrated near the lesion site (Boruch et al., 2001; Li et al., 1998; Perez-Bouza et al., 1998). Although the number of axons regenerating across the lesion site was often found to be limited, they were capable of rapid impulse conduction (Imaizumi et al., 2000b; Ramon-Cueto et al., 2000). This is in part due to the fact that the ensheathing cells were shown to be capable of remyelinating central axons (Kato et al., 2000; Li et al., 1998).

Nevertheless, there are unanswered questions regarding ensheathing cell behaviour in a novel environment. In a study in which ensheathing cells were injected into hippocampal CA1 region, it was found that ensheathing cells migrated extensively and could be found one month later in several distant loci, e.g., the laterodorsal thalamic nuclei, internal capsule, and arcuate nucleus (Gudino-Cabrera and Nieto-Sampedro, 1996). Hence, ensheathing cells are able to move through white matter, gray matter, and even glial scars (Ramon Cueto et al., 1998). Exactly which molecules are involved and how they regulate ensheathing cell migration in a novel environment are important questions that need to be addressed if ensheathing cells are to be successfully applied as a therapeutic agent for CNS repair.

Perhaps what is most remarkable in these regeneration studies is the functional recovery attained by previously paraplegic rats that had been subjected to total spinal cord transection (Ramon-Cueto et al., 2000). Histological analysis of the spinal cord of these animals reveals the presence of newly regenerated motor axons elongating across the lesion site amidst the presence of ensheathing cells and a glial scar. The data suggest that ensheathing cells are somehow able to alter the restrictive environment of the central nervous system into a positive milieu that promotes axon regeneration. Possible mechanisms underpinning the regeneration include direct membrane interaction between ensheathing cells and surrounding host tissue, and the production of optimal levels of certain growth factors.

It is well documented that growth factors such as the neurotrophins, GDNF and neuregulins produced by ensheathing cells exert a positive survival effect on neurons and Schwann cells (e.g., Barde, 1989; Davies, 1994; Mahanthappa et al., 1996). What is less known is the response of central glia to soluble factors produced by ensheathing cells. Oligodendrocytes, reactive astrocytes, and microglia exert inhibitory influences on ma-

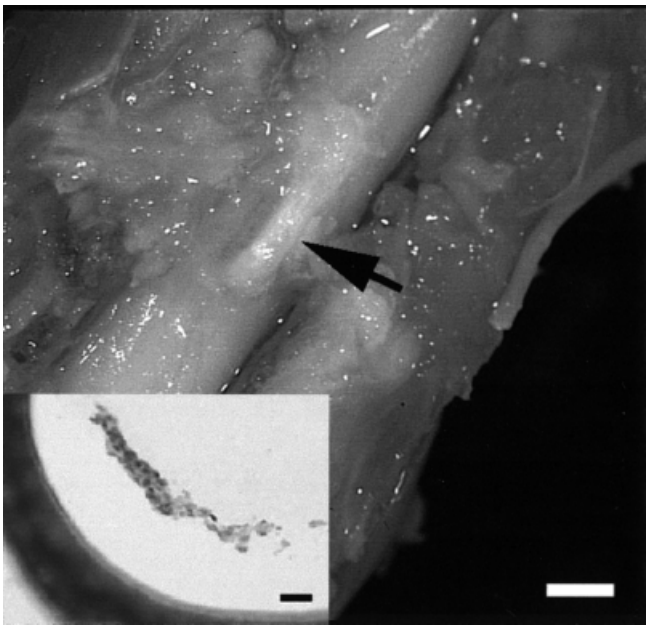

Fig. 3. Lesioned spinal cord implanted with polymer capsule (arrow) containing ensheathing cells. The capsule is placed beneath the dura and immediately above the dorsal columns. **Inset:** A capsule that has been sectioned to reveal some of the ensheathing cells present within it. Bar = 4 mm; bar in inset = 50 μm.

ture axonal growth, thus presenting a major obstacle to the process of central nerve repair (Giulian, 1993; Schwab et al., 1993). Two factors known to be secreted by ensheathing cells, namely NGF and BDNF (Woodhall et al., 2001), have been shown to block the inhibitory influences of myelin-associated glycoprotein (MAG) on axonal growth (Cai et al., 1999). On the other hand, GGF is shown to promote the proliferation and survival of oligodendrocytes but to inhibit their differentiation (Canoll et al., 1996). It is highly plausible that, in addition to these known growth factors, ensheathing cells implanted into a lesion site in the central nervous system are stimulated to provide a number of other yet to be identified soluble factors.

In order to gain further insight into the physiology of ensheathing cells and to investigate how they are promoting repair in the injured central nervous system, we have packed ensheathing cells into porous polymer capsules and implanted them into lesioned spinal cords (Chung et al., 2000) (Fig. 3). The polymer capsule allows the exit and entry of soluble factors out of and into the capsule. It also has the advantage of permitting easy retrieval of ensheathing cells for subsequent analysis, for example, to characterise using genetic assays the expression profile of ensheathing cells after implantation into the spinal cord. It is highly likely that ensheathing cells undergo a phenotypic shift in a new environment given that they are known to exhibit different phenotypes under different culture conditions. This experimental paradigm will also enable us to determine whether or not soluble factors, be they known growth factors or novel molecules, produced by ensheathing cells are crucial in promoting the regrowth of previously injured axons.

Recent studies have proposed the alternative of xenotransplantation in which genetically engineered pig cells are used as a source for human cell therapies (Imaizumi et al., 2000a). Whilst pigs are generally considered an ethical source of cells for transplantation into humans, there are risks associated with the transmission of viral antigens. Hence it is not surprising that research efforts are also directed at the use of human ensheathing cells in transplantation (Barnett et al., 2000; Kato et al., 2000). It is imperative that a deeper understanding of the biology of ensheathing cells is attained if in vitro stock populations are to be successfully expanded for potential use in clinical trials aimed at promoting regeneration.

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