Cellular and molecular studies on olfactory bulb ensheathing cells

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Abstract

Olfactory bulb ensheathing cells (OBECs) are a specialised type of glial cell that reside in the outer layer of the olfactory bulb, where they are thought to play a role in the exceptional ability of this central nervous system tissue to support re-enervertion throughout life. This thesis reports on the characterisation of the antigenic expression and growth and functional properties of OBECs *in vitro*. OBECs were purified from perinatal rat olfactory bulbs by fluorescence activated cell sorting using the O4 antibody (Barnett et al., 1993a).

Immunofluorescence labelling of the cultures and of tissue sections of the olfactory bulb with a large panel of neural marker antibodies showed that OBECs represent a heterogeneous population of cells. The antigenic profile of the sorted cell population initially correlated with their antigenic profile *in vivo*, although expression of some of the markers was either lost or gained during time in culture. These changes were influenced by the culture conditions, with a greater loss of "typical" OBEC markers in serum-containing medium. In serum-free medium, which maintained the cells in a phenotype that closely resembled their *in vivo* counterparts, two antigenically and morphologically distinct cell types were observed, reminescent of non-myelinating Schwann cells and type-1 astrocytes, respectively. Two similar phenotypes were detected in a clonal OBEC cell line generated by retroviral infection with the temperature sensitive mutant gene of the large T antigen, supporting the view that the astrocyte-like and Schwann cell-like cells belong to the same lineage. A detailed immunocytochemical analysis of the developing olfactory system suggested that the diversification of OBEC antigenic phenotype occurs prior to embryonic day 14, along both, the peripheral and central portion of the olfactory nerves, is maintained throughout development, and is controled by microenvironmental cues.

Astrocyte conditioned medium (ACM) was shown to exibit potent mitogenic activity for cultured OBECs but most of the known growth factors present in ACM were not mitogenic when applyed individually using recombinant or highly purified preparations. The mitogenic activity of ACM could be reproduced with a glial growth factor (GGF)-enriched preparation of bovine pituitary extract. However, preliminary biochemical characterisation of the ACM-derived mitogenic activity did not correlate with that of any of the previously purified neuregulins and suggested that it may be confered by a novel member of this family of polypeptide growth factors.

The ability of OBECs to promote neuronal attachment/survival, neurite initiation and neurite elongation was compared to that of astrocytes and Schwann cells in an *in vitro* system consisting of plating cerebellar granule neurons onto monolayers enriched for each glial cell type. OBEC monolayers grown in the presence or absence of serum exerted similar neurite-outgrowth promoting activity. They were similar than Schwann cells and astrocytes at supporting neuronal attachment/survival and at initiating neurite outgrowth. However, the extent of neurite elongation on OBECs was greater than on Schwann cells and smaller than on astrocytes. When neurons were plated onto sparse cultures of OBECs, neurite initiation and elongation was greater among neurons in contact with glial cells, suggesting a role for surface interactions.

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Abbreviations

Abbreviated words

ACM:	Astrocyte conditioned media
ALOBEC:	Astrocyte-like OBEC
AMOG:	Adhesion molecule on glia
ARIA:	Acetylcholine receptor inducing activity
cAMP:	Cyclic 3' 5' adenosine mono phosphate
cDNA:	Complementary DNA
FGF:	Fibroblast growth factor
BDNF:	Brain derived growth factor
BSA:	Bovine serum albumin
BrdU:	5-Bromo-2'-deoxy-uridine
CNS:	Central nervous system
CNTF:	Cilliary neurotrophic factor
COF:	Central olfactory fascicle
dbcAMP	Dibutyryl cAMP
DMEM:	Dulbecco's modified eagle medium
DNA:	Deoxyribonucleic acid
E:	Embryonic day
ECM:	Extracellular matrix
EDTA:	Ethylenediamine tetra-acetic acid
EGF:	Epidermial growth factor
EPL:	External plexiform layer
DCS:	Donor calf serum
FACS:	Fluorescence activated cell sorter
FCS:	Foetal calf serum
Fl:	Fluorescein
GalC:	Galactocerebroside
GAP-43:	Growth associated protein 43
GCL:	Granule cell layer
GFAP:	Glial fibrillary acidic protein
GGF:	Glial growth factor
GL:	Glomerular layer
HBSS:	Hank's balanced salt solution
HRPO:	Horseradish peroxidase
Ig:	Immunoglobulin
IGF	Insulin-like growth factor
IPL:	Internal plexiform layer
IR:	Immunoreactivity
LH-RH:	Luteinizing hormone-releasing hormone
L-NGFr:	Low affinity nerve growth factor receptor
MAG:	Myelin associated glycoprotein
MAP:	Microtubule associated protein
MBP:	Myelin basic protein
MM:	Migratory mass (prospective olfactory bulb)
NCAM:	Neural cell adhesion molecule
NDF:	Neu differentiation factor
NF:	Neurofilament
NGF:	Nerve growth factor
NF:	Neurofilament
NSE:	Neuron-specific enolase
OB:	Olfactory bulb
OBEC:	Olfactory bulb ensheathing cell
OMP:	Olfactory marker protein

ONEC:	Olfactory nerve ensheathing cell
ONL:	Olfactory nerve layer
P:	Postnatal day
PBS:	Phosphate buffer saline
PCR:	Polymerase chain reaction
PDGF:	Platelet derived growth factor
PE:	Phycoerythrin
PLL:	Poly-L-lysine
PNS:	Peripheral nervous system
POF:	Peripheral olfactory fascicle
PSA:	Polysialic acid
PSA-NCAM:	Polysialilated (embryonic form of) neural cell adhesion molecule
rh:	Recombinant form of
Rd:	Rhodamine
RNA:	Ribonucleic acid
RT:	Room temperature
SDS:	Sodium dodecyl sulphate
SLOBEC:	Schwann cell-like OBEC
Tag:	Large T antigen
TGF:	Transforming growth factor
trK:	Tyrosine kinase
Vm:	Vimentin

Abreviated measures

g:	grammes
mg:	milligrammes
μg:	microgrammes
pg:	picogrammes
M:	molar
mM:	millimolar
μM:	micromolar
l:	litres
ml:	millilitres
μl:	microlitres
mm:	millimetres
cm:	centimetres
A:	amperes
V:	volts
W:	watts
bp:	base pairs
Kb:	kilobase pairs
Kda:	kilodaltons
v:	volume
w:	weight
min:	minutes
h:	hours
^{o}C:	degrees centigrade

CHAPTER ONE

General introduction

1.1 General structure of the nervous system

1.1.1 Central and peripheral nervous system

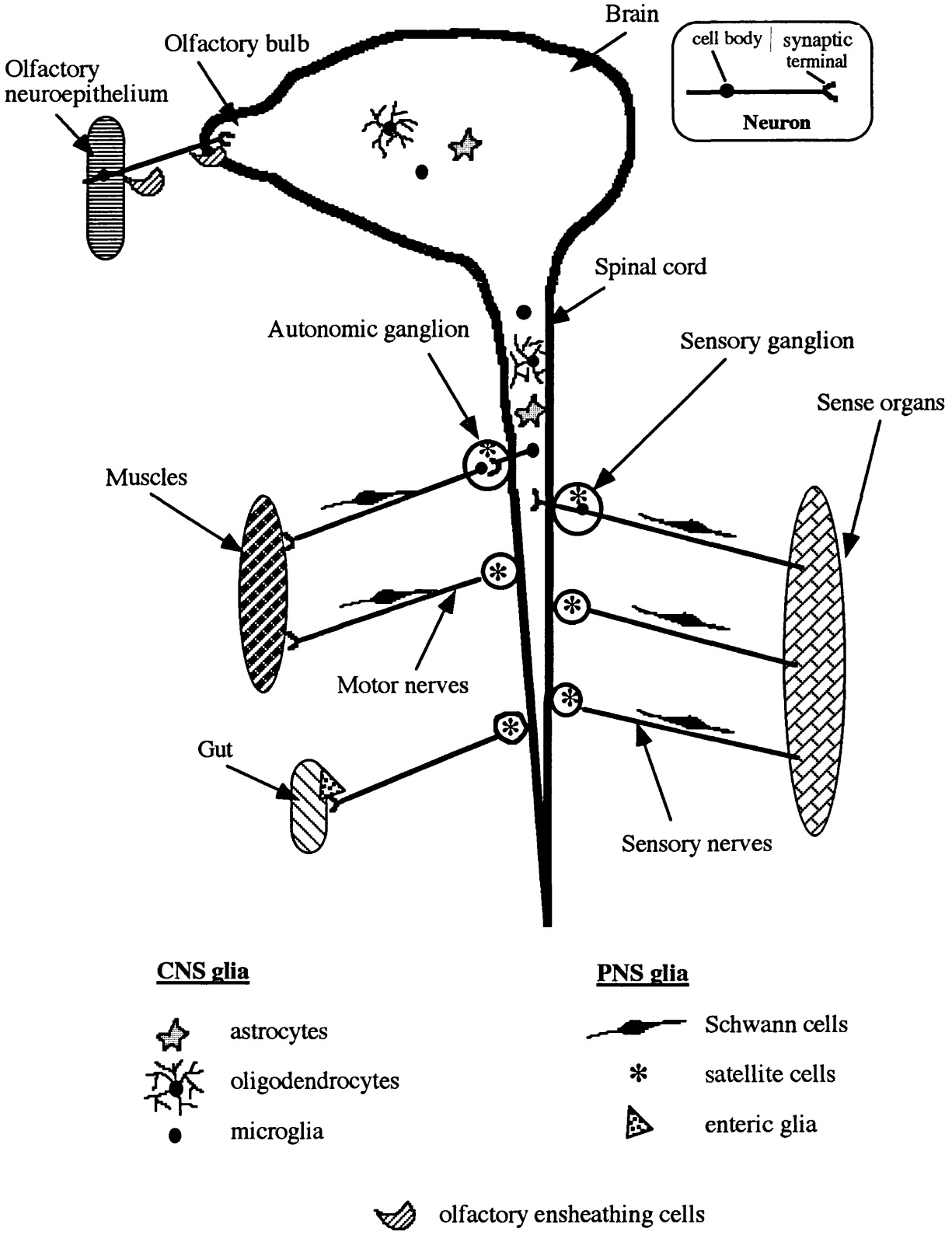
The nervous system of adult vertebrates can be subdivided into two main compartments: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises the brain and the spinal cord. The PNS comprises the sensory ganglia, the autonomic ganglia, the olfactory neuroepithelium, and the network of peripheral nerves which link the CNS to a large number of peripheral structures including sense organs and muscles (Fig. 1.1). With the exception of olfactory nerves, all peripheral nerves connect with the CNS in the spinal cord or at the base of the brain (medulla and pons). The olfactory nerves, which arise from the olfactory neuroepithelium in the nasal cavity, connect with the CNS in the olfactory bulb, an elongated tissue at the rostral end of each telencephalic vesicle.

1.1.2 Neurons and glial cells

Neurons and glial cells are the main cellular components of the nervous system. The fundamental task of neurons is to receive, conduct, and transmit information. Most neurons possess a network of highly branched short cellular processes called dendrites and one or two long cellular processes called axons. Dendrites and axons are often commonly referred to as "neurites". Dendrites receive chemical signals from other neurons at sites of functional contacts called synapses. These chemical signals are integrated in the neuronal cell body and converted into electrical signals that are sent along the axon. At the end of the axon, the signals reach a bulbous region called pre-synaptic nerve terminal and reconverted into chemical signals which are releases onto the next cell. Glial cells are the support cells of the nervous system. They fill interstices between neuronal cell bodies,

Figure 1.1

Schematic view of the nervous system



ensheath axons, surround the blood vessels of the brain, and line its internal and external surfaces. In the mammalian brain, glial cells outnumber neurons by at least 10 to 1.

1.1.3 The different types of glial cells

Glial cells have been classified into different types on the basis of their location, developmental origin, morphology, ultrastructure, intracellular and surface chemistry, survival, growth, and functional properties. In the CNS of adult vertebrates, there are three main types: type 1 astrocytes, oligodendrocytes and microglia. A second type of astrocyte, referred to as type 2 astrocyte, which shares a common progenitor with oligodendrocytes, has been identified *in vitro,* but its presence *in vivo* is at present controversial. Peripheral glial cells fall into three main categories: Schwann cells in peripheral nerves, satellite cells in sensory and autonomic ganglia, and enteric glial cells in the nerve plexuses of the gut wall. In addition, specialised glial cells, which share properties with both type-1 astrocytes and Schwann cells, are found in the olfactory nerves along both, the PNS portion and CNS portion (olfactory bulb) of their course. This is in marked contrast with the glial cell environment of other peripheral nerves, which changes abruptly from Schwann cells to astrocytes and oligodendrocytes at the PNS/CNS transitional zone of the spinal cord. After numerous attempts to classify the glial cells of the olfactory nerves as either Schwann cells or astrocytes, it appears that they do in fact not belong to any previously defined glial cell types and have an identity of their own (Barnett et al., 1993a; reviewed by Ramon-Cueto and Valverde, 1995). Their nomenclature is still ill-defined and in this thesis, the latest nomenclature of Doucette (1995) has been adopted: they will be referred to as olfactory ensheathing cells. The respective anatomical locations of the different glial cell types within the nervous system is summarised in Fig. 1.1.

1.2 Neurogenesis, axonal outgrowth and axonal regeneration

1.2.1 Dichotomy between embryo and adult / PNS and CNS

Neurogenesis and axonal outgrowth are common throughout the nervous system at embryonic and early postnatal stages. As neurons differentiate, they irreversibly loose their ability to divide and neurogenesis is absent in most of the adult nervous system. The alternative response of neurons to injury, is to re-grow their axon (a process called axonal regeneration), provided their cell body has been spared. Damaged PNS axons often regenerate spontaneously and re-innervate the sensory and effector organs so that almost normal function is restored. In contrast, axonal regeneration is generally not successful in the CNS. This is well demonstrated at the PNS/CNS junction of the spinal cord. Following transection of dorsal root neurons central to the ganglia, axons will regenerate within the PNS portion of their course but fail to cross the PNS/CNS transitional zone to gain entry into the spinal cord (Carlstedt et al., 1987; Liuzzi and Lasek, 1987).

1.2.2 Exceptional nervous tissues where neurogenesis persists throughout life

There are a few exceptional areas of the nervous system where neurogenesis persists during adulthood. In the PNS there is the olfactory neuroepithelium (Graziadei and Monti-Graziadei, 1978a, b; Barber, 1982; Costanzo, 1991) and in the CNS, the subventricular zone of the anterior portion of the lateral ventricles, extending to the subventricular zone of the olfactory bulb (Altman, 1969; Kaplan and Hinds, 1977; Bayer, 1983; Corotto et al., 1993; Lois and Alvarez-Buylla, 1994), and the hippocampal dentate gyrus (Altman and Das, 1965; Kaplan and Hinds, 1977; Bayer et al., 1982; Altman and Bayer, 1990). The most understood is the olfactory neuroepithelium, which is easily accessible for experimental manipulation due to its peripheral location. It contains a pool of (as yet unidentified) stem cells, that continuously generate olfactory neurons throughout life (reviewed by Crews and Hunter, 1994). In adult rodents, olfactory neurons replace themselves in approximately one month under "normal" conditions (Graziadei and Monti-

Graziadei, 1979). The rate of this turnover can however be modified by experimental manipulations such as olfactory bulbectomy (Carr and Farbman, 1992; Schwob et al., 1992) or naris occlusion (Farbman et al., 1988). Unlike other peripheral axons, olfactory axons do not regenerate following injury. Instead, the olfactory neurons die and are replaced from the pool of stem cells in the olfactory epithelium.

1.2.3 The olfactory bulb: an exceptional CNS tissue that supports axonal growth throughout life

As olfactory neurons differentiate from the pool of stem cells in the olfactory epithelium, they extend axonal processes up to the olfactory bulb. The anatomy of this pathway will be described in detail in Section 1.4.1. Unlike other peripheral nerves, these axons have the unique ability to cross the PNS/CNS junction at the surface of the olfactory bulb and re-establish functional synaptic connections with CNS neurons within this tissue. Due to the continual turnover of olfactory neurons, the olfactory bulb can thus be regarded as a CNS tissue that undergoes continuous repair throughout life. The permissivity of the olfactory bulb to axonal growth persists after transection of the olfactory nerve (Graziadei and Monti-Graziadei, 1980; Doucette et al., 1983). The hypothesis has been put forward that the glial cells associated with olfactory nerves, the olfactory ensheathing cells, play a role of utmost importance in the continuous repair process that takes place in the olfactory bulb throughout adult life (Barber and Lindsay, 1982; Doucette, 1984; Raisman, 1985; reviewed by Doucette, 1990). Before reviewing the current literature on olfactory ensheathing cells (Section 1.5. and 1.6.), it is relevant to give some background on glial cells in general.

1.3 Antigenic and functional properties of glial cells

Glial cells were first described by R. Virchow in 1853. He perceived them as a syncytial mass and believed that their function was to glue nerve cells together and thereby maintain the cohesive form of the nervous system. Their identity as single cells was definitively shown by Ramon y Cajal at the beginning of this century. Glial cells have received particular interest since it was shown that they can regulate the environment and the behaviour of neurons. In this section, particular emphasis will be made on the antigenic and functional properties of Schwann cells, astrocytes and oligodendrocytes, which represent the major macroglial cell types. Much of this knowledge has been gained from the use of immunocytochemistry, a technique which has also been extensively used throughout the present thesis to characterise olfactory ensheathing cells. I will begin by summarising the principle of immunocytochemistry.

1.3.1 Principle of immunocytochemistry

Together with the more recent method of *in situ* hybridisation, immunocytochemistry is presently one of the most powerful methods available for distinguishing various cell populations. This method is based on the use of antibodies coupled with a marker molecule, enabling the localisation of the corresponding antigen in cells or tissues. Commonly used marker molecules include fluorescein and rhodamine (for fluorescence microscopy), the enzyme horseradish peroxidase (for either bright field microscopy or electron microscopy) and colloidal gold spheres (for electron microscopy). Antigen-specific antibodies are referred to as primary antibodies. The marker molecules can be covalently linked to the primary antibodies (direct immunocytochemistry). Alternatively, the marker molecules can be covalently linked to secondary class specific antibodies that bind to unlabelled primary antibodies (indirect immunofluorescent labelling). Detailed antigenic profiles of cells can be established by using 2 or 3 primary antibodies of different

classes in conjunction with secondary class specific antibodies labelled with different markers (double or triple indirect immunocytochemistry).

1.3.2 Antibody markers for glial cells

During the last two decades a large variety of antibodies have been generated against cell surface, ECM and intracellular molecules, that allow the discrimination between the diverse tissues and cellular elements that together form the nervous system. Although most of these antibody markers bind to antigens whose function is as yet ill-defined, they nevertheless are powerful tools to characterise cells and tissues. Commonly used glial markers are described in Table 1.1. From this table it can be seen that there are in fact very few clear cut markers specific for one glial cell type. For example GFAP, which was initially considered as an astrocyte-specific marker (Bignami et al., 1977) is also present in some Schwann cells (Yen and Fields, 1981; Dahl et al., 1982) and enteric glia (Jessen and Mirsky, 1980). In the PNS, L-NGFr is specifically found on the surface of Schwann cells and enteric glial cells. However in the CNS, it is expressed by many neurons and has also been detected on specialised astrocyte populations such as pituicytes in the neurohypophysis (Yan and Johnson, 1989; Borson et al., 1994). MAG, MBP, GalC, and O4 are found on both, Schwann cells and oligodendrocytes. Thus, the different glial cell types are usually distinguished by a combination of antigens. Immunocytochemical analysis of olfactory ensheathing cells has largely contributed to its classification as a unique glial cell type (see Section 1.5.2).

1.3.3 Developmental origin of glial cells and examples of glial cell lineages

With the exception of microglial cells, which originate from hemopoietic tissue, all the glial cells share a common embryonic origin with the neurons with which they associate. Type-1 astrocytes and oligodendrocytes derive from the neural tube while Schwann cells, satellite cells and enteric glial cells derive from the neural

Table 1.1
Examples of commonly used glial markers

Usual name	Full name (when applies)	Cell specificity in the nervous system	References
NCAM	Neural cell adhesion molecule	Schwann cells, astrocytes, neurons	Goridis et al, 1983; Keilhauer et al., 1985; Noble et al., 1985 Mirsky et al., 1986
L1		Schwann cells, neurons	Rathden and Schachner, 1984; Keilhauer et al., 1985 Mirsky et al., 1986
Po	Protein o	Schwann cells	Roomi et al., 1978; Mirsky et al., 1980
MAG	Myelin associated glycoprotein	Schwann cells, oligodendrocytes	Trapp and Quarles, 1984
MBP	Myelin basic protein	Schwann cells, oligodendrocytes	Mirsky et al., 1980
L-NGFr	Low affinity nerve growth factor	Schwann cells, neurons receptor	Chandler et al., 1984; Taniuchi et al., 1986; Distefano and Johnson, 1988; Yan and Johnson, 1988, 1989
A2B5		Oligodendrocyte progenitors, astrocytes, neurons	Eisenbarth et al., 1979; Schnitzer and Schachner, 1982; Raff et al., 1983
GalC	Galactocerebroside	Schwann cells, oligodendrocytes	Raff et al., 1978; Mirsky et al., 1980
O4		Schwann cells, oligodendrocytes	Sommer and Schachner, 1981; Bansal et al., 1989; Mirsky et al., 1990
GFAP	Glial fibrillary acidic protein	Schwann cells, astrocytes, enteric glia	Bignami and Dahl, 1977; Yen and Field, 1981; Jessen and Mirsky, 1980
Vimentin		Schwann cells, astrocytes, neurons	Bignami et al., 1982; Schnitzer et al., 1981
S100		All types of glial cells	Kligman and Hilt, 1988

crest (Jacobson, 1991). There is increasing evidence that olfactory ensheathing cells, like olfactory neurons, derive from the olfactory placodes (see Section 1.5.1.2.).

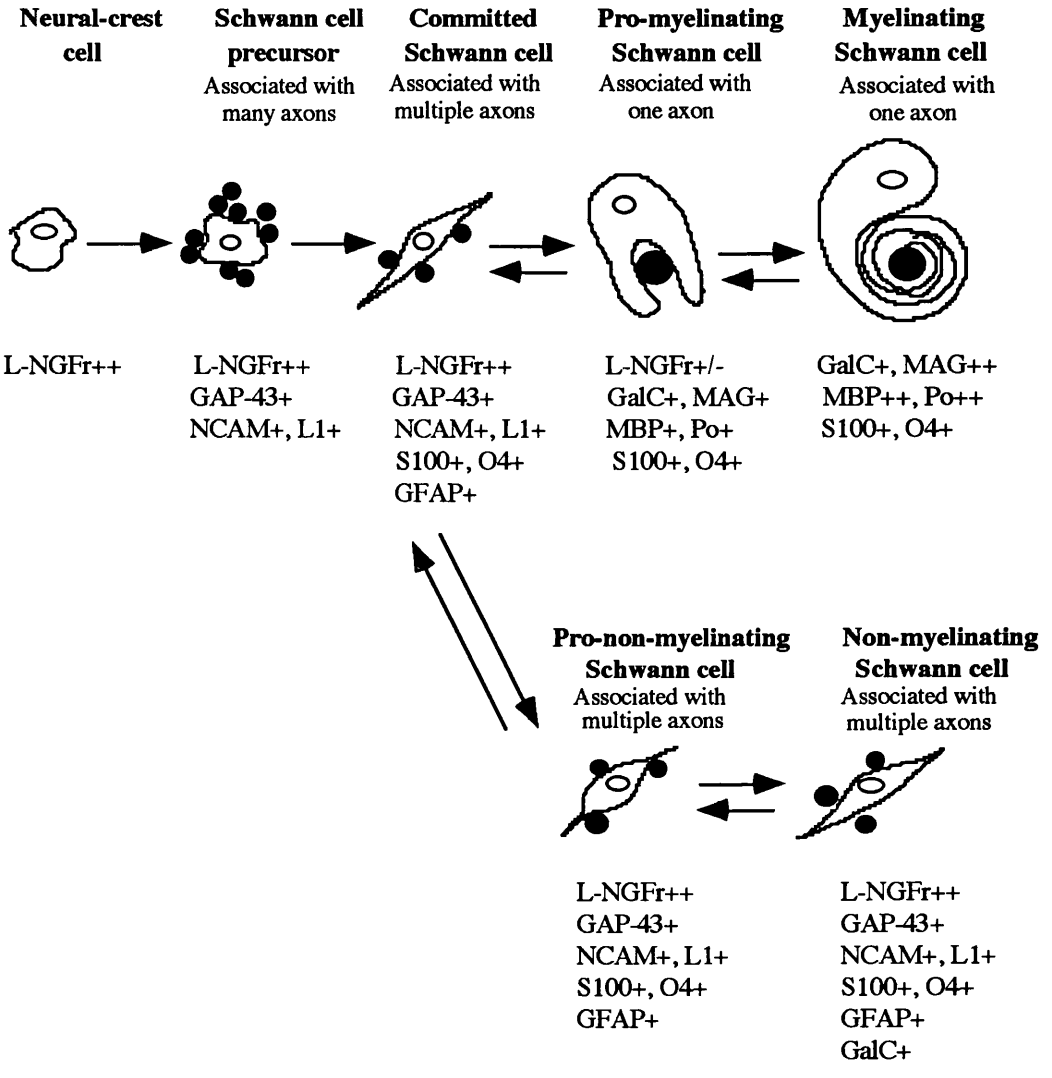
Since the development of hybridoma technology (Köhler and Milstein, 1975) and with the progress in techniques of cell purification, numerous differentiation stage-specific monoclonal antibodies have been produced that have greatly facilitated the study of many cell lineages. The most understood glial cell lineages are the Schwann cell and O-2A lineages which are described below. Very little is known about the lineage(s) of the other glial cell types. Type-1 astrocytes are antigenically and functionally very diverse and may arise from a number of separate lineages.

Schwann cell lineage

Antibodies against GAP-43, L-NGFr, S100, O4, GFAP, L1, N-CAM, GalC and against the myelin proteins Po, MAG, and MBP have been used both, *in vitro* and *in vivo*, to define different stages of the Schwann cell lineage (Fig 1.2). This lineage comprises two forms of mature Schwann cells, myelinating- and non-myelinating-Schwann cells, which originate from a common Schwann cell precursor (reviewed by Jessen and Mirsky, 1991). *In vivo*, Schwann cell precursors differentiate into a bipotential Schwann cell at around approximately E16 in rats. This bipotential Schwann cell then differentiates along either the myelinating- pathway or the non-myelinating-Schwann cell pathway during early postnatal development. In the adult, myelinating and non-myelinating Schwann cells are found along large calibre and small calibre axons, respectively. Following peripheral nerve injury, Schwann cells revert to a developmentally less mature phenotype and re-enter the cell cycle, proliferating distal to the lesion site.

Figure 1.2

Simplified scheme of the Schwann cell lineage



Stages in Schwann cell differentiation. This diagram summarises the profile of markers used to define distinct developmental stages in both the myelinating and the non-myelinating Schwann cell lineages. It is based on observations made *in vivo*, and in cultured cells. Although most of the forward differentiation of Schwann cells is reversible (for example, following disruption of axonal contact), it is not established in mammals that Schwann cells can revert back all the way to a multipotent neural crest cell phenotype. (Modified from Zorick and Lemke, 1996).

O-2A lineage

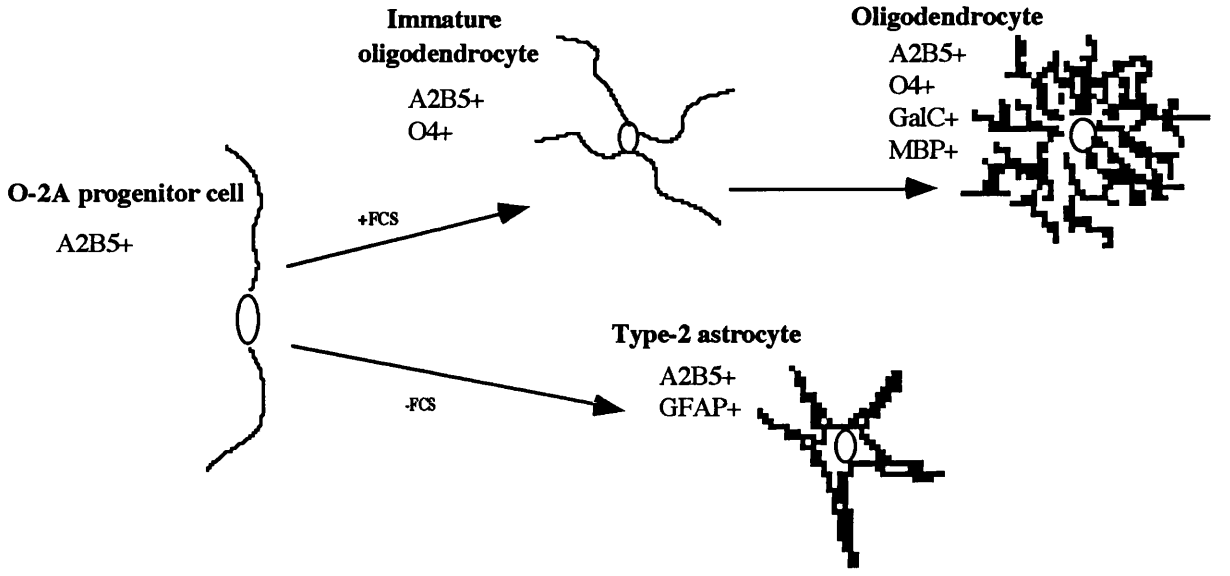
The series of "O" antibodies (Sommer and Schachner, 1981), the A2B5 antibody and antibodies against GalC, GFAP, and MBP have been used to define different stages of the O-2A lineage *in vitro* (Fig. 1.3). This lineage comprises oligodendrocytes and type-2 astrocytes, which *in vitro*, can be derived from a common progenitor cell, called the oligodendrocyte-type 2 astrocyte (O-2A) progenitor (Raff et al., 1983). Initially identified in cultures of perinatal rat optic nerves, the O-2A progenitor has since been isolated from other brain regions and identified *in vivo*. Differentiation of the O-2A progenitor into an oligodendrocyte is the default pathway. In contrast, its differentiation into a type-2 astrocyte has to be induced by appropriate factors *in vitro*. During development, oligodendrocytes are essentially generated during the first postnatal week in rats. In the adult, oligodendrocytes are thought to be replaced from a persistent pool of progenitors, called O-2A^{adult} progenitors, also derived from the O-2A progenitors (ffrench-Constant and Raff, 1986a; Wolswijk and Noble, 1989; Wren et al., 1992). These progenitors have a lesser ability to migrate than their perinatal counterparts. The identification of type-2 astrocytes has proved difficult under "normal" conditions *in vivo*, mainly due to the lack of appropriate specific markers for these cells. Recent studies suggest that O-2A progenitors are normally not exposed to factors enabling their differentiation into type-2 astrocytes during development but can differentiate along this pathway under certain pathological conditions (Barnett et al., 1993b; reviewed by Franklin and Blakemore, 1995).

1.3.4 Function of glial cells in the healthy nervous system

In the adult nervous system, the main function of Schwann cells and oligodendrocytes (which enwrap PNS and CNS axon, respectively), is to provide electrical insulation in the form of a myelin sheath (Peters and Vaughn, 1970; ffrench-Constant and Raff, 1986b). The astrocytes are the largest, the most plentiful, and diverse of the glial cells. Their high content of filaments suggests that they play a major role in the structural support of the CNS. They have also been assigned the

Figure 1.3

Simplified scheme of the oligodendrocyte-type-2 astrocyte (O-2A) cell lineage



Summary of the lineage relationships, morphologies and antigenic expression of O-2A lineage cells. The various perinatal optic-nerve-derived glial cells can be identified unambiguously by their morphology and antigenic phenotype (see text for specific details).

A2B5 (set of ganglioside) characterises the entire O-2A lineage

O4 (set of lipids, including sulphatide) characterises pro-oligodendrocytes and oligodendrocytes

GalC (galactocerebroside; lipid component of myelin) and MBP (myelin basic protein) characterise oligodendrocytes

GFAP (glial fibrillary acidic protein; glial specific intermediate filament) characterises astrocytes

role of controlling the chemical and ionic environment of the neurons, taking up and degrading neurotransmitters that overflow from the synapse, and providing glutamatergic and GABAergique neurons with glutamine. Some astrocytes extend processes that are expanded into "end-feet", which, linked by junctional complexes, form a sealed barrier called glia limitans, at the external surface of the brain and spinal cord. Some astrocytes also form end-feet on blood vessels, where they interact with endothelial cells for the formation of the blood brain barrier (Janzer and Raff, 1987). The counterparts of astrocytes in early development, the radial glial cells, play an important role in the morphogenesis of the nervous system, by providing an attractive substratum for migratory neurons and growing axons (Silver et al., 1982).

1.3.5 The response of glial cells following damage to the nervous system

The concept that the ability of axons to regenerate following injury might be conditioned by environmental factors arose from transplantation experiments which showed that a segment of peripheral nerve can serve as a conduct for the regeneration of both, CNS and PNS axons, whereas a graft of CNS tissue blocks the regeneration of both (Tello, 1907; Ramon y Cajal, 1928; Richardson et al., 1980; David and Aguayo, 1981). The dichotomy between the ability of CNS and PNS axons to regenerate following injury can be attributed in part to their different glial cell environment (reviewed by Bähr and Bonhoeffer, 1994). There is an extensive list of molecules expressed by glial cells, that have been implicated in the control of neuronal survival and axonal growth (for reviews see Fawcett, 1991; Martin, 1992; Kapfhammer and Schwab, 1992; Johnson, 1993; Schachner, 1994; Aubert et al., 1995). It should be noted, that neurons from different regions of the nervous system can differ in their responsiveness to these factors. These molecules fall into three main types: cell surface molecules, extracellular matrix (ECM) molecules and secreted molecules that diffuse into the environment. Many of these molecules are large glycoproteins with multiple functional domains, can interact to modulate each others function, and are found on both glial cells and neurons. Thus, axonal growth

in vivo is likely to depend on the integration of a large variety of signals. Except for relatively few *in vivo* studies, most of the functional data are based on *in vitro* observations.

Following injury of a peripheral nerve, Schwann cells proliferate at the lesion site, and re-express cell surface adhesion molecules (e.g., NCAM and L1) and extracellular matrix components (e.g., laminin) shown to promote neurite outgrowth *in vitro* (reviewed by Martini, 1994). In marked contrast, oligodendrocytes in the CNS are believed to inhibit axonal regeneration. Two CNS myelin-specific proteins (NI-35/250) have been detected that arrest axonal growth both *in vitro* and *in vivo* (Caroni and Schwab, 1988; Schnell and Schwab, 1990). In addition, recent *in vitro* studies suggest a role for MAG as an inhibitor of axonal regeneration in the CNS (Mukhopadhyay et al., 1994). The response of astrocytes to CNS damage is a phenomenon called reactive gliosis: Reactive astrocytes proliferate and invade the site of CNS injury where they form a tightly packed glial scar (reviewed by Reier et al., 1983). The effect of reactive gliosis after injury is controversial and probably mixed (reviewed by Reier et al., 1989). It has been suggested that this glial scar represents a physical obstruction to regenerating axons. Another possibility is that it protects the injured area by preventing its invasion by neurotoxic stimuli. It has also been suggested that reactive astrocytes mediate contact inhibition of axonal growth by expressing inhibitory molecules, including sulphated proteoglycans (Snow et al., 1990; McKeon et al., 1991). However, the molecular profile of reactive astrocytes indicates that they also express potentially beneficial molecules for neuronal survival and axonal regeneration (reviewed by Eddleston and Mucke, 1993).

1.4 The primary olfactory pathway

1.4.1 Anatomy

The primary olfactory pathway can be divided into three compartments, based on the clear anatomical segregation of i) the olfactory epithelium, ii) the lamina propria, and iii) the olfactory bulb (Fig. 1.4; reviewed by Greer, 1991).

The olfactory epithelium

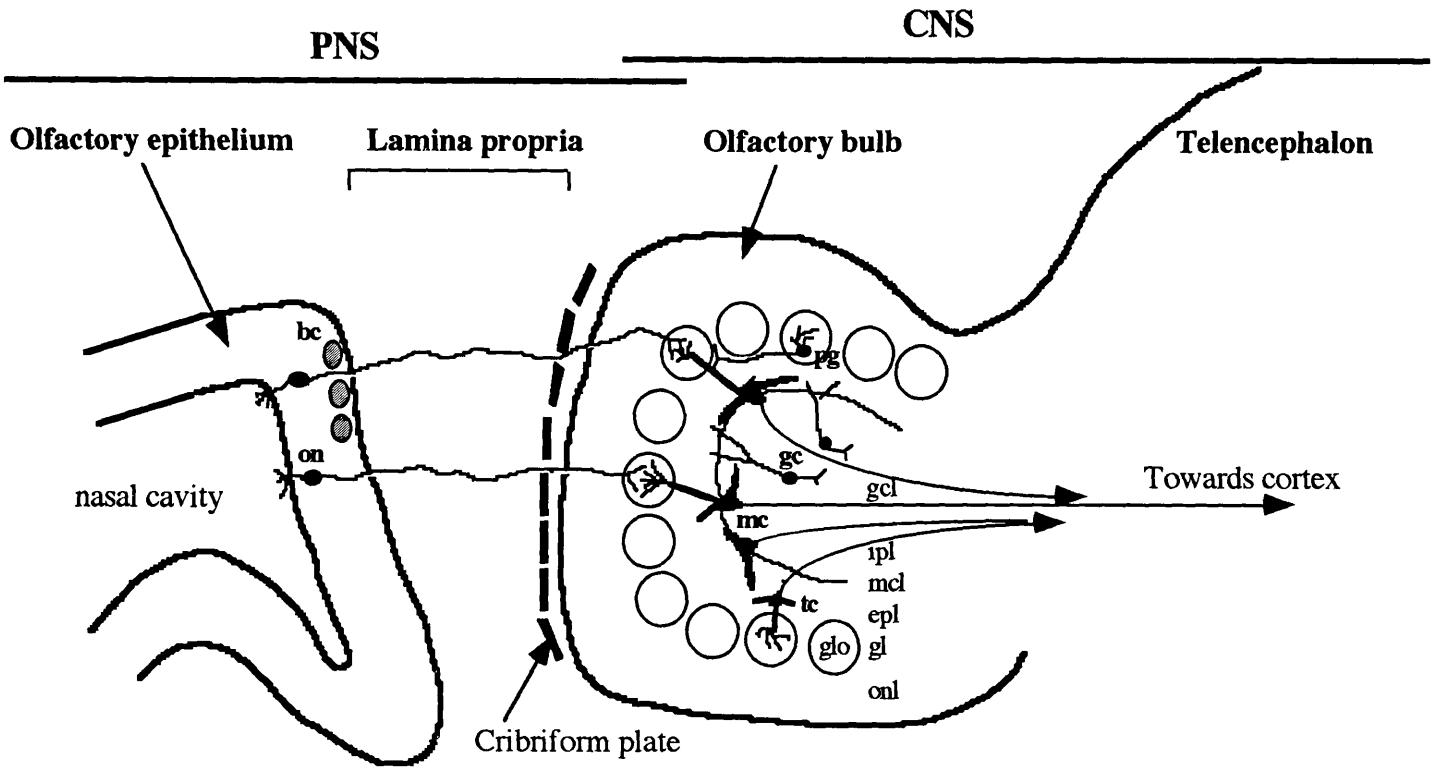
In rodents, the olfactory epithelium extends throughout the rostral-caudal extension of the nasal cavity, occupying the superior and lateral portions of all nasal turbinates. The olfactory epithelium contains olfactory neurons, supporting cells, and basal cells, organised in a pseudo-stratified manner. The supporting cells form a single layer close to the luminal surface of the epithelium. The olfactory neurons usually make up 6 to 8 or more layers in the lower two thirds of the epithelium. The basal cells are apposed to the internal surface of the epithelium, which is underlined by a basal lamina. Olfactory neurons are bipolar, with one short cytoplasmic process (dendrite) extending towards the luminal surface of the epithelium and one unmyelinated axon extending towards the basal membrane of the epithelium. Olfactory axons exit the olfactory epithelium in small bundles, through perforations of the epithelial basal membrane.

The lamina propria

The lamina propria is a connective tissue lying in between the olfactory epithelium and the olfactory bulb. It contains most of the cellular components of connective tissue, including fibroblasts, macrophages, mast cells and leukocytes. It is well irrigated by blood vessels of relatively large calibre and contains exocrine glands (Bowman glands). It is through the lamina propria that olfactory neurons (their cell bodies remaining in the epithelium) extend their axonal processes towards the main olfactory bulbs. This is accompanied by a progressive coalescence of small bundles

Figure 1.4

Schematic view of the primary olfactory pathway



An overview of the primary olfactory pathway. The olfactory bulb receives imput from the receptor neurons in the olfactory epithelium and projects to the olfactory cortex. The diagram indicates some essential aspects of the projection patterns between the regions as well as the main neural elements within the olfactory bulb.
bc, basal cell; epl, external plexiform layer; ipl, internal plexiform layer; gc, granule cell; gcl, granule cell layer; gl, glomerular layer; glo, glomerulus; mc, mitral cell; mcl, mitral cell layer; on, olfactory neuron; onl, olfactory nerve layer; pg, periglomerular cell; tc, tufted cell (modified from Greer, 1991).

of axons into larger fascicles, which will be referred to as peripheral olfactory fascicles (POFs).

The olfactory bulb

Shortly after crossing the cribriform plate of the ethmoid bone, olfactory axon fascicles reach the olfactory bulb (OB), an elongated structure lying on the ventral, anterior end of the forebrain. The OB encloses the initial integrative network for the sensory information coming from the olfactory epithelium and sends projections for further information processing to higher olfactory centres in the brain. The OB contains various populations of neurons and glial cells, arranged in a concentric laminar fashion. This laminar pattern comprises the following layers from the pial surface inwards : i) olfactory nerve layer (ONL), ii) glomerular layer (GL), iii) external plexiform layer, iv) mitral cell layer, v) internal plexiform layer, vi) granule cell layer and, vii) subependymal layer. In this thesis, the focus will be on the ONL and GL which are the only layers occupied by olfactory axons. The peripheral olfactory axon fascicles penetrate the OB on its anterior and ventral aspect, through disruptions of its meningeal covering, and course around its surface, in the olfactory nerve layer, before entering the glomerular layer where they establish synaptic connections with the secondary projection neurons, mitral and tufted cells. These synaptic connections take place in specialised structures called glomeruli (Shepard, 1972). The ONL can be considered as part of the CNS by the fact that it is enclosed by the same meningeal coverings that surround the entire brain. For convenience, the olfactory axon fascicles will be referred to as central olfactory fascicles (COFs) within the OB as opposed to the peripheral olfactory fascicles (POFs) defined above. Recent studies have shown that the olfactory nerve layer represents a critical choice point in the trajectory of olfactory axons: within this tissue, olfactory axons sort out and refasciculate into discrete bundles that selectively terminate in specific glomeruli (Key and Akeson, 1993).

1.4.2. Development

The gross anatomical changes occurring during the development of the primary olfactory pathway can be described in 3 phases. The following developmental stages are given in embryonic days (E) for the rat (the animal model used in this thesis). 1) During E11.5-12.5, the olfactory placodes form, as bilateral areas of thick cranial ectoderm, located rostrolateral to each developing telencephalic vesicle. At this stage, the telencephalic vesicle consists entirely of ventricular cells. 2) During E12.5-14, the olfactory placode evaginates to form the olfactory pit followed by the olfactory epithelium. The telencephalic vesicle consists essentially of ventricular cells, there being only a very thin marginal zone. The olfactory bulb primordium has been defined as the transitional zone between a thick-walled ventral part and a thin-walled dorsal part of the telencephalic vesicle (Hinds, 1972). During this period, small bundles of olfactory axons exit the developing olfactory epithelium and progress towards the olfactory bulb primordium, which they first reach at E14. 3) During E14.5-15.5, the olfactory axons form a thin, but distinct, superficial layer on the olfactory bulb primordium. The telencephalic vesicle evaginates in the region of contact with the olfactory axons and develops into the OB.

1.4.3 The glial cells of the primary olfactory pathway: ONECs and OBECs

From the exit of the olfactory epithelium to the glomeruli of the olfactory bulb, olfactory axons are ensheathed by olfactory ensheathing cells. The perikarya of these glial cells are usually found near the surface of each axon fascicle. Their cytoplasmic processes ensheath a variable number of close-packed axons within each fascicle in a similar manner as enteric or embryonic glial cells (Gasser, 1956; De Lorenzo, 1957; Frish, 1967; Kreutzberg and Gross, 1977; Barber and Lindsay, 1982; Doucette, 1990). From the exit of the olfactory epithelium to the surface of the olfactory bulb, i.e. in the peripheral olfactory fascicles (POFs), olfactory ensheathing cells are apposed to a basal lamina on the side of their plasma membrane facing away from the axons they ensheath (De Lorenzo, 1957; Frish, 1967). However

within the olfactory bulb, i.e. in the central olfactory fascicles (COFs), they are not apposed to a basal lamina, unless in areas where they form part of the glia limitans at the surface of the bulb (Berger, 1969; Barber and Lindsay, 1982; Doucette, 1991). The olfactory ensheathing cells in the COFs also differ from those in the POFs in terms of antigenic profile (see Section 1.5.2.2). For convenience, the olfactory ensheathing cells in the POFs will be referred to as olfactory nerve ensheathing cells (ONECs) and those in the COFs as olfactory bulb ensheathing cells (OBECs). The exact relationship between ONECs and OBECs is still unclear although developmental studies suggest that they both originate from the olfactory placode (Section 1.5.1.2). The following paragraph will focus on OBECs, which are of particular interest since they reside in the olfactory bulb, a CNS tissue in which axonal growth is possible throughout adult life. The hypothesis has been put forward, that they are responsible, at least partially for the exceptional ability of olfactory axons to penetrate, grow and synapse within the olfactory bulb throughout life (Barber and Lindsay, 1982; Doucette, 1984; Raisman, 1985; Doucette, 1990).

1.5 Studies of olfactory bulb ensheathing cells *in vivo*

1.5.1 Morphological and ultrastructural characterisation of OBECs *in vivo*

1.5.1.1 OBECs in the adult olfactory bulb

The presence of an unusual glial cell type along the pathway of olfactory axons was first reported in the cat olfactory bulb (Golgi, 1875; Blanes, 1898). These authors identified cells of fusiform morphology, exclusively present in the ONL. Blanes described them as having a superficially located pyramidal cell body which generated two types of processes - basal processes giving rise to the ensheathment of the olfactory nerve fibres, and a single, stout, apical process directed towards the deeper layers of the olfactory bulb. It was referred to as "Blanes glia", was assumed to be a morphological variant of astrocytes, and received very little attention for almost a century. With the development of electron microscopy the cellular composition of the olfactory bulb has since been investigated at the ultrastructural

level in several mammals including the rabbit (e.g., Berger, 1969, 1971), cat (e.g., Willey, 1973), rat (e.g., Andres, 1965; Pinching and Powell, 1971a, b; Barber and Lindsay, 1982; Burd et al., 1993), mouse (e.g. Doucette, 1984, 1991; Raisman, 1985), and hedgehog (e.g. Valverde and Lopez-Mascaraque, 1991).

On the basis of morphological and ultrastructural criteria, OBECs are clearly distinct from oligodendrocytes, but share a number of features with Schwann cells and especially with astrocytes (Table 1.2; reviewed by Doucette, 1993a). Like typical astrocytes, OBECs possess numerous cytoplasmic processes which occasionally terminate in end-feet at contact with blood vessels or leptomeningeal cells. They are not apposed to basal lamina, unless in areas where they contribute to the formation of the glia limitans at the surface of the olfactory bulb. They possess an ovoid or lobulated nucleus which shows a homogeneous pale granular staining without prominent condensation of heterochromatin. Their cytoplasmic matrix is of variable electron density and contains numerous organelles and glycogen particles. The additional presence of bundles of filaments remains a matter of debate. While some authors described this cell as containing bundles of filaments (Barber and Lindsay, 1982; Raisman, 1985; Valverde and Lopez-Masquaraque, 1991), others observed only a few filaments, scattered throughout the cytoplasm (Berger, 1969; Doucette, 1984, 1991).

The characteristics mentioned above makes it very difficult to distinguish OBECs from typical astrocytes on the basis of morphological and ultrastructural criteria. This may explain the unclarity of the literature as to the exact cellular composition of the ONL. For instance, the ultrastructural studies of Doucette (1984, 1989, 1991) have been interpreted in support of the view that the ONL contains an homogeneous population of OBECs intermingled with a distinct population of typical type-1 astrocytes. In contrast, other ultrastructural studies have been interpreted in support of the view that OBECs are the only cellular components of

Table 1.2
Ultrastructural characteristics of OBECs *in vivo*. Comparison with astrocytes and Schwann cells

	OBECs	Schwann cells	Astrocytes
Morphology	Multipolar or bipolar	Bipolar	Multipolar
Processes	Multiple thin, long + 1 deep apical process[1]	Two thin, long	Multiple, thick, short
Form of association with axons (A.)	Ensheath bundles of tightly packed A.	Ensheath one or a few A.	No ensheathment of A.
Direct contact with astrocytes	Yes[2]	No	Yes
Laminin enclosure	No	Yes	No
Form glia limitans	Yes[3]	No	Yes
End-feet at vessels	Yes	No	Yes
Cytoplasmic matrix	Variable[4]	Electron-dense	Electon-lucent
Cytoplasmic filaments	Variable[5]	Few, scattered	Numerous bundles
Nucleus shape	Ovoid or lobulated	Elongated	Ovoide
Nucleus matrix	Electron lucent	Electron dense	Electron lucent
Chromatin distribution	Homogeneous with slight peripheral clumping	Heterogeneous with strong peripheral clumping	Homogeneous with slight peripheral clumping

1. The deep apical process has only be described in the cat main OB (Blanes, 1898) and rat accessory OB (Raisman, 1985).
2. Only Doucette(1984) and Raisman (1985) reported the contact of OBECs with astrocytes throughout the ONL, in the mouse main OB and rat accessory OB, respectively. Valverde and Lopez Mascaraque (1991) reported that this contact occurs exclusively in the GL of the hedgehock OB.
3. The formation of a glia limitans by OBECs has only been shown by Doucette (1991) in the mouse main OB. In the cat accessory OB, Raisman considered that the glia limitans was exclusively formed by astrocytes.
4. In the cat accessory OB, the cytoplasmic matrix of OBECs was described as very electron dense (Raisman, 1985). In the mouse main OB, it was initially described as electron lucent (Doucette, 1984) and later as variable (Doucette, 1990, 1993).
5. Some authors have reported abundant cytoplasmic filaments (Barber and Lindsay, 1982; Valverde and Lopez Mascaraque, 1991) while others showed only occasional bundle scattered throughout the cytoplasm (Doucette, 1984).

this layer (Raisman, 1985; Valverde and Lopez-Mascaraque, 1991). Although some processes of astrocytes were identified within the ONL, their pericarya were exclusively located in the glomerular layer. A number of ultrastructural studies have even classified the cells of the ONL into OBECs and microglia (Berger, 1971; Barber and Lindsay, 1982; Burd, 1993).

1.5.1.2 Developmental origin of OBECs

It has been known for many years that migratory cells, derived from the olfactory placode, accompany olfactory axons in their course towards the telencephalic vesicle (Bedford, 1904; Van Campenhout, 1956; Gasser, 1956; De Lorenzo, 1957; Frish, 1967; Cuschieri and Bannister, 1975; Farbman and Squinto, 1985). It was suggested that some of these migratory cells represent the precursors of ONECs and until recently, their migration was never studied beyond the cribriform plate. It was assumed that OBECs, by virtue of the fact that they were located inside the brain and resembled astrocytes, were derived from the neural tube (Golgi, 1875; Blanes, 1898; Berger, 1969, 1971; Doucette, 1984).

In recent years, new evidence has emerged from detailed ultrastructural analysis of the developing olfactory system, that OBECs, like ONECs, are derived from the olfactory placodes (Doucette, 1989; Marin-Padilla and Amieva, 1989; Valverde et al., 1992). According to these studies, a presumptive ONL forms, superficial to the marginal zone of the telencephalic vesicle, by a local accumulation of olfactory axons and cells derived from the olfactory placode. These cells have been classified into two main types on the basis of ultrastructural criteria and into 4 cell types on the basis of morphological criteria (Farbman and Squinto, 1985; Valverde et al., 1992). Some of these cells have been identified as the precursors of OBECs, based on the observation that they ensheathed bundles of olfactory axons in a manner similar to that observed in adult OBECs. In addition, it has been suggested that some of these migrating cells are neurons, based on the basis of their

immunoreactivity with antibodies against the neuron-specific molecules LH-RH, OMP, and b tubulin (Wray et al., 1989; Schwanzel-Fukuda and Pfaff, 1989; Valverde et al., 1993; De Carlos et al., 1995). The presumptive ONL, also referred to as migratory mass, becomes intercalated between the glia limitans of the telencephalon and the meninges. The glia limitans of the telencephalon is then progressively disrupted by an invasion of olfactory axons inside the telencephalon (Doucette, 1989; Marin-Padilla and Amieva, 1989; Valverde et al., 1992). This is accompanied by the formation of a new glia limitans by OBEC precursors (Doucette, 1989).

From developmental studies, the question remains open whether OBECs are present in the GL of adult rats and whether astrocytes are present in the ONL. First, it is not clear whether the cells of the migratory mass follow olfactory axons up to their synaptic terminals. Doucette (1984) reported that these cells always stayed superficial to the marginal zone of the telencephalon. Valverde et al. (1992) reported that a small proportion of these cells followed the olfactory axons up to their synaptic terminals. Marin-Padilla and Amieva (1989) reported that all of these cells followed the olfactory axons beyond the marginal zone of the telencephalon, resulting in a total integration of the migratory mass into the developing telencephalon. Second, migration of cells into the ONL from deeper layers of the olfactory bulb has not yet been successfully demonstrated. Some authors have suggested the migration of astrocytes on the basis of an increase of GFAP-immunoreactivity in the ONL during postnatal development. However, the use of anti-GFAP as an astrocyte-specific marker is controversial (see Section 1.5.2).

1.5.2 Immunocytochemical studies *in vivo*

A number of studies have attempted to localise specific antigenic determinants on the glial cells of the primary olfactory pathway, using immuno-electron microscopy or immunocytochemistry at the light microscopic level. In the latter case, glial cells have been distinguished from olfactory axons by the pattern of immunoreactivity, by the use of a glia-specific marker, or by double immunocytochemistry including a glia- or neuron-specific marker. The antigens mentioned below are described in Table 1.3. Most of them have also been shown on OBECs *in vitro* (see Section 1.6.1).

Table 1.3 List of antigens whose expression has been investigated in OBECs *in vivo* and/or *in vitro*.

In vitro studies are indicated with an asterix.
CAM: cell adhesion molecule
ECM: extracellular matrix
Ig: immunoglobulin

Antigens	Description	References
Cell adhesion molecules		
NCAM (Neural cell adhesion molecule)	Member of the Ig supergene family of Ca^{2+} independant CAMs	Miragall et al., 1988, 1989 Chung et al., 1991; Miragall and Dermiedzel, 1992; Bonfanti et al., 1992 Chuah and Au, 1993*
L1	Same as above	Same as above Ramon-Cueto and Nieto-Sampedro, 1992*; Barnett et al., 1993a*
Po (1E8 antibody)	Same as above	Norgren et al., 1992
AMOG (Adhesion molecule on glia)	Ca^{2+} independent CAM, homologue of the β1 subunit of ATPase	Miragall and Dermiedzel, 1992
N-Cadherin	Member of the cadherin family of Ca^{2+} dependant CAMs	Chuah and Au, 1994*

Table 1.3 (continued)

Antigens	Description	References
Carbohydrate moities		
HNK-1 (Human natural killer protein 1)	Sulphated glucuronic acid, attached to a variety of CAMs	Miragall and Dermiedzel, 1992 Ramon-Cueto and Nieto-Sampedro, 1992*; Barnett et al., 1993a*
PSA (Polysialic acid)	Large negativelly charged carbohydrate attached to NCAM (PSA-NCAM)	Miragall et al., 1988, 1989 Bonfanti et al., 1992
Growth factor receptors		
L-NGFr (Low affinity nerve growth factor receptor)	Also acts as a low affinity receptor for the neurotrophins NT3, BDNF	Yan and Johnson, 1988, 1989 Vickland et al., 1991; Gong et al., 1994; Turner and Perez-Polo, 1994; Ramon-Cueto and Nieto-Sampedro, 1992*; Barnett et al., 1993a*; Doucette, 1993b*
GAP/tight junctions		
Connexin 43	GAP junction protein	Miragall et al., 1992
ZO-1	Phosphoprotein of unknown function associated with tight junctions and possibly cadherins	Miragall et al., 1994
Other membrane proteins		
MBP (myelin basic protein)	Myelin protein	Ramon-Cueto and Nieto-Sampedro, 1992*; Devon and Doucette, 1992*; Doucette and Devon, 1994*, 1995*; Devon and Doucette, 1995
A5E3	Protein of unknown function	Barnett et al., 1993a*
Ran-2	Protein of unknown function	Barnett et al., 1993a*
A4 antigen	Protein of unknown function	Barnett et al., 1993a*
Surface lipids		
A2B5	Group of gangliosides of unknown function	Ramon-Cueto and Nieto-Sampedro, 1992*; Barnett et al., 1993a*
GD3	Ganglioside of unknown function	Barnett et al., 1993a*

Table 1.3 (continued)

Antigens	Description	References
GalC (Galactocerebroside)	Cerebroside of unknown function	Devon and Doucette, 1992* Barnett et al., 1993a*; Doucette and Devon, 1994*, 1995*; Devon and Doucette, 1995*
O4	Group of lipids of unknown function, including sulphatide and seminolipid	Barnett et al., 1993a*
Cytoskeletal proteins		
GFAP (Glial fibrillary acidic protein)	Intermediate filament subunit	Barber and Lindsay, 1982 Anders and Johnson, 1990 Bailey and Shipley, 1993 Ramon-Cueto and Nieto-Sampedro, 1992*; Barnett et al., 1993a*; Chuah and Au, 1993*; Doucette,1993*; Doucette and Devon, 1994*
Vimentin	Intermediate filament subunit	Ramon-Cueto and Nieto-Sampedro, 1992*; Doucette, 1993*
Nestin	Intermediate filament subunit	Doucette, 1993*
Diffusible molecules		
PDGF (Platelet derivedgrowth fator)	Polypeptide growth factor	Kott et al., 1994
FGF (Fibroblast growth factor)	Polypeptide growth factor	Lee et al., 1995*
CNTF (Cilliary neurotrophic factor)	Polypeptide growth factor	
GDN (Glia derived nexin)	Serine protease inhibitor of the serpin superfamilly	Reinhart et al., 1988 Scotti et al., 1994
Neuropeptide Y	Neuropeptide	Ubink et al., 1994
S100	Small acidic Ca^{2+}-binding protein	Chuah, 1993*; Doucette, 1993*; Doucette and Devon, 1994*, 1995*
ECM molecules		
Laminin		Denis-Donini, 1988* Ramon-Cueto and Nieto-Sampedro, 1992*
Fibronectin		Ramon Cueto et al., 1992*
J1		Miragall and Dermiedzel, 1992

1.5.2.1 Comparison of OBECs with astrocytes and Schwann cells *in vivo*

Table 1.4 compares the *in vivo* antigenic profile of OBECs with that of Schwann cells and astrocytes.

Table 1.4 **Antigenic profile of OBECs *in vivo*. Comparison with astrocytes and Schwann cells**

Antigen	OBECs	Non-myelinating Schwann cells	Astrocytes
A.			
NCAM	+	+	+
GFAP	+	+	+
Vimentin	+	+	+
S100	+	+	+
B.			
O4	+	+	-
Po (1E8[1])	+*	+	-
L-NGFr	+*	+	-
C.			
AMOG	+	-	+
Connexin-43	+	-	+
"central type" GFAP	+?	-	+
L1	-?	+	-

A: expressed by both astrocytes and Schwann cells
B: expressed by Schwann cells but mot astrocytes
C: expressed by astrocytes but not Schwann cells

(1): The 1E8 antibody is believed to recognise an antigenic determinant on Po, early in the chick Schwann cell lineage (Bhattacharyya et al., 1991)
?: suggested but not yet fully demonstrated
*: only expressed by a subpopulation of OBECs

From this table, it can be seen that OBECs share a number of antigenic characteristics common to both, type-1 astrocytes and non-myelinating Schwann cells, including NCAM (Miragall et al., 1988, 1989), S100 (Ubink et al., 1994), vimentin (Schwob et al., 1986) and GFAP (Barber and Lindsay, 1982). However, OBECs have a unique antigenic profile in being labelled by both Schwann cell-specific and astrocyte-specific markers. For instance, OBECs have been labelled with the O4 antibody (Barnett et al., 1993a), the antibody 1E8 against chick Po (Norgren et al., 1992), and with antibodies against L-NGFr (Vickland et al., 1991), which label Schwann cells but not typical type-1 astrocytes. On the other hand, they

have been labelled with antibodies against AMOG (Miragall and Dermietzel, 1992), and connexin-43 (Miragall et al., 1992), which label astrocytes but not typical Schwann cells. OBECs also differ from typical Schwann cells by not expressing the HNK-1 antigen at any developmental stage (Miragall and Dermietzel, 1992). In addition, it is very likely that OBECs express the "central type" GFAP as has been shown for ONECs *in vivo* (Barber and Dahl, 1987). The monoclonal antibody used by these authors is thought to recognise an antigenic determinant present on astrocytic GFAP (central-type) but not on Schwann cell GFAP (peripheral-type) as it binds a protein co-migrating with GFAP on SDS-PAGE and labels astrocytes but not Schwann cells (Jessen et al., 1984). The co-expression of astrocytic and Schwann cell markers makes OBECs similar to enteric glia (Jessen and Mirsky, 1983). However enteric glia, unlike OBECs, are not labelled with the 1E8 antibody (Bhattacharyya et al., 1991).

1.5.2.2 Comparison of OBECs with ONECs

Most of the antigens detected on OBECs in the ONL have also been detected on ONECs in the peripheral olfactory fascicles (POFs), including N-CAM (Miragall et al, 1988, 1989), L-NGFr (Yan and Johnson, 1988; Gong at al., 1994; Turner and Perez-Polo, 1994), Po (Norgren et al, 1992), AMOG (Miragall and Dermietzel, 1992), Connexin-43 (Miragall et al, 1992), GFAP (Barber and Lindsay, 1982), S100, PDGF (Kott et al., 1994), and Neuropeptide Y (Ubink et al., 1994). However, both cell types appear to differ *in vivo* in their immunoreactivity to antibodies against the cell adhesion molecule L1 and the tight junction-associated protein ZO-1. L1 has been detected on ONEC homeotypic contacts but not on OBEC homeotypic contacts (Miragall et al., 1988, 1989). ZO-1 has been shown to co-localise with GFAP in the POFs but was completely absent in the ONL (Miragall et al, 1994). In addition, the extracellular matrix glycoproteins laminin, fibronectin and J1 have been found in high amounts along the POFs but not in the ONL of developing and adult rats (Reinhard et al., 1988; Miragall and Dermietzel, 1992).

1.5.2.3 Antigenic heterogeneity of the olfactory nerve layer

A number of glial markers have been shown to label restricted areas of the olfactory layer (ONL), suggesting a heterogeneity among the OBEC and/or astrocyte population of this tissue. These include antibodies against Po, L-NGFr, PSA-NCAM, neuropeptide Y and GFAP.

Po

Antibody 1E8 was found to label only the periphery of the ONL in developing chicks (Norgren et al, 1992). This antibody is believed to recognise an antigenic determinant on Po, that appears early in the chick Schwann cell lineage (Bhattacharyya et al., 1991).

L-NGFr

In developing rats, L-NGFr-IR was exclusively detected in the outer 70-80% of the ONL (Vickland et al, 1991; Gong et al, 1994). In adult rats it was exclusively detected in the glomerular layer (Gomez-Pinilla et al., 1989; Yan and Johnson, 1989; Vickland et al., 1991; Turner and Perez-Polo, 1994; Gong et al., 1994). The precise cellular origin of the L-NGFr-IR in the glomerular layer remains a matter of debate. Vickland et al. (1991) assigned it to OBECs, but others believe it may be derived from cholinergic input neurons of the diagonal band (Gomez-Pinilla et al., 1989; Turner and Perez-Polo, 1994), or from juxtaglomerular astrocytes (Gong et al., 1994).

PSA-NCAM

A small number of PSA-NCAM+ cells have been detected at the glia limitans of the olfactory bulb of adult mice by immunoelectron microscopy but the question remains open whether these represent a subpopulation of OBECs or of astrocytes (Miragall et al., 1988; Bonfanti et al., 1992).

Neuropeptide Y

Antibodies against neuropeptide Y predominately labelled the deep zone of the ONL close to the GL (Ubink et al., 1994). Neuropeptide Y transcripts were detected in OBECs by *in situ* hybridisation.

GFAP

A recent study of the adult rat olfactory bulb using GFAP immunocytochemistry and the gold sublimate method, showed a heterogeneity among the cells expressing GFAP in the ONL and GL (Bailey and Shipley, 1993). These authors attributed low levels of diffuse GFAP-IR to OBECs and high levels of filamentous-like GFAP-IR to astrocytes. Olfactory bulb astrocytes where further classified into 6 morphological subtypes, two of which were exclusively detected in the deepest one third of the ONL and one of which was exclusively detected in the GL. However, the pattern of GFAP-IR may not be a good criteria for distinguishing OBECs from astrocytes (OBECs express high levels of filamentous GFAP *in vitro*, Barnett at al, 1993a) and these results may therefore reflect an heterogeneity among OBECs *in vivo*. Noteworthy, ONECs (in the peripheral olfactory fascicles) have been shown to express variable levels of GFAP (Pixley, 1992).

1.5.2.4 Changes of OBEC antigenic expression during development and after experimental manipulation

Immunocytochemical analysis of the olfactory system during development or after experimental manipulation such as chemical lesion, axotomy, odour deprivation and OB transplantation, have provided some insight into the regulation of antigenic expression in OBECs. The next paragraphs describe changes in expression of some antigens that appear prominent in the olfactory bulb during development or following experimental manipulation. Antibodies against these antigens will be used for detailed studies on OBECs in this thesis.

L-NGFr

L-NGFr expression in the ONL is strong during embryonic development and decreases postnatally to reach very low levels in the adult (Yan and Johnson, 1988; Vickland et al., 1991; Gong et al., 1994). Chemical lesion of the adult olfactory epithelium with triton-X or zinc sulphates, results in a robust re-expression of L-NGFr in the ONL, followed by a return to pre-lesioned levels (Turner and Perez-Polo, 1994; Gong et al., 1994). The time course and transience of increased L-NGFr-immunoreactivity was shown to closely correlate with the time course of olfactory neuronal degeneration and regeneration (Turner and Perez-Polo, 1994). Similarly, L-NGFr expression is down-regulated with maturation in myelin-forming Schwann cells, and is transiently re-induced in adults, following sciatic nerve axotomy (Taniuchi et al., 1986; Jessen et al., 1990). These observations suggest that L-NGFr expression by OBECs, like that of sciatic nerve Schwann cells, may be reversibly down-regulated by axonal contact. In the glomerular layer, L-NGFr expression is differently regulated. It is absent during embryonic development and increases postnatally to reach moderate levels in adults. Lesioning of the adult olfactory epithelium or olfactory nerves as they cross the cribriform plate, results in a transient decrease of glomerular L-NGFr-immunoreactivity (Turner and Perez-Polo 1994; Gong et al., 1994). Neonatal naris closure (which increases the life span of olfactory neurons) results in an increase of glomerular L-NGFr-immunoreactivity (Gomez-Pinilla et al., 1989).

GFAP

During postnatal stages, GFAP-IR increases in the ONL to reach moderate levels in the adult (Barber and Lindsay, 1982; Valverde et al., 1992; Gonzalez et al., 1993). It is still unclear whether this phenomenon is due to a migration of astrocytes from deeper regions of the olfactory bulb, to an up-regulation of GFAP expression by OBECs, or both. The intensity of GFAP-IR in the adult ONL has been shown to increase following transection of olfactory axons at the cribriform plate (Barber and

Lindsay, 1982; Anders and Johnson, 1990). Anders and Johnson (1990) suggested that this reflected an increased synthesis of GFAP by astrocytes. However, the *in vitro* study of Barber and Lindsay (1982) demonstrated that the processes of ONECs derived from lesioned olfactory mucosa were substantially thicker than those of the normal adult, resulting in an increased GFAP staining intensity. The same might apply for OBECs.

1.5.3 Studies of cell proliferation and migration

Studies on the dynamic aspect of the primary olfactory pathway have so far mainly focused on neurogenesis in the olfactory epithelium and they are very few reports in the literature about the proliferation of olfactory ensheathing cells. Morphological studies of the developing olfactory system have shown the presence of mitotic cells from the point of exit of the olfactory epithelium to the olfactory nerve layer (Marin-Padilla and Amieva, 1989; Valverde et al., 1992). This has been interpreted in support of the view that olfactory epithelium-derived OBEC precursor cells actively proliferate during their migratory route from the olfactory epithelium to the olfactory bulb. To that respect, the proliferative behaviour of olfactory ensheathing cell precursors is similar to that of Schwann cell precursors and radial glial cells, both of which actively proliferate while migrating along developing axons. In addition, active cell proliferation was shown in the intracranial portion of the olfactory nerves, at the base of the olfactory bulb primordium. Valverde et al. (1992) reported that the number of cells (presumably representing OBEC precursors) in the prospective olfactory nerve layer/migratory mass increased considerably around E18 (in rats) by addition of indigenous cells resulting from local mitotic divisions. An autoradiographic study showed that these cells reach their final cell division chiefly between E17 and P10 in mice (Hinds, 1968).

The response of OBECs to olfactory nerve injury is still ill-defined. There is as yet no evidence for their re-entrance into the cell cycle as usually occurs for Schwann cells after peripheral nerve damage. Berger (1971) detected very rare mitotic figures in the ONL following lesion of peripheral olfactory nerves. The main glial response to olfactory nerve injury appears to be a migration towards the olfactory bulb, followed by active phagocytosis of cellular debris within the ONL (Berger, 1971; Burd, 1993; Chuah et al., 1995). Relevant to the intracranial migration of olfactory ensheathing cells are the results obtained from an ultrastructural study of normal and partially bulbectomised adult rats (Monti-Graziadei, 1992). This study showed that cell migration from the olfactory epithelium towards the olfactory nerve layer persists to a small extent throughout adult life and increases following partial bulbectomy. The author presumed that these migrating cells represent neuroblasts but their ultrastructural characteristics are very similar to that of olfactory ensheathing cells, raising the hypothesis that OBECs may be replaced by de-novo gliogenesis in the olfactory epithelium rather than by a re-entrance into the cell cycle.

1.5.4 Functional studies

The ultrastructural study of Doucette (1991) showed that, like astrocytes, OBECs participate in the formation of the glia limitans of the olfactory bulb. Their role in the continuous repair processes taking place in the adult olfactory bulb (see Section 1.2.3.) is still ill defined and largely hypothetical. Ultrastructural analysis of the primary olfactory pathway following experimentally induced olfactory nerve injury suggests that OBECs, like microglial cells, assist in the removal of cell debris by phagocytosis (Berger, 1971; Burd, 1993; Chuah et al., 1995). Of particular interest is the hypothesis that OBECs furthermore promote and guide the outgrowth of olfactory axons within the olfactory bulb. The neurite outgrowth promoting properties of OBECs within the olfactory bulb is indirectly supported by the recent study of Ramon Cueto and Nieto-Sampedro (1994). These authors took the approach

of transplanting highly enriched OBEC cultures in the spinal cord transitional zone, after dorsal root transection. The conclusion of this study was that "OBECs made possible the ingrowth of regenerating dorsal root axons into the spinal cord and promoted elongation within the CNS".

Immunocytochemical analysis of OBECs *in vivo*, have provided some support to this hypothesis. For example, N-CAM, Po and AMOG are cell adhesion molecules that have been directly implicated in developmental and regenerative processes of the nervous system, including cell migration, neurite outgrowth and fasciculation, growth cone guidance and motility, and/or synapse formation (Antonicek et al. 1987; Doherty et al., 1990; Schneider-Shaulies et al., 1990; Muller-Hussmann et al., 1993). L-NGFr on OBECs may serve similar functions as those proposed by Taniuchi et al., (1986) for Schwann cells in the sciatic nerve, i.e., binding of neurotrophins and/or guidance for regenerating axons. Ubink et al. (1994) discussed the possibility that OBEC-derived neuropeptide Y acts as a trophic molecule for olfactory neurons. Of particular interest is the possibility that OBECs retain expression of PSA-NCAM (Miragall et al, 1988; Bonfanti et al., 1992), and glia-derived nexin (GDN, Reinhard et al., 1988; Scotti et al., 1994) throughout adult life. These molecules are usually characteristic of dynamic embryonic tissues. The retention of these embryonic features in the adult olfactory system could be related to its regenerative capacities. PSA-NCAM and GDN have both been shown to exert a neurite outgrowth promoting activity *in vitro* (Zurn et al, 1988; Nakayama et al, 1995).

1.6. Studies of olfactory bulb ensheathing cells *in vitro*

OBECs *in vitro* were initially characterised in mixed cultures of neonatal rat olfactory bulbs (Barber and Lindsay, 1982; Denis-Donini and Estenoz, 1988). During the past 5 years various purification procedures have been developed to produce highly enriched populations of OBECs from embryonic (Devon and Doucette, 1992; Doucette, 1993b), neonatal (Chuah and Au, 1993), perinatal (Barnett et al., 1993a) and adult (Ramon-Cueto and Nieto-Sampedro, 1992) rat olfactory bulbs. These have mainly been characterised after prolonged culture time in serum containing media (except for the study of Barnett et al., 1993a). Table 1.5 summarises the *in vitro* antigenic profile of OBECs and relates it to that of cultured Schwann cells and astrocytes.

Table 1.5 **Antigens shown on OBECs *in vitro*. Comparison with astrocytes and Schwann cells**

Antigen	OBECs	Schwann cells	Astrocytes
NCAM	+	+	+
GFAP	+	+	+
Vimentin	+	+	+
S100	+	+	+
L1	+	+	+
O4	+	+	-
L-NGFr	+	+	-
A5E3	+	+	-
MBP	+*	+	-
GalC	+*	+	-
Laminin	+	+	-
7B11A6-C7 antigen	+	-	+

* : Induced under specific conditions

1.6.1 Comparison of OBECs *in vivo* and *in vitro*

The expression of S100, NCAM, L-NGFr, O4, GFAP and vimentin by OBECs, has been confirmed *in vitro* (Ramon-Cueto and Nieto-Sampedro, 1992; Chuah and Au, 1993; Barnett et al., 1993a; Goodman et al, 1993). Cultured OBECs

have also been shown to express a number of antigens that do not appear to be expressed by these cells *in vivo*. These include L1 (Barnett et al., 1993a; Chuah and Au, 1994), laminin (Denis-Donini and Estenoz, 1988; Ramon-Cueto and Nieto-Sampedro, 1992), fibronectin (Ramon-Cueto and Nieto-Sampedro, 1992) and possibly ZO-1 (Miragall et al., 1994). In addition, a number of antigens have been localised on OBECs *in vitro* but not yet examined *in vivo*. These include N-cadherin (Chuah and Au, 1994), Ran-2 and A5E3 (Barnett et al, 1993a), nestin (Doucette, 1993b), GalC (Devon and Doucette, 1995; Doucette and Devon, 1994, 1995), MBP (Ramon-Cueto and Nieto-Sampedro, 1992; Devon and Doucette, 1992, 1995) and the antigenic determinant recognised by the monoclonal antibody 7B11A6-C7 (Goodman et al., 1993). This antibody has been shown to label subsets of immature astrocytes and O-2A progenitor cells but does not label Schwann cells from the sciatic nerve (Szigeti and Miller, 1994).

Noteworthy, many OBEC cultures appeared heterogeneous in terms of cell morphology and/or antigenic expression (Ramon-Cueto and Nieto-Sampedro, 1992; Devon and Doucette, 1993; Doucette, 1993b; Doucette and Devon, 1995; Chuah and Au, 1993, 1994). The relative proportions between morphological and/or antigenic subtypes of OBECs however differs between studies.

1.6.2 Modulation of OBEC antigenic expression *in vitro*

Most OBEC cultures have been characterised after prolonged culture time in a serum-containing medium but a few authors have studied the evolution of OBEC phenotype under specific culture conditions, in order to compare their differentiation capacity with that of other known glial cell types (Barnett et al., 1993a; Doucette, 1993b; Doucette and Devon, 1994, 1995; Devon and Doucette, 1995). These studies have largely contributed to the classification of OBECs as a unique glial cell type and deserve particular attention.

Barnett et al., 1993

The OBEC purification procedure developed by these authors was based on the discovery that the ONL was the only region of the perinatal rat olfactory bulb to label with the O4 antibody, a marker for Schwann cells, O-2A progenitors and oligodendrocytes, which does not label typical astrocytes (Sommer and Schachner, 1981, Bansal et al., 1989). Purification of the O4+/GalC- cells from postnatal day 7 rat olfactory bulbs by fluorescence activated cell sorting (FACS), and subsequent detailed immunocytochemical analysis on the first day *in vitro* showed that these cells were distinct from typical Schwann cells, type-1 astrocytes and cells of the O-2A lineage. They did not differentiate along the same pathway as O-2A progenitor cells or immature Schwann cells (other O4+ cells) when grown under similar culture conditions. In particular, they did not differentiate into O4+/GalC+/GFAP- oligodendrocytes when grown in DMEM-BS, a serum-free defined medium (Bottenstein and Sato, 1984) which allows such differentiation in O-2A progenitor cells (Raff et al., 1983). Instead, OBECs lost O4 expression and acquired GFAP expression. In further contrast with O-2A progenitor cells (Raff et al., 1983), OBECs grown in the presence of FCS did not differentiate into A2B5+/GFAP+ type-2 astrocytes, but instead developed into O4-/GFAP-/GC-/A2B5- cells. When grown in astrocyte-conditioned medium, a medium in which O-2A progenitor cells either divide as bipolar cells or differentiate into multipolar oligodendrocytes (Noble and Murray, 1984), OBECs developed into GFAP+ cells of flattened morphology. OBECs differed from perinatal sciatic nerve Schwann cells (Morgan et al., 1991) in that they could not be induced to express GalC or Po when grown at high density in the presence of dbcAMP.

Doucette, 1993; Doucette and Devon, 1994, 1995; Devon and Doucette , 1995;

OBECs derived from embryonic rat ONL could not be induced to express GFAP under *in vitro* conditions which promoted such differentiation in astrocyte progenitors (Doucette, 1993b). Of particular interest was the finding that these cells

acquired expression of GalC and the myelin-specific protein MBP when co-cultured with dorsal root ganglion neurons (Devon and Doucette, 1992). Expression of these antigens did not appear to be controlled by the same regulatory mechanisms as in oligodendrocytes. Oligodendrocytes constitutively express GalC and MBP *in vitro* in the absence of axonal contact (Mirsky et al. 1980). Doucette and Devon (1994) showed that growth media that supported this constitutive expression of GalC and MBP by oligodendrocytes *in vitro*, failed to induce OBECs to express either molecules. In addition, experiments have recently been carried out, in which dbcAMP was added to embryonic OBECs grown in a serum-free defined medium or a serum-containing medium (Doucette and Devon, 1995). This cyclic AMP analogue has been shown to play an important role in the control of Schwann cell growth and differentiation (Morgan et al., 1991; reviewed by Jessen and Mirsky, 1991). In particular, dbcAMP has been shown to reverse the down-regulation of O4, GalC and Po and up-regulation of L-NGFr, NCAM and GFAP expression affecting neonatal myelinating Schwann cells cultured under neuron-free conditions. Embryonic OBECs responded to dbcAMP differently: GFAP expression was induced in most of the cells, GalC expression in a small proportion of the cells, and MBP expression in none of the cells. It has also been reported that OBECs do not require L-ascorbic acid to assemble basal lamina *in vitro* , providing further evidence that OBECs differ fundamentally from Schwann cells (Devon and Doucette; 1995).

1.6.3 Characterisation of OBEC-neuron interactions *in vitro*

A few authors have investigated the ability of OBECs to promote neurite outgrowth *in vitro* (Denis Donini and Estenoz, 1988; Goodman et al., 1993; Chuah and Au, 1994). It has been shown that OBECs are a good substrate for the *in vitro* growth of various neuronal populations. These studies are summarised in Table 1.6. OBECs have also been shown to ensheath olfactory neurites (Ramon-Cueto and Nieto-Sampedro, 1993; Chuah and Au, 1994) and to myelinate dorsal root ganglion neurons *in vitro* (Devon and Doucette, 1992).

Table 1.6
Summary of published data analysing the influence of OBECs on the *in vitro* growth of neurons

Authors	Glial cell monolayers	Neuron source	OBEC identification	Neuron identification	Conclusions
Denis-Donini and Estenoz, 1988	E21 rat - mixed OB glia - mixed Str glia - mixed Mes glia	E14 rat SN (dissociated)	GFAP+ fusiform	TH+ (Tyrosine hydroxylase)	OB glial cells supported higher neurite outgrowth (mean neurite length) than Mes or Str glia
Chuah and Au, 1993	P0 rat - OBECs - Ctx astrocytes	1 month-old rat OE (dissociated)	NCAM+ L1+ N-cadherin+ Ultrastructural criteria	NCAM++ Ultrastructural criteria	OBECs supported slightly higher neurite outgrowth (percentage of neurite-bearing neurons) than Ctx astrocytes Some neurons were ensheathed by OBECs
Goodman et al., 1993	P0 and adult rat lgT-immortalised - OBECs - OB astrocytes - Ctx astrocytes	Emb chick Ret (dissociated)	GFAP+ L-NGFr+ Vim+ fusiform	TuJ1+ (β3 tubulin)	Neonatal and adult OBEC lines, as well as some neonatal OB astrocyte lines, supported higher neurite outgrowth (mean neurite lentgh and percentage of neurite-bearing neurons) than Ctx astrocyte lines.
Ramon-Cueto et al., 1993	Adult rat - mixed OB glia	E17 rat OE (explants)	L-NGFr+ stelate or fusiform	Ultrastructural criteria	Olfactory neurites grew preferentially over OBECs Some neurons were ensheathed by OBECs

Ctx, cortex; OB, olfactory bulb; OE, olfactory epithelium; Mes, mesencephalon, Ret, retina; Str, striatum; SN, substantia nigra
lgT, large T antigen

1.7 Aims of the project

The first aim of this thesis is to resolve the controversy that exists in the literature as to whether OBECs represent a homogeneous population of cells (Barber and Lindsay, 1982; Doucette, 1984; Valverde and Lopez-Mascaraque, 1991; Bailey et al., 1993; Gonzalez et al., 1993), or a heterogeneous population of cells (Vickland et al., 1991; Norgren et al., 1992; Ramon Cueto and Nieto-Sampedro, 1992; Chuah and Au, 1994; Gong et al., 1994; Ubink et al., 1994; Devon and Doucette, 1994, 1995). The approach that will be taken is a detailed comparative immunocytochemical analysis of i) FACS purified OBECs grown under various culture conditions and of ii) tissue sections of the developing olfactory system. If this study shows a heterogeneity among the FACS sorted cell population, clonal OBEC cell lines will be constructed by retroviral infection of FACS purified OBECs with the temperature mutant gene of the large T antigen and characterised using the same panel of neural antibodies as an attempt to verify that the different cell types identified in primary cultures belong to the same lineage.

The second aim is to define mitogenic factors that will enable to expand FACS purified OBECs *in vitro* for further studies. FACS purified OBECs will be grown in the presence of a astrocyte conditioned medium and a variety of known growth factors, and DNA synthesis will be assessed by immunocytochemical detection of BrdU incorporation.

The third aim is to compare the ability of OBECs to support the *in vitro* growth of CNS neurons with that of astrocytes and Schwann cells.

CHAPTER TWO

Materials and Methods

Part A. Materials

The following section lists all routinely used materials. Less frequently used materials are described within the appropriate method's section.

2.1 Chemicals and biochemicals

2.1.1 Chemicals

All chemicals mentioned below were purchased from Sigma and diluted in doubled distilled water (ddH_2O), unless otherwise stated.

2.1.2 Antibodies

Primary antibodies were selected for their specificity for neuronal or glial cells. The source and specificity of the antibodies used are specified in Table 2.1. This table also indicates the dilutions that have been used for immunolabelling of tissues and cells.

2.1.3 Growth factors

Recombinant human (rh) growth factors rhFGF2, rhPDGFBB, and rhTGFβ were purchased from Boehringer Mannheim, rhPDGFAA from British Biotechnology, and rhNT-3, rhBDNF and rhCNTF from Regeneron Pharmaceuticals. Purified NGF7S was purchased from Sigma. Bovine pituitary extract enriched for GGF was a gift from Dr. Stroobant (LICR London).

Table 2.1
List of antibodies.

Antibodies (Ab)	Class &Species of origin	Source	Dilution used for labelling of Tissue sections	Cultured cells	References
O4	Mouse IgM	Gift from Dr Sommer	1:2	1:5	Sommer & Schachner, 1981
anti-PSA-NCAM	Mouse IgM	Cambio Gift from Dr Rougon	1:100 1:250	1:100 1:250	Doherty et al., 1990 Rougon et al., 1986
HNK-1	Mouse IgM	Gift from Dr Beveridge	1:2	1:5	McGarry et al, 1983
anti-L-NGFr	Mouse IgG1	Gift from Dr. Johnson Boehringer Manheim	1:200 1:10	1:200 1:10	Chandler et al, 1984
anti-Vm	Mouse IgG1	Dakopatts	1:50	1:50	
anti-Thy-1.1	Mouse IgG1	Serotec	nd	1:100	
anti-BrdU	Mouse IgG1	Gift from Dr D. Mason	nd	1:10	
anti-GalC	Mouse IgG3	Gift from Dr Noble	1:2	1:5	Ranscht et al, 1982
anti-GFAP	Rabbit Ig	Dakopatts	1:100	1:100	
anti-NCAM	Rabbit Ig	Affinity	1:500	1:500	
L1	Rabbit Ig	Gift from Dr. Schachner	nd	1:50	Rathden & Schachner, 1984
anti-S100	Rabbit Ig	Dakopatts	1:50	1:50	
anti-GAP43	Rabbit Ig	Gift from Dr Curtis	1:50	1:50	

2.2 Tissue culture solutions and media

All aqueous solutions were autoclaved and stored at room temperature.

2.2.1 General solutions

Phosphate Buffered Saline (PBS)

140 mM NaCl

10 mM Na_2HPO4

2 mM KCl

1 mM KH_2PO_4

2.2.2 General media

The media listed below were supplemented with gentamycin (50 mg ml^{-1}, Gibco) to a final concentration of 25 μg ml^{-1}, and stored at 4°C.

L-15

Leibowitz L-15 medium (Flow Laboratories)

HBSS

Hank's balanced salt solution, Ca^{2+} and Mg^{2+} free (Gibco)

DMEM

Dulbecco's modified Eagle's Medium containing 4.5 g l^{-1} glucose (Gibco).

1X and 10X solutions were used. Only the 1X solution was supplemented with gentamycin.

2.2.3 Stock solutions

The stock solutions listed below, if not sterile on purchase, were sterilised by passing through a 0.22 μm filter (Millipore), aliquoted, and stored at - 20°C.

Trypsin stock

HBSS containing 2.5 mg ml^{-1} trypsin (bovine pancreas, Sigma), stored as 1 and 2 ml aliquots.

SBTI-DNAse (SD) stock

L-15 containing:

0.52 mg ml^{-1} soybean trypsin inhibitor (Sigma)

0.04 mg ml^{-1} bovine pancreas DNAse (Sigma)

3.0 mg ml^{-1} bovine serum albumin, BSA fraction v (Sigma)

Stored as 1 and 2 ml aliquots.

Collagenase stock

L-15 containing 13.3 mg ml^{-1} (2000 U ml^{-1}) collagenase (ICN Biochemicals), stored as 0.5 and 1 ml aliquots.

Cytosine arabinoside (AraC) stock

HBSS containing 279.7 μg ml^{-1} (1 mM) Ara C, stored as 1 ml aliquots.

Poly-L-Lysine (PLL) stock

H20 containing 4 mg ml^{-1} PLL (Sigma), stored as 66 μl aliquots.

Insulin stock

10 mM HCL containing 0.5 mg ml^{-1} insulin (bovine pancreas, Sigma), stored as 4 ml aliquots.

Transferrin stock

H_2O containing 10 mg ml^{-1} human transferrin (Sigma), stored as 2 ml aliquots.

Sato mix stock

200 ml PBS containing 5.72 ml BSA path-o-cyte 4 (Miles SCI, PENTEX)

200 ml H_20 containing 322 mg putrescine (Sigma)

20 ml ETOH containing 8.0 mg thyroxine, T4 (Sigma)

20 ml ETOH containing 6.74 mg tri-iodo-thyronine, T3 (sigma)

2 ml ETOH containing 1.246 mg progesterone (Sigma)

2 ml H_2O containing 0.774 mg selenium (Sigma)

Stored as 4.4 ml aliquots

Glutamine stock

200 mM L-glutamine (Gibco), stored as 2 and 5 ml aliquots.

Foetal calf serum (FCS)

Heat inactivated foetal calf serum (GlobePharm), stored as 50 ml aliquots.

2.2.4 Specific media

The media listed below were stored at 4°C for up to 2 weeks.

DMEM-BS

DMEM containing:	<u>for 200 ml:</u>
2 mM glutamine	200 ml DMEM 1X
0.5 mg ml^{-1} bovine pancreas insulin	2 ml glutamine stock
0.1 mg ml^{-1} transferrin	4 ml insulin stock
O.0286 % (v/v) BSA pathocyte	2 ml transferrin stock
0.2 μM progesterone	4.4 ml Sato mix stock
0.10 μM putrescine	
0.45 μM L-thyroxine	
0.224 μM selenium	
0.49 μM 3,3',5-triiodo-L-thyronine	

DMEM-FCS

DMEM containing:	<u>for 500 ml:</u>
10 % FCS	360 ml ddH_20
0.075 % sodium bicarbonate	50 ml DMEM 10X
2 mM L-glutamine	50 ml FCS
1mM sodium pyruvate	25 ml sodium bicarbonate (7.5 %, Gibco)
5 ml sodium pyruvate (100 mM, Gibco)	5 ml glutamine stock
0.25 ml gentamycin (50 mg ml^{-1}, Gibco)	Brought to physiological pH with 6-8 ml NaOH (1M)

2.3 Solutions for immunofluorescent labelling procedures

The solutions listed below were stored at 4°C.

Staining solution

DMEM containing:	<u>For 1 litre:</u>
5 % donor calf serum (DCS, Imperial Laboratories)	830 ml ddH_20
4.76 g l^{-1} Hepes/MW 238.3	100 ml DMEM (10X)
5.2 g l^{-1} Hepes/MW 260.3	50 ml DCS
0.05 % sodium azide	5 ml sodium azide 10 %
	4.76 g Hepes/MW 238.3
	5.2 g Hepes/MW 260.3
	Brought to physiological pH with 15 ml NaOH (1M)

Anti-fade

Glycerol containing 2.5% 1,4-diazabicyclo-2.2.2 octane (DABCO)

2.4 Solutions for Southern blotting

20 x SSC

3 M NaCl

0.3 M Tri-sodium citrate

Brought to pH 7.0 with NaOH

1 x TBE

89 mM Tris borate

89 mM Boric acid

2.5 mM EDTA

1 x TAE

40 mM Tris base

2 mM EDTA

20 mM NaCl

20 mM Na Acetate

Brought to pH 8.15 with glacial acetic acid

DNA lysis buffer

0.3 M sodium acetate, pH 8.0

0.5 % SDS

5 mM EDTA

DNA loading dye

0.25 % bromophenol blue

0.25 % xylene cyanol FF

30 % glycerol

20 x Genescreen

0.5 M Na_2HPO_4

0.5 M NaH_2PO_4

Denaturation solution

2.5 M NaOH

7.5 M NaCl

Neutralisation solution

1 M Tris base (pH 7.4)

1.5 M NaCl

Hybridisation buffer

50 mM PIPES

50 mM $NaH_2PO4.2H_2O$

50 mM Na_2HPO_4

100 mM NaCl

1 mM EDTA

5 % SDS

Wash solution

1x SSC

5 % SDS

Part B. Methods

2.5 Preparation of tissue sections for immunocytochemistry

Sprague-Dawley rats were used for all preparation of tissues and cells. Embryos from embryonic day (E) E14, E16, E17, E18, E20 were obtained from timed pregnant rats killed by carbon dioxide inhalation. Animals under 2 weeks old (birth (P0), postnatal day (P) P1, P2, P4, P7, P14) were killed by decapitation. Older animals (P17, 1 month-old, 3 month-old) were killed by carbon dioxide inhalation and their brain pre-fixed by transcardial perfusion with a freshly prepared solution of 4% paraformaldehyde, pH 7.3. The brains of postnatal animals were dissected out, with great care taken not to damage the olfactory bulbs. The entire embryos and the brains of postnatal rats were fixed by immersion in 4% paraformaldehyde/pH 7.3 for approximately 24 hours at 4°C. Tissues were then cryoprotected by immersion in a 30 % sucrose solution in PBS until they sank. The olfactory bulbs, attached to a third of the cortex for orientation, were finally mounted in O.C.T. compound (BDH Ltd.) and quickly frozen on dry ice. The frozen tissues were stored at -80°C for up to 3 months.

For preparation of gelatine-coated slides, 2 g of gelatine and a spatula tip-full of chrome aluminium were added to 200 ml H_2O. This solution was heated to dissolve both compounds. Glass slides were then rapidly immersed into this warm solution and allowed to dry at room temperature overnight. 5-7 μm-thick corronal, transverse or longitudinal sections were cut on a Bright cryostat between -22°C and -28°C. The sections were immediately placed on gelatine-coated slides, kept in a humidified chamber for at least an hour, and processed for immunolabelling.

2.6 Cell culture

2.6.1 General cell culture methods

Aseptic technique

All cell culture manipulations were carried out in a class II microbiological safety cabinet with vertical air flow. All glassware was dry heat sterilised at 160°C for a minimum of 1 hour. Heat stable solutions were autoclaved before use and heat labile solutions were either purchased sterile or filtered through 0.22 μm Millipore filters.

Incubation

All the cells used grew as monolayers and were maintained either on cover slips in a 24 well-tray or in Nucleon flasks or dishes with the appropriate medium in a humidified incubator under 7 % CO_2/ 93 % air. All primary cultures, fibroblast cell line 3T3 and hybridoma lines O4, GalC, A2B5, HNK1 were incubated at 37°C. tsT-astrocyte cell lines and tsT-OBEC cell lines were maintained either at 33°C or 39°C.

Cell passaging

After removal of all the medium, a sufficient amount of trypsin solution was added to cover the entire cell monolayer. Cells cultured in the presence of serum were prewashed briefly with PBS prior to the addition of trypsin. The cells were then incubated for approximately 5 min at 37°C. The extent of cell detachment from the surface was assessed under a microscope. If necessary, the cells were shaken or further incubated. Once all the cells had detached from the surface, the cell suspension was transferred in a 12 ml falcon tube on top of a 2x amount of SD solution or 5x amount of DMEM-FCS if the cells were to be cultured in the absence or presence of serum, respectively. After 5 min centrifugation at 800g, the cell pellet was ressuspended in the appropriate culture medium. The cells were replated onto new coverslips, flasks or dishes at the appropriate density.

Cell freezing and thawing

All the cell lines used were stored as frozen stocks in 1 ml vials placed in liquid nitrogen. Cells were thawed by rapid warming of a vial in a covered container with warm water and subsequently transferred with a pipette in a 12 ml falcon tube on top of 10 ml DMEM-FCS. DMEM-BS was used instead of DMEM-FCS if the cells were to be maintained in a serum-free medium. After 5 min centrifugation at 800 g the cell pellet was resuspended in 3 ml of appropriate medium, plated in a 25 cm^2 flask and incubated at the appropriate temperature. Most of these cells were expanded for study by successive passages while a fraction was used to replace the frozen stock. Cells for freezing were detached from the flask by trypsinisation or shaking in the presence of EDTA (hybridoma), pelleted by centrifugation at 800g and resuspended in a mixture of 45 % DMEM-FCS, 45 % FCS and 10 % DMSO at a concentration of at least 106 cells per ml. They were frozen in at -70°C freezer prior storage in liquid nitrogen.

Preparation of the culture substrate

A 13.3 $\mu g\ ml^{-1}$ poly-L-lysine (PLL) solution was prepared by adding 66.6 µl PLL stock solution per 20 ml H_2O or 1M sodium borite solution (pH 8.0). Sodium borite was used for the coating of coverslips on which tsT-OBEC cell lines were to be grown, as it was found that this enhanced their attachment to the substrate. For the coating of 13 mm-diameter round glass coverslips, one hundred oven-sterilised coverslips were placed in a petri dish containing 20 ml PLL solution. For the coating of plastic tissue culture flasks or dishes, a sufficient amount of PLL solution was added to cover the surface of each flask or dish. Coverslips and flasks were incubated in a 33°C or 37°C incubator for a period of time ranging from 1 to 24 hours. Each coverslip was then positioned inside the well of a 24 well-tray using forceps. After aspirating the remaining PLL solution, coverslips and flasks were allowed to dry in the hood and subsequently stored at room temperature for up to 1

week. After application of sodium borite, coverslips were rinsed 3 times with H_2O before drying.

2.6.2 Isolation of antibodies from hybridoma lines

O4- or GalC-producing hybridoma lines (gift from Dr. I. Sommer, Glasgow University) were obtained from frozen stocks in liquid nitrogen. The cells were fed every 2 day with DMEM-FCS and expanded by successive passage from 25 cm^2 flasks to 175 cm^2 flasks. When the cells reached confluency the medium was replaced by 20 ml DMEM-BS. After 48 hour incubation the conditioned medium was collected and briefly centrifuged at 800 g to pellet down contaminating cells. The supernatant was gently pippeted out and filtered through a 0.22 µl Millipore filter. The presence of O4 or Gal-C antibodies in the supernatant was tested by immunofluorescent labelling of oligodendrocytes isolated from neonatal rat optic nerves and grown on PLL-coated coverslips in DMEM-BS (provided by Dr. Barnett). The supernatants were then aliquoted and stored at -20°C for up to 6 months.

2.6.3 Purification and culture of cortical astrocytes

Purified type-1 cortical astrocytes were prepared from 1 to 2-day-old Sprague-Dawley rats using the following modification of the method of Noble and Murray (1984). The brains were isolated and flipped over in a petri dish containing L-15, after removal of the cerebellum and olfactory bulbs. All the midbrain structures were then removed using a microscope under sterile conditions. These include the hypocampus, caudate and corpus collosum. The remaining cortices were turned over again and the meninges peeled off. The cerebral cortices were then pooled in a small volume of L-15 and finely chopped with a scalpel blade. The following amounts of enzymes apply for a litter of 10 to 15 rats. The tissue fragments were digested in 3 ml of a 666.6 U ml^{-1} collagenase solution (2 ml L-15 + 1 ml collagenase stock) at 37°C for 30 min. 1 ml of trypsin stock was then added

and the tissue fragments incubated at 37°C for a further 15 min. After a 5 min centrifugation at 800 g, the supernatant was discarded and the pellet resuspended in a 0.125 % trypsin solution (1 ml EDTA stock + 1ml trypsin stock). After a further 15 min incubation at 37°C, the trypsin was inhibited by addition of 1 ml SD stock. The preparation was again centrifuged 5 min at 800 g, the supernatant discarded, and the pellet resuspended in 2 ml of HBSS, Ca^{2+}, Mg^{2+}-free followed by trituration through a 1 ml pipette and 25G hypodermic needle successively. The cells were then diluted in DMEM-FCS and plated in PLL-coated flasks at a density equivalent to 2.5 cerebral cortices (1.5 -2 x 10^7 cells) per 75 cm2 flask. After an overnight incubation at 37°C most of the culture medium was removed and replaced by 10 ml of fresh DMEM-FCS. From then on, the cells were fed every 2-3 day with DMEM-FCS. After 1 to 2 weeks the astrocytes reached confluency and the monolayers were treated with 2 x 10^5 M cytosine arabinoside (200 µl AraC stock in 10 ml DMEM-FCS) for 48 hours to remove dividing contaminating cells of the oligodendrocyte-type 2 astrocyte lineage which grow on the astrocytes.

2.6.4 Preparation of astrocyte conditioned medium

Astrocyte conditioned medium (ACM) was prepared from 2 weeks to 3 month-old astrocyte monolayers prepared as above. Cells were rinsed briefly with PBS and incubated at 37°C with 10 ml of DMEM-BS. After 48 hours the cell supernatant was collected, filtrated through a 0.22 mm Millipore filter and stored at -20°C for up to 3 months. Cells were reincubated in DMEM- FCS for at least 24 hours before the next ACM preparation.

2.6.5 Purification and culture of sciatic nerve Schwann cells

Schwann cells were prepared from the sciatic nerves of 4-6-day-old rats according to the technique of Brockes et al. (1979). Briefly, the sciatic nerves from 10-15 animals were dissected, and collected in a petri dish containing L-15. After removal of most of the medium with a glass pipette, the nerves were carefully

chopped with a scalpel blade and ressuspended in a 1000 U ml^{-1} collagenase solution (1 ml L-15 + 1 ml collagenase stock). After approximately 45 min, the cell suspension was centrifuged for 5 min at 800 g and the cell pellet resuspended in 1 ml HBSS, Ca^{2+}, Mg^{2+}-free, followed by trituration through a 25G and 27G hypodermic needle successively. The cell suspension was then washed with DMEM-FCS and centrifuged for 5 min at 800 g. The cells were ressuspended in 0.5 ml DMEM-FCS, plated onto the centre of a 75 cm^2 tissue culture flask, and incubated at 37°C for approximately 1 hour, until they attached. An additional 2 ml of DMEM-FCS was then added to the culture. After 1-2 days, the cultures were treated with 2 x 10^5 M cytosine arabinoside (50 µl AraC stock in 2.5 ml DMEM-FCS) for 48 hours, to suppress the faster growing fibroblasts. The cells were then fed every 2-3 days with DMEM-FCS.

2.6.6 Purification and culture of olfactory bulb ensheathing cells

Olfactory bulb ensheathing cells were purified following the method of Barnett et al. (1993a). The following quantities of enzymes and antibodies apply for a litter of 10 to 15 rats.

Preparation of olfactory bulb cell suspension

Olfactory bulbs were dissected from 7-day-old Sprague-Dawley rats, washed in L-15, and diced in small fragments with a scalpel blade. The tissue fragments were digested in a 1000 U ml^{-1} collagenase solution (1 ml L-15 + 1 ml collagenase stock) at 37°C. After 1 hour incubation, After 1 hour incubation at 37°C, 0.5 ml of SBTI-DNAse (SD) stock was added and the cells incubated for a further 5 min. The suspension was then centrifuged for 5 min at 800 g and the cell pellet resuspended in 1 ml HBSS, Ca^{2+}, Mg^{2+}-free, followed by trituration through a 25G, 27G, and 29G hypodermic needle successively. The cell suspension was then washed with L-15 containing 1% FCS and centrifuged for 5 min at 800 g.

Immunofluorescent labelling of olfactory bulb cells

The cells were labelled in suspension for 40 min on ice with a mixture of O4 and anti-GalC hybridoma supernatant (3:4, 1:4, v/v). The primary antibodies were then washed out by centrifuging the cells for 5 min at 800 g through 10 ml L-15 containing 1 % FCS (L-15/FCS). The cell pellet was resuspended incubated on ice for a further 30 min in DMEM-BS containing 1 % FCS (DMEM/FCS) and a 1:100 dilution of both goat anti-mouse IgM-Fl and goat anti-mouse IgG3-R-PE. The cells were then washed twice in L-15/FCS and resuspended in DMEM/FCS.

Fluorescence activated cell sorting

The olfactory nerve ensheathing cells, previously defined as O4+/GC-(Barnett et al., 93a), were sorted using a FACStar fluorescence-activated cell sorter (Becton Dickinson UK Ltd., Oxford, England). The argon laser was attuned for 488 nm excitation and sorts were carried out at 100-150 mW. Fluorescence compensation was set to negate the spillover of the fluorophores into each channel. The sort window was set around a tight population of fluorescein (O4)+/ phycoerythrine (GalC)- cells.

Culture of olfactory bulb ensheathing cells

After 15 min centrifugation at 800 g, the sorted cell population was resuspended in ACM diluted 1:5 with DMEM-BS (DMEM-BS/ACM). The cells were plated onto the centre of 13 mm PLL-coated coverslips at a density of 7000 cells/20-30 µl and incubated for 30-45 min at 37°C until they attached. As it was found that ACM greatly enhanced the survival of the FACS sorted cells, these were initially incubated overnight in 500 µl of DMEM-BS/ACM (1:5). The following morning, this medium was replaced by 500 µl of either fresh DMEM-BS/ACM, DMEM-BS, or DMEM-FCS. Half of the media was renewed every other day during the first week an every 2 or 3 day for longer term cultures. When cells reached confluency, they were trypsinised, diluted, and passaged onto new PLL-coated coverslips.

2.6.7 Generation of ts-T OBEC cell lines

O4+ FACS sorted OBECs were plated onto a 35 mm-diameter-PLL-coated tissue culture dish and incubated in DMEM-BS/ACM overnight. The OBECs were infected with retroviruses containing the temperature sensitive (ts) mutant gene of the large T antigen (Tag) and the selection marker, hygromycin. The construction of this retrovirus has previously been described (Barnett et al., 1993b). The large T antigen gene product is functional at the permissive temperature of 33°C but is rapidly degraded at the non-permissive temperature of 39°C. After infection the cells were placed in selection medium containing hygromycin (100 $\mu g\ ml^{-1}$) for 7-10 days. Colonies that grew out were marked on the bottom of the dish and then picked using a disposable plastic Gilman pipette tip. These cells were expanded in culture in DMEM-BS/ACM at 33°C. Clones were recloned by limited dilution and the clonality of the cell lines tested by Southern blotting.

2.6.8 Purification and culture of cerebellar granule neurons

Cerebella from 8 to 10-day-old rats were removed and dissected free of meninges, minced with a scalpel blade and subsequently digested with trypsin (3000 $U\ ml^{-1}$ in L-15) for 15 min at 37°C. SBTI-DNAse was then added and the cell suspension was centrifuged for 5 min at 800 g. The cell pellet was resuspended in HBSS, Ca^{2+}, Mg^{2+}-free and dissociated by trituration through 21G and 25G needles. The dissociated cells were centrifuged through a 3 ml cushion of DMEM-FCS containing 4% BSA. The pellet was resuspended in DMEM-FCS and the cells seeded out on PLL-coated flask, and incubated at 37°C for 30 min. The flask was then gently agitated and the medium, containing a population enriched for granule neurons was removed. After a 5 min centrifugation at 800 g, the pellet was resuspended in DMEM-BS.

2.7 Indirect immunofluorescent labelling

All antibodies were diluted in staining solution for immunolabelling of cells and tissues. The dilutions for each antibody are indicated in Table 2.1.

2.7.1 Immunolabelling of tissue sections

For immunolabelling of olfactory bulb frozen sections, freshly sectioned tissues were incubated overnight at 4°C with cell surface and intracellular antibodies. The sections were then rinsed 3 times for 5 min in staining solution and incubated with the appropriate class-specific fluorochrome-conjugated second antibodies for 2.5 hours at room temperature. After three 5 min washes in staining solution, the sections were mounted in anti-fade solution, sealed with nail varnish, stored in the dark at 4°C and examined under a fluorescent microscope.

2.7.2 Immunolabelling of cultured cells

All antibody incubations of cultured cells grown on PLL-coated coverslips were carried out at room temperature and the cells were rinsed 3 times with staining solution in between each antibody application. Double immunolabelling was carried out using two antibodies of different class in conjunction with their corresponding class specific second antibodies conjugated to different fluorochromes (fluorescein and tetrametyl rhodamine). For double immunolabelling with two antibodies against cell surface antigens (O4, A2B5, HNK1, 192 IgG, anti-GalC, anti-NCAM or anti-PSA-NCAM), living cells were incubated for 20-30 min with both antibodies simultaneously, followed by 20-25 min incubation with the corresponding class-specific fluorescein and tetramethyl rhodamine-conjugated second antibodies. The cells were then fixed in methanol at -20°C for 10-15 min. For double immunolabelling with one cell surface and one intracellular antibody, the cells were first labelled with the cell surface antibody as described previously, fixed, and then labelled with the intracellular antibody. Fixation was carried out in methanol (-20°C, 10 min) for the labelling with anti-GFAP or anti-vimentin, in 4% paraformaldehyde

(room temperature, 20 min) followed by 95% ethanol/5% acetic acid (-20°C, 10 min) for the labelling with anti-S100. Anti-GFAP, anti-vimentin or anti-S100 were then applied for 40 min followed by 20-25 min incubation with the corresponding class specific fluorochrome-conjugated antibody. For labelling with anti-BrdU, cells were fixed in methanol (-20°C, 10 min), incubated in 0.02% paraformaldehyde for 1 min at room temperature, and treated with 0.07% NaOH for about 10 min at room temperature to denature DNA. Anti-BrdU was then applied for 40 min followed by 20-25 min incubation with an anti mouse IgG1 fluorochrome-conjugated antibody. After immunolabelling, coverslips were washed, mounted in a drop of anti-fade solution, sealed with nail varnish, stored in the dark at 4°C and examined under a fluorescent microscope. The proportion of cells labelled with each antibody was estimated from the counting of at least 300 cells per coverslip.

2.8 Screening of candidate OBEC mitogens using a DNA synthesis assay

BrdU uptake and its subsequent detection using an anti-BrdU antibody was used to assess cell proliferation. After an overnight incubation in DMEM-BS/ACM (4:1, v/v), the sorted cells were washed twice by replacement of half the medium with fresh DMEM-BS and further incubated in the presence of potential mitogens including rhFGF2 (10-100 ng ml^{-1}), rhPDGFAA (10 ng ml^{-1}), rhPDGFBB (5 ng ml^{-1}), NGF7S (50-150 ng ml^{-1}), TGFβ (1 ng ml^{-1}), rhNT3 (10 ng ml^{-1}), rhBDNF (10 ng ml^{-1}), rhCNTF (10 ng ml^{-1}) and BPE-GGF (1:50-1:250), DMEM-BS/ACM (4:1). The cells were refed on day 3, by replacement of half the media with fresh DMEM-BS containing the potential mitogens at double concentration. For the last 16 hours of culture the cells were incubated in the presence of BrdU (20 μM). On day 4, the cells were washed with staining solution and processed for immunolabelling.

2.9 Assay of neuron-glia interactions

Five hundred microlitre of DMEM-BS, containing 10 x 10^3 cerebellar granule neurons prepared as above, was added onto confluent monolayers of OBECs, Schwann cells, astrocytes and 3T3 fibroblasts grown on PLL-coated coverslips for the past week. Each monolayer was washed 3 times with DMEM-BS prior to the addition of the neurons. After a 16 hour incubation period, the co-cultures were fixed with 2% paraformaldehyde and processed for immunofluorescent labelling (as described in section 2.8.2). Anti-L-NGFr was used as a marker for OBECs and Schwann cells and anti-GFAP as a marker for astrocytes. A large panel of neuron markers were tested (see Chapter 6). Anti-GAP43 was shown to strongly label the cell body and the entire length of the neurites and was thus used to assess the extent of neurite outgrowth. The cells were examined on the confocal laser scanning microscope system at a final image magnification of X 200. For each substrate, three parameters were analysed; the average number of neurons per field, the percentage of neurite-bearing neurons and the mean total neurite length per neurite-bearing neurons, as an estimation of neuronal attachment/survival-, neurite initiation, and neurite elongation , respectively. For each confluent monolayer, neurons from 20-30 fields, from two separate coverslips, were counted for quantification of the average number of neurons per field and of the percentage of neurite bearing neurons. Neurites were defined as those processes with a length of at least one cell body diameter. At least 50 neurite-bearing neurons, from two separate coverslips, were subjected to neurite length measurements, using software supplied with the confocal microscope. The total length of all neurites emerging from each neurons was recorded and used to generate a grand mean and standard deviation for parametric tests.

In one experiment, neurons were plated onto sparse cultures of OBECs. For each sparse glial cell culture, neurons from 30-50 consecutive fields (covering at least half of a coverslip), were counted for quantification of the average number of

neurons per field and of the percentage of neurite bearing neurons. At least 100 neurite-bearing neurons, from two separate coverslips, were subjected to neurite length measurements. The number of glial cells within each field was recorded under rhodamine optics, and used as estimate for local glial cell density. The average number of neurons per field was analysed in function of local glial cell density. The population of neurons in contact with glial cells and the population of neurons not in contact with glial cells were analysed separately for quantification of the two other parameters.

The significance of differences between experimental values was analysed using the t-test.

2.10 Microscopy and photography

Immunolabelled tissue sections and cells were viewed on a fluorescent microscope (Zeiss, Nikon) or alternatively on a Bio-Rad MRC-600 laser scanning confocal microscope equipped with a krypton/argon ion laser. For the detection of fluorescein and rhodamine, 488/568 nm line excitation and dual channel 522 and 585 nm emission filters were used. Image analysis (measurement of neurite length) was performed using Bio-Rad software.

2.11 Characterisation of the sensitivity of the mitogenic activity of ACM to various treatments

2.11.1 Heat treatment of ACM

Heat stability of ACM mitogenic activity was assessed by incubating aliquots of ACM for 2 hour at 37°C, 30 min at 55°C, 10 min at 75°C and 2 min at 100°C.

2.11.2 Protease treatment of ACM

Proteolytic stability was assessed by incubating ACM in 0.2 mg ml^{-1} trypsin or 0.2 mg ml^{-1} chymotrypsin for 1 hour at 37°C following which both enzymes were

neutralised by trypsin/chymotrypsin inhibitor. Activity of the heat treated or protease treated ACM was then measured using the DNA synthesis assay described above.

2.12 Chromatography

All the purification steps were performed by using a Phamacia fast protein liquid chromatography system (FPLC). All columns were equilibrated and washed in 0.02 M Tris/HCl, pH 7.6, containing 0.1 M NaCl (buffer A). The material recovered from the columns were filter sterilised through 0.22 μl Millipore filters, aliquoted, and either directly tested for mitogenic activity on FACS-sorted OBECs or stored at -20°C for a later assay of mitogenic activity. The fractions recovered from elution of the anion exchange and heparin-Sepharose columns were desalted by dialysis against DMEM-BS using a Sephadex G21 column (HR 10/10) before being filter sterilised.

2.12.1 Heparin-affinity chromatography

Fifteen millilitre of ACM was loaded onto a 1 ml pre-equilibrated heparin-Sepharose column. The column was washed with 17 ml of buffer A, after which no absorbance at 280 nm wavelength could be detected. Bound proteins were then eluted with 6 ml of 2M NaCl at a flow rate of 0.5 ml/min. Both the unbound (H1, 17 ml) and bound (H2, 6 ml) material were collected and tested for mitogenic activity.

2.12.2 Anion exchange chromatography

Anion exchange chromatography was performed using a MonoQ anion exchange column. Following the loading of the sample, the column was washed with buffer A, until the ultraviolet light absorbance returned to baseline (indicating complete flow-through of unbound proteins) before elution of the bound proteins.

The first chromatography was performed by loading 12 ml of ACM onto the column. The flow rate of the running buffer was adjusted to 0.5 ml/min. After washing the column with 30 ml of buffer, the bound proteins were eluted with 10 ml,

using a linear salt gradient to 1.0 M NaCl, followed by 4 ml of 2.0 M NaCl. Seven fractions of 2 ml were collected .

For the second chromatography, pooled conditioned medium from 6 harvest (60 ml total) was cleared by filtration through 0.22 µl Millipore filters and concentrated 30-fold with an Amicon ultrafiltration system using membranes with a 10 kd molecular size cut-off. A 0.5-ml-sample of concentrated ACM was loaded onto the column. The flow rate of the running buffer was adjusted to 0.2 ml/min. After washing the column with 125 ml of running buffer, the bound proteins were eluted with 10 ml using a linear salt gradient to 2.0 M NaCl followed by 5 ml of 2M NaCl. Fifteen fractions of 1 ml were collected.

2.12.3 Gel filtration

For gel filtration chromatography of concentrated conditioned medium, 500 µl of concentrated ACM was loaded onto a pre-equilibrated Sephacryl S200 (HR 10/30) column The flow rate was adjusted to 0.5 ml/min. Fifteen fractions of 1 ml were collected.

2.13 Southern blot analysis

2.13.1 DNA extraction

Genomic DNA was harvested from a confluent 75 cm^2 tissue culture flask of tsT-OBEC cells for the preparation of Southern blots. Extraction of DNA was achieved with a nucleon I DNA extraction kit (Scotlab) or alternatively by using the following method. Cells were briefly rinsed with PBS and 10 ml of equilibrated phenol was added to each flask. Cells were scraped from the bottom of the flask using a Costar disposable cell scraper and the mixture left, shaking gently at 37°C for 10 min. 10 ml of DNA lysis buffer was then added and the flask again incubated, shaking gently at 37°C for 10 min. The content of the flask was then transferred into a 50 ml Falcon tube (Becton Dickinson) and 10 ml of chloroform/isoamyl alcohol

(25:1) was added. The tube was then incubated at 37°C on a rotating rack for at least 30 min and up to 2 hours. The solution was then centrifuged for 15 min at 800 g and 22°C, following which the upper phenol phase was removed and the genomic DNA precipitated by adding an equal volume of isopropyl alcohol to the aqueous phase. This solution was placed at -20°C for at least 30 min and then centrifuged as above. The DNA pellet was air dried for approximately 10 min and resuspended in 100-200 µl of distilled H_2O. All genomic DNA samples were stored at 4°C. An aliquot of each sample was used for estimation of DNA concentration and purity. This was performed using a spectrophotometer under 260 and 280 nm excitation, an absorbance A_{260} of 1.0 being equivalent to a DNA concentration of 50 µg ml^{-1}.

2.13.2 Digestion of genomic DNA

Twenty microlitre of DNA was digested overnight at 37°C with *Bam*H1, *Xba* I (Gibco, BRL) or *Bgl* II (Pharmacia LKB), according to manufacturers' instructions, in a total volume of 150 µl. The DNA was cut with *Bam*H1 to detect the large T antigen gene alone, and *Bgl* II for the clonality of the cell line since there was only one *Bgl* II site in the plasmid (Barnett et al., 1993b). Completion of the digestion was tested by running a 10 µl aliquot of digestion mixture on a 0.8 % agarose/TBE minigel. The digested DNA was precipitated by addition of 15 µl of 3M NaOAc and 200 µl cold isopropanol to each sample following which the mixture was placed at -20°C for at least 1.5 hours. DNA was pelleted at 13,000 rpm for 25 min, air dried for 30 min and finally resuspended in 30 µl distilled H_2O, overnight at 4°C.

2.13.3 Electrophoresis of DNA restriction fragments

Five microlitre of DNA loading dye was mixed to the 30 µl samples of digested DNA. The samples were then run on a 0.8 % agarose/TEA gel for approximately 24 hours at 50 V using gel tanks from IBM Ltd., Cambridge. Following electrophoresis, the digested DNA was stained for 45 min with ethidium bromide (0.5 µg.ml-1) and the gel examined under UV illumination to check the

extent of DNA migration. Using an Appligene "Imager" CCD camera with supplied software an image of the gel was printed on thermal paper.

2.13.4. Southern blotting

DNA on the gel was denaturated by two successive 15 min incubation in denaturation solution and subsequently neutralised by two successive 30 min incubation in neutralisation solution. Both incubations were carried out on the bench at room temperature, with occasional shaking. Gels were then blotted overnight onto nylon filters (Hybond Nfp, Amersham, UK) using 1 x Genescreen buffer. Filters were then rinced briefly in Genescreen and dried. DNA was subsequently cross-linked to the filter by exposure to a UV source.

2.13.5 Radiolabelling of tsT DNA probe

^{32}P-labelled tsT dsDNA probe (gift from Dr. S. Barnett, Glasgow University) was prepared using the Stratagene "Prime-It" kit with 100 ng of template tsT DNA. The labelling protocol was carried out using manufacturer's instructions. Following labelling, the probe was purified using Sephadex-containing NICK columns (Pharmacia) and chemically denatured before being applied to the nylon filters.

2.13.6 Hybridisation and washing of filters

Hybridisation and washing of filters was carried out in an Hybaid oven set at 65°C. Filters were rolled in 20 x 20 cm squares of nylon mesh (Hybaid), placed in glass hybridisation bottles and incubated on a rotating rack in the oven. Filters were prehybridised for 1-2 hours in approximately 20 ml of hybridisation solution before the addition of radiolabelled probe. Labelled probes were applied to filters in a 10 ml volume of hybridisation buffer and hybridisation carried out for at least 24 hours in the 65°C oven. Filters were subsequently washed in 20-30 ml of wash solution for several hours in the rotisserie oven with repeated changes of solution.

2.13.7 Autoradiography

Probed filters were exposed to Kodak XAR 5 or FUJI RX autoradiography film in sealed cassettes with fast tungsten intensifying screens at -70°C for varying length of time depending on the signal density.

CHAPTER THREE

In vivo and *in vitro* immunocytochemical analysis of OBECs from perinatal rats

3.1 Introduction

The hypothesis has been put forward that OBECs play a role of utmost importance in the exceptional ability of the olfactory bulb to support re-innervation throughout life (Barber and Lindsay, 1982; Doucette, 1984; Raisman, 1985; Doucette, 1990). It is therefore of particular interest to establish the cellular and molecular properties of these glial cells. To this aim, the development of appropriate methods to purify and grow these cells *in vitro* is paramount. To devise an accurate culture system, it is important to define conditions which allow the cultured cells to closely resemble their *in vivo* counterparts. *In vivo* (see Section 1.5.) and *in vitro* (see Section 1.6) studies of OBECs have mostly been undertaken separately in the past and the relationship between both conditions have rarely been made.

In the present study, the antigenic properties of cultured OBECs have been investigated under different culture media, and directly compared to the antigenic profile of the olfactory nerve layer, the tissue from which they are derived. The OBEC purification procedure chosen for this study is fluorescence activated cell sorting (FACS) using the O4 antibody (Barnett et al., 1993a). This method has the advantage to ascertain the purity of the cultures at a very early stage (directly after the FACS sorting procedure, i.e. only a few hours after the cells have been removed from their *in vivo* environment). Their antigenic profile can be precisely assessed using a large panel of glial markers on the first day *in vitro*, a time at which it is expected to closely mimic their *in vivo* phenotype. It can then be assessed how this phenotype changes in response to various culture conditions. This is in contrast to other published OBEC purification procedures that, due to their numerous steps,

require prolonged time in culture prior to immunocytochemical analysis (Ramon-Cueto and Nieto-Sampedro, 1992; Chuah and Au, 1993).

By using double immunofluorescent labelling with a large panel of glial markers, both *in vivo* and *in vitro*, this study has helped to resolve the controversy that exists in the literature as to whether OBECs represent a homogeneous population of cells co-expressing astrocytic and Schwann cell features or whether antigenically different, but related, subtypes exist.

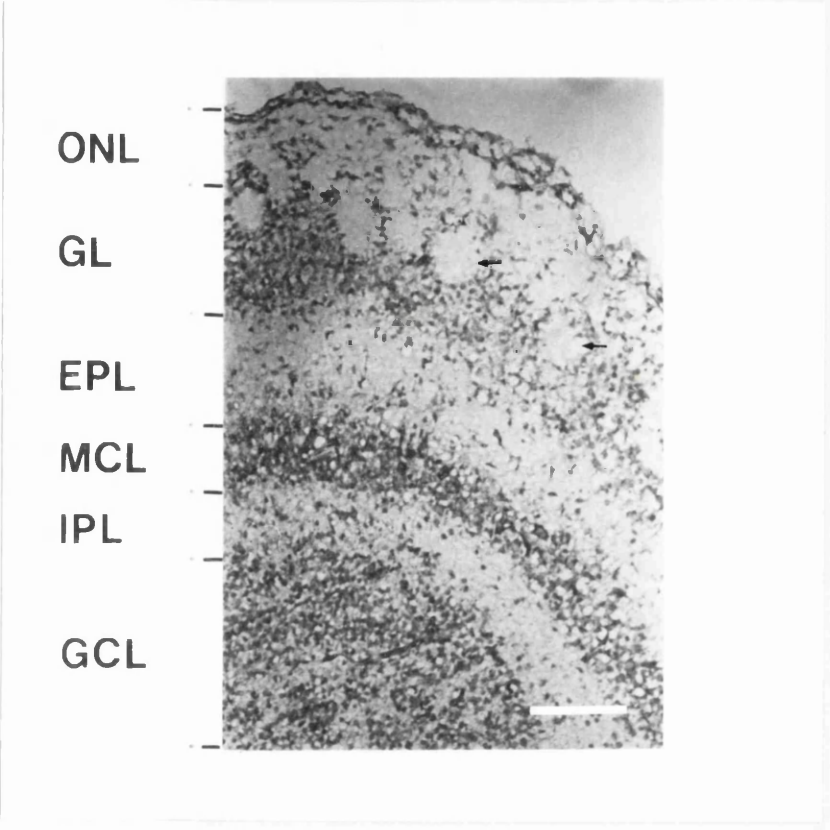
3.2 Results

3.2.1 Immunocytochemical analysis of the 7-day-old rat olfactory bulb

Cresyl violet staining of frozen sections of the olfactory bulb demonstrated a laminar organisation as previously described (Shepard, 1972). 5 distinctive layers were identified (Fig. 3.1). This paragraph will focus on the description of the two outermost layers of the olfactory bulb which are i) the olfactory nerve layer (ONL), the main tissue in which OBECs reside and ii) the glomerular layer (GL) which may also comprise some OBECs (Raisman, 1985; Marin Padilla and Amieva, 1989; Valverde and Lopez-Mascaraque, 1991). Each time a section was immunolabelled, a consecutive section was stained with cresyl violet to define the location of immunolabelling. Incubation of olfactory bulb sections with the O4 antibody resulted in the exclusive staining of the ONL from the pial surface to the ONL/GL interface and confirmed previous work (Barnett *et al.*, 1993a). The entire width of the ONL also labelled with anti-Vm and anti-NCAM and was unlabelled with anti-GalC and HNK-1 antibodies. The use of a larger panel of neural markers showed that this layer could be further sub-divided into antigenically distinct areas: i) a thin O4+/L-NGFr+/PSA-NCAM-/GFAP-/S100-/GAP-43- layer at the surface of the olfactory bulb which also encompassed ii) larger bundles of O4+/L-NGFr-/PSA-NCAM+/GFAP-/S100+/GAP-43+ tissue in the central part of the ONL (termed outer ONL), iii) an O4+/L-NGFr-/PSA-NCAM-/GFAP-/S100+/GAP-43+ area in the

Figure 3.1

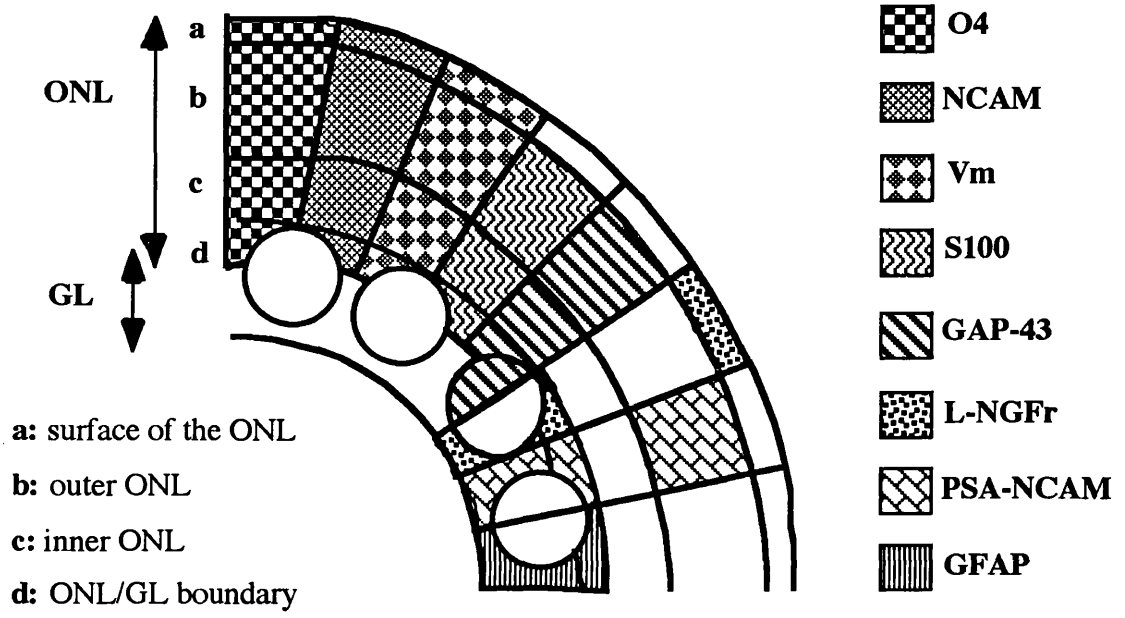
Nissl-stained section of a 7-day-old rat olfactory bulb, illustrating its laminated cytoarchitecture



Methyl-red staining was carried out on a 5-8 μm-thick coronal cryostat section through a 7-day-old rat olfactory bulb. 6 concentric layers can be seen: the olfactory nerve layer (ONL), the glomerular layer (GL), the external plexiform layer (EPL), the mitral cell layer (MCL), the internal plexiform layer (IPL) and the granule cell layer (GCL). The Nissl-stained material in the ONL represent the cell bodies of glial cells, consisting predominately of OBECs and possibly a few astrocytes. Several glomeruli can be seen in the GL as round Nissl-free regions (arrows). Photomicrograph, scale bar = 1100 μm.

Figure 3.2

Diagrammatic scheme summarising the antigenic profile of the 7-day-old-rat olfactory nerve layer



Immunofluorescent labelling was carried out on 5-8 μm-thick coronal cryostat sections through a 7-day-old rat olfactory bulb with a panel of neural markers. The olfactory nerve layer (ONL) labels entirely with O4, anti-NCAM and anti-Vm but can be subdivided into four antigenically distinct layers with antibodies against S100, GAP-43, L-NGFr, PSA-NCAM and GFAP: (a) a thin O4+/L-NGFr+/PSA-NCAM-/GFAP-/S100-/GAP-43- layer which generally defines the surface of the olfactory bulb but also encompasses (b) larger bundles of O4+/L-NGFr-/PSA-NCAM+/GFAP-/S100+/GAP-43+ tissues in the outer ONL ; (c) an O4+/L-NGFr-/PSA-NCAM-/ GFAP-/S100+/GAP-43+ area deeper within the ONL; and (d) an O4+/L-NGFr±/PSA-NCAM+/GFAP+/ S100+/GAP-43+ area at the ONL/GL boundary . L-NGFr immunolabelling at the ONL/GL boundary is faint and variable. NCAM and Vm immunolabelling decreases towards the centre of the bulb becoming faint and variable in the GL.

deeper part of the ONL (termed inner ONL) and iv) a O4+/L-NGFr±/PSA-NCAM+/GFAP+/S100+/GAP-43+ area at the ONL/GL boundary (summarised diagramatically in Fig. 3.2). Although GFAP-immunoreactivity was mainly detected at the ONL/GL boundary, some faint GFAP staining was occasionally detected throughout the ONL. Fig. 3.3 illustrates the four O4+ sublayers, distinguished on the basis of their labelling with anti-L-NGFr, anti-PSA-NCAM and anti-GFAP.

Sections of turbinates from the same developmental stage were also processed for immunolabelling and showed that O4+ cells were equally present in the peripheral portions of the olfactory nerves (identified by their immunoreactivity to anti-GAP-43; Fig. 3.4). These appeared to comprise 2 antigenically distinct cell population: L-NGFr+ cells at the periphery of olfactory axon fascicles, and GFAP+ cells within their interior (compare Fig. 3.4 C and 3.4 E).

3.2.2 Fluorescence activated cell sorting of the O4+ cells of 6-8-day-old rat olfactory bulbs

O4+/GalC- cells were purified from 6-8-day-old rat olfactory bulbs by fluorescence activated cell sorting (FACS) as described in the corresponding method's section. Although no GalC immunoreactivity could be detected in olfactory bulb frozen sections, double-immunolabelling was used to avoid enrichment for any O4+/GalC+ oligodendrocytes which might still be present in minute quantities in the olfactory bulb. The sort window was set around a tight population of O4+/GalC- cells as exemplified in Fig. 3.5. 2 x 10^4 events were recorded for FACS analysis. The percentage of sorted cells averaged 14.4 ± 1.3% between different sorts. The non-sorted cell population comprised an average of 5% O4+/GalC+ cells likely to represent developing oligodendrocytes present in the olfactory bulb at this developmental stage (Philpot et al., 1995). Aliquots of cells pre- and post-sorting were examined by fluorescence microscopy to determine the purity of each sort. olfactory bulb-derived O4+/GalC- cells were enriched from an initial prevalence of

Figure 3.3

Differential labelling of the 7-day-old rat ONL and GL with antibodies against O4, GFAP, L-NGFr and PSA-NCAM

Immunofluorescent labelling was carried out on 5-8 μm-thick coronal cryostat sections through a 7-day-old rat olfactory bulb.

A, D, and **G** represent consecutive sections of B/C, E /F, and H, respectively, stained with the Nissl stain cresyl violet to illustrate the positions of the olfactory nerve layer (ONL) and glomerular layer (GL).

B, C: Double immunofluorescent labelling of a section with O4/rhodamine (B) and anti-GFAP/fluorescein (C). GFAP-immunoreactivity (IR) is mainly seen at the ONL/GL boundary but occasional GFAP-positive elements are also seen within the ONL.

E, F: Double immunofluorescent labelling of a section with O4/rhodamine (E) and anti-L-NGFr/fluorescein (F). L-NGFr-IR is mainly present at the surface of the olfactory bulb and in the outer ONL around olfactory nerve fascicles. Some faint L-NGFr-IR can be seen in the GL. The O4 antibody labels the entire ONL; some O4-IR is also seen at the ONL/GL boundary.

H: Immunofluorescent labelling of a section with anti-PSA-NCAM/rhodamine. PSA-NCAM-IR is seen in the outer ONL and in the glomerular layer, including the ONL/GL boundary. Note that the PSA-NCAM-IR in the outer ONL decreases gradually as one progresses deeper within this layer.

Photomicrographs, scale bar: 100 μm.

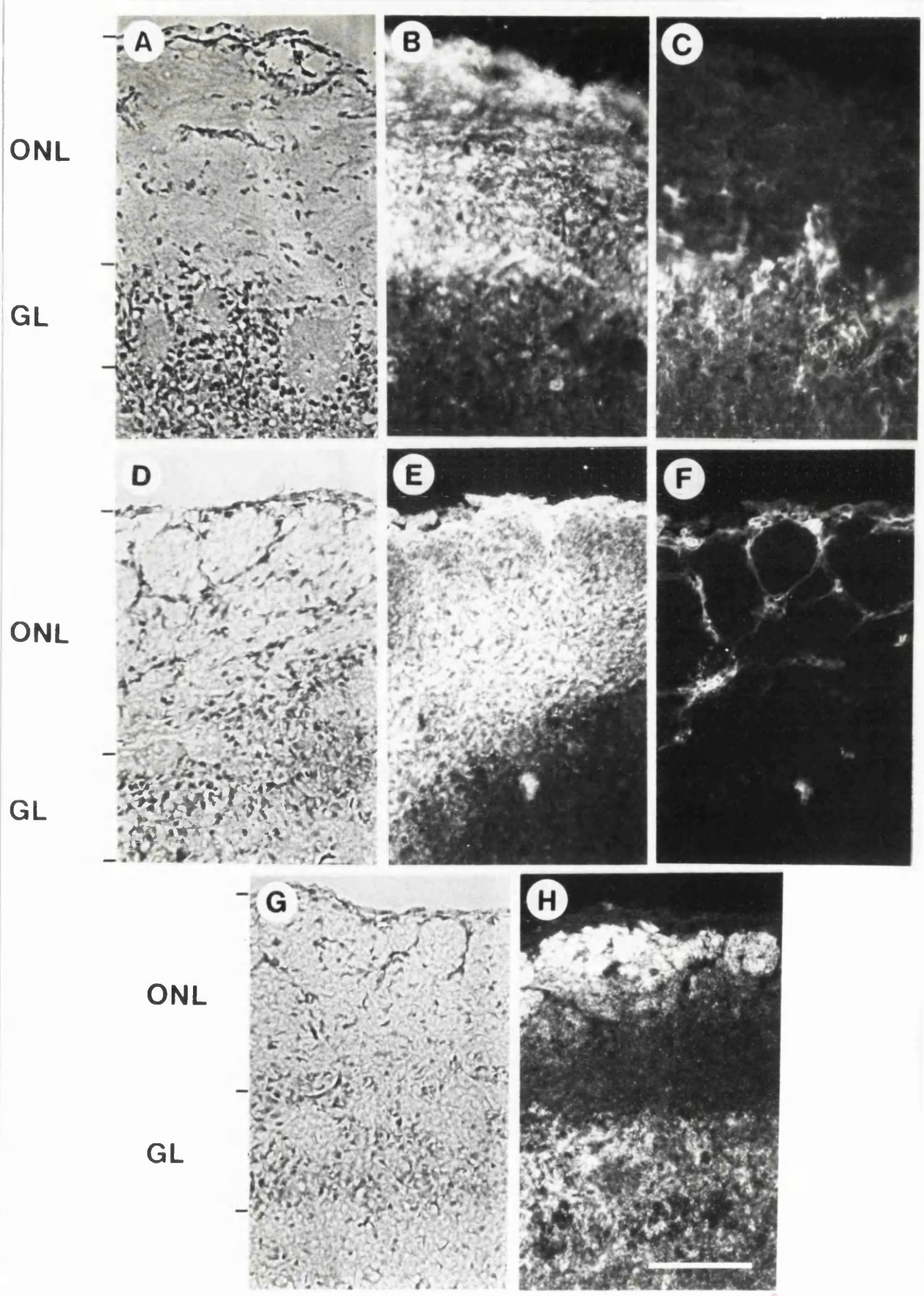

A
B
C
ONL
GL
D
E
F
ONL
GL
G
H
ONL
GL

Figure 3.4

Differential labelling of the 7-day-old rat peripheral olfactory system with antibodies against O4, GFAP, GAP-43 and L-NGFr

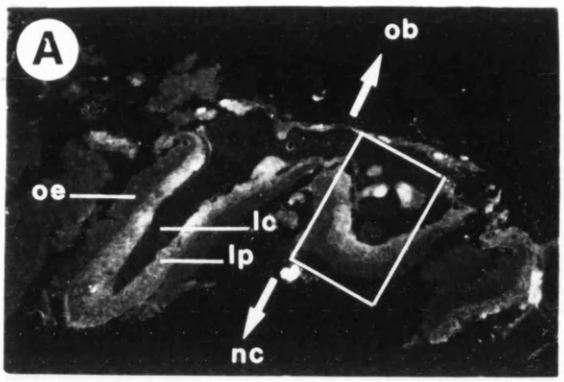
Immunofluorescent labelling was carried out on 5-8 μm-thick coronal cryostat sections through the nasal cavity of a 7-day-old rat olfactory bulb.

A: Immunofluorescent labelling of a section with O4/rhodamine.
This low magnification photomicrograph shows an overview of the peripheral olfactory system including the olfactory epithelium (oe), lamina propria (lp) and lamina cribrosa (lc). Arrows have been traced for orientation, indicating the directions towards the olfactory bulb (ob) and towards the lumen of the nasal cavity (nc). O4-IR is exclusively found in the lamina propria. Regions consecutive to the boxed area are viewed at higher magnification in B, C, D and E.

B, C: Double immunofluorescent labelling of a section with O4/rhodamine (B) and anti-GFAP/fluorescein (C). O4-IR (B) is present in the submucosa underlying the olfactory epithelium (asterix) and within round profiles (arrow), identified with anti-GAP-43 (D) as bundles of olfactory axons (in cross sections) leaving the neuroepithelium. Some filamentous GFAP-IR is found within the O4+ bundles of olfactory axons (C).

D, E: Double immunofluorescent labelling of a section consecutive to section B/C, with anti-GAP-43/fluorescein (D) and anti-L-NGFr/rhodamine (E). Anti GAP-43 clearly labels olfactory neurons within the olfactory epithelium, their axonal projections within the underlying submucosa, and bundles of olfactory axons. Note that anti-GFAP and L-NGFr appear to label distinct elements.

Photomicrographs, scale bar: 100 μm


A
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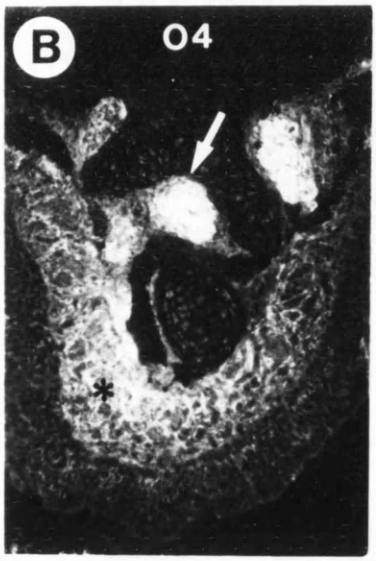
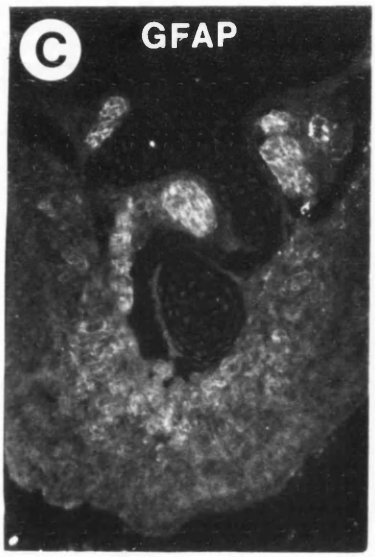

B
O4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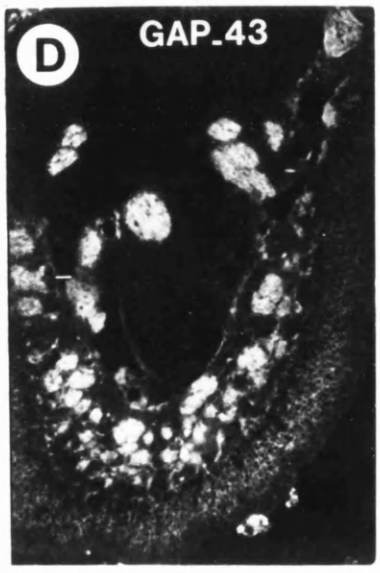
GFAP


D
GAP-43

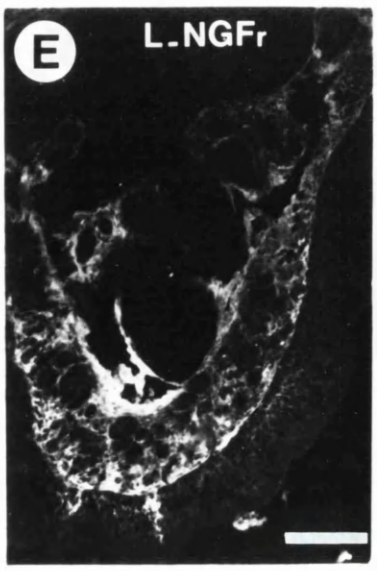
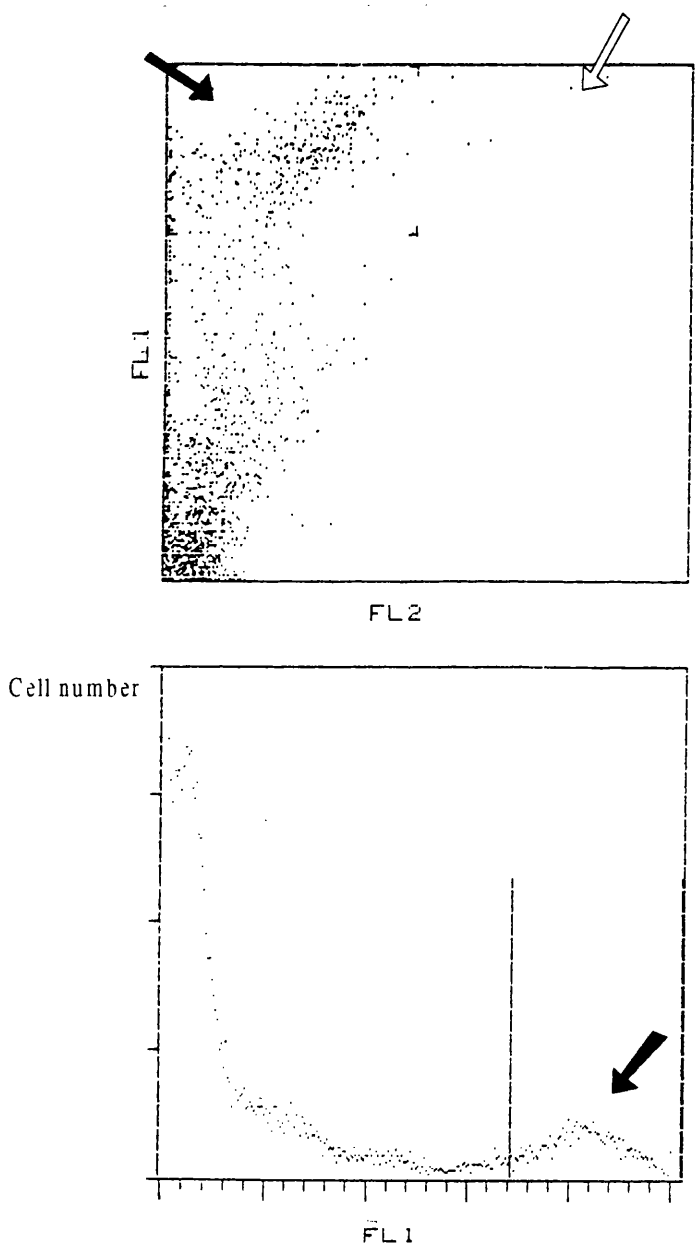

E
L-NGFr

Figure 3.5
FACS profile



Purification of OBECs from perinatal rat olfactory bulbs using FACS.
The upper panel represents a two dimentional FACS profile (flluorescence 1, fluorescein against fluorescence 2, phycoerythrin).
The lower panel represents a one dimentional FACS profile (fluorescence 1, fluorescein against cell number).
The black arrow points to the gated O4+/GalC-cells which represent the OBEC population that is sorted.
The white arrow points to O4+/GalC+ cells that are excluded from the sort and contain cells of the O-2A lineage.

15.3 ± 2.7% to over 90%. A litter of 12 rats yielded to an average of 1 x 10^5 cells as estimated with the haemocytometer prior to plating. The FACS sorted cells were plated onto PLL-coated coverslips and immunolabelled after various time in culture. Out of the 7 x 10^3 cells plated per coverslip, only a few hundred were present when examined for immunofluorescence after an overnight incubation, indicating that cell viability and/or attachment was poor.

3.2.3 Antigenic profile of O4+ FACS sorted cells on the first day *in vitro*

FACS sorted OBECs incubated in DMEM-BS/ACM overnight expressed a similar pattern of antigenic heterogeneity as demonstrated for the cryostat sections of the olfactory bulb (Table 3.1). Over 91.6% of the cells were labelled with O4, 51.2% with anti-PSA-NCAM, 11.8% with anti-GFAP and under 2% with anti-L-NGFr. The occasional L-NGFr+ cells in these cultures were always PSA-NCAM- as assessed by double immunofluorescence. The proportion of cells labelled with anti-GFAP or anti-PSA-NCAM varied greatly among sorts, ranging from 3.6% to 23.8% (n=7) and from 25.5% to 75.8% (n=13), respectively. This variability may reflect a developmental difference. Double immunofluorescent labelling with anti-PSA-NCAM and anti-GFAP showed that there was an overlap between the PSA-NCAM+ and GFAP+ cell populations. The entire cell population was GalC-, HNK-1-, GAP-43-, Vm+, NCAM+. Immunoreactivity of the cells to S100 was of variable intensity and much weaker than that of sciatic nerve Schwann cells cultured under the same conditions. Some cells were so weakly immunofluorescent that it was difficult to be sure whether this was due to background staining or truly reflected S100 expression.

3.2.4 Evolution of antigenic expression over time in culture

Three culture media were used in this study; DMEM-BS, DMEM-BS/ACM and DMEM/FCS. The cultures remained 100% NCAM+, Vm+, GalC- and HNK-1- in any of these media. However, the antigenic profile of the sorted cell population changed with culture time in a medium-dependant manner, as shown by

Table 3.1.

Evolution of OBEC antigenic profile with culture time

Antibody	Overnight culture	DMEM-BS		DMEM-BS/ACM		DMEM-FCS	
		DIV 3-4	DIV 6-7	DIV 3-4	DIV 6-7	Day 3-4	DIV 6-7
O4	91.6 ± 1.4	69.5 ± 3.4	31.8 ± 15.8	31.3 ± 3.7	5.1 ± 1.5	nd	0
Anti-L-NGFr	< 2	74.9 ± 4.0	87.5 ± 8.9	62.6 ± 7.5	89.9 ± 2.8	nd	81.6 ± 9.4
Anti-PSA-NCAM	51.2 ± 5.2		49.8 ± 3.2	43.0 ± 6.2	32.6 ± 4.3	nd	5.1 ± 1.0
Anti-GFAP	11.8 ± 2.7	51.7 ± 7.1	57.2 ± 12.7	35.3 ± 4	45.2 ± 6.4	nd	0
L1	2.9 ± 1.3	42.3	nd	81.1 ± 1.6	nd	nd	nd
Anti-GAP-43	<2	7.6 + 1.5	12.3 + 1.5	8.6 + 1.1	7.8 + 2.2	nd	<2

FACS sorted O4-positive cells of the olfactory bulbs of 7-day-old rats were plated at 7000 cells/PLL coated-coverslips and labelled with a panel of antibodies after an overnight culture in DMEM-BS/ACM, after 3-4 and 6-7 days in DMEM-BS, ACM (diluted 1:2 with DMEM-BS) or 7 days in DMEM-FCS. The values represent the mean ± standard error of duplicate samples from at least three experiments.

immunofluorescent labelling with O4, anti-L-NGFr, anti-PSA-NCAM, anti-GFAP, anti-L1 and anti-GAP-43 (Table 3.1).

O4

In all 3 media, the proportion of cells labelled with O4 decreased with culture time. The rate of this decrease differed between the culture media. After 1 week of ***in vitro*** growth, the entire cell population lost O4 expression in DMEM-FCS and only 5.1% remained O4+ in DMEM-BS/ACM. In DMEM-BS, this decrease was slower, with still 69.5% of the cells labelled after 3-4 days, as compared to 31.3% in DMEM-BS/ACM. The proportion of O4+ cells after 1 week of ***in vitro*** growth in DMEM-BS was highly variable, ranging from 15.7% to 63 % and averaging 31.8%.

L-NGFr

The proportion of L-NGFr+ cells increased with culture time. After 1 week of ***in vitro*** growth in any of the media used in this study, over 80% of the cell population labelled with anti-L-NGFr, as compared with under 2% on the first day ***in vitro.*** Cellular-immunoreactivity to this antibody in DMEM-FCS was of lower intensity compared to cells grown in DMEM-BS or DMEM-BS/ACM.

PSA-NCAM

The proportion of PSA-NCAM+ cells did not change consistently after 1 week of ***in vitro*** growth in DMEM-BS but was reduced from an initial 51.2% on day 1 to 5.1% in DMEM-FCS and to 32.6% in DMEM-BS/ACM.

GFAP

In all three media, the entire cell population of cells labelled with anti-GFAP after a week in culture (confirming that the cultures consisted entirely of glial cells), as compared to 11.8% in the overnight cultures. However, the intensity and pattern of this immunoreactivity varied between the media. In DMEM-FCS, GFAP-

immunoreactivity was faint and diffuse throughout the cytoplasm. Although faint, this immunoreactivity was well above background level as tested by omitting the primary antibody from the anti-GFAP labelling procedure. In contrast, when cells were grown in DMEM-BS or DMEM-BS/ACM (serum-free media), a proportion of cells acquired an intense and filamentous GFAP immunoreactivity (compare Fig. 3.6 B and 3.6 D). This proportion (averaged in Table 3.1) varied greatly between experiments. After 4 days in culture, it ranged from 30.9% to 73.2% in DMEM-BS and from 14.3% to 59% in DMEM-BS/ACM. After 7 days, the percentage of filamentous GFAP ranged from 23.5% to 75.1% in DMEM-BS and from 24.7% to 65.9% in DMEM-BS/ACM.

L1

The proportion of L1+ cells increased during the first 3 days in culture in both DMEM-BS and DMEM-BS/ACM. Due to a shortage of antibody (gift from Dr Schachner, Zurich), the L1 immunoreactivity of these cultures was not assessed beyond that stage nor was that of cultures grown in DMEM-FCS.

GAP-43

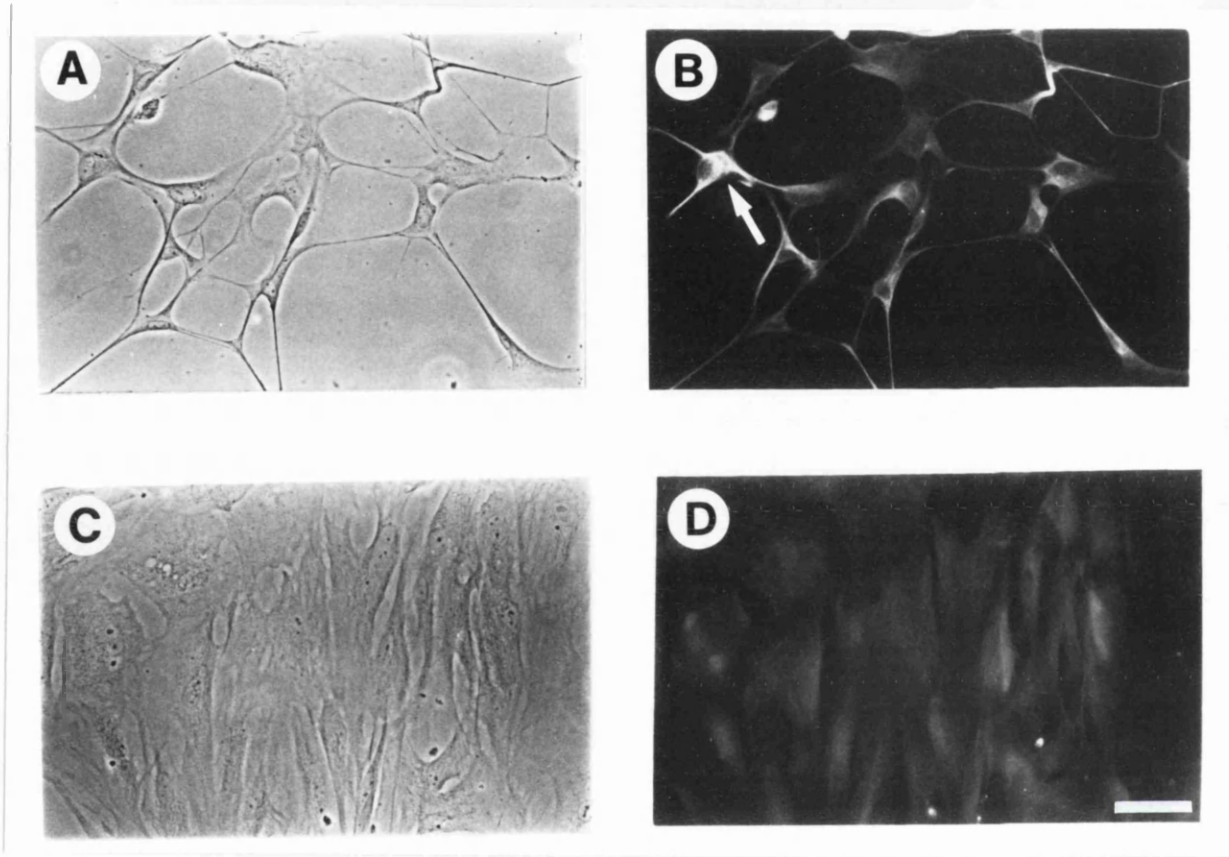
In both DMEM-BS and DMEM-BS/ACM but not in DMEM-FCS, approximately a tenth of the cell population acquired GAP-43 after 6-7 days *in vitro*. Double immunofluorescence studies showed that all GAP-43+ cells also expressed L-NGFr.

Longer term cultures

Cells were passaged between the first and second week in culture. After 3 weeks in culture the change in antigenic expression continued in a similar pattern as described above. PSA-NCAM expression was highest in cells grown in DMEM-BS. In one experiment, 68.4% of the cells labelled with anti-PSA-NCAM after 2 weeks in DMEM-BS. Expression of L-NGFr was constant at around 90% and O4 disappeared in DMEM-BS/ACM and DMEM-FCS. GFAP expression was more variable in

Figure 3.6

Differences in cell morphology and pattern of GFAP expression between OBECs grown in DMEM-BS/ACM and DMEM-FCS



Immunofluorescent labelling was carried out after 7 days of culture.

A: OBECs grown in DMEM-BS/ACM, viewed in phase contrast.
B: Immunofluorescent labelling of the field shown in (A) with anti-GFAP/fluorescein.
C: OBECs grown in DMEM-FCS, viewed in phase contrast.
D: Immunofluorescent labelling of the field shown in (C) with anti-GFAP/fluorescein.

Note the differences in cell morphology and GFAP-IR between the two cultures (compare A and C; B and D). Arrow in B points to a cell with intense and fibrous GFAP-immunoreactivity.

Photomicrographs, scale bar: 50 μm

FACS sorts over the longer term cultures. In DMEM-BS and DMEM-BS/ACM there was a mixed GFAP staining ranging from filamentous to diffuse. In DMEM-FCS, all of the filamentous GFAP staining disappeared leaving a faint diffuse label and the intensity of L-NGFr immunoreactivity was similarly reduced. Cells maintained in DMEM-BS rarely survived beyond 2-3 weeks in culture although cell viability appeared to increase when cells were plated at high density.

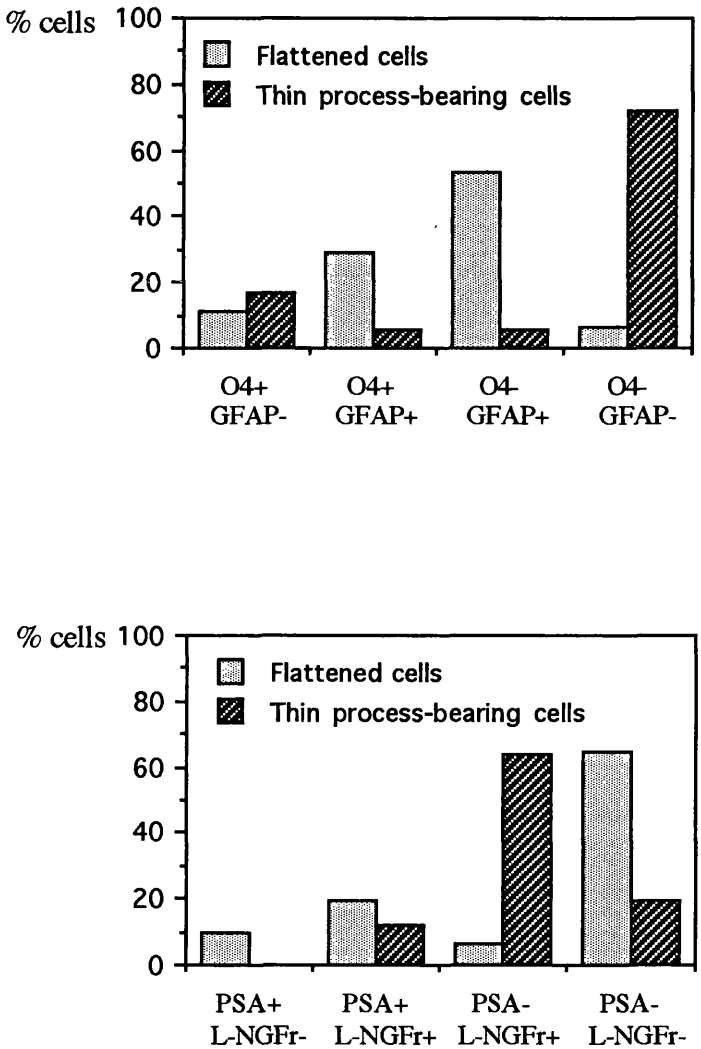
3.2.5 Heterogeneity of the cultures

Although the proportion of cells expressing O4, GFAP, L-NGFr and PSA-NCAM changed with time in culture, heterogeneity of the cultures was maintained for several weeks of *in vitro* growth in the serum-free media (DMEM-BS or DMEM-BS-ACM).

After 3-4 days of *in vitro* growth, it was possible to classify the cells not only according to their immunocytochemical profile but also according to their morphology. Two well defined morphological types, together with intermediate forms, could be identified in those cultures: i) phase bright, thin process-bearing cells, reminiscent of Schwann cells and ii) larger, less phase bright cells with a flattened morphology reminiscent of astrocytes. Most of the L-NGFr+ cells fall into the first category while most of the filamentous GFAP+ cells fall into the second (Fig. 3.7). Antibodies to PSA-NCAM or O4 did not as obviously discriminate between the two morphological types, although a slightly higher proportion of the flattened cells than of the process-bearing cells were labelled with O4 or with anti-PSA-NCAM (Fig. 3.7). Double immunolabelling of the cultures with anti-GFAP and anti-L-NGFr after 3 days in DMEM-BS/ACM showed that the L-NGFr+ and the filamentous GFAP+ cell populations overlapped slightly (Fig. 3.8).

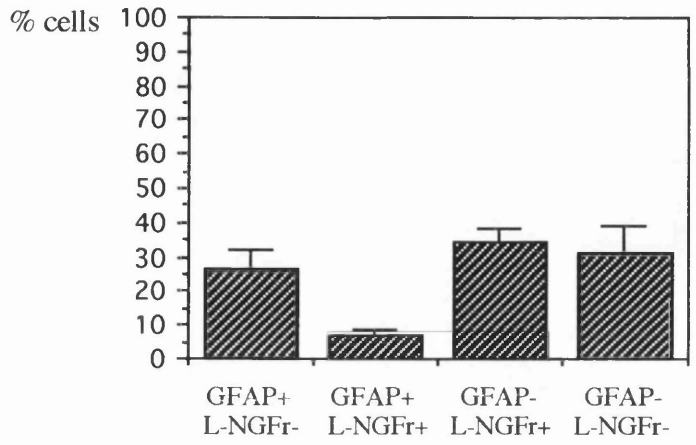
Figure 3.7

Antigenic profile of the flattened cell population and of the thin-process bearing cell population of 3-day-old OBEC cultures



Double immunofluorescent labeling was carried out on FACS-sorted OBECs grown for 3 days in DMEM-BS/ACM. The antibodies used were O4/rhodamine in conjunction with GFAP/fluorescein (upper histogram) and anti-PSA-NCAM/rhodamine in conjunction with anti-L-NGFr/fluorescein (lower histogram). The population of flattened cells (14% of total) and of thin process-bearing cells (23% of total) were counted separately. 200 cells from each population were counted.The data is derived from 1 representative sort.

Figure 3.8 **Antigenic profile of 3-day-old OBEC cultures (in DMEM-BS/ACM) using double immunofluorescence with anti-GFAP and anti-L-NGFr**

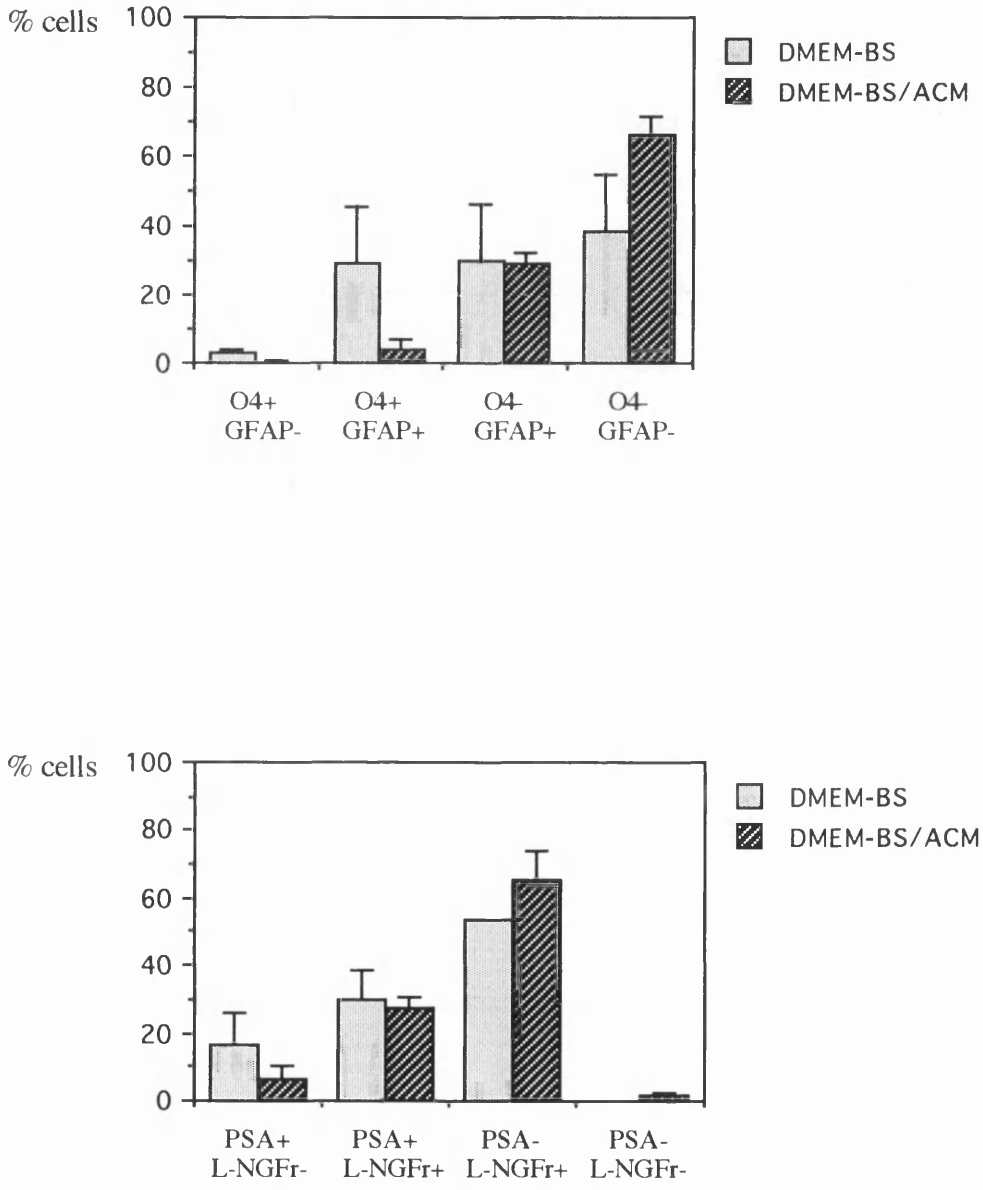


Double immunofluorescent labelling was carried out on FACS-sorted OBECs grown for 3 days in DMEM-BS/ACM. The antibodies used were anti-GFAP/fluorescein and anti-L-NGFr/rhodamine.

After 7 days of *in vitro* growth in the serum-free media, most of the cell population lost O4 but the cultures remained heterogeneous in terms of morphology and immunoreactivity to GFAP, L-NGFr, and PSA-NCAM. Double or triple immunofluorescent labelling with these markers showed the presence of a large variety of phenotypes (Fig. 3.9). Triple immunofluorescence labelling of a culture after 14 days in DMEM-BS/ACM is shown in Fig. 3.10. The thin process-bearing cells were always strongly L-NGFr+ and mostly PSA-NCAM- and diffuse GFAP+. The flattened cells were mostly filamentous GFAP+, and of variable PSA-NCAM-IR and L-NGFr-IR. The small proportion of L-NGFr- (approximately 10%) cells were always of flattened morphology, filamentous GFAP+ and of variable PSA-NCAM-IR. Cells of intermediate morphology were also present, which were L-NGFr+ and of variable GFAP-IR and PSA-NCAM-IR. Diffuse GFAP+/NGFr-/PSA-NCAM- and diffuse GFAP+/L-NGFr-/PSA-NCAM+ cells were never observed in these cultures.

Figure 3.9

Antigenic profile of 7 day-old OBEC cultures defined by double immunofluorescence with O4/anti-GFAP and with anti-L-NGFr/anti-PSA-NCAM



Double immunofluorescent labeling was carried out on FACS sorted OBECs grown for 7 days in either DMEM-BS or DMEM-BS/ACM. The antibodies used were O4/rhodamine in conjunction with GFAP/fluorescein (upper histogram) and anti-PSA-NCAM/rhodamine in conjunction with anti-L-NGFr/fluorescein (lower histogram). Each point represents the mean of at least three separate experiments.

Figure 3.10

Triple immunofluorescent labelling of OBEC cultures with antibodies against GFAP, PSA-NCAM, and L-NGFr

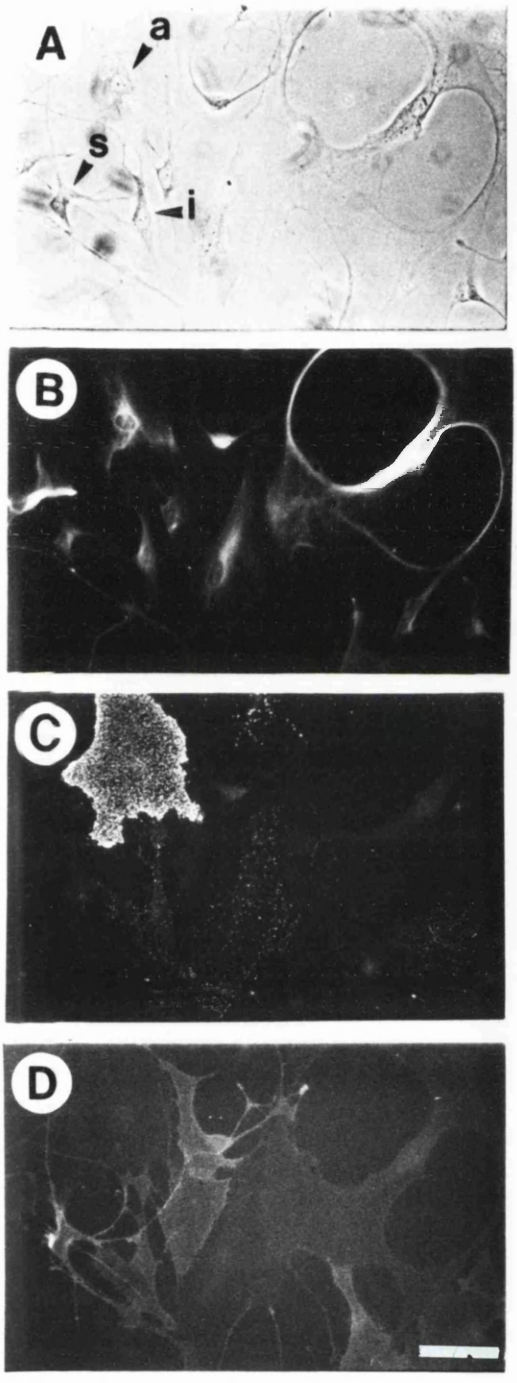
Immunofluorescent labelling was carried out on FACS-sorted OBECs after 2 weeks in DMEM-BS/ACM

A: OBECs viewed in phase contrast.

B, C, D: Triple immunofluorescent labelling of the field shown in (A) with anti-GFAP/AMCA (B), anti-PSA-NCAM/rhodamine (C), and anti-L-NGFr/fluorescein (D).

Astrocyte-like cells (a) are of flattened morphology, express fibrous GFAP, intense PSA and low or no levels of L-NGFr; Schwann cell-like cells (s) are thin with 2 to 4 processes, express diffuse GFAP, high levels of L-NGFr and no PSA. Cells of an intermediate morphology (i) can be observed that express various levels of the three antigens.

Photomicrographs, scale bar: 50 μm


A
a
s
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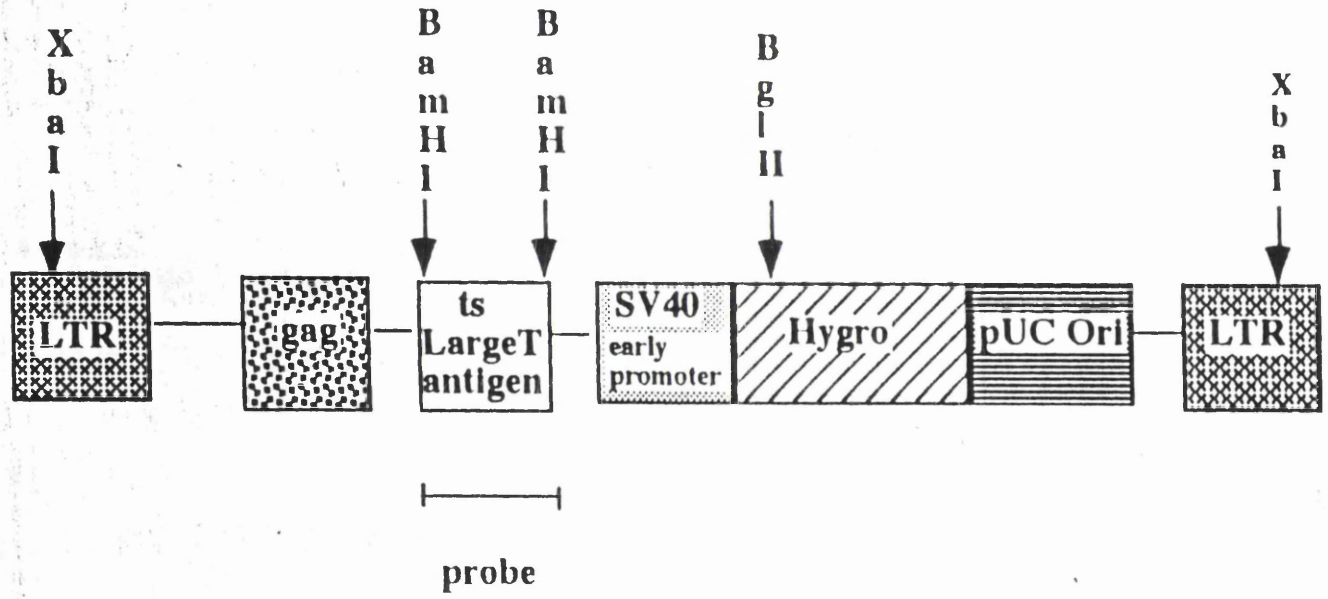
B
C
D

In the serum-containing medium, the majority of cells possessed a flattened morphology, PSA-NCAM expression was hardly present and GFAP and L-NGFr expression was faint resulting in a less obvious discrimination between cells.

3.2.6 Characterisation of the tsT cl 1.6 OBEC cell line.

It is apparent that two extreme OBEC cell types with Schwann cell-like and astrocyte-like phenotypes co-exist both *in vitro* and *in vivo*. To address the lineage relationship of these cells OBEC cell lines were generated by retroviral infection with the temperature sensitive (ts) mutant gene of Tag, thereby allowing to assess the clonality of the cell lines by Southern blot analysis using Tag as probe. Since the integration of retroviral DNA into the cell genome is thought to be random (Varmus and Swanstrom, 1984), a Southern blot analysis of a cell line showing a single site of viral integration is strong evidence that the clone originates from a single infected cell (Ryder *et al.* 1990). After retroviral infection and subsequent selection in 100 μg/ml of hygromycin, several tsT OBEC clones were established. After freezing stocks, DNA was isolated and cut with the restriction enzyme *Bam*H1 to illustrate the presence of Tag alone and with *Bgl II* to demonstrate the clonality of the cells, since *Bgl* II has a unique restriction site in the pBabe vector. The clone 1.6 (tsT cl 1.6) was shown to be of clonal origin from Southern blot analysis (Fig. 3.11).

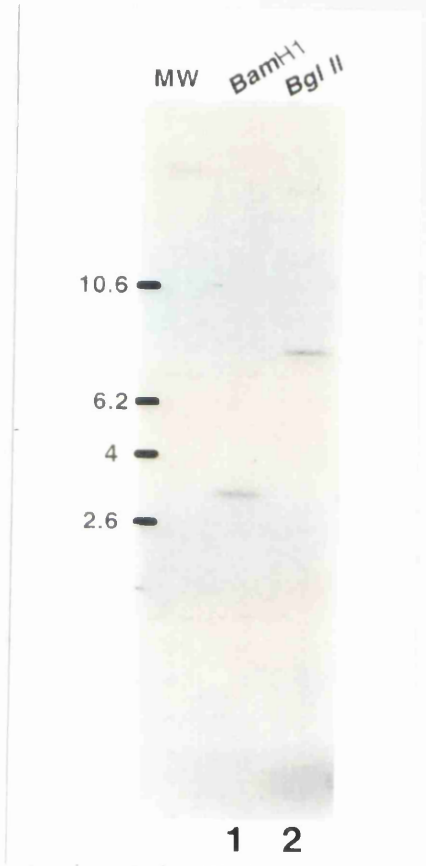
This cell line possessed a similar antigenic profile as long-term cultures of sorted OBECs (Table 3.2). DMEM-BS/ACM and activated Tag increased cell growth and viability. At both the permissive and non permissive temperature the entire cell population expressed GFAP, NCAM and Vm. The morphological and antigenic profile of the cultures however varied depending on cell density: at low density (where cell contact is nil) the great majority of the cells exhibited a flattened morphology, expressed low levels of diffuse GFAP, while only a few cells were of spindle morphology and expressed L-NGFr and S100. None of the cells expressed



Restriction map of the pBabe−tsU19−H vector. The *Bgl*II site is a unique site within the vector allowing the assessment of the clonality of cell lines.

Figure 3.11

Southern blot of the tsT cll 1.6. OBEC cell line genomic DNA with a probe against Tag, illustrating its clonality



Autoradiographic scan.

Lane 1 is a *Bam* HI digest, lane 2 is a *Bgl* II digest of 20 μg DNA of tsT cl1.6. The single band obtained after digestion with *Bgl* II illustrates the clonality of the cell line.

O4 or GAP-43. The proportion of cells that expressed L-NGFr, S100, GAP-43, or O4 increased with cell density.

Table 3.2 **Antigenic profile of the tsT cl. 1.6 OBEC cell line.**

Antibody	Percentage of cells labelled				
	OBEC cell line cultured				Sorted OBECs cultured long -term
	at low density		after spheroid formation		
	33°C	39°C	33°C	39°C	
O4	0	0	<5	0	<5
anti-L-NGFr	<5	<5	>95	>95	>95
anti-PSA-NCAM	0	<5	0	0	<5
anti-GFAP*	100	100	100	100	100
anti-GAP-43	0	0	31±6	28±8	41±8
anti-S100	<5	<5	>95	>95	>95
anti-Vm	100	100	100	100	100
anti-N-CAM	100	100	100	100	100

*GFAP immunoreactivity ranged from weak to intense and was generally diffuse throughout the cytoplasm. In spindle-like cells GFAP staining was intense around the nucleus and occasionally filamentous.

Fig 3.12 illustrates immunofluorescent labelling of the tsT cl. 1.6. cell line with anti-L-NGFr, anti-GFAP and O4. At confluency the cells initially formed aggregates and then spheroids that eventually detached from the cover slips (this phenomenon was not observed with primary cultures of OBECs, which seemed to adhere more strongly to the PLL substrate). A population of over 95% L-NGFr+/S100+ could be generated by replating these spheroids onto fresh PLL-coated cover slips (Fig. 3.12 D). The morphology of these cells ranged from spindle-like to the intermediate form (see definition in Fig. 3.10). Double immunofluorescence demonstrated that 31% of these L-NGFr+ co-expressed GAP-43. PSA-NCAM immunoreactivity could not be detected at 33°C but was induced in some cells by inactivation of Tag at 39°C (Fig. 3.13). The loss of a differentiation phenotype in the presence of Tag has been reported for many cell types and illustrates the usefulness of creating cell lines with a conditional gene product (Frederiksen *et al.* 1988; Barnett *et al.* 1993b). It is clear that the L-NGFr+ and PSA-NCAM+ phenotypes can be induced in the appropriate culture conditions and in the absence of Tag. Since this cell line is clonal, it is likely

Figure 3.12

Immunofluorescent labelling of the tsT cl 1.6. OBEC cell line with anti-GFAP, O4 and anti-L-NGFr (33°C)

A: TsT cl .6 OBECs viewed in phase contrast

B: Double immunofluorecent labelling of the cells showed in (A) with anti-GFAP/fluorescein (green) and O4/rhodamine (red).

C: TsT cl 1.6 OBECs after spheroid formation. Phase contrast.

D: Immunofluorescent labelling of the cells shown in C with L-NGFr/fluorescein.

Confocal image scan. Scale bar: 50 μm

Figure 3.12

Immunofluorescent labelling of the tsT cl 1.6. OBEC cell line with O4. anti-GFAP, and anti-L-NGFr

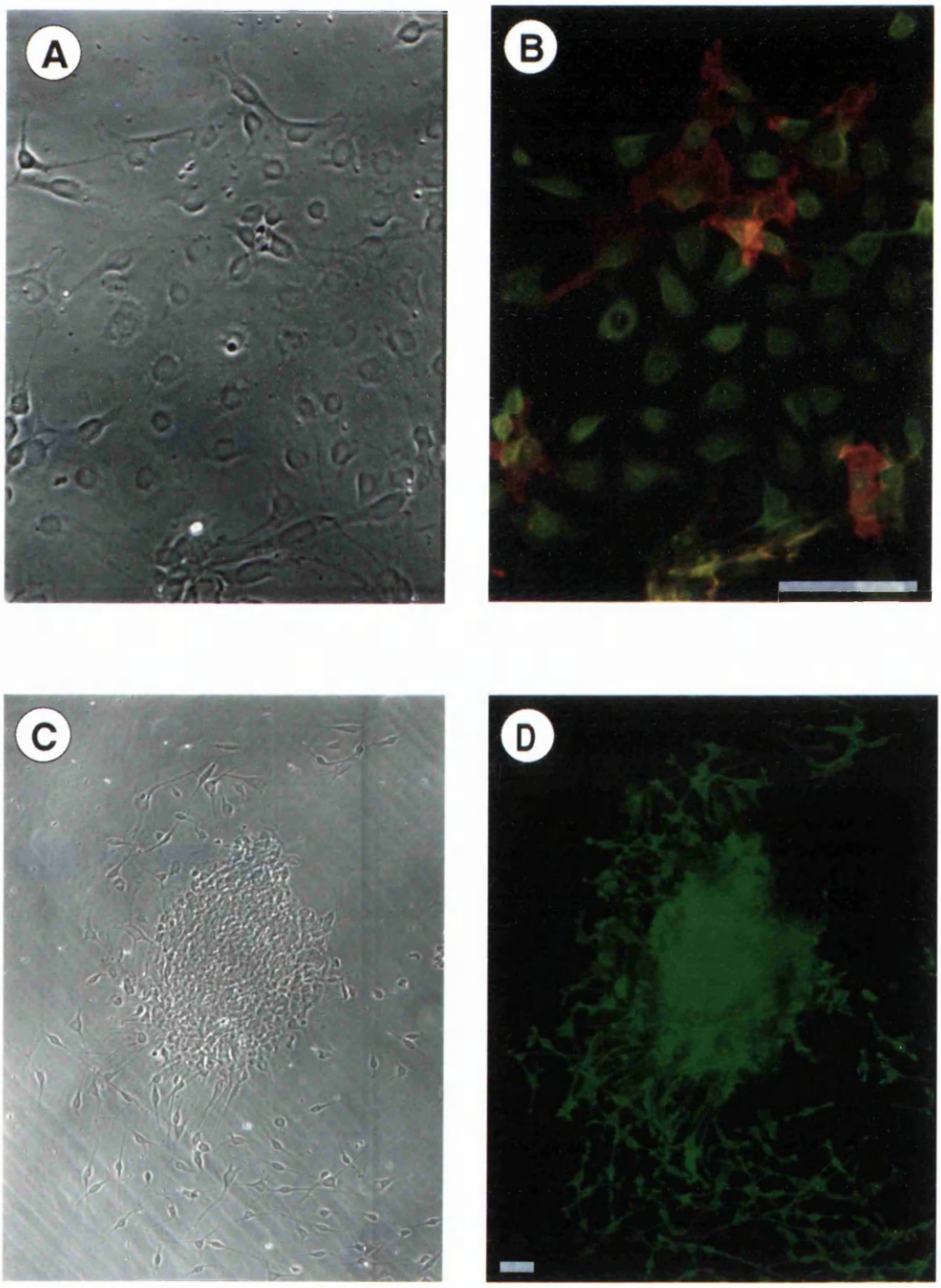
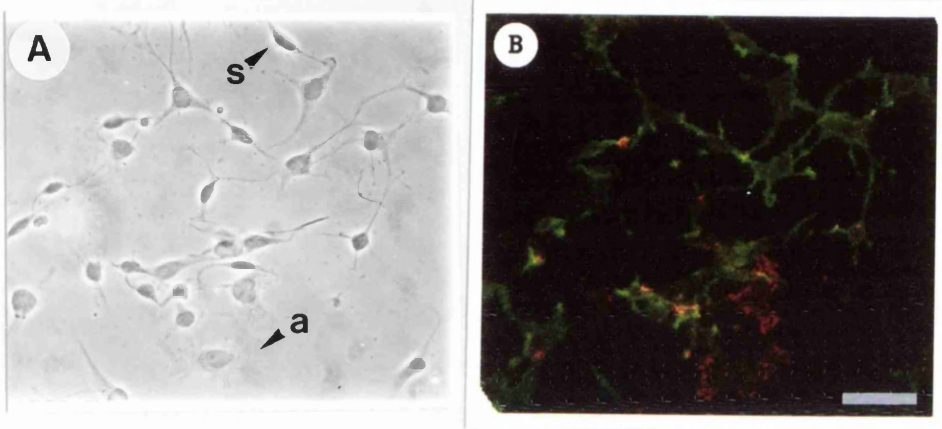


Figure 3.13

Double immunofluorescent labelling of the tsT cl 1.6. OBEC cell line with antibodies against NGFr and PSA-NCAM (39°C)



A: Phase contrast of a field, showing Schwann cell-like (s) and astrocyte-like (a) cells.

B: Double immunolabelling of the cells shown in (A) with anti-L NGFr/fluorescein (green) and anti-PSA-NCAM/rhodamine (red).

Confocal image, scale bar = 50 μm.

that these two antigenic phenotypes are characteristic of two cell types from the same lineage.

3.3 Discussion

3.3.1 OBECs *in vivo* represent a heterogeneous population of related cells

In order to precisely define the location and antigenic profile of OBECs *in vivo*, immunofluorescence analysis of olfactory bulb (OB) frozen sections was carried out using the O4 antibody in conjunction with a large panel of glial markers, and compared to the antigenic profile of the O4+ sorted cell population on the first day *in vitro*.

Immunoreactivity of OB sections to the O4 antibody was uniform throughout the entire ONL and extended up to periglomerular regions at the ONL/GL boundary, supporting the view that OBECs participate in the encapsulation of olfactory glomeruli (Marin-Padilla and Amieva, 1989; Valverde et al., 1992).

Antibodies against NCAM, Vm and GAP-43 labelled similar areas as O4. Immunocytochemical analysis of the O4+ sorted cells after an overnight culture confirmed previous immuno-electron microscopy studies that the NCAM- and Vim-immunoreactivity (IR) of the ONL was not only derived from olfactory axons but also from OBECs (Schwob et al., 1986; Miragall et al., 1988, 1989). The GAP-43-IR of the ONL, on the other hand, is likely to derive mainly, if not only, from olfactory axons as less than 2% of the sorted OBEC population expressed this antigen after an overnight culture. GAP-43 expression by olfactory axons has been shown previously (Verhaagen et al., 1989).

It is difficult to ascertain whether the few GFAP+ cells detected within the ONL on tissue sections represent OBECs, astrocytes or both. It has been suggested that the entire population of OBECs express GFAP in the adult rat OB (Barber and Lindsay, 1982).

Of particular interest was the finding that anti-L-NGFr and anti-PSA-NCAM labelled restricted areas of the O4+ ONL (Fig. 3.3). The cellular origin of PSA-NCAM and L-NGFr in the ONL has previously been investigated at the ultrastructural level. In developing mice (up to P7), PSA-NCAM-IR has been assigned to both immature OBECs and olfactory axons (Miragall et al., 1989). In 5-day-old rats, L-NGFr-IR has been assigned to OBECs exclusively (Vickland et al, 1991). The present study further shows, by double immunofluorescent labelling of OB tissue sections and of overnight cultures of FACS sorted O4+/GalC- cells, that antibodies to PSA-NCAM and L-NGFr label distinct populations of OBECs. Under 2% of the cells were L-NGFr+/PSA-NCAM-, 51% were PSA-NCAM+/L-NGFr- and the rest of the cell population was L-NGFr-/PSA-NCAM-, which correlated with the corresponding amounts of immunolabelling observed within the ONL on tissue sections. This observation raised the question whether these three antigenically distinct types of sorted cells share a common lineage or have distinct developmental origin. Several lines of evidence support the first hypothesis: 1) The 3 cell types possess common antigenic characteristics including O4, NCAM and Vim expression. 2) In cultures older than 1 week, all the cells could be labelled with either anti-L-NGFr or anti-PSA-NCAM and cells of intermediate phenotype, i.e. expressing both L-NGFr and PSA-NCAM, were also present. 3) Both L-NGFr+ cells and PSA-NCAM+ cells could be induced in the tsT cl.1.6 OBEC cell line, shown to be clonal by Southern blot analysis. From double immunofluorescent labelling of OB tissue sections, these 3 antigenically distinct populations presumably arise from restricted but well defined areas of the ONL. Thus the antigenic phenotype of OBECs is likely

to be controlled by important environmental cues *in vivo*. This will be discussed in detail in Chapter 4.

3.3.2 Insight into the regulation of OBEC antigenic expression

The decrease in the proportion of O4+ cells and increase in the proportion of L-NGFr+, GFAP+, or L1+ cells observed over time in culture in any of the 3 media used in this study, is likely to reflect a down- and up-regulation of these antigens, respectively, rather than differences in growth rates or viability between antigenically distinct subpopulations. For, these changes not only occurred under potent mitogenic conditions (DMEM-ACM and DMEM-FCS) but also in DMEM-BS, a medium which promoted less OBEC proliferation and double immunofluorescent labelling with anti-BrdU and different antigenic markers did not enable to identify a predominant subpopulation of dividing cells (see Chapter 5). Cell death, although not precisely assessed, did not appear consistent in any of the media, as judged from the amount of cellular debris. Several hypothesis can be made on mechanism regulating these antigenic changes, which are discussed below.

By analogy with previous studies on cultured sciatic nerve Schwann cells, the down-regulation of O4 expression and up-regulation of L-NGFr, GFAP and L1 may be due to the absence in the media of regulatory signals provided by neurons *in vivo* (Seilheimer et al., 1989; Jessen et al., 1990; reviewed by Mirsky and Jessen, 1990). Previous experiments in which olfactory neurons have been lesioned *in vivo*, have provided strong evidence that expression of L-NGFr (Turner and Perez-Polo, 1994; Gong and al., 1994) and GFAP (Barber and Dahl, 1987; Anders and Johnson, 1990) by the glial cells of the primary olfactory pathway depend on axon-glia signalling, involving membrane-membrane interactions and/or the secretion of very short range soluble factors. It has also been shown that OBECs in contact with olfactory neurites *in vitro*, down-regulate L-NGFr expression on the side of their membrane apposed to the neurite (Ramon-Cueto et al., 1993).

Part of the phenotypic changes observed on cultured OBECs may have been induced by exogenous factors present in the culture medium. This could account for the differences observed between the three culture media (Table 1). DMEM-BS is a defined serum-free medium containing a number of hormones which may act as signalling molecules for OBECs. DMEM-FCS and DMEM-ACM are ill-defined media containing a multitude of growth factors which may influence the growth and differentiation of OBECs. Factors produced by astrocytes have been partially characterised and include NGF (Furukawa et al., 1986; Assouline et al., 1987), FGF2 (Araujo et al., 1992), PDGF (Richardson et al., 1988), TGFβ (Saad et al., 1991), and CNTF (Rudge et al., 1992).

The antigenic phenotype of cultured OBECs might be partially controlled by autocrine signalling. Evidence for an autocrine regulation of L-NGFr and GAP-43 expression comes from observations made on the tsT cl.1.6 OBEC cell line. When these cells were grown in DMEM-BS/ACM at either the permissive or non-permissive temperature, the proportion of cells expressing either of these antigen significantly increased with cell density. The involvement of an autocrine mechanism in the regulation of GAP-43 expression has also been proposed for Schwann cells (Curtis et al., 1992). Autocrine signalling can be mediated by 3 types of interactions: secreted soluble factor/receptor, membrane/membrane or membrane/ECM interactions. Soluble factors that may be produced by OBECs include glial derived nexin (Reinhard et al. 1988; Scotti et al., 1994), PDGF (Kott et al., 1994), FGF1 (Lee et al; 1995) and neuropeptide Y (Ubink et al., 1994) which have been detected in these cells *in vivo.* It is however not known whether these factors are released by OBECs *in vitro* nor if OBECs express the corresponding receptor molecules. It has also been suggested that cultured OBECs secrete NGF-like molecules (Ramon-Cueto et al., 1993). This growth factor has previously been implicated in autocrine regulation of L1 expression by Schwann cells (Seilheimer and Schachner, 1987). ECM molecules produced by OBECs *in vitro* include laminin

and fibronectin (Denis-Donini and Estenoz, 1988; Ramon-Cueto and Nieto-Sampedro, 1992) but it is not known whether OBECs also express the corresponding integrin receptors. OBECs might also signal each other via homophilic and/or heterophilic binding involving the cell adhesion molecules NCAM and L1.

Some of the phenotypic changes affecting cultured OBECs may involve an intrinsic genetic programme. A detailed immunocytochemical analysis of the developing olfactory system has been undertaken to assess whether any of the changes in OBEC antigenic expression observed *in vitro* correlate with developmental changes *in situ* (see Chapter 4).

3.3.3 Emphasis on PSA-NCAM expression

This thesis demonstrates for the first time that OBECs express PSA-NCAM, a molecule that has been associated with structural plasticity (revied by Fryer and Hockfield, 1996). This characteristic therefore deserves particular attention. During the first week of *in vitro* growth, the proportion of PSA-NCAM+ cells did not change consistently in DMEM-BS (non mitogenic) and decreased slightly in DMEM-BS/ACM (mitogenic). It is not possible to ascertain from these data whether this decrease reflects a down-regulation of PSA-NCAM expression or a slightly higher growth rate and/or viability of the PSA-NCAM- cells in DMEM-BS/ACM. The presence of cells with variable intensities of PSA-NCAM-IR in DMEM-BS/ACM however suggest that some cells do down-regulate PSA-NCAM expression in this medium (see Fig. 3.11). Factors involved in the regulation of PSA-NCAM expression are unknown. A differentiation-related conversion of PSA-NCAM into the less sialylated adult form of NCAM occurs in most PSA-NCAM+ tissues during postnatal development and has also been shown in some glial cells *in vitro*. In the O-2A lineage, the loss in the expression of PSA-NCAM was shown to take place at the time when oligodendrocyte precursor cells start to express the O4 antigen (Trotter et al., 1989) and in astrocyte precursors, soon after the onset of GFAP expression

(Blass-Kampmann et al., 1994). It has also been shown that PSA-NCAM can be transiently induced in reactive astrocytes from discrete CNS regions following an experimentally created lesion (e.g. Le Gal la Salle et al., 1992; Lehman et al., 1993; Alonso and Privat, 1993; Nait Oumesmar et al., 1995). Whether these spatial differences in astrocytic PSA-NCAM expression result from micro-environmental differences or reflect intrinsic differences between astrocyte subtypes is not clear. A recent study suggests that PSA-NCAM expression by neurohypophyseal astrocytes of adult rats is dependant on axonal contact (Kiss et al., 1993). From the present *in vitro* data, it is unlikely that PSA-NCAM expression by OBECs strictly depends on axonal factors as it could be maintained in DMEM-BS after prolonged culture time in the absence of neurons.

3.3.4 Serum-free OBEC cultures contain astrocyte-like and Schwann cell-like cells

Despite considerable antigenic changes with culture time, the heterogeneous antigenic phenotype of OBECs described *in vivo* could be maintained to a certain extent *in vitro* with the appropriate culture condition. DMEM-BS, followed by DMEM-BS/ACM were the best conditions identified, for maintaining antigenic heterogeneity of the OBEC population as measured by PSA-NCAM expression. After a week of *in vitro* growth in these media, the culture where also heterogeneous, in terms of cell morphology, pattern of GFAP expression, L-NGFr and GAP-43 expression (Table 3.1 and Fig. 3.7). Double and triple-immunofluorescence labelling in conjunction with morphological analysis of the cultures showed the presence of a large variety of phenotypes and demonstrated a certain amount of correlation between PSA-NCAM, L-NGFr, GAP-43 and GFAP expression and/or cell morphology. In particular, two interesting phenotypes could be detected. The first cell type resembled cultured Schwann cells purified from neonatal rat sciatic nerves (Jessen et al., 1990), possessing a spindle-like morphology and expressing high levels of L-NGFr, diffuse GFAP, variable levels of GAP-43 and no PSA-

NCAM. The second cell type resembled cultured immature astrocytes (Blass Kampmann et al., 1993; Yokohama et al., 1993; Valles et al., 1994), possessing a flattened morphology and expressing filamentous GFAP, PSA-NCAM, variable levels of GAP-43 and no or low levels of L-NGFr. From now on, these cells will be referred to as Schwann cell-like OBECs (SLOBECs) and astrocyte-like OBECs (ALOBECs), respectively. Noteworthy, it has previously been shown that a subpopulation of Schwann cells can develop a flattened morphology and express filamentous GFAP under certain culture conditions (Morgan et al., 1991). However PSA-NCAM expression has so far not been detected in the Schwann cell lineage *in vitro* (Mirsky, personal communication) and its expression by Schwann cells *in vivo* is unclear (Boisseau et al., 1991; Zhang et al., 1995; Nait Oumesmar et al., 1995). For this reason, we believe ALOBECs are akin to astrocytes rather than to a subtype of Schwann cells.

The existence of OBECs that have inherent Schwann cell-like and astrocyte-like properties has been a matter of debate for many years (e.g. Willey et al. 1973; Barber and Lindsay, 1982; reviewed by Doucette, 1990). The present data suggests that the astrocytic and Schwann cell characteristics of OBECs are found in separate populations of cells. This may explain why only a few authors reported the presence of "true" astrocytes within the ONL (Doucette, 1984; 1991; Anders and Johnson, 1990; Bailey and Shipley, 1993). These may have been incorrectly identified and may in fact be ALOBECs. Two similar cell types have also been reported in serum-free cultures of newborn rat olfactory mucosa (Pixley, 1992) although PSA-NCAM expression was not investigated in that study. The immunocytochemical analysis of peripheral olfactory fascicle (POF) sections performed in the present study also suggest that GFAP and L-NGFr are found in separate population of cells: L-NGFr+ cells at the periphery of POFs and GFAP+ cells in the interior of POFs.

3.3.5 Possible lineage relationship between the astrocyte-like and Schwann cell-like OBECs

As discussed earlier, SLOBECs and ALOBECs are likely to belong to the same cell lineage. Considering the similarity between ALOBECs and immature astrocytes, it is tempting to speculate that these cells represent an immature stage of the OBEC lineage, possibly the precursors of SLOBECs. Alternatively, SLOBECs and ALOBECs may both represent mature stages of the OBEC lineage, derived from a common precursor cell. The fact that two similar cell types can be derived from PSA-NCAM-/L-NGFr- cells of the tsT cl. 1.6. OBEC cell line, supports this second hypothesis. A detailed immunocytochemical analysis of the developing olfactory system has been undertaken as an attempt to clarify this issue (Chapter 4).

CHAPTER FOUR

Immunocytochemical analysis of the developing olfactory system

4.1 Introduction

The results presented in chapter three have demonstrated the existence of three antigenic variants of OBECs in the P7 rat olfactory bulb: O4+/PSA-NCAM-/L-NGFr- cells, O4+/ PSA-NCAM+/ L-NGFr- cells and O4+/ PSA-NCAM-/ L-NGFr+ cells. When isolated by FACS sorting with the O4 antibody, and cultured in DMEM-BS or DMEM-BS/ACM, these cells gave rise to two morphologically and antigenically distinct cell types reminiscent of Schwann cells and astrocytes, respectively. In order to establish the relevance of these *in vitro* observations with the phenotype of these cells *in vivo*, an immunocytochemical analysis of the developing primary olfactory system, from embryonic day 14 (E14) to postnatal month 3 has been performed. This includes the olfactory epithelium, the olfactory nerves and the olfactory bulb. The results of this study also shed some light on the developmental processes (cell migration and differentiation) leading to the formation of the olfactory nerve layer (ONL).

4.2 Results

O4-immunoreactivity in the primary olfactory system was not detected until E17 and was continually present thereafter, until the third postnatal month (after which no further animals were examined). A number of OBEC markers however labelled the primary olfactory pathway prior to this developmental stage, including anti-GAP-43, anti-Vm, anti-NCAM, anti-PSA-NCAM and anti-L-NGFr. The time course and localisation of antigen expression is summarised in Tables 4.1 and 4.2.

Table 4.1 **Immunocytochemical analysis of the primary olfactory pathway during embryonic development**

	PNS										CNS																	
Antigen	Centre of ONF					Surface of ONF					Surface of MM/ONL					Outer MM/ONL					Inner MM/ONL					MM/ONL-brain boundary[1]				
	E14	E16	E17	E18	P0	E14	E16	E17	E18	P0	E14	E16	E17	E18	P0	E14	E16	E17	E18	P0	E14	E16	E17	E18	P0	E14	E16	E17	E18	P0
O4	-	-	±	±	nd	-	-	±	±	nd	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
L-NGFr	-	-	-	-	nd	+	+	+	+	nd	+	+	+	+	+	-	-	±	±	±	-	-	-	-	-	-	+	±	±	-
PSA-NCAM	+	nd	+	+	nd	-	nd	-	-	nd	-	nd	-	-	-	+	nd	+	+	+	-	nd	-	-	-	-	-	-	-	+
GFAP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.2 **Immunohistochemical analysis of the olfactory nerve layer during postnatal development.**

Antigen	Surface of ONL					Outer ONL					Inner ONL					ONL/GL boundary				
	P2	P7	P14	P28	Ad	P2	P7	P14	P28	Ad	P2	P7	P14	P28	Ad	P2	P7	P14	P28	Ad
O4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	++
L-NGFr	+	+	±	-	-	±	±	±	-	-	-	-	-	-	-	-	±[2]	+	+	+
PSA-NCAM	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+
GFAP	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+

Note. Expression of GAP-43, NCAM, and Vm was constant throughout development.
ONF: olfactory nerve fascicle; MM: migratory mass; ONL: olfactory nerve layer; Ad= adult rat (3 months old).
1: The MM-brain boundary is defined, at E16, by a layer of L-NGFr+ cells ("deep" L-NGFr+ layer). This boundary becomes less clear at later developmental stages as the ONL becomes partially incorporated into the telencephalon, resulting in the distinction between an inner ONL and an outer ONL.
2: L-NGFr-IR around the glomeruli was very faint and variable at P7.

4.2.1 Immunocytochemical analysis of the E14-15 rat olfactory system

At E14, the presumptive ONL was identified as a GAP-43+ tissue at the medio-ventral surface of the telencephalon (Fig. 4.1 A). This tissue has been referred to in the literature as "migratory mass" (MM) because it ultrastructurally consists of axons and cells that have migrated from the olfactory epithelium to the olfactory bulb (Valverde *et al.* 1992). At E15, the olfactory bulb primordium appeared as an evagination of the telencephalon, located dorsal to the MM (Fig. 4.1 B).

The MM and the olfactory axons, which extended from the olfactory epithelium to the MM, were labelled with antibodies to GAP-43, NCAM and Vm. No GAP-43-immunoreactivity was detected within the developing telencephalon (Fig. 4.1), confirming previous work (Dani et al., 1990). Along the entire pathway of the olfactory nerves PSA-NCAM-immunoreactivity (IR) and L-NGFr-IR were associated with distinct areas (Fig 4.2): a thin L-NGFr+ layer encompassing larger PSA-NCAM+ area. L-NGFr-immunoreactivity was detected as a thin, filamentous layer that enclosed the MM and extended towards the dorsal surface of the cerebral vesicle (Fig. 4.2). Along the peripheral parts of the olfactory nerves, L-NGFr-immunoreactivity was more scattered and less intense than at the surface of the MM. The L-NGFr+ layer on the surface of the MM ("peripheral" L-NGFr layer) was disrupted occasionally and double immunofluorescent labelling with anti-PSA-NCAM and anti-L-NGFr showed that PSA-NCAM+ elements extended through these openings (Fig. 4.2). The PSA-NCAM+ structures of the MM were intensively immunoreactive on its ventral side. Their immunoreactivity to anti-PSA-NCAM appeared to diminish as they progressed towards the telencephalon.

4.2.2 Immunocytochemical analysis of the E15-18 rat olfactory system

Between E15 and E18, the size of the primary olfactory system increased, the evagination of the telencephalon became accentuated and the MM extended towards the dorsal and lateral sides of each olfactory bulb, giving rise to the definitive ONL

Figure 4.1

Immunofluorescent labelling of the E14 and E15 primary olfactory pathway with anti-GAP-43

Immunofluorescent labelling was carried out on 5-8 μm-thick sagittal cryostat sections through the nasal and cranial cavity of an E14 (**A**) and E15 rat (**B**).

These low magnification photomicrographs show an overview of the developing telencephalon (T) and primary olfactory projection. GAP-43-IR is found i) along longitudinal elements (arrows) identified as bundles of olfactory axons (POFs), extending from the olfactory epithelium to the ventral surface of the developing telencephalon, and ii) on a thick tissue apposed the ventral surface of the telencephalon, identified as the migratory mass (MM). No GAP-43-IR was found within the telencephalon at these developmental stages. The evagination of the telencephalon, giving rise to the prospective olfactory bulb occurs between E14 and E15 (compare A and B).

Photomicrographs, scale bar: 200 μm

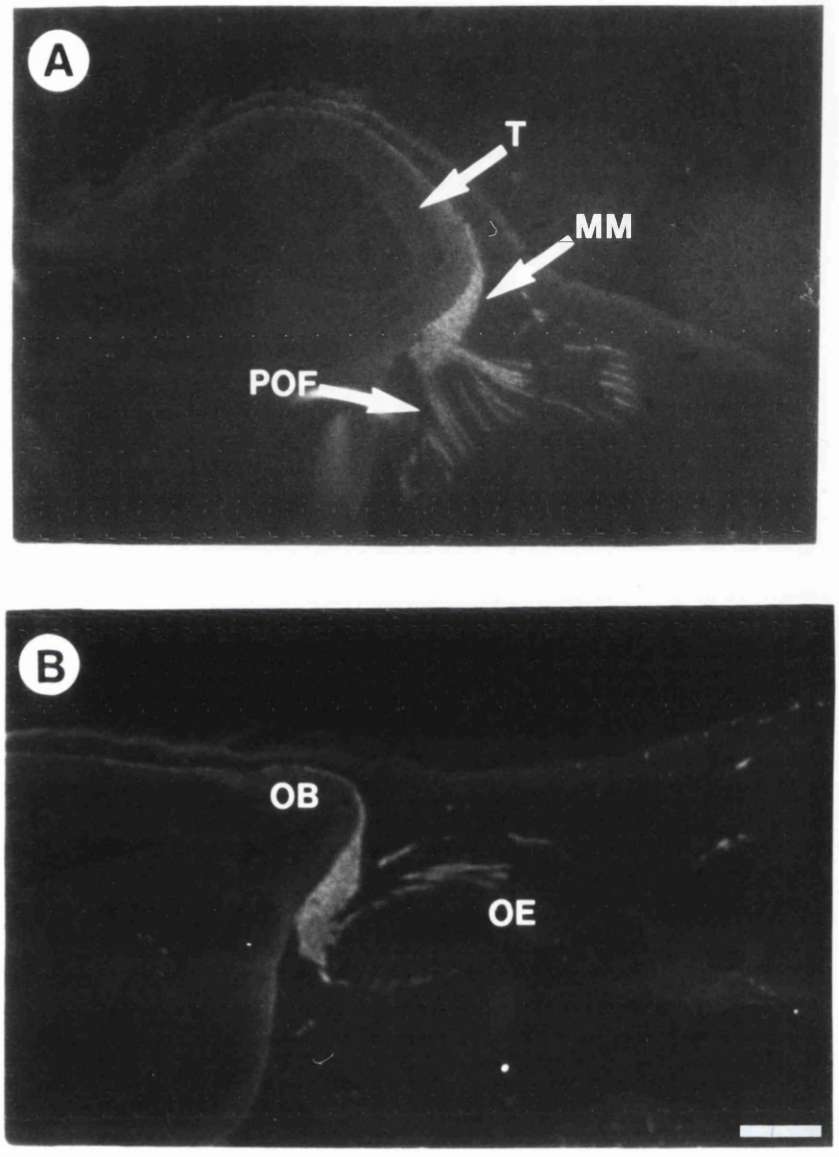
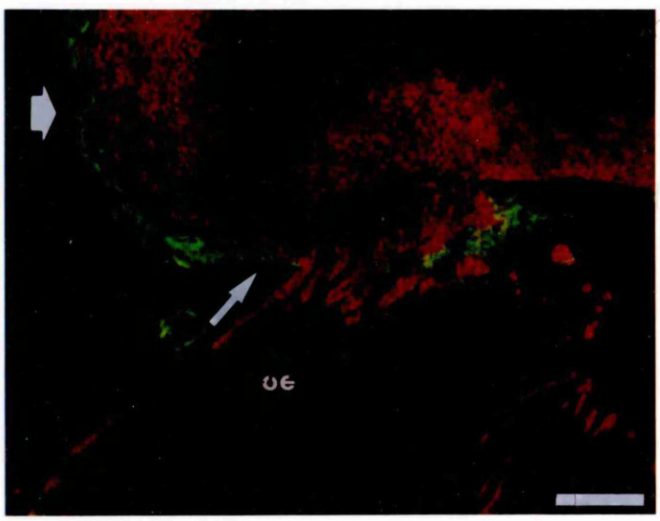

A
T
MM
POF
B
OB
OE

Figure 4.2

Double immunofluorescent labelling of the E14 primary olfactory pathway with antibodies against PSA-NCAM and L-NGFr



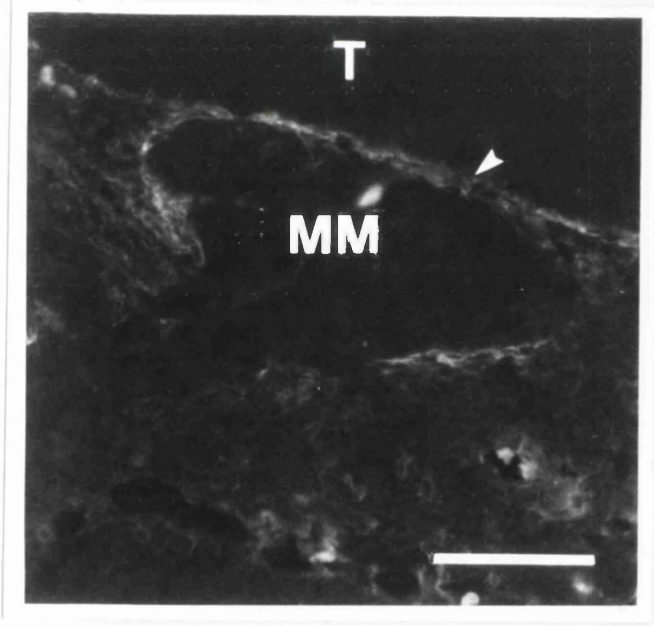
Immunofluorescent labelling was carried out on a 5-8 μm-thick sagittal cryostat section through the nasal and cranial cavity of an E14 rat.

Double immunofluorescent with anti-L-NGFr/fluorescein (green) and anti-PSA-NCAM/rhodamine (red). Note that anti-L-NGFr and anti-PSA-NCAM label distinct elements within the primary olfactory projection. L-NGFr-IR defines a thin layer at the periphery of the migratory mass (arrow) and extends along the dorsal surface of the telencephalon (arrowhead). In some places, the L-NGFr+ covering of the MM is disrupted by PSA-NCAM+ elements that extend from the olfactory epithelium (oe) to the centre of the MM.

Confocal image scan, scale bar: 100 μm

at E18. The same general pattern of immunoreactivity as described for E14 was detected along the peripheral olfactory nerves and in the MM with antibodies to GAP-43, Vm, NCAM and PSA-NCAM. The main changes to occur were: i) at E16 an additional L-NGFr labelled sublayer could be detected at the junction between the MM and the cortex (Fig. 4.3). This layer will be referred to as "deep" L-NGFr+ layer as opposed to the previously defined "peripheral" L-NGFr+ layer. ii) At E17, O4-immunoreactivity was first detected in the primary olfactory system predominantly in the MM, although it could also be detected in the peripheral olfactory nerve fascicles, albeit weaker and scattered.

Figure 4.3 **Immunofluorescent labelling of the MM with anti-L-NGFr at E16**



Immunofluorescent labelling was carried out on a 5-8 mm-thick horizontal cryostat section through the nasal and cranial cavity of an E16 rat.
MM: migratory mass
T: telencephalon
Photomicrographs, scale bar:100um

At E18, the olfactory bulb could be clearly identified as a pronounced evagination of the cerebral vesicle, and the MM had developed into the ONL by expansion towards its caudal, rostral, lateral, medial and dorsal sides as seen from sagittal (Fig. 4.4 A) and coronal (Fig. 4.5 A) sections labelled with O4 or anti-GAP-43. The ONL appeared thicker at the ventral surface of the olfactory bulb (former MM) than on its dorsal side. In this ventral region, O4 and anti-GAP-43 showed a similar pattern of immunoreactivity, except that there was broken a line of GAP-43- tissue halfway throughout the ONL (Fig. 4.4 B, arrow), whereas O4 showed a continuous pattern of immunolabelling (Fig. 4.4 C). This O4+/GAP-43- tissue marked the limit between a GAP-43+/PSA-NCAM+ "outer ONL" and a GAP-43+/PSA-NCAM- ONL (Fig. 4.4, D, E, star and asterix, respectively). Double immunofluorescent labelling with anti-PSA-NCAM and anti-L-NGFr showed that it corresponded to the previously defined deep L-NGFr+ layer (Fig. 4.4 F, G). Double immunofluorescent labelling with O4 and anti-L-NGFr demonstrated that both, the deep and peripheral L-NGFr+ layers, were included in the O4+ area (Fig. 4.5 B, C). The deep L-NGFr+ layer appeared disrupted (arrowhead in 4.4 F and 4.5.C).

4.2.3 Immunocytochemical analysis of the ONL and GL from E18 onwards.

From E18 onwards, the main anatomical changes detected in the primary olfactory system were an increase in size and a progressive lamination of the olfactory bulb into its different cellular compartments. Glomeruli could be clearly recognised at birth, as round GAP-43+ structures adjacent to the GAP-43+ ONL. The pattern of immunoreactivity (IR) in the ONL with antibodies to HNK-1, GAP-43, NCAM and Vm was similar during all postnatal stages studied (Fig. 4.6). O4-IR was continually expressed throughout the entire width of the ONL, from the pial surface to the ONL/GL boundary (Fig. 4.7). This O4-IR was intense and homogeneous at embryonic and neonatal stages. In rats older than 1 month, the O4-IR became more scattered throughout the ONL and more intense at the ONL/GL boundary (Fig. 4.8 A). Curiously, some O4-IR was temporarily seen within the

Figure 4.4

Differential labelling of the MM/ONL with antibodies against GAP-43, O4, PSA-NCAM and L-NGFr at E18

Immunofluorescent labelling was carried out on 5-8 μm-thick sagittal cryostat sections through the nasal and cranial cavity of an E18 rat.

A: Immunofluorescent labelling of a section with O4/rhodamine. This low magnification photomicrograph shows an overview of the olfactory bulb and underlying nasal cavity. ob: olfactory bulb; mm/onl: migratory mass/olfactory nerve layer; oe: olfactory epithelium. Note the pronounced evagination of the telencephalon that has given rise to the olfactory bulb. A layer of O4+ cells can be seen around the olfactory bulb. This layer appears thicker on its rostro-ventral surface. 2 regions consecutive to the plain boxed area are viewed at higher magnification in sections B/C and D/E. Section G/H is through a more caudal area of the olfactory nerve layer.

B, C: Double immunofluorescent labelling of a section with anti-GAP-43/fluorescein (C) and O4/rhodamine (D)

D, E: Double immunofluorescent labelling of a section with anti-GAP-43/fluorescein (E) and anti-PSA-NCAM/rhodamine (F)

F, G: Double immunofluorescent labelling of a section with anti-L-NGFr/fluorescein (G) and anti-PSA-NCAM/rhodamine (H)

Arrows in B-G point to the limit between an inner (asterix) and outer (star) ONL.

Photomicrographs, scale bar: 100 μm

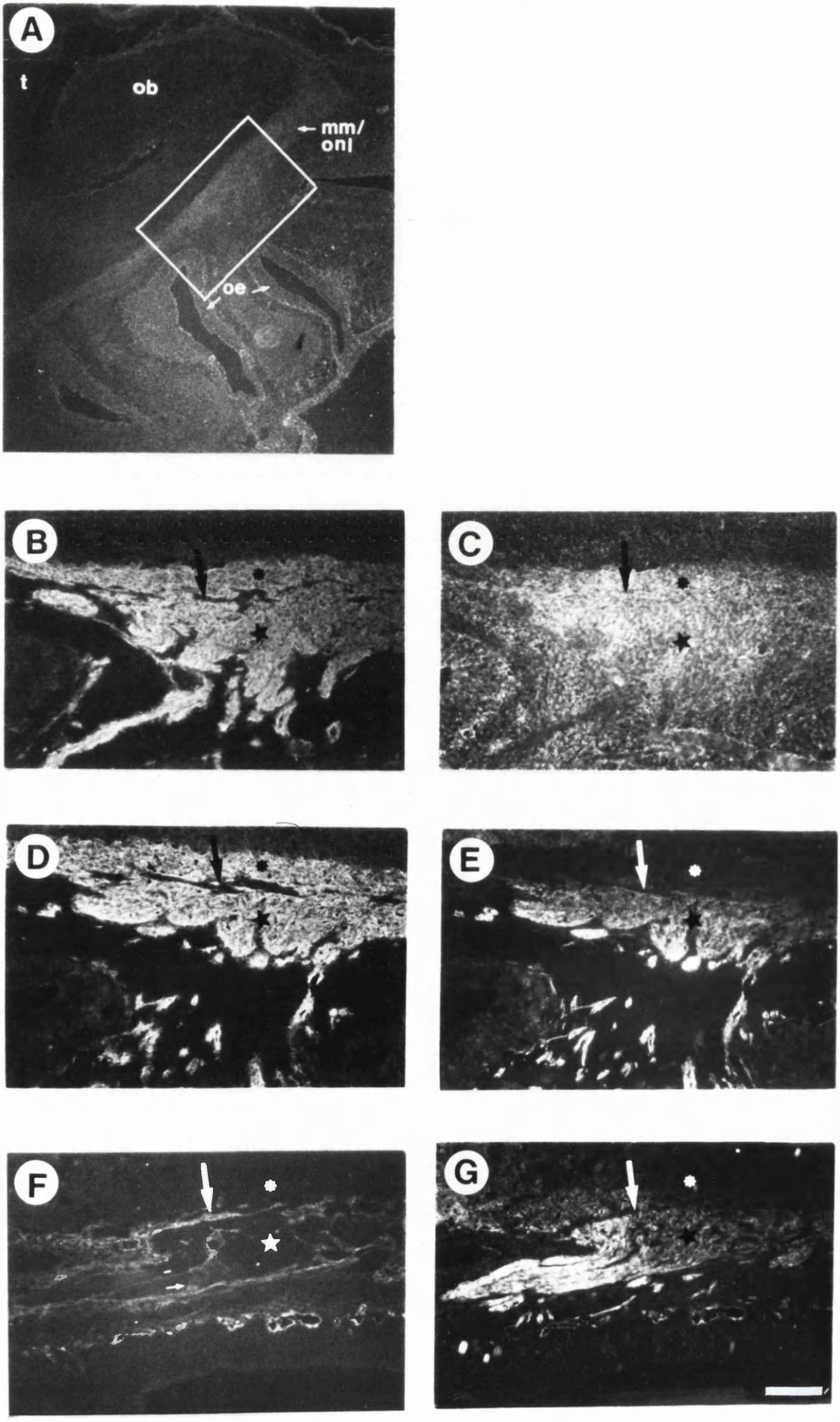
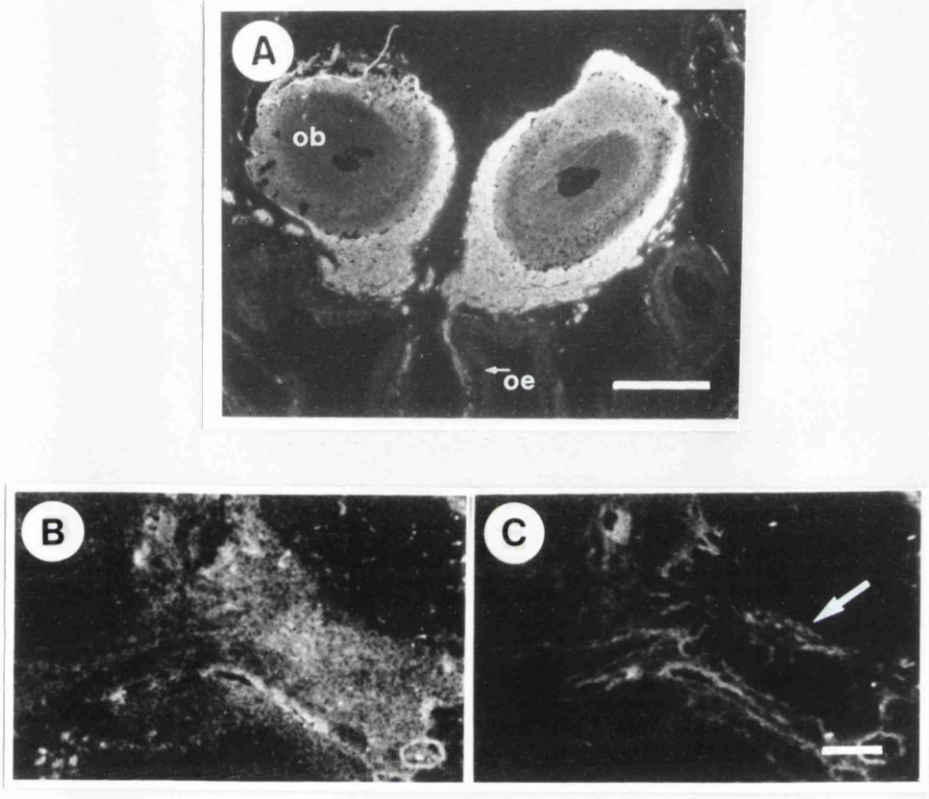

A
t
ob
mm/
onl
oe
B
C
D
E
F
G

Figure 4.5

Double immunofluorescent labelling of the MM/ONL with antibodies against O4 and L-NGFr at E18.



Immunofluorescent labelling was carried out on 5-8 μm-thick coronal cryostat sections through the nasal and cranial cavity of an E18 rat.

A: Immunofluorescent labelling of a section with GAP 43/fluorescein. This low magnification photomicrograph shows an overview of the olfactory bulbs and underlying nasal cavity. ob: olfactory bulb; oe: olfactory epithelium

B, C: Double immunofluorescent labelling of a section with O4/rhodamine (B) and anti-L-NGFr/fluorescein (C).

Arrow in C points to the deep L-NGFr+ layer.

Photomicrographs, scale bar: 100 μm

mitral cell layer during early neonatal stages (Fig. 4.7, A). Several authors have reported that some OBECs possess a deep apical process that extends radially towards the mitral cell layer (Blanes, 1898; Raisman, 1985; Doucette, 1989). The O4-IR of the mitral cell layer may be derived from these deep OBEC processes. O4-IR appeared in two additional areas of the olfactory bulb between P14 and postnatal month 1: i) along the deep borders of olfactory glomeruli and ii) within submitral areas. This immunoreactivity correlated with GalC-IR (4.8) and with the spatial and temporal appearance of oligodendrocyte/myelin immunoreactivity in the developing olfactory system (Philpot et al., 1995) and can therefore be attributed to differentiating oligodendrocytes. GFAP-IR was strongly expressed in the glomerular layer during the neonatal period (Fig. 4.7). Faint, scattered GFAP-IR was detected within the ONL from P7 and continued to increase into adulthood (Fig. 4.7). Fig. 4.9 illustrates double immunofluorescent labelling of olfactory bulb tissue sections with anti-PSA-NCAM and anti-L-NGFr throughout postnatal development. These antibodies labelled restricted areas of the olfactory bulb until P7. From P7 to postnatal month 3, both antigens were detected in the glomerular layer but it was difficult to assess at the light microscopic level whether they were derived from the same cells. The outer and inner L-NGFr+ layers were no longer detected in animals older than 1 month. At P7, L-NGFr-IR started to appear around glomeruli, albeit very weakly, and increased from P7 onwards. PSA-NCAM-IR was restricted to the outer ONL during embryonic development. In newborn rats, it was also detected in the GL, including the ONL/GL boundary. This correlates with the time at which olfactory glomeruli are first identified. The PSA-NCAM-IR in these two areas was maintained until adulthood.

Figure 4.6

Postnatal evolution of GAP-43-, Vm-, HNK-1- and S100- and O4-immunoreactivity in the olfactory bulb

Immunofluorescent labelling was carried out on 5-8 μm-thick coronal cryostat sections through the olfactory bulbs of rats from various postnatal stages. Immunofluorescent labelling of olfactory bulb sections from postnatal ages P7 (A-E) and 3 month (F-J) with anti-GAP-43 (A, F), anti-Vm (B, G), HNK-1 (C, H), anti-S100 (D, I), and O4 (E, J).
The ONL/GL boundary is indicated by an arrowhead in all pictures.

Photomicrographs, scale bar: 100 μm

GAP_43
VM
HNK_1
S100
O4
A
B
C
D
E
F
G
H
I
J

Figure 4.7

Postnatal evolution of O4- and GFAP-immunoreactivity in the olfactory nerve layer

Immunofluorescent labelling was carried out on 5-8 μm-thick coronal cryostat sections through the olfactory bulbs of rats from various postnatal stages.

Double immunofluorescent labelling of olfactory bulb sections from postnatal ages P0 (**A, B**), P7 (**C, D**), P14 (**E, F**) and Postnatal month 1 (**G, H**) with O4/rhodamine (A, C, E, G) and GFAP/fluorescein (B, D, F, H).

I, J: Two sections through a 3-month-old rat olfactory bulb labelled with anti-GFAP/fluorescein. In section I, GFAP-IR in the ONL is seen on fine lines oriented parallel to the surface of the olfactory bulb. These GFAP+ structures may represent processes of OBECs and/or astrocytes, running along an olfactory nerve fascicle sectioned longitudinally. In section J, GFAP-IR is seen scattered within round structures representing cross sections of olfactory nerve fascicles.

onl: olfactory nerve layer
gl: glomerular layer
mcl: mitral cell layer

Photomicrographs, scale bar: 100 μm

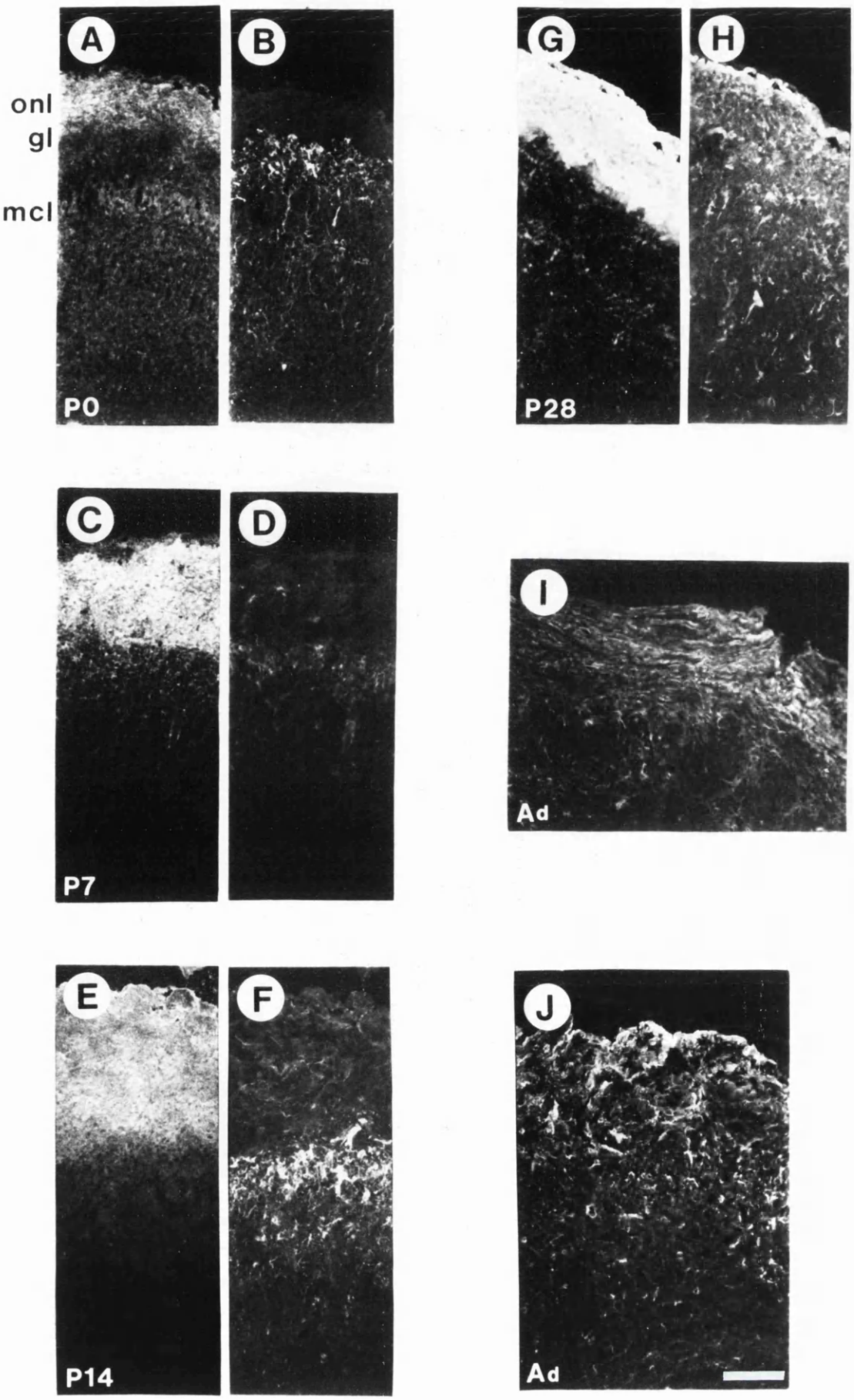
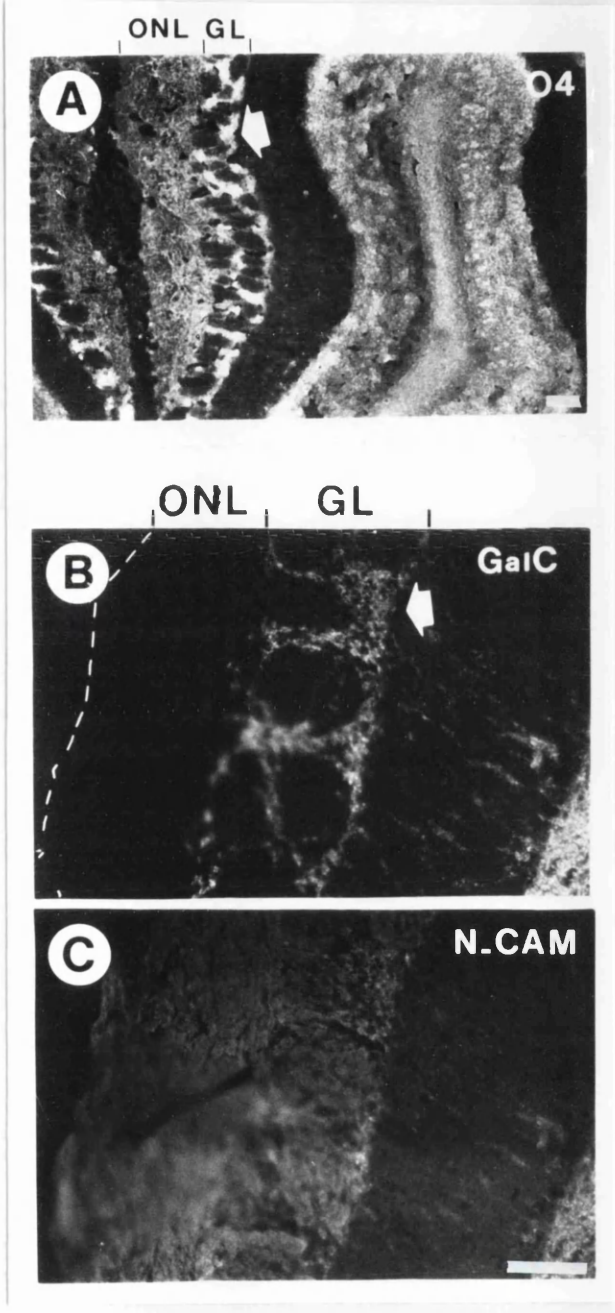

A
B
onl
gl
mcl
P0
G
H
P28
C
D
P7
I
Ad
E
F
P14
J
Ad

Figure 4.8

Co-localisation of O4- and GalC-immunoreactivity in the glomerular layer of adult rats



Immunofluorescent labelling was carried out on 5-8 µm-thick coronal cryostat sections through 3-month-old-rat olfactory bulbs.

A: Immunofluorescent labelling of a section with O4/rhodamine.

B, C: Double immunofluorescent labelling of a section with anti-GalC/fluorescein (B) and with anti-NCAM/rhodamine (C), to visualise the position of the ONL.

Note the colocalisation of O4 and GalC in the glomerular layer (arrowhead).

Photomicrographs, scale bar: 100 µm

Figure 4.9

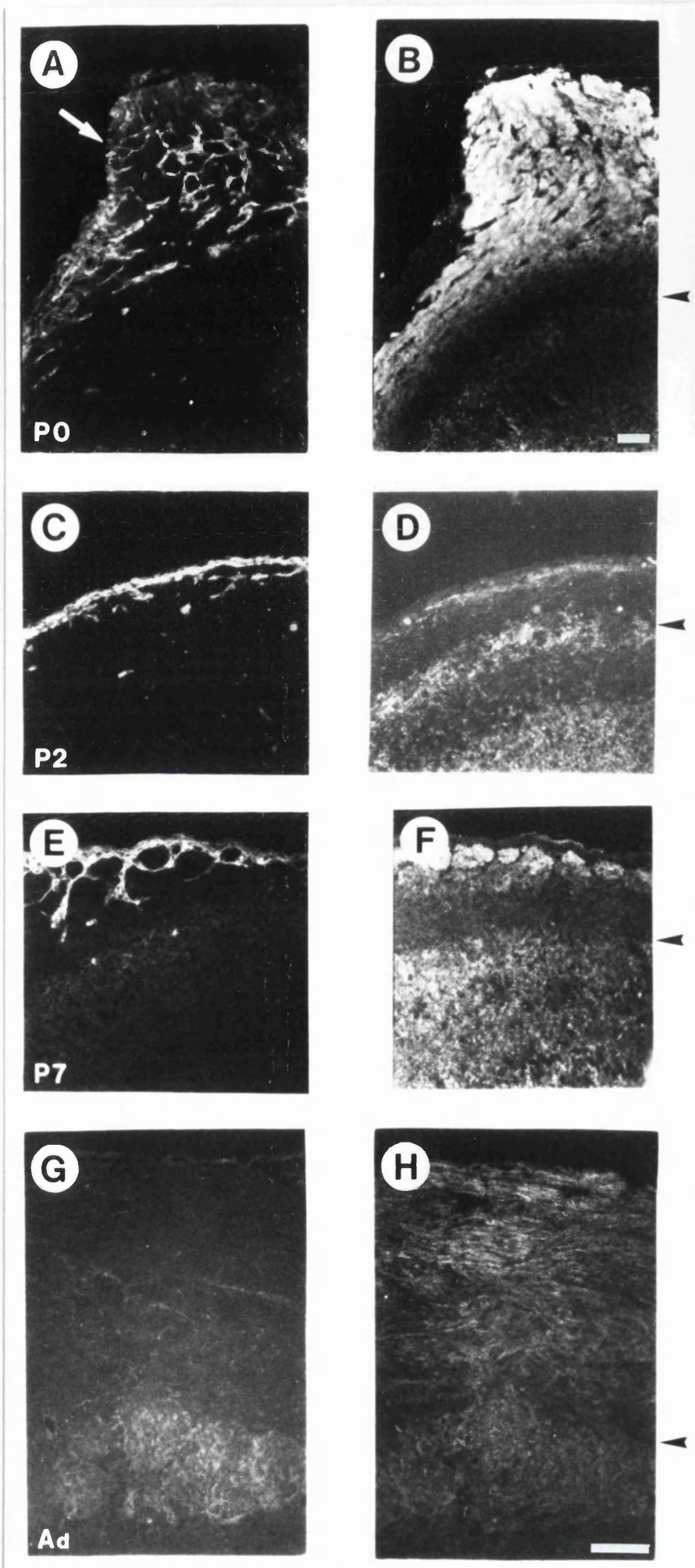
Postnatal evolution of PSA-NCAM- and L-NGFr-immunoreactivity in the olfactory nerve layer

Immunofluorescent labelling was carried out on 5-8 μm-thick coronal cryostat sections through the olfactory bulbs of rats from various postnatal stages.

Double immunofluorescent labelling of olfactory bulb sections from postnatal ages P0 (**A, B**), P2 (**C, D**), and postnatal month 3 (**G, H**) with anti-L-NGFr/fluorescein (A, C, G) and anti-PSA-NCAM/rhodamine (B, D, H). Arrow in A points to an olfactory nerve rootlet at its point of entry in the ONL.
E: A coronal section of a P7 olfactory bulb labelled with anti-L-NGFr/fluorescein.
F: A coronal section from the same region as section E, labelled with anti-PSA-NCAM/rhodamine.

Arrowheads in B, D, F, H, indicate the position of the ONL/GL boundary

Photomicrographs, scale bar: 100 μm


A
P0
B
C
P2
D
E
P7
F
G
Ad
H

4.3 Discussion

4.3.1 Antigens expressed by embryonic olfactory ensheathing cells prior to O4

The earliest OBEC markers to label the primary olfactory pathway were those against GAP-43, Vm, NCAM, PSA-NCAM and L-NGFr. These antigens were detected at E14 (the earliest stage studied), i.e. 3 days before the appearance of O4-immunoreactivity, from the exit of the developing olfactory epithelium to the presumptive ONL/MM. Previous ultrastructural and immunocytochemical studies have shown that during embryonic development, this pathway consists of olfactory axons and various types of migrating cells including embryonic olfactory ensheathing cells (which are believed to give rise to both OBECs and ONECs), LHRH-producing cells and OMP+ cells (Farbman and Squinto, 1985; Doucette, 1989; Marin-Padilla and Amieva, 1989; Wray et al., 1989; Schwantzel-Fukuda and Pfaff, 1989; Valverde et al., 1992, 1993; De Carlos et al., 1995).

The pattern of L-NGFr immunoreactivity observed in the present study during embryonic development supports previous reports that it is essentially derived from embryonic olfactory ensheathing cells (Yan and Johnson, 1988; Gong et al., 1994). In addition, immature leptomeningeal cells may contribute to the particularly intense L-NGFr-IR seen at the surface of the MM (Yan and Johnson, 1988).

In contrast, the pattern of immunoreactivity to GAP-43, Vm, NCAM and PSA-NCAM suggests that the corresponding antigens are, at least partially, derived from olfactory axons. The possibility that embryonic olfactory ensheathing cells also contribute to this immunoreactivity is discussed below in relation to previous reports in the literature:

GAP 43

The results of immunolabelling with anti-GAP-43 confirm and extend previous immunocytochemical analysis of the newborn and adult (Verhaagen et al., 1989) rat

olfactory system. These authors assigned the GAP-43-IR of the primary olfactory pathway to immature olfactory axons. However, in view of the present finding that some OBECs express GAP-43 *in vitro* (see chapter 3), the possibility has to be considered that OBEC precursors, which migrate from the olfactory epithelium to the olfactory bulb during embryonic development, contribute, in part, to the GAP-43-immunoreactivity of this pathway at early developmental stages. GAP-43 has already been shown on the progenitors of all major macroglial cell types including Schwann cells, astrocytes and oligodendrocytes (Cunha and Vikovic, 1990; Curtis et al., 1992).

Vimentin

The results of immunolabelling with anti-Vm confirm and extend previous immunocytochemical analysis of the developing (E16 to P10; Gonzalez et al., 1993) and adult (Schwob et al., 1986) rat olfactory system. In the adult rat, immunoelectron microscopic analysis have established that the Vm-immunoreactivity derives not only from olfactory axons but also from olfactory ensheathing cells (Schwob et al., 1986) and it is likely that this also applies to the earlier developmental stages studied here. Other glial cell types, including astrocytes and Schwann cells synthesise Vm early in mammalian embryogenesis (Schnitzer et al., 1981; Jessen et al., 1994).

NCAM and PSA-NCAM.

The results of immunolabelling with anti-NCAM and anti-PSA-NCAM are in accordance with the studies of Miragall et al. (1988, 1989) on the developing and adult mouse olfactory system. These authors showed by immunoelectron microscopy that during embryonic development, the NCAM and PSA-NCAM-immunoreactivity of the primary olfactory pathway derives not only from olfactory axons but also from embryonic olfactory ensheathing cells. In contrast, Chung et al. (1991) reported that the olfactory nerves and the ONL are essentially negative for PSA-NCAM throughout development. This discrepancy may be due to the use of different

antibodies. Other glial cell precursors that express PSA-NCAM include oligodendrocyte progenitors (Trotter et al., 1989) and immature astrocytes (Blass-Kampmann et al., 1994).

In conclusion, it is most likely that embryonic olfactory ensheathing cells express L-NGFr, NCAM, PSA-NCAM and Vm from as early as E14 in the rat. Expression of GAP-43 needs to be confirmed by further immunoelectron-microscopic and/or *in vitro* analysis.

4.3.2 Evidences for antigenically distinct subtypes of embryonic olfactory ensheathing cells

L-NGFr-IR and PSA-NCAM-IR did not overlap at any embryonic stages examined here, implying that these antigens are expressed by distinct populations of embryonic olfactory ensheathing cells. From E14 to P7, immunoreactivity to these antibodies was continuous between the POFs and the MM/ONL but not between the MM/ONL and the rest of the olfactory bulb, suggesting that both types of embryonic olfactory ensheathing cells originate from the olfactory placode (rather than from the neural tube) and give rise to the PSA-NCAM+ and the L-NGFr+ OBECs defined in the P6-8 olfactory bulb, respectively (see Chapter 3). The lack of immunoreactivity for both antibodies in the "inner" portion of the MM (apposed to the telencephalic surface) could either reflect the absence of embryonic ensheathing cells or the presence of PSA-NCAM-/L-NGFr- embryonic ensheathing cells in this tissue. Previous ultrastructural studies of the MM support the second hypothesis (Doucette, 1989; Marin-Padilla and Amieva, 1989; Valverde et al., 1992). Thus, these observations suggest that the diversification of OBEC phenotype occurs prior to E14.

4.3.3 Possible lineage relationship between the PSA-NCAM+ and the L-NGFr+ embryonic olfactory ensheathing cells

From their respective location *in vivo* the PSA-NCAM+ and L-NGFr+ embryonic olfactory ensheathing cells can be likened to two morphological cell types identified by Valverde et al. (1992) in the peripheral olfactory fascicles of E14 rats; these authors suggested that the cells seen at the periphery of olfactory fascicles (the location of L-NGFr-IR in the present study) represented non-migrating mature ensheathing cells while the cells seen inside the fascicles (the location of the PSA-NCAM-IR in the present study), represented migrating immature ensheathing cells. Expression of PSA-NCAM by the more immature cells is consistent with reports in the literature that this antigen is predominantly expressed in embryonic nervous tissues and is down-regulated during development (Chuong and Edelman, 1984). The hypothesis that some ensheathing cell precursors migrate towards the telencephalic vesicle prior to their expression of L-NGFr is further supported by the study of Gong et al. (1994) which showed that L-NGFr was already present along the entire length of the POFs at the time it first appeared (E13). Of particular relevance, PSA-NCAM expression has recently been associated with the ability of O-2A progenitors to migrate out of neurohypophyseal explants *in vitro* (Wang et al., 1994) and of olfactory interneuron progenitors to migrate from the subventricular zone of the cerebral cortex to the olfactory bulb *in vivo* (Ono et al., 1994; Hu et al., 1996; reviewed by O'Rourke, 1996).

4.3.4 Possible regulation of antigenic expression by environmental cues

The restricted location of the L-NGFr+ cells at i) the surface of the peripheral olfactory fascicles and ii) the surface of the MM, raises the hypothesis that expression of this antigen is related to the presence of a basal lamina (Doucette, 1989, 1991). Of particular relevance to this hypothesis was the appearance at E16, of a "deep" L-NGFr+ layer at the MM/telencephalon boundary. Previous ultrastructural studies have reported the presence of a single row of elongated cells in contact with

the external glial limiting membrane of the telencephalon, during the early stages of ONL development (Marin-Padilla and Amieva, 1989; Valverde et al., 1992). These were first identified at E15 in the rat (Valverde et al., 1992). It is tempting to speculate that these cells are induced to express L-NGFr by contact with the basal lamina of the glial limiting membrane of the telencephalon. As development proceeds, this initial glial limitans and its basal lamina become progressively disrupted by the penetration of olfactory axons within the telencephalon (Doucette, 1989; Marin-Padilla and Amieva, 1989; Valverde et al., 1992), correlating with the progressive disruption and subsequent disappearance of the "deep" L-NGFr+ layer observed in the present study.

4.3.5 Insight into the developmental processes leading to the formation of the ONL

As discussed above, the "deep" L-NGFr+ layer may be used as a marker to denote the initial boundary of the telencephalon. At E16, GAP-43-IR did not extend beyond the inner L-NGFr+ layer, indicating that olfactory axons have not yet invaded the CNS compartment. At E18, GAP-43-IR and O4-IR clearly extended beyond this limit, consistent with the view that both olfactory axons and embryonic olfactory ensheathing cells had penetrated the telencephalon (Marin-Padilla and Amieva, 1989; Valverde et al., 1992). PSA-NCAM-IR, however, always stayed superficial to this limit, indicating that PSA-NCAM+ cells do not follow olfactory axons inside the telencephalon or loose PSA-NCAM expression as they migrate into the CNS. The observation of a radial gradient of PSA-NCAM intensity supports the second hypothesis. The pattern of PSA-NCAM-IR within the O4+ MM/ONL did not change consistently from E18 to postnatal month 3, defining a PSA-NCAM+ "outer ONL" and an PSA-NCAM- "inner ONL". Hence the proposal that the inner ONL is derived from the portion of the MM that has penetrated inside the telencephalon during embryonic development, and the outer ONL from the portion of the MM that has stayed peripheral to the CNS compartment. This could account for the recent

report of Bailey and Shipley (1993), that astrocytes are not homogeneously distributed within the adult ONL and reside essentially in its deeper 1/3. It also supports the ultrastructural study of Valverde et al. (1992) suggesting that only a portion of ensheathing cell precursors enter the telencephalon during the development of the ONL. In contrast, Marin-Padilla and Amieva (1989) reported that the MM becomes entirely incorporated within the telencephalon and Doucette (1989) reported that all ensheathing cell precursors stay behind the marginal zone of the telencephalon and that the ONL consequently forms superficial to the CNS compartment. The adult ONL has similarly been subdivided into an inner and outer tissue with antibodies to GFAP (Bailey and Shipley, 1993) and neuropeptide Y (Ubink et al., 1994). This separation may also be correlated with a defasciculation of olfactory axons halfway within the ONL (Key and Akeson, 1993).

4.3.6 Comparison of OBEC antigenic expression*in vivo* and *in vitro*

This immunocytochemical analysis of the developing olfactory system *in vivo* enabled to establish some correlation with the antigenic profile of OBECs observed *in vitro*. For example, the entire olfactory pathway was NCAM+, Vm+, GalC-, HNK-1- throughout all developmental stages examined, as were OBECs *in vitro*, in any of the media and at all culture times studied. GFAP-IR increased in the ONL between P7 and P14, correlating with an increased GFAP expression by P6-8-olfactory bulb-derived-OBECs during the first week *in vitro*. GFAP expression by OBECs may therefore represent an intrinsically-programmed developmental event, as proposed for immature sciatic nerve Schwann cells (Jessen et al., 1990). Part of the GFAP-IR of the ONL may also be due to a postnatal migration of "true" astrocytes within this layer as suggested by some authors (Doucette et al. 1984; Bailey and Shipley, 1993; Gonzalez et al., 1993). The PSA-NCAM-IR of the ONL underwent little changes during development, as that of OBEC cultures maintained in the serum-free media.

However some antigens appeared to be differently expressed *in vivo* and *in vitro*, reflecting the importance of environmental signals in the control of OBEC phenotype. For instance, O4 expression was maintained in the ONL from E17 to postnatal month 3 but decreased *in vitro*, in any of the media. Spatial differences were observed in the evolution of L-NGFr expression during development *in vivo*, confirming previous studies (Vickland et al., 1991; Gong et al., 1994). L-NGFr-IR decreased at the ONL periphery but increased in the GL, including the ONL/GL boundary during postnatal development. In contrast, the majority of cultured OBECs acquired expression of this marker *in vitro*, in any of the media. Noteworthy, most of the PSA-NCAM+ OBECs acquired L-NGFr expression *in vitro*. The existence of PSA-NCAM+/L-NGFr+ OBECs *in vivo* is however uncertain. Both antigens were essentially restricted to distinct regions of the olfactory system during development. From P7, they were both detected in the glomerular layer including the ONL/GL boundary, but it is not known whether they were expressed by the same cell.

CHAPTER FIVE

OBEC mitogens

5.1 Introduction

The search for OBEC mitogens was stimulated by the necessity to amplify the small number of viable cells recovered from the FACS purification procedure for further studies. In addition, understanding the factors that control the proliferation of OBECs is of utmost importance when considering the development and continuous repair processes of the olfactory nerve layer. Cell culture has already provided a very successful model for elucidating the factors regulating the growth and differentiation of Schwann cells (e.g. Morgan et al., 1991; Stewart et al., 1991; 1996). The proliferation of olfactory bulb ensheathing cells has so far not been precisely investigated *in vitro*. Chuah and Au (1993) reported that the addition of bovine pituitary extract increased the proportion of OBECs in partially purified neonatal OBEC cultures maintained in a serum-containing medium. However, neither cell viability, DNA synthesis nor cell differentiation was assessed in that study. Furthermore, cultures maintained in the presence of serum are not very good models for the analysis of agents required for cell division since serum contains an ill-defined mixture of hormones and growth factors.

In the present study, the serum-free medium DMEM-BS has therefore been used to analyse the response of OBECs to a range of potential mitogens. Mitogenic activity was quantified using an assay of DNA synthesis based on immunocytochemical detection of nuclear BrdU incorporation of cells having entered the S phase during a set period of exposure to this agent. Using this assay, the potent mitogenic activity of astrocyte conditioned medium (ACM), initially suspected on the basis of an increase in cell number during a study of OBEC differentiation (Dr. Barnett, personal communication; Barnett et al., 1993a), was

confirmed. ACM contains an ill-defined mixture of growth factors. Some of these growth factors have previously been identified, including FGF2 (Araujo et al., 1992), PDGF (Richardson et al., 1988), TGFβ (Saad et al., 1991), CNTF (Stockli et al., 1989), NGF (Furukawa et al., 1986; Houlgatte et al., 1989), and NT-3 (Rudge et al., 1992). The mitogenic activity of recombinant forms or highly purified preparations of the factors listed above was therefore assessed on FACS-purified OBECs and compared to that of ACM, in an attempt to identify the factor(s) responsible for the mitogenic activity of this conditioned medium. None of these growth factors was able to reproduce the mitogenic activity of ACM when applied individually. A semi-purified preparation of GGF from bovine pituitary extract (BPE-GGF, gift from Dr. Stroobant, LICR London) was also considered as a candidate OBEC mitogen as GGF has previously been implicated in the control of Schwann cell proliferation (Lemke and Brokes, 1984; Ridley et al., 1989; Goodearl et al., 1993; Rutkowski et al., 1996), with whom OBECs share many immunocytochemical and morphological characteristics (see Chapter 3). This factor was shown to be as active as ACM for the stimulation of OBEC DNA synthesis. However, preliminary biochemical characterisation of the mitogenic activity of ACM using heparin-affinity, anion exchange- and size exclusion chromatography indicated that it might be conferred by a novel growth factor (in collaboration with Dr. G. Graham, Beatson Institute, Glasgow).

5.2 Results

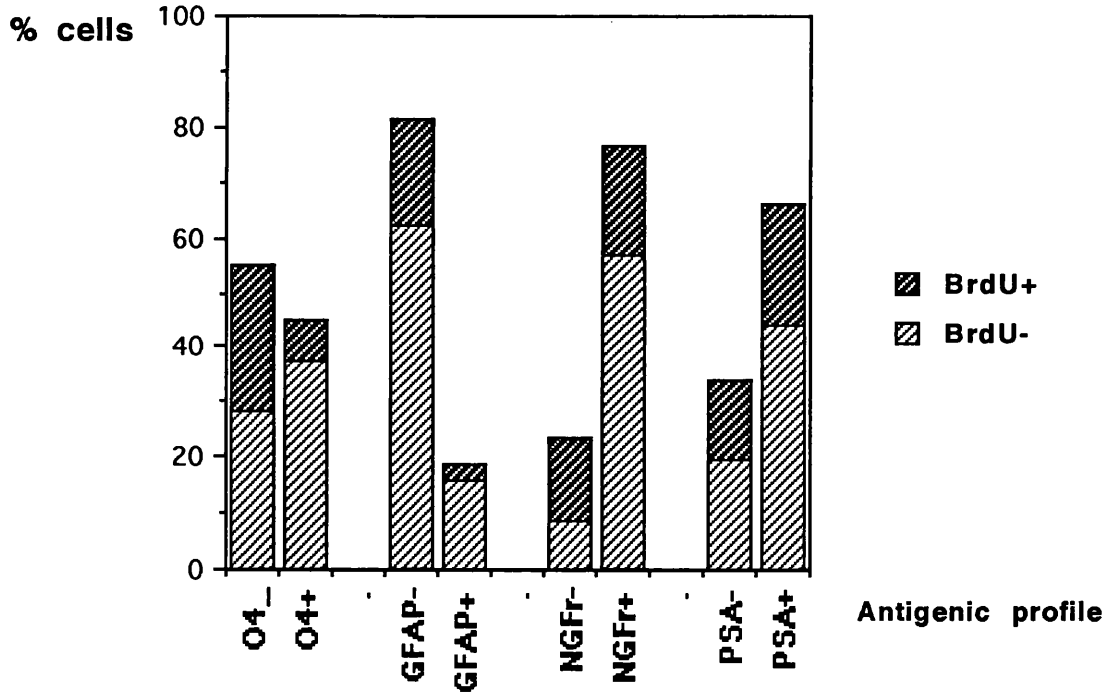
5.2.1 Mitogenic activity of DMEM-BS/ACM

FACS-sorted OBECs proliferated slowly in the control medium DMEM-BS; the percentage of cells incorporating BrdU over a 16 hr exposure on the 4th day *in vitro* was 15.8 % + 2. In one experiment, FACS sorted OBECs were not re-fed with fresh DMEM-BS on the third day of culture. This increased the BrdU labelling indices of the cells by a factor 1.6, suggesting that the small mitogenic activity of

the control may in part be due to autocrine factors. Treatment of OBECs with ACM increased the BrdU labelling indices of these cells by a factor 2.5.

Double immunofluorescent labelling of OBEC cultures using anti-BrdU in conjunction with the OBEC markers O4, anti-GFAP, anti-L-NGFr or anti-PSA-NCAM was carried out to get an insight to whether ACM preferentially stimulated the division of a particular OBEC phenotype. It appeared that OBECs divided irrespective of the expression of any of these antigens (Fig. 5.1).

Figure 5.1 **Histogram showing the proportion of BrdU+ cells among various antigenic subpopulations of OBECs**

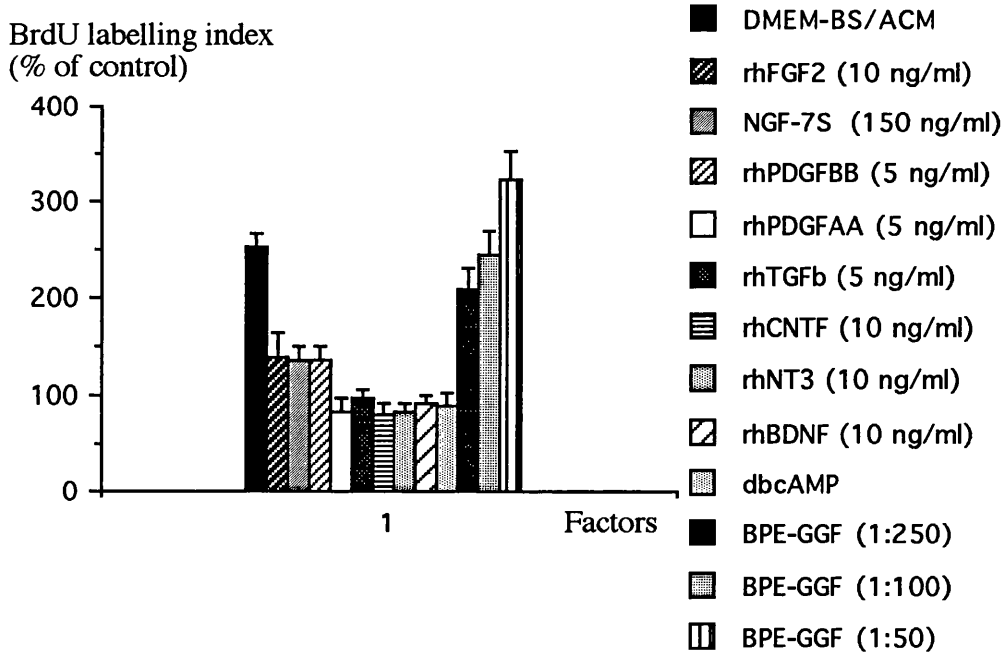


Medium conditioned by an astrocyte cell line generated by retroviral infection with the temperature sensitive mutant gene of the large T antigen (tsT ACM; gift from Dr. S Barnett) was also mitogenic, although less than the conditioned medium from primary astrocyte cultures.

5.2.2 Mitogenic activity of previously defined growth factors

In an attempt to identify the mitogenic factor(s) produced by astrocyte cultures, the mitogenic activity of ACM was compared to that of a number of previously defined growth factors. None of the growth factors tested were able to reproduce the mitogenic activity detected in ACM when tested individually at concentrations previously shown to stimulate the division of other cell types, although rhFGF2 and rhPDGFBB and NGF were weakly mitogenic (Fig. 5.2). The mitogenic response of OBECs could not be enhanced by using higher concentrations of rhFGF2 (100 ng/ml) and NGF (200 ng/ml). In addition, none of these factors was able to maintain the predominant O4+/L-NGFr-/GFAP- phenotype observed on the first day *in vitro*. The response of OBECs to dbcAMP was also analysed. This agent elevates intracellular levels of cAMP and has previously been shown to modulate the growth and differentiation of Schwann cells *in vitro* (Morgan et al., 1991). dbcAMP did not stimulate OBEC DNA synthesis but appeared to significantly decrease the proportion of cells expressing fibrous GFAP.

Figure 5.2 **Histogram showing the mitogenicity of ACM and a panel of growth factors on OBECs**



BPE-GGF acted as a potent OBEC mitogen. This activity was dose dependent (Fig 5.1). The possibility had to be considered that this mitogenic activity was due to a synergistic action of FGF2 and TGFβ rather than to GGF, since this BPE-GGF preparation also contains traces of FGF2 and TGFβ (A. Goodearl, personal communication). Combination of these growth factors did not reproduce the mitogenic activity of BPE-GGF.

5.2.3 Biochemical characterisation of the mitogenic activity of ACM

The heat and trypsin sensitivity of the ACM mitogenic activity was tested (Table 5.1). The activity was lost completely by heating ACM in boiling water for 5 min and was decreased markedly by heat treatment at 55°C for 30 min. It was inactivated by trypsin.

Table 5.1 **Temperature and trypsin sensitivity of the mitogenic activity of ACM**

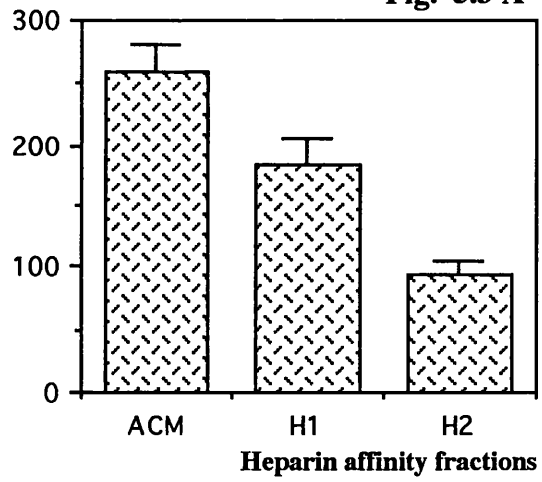
Property	Treatment	% ACM activity (untreated control)
Temperature sensitivity	2 hr 37°C	88 %
	30 min 55°C	74 %
	10 min 75°C	63 %
	2 min 100°C	48 %
Trypsin sensitivity	Trypsin (0.2 mg/ml), 1hr 37°C	60 %
	Trypsin control (Trypsin + soybean inhibitor)	93 %

To gain further insight into the molecular nature of this mitogenic activity, ACM was separated by various chromatographic methods and the mitogenic activities of the resulting fractions assayed. The mitogenic activity of ACM did not bind the heparin-Sepharose column and was recovered in the 0.1M NaCl flow through of this column (Fig 5.3 A). One activity peak was identified by gel filtration, eluting at approximately 80 kd (Fig. 5.3 C). FPLC-anion exchange chromatography was initially performed using a linear salt gradient up to 1 M NaCl as most negatively charged proteins elute within this range. A first experiment in which the activity of the eluted fractions was tested the day following the chromatography showed a potent mitogenic activity eluting at approximately 0.5 M NaCl. Fractions eluted at higher salt concentration appeared cytotoxic.

FPLC anion-exchange chromatography of concentrated ACM separated one major peak of activity, eluting at approximately 1.5 M NaCl (Fig. 5.3 B).

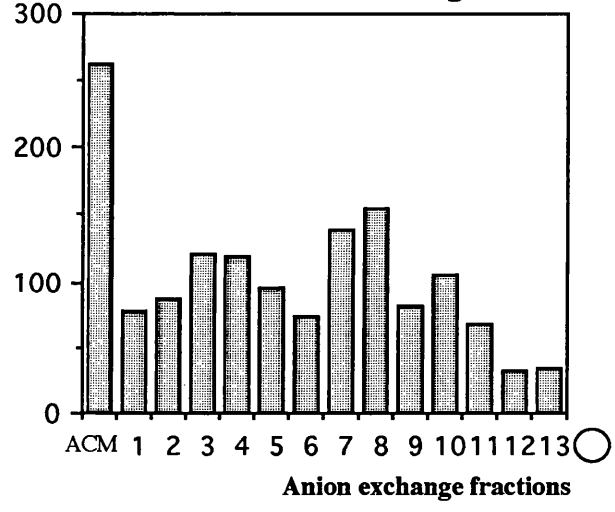
BrdU labelling index

Fig. 5.3 A



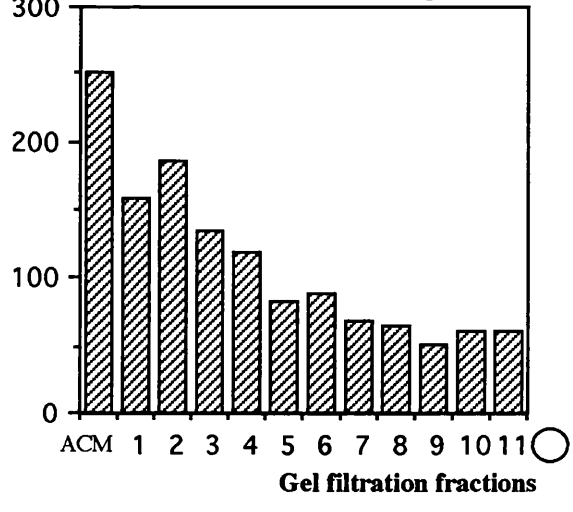
BrdU labelling index
(% of contol)

Fig. 5.3 B



BrdU labelling index
(% of control)

Fig. 5.3 C



5.3 Discussion

5.3.1 The proliferative behaviour of OBECs shows similarities with that of Schwann cells and enteric glial cells

This is the first investigation into the factors controlling OBEC proliferation. Experiments have been set up in a similar manner as previously described for the study of Schwann cell and enteric glial cell mitogens (Ecclesson et al., 1987; Morgan et al., 1991; Stewart et al., 1991, 1996). From these reports, it is possible to draw similarities and differences between the proliferative behaviour of OBECs and that of the peripheral glial cell types.

Basal rate of division

A notable feature of OBECs revealed in this study was their relatively high proliferation rate in DMEM-BS. This result further highlights their similarity with enteric glial cells (Ecclesson et al., 1987). In contrast, Schwann cells purified from perinatal sciatic nerves are essentially quiescent in the absence of exogeneously added growth factors (Ecclesson et al., 1987; Stewart et al, 1991; 1996). However, direct comparison between the proliferative behaviour of OBECs, Schwann cells and enteric glia will be necessary to confirm this idea, as differences between the present study and previous results on Schwann cells and enteric glial cells proliferation, may be due to the use of slightly different experimental regimes.

It is unlikely that the significant division rate of OBECs maintained in DMEM-BS is due to a carry over of mitogenic signals from *in vivo*, as DNA synthesis was assessed after 4 days *in vitro*. DMEM-BS contains a relatively high concentration of insulin, sufficient to activate type-1 IGF receptors (Neely et al., 1991). The possibility has therefore to be considered that this growth factor plays a role in the basal division rate of OBECs. It is also possible that the relatively high rate of DNA synthesis of OBECs in DMEM-BS is due to some mechanism intrinsic to OBECs, possibly the release of autocrine growth factors by the cells. In support of

this view was the finding that the percentage of cells incorporating BrdU was enhanced in i) cultures where the medium was not replaced during the 4 day culture period and ii) cultures that were fed with medium conditioned by OBECs. PDGFBB is a strong candidate for acting as an OBEC autocrine growth factor, in view of the present finding that exogeneously added rhPDGFBB enhanced OBEC DNA synthesis and a previous report showing PDGFB-chain-immunoreactivity in OBECs *in vivo* (Kott et al., 1994). It has also been suggested that PDGF acts as an autocrine growth factor for long term cultured Schwann cells (Ecclesson et al., 1990).

Response to soluble growth factors

Among the recombinant growth factors tested, only rhFGF2 and rhPDGFBB were able to stimulate OBEC DNA synthesis. Both growth factors have previously been shown to be mitogenic for Schwann cells (Ecclesson et al., 1987, 1990; Davis and Stroobant, 1990; Stewart et al., 1991, 1996). In the case of short term rat Schwann cell cultures however, these polypeptide growth factors are only mitogenic in the presence of both, i) agents elevating intracellular levels of cAMP and ii) either serum (Davis and Stroobant, 1990) , IGF1, IGF2 (Stewart et al., 1996) or high concentrations of insulin (Stewart et al., 1991). The present study suggest that OBECs are different from Schwann cells in that their mitogenic response to rhFGF2 and rhPDGFBB does not require elevation of intracellular cAMP levels.

The mitogenic response of OBECs to NGF was of particular interest since this factor does not appear to be mitogenic for any other glial cell populations. However, NGF has been reported to act as a mitogen for certain neural crest-derived cells (Lillien and Claude, 1985) and CNS neuroepithelial stem cells (Cattaneo and McKay, 1990). The mitogenic effect of NGF is thought to be mediated by its binding to the high affinity trkA receptor tyrosine kinase (Cordon-Cardo et al., 1991). Schwann cells, which do not proliferate in response to NGF, do not express trkA

(Lemke, 1990). It will be interesting to test whether OBECs, unlike Schwann cells, do express trkA.

The semi purified preparation of GGF from bovine pituitary extracts (BPE-GGF) elicited the most potent mitogenic response in OBECs. Since a combination of FGF2 and TGFβ (both of which are present in trace amount in BPE-GGF) did not reproduce the mitogenic activity of this preparation, it is most likely that this activity was conferred by GGF. GGF has also been shown to act as a Schwann cell mitogen *in vitro* and was purified from bovine pituitaries on the basis of this activity (Lemke and Brokes, 1984; Brokes et al., 1987). It is not clear however whether Schwann cells respond to this mitogen in the absence of serum and cAMP-elevating agents as conflicting results have been reported (Ecclesson et al., 1987; Stewart et al., 1991).

5.3.2 The mitogenic activity of ACM might be conferred by a novel neuregulin isoform

Among the extensive list of growth factors tested, BPE-GGF was able to reproduce the mitogenic activity ACM, raising the hypothesis that ACM contains GGF or a closely related growth factor. GGF belongs to the neuregulin family of proteins that have recently been cloned from rat (NDF, Wen et al., 1992), human (heregulin; Holmes et al., 1992), bovine (GGF; Marchionni et al., 1993), and chick (ARIA; Falls et al., 1993). They are encoded by a single gene, but exist in an unusual variety of forms generated by alternative splicing. At least 15 neuregulin isoforms have been purified to date, all of which contain two functional domains recognised in known superfamilies: an immunoglobulin (Ig)-like domain and an epidermal growth factor (EGF)-like domain. Their biochemical and functional properties are currently under intensive investigation (reviewed by Peles and Yarden, 1993; Carraway and Burden, 1995). They have been classified into α and β forms on the basis of differences between their EGF-like domain. Neuregulins belong to the EGF-like superfamily of polypeptide growth factors which bind to and activate members of a

subfamily of receptor tyrosine kinases that include erbB1, erbB2, erbB3 and erbB4 (Beerli and Hynes, 1996; reviewed by Carraway and Burden, 1995). The formation of erbB heterodimers can modulate the binding activities of neuregulins to their receptors.

Several isoforms of neuregulin have been identified as soluble secreted proteins (some of which are derived from transmembrane precursors) with mitogenic activity for cultured Schwann cells, including the β1, 2 and 3 isoforms of rat NDF (Dong et al., 1995; Stewart et al., 1996). Unlike most other Schwann cell mitogens, these neuregulins exhibit potent mitogenic activity even in the absence of cAMP-elevating agents and IGFs (or with low insulin concentrations). The EGF-like domain of β-type neuregulins is sufficient for mitogenic activity (Stewart et al., 1996).

In support of the view that ACM contains one or several neuregulin isoforms, neuregulin transcripts have recently been detected newborn rat astrocyte cultures using reverse transcription PCR analysis (Pinkas-Kramarski et al., 1994).

The present preliminary biochemical characterisation of the ACM-derived mitogen(s) did however not correlate with that of any previously described neuregulin. In particular, the mitogenic activity of ACM did not bind to the heparin-Sepharose column and bound to the anion exchange column under physiological pH. In contrast, all published neuregulin isoforms have been purified using cation exchange columns and have heparin-binding properties. These data are however not sufficient to completely rule out the possibility that a neuregulin is responsible for the mitogenic activity detected in ACM. Several hypothesis can be made, which are outlined below.

Since neuregulins are rich in glycosylation sites, it is possible that the ACM-derived OBEC mitogen is a novel glycosylated form of neuregulin with different biochemical properties. Negatively charged carbohydrate moieties could account for its anion exchange-binding properties.

The ACM-derived OBEC mitogen could also be a neuregulin associated with glycosaminoglycan (GAG) chain(s), possibly a neuregulin-proteoglycan complex. GAGs are highly negatively charged polysacharides which tend to dominate the biochemical behaviour of the molecules to which they are attached. They strongly bind to anion exchange columns and do not bind heparin under physiological pH. In support of this hypothesis, various proteoglycans have been detected in ACM (Matthiesen et al., 1989; Johnson-Green et al., 1992) and putative GAG attachment sites have been identified on the amino acid sequences of several neuregulins (Wen et al., 1992; Marchionni et al., 1993; Loeb and Fishbach, 1995). Several other polypeptide growth factors, including FGF1, 2, 5 and TGFβ, are known to bind to GAGs or proteoglycans (Vlodavsky et al., 1987; Andres et al., 1989). Such growth factor-proteoglycan complexes are mostly associated with the extracellular matrix but also exist as soluble forms which can be recovered from conditioned media of cultured cells. For example, a molecular complex containing FGF2 and an HSPG has been purified from endothelial cell conditioned medium (Saksela et al., 1988, 1990). The possible association of neuregulins with proteoglycans has previously been discussed (Meyer and Birchmeyer, 1995; Loeb and Fishbach, 1995; Sudhalter et al., 1996).

The ACM-derived OBEC mitogen(s) may also be a novel splice variant of neuregulin. Since at least 13 exons have been identified within the neuregulin gene (Marchionni et al., 1993), this family is likely to comprise many more, as yet unidentified proteins derived from alternative splicing. In support of this hypothesis, neuregulin transcripts without Ig-like domain have recently been detected by *in situ*

hybridisation on rat brain tissue sections (Corfas et al., 1995). It is likely that the corresponding proteins, which have not yet been purified, do not bind heparin, since the heparin binding sites of the known neuregulins are presumably located within their immunoglobulin (Ig)-like domain. This region contains a particularly high density of positively charged residues and includes an amino acid sequence conforming to a consensus sequence for heparin binding found in a human heparan sulphate proteoglycan core protein (Wen et al., 1992; Marchionni et al., 1993). An additional putative heparin-binding site has been reported within the Ig-like domain of ARIA (Loeb and Fischbach, 1995). Such an Ig-domain-free neuregulin cDNA has recently been cloned from two human brain cDNA libraries (Ho et al., 1995). The corresponding protein (called SMDF) has a predicted amino acid sequence which, instead of an Ig-like domain, contains an unique N-terminal region rich in negatively charged residues. It is therefore possible that SMDF binds to anion exchange columns and the ACM-derived mitogen may be the rat homologue of SMDF.

The result from the size exclusion chromatography does not enable to rule out any of these three hypothesis. The apparent molecular weight of the activity was relatively high (80-100 kd; the sizes of the known neuregulins, predicted from their amino acid sequence, fall in the range 30-50 kd), and may be due to a high degree of glycosylation, complex formation, or dimerisation. Moreover, it has recently been shown that mammalian cell-derived recombinant forms of NDF have an unusual shape in solution, conferring them with an apparent molecular weight of 100-120 kd, which is much larger than that estimated by SDS-PAGE (Lu et al., 1995).

During the writing of this thesis, various results have been produced by Dr G. Pollock, a postdoctoral fellow pursuing this work in the laboratory, which further support a role for a neuregulin-like growth factor in the mitogenic activity of ACM: 1) the mitogenic activity of ACM has been neutralised with an anti-NDF antibody. 2) neuregulin-like proteins have been detected in ACM and its non-heparin binding

fraction, by western blotting. 3) ACM has been shown to induce phosphorylation of a tsT cl 1.6 OBEC cell line-derived protein co-migrating with erbB receptor tyrosine kinases (185 kd). 4) primary OBECs and tsT cl 1.6. OBECs have been shown to express the high affinity neuregulin receptor erbB4, using immunocytochemistry. 5) neuregulin transcripts have been detected in primary cultures of cortical astrocytes using reverse transcription PCR analysis.

CHAPTER SIX

OBEC-neuron interactions *in vitro*

6.1 Introduction

It has been proposed that OBECs play an important role in the exceptional ability of olfactory axons to grow throughout adult life within the olfactory bulb and could also promote regeneration of non-olfactory axons upon transplantation into other CNS tissues (Barber and Lindsay, 1982; Doucette, 1984; Raisman, 1985; reviewed by Ramon-Cueto and Valverde, 1995; Doucette, 1995). Ramon-Cueto and Nieto-Sampedro (1994) showed substantial regeneration of dorsal root axons into the spinal cord following transplantation of purified OBECs within the dorsal root entry zone of the spinal cord. Studying the mechanisms of axonal growth *in vivo* is however impeded by the confusing variety of cell types, cell interactions, and trophic factors that may influence the behaviour of neurons. In this chapter, the interaction of OBECs with neurons has been studied in dissociated cell cultures. The advantage of cell culture, and of dissociated cell culture in particular, is that it makes individual living cells accessible and amenable to manipulations under controlled conditions. Neurons can be distinguished from glial cells on the basis of morphological and immunocytochemical criteria, enabling direct observation of growing neurites and their mode of elongation and branching.

Thus, neuro-glia co-culture systems have largely contributed to our understanding of neuro-glia interactions. Examples in the literature from *in vitro* studies have led to the identification of surface and/or ECM molecules associated with the neurite-outgrowth- promoting and neurite-outgrowth-inhibiting activities of Schwann cells and oligodendrocytes, respectively (Bixby and Reichardt, 1988; Caroni and Schwab, 1988). The development of OB-derived cell culture systems are

relatively recent, so there are very few reports in the literature on the ability of OBECs to function as substrate for the growth of neurons *in vitro*. These studies have been summarised in Table 1.7 and, taken together, suggest that OBECs can promote substantial neurite outgrowth from olfactory neurons and also from various types of CNS neurons *in vitro* (Denis-Donini et al. 1988; Goodman et al. 1993; Chuah and Au, 1994).

In the present study, FACS-purified OBECs have been tested for their interaction with CNS neurons derived from the cerebellum of perinatal rats. The cerebellum has the advantage to be an easily accessible CNS tissue; it contains a single dominant cell type, the cerebellar granule neurons, which can be prepared as a highly purified neuronal population (reviewed by Burgoyne and Cambray-Deakin, 1988). They are morphologically distinct in size and shape, facilitating their identification in culture. These neurons have widely been used to assess the ability of various cell types to support neuronal attachment, survival, migration, and neurite outgrowth and to dissect out the molecular mechanisms underlying this support (e.g. Noble, 1984; Doherty et al., 1990; Groves et al., 1993; Muller-Hussmann et al., 1993; Mukhopadhyay et al., 1994; Williams et al., 1994).

6.2 Results

In order to identify an appropriate marker for the identification and length measurements of cerebellar granule neurons over OBECs, these neurons were plated over a substrate of 3T3 fibroblasts and labelled with a large panel of neural marker antibodies (Table 6.1).

Table 6.1 **Molecular profile of cerebellar granule cells**

	Antigenic profile	Source of Antibody
Neurofilament, MW 68 kd (NF-68)	-	Sigma, Affiniti
NF-160	-	Sigma, Affiniti
NF-200	-	Sigma, Affiniti
Neuron specific enolase (NSE)	-	Affiniti
Synaptophysin	-	Sigma
GAP-43	++	See Table 2.1
L1	+	//
NCAM	++	//
PSA-NCAM	+	//
HNK-1	+/-	//
A2B5	+/-	//
L-NGFr	+/-	//
Vm	+/-	//

+/- indicates partial or faint labelling of neurons

To generate neuron-specific markers turned out to be quite difficult since the best neuron markers also labelled OBECs (See Chapter 3). Antibodies known to be neuron-specific, e.g. anti-NFs and anti-NSE were technically difficult to produce good reproducible results and a lot of time was spent trying to improve the fixation procedure. After much effort, these antibodies turned out to be unsuccessful. Anti-GAP-43, which strongly labelled cerebellar granule neurons, including cell bodies and neurites, was finally used. Based on the intensity of immunofluorescence, GAP-43 was usually expressed at much higher levels on the neurons than on OBECs. Whenever there was an ambiguity at identifying the two cell types (a minority of OBECs were intensively labelled with anti-GAP-43), neurons were excluded from the analysis.

Cerebellar granule neurons were plated onto poly-L-lysine and onto dense monolayers of OBECs, Schwann cells, astrocytes, and 3T3 fibroblasts, incubated for 16 hours, fixed, stained, photographed and evaluated for neuron survival/attachment, neurite initiation and neurite elongation, as described in Material and Methods. Poly-L-lysine was unable to support neurite outgrowth. In contrast, all the cell monolayers tested were able to support neurite outgrowth (Fig. 6.1). Neuronal cell bodies and processes tended to remain as discrete units, with only a limited amount of cell body aggregation or process fasciculation being observed. On 3T3 fibroblasts, the extent of neurite outgrowth was very limited as compared to the glial cell monolayers (Fig 6.1 B). A detailed quantitative analysis of the behaviour of neurons on the different glial cell monolayers was undertaken (Table 6.2).

Table 6.2 **Cerebellar granule neuron attachment/survival and neurite outgrowth over various glial cell monolayers**

Substrate	Neurons per field	% Neurons with neurites	Total neurite length (μm)
OBECs (ACM)	14.6 ± 0.9	80%	184 ± 15
OBECs (FCS)	13.5 ± 0.7	67%	182 ± 16
Astrocytes	12.3 ± 0.8	65.8%	249 ± 22
Schwann cells	12.3 ± 1.0	74.5%	103 ± 8

The number of neurons per field and total neurite length are expressed as means ± standard deviation from two coverslips per condition in one representative experiment. OBEC (ACM) and OBEC (FCS) indicate culture conditions for OBECs, which may select for subtypes of OBECs (see Chapter 3).

Neuron survival/attachment and neurite initiation was similar on all cell monolayers. OBECs (especially when grown in DMEM-BS/ACM), may support

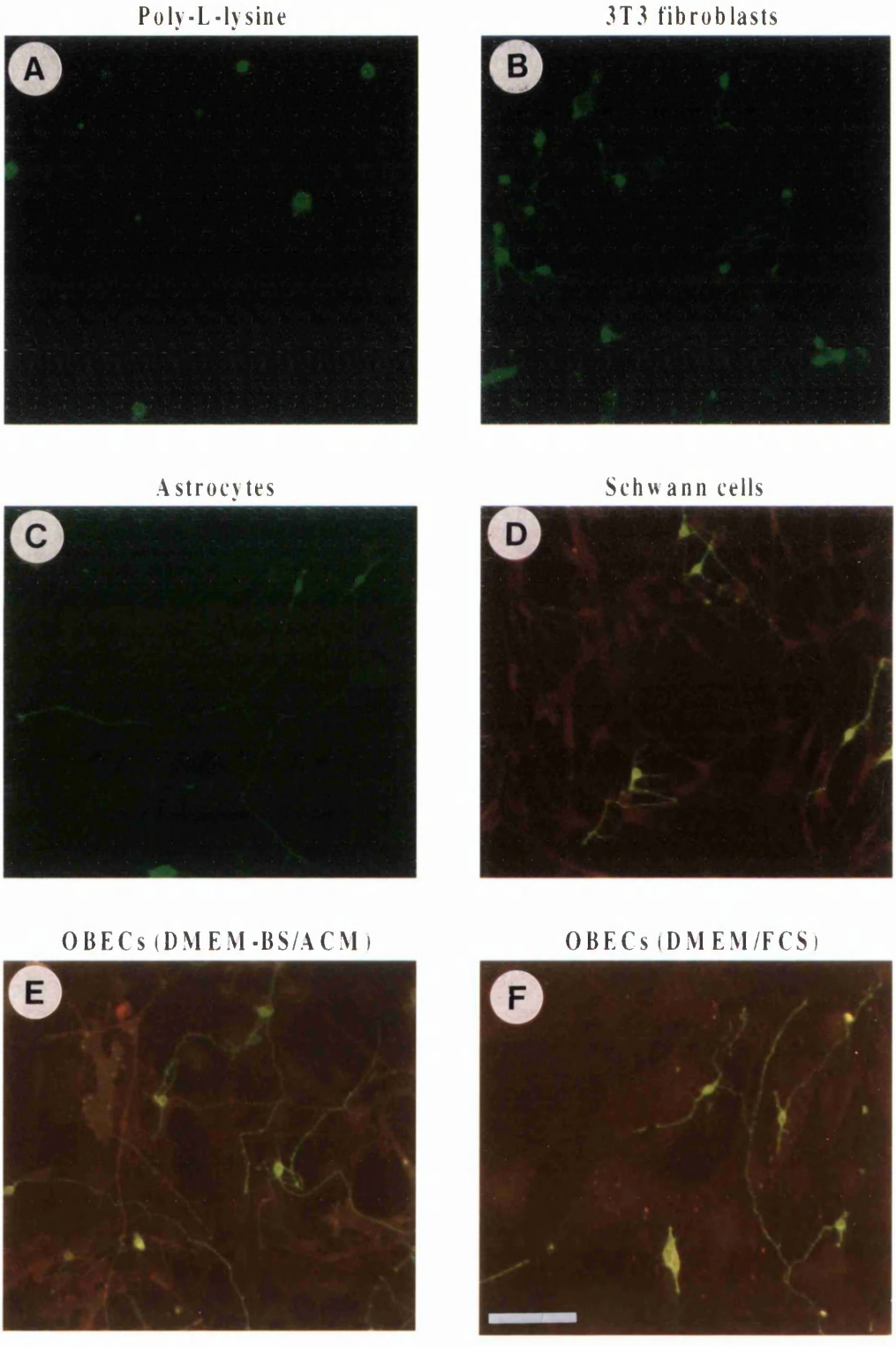
Figure 6.1

Cerebellar granule neurons plated over various substrates.
Double immunofluorescent labelling for GAP-43 and L-NGFr

Neurite outgrowth of perinatal rat cerebellar granule neurons on Poly-L-lysine (**A**), and on confluent monolayers of 3T3 fibroblasts (**B**), cortical astrocytes (**C**), sciatic nerve Schwann cells (**D**), OBECs grown in DMEM-BS/ACM (**E**), and OBECs grown in DMEM/FCS (**F**). Ten thousand neurons were plated onto the various substrates in DMEM-BS and cultured for 16 hr at 37°C. Cultures were fixed with 2 % paraformaldehyde and processed for immunofluorescent labelling with anti-GAP-43/fluorescein (green) to visualise neurites, and with anti-L-NGFr/rhodamine (red) to visualise OBEC and Schwann cell monolayers.
Photomicrograph. Scale bar = 50 μm

Figure 6.1

Cerebellar granule neurons plated over various substrates.
Double immunofluorescent labelling for L-NGFr and GAP-43



neuron survival/attachment and neurite initiation slightly better than astrocytes or Schwann cells, but these are preliminary data and additional experiments are required. The major difference observed between the various cell monolayers tested was the extent of neurite elongation. Astrocytes were the best at supporting neurite elongation, followed by OBECs, followed by Schwann cells. These differences were quite significant ($p = 0.015$ for OBECs in DMEM-FCS versus astrocytes; $p= 0.004$ for OBECs versus Schwann cells). OBECs grown in DMEM-FCS and DMEM-BS/ACM supported neurite elongation to a similar extent ($p= 0.916$). It should be noted that the values calculated for the mean total neurite length on OBECs and astrocytes may represent an underestimation since a small number of neurons, whose neurites were particularly long and intermingled with other neurites were difficult to measure and therefore were excluded from the analysis.

Experiments in which cerebellar granule neurons were plated onto sparse cultures of OBECs, that had been grown in DMEM-BS, showed that neurons in contact with OBECs were 3 times more likely to extend neurites than those not in contact (Table 6.3). Mean neurite length was also significantly greater for neurons in contact with OBECs.

Table 6.3 **Neurite outgrowth as a function of neuron-OBEC contact**

Analysed neuronal population	% Neurons with neurites	Total neurite length (μm)
Contacting glial cells (55.8%)	76	81 ± 4
Not contacting glial cells (44.2%)	24	49 ± 7

In this experiment the time of neuro-glia co-culture was reduced to 10 hours, as experiments with longer incubation periods imposed the exclusion of some neurons with particularly long neurites from the analysis.

Figure 6.2

Cerebellar granule neurons plated over a sparse culture of OBECs. Double immunofluorescent labelling for L-NGFr and GAP-43

Neurite outgrowth of perinatal rat cerebellar granule neurons on sparse monolayers of FACS-sorted OBECs. Ten thousand neurons were plated onto 1 week-old cultures of FACS sorted OBECs and cultured for 12 hr at 37°C. Cultures were fixed with 2 % paraformaldehyde and processed for immunofluorescent labelling with anti-GAP-43/fluorescein (green) to visualise neurites, and with anti-L-NGFr/rhodamine (red) to visualise OBECs.

A: OBECs grown in DMEM-FCS; **B:** OBECs grown in DMEM-BS.

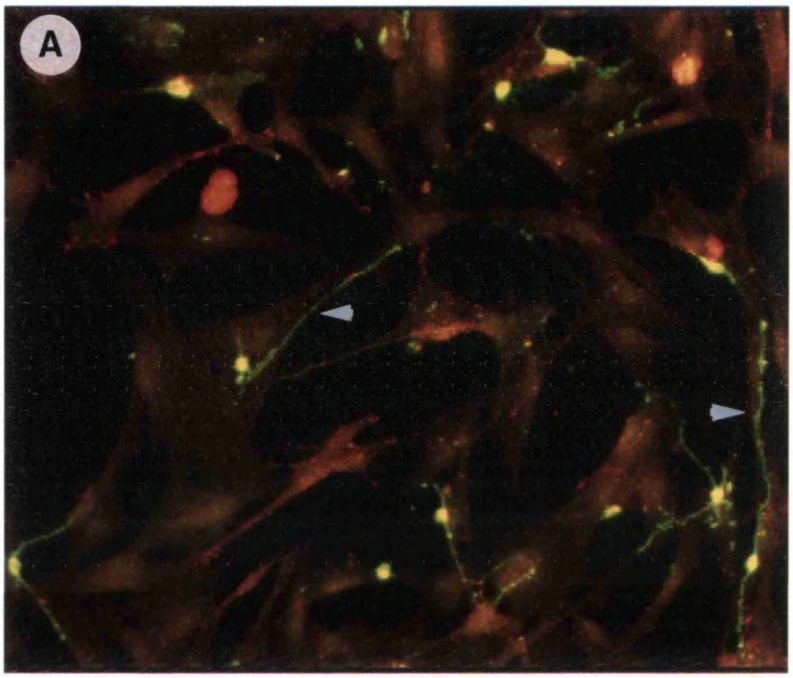
Many neurites are seen following OBEC processes (arrowheads).

Photomicrograph. Scale bar = 50 μm

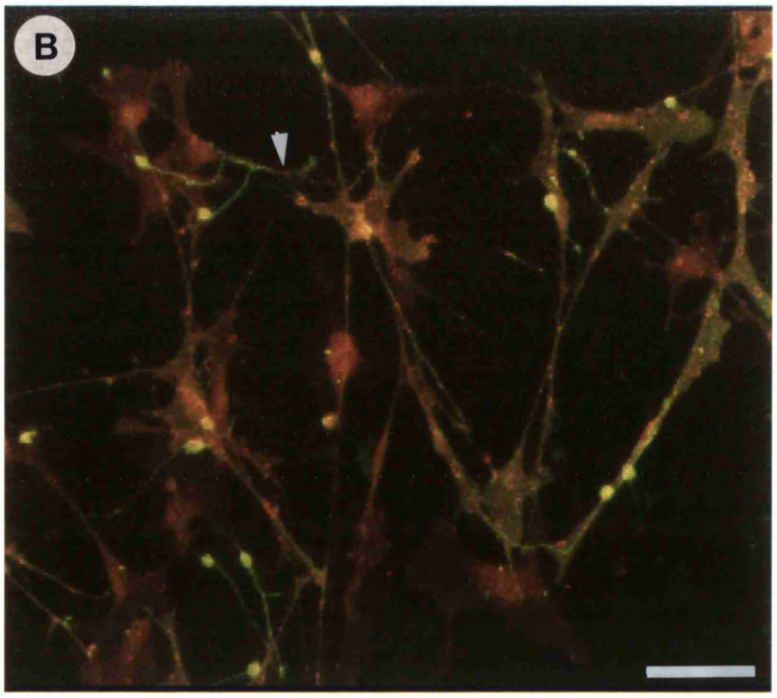
Figure. 6.2

Cerebellar granule neurons plated over sparse cultures of OBECs. Double immunofluorescent labelling for L-NGFr and GAP-43.

OBECs (DMEM/FCS)



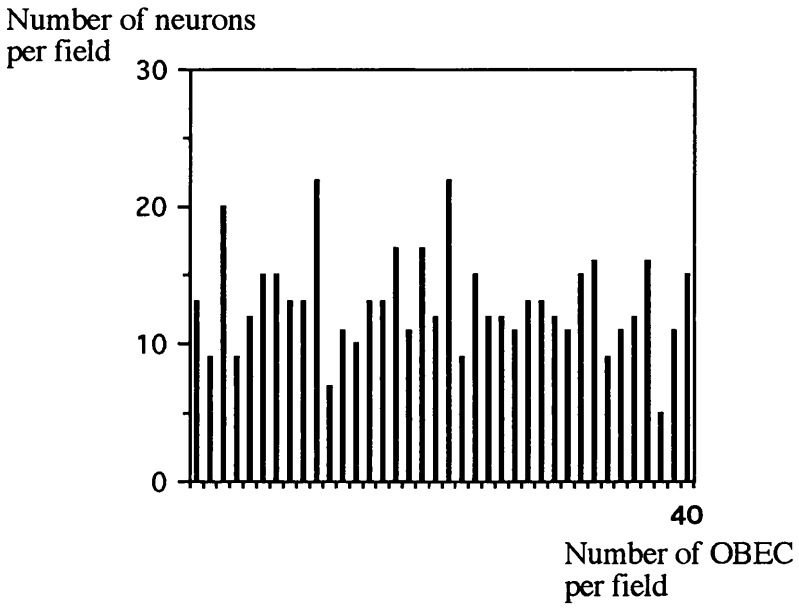
OBECs (DMEM-BS)



In such sparse cultures, neurites were often seen following OBEC processes (Fig. 6.2). These observations strongly suggest a role for membrane molecules in the neurite outgrowth promoting activity of OBECs.

On the other hand, neuron attachment/survival did not appear to depend on the local OBEC density within a same coverslip, suggesting a role for soluble neurotrophic factors (Fig. 6.3).

Fig. 6.3 **Neuronal survival/attachment as a function of local glial density**



To gain further insight into the molecular mechanism of OBEC support, a protocol was used, which has previously been described to isolate extracellular matrix (ECM) molecules. Neurons were plated onto a monolayer of confluent OBECs (grown for 1 week in DMEM-BS/ACM), that had been incubated in water, followed by extensive washing in PBS to remove lysis material. Results from this study are presented in Table 6.4.

Table 6.4 **Influence of OBEC-derived ECM on neurite outgrowth**

Substrate	Neurons per field	% Neurons with neurites	Total neurite length (μm)
OBECs	11.6 ± 0.9	75%	110 ± 6
OBEC ECM	13.7 ± 0.9	43.2%	37 ± 2

From this table it can be seen that both components of neurite outgrowth (neurite initiation and elongation) were significantly reduced when neurons were plated over OBEC ECM as compared to the untreated OBEC monolayer. In contrast, neuronal survival/attachment was similar under both conditions.

6.3 Discussion

These results can be related to previous studies on OBEC-neuron interactions *in vitro*. From the study of Chuah and Au (1994), it appears that cerebellar granule neurons responded similarly to olfactory neurons in terms of neurite initiation over neonatal cortical astrocytes. Neurite initiation over OBECs was also similar, despite the fact that we used OBECs from a later developmental stage (P7 as compared to P0). This comparison indirectly suggests that OBECs, unlike astrocytes from other CNS regions (Geisert and Stewart, 1991), maintain neurite-outgrowth promoting activity beyond the critical neonatal periods. Further experiments, comparing the extent of neurite outgrowth (from a same population of cerebellar granule neurons) over OBEC monolayers derived from a wider range of developmental stages will be required to confirm this hypothesis. In contrast to the study of Goodman et al, (1993) the present work shows that neonatal cortical astrocytes are better than OBECs at promoting neurite outgrowth. This difference may result from 2 factors. First, in contrast to our study, these authors analysed astrocytes and OBECs cell lines transfected with the large T antigen. It is known that some aspect of phenotype, including the neurite outgrowth promoting activity of astrocytes, can be affected by immortalisation with SV40 T-antigen (Groves et al., 1993). Thus, SV40 Tag may have selectively down regulated the neurite outgrowth promoting activity of astrocytes and not that of OBECs, by an unknown mechanism. Second, in the study of Goodman et al. (1993), the neurite outgrowth promoting activity of OBECs was assayed using a different type of neuron (embryonic chick retinal ganglion neuron) and it is now well established that neurons from different regions of the nervous system differ in their responsiveness to the same factors (Fawcett, 1992).

The results from the sparse OBEC monolayer experiment provide some insight into the mechanism of OBEC support. The extent of neurite outgrowth was largely dependent on OBEC-neuron surface interactions. The result of neurite outgrowth over osmotically lysed OBECs suggests that it is not mediated by ECM

although ECM-associated molecules may be involved in neuron survival/attachment. Strong candidate molecules for the neurite outgrowth promoting activity of OBECs include the cell adhesion molecules NCAM and L1 (both of which are expressed by OBECs in our culture conditions, see Chapter 3). These molecules have previously been shown to promote substantial neurite outgrowth for cerebellar granule cells when compared to 3T3 fibroblasts (Doherty et al., 1990). L-NGFr, which is expressed by the great majority of OBECs may also be involved by presenting NGF molecules to the neurons as proposed by Taniuchi et al. (1988) for Schwann cells. The culture conditions in which the OBECs were grown prior to the addition of neurons did not appear to substantially affect their neurite outgrowth promoting activity, suggesting that PSA-NCAM (expressed in DMEM-ACM but not in DMEM-FCS, see Chapter 3) does not play a major role in this activity.

CHAPTER SEVEN

General discussion and future prospects

The work described in this thesis was concerned with a detailed cellular and molecular study of the olfactory system with particular emphasis on the olfactory bulb glial cells. The long term aim is to understand the mechanisms underlying the exceptional regenerative capacity of the olfactory bulb. In this last chapter, I would like to summarise my present findings in relation to previous work and propose experiments that may be carried on in the near future. It is hoped that these current findings will lead to a greater understanding of the biology of OBECs and have use in future work on repair and regeneration in the CNS.

7.1 The OBEC lineage

7.1.1 The past

Distinct cell phenotypes have been detected in the olfactory nerve layer of the olfactory bulb by electron microscopy (Doucette, 1984, 1991), immunocytochemistry (Miragall et al., 1988; Vickland et al., 1991; Norgren et al., 1992; Bailey and Shipley, 1993; Gonzalez et al., 1993; Ubink et al., 1994) or *in situ* hybridisation (Ubink et al., 1994), but the identity of each cell phenotype as either astrocyte or OBEC is not clear. This disparity appears to stem from the fact that OBECs possess many features which are common to astrocytes, including ultrastructural features, expression of GFAP and formation of a glia limitans (Barber and Lindsay, 1982; Doucette et al., 1984, 1991). The lineage of olfactory bulb astrocytes and of OBECs are poorly understood. There is increasing evidence that the astrocytic population of the brain comprises many different cell phenotypes that may arise from separate lineages (e.g. Prochiantz and Mallat, 1988; Wilkin et al., 1990; Hoke and Silver, 1994; Miller et al., 1994). Purification procedures have recently been developed to characterise OBECs *in vitro* and some of these studies

have shown a morphological and antigenical heterogeneity among these cells (Ramon-Cueto and Nieto-Sampedro, 1992; Doucette, 1993; Chuah and Au, 1994; Doucette and Devon 1994,1995). However the relative proportion of each cell type varies between studies and it is not clear whether this is due to an *in vitro* artefact (the cells were characterised after prolonged time in culture), to the use of different culture conditions, or to the use of rats from different developmental stages.

To resolve this issue, a detailed comparative immunocytochemical analysis of short term OBEC cultures and of tissue sections of the developing olfactory nerve layer has been undertaken in this thesis. The *in vitro* study of OBECs was based on the recent development of a purification procedure by fluorescence activated cell sorting using the O4 antibody (Barnett et al., 1993a).

7.1.2 **The present** (Franceschini and Barnett, 1996)

The present study demonstrates that the O4+ cell population of perinatal rat olfactory bulbs (OB) does not represent a homogeneous cell population; when O4+ cells were purified by FACS sorting and immunolabelled on the first day *in vitro* with a panel of neural marker antibodies, it was found that approximately half of the sorted cell population expressed PSA-NCAM, 11 % GFAP and under 2% L-NGFr, correlating with the relative amounts of immunoreactivity detected *in vivo* by immunocytochemical analysis of OB tissue sections. Immunocytochemical analysis of the developing olfactory pathway suggested that this heterogeneity is maintained, to a certain extent, throughout postnatal development; of particular interest, the L-NGFr+ and PSA-NCAM+ cell populations of the ONL did not appear to overlap at any developmental stages examined. Furthermore, these two markers could be used to characterise two morphological subtypes of OBECs *in vitro*: astrocyte-like OBECs (ALOBECs) and Schwann cell like OBECs (SLOBECs). These observations raised the question as to whether the two cell types belong to the same lineage. To address this question, a clonal OBEC cell line was constructed by

retroviral infection with the temperature mutant gene of the large T antigen, and characterised with the same panel of antibodies used to characterise OBECs *in vivo* and in primary cultures. Morphological and immunocytochemical analysis of this cell line showed the presence of both flattened PSA-NCAM+ cells and process-bearing L-NGFr+ cells, similar to ALOBECs and SLOBECs, respectively. This strongly supports the view that ALOBECs and SLOBECs share a common lineage.

The present discovery that some of the astrocytic and Schwann cell characteristics of OBECs are found in separate populations of cells of the olfactory nerve layer may explain the controversy that has been going on for almost a century as to whether OBECs represent astrocytes (e.g. Kreutzberg and Gross, 1977; Barber and Lindsay, 1982) or Schwann cells (e.g. Willey, 1973). These authors may have characterised different OBEC subtypes. It may also explain the ongoing controversy about the presence of "true" (neural tube-derived) astrocytes within this tissue. ALOBECs might have been mistaken for astrocytes by some authors.

The immunocytochemical analysis of the olfactory system during embryonic development confirms previous ultrastructural studies showing that the ONL initially forms superficially to the marginal zone of the telencephalic vesicle (olfactory bulb primordium) and that its constituents are the unique source of the OBECs of the adult (Doucette et al., 1989; Marin-Padilla and Amieva, 1989; Valverde et al., 1992). It favours the interpretation of Valverde et al. (1992) as opposed to those of Doucette (1989), and Marin-Padilla and Amieva (1989), that the ONL becomes partially incorporated into the telencephalic vesicle during late embryonic stages, by the penetration of olfactory axons and of a portion of embryonic olfactory ensheathing cells into this tissue.

This thesis also highlights the fact that OBECs have a highly plastic phenotype. The antigenic profile of the sorted cell population changed with time in culture. Except for the up-regulation of GFAP, these changes did not correlate with the antigenic changes observed *in vivo* during the postnatal development of the ONL and are most likely to be due to a change in the environmental signals of the cells upon culture. Differences were observed between the culture media used. Antigenic heterogeneity became less obvious in the serum containing medium but could be maintained in DMEM-BS and DMEM-BS/ACM. Of particular interest was the appearance of a variety of phenotypes that were intermediate between ALOBECs and SLOBECs, suggesting that both cell types are interconvertible or that one gives rise to the other. Noteworthy, cells expressing both, PSA-NCAM and L-NGFr, were abundant *in vitro* but not apparent at any developmental stage *in vivo.* It may be that the signals inducing such a phenotype are generally not encountered by OBECs *in vivo*. Another explanation could be that these antigens are polarised on the cell membrane *in vivo*, giving the impression, at the light microscopic level, that they originate from different cells.

Further evidence of the plasticity of OBECs stems from the recent finding that the tsT cl. 1.6. OBEC cell line could be induced to differentiate into a Schwann cell-like myelinating phenotype upon transplantation into experimentally induced CNS lesions devoid of endogenous glial cells (Franklin et al., 1996). In addition, two non-myelinating cell types were observed within the transplanted area, presumably reflecting the *in vitro* phenotypic plasticity of OBECs shown in this thesis. One of these cell types formed little or no association with host axons, contained intermediate filaments, had a particularly large cytoplasm, was incompletely covered by a basal lamina, and occurred within areas of the lesion which also contained GFAP+ cells. The other cell type had long thin processes which often loosely enwrapped single or groups of myelinating cells and occurred within collagen-rich areas of the lesions (Franklin et al., 1996). The morphological features of these two

cell types makes them very similar to the ALOBECs and SLOBECs defined in this thesis, respectively. It will be interesting to test whether these two types of transplanted cells can also be distinguished from each other by their immunoreactivity for PSA-NCAM and L-NGFr.

7.1.3 The future

Future studies will concentrate on dissecting out the mechanisms by which the different cellular forms of OBECs are regulated and on defining their relationship.

The 3 different OBEC phenotypes observed in the perinatal olfactory bulb using double immunolabelling with anti-PSA-NCAM and anti-L-NGFr (PSA-NCAM+/L-NGFr-, PSA-NCAM-/L-NGFr+, and PSA-NCAM-/L-NGFr- cells) might all represent differentiated but interconvertible phenotypes and the diversity of phenotypes due to differences in local environmental cues. This situation can be compared to the Schwann cell lineage. Most of the overt phenotypic changes seen in Schwann cell development are extrinsically signalled rather than intrinsically programmed (see Fig. 1.2). Thus, when Schwann cells are isolated from their *in vivo* environment and cultured in the absence of neurons, both, myelinating- and non-myelinating Schwann cells revert to a developmentally less mature phenotype that can be induced along either pathway with appropriate factors (reviewed by Mirsky and Jessen, 1996). If the phenotype of OBECs is entirely dependent on extrinsic signalling one may be surprised that heterogeneity of the cultures persisted after prolonged time within the same culture dish. This may underlie the fact that local signals derived from OBECs themselves play a role in the regulation of their phenotype.

The phenotypic plasticity of the sorted OBEC population could alternatively be explained by the retention of a subpopulation of OBECs that maintain some

degree of immaturity over aprotractedperiod, possibly a multipotential progenitor cell. This situation can be related to the O-2A lineage (Fig. 1.3). Perinatal, as well as adult, O-2A progenitors are able to differentiate into both oligodendrocytes and type-2 astrocytes. Similarly, ALOBECs and SLOBECs may be derived from an (as yet unidentified) bipotential progenitor cell.

The PSA-NCAM+/L-NGFr- OBECs, identified for the first time in this thesis, are good candidates for progenitor cells since PSA-NCAM is generally a characteristic of immature embryonic tissues and is down-regulated during development (Chuong and Edelman, 1984). Among glial cells, PSA-NCAM is expressed by both, immature astrocytes and O-2A progenitor cells (Trotter et al., 1989; Blass Kampman et al., 1993). In the O-2A lineage, the loss of PSA-NCAM expression was shown to correlate with differentiation *in vitro* (Trotter et al., 1989). Interestingly, PSA-NCAM expression has been related to the ability of neurohypophysial O-2A progenitors to migrate *in vitro* (Wang et al., 1994) and to the ability of some neuroblasts to migrate *in vivo* (Ono et al., 1994; Hu et al., 1996; reviewed by O'Rourke et al., 1996), raising the hypothesis that the PSA-NCAM+ OBECs represent migrating OBEC progenitors cells. The idea that OBECs are renewed during regeneration, by a migration into the ONL of immature progenitor cells derived from the olfactory epithelium, is supported by recent ultrastructural studies (Monti-Graziadei et al., 1992; Chuah et al., 1995). The first of these studies detected a small population of migratory cells along the primary olfactory pathway of adult rats. Although these cells were tentatively identified as migrating neuroblasts, their ultrastructural description is very similar to that reported for OBEC precursors (Doucette, 1989; Marin-Padilla and Amieva, 1989; Valverde et al., 1992). According to the study of Chuah et al. (1995), the primary response of olfactory ensheathing cells to chemically induced olfactory nerve injury (an experimental paradigm that has been widely used to study the regenerative processes of this tissue) is an abundant migration of these cells towards the olfactory bulb. The retained

ability of the olfactory epithelial cell matrix to generate olfactory ensheathing cells beyond the embryonic period is further supported by the demonstration of GFAP+ cells migrating out of olfactory epithelial explants from late postnatal stages (Chuah and Au; 1991). The precursors of OBECs in the olfactory epithelium have not yet been identified. One interesting hypothesis is that the olfactory neuroepithelial stem cells from which olfactory neurons are derived are pluripotential, giving rise to both glia and neurons. These stem cells are at present poorly understood although the possibility of pluripotentiality has recently been raised following the discovery of multiple precursor stages in the olfactory neuron lineage (Gordon et al., 1995). Multipotential neural precursor cells that are able to generate both, glia and neurons exist in other regions of the adult nervous system (shown *in vivo* by Altman, 1969; Bayer, 1983; Miragall et al., 1990; Lois and Alvarez-Buylla, 1994; and *in vitro* by Reynolds and Weiss, 1992). Some of these cells have recently been identified as adult neural stem cells (Morshead et al., 1994; Gritti et al., 1996).

The PSA-NCAM+/L-NGFr- OBEC subtype could be isolated from the olfactory bulb by fluorescence activated cell sorting using O4 in conjunction with an anti-PSA-NCAM antibody. The anti-PSA-NCAM antibody used in the present study was of the same class specificity as O4 (IgM), and it would be more appropriate to use an antibody of different class specificity such as Ak 735 (IgG2, Finne et al., 1987). The survival, growth and differentiation properties of these cells could then be analysed in single cell microcultures, an approach that has already proved very useful for the understanding of the O-2A lineage (Temple and Raff, 1985, 1986). The sorted cells would be plated individually in microwells and exposed to a variety of signals. Immunocytochemical and morphological analysis of these cultures would be performed after various times *in vitro*. The potential of the PSA-NCAM+/L-NGFr- OBECs to migrate could be assessed *in vivo*, by transplanting them in the olfactory nerves of a host animal.

Similar experiments as those outlined above could be performed to study the differentiation and migrating properties of the two other OBEC phenotypes detected *in vivo*. The PSA-NCAM-/L-NGFr- OBECs could be isolated by complement mediated kill of the two other phenotypes in OB cell suspensions (using anti-PSA-NCAM and anti-L-NGFr) followed by FACS sorting with the O4 antibody.

A complementary approach to address the lineage relationship between the various OBEC subtypes described in this thesis would be to perform immunocytochemical analysis of tissue sections of the olfactory system at earlier developmental stages and following experimentally induced olfactory nerve injury. The relative proportions of each antigenic phenotype could be precisely followed throughout development and regeneration by FACS analysis.

The differentiation potential of OBECs could also be further investigated using transplantation strategies. During the past decade, glial cell transplantation has emerged as a powerful tool for bridging the gap between *in vitro* and *in vivo* studies. In particular, it allows hypotheses about glial cell lineages developed by studies *in vitro* to be tested in the more complex *in vivo* environment (reviewed by Franklin and Blakemore, 1995). The cl. 1.6 OBEC cell line generated in this thesis may be a useful tool for investigating the full differentiation potential of OBECs. This cell line offers the advantage over primary cultures of being clonal (as demonstrated by Southern blot analysis). This is particularly important as a small subpopulation of contaminant cells within primary cultures at the time of transplantation, however small, may rapidly expand following transplantation *in vivo* (Franklin et al., 1993), making studies addressing the differentiation and phenotypic properties of the cells of interest difficult to interpret.

7.2 OBEC mitogens

7.2.1 The present

This is the first investigation into the factors regulating OBEC proliferation. This investigation was undertaken using an *in vitro* approach. A semipurified preparation of GGF (a member of the neuregulin family of polypeptide growth factors) and ACM were shown to act as potent OBEC mitogens. However, most of the known growth factors present in ACM were not able to induce OBEC DNA synthesis. In collaboration with Dr. G. Graham, biochemical characterisation of the ACM-derived OBEC mitogenic activity was therefore performed and preliminary data suggests that this activity is conferred by a single factor, different from GGF and from any other previously purified neuregulins.

7.2.2 The future

Future work will concentrate on the identification of the ACM-derived mitogen and its implication in the development and regenerative processes of the olfactory system *in vivo*.

There is increasing evidence from the ongoing work of Graham Pollock, the postdoctoral fellow pursuing this work in the laboratory, that it is a neuregulin-related growth factor, which lacks heparin-binding capacity. A novel splice variant of neuregulin, which lacks the Ig-domain (the heparin-binding region), has recently been cloned from human brain libraries and detected *in vivo* by *in situ* hybridisation (SMDF, Ho et al., 1995; Corfas et al., 1995). It will be interesting to test for the presence of SMDF transcripts in cultured astrocytes, using reverse transcription PCR with two oligonucleotide sequences from the published, as yet unique, N-terminal sequence of human SMDF cDNA (Ho et al., 1995). The possibility that the unusual biochemical properties of the ACM-derived mitogen are due to a particular post-translational modification or complex formation (with a proteoglycan) of a known neuregulin could also be investigated. For example, the heparin-binding properties

and anion exchange elution profiles of the ACM-derived activity could be compared with that of ACM pre-treated with glycosidases or glycosaminoglycan-degrading enzymes, respectively. If these possibilities are ruled out, it will be reasonable to assume that the ACM-derived mitogen is a novel growth factor, possibly a new neuregulin isoform, and efforts will concentrate on the cloning of this novel isoform. An astrocyte λgt11 cDNA library would be constructed and screened using labelled oligonucleotides spanning the EGF domain sequence common to all neuregulins, as previously described for the cloning of SMDF (Ho et al., 1995).

Once the ACM-derived OBEC mitogen is identified, *in situ* hybridisation could be performed on tissue sections of the olfactory system throughout development and following experimentally induced olfactory nerve injury to shed some light on the implication of this factor in the development and regeneration of the olfactory system. An interesting possibility is that olfactory bulb-derived astrocytes regulate the cell cycle of OBECs. Relevant to this hypothesis, active cell proliferation has been reported to occur within the migratory mass (prospective ONL) during embryonic development (Marin-Padilla and Amieva, 1989; Valverde et al., 1992). It may be that OBEC precursors are stimulated to divide under the influence of astrocyte precursors from the telencephalon.

7.3 OBEC-neuron interactions *in vitro*

7.3.1 The past

Since the development of purification procedures for OBECs, the functional properties these cells has been investigated *in vitro*. Cultured embryonic rat OBECs have been shown to myelinate dorsal root ganglia neurons (Devon and Doucette, 1992, 1995). Neonatal rat OBECs have been shown to support neurite initiation from 5 week-old rat olfactory neurons (Chuah and Au, 1994). Several oncogene-immortalised neonatal and adult OBEC cell lines have been produced and shown to

promote neurite initiation and elongation from embryonic chick retinal ganglion neurons (Goodman et al., 1993).

7.3.2 The present

This thesis further shows that primary cultures of perinatal OBECs, purified by FACS sorting, support neurite outgrowth from perinatal rat cerebellar granule neurons and that this is likely to be mediated by cell surface interactions rather than by soluble factors. No significant differences in neurite initiation and elongation were observed between the OBEC monolayers that had been grown in DMEM-FCS and those that had been grown in DMEM-BS/ACM. These activities were compared to that of neonatal sciatic nerve Schwann cells and cortical astrocytes. The extent of neurite elongation over OBEC monolayers was greater than over Schwann cell monolayers but smaller than over astrocyte monolayers, further supporting the hypothesis of the unique phenotype of OBECs.

7.3.3 The future

In the present study the neurite-outgrowth promoting activities of OBECs was assessed using mixed OBEC cultures consisting of SLOBECs, ALOBECs and intermediate phenotypes, therefore it was difficult highlight specific roles for each cell types. Once appropriate culture conditions are found for the generation of individual phenotypes, it will be possible to assess their respective effect on neurons. It will also be important to test if OBECs can support the *in vitro* growth of adult neurons as these glial cells may subsequently be used to promote axonal regeneration in the adult CNS. Such dissociated OBEC culture systems should prove very useful for analysis of the molecular mechanisms by which these cells interact with neurons. To gain a more accurate picture on how OBECs function *in vivo*, olfactory bulb cryocultures or organ cultures could be used, which allow the study of neurite regeneration by dissociated neurons *in vitro* over intact, physiologically relevant substrates. Cryoculture of peripheral nerves and of various CNS tissues have already

been developed, and used for neurite outgrowth assays (Sandrock and Matthew, 1987). An olfactory bulb serum-free organ culture system such as the one recently described by Whether et al. (1993) would allow neurite outgrowth analysis in a more "*in vivo* -like" three-dimensional environment.

The clonal OBEC cell line generated in this thesis will be particularly useful for the development of transplantation strategies aimed at promoting CNS repair. Transplantation of Schwann cells, immature astrocytes and genetically engineered fibroblasts are currently being developed as a means to promote axonal regeneration in the CNS. In addition, transplantation of Schwann cells, oligodendrocytes and their precursors are being developed as a means to promote remyelination. These are still at an experimental stage in animal models (reviewed by Franklin and Blakemore, 1990; Blakemore and Franklin, 1991; Comptson, 1995). From the present *in vitro* study of OBEC-neuron interactions, this glial cell type does not appear to be better than Schwann cells or neonatal astrocytes in promoting neurite outgrowth. However, the foreseen advantages of OBECs over the cell types mentioned above are that 1) they may be easily integrated into the host CNS tissue, with a retained ability to migrate away from the point of injection (supported by Ramon-Cueto and Nieto-Sampedro, 1994), unlike Schwann cells or fibroblasts (Franklin and Blakemore, 1993) and 2) they may be able to assume multiple repair functions within a lesion, including promotion of axonal regeneration (Ramon-Cueto and Nieto-Sampedro, 1994), remyelination (Devon and Doucette, 1992; Franklin et al., 1996), phagocytosis of necrotic material (Burd et al., 1993; Chuah et al., 1995), and reconstitution of a glia limitans and/or a blood brain barrier (Doucette, 1991; reviewed by Doucette, 1995). It will be important to analyse how the cl. 1.6 OBEC cell line interacts and competes with other glial cell types and its ability to migrate away from the point of injection, in order to develop the best transplantation strategy for repair.

7.4 The ultimate goal of the project: clinical applications

Once the lineage of OBECs is well understood and successful transplantation strategies for CNS repair are established in animal models, OBECs could find their way forward to the clinic. Patients with the, as yet irreversible, severe disabilities caused by spinal cord injuries, brain trauma and demyelinating diseases could ultimately benefit from OBEC transplantation. Oncogene-immortalised cell lines are probably not well suited for transplantation into humans as they provide a risk of tumour formation. It is therefore more likely that OBECs would be expanded in culture using growth factors, possibly of the neuregulin family as shown in this thesis. Preliminary experiments, unfortunately, suggest that NDF is not able to sustain OBEC proliferation over prolonged culture times (Barnett, personal communication). Moreover, it may be technically difficult to perform olfactory bulb biopsies for the isolation of OBECs. The idea that OBEC progenitors are continually produced from stem cells in the olfactory epithelium is particularly exciting as this would offer an easily accessible and inexhaustible source of OBECs. An autologous nasal mucosal biopsy could be performed on the disabled patient and this tissue grown under culture conditions containing stem cell mitogens. Multipotential stem cells from other regions of the adult nervous system have been shown to proliferate *in vitro* in response to FGF (Gritti et al., 1995) and there is some indirect evidence that this growth factor also induces the proliferation of olfactory epithelial stem cells (DeHamer et al., 1994). Once sufficient stem cells are obtained, they would be grown under culture conditions which induce their differentiation into OBEC progenitors. These cells could then be purified by FACS sorting using specific antibodies (possibly O4 and PSA-NCAM) and subsequently transplanted into the patient for CNS repair.

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