From the Department of Neuroscience
Karolinska Institutet, Stockholm, Sweden

FACTORS INFLUENCING THE TURNOVER OF OLFACTORY RECEPTOR NEURONS

MAJA-LENA DECKNER

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To my parents Eva och John-Ivar

CONTENTS

PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. Maja-Lena Deckner, Mårten Risling, Jonas Frisén. 1997. Apoptotic death of olfactory sensory neurons in the adult rat. *Experimental Neurology* 143, 132-140

II. Maja-Lena Deckner, Jonas Frisén, Valerie M. K. Verge, Staffan Cullheim, Tomas Hökfelt, Mårten Risling.1993. Localization of neurotrophin receptors in olfactory epithelium and bulb. *Neuroreport* 5, 301-304

III. Maja Deckner, Tomas Lindholm, Staffan Cullheim, Mårten Risling. 2000. Differential Expression of Tenascin-C, Tenascin-R, Tenascin/J1 and Tenascin-X in Spinal Cord Scar Tissue and in the Olfactory System. *Experimental Neurology* 166, 350-362

IV. Maja-Lena Deckner. Perinatal and Adult Expression of VEGF and VEGF-receptors in the Rat Olfactory Mucosa and Bulb. Manuscript

V. Maja-Lena Deckner. An Immunohistochemical, Functional and Morphological Study of the Blood-Brain Barrier in the Olfactory Bulb. Manuscript

ABBREVIATIONS

BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BNB	blood-nerve barrier
BSA	bovine serum albumin
CNS	central nervous system
E	embryonic day
E-N-CAM	embryonic neuronal cell adhesion molecule
FITC	Fluorescein isothiocyanate
Flk-1	fetal liver kinase-1
Flt-1	fms-like tyrosine kinase-1
GAP43	Growth associated protein 43 kDa
HRP	horseradish peroxidase
IR	immunoreactivity
mRNA	messenger ribonucelic acid
N-CAM	neuronal cell adhesion molecule
O-CAM	olfactory cell adhesion molecule
NGF	nerve growth factor
NT3	neurotrophin-3
NT4	neurotrophin-4
OMP	olfactory marker proten
P	postnatal day
P75(NTR)	low affinity neurotrophin receptor
PBS	phosphate buffered saline
PNS	periferal nervous system
TGF	transforming growth factor
trk	tropomyosin receptor kinase
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

INTRODUCTION

THE PRIMARY OLFACTORY SYSTEM

Neuronal regeneration

Studies of traumatic lesions in the nervous system have shown that successful neuronal regeneration is much more likely to occur in the peripheral nervous system (PNS) than in the central nervous system (CNS). However, one part of the CNS, the olfactory system, has a striking capacity for neuronal plasticity throughout life (Weiler and Farbman 1997). The olfactory epithelium is able to generate both neuronal and non-neuronal cells in the normal adult animal as well as after experimental injury. (For review, see Murray and Calof 1999). While regenerating neurites from the dorsal root ganglion cannot grow through the transitional zone and re enter the spinal cord after transection of the dorsal root (Carlstedt 1991) the olfactory axons are able to cross the border between the PNS and CNS and establish synapses with their target cells in the olfactory bulb (Monti Graziadei et al. 1980; Doucette et al. 1983). (For review, see Fraher 1999). Thus, in contrast to other types of primary sensory neurons, olfactory axons are able to cross the olfactory nerve layer, the olfactory bulb transitional zone, after transection in the adult animal (Doucette 1991).

The primary olfactory system includes the olfactory sensory neurons and their connections in the olfactory bulb. The main reason for studying the turnover of olfactory receptor neurons is that knowledge about factors influencing neuronal regeneration in the primary olfactory system could be of help to understand why neuronal regeneration in general does not occur in the CNS and how it could be stimulated. Up to date, scientists in the field have not been able to the reveal the "secret formula" enabling neuronal regeneration in the primary olfactory system. Obviously, there is not one single factor that enables this neuronal turnover, but a complex network of contributing factors. This is not surprising, since the olfactory system, from an evolutionary point of view, is a very old part of the brain and the factors that promote neuronal turnover in the primary olfactory system have evolved during millions of years.

Development

The olfactory epithelium is derived from the olfactory placode and is thus of ektodermal origin. In the rat, the first olfactory axons grow from the olfactory epithelium, through the mesenchymal tissue, to the cerebral vesicle at embryonic day 12 and 13 (E12-13). At E14 to E16 the arriving olfactory axons grow superficially along the external surface of the olfactory bulb primordium to cover the surface of the bulb and at E16 the thin layer of glia limitans is also disrupted and the axons can reach their target, the neuroepithelium of the rostral telencephalic wall (Doucette 1989), (Doucette 1991). This is an important event for the induction of the olfactory bulb formation (Gong and Shipley 1995). Eventually, a new superficial layer of glia limitans is formed and completes the formation of the olfactory nerve layer. This layer is generally believed to constitute a transitional zone between the periferal and central nevous system in this region (Doucette 1991). Olfactory nerve ensheathing cells also derived from the olfactory placode migrate along with the olfactory axons into the olfactory bulb primordium (Doucette 1989; Chuah and Au 1991). From E18 the olfactory axons eventually establish contact with their target cells in the olfactory bulb. The penetration of axons and migratory cells into the bulb has been suggested to induce the formation of the synaptic neuropil into glomeruli, a process that continues also after birth (Valverde et al. 1992; Gonzalez et al. 1993). As a result the olfactory nerve layer contains a mixture of periferally derived ensheathing cells and centrally derived astrocytes (Doucette 1989; Pixley 1992).

Morphology of the olfactory bulb

The distinct layers of the olfactory bulb can easily be distinguished by ordinary cell staining or with immunohistochemistry (fig. 1b). The outermost layer, the olfactory nerve layer contains olfactory axons, olfactory nerve ensheathing cells and astrocytes. The olfactory axons extend into the glomerular layer, where the glomeruli are located. In the glomeruli olfactory receptor neurons have synaptic contact with mitral and tufted cells. The synaptic acitivity in the is modulated by the periglomerular cells surrounding the glomeruli. The next layer, the external plexiform layer, is

mainly constituted by axons and dendrites but also contains tufted cells and glia. The mitral cell layer is a thin layer inside the external plexiform layer, constituted by glia and mitral cells. The innermost layer is the granular cell layer which contains glia and granular cells. A thin layer called the internal plexiform layer can also be distinguished between the mitral and the granular cell layer. In rat and cat the anterior horn of the lateral ventricle extends into the olfactory bulb and forms the olfactory ventricle. Thus, ependyma and subependymal layer can also be found in the core of the olfactory bulb.

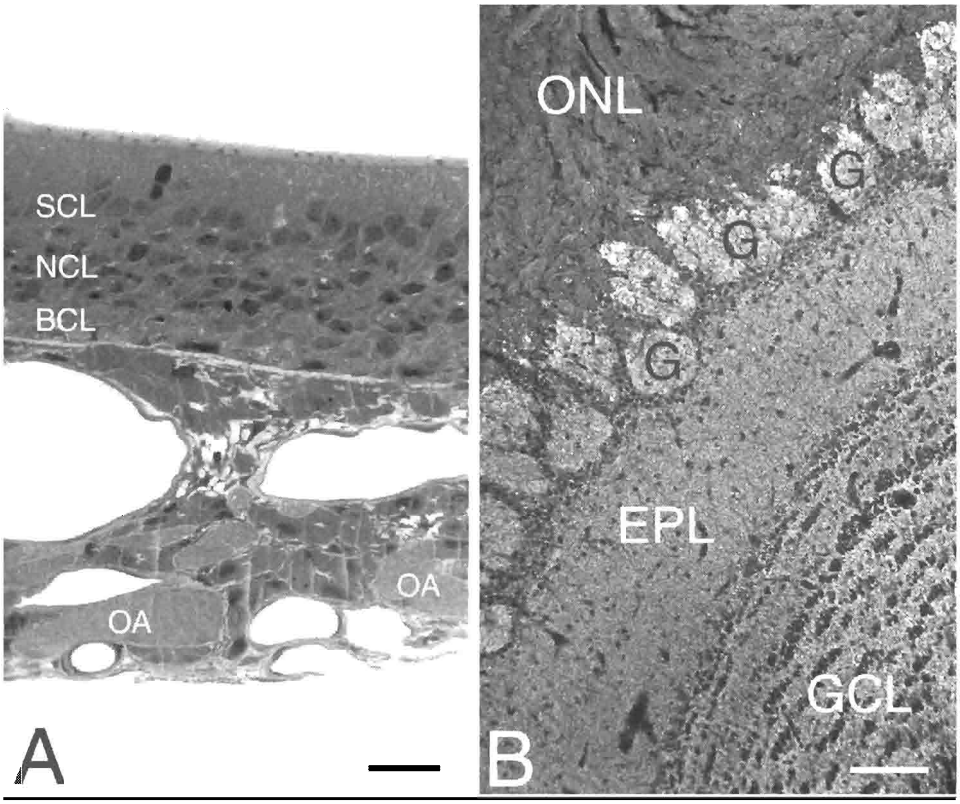

FIG. 1. Bright-field (A) and dark-field (B) photomicrographs illustrating the morphology of the the olfactory epithelium and bulb. (A) Plastic embedded section (0.5 µm) from the olfactory epithelium stained with toluidine. (B) Olfactory bulb immunostained with antibodies against synaptophysin. Abbreviations: BCL – basal cell layer, GCL – granular cell layer, G – glomerus, NCL – neuronal cell layer, OA – olfactory axons, ONL – olfactory nerve layer, SCL – sustentacular cell layer. Scale bars: (A) 20 µm, (B) 80 µm.

Morphology of the olfactory epithelium

The olfactory epithelium is located in the nasal cavity. In humans, the suface area of the olfactory epithelium is about 5 cm^2 and is located in the upper part of the nasal cavity. The cell bodies of the olfactory receptor neurons are located in the olfactory epithelium and their axons extend through the small holes of the cribiform plate, to reach the olfactory bulb. In rat and mice, the olfactory epithelium covers the caudal half of septum and most of the nasal turbinates. As all

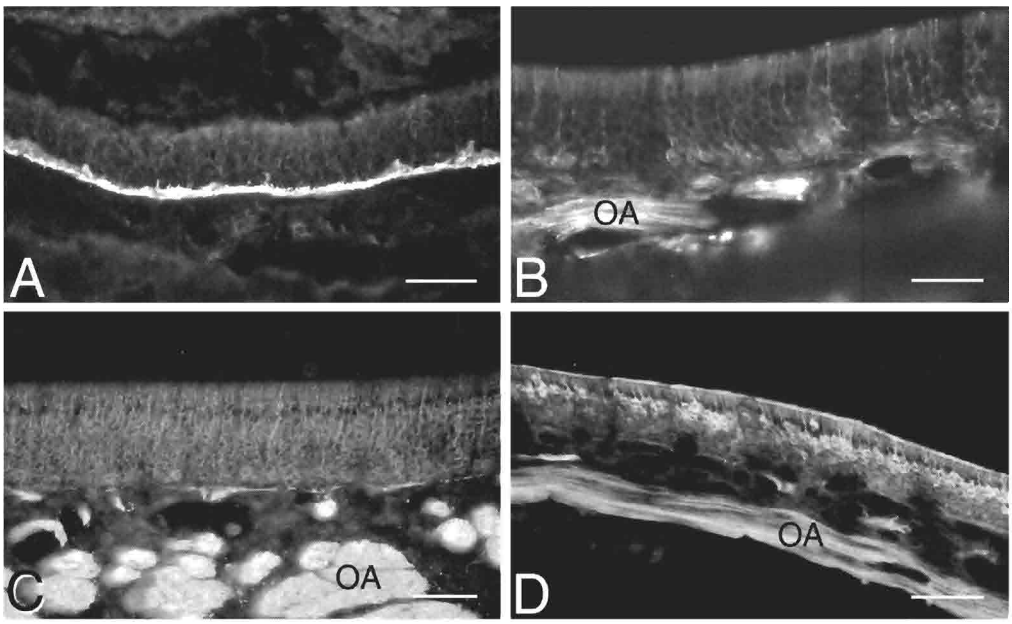

FIG. 2. Dark-field photomicrographs illustrating the different layers in the olfactory epithelium and the location of the olfactory nerve bundles in the underlying lamina propria. (A) Horizontal basal cells residing on the basal membrane visualized by antiserum against keratin. (B) Immature olfactory receptor neurons in the basal part of the epithelium, visualized by the use of antibodies recognizing GAP43. (C) Immunostaining of all olfactory receptor neurons by antibodies against N-CAM. (D) Mature olfactory receptor neurons showing strong OMP immunoreactivity. (B-D) The olfactory axons in the lamina propria underlying the olfactory epithelium are immunstained with antibodies against GAP43 (B), N-CAM (C) and OMP (D). Sections from rat (A-C) and cat (D). Abbreviation: OA – olfactory axons. Scale bars: (A and D) 50 µm, (B and C) 30 µm.

other epithelia, the olfactory epithelium is avascular and resides on a basal membrane (fig 1A). The cells of the olfactory epithelium attached to the basal membrane are often refered to as basal

cells. The vast majority of the basal cells are horizontal basal cell, also designated dark or flat basal cells (Holbrook et al. 1995). These cells can be immunolabelled by antibodies against keratin (fig 2A). Intermingled between the horizontal basal cells are the globose (round or light) basal cells located. The globose basal cells also adhere to the basal membrane. After mitotic division the globose basal cells give rise to immature olfactory sensory neurons (Suzuki and Takeda 1991; Caggiano et al. 1994; Hunter et al. 1994; Huard and Schwob 1995). There is yet another cell type that attaches to the basal membrane and that is the supporting or sustentacular cells (Hempstead and Morgan 1983). The sustentacular cells have their soma located in the most superficial cell layer of the olfactory epithelium but are still attached to the basal membrane by a thin foot-process (Costanzo and Morrison 1989).

The immature and the mature olfactory receptor neurons located in the middle compartment of the olfactory epithelium. The olfactory receptor neurons are bipolar neurons that constitute the majority of the total cellpopulation in the olfactory epithelium. The immature olfactory receptor neurons, which are more basally located within the neuronal compartment, express a cell surface protein called neuronal-cell adhesion molecule (N-CAM) (fig. 2C) as well as calmodulin/growth associated protein 43 (GAP43) (fig. 2B). As the immature olfactory receptor neurons differentiate, their dendrites extend toward the apical surface of the epithelium, while the axons grow toward olfactory bulb. The mature olfactory receptor neurons, which are located in the middle and apical part of the neuronal compartment, not only express N-CAM but also a protein designated olfactory marker protein (OMP) (Margolis 1972; Keller and Margolis 1976) which is involved in regulation of the signal transduction after odor stimulation (Buiakova et al. 1996; Ivic et al. 2000) (fig. 2D). This means that a ”maturation gradient” can be found within the neuronal compartment of the olfactory epithelium, with the younger neurons located basally and the older neurons apically. The mature olfactory sensory neuron is charactarized by an apical dendrite that terminates in an olfactory knob with cilia that are in contact with the environment and the odorant molecules. Therefore the localization of odorant binding receptors to these cilia seems adeqate.

The olfactory map

Approximately 1000 different odorant receptors are expressed in the mammalian olfactory system. One specific odorant receptor can only be expressed in one out of four spatial zones in the olfactory epithelium. All olfactory receptor neurons expressing the same odorant receptor molecule project to only a few specific glomeruli in each bulb. The location of the glomeruli connected with one specific odorant receptor is approximately the same in different animals of the same species. Binding of an odorant to its specific olfactory receptors will lead to the activation of a limited number of glomeruli with fixed location. The spatial pattern of activated glomeruli will be interpreted and recognized as a specific smell (Vassar et al. 1994; Fulle et al. 1995), (For reviews, see Ressler et al. 1994; Mombaerts 1996). Genetic deletions of specific odorant receptors alter the ability of the olfactory axons to find their right target glomerulus in the olfactory bulb (For reviews, see Mombaerts et al. 1996; Wang et al. 1998). Therefor, it has been suggested that the odorant receptor-molecules are involved in the process of axon guidance.

Retained expression of embryonic proteins

Most neurons use vimentin as a cytoskeleton component only during the embryonic period and whereas the adult cytoskeleton is constituted by neurofilaments. In contrast, the olfactory receptor neurons use vimentin to build up their cytoskeleton also in the adult animal (Schwob et al. 1986) (fig. 3a) and no switch to neurofilament expression is seen during their maturation (fig. 3b). Sustained expression of the embryonic, polysialylated form of the cell adhesion molecule N-CAM is seen in the primary olfactory system (Miragall et al. 1988) and has been shown to be essential for the development of the granular cell layer of the olfactory bulb (Tomasiewicz et al. 1993).

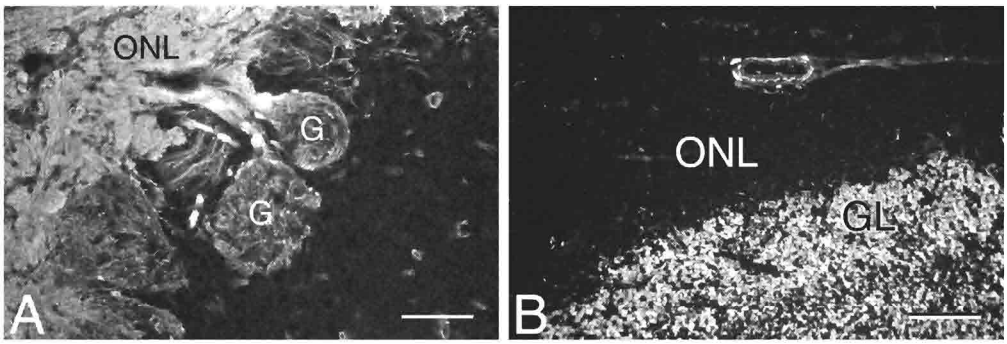

FIG. 3. Dark-field photomicrographs showing immunostaining with antibodies against vimentin (A) and pan-neurofilament (B) in the olfactory bulb. (A) Vimentin immunoreactivity is seen in olfactory axons but not in olfactory bulb neurons. (B) Pan-neurofilament immunoreactivity is seen in olfactory bulb neurons but not in olfactory axons. Abbreviations: G – glomerulus, GL – glomerular layer, ONL – olfactory nerve layer. Scale bars: (A) 50 μm, (B) 100 μm.

Neuronal turnover rate

The olfactory receptor neurons were initially believed to have a life span of 30-40 days (Moulton 1974; Graziadei and Graziadei 1979b). However, additional studies have shown that olfactory receptor neurons can live considerably longer (Mackay-Sim and Kittel 1991). In fact, it has been shown that the OSN life span can reach at least 12 months if the animals are bred in a pollution-free environment (Hinds et al. 1984). Yet, the olfactory receptor neurons do have a short life span compared to most other neurons. The rate of their turnover is most likely to be affected by both genetic and environmental influences (For review, see Farbman 1990).

Regeneration of olfactory receptor neurons after deafferentation

Much of the knowledge about neuronal turnover in the olfactory epithelium has been obtained from studies on olfactory deafferentation, which can be achieved by three different methods.

1) *Chemical deafferentation* (Harding et al. 1978; Nadi et al. 1981; Verhaagen et al. 1990; Schwob et al. 1995). By this method deafferentation is obtained by subjecting the olfactory epithelium to a fluid or a gas that destroys the cells in the olfactory epithelium. The epithelial damage can be more or less severe depending on the type and concentration of the solution or gas used, but the olfactory bulb is not directly affected. Still, secondary changes can be seen in the olfactory bulb as a consequence of the loss of synaptic contact with the olfactory receptor neurons. The method is not selective to neuronal cells since it also kills the sustentacular cells. The ability for regeneration after chemical lesions depends on how deep down in the epithelium the cells are destroyed. If the basal cells are lesioned, the prognosis for regeneration is poor and the olfactory epithelium will most likely be replaced by an epithelium of respiratory type (Schwob et al. 1995). A disadvantage with the use of intranasal irrigation with liquid solvents is the uneven distribution of fluid in the nasal cavity.

2) *Axotomy* (Harding et al. 1977; Graziadei and Graziadei 1979b; Monti Graziadei et al. 1980; Doucette et al. 1983)

Deafferentation can also be obtained by surgically removing the bone overlying the olfactory bulb, therebye exposing the region where the olfactory nerves enter into the olfactory bulb. The olfactory nerves are then cut between the lamina cribrosa and the olfactory bulb. Great care must be taken to avoid damage to the olfactory bulb. This method selectively destroys the olfactory receptor neurons that are in contact with the olfactory bulb, thus the mature population mainly. The potential for reinnervation is good since the basal cells and the immature neurons are unaffected by the lesion. A disadvantage of this method is the difficulty to cut all the olfactory nerves, since many of them enter the olfactory bulb from the inferior side. For studies with short survival times this is not a major problem, since the affected areas of the epithelium are easily detected because of the extensively occuring neuronal cell death and the characteristic changes in GAP43 and OMP expression. However, for studies on regeneration with long survival times, there is still no reliable method to prove that the part of the olfactory epithelium studied was really damaged initially.

3) *Bulbectomy* (Costanzo and Graziadei 1983; Verhaagen et al. 1990; Carr and Farbman 1992; Carr and Farbman 1993; Suzuki et al. 1996)
The procedure of the bulbectomy method is initially the same as that of the axotomy, but instead of cutting the olfactory axons, the whole or at least the anterior part of the olfactory bulb is removed. For long-time studies the produced cavity is often filled in with gel or foam to prevent regenerating axons from reaching the remaining parts of the olfactory bulb or the anterior forebrain, which in fact can occur (Graziadei et al. 1979; Graziadei and Monti Graziadei 1986). This is done since the point with this method is to study how the olfactory bulb influences olfactory receptor neuron regeneration.

All above mentioned methods lead to extensive cell death, rapid loss of OMP-immunoreactivity (-IR), increased mitotic activity of globose basal cells, increased numbers of GAP43 positive cells and morphological changes in the dark/horizontal basal cells (Verhaagen et al. 1990; Suzuki and Takeda 1991; Carr and Farbman 1992; Schwob et al. 1992; Deckner et al. 1997). Comparisons of results obtained by these three different methods have enabled researchers to answer the question whether the olfactory receptor neurons are dependent on trophic factors from the olfactory bulb for survival and maturation.

Olfactory stem cells

The search for the olfactory stem cells has been intense for a long time. In the late seventies Graziadei and Graziadei assumed that the horizontal basal cells were stem cells with capacity to generate new olfactory receptor neurons (Graziadei and Graziadei 1979a). This cell type is attached to the basal membrane and indeed has the same basal location as the stem cells in other types of epithelia. Even if a few articles claim to have proved that the horizontal basal cells are the stem cell of the olfactory sensory neuron (Satoh and Takeuchi 1995; Sicard et al. 1998), other studies on the subject indicate that they are not (Suzuki and Takeda 1991; Caggiano et al. 1994; Calof et al. 1998). What has been shown is that progenitor cells, or immediate neuronal precursors, can be found among the globose basal cell population. These precursor cells are

determined to give rise to olfactory sensory neurons (Calof and Chikaraishi 1989). Even if *in vitro* studies have shown that cells with colony forming-potential reside in the olfactory mucosa (Mumm et al. 1996) there is yet no conclusive evidence that the globose basal cells are self-renewing stem cells (Calof et al. 1991; Gordon et al. 1995). The ability for asymmetric division into one new stem cell and one cell that have capacity for clonal expansion is one of the criteria for a true stem cell (Morrison et al. 1997). Furthermore, a true stem cell is able to give rise to all the different cell types of the tissue it resides in (Morrison et al. 1997).

Cell death

Continuous mitosis and cell death occurs in the normal adult olfactory epithelium as a consequence of the short life span of olfactory receptor neurons. Transection of the olfactory nerve leads to extensive cell death in the olfactory epithelium (Carr and Farbman 1992). Cell death was for a long time considered to occur in to different ways. The first type of cell death, *nekrosis* is morphologically distinguished by cell swelling, membrane blebbing and general signs of inflammation in the surrounding tissue. The second type of cell death, *apoptosis* is morphologically characterized by cell shrinkage, chromatin condensation and loss of contact with neighboring cells. Apoptosis is considered to be a physiologically proper way of eliminating cells for example during embryonic development, since signs of inflammation are absent in the surrounding tissue. Further studies revealed that the cell death seen during development is genetically programmed mainly and this type of cell death is therefore often designated ”programmed cell death”. However, apoptotic cell death can also be induced also by exogenous factors like radiation, toxic compounds or by lack of growth factors. During the last decade the knowledge about cell death appears to have increased exponentially. However, the mechanisms of cell death have turned out to be much more complex than initially presumed. The two suggested ways leading to cell death, apoptosis and nekrosis, have now been ”transformed” into a complicated network of interacting pathways and the previous sharp border between apoptosis and necrosis is no longer very distinct. (For review, see McConkey et al. 1996).

The DNA of a cell undergoing apoptosis is cleaved in nucleosome-sized fragments (Berkelaar et al. 1994). Axotomy of the olfactory nerves, as well as bulbectomy, lead to an acute apoptotic wave with a maximal level of DNA fragmentation seen 24-40 hours after the operation (Michel et al. 1994). The phenomenon of apoptosis associated DNA-fragmentation was used to develop a technique called TUNEL, (see material and methods) which enables fast and convenient studies of DNA fragmentation on tissue sections (Gavrieli et al. 1992).

FACTORS INFLUENCING THE TURNOVER OF OLFACTORY RECEPTOR NEURONS

Olfactory nerve ensheathing cells

Even if the generation of new olfactory neurons from precursor cells in the olfactory epithelium is a prerequisite for the neuronal turnover in the primary olfactory system, the environment where the axon growth takes place is probably as important. The glial cells that ensheath the olfactory axons are believed to be crucial for axon outgrowth in the primary olfactory system. The olfactory nerve ensheathing cells (ensheathing cells) resemble non-myelinating Schwann cells but express the astrocyte related component glial fibrillary acidic protein. The ensheathing cells are believed to be derived from the olfactory placode, but if and how they are developmentally related to astrocytes and Schwann cells is still unclear. The ensheathing cells have a malleable phenotype and can be induced to produce myelin *in vitro* as well as *in vivo*. This mixture of astrocyte-specific and Schwann cell-specific characteristics has been suggested to be crucial to enable the guidance of olfactory axons through the transitional zone in the CNS (reviewed in ref. (Doucette 1995)). In addition, the ensheathing cells produce a number of adhesion and matrix molecules suggested to support axon growth (Doucette 1990; Miragall and Dermietzel 1992). For example, transplantation experiments in rat have shown that olfactory ensheathing cells exhibit an exceptional capability to promote neurite extension after fimbria-fornix transection (Smale et al. 1996), lesions in the spinal cord (Li et al. 1997; Li et al. 1998; Imaizumi et al. 2000) as well as after complete spinal cord transection (Ramon-Cueto et al. 1998; Ramon-Cueto et al. 2000). Ensheathing cells have also been shown to promote neurite outgrowth from primary sensory neurons through the transitional zone after dorsal root transection (Ramon-Cueto and Nieto-Sampedro 1994). Human olfactory ensheathing cells transplanted into demyelinated spinal cord of immunosuppressed rats have also been shown to remyelinate spinal cord axons (Kato et al. 2000). (For review see ref. (Ramon-Cueto and Avila 1998)).

Trophic dependence

Generation of new olfactory receptor neurons during the life is of course a pre-requisite for maintenance of a functional olfactory epithelium since olfactory receptor neurons have a very short life span compared to most other neurons. Many factors have been suggested to enhance the generation of immature olfactory receptor neurons by acting as mitogenic factors for globose basal cells (Mahanthappa and Schwarting 1993; DeHamer et al. 1994; Farbman and Buchholz 1996; MacDonald et al. 1996; Salehi-Ashtiani and Farbman 1996). For review see ref. (Calof et al. 1996).

After axotomy the immature neurons eventually make synaptic contact with their target cells in the olfactory bulb and mature, as indicated by the development of an olfactory knob and expression of OMP. In contrast, the same immature olfactory receptor neurons after bulbectomy, being unable to find their target, remain in a state of incomplete maturation as indicated by lack of OMP expression (Verhaagen et al. 1990). The life span of the olfactory receptor neurons is substantially reduced without their target organ and apoptotic waves can be seen with an interval of approximately seven days (Schwob et al. 1992; Carr and Farbman 1993). These findings have led to the assumption that olfactory receptor neurons are trophically dependent on the olfactory bulb for maturation and survival.

Many trophic factors have been implicated to affect the survival and maturation of olfactory receptor neurons (Mackay-Sim and Beard 1987; Mahanthappa and Schwarting 1993; MacDonald et al. 1996; Paternostro and Meisami 1996; Ubink and Hokfelt 2000), which have been shown to express receptors for cytokines from the neurotrophin family (Balboni et al. 1991; Deckner et al. 1993; Gong et al. 1994; Roskams et al. 1996) as well as receptors for TGF-alpha/EGF (Balboni et al. 1991; Krishna et al. 1996) and members from the PDGF-family (Ding et al. 2000). (For review, see Murray and Calof 1999).

The neurotrophin family

The first neurotrophic molecule to be described was the nerve growth factor (NGF) (reviewed in ref. (Levi-Montalcini 1998)). NGF was later found to be only one of the members in a family of molecules with neurotrophic activity. These molecules are often referred to as the neurotrophins and the family also includes brain-derived neurotrophic factor (BDNF) (Leibrock et al. 1989; Ernfors et al. 1990), neurotrophin-4 (NT4) (Hohn et al. 1990; Maisonpierre et al. 1990; Rosenthal et al. 1990) and neurotrophin-4/5 (NT4) (Berkemeier et al. 1991; Hallbook et al. 1991; Ip et al. 1992). Neurotrophin signaling has been suggested to be involved in a number of cellular processes such as development (Ibanez et al. 1993; Davies 1994; von Bartheld 1998), differentiation (Lewin 1996) growth, survival and neuronal regeneration (Aguayo et al. 1996; Yin et al. 1998), synaptic plasticity (McAllister et al. 1999; Gallo and Letourneau 2000) and memory (Fischer et al. 1994; Xie et al. 2000). In addition to the well-known action on neurons, BDNF has recently been found to be produced by endothelial cells (Leventhal et al. 1999) and to be required for intramyocardial vessel survival and stabilization (Donovan et al. 2000).

To exert their actions the neurotrophins bind to specific receptors (reviewed in (Bothwell 1995)). The first receptor was designated NGF-receptor since NGF was its only known ligand (Johnson et al. 1986; Radeke et al. 1987). When also the other members of the neurotrophin family were found to bind to this receptor (Rodriguez-Tebar et al. 1990; Ibanez et al. 1991; Rodriguez-Tebar et al. 1992) the molecule was renamed and is now often referred to as the low affinity neurotrophin receptor or p75 neurotrophin receptor (p75(NTR)). Additional neurotrophin receptors binding the neurotrophins with higher affinity than the "NGF-receptor" also exist. These high affinity neurotrophin receptors are members of the tropomyosin related kinase (trk) family of tyrosine kinase receptors. The high affinity neurotrophin receptors are not as promiscuous as p75(NTR). NGF binds only to trkA; BDNF and NT4 bind to trkB while NT3 is the ligand for trkC. (For reviews, see Snider 1994; McInnes and Sykes 1997; Ibanez 1998).

At least some of the neurotrophic effects of NGF on neurons are believed to be mediated via trkA and potentiated by the interaction between trkA and p75(NTR) (Gallo et al. 1997; Gargano et al. 1997; Kimpinski et al. 1999). A possible interaction between p75(NTR) and trkB has also been reported (Bibel et al. 1999). On the other hand binding of neurotrophins to the

p75(NTR) can also induce cell death (Barrett and Bartlett 1994; Frade et al. 1996; McInnes and Sykes 1997; Kuner and Hertel 1998; Friedman 2000).

The trkB and trkC-receptors also occur in additional forms. The physiological role for these truncated high affinity neurotrophin receptors is not fully understood. A local increase of truncated trkB receptors produced by glial cells have previously been observed in the scar tissue after sciatic nerve transection (Funakoshi et al. 1993) and penetrating lesions in the spinal cord (Frisen et al. 1993). These truncated trkB receptors have been suggested to act by locally increasing the concentration of neurotrophins on the surface of the glial cells, thereby stimulating outgrowth of axons dependent on BDNF/NT4. Signalling via truncated trkB receptors (Baxter et al. 1997) as well as differential effects on dendrite outgrowth (Baxter et al. 1997) have also been reported. Also trkC exists in multiple forms with different biological activities (Lamballe et al. 1993; Valenzuela et al. 1993; Hapner et al. 1998; Menn et al. 1998; Palko et al. 1999).

Matrix and adhesion molecules

Many adhesion and matrix molecules are believed to influence fasciculation, guidance and extension of olfactory axons. Molecules like N-CAM/E-N-CAM, L1, (Miragall et al. 1989; Doucette 1990; Chuah et al. 1991; Gong and Shipley 1996) Neuropilin-1, RN-CAM/O-CAM (Alenius and Bohm 1997; Yoshihara et al. 1997), N-cadherin (Chuah et al. 1991), protease nexin-1 (Reinhard et al. 1988) and tenascins (Miragall et al. 1990; Gonzalez and Silver 1994) have been implicated to influence the outgrowth of olfactory axons.

Defects in the development of the olfactory nervous system seen after targeted deletion of the gene for neuropilin-1 has been ascribed to the binding of the Semaphorin 3A (Pasterkamp et al. 1998; Schwarting et al. 2000). Neuropilin-1 has also been reported to interact with the adhesion molecule L1 to guide axon outgrowth during spinal cord development (Castellani et al. 2000). Since these two molecules are co-expressed in the primary olfactory system Miragall, 1988 #485](Gong and Shipley 1996; Kawakami et al. 1996; Pasterkamp et al. 1998) this finding might be worth bearing in mind when studying olfactory axon outgrowth. An additional molecule that should also be mentioned in this context is the adhesion molecule RN-

CAM/O-CAM that is related to zone-to-zone projection of olfactory axons (Alenius and Bohm 1997; Yoshihara et al. 1997). (For review, see Yoshihara and Mori 1997).

Integrins are receptors for matrix molecules like laminins, collagens, vitronectin and tenascin (Hynes 1992; Sonnenberg 1993), involved in regulation of cell migration, differentiation, survival and process outgrowth (Jones 1996; Howe et al. 1998). Cell-cell and cell-extracellular matrix interactions mediated by integrins are essential not only for cell survival in many different kinds of tissues (Ruoslahti and Reed 1994; Frisch and Ruoslahti 1997) but probably also for axon outgrowth (Hammarberg et al. 2000).

The tenascin family

Neuronal development depends on the balance of adhesion-promoting and adhesion-inhibitory molecules present in the extra cellular matrix or on the plasma membrane surface (Goodman 1996). The tenascins are large extracellular glycoproteins that bind to heparin, cell surface and matrix proteoglycans. The members of the tenascin family, tenascin-X, tenascin-C (cytotactin) and tenascin-R (restrictin) are involved in the regulation of numerous developmental processes, such as morphogenetic cell migration, somite organization and organogenesis (Poelmann et al. 1994; Goodman 1996; Faissner 1997; Schachner 1997). Tenascin-C, the first tenascin to be discovered, is an adhesion-modulating protein (Dorries et al. 1996; Gotz et al. 1996) associated with morphogenetic tissue interactions during organogenesis (Bartsch 1996). Schwann cells in the distal stumps of lesioned peripheral nerves strongly express tenascin-C (Martini et al. 1990) and up-regulation of tenascin gene expression usually follows after lesions in the central nervous system (McKeon et al. 1991; Zhang et al. 1995; Zhang et al. 1997).

Tenascin-R is anti-adhesive for activated microglia, promotes axon growth *in vitro* and seems to be specific for the nervous system (Angelov et al. 1998). The oligodendrocyte-derived extracellular matrix glycoprotein J1-160/180 (tenascin/J1 or janusin) is exclusively expressed in the CNS and has been shown to be adhesive for astrocytes but repellent towards neurons and growth cones (Schachner et al. 1994). Due to their structural similarities tenascin/J1 is often considered as a tenascin-R isoform (Pesheva and Probstmeier 2000).

Tenascin-X expression has been reported in the peripheral but not the central nervous system (Burch et al. 1995; Geffrotin et al. 1995). Still, the implication of tenascin expression during development and after lesions is unclear, since the members of the tenascin family have both stimulatory and anti-adhesive or inhibitory properties for axon growth (Faissner 1997).

Vascular permeability and neuronal regeneration

The composition of the interstitial fluid surrounding neurons and glial cells in the CNS is regulated by the blood-brain barrier (BBB). Endothelium with BBB function is characterized by tight interendothelial cell junctions, sparse fluid phase endocytosis and absence of pores or fenestrations (Reese and Karnovsky 1967; Brightman and Reese 1969). The tight junctions are arranged in a belt like fashion, which restricts intercellular passage to molecules with a diameter less than 2-3 nm (Claude and Goodenough 1973). It is known that the surrounding central nervous tissue induces the blood vessels to form the BBB (Stewart and Wiley 1981). Formation of tight junctions can be induced *in vitro* by astrocytes and probably also pericytes (Janzer and Raff 1987; Broadwell et al. 1991; Minakawa et al. 1991). The membrane protein occludin is a part of the large protein complex that builds up the tight junction between adjacent endothelial cells within the BBB (Anderson and Van Itallie 1995). The localization of occludin molecules can be detected immunohistochemically. Blood vessels lacking occludin-IR can also be considered to lack a BBB. Except from the circumventricular organs, which contain vessels lined with thin, fenestrated endothelia (Gotow and Hashimoto 1979; Van Houten and Posner 1983), the subpial penetrating arterioles are the only blood vessels supplying the brain that appear to be slightly permeable for large macromolecules (Balin et al. 1986; Broadwell and Sofroniew 1993).

Our knowledge about the molecular mechanisms regulating blood vessel-permeability is currently increasing rapidly. Adhesion molecules from the integrin, immunoglobulin and cadherin families binding to matrix molecules in the basal membrane surrounding the blood vessels, are likely to be involved in the formation of the BBB(reviewed in ref. (Vestweber 2000)). Cytokines like TGF-beta and VEGF are also believed to be important regulators of vascular permeability (Nag et al. 1997).

To support the brain tissue with nutrients the BBB must permit passage of glucose, amino acids, proteins, etc. These molecules are actively transported through the endothelium by receptor mediated transcytosis mainly (Jefferies et al. 1984; Frank et al. 1986; Fishman et al. 1987; Pardridge et al. 1987). The glucose transporter-1 (Glut1)(Mueckler et al. 1985; Birnbaum et al. 1986) is a protein involved in the active transport of D-glucose from the vascular to the neural compartment. Glut1 is expressed by endothelial cells within blood-brain and blood-nerve barriers (BNB)(Takata et al. 1990) and has been considered as a specific marker for cells possessing barrier quality (Harik et al. 1990).

Penetrating lesions in the central or peripheral nervous system lead to the break down of the BBB or BNB (Kiernan 1979; Kiernan and Contestabile 1980; Kiernan 1985; Risling et al. 1989). Functional studies indicate that after CNS trauma the normal function of the BBB is restored within one month (Kiernan 1979). Likewise, lesion-induced axonal sprouts are known to degenerate within four weeks (Risling et al. 1989). There are some interesting exceptions to this rule. Increased duration of post-traumatic BBB defects has been reported in the regenerating optic nerve of the goldfish (Kiernan and Contestabile 1980), after grafting of embryonic CNS tissue to the mammalian brain (Rosenstein 1987) and after lesions in the ventral funiculus of the spinal cord of adult cats and after re-implantation of avulsed ventral roots (Risling et al. 1989; Sjogren et al. 1991). These examples show that axon regeneration after CNS trauma take place in an environment that differs from the normal CNS with regards to the exposure to blood-borne molecules.

VEGF and VEGF receptors

Vascular Endothelial Growth Factor (VEGF) (Ferrara and Henzel 1989), a homodimeric protein structurally related to PDGF is a mitogenic and chemotactic factor for endothelial cells (Gospodarowicz et al. 1989) (Connolly et al. 1989; Ferrara and Henzel 1989; Keck et al. 1989; Leung et al. 1989; Plouet et al. 1989; Tischer et al. 1989; Conn et al. 1990). Targeted inactivation of the VEGF gene reveals that VEGF is essential for embryonic angiogenesis and that modest changes in the VEGF-expression level lead to aberrant embryonic development (Carmeliet et al.

1996; Ferrara et al. 1996; Gerber et al. 1999; Miquerol et al. 2000). VEGF is also designated vascular permeability factor (Senger et al. 1983) because of its ability to increase the permeability of mature blood vessels (Senger et al. 1986; Roberts and Palade 1995; Esser et al. 1998; Pham et al. 1998). In agreement with its permeabilizing properties, VEGF is believed to be involved in the BBB breakdown seen in the scar tissue after CNS-lesions (Bartholdi et al. 1997; Nag et al. 1997). VEGF has also been suggested to be involved in the maintenance of blood vessel structure and endothelial cell survival (Yamane et al. 1994; Gerber et al. 1999). VEGF was isolated and characterized in 1989 (Ferrara and Henzel 1989; Gospodarowicz et al. 1989) but is already used in clinical medicine with promising results. By local injection or by gene transfer, VEGF has been distributed to humans with impaired blood supply to enhance angiogenesis (reviewed in ref. (Neufeld et al. 1999)). Yet, VEGF is not the only factor of major importance for normal blood vessel development. Deletion of endothelial cell-specific receptors Tie-1 and Tie-2 gives rise to severe abnormalities in the vascular system (Puri et al. 1995; Sato et al. 1995). Even if VEGF probably acts as a paracrine angiogenetic factor during development (Breier et al. 1995), it is possible that VEGF is also a mediator for indirect-acting angiogenetic agents such as TGF-beta (Pertovaara et al. 1994; Berse et al. 1999) and basic fibroblast growth factor (Pepper and Mandriota 1998; Seghezzi et al. 1998). Targeted disruption of the gene encoding TGF-beta1 results in defective vasculogenesis due to incomplete endothelial cell differentiation (Dickson et al. 1995).

The human VEGF gene can be spliced into five different isoforms; $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (Neufeld et al. 1999). However, in rat only three isoforms have been definitively demonstrated, namely $VEGF_{188}$, $VEGF_{164}$ and $VEGF_{120}$ (Leung et al. 1989; Tischer et al. 1989; Conn et al. 1990; Breier et al. 1992; Garrido et al. 1993; Ladoux and Frelin 1993). (For review, see Neufeld et al. 1996). The VEGF family also includes four other members: placenta growth factor (Maglione et al. 1991; Hauser and Weich 1993; Maglione et al. 1993), VEGF-B (Grimmond et al. 1996; Olofsson et al. 1996), VEGF-C (Joukov et al. 1996; Lee et al. 1996) VEGF-D (Orlandini et al. 1996), molecules that have also been suggested to be involved in regulation of blood vessel growth (For reviews, see Joukov et al. 1997; Korpelainen and Alitalo 1998).

Up to date, three different tyrosine kinase type of receptors for the VEGF-family have been characterized. The fms-like tyrosine kinase receptor (flt-1) also designated VEGF-R1 (Shibuya et al. 1989; Shibuya et al. 1990; de Vries et al. 1992) and the murine KDR homolog flk-1/VEGFR-2 (Matthews et al. 1991; Terman et al. 1991; Terman et al. 1992; Millauer et al. 1993; Quinn et al. 1993) which are mainly expressed in blood vessel endothelial cells (Breier et al. 1995; Flamme et al. 1995a), and the structurally related tyrosine kinase receptor flt-4/VEGFR-3 which is expressed in lymph vessels (Kaipainen et al. 1995; Jussila et al. 1998). VEGF signalling via VEGFR-1 and VEGFR-2 has been shown to be essential for angiogenesis and vasculogenesis (Flamme et al. 1995b; Fong et al. 1995; Shalaby et al. 1995; Carmeliet et al. 1996; Ferrara et al. 1996). The mitotic activity on endothelial cells of VEGF has been suggested to be mediated by the VEGFR-2 (Waltenberger et al. 1994) but not via the VEGFR-1(Seetharam et al. 1995). VEGFR-1 also exists in a soluble form (sFLT-1) which has been suggested to bind and inactivate VEGF by forming a dominant negative complex with VEGFR-2 (Kendall et al. 1996; Goldman et al. 1998).

Even if VEGF initially was thought to be specific for endothelial cells, the number of studies showing that VEGF also acts on non-endothelial cells is increasing. VEGF has neurotrophic activity, stimulates axonal outgrowth (Sondell et al. 1999; Jin et al. 2000) and is involved in neurogenesis during retinal development (Yang and Cepko 1996; Yourey et al. 2000). An intimate association between angiogenesis and neurogenesis is thus possible (Palmer et al. 2000; Shima and Mailhos 2000).

In addition to these tyrosine kinase receptors the Semaphorin III-receptor Neuropilin-1 (He and Tessier-Lavigne 1997; Kolodkin et al. 1997) is also capable of binding to $VEGF_{165}$, VEGF-B_{167} and VEGF-B_{186} (Soker et al. 1998; Makinen et al. 1999). Neuropilin-1 is not capable of transmitting VEGF signalling alone, but has been shown to act as a co-receptor to VEGFR-1 (Fuh et al. 2000) and VEGFR-2 (Soker et al. 1998). Targeted inactivation of the neuropilin-1 gene in mice gives rise to severe abnormalities in the development of the vascular system and a possible role for VEGF in this context has been discussed (Kawasaki et al. 1999). (For review, see Neufeld et al. 1999).

AIMS OF THE STUDY

The general aim of this study has been to shed light on neuronal turnover in the primary olfactory system from many different angles to further increase the understanding of the complex pattern of factors that contribute to the ability of neuronal regeneration.

This task was subdivided by posing three different questions:

i) Are olfactory receptor neurons dependent on neurotrophins produced by the olfacory bulb?
Since it has been shown that neurotrophic growth factor withdrawal leads to apoptosis olfactory axons were unilaterally axotomized and the occurrence of apoptotic cell death in the olfactory epithelium was studied (paper I). Additionally, the distribution of high affinity neurotrophin receptors, trk, trkB and trkC, in the olfactory bulb was investigated (paper II).

ii) Which of the tenascin family members influence the development and the adult neuronal turnover in the primary olfactory system?
To address this question the expression patterns of tenascin-C, tenascin-X and two variants of tenascin-R mRNA in the perinatal and adult primary olfactory system were analyzed (paper III).

iii) Are olfactory axons in the olfactory nerve layer exposed to blood-borne molecules that cannot pass an intact BBB?
To address this question distribution of VEGF and its receptors in the olfactory mucosa and bulb was studied in perinatal and adult rats (paper IV). An additional study on the immunohistochemical distribution of BBB-associated proteins was also performed. This paper also includes a study on the distribution of two different molecules, both unable to pass an intact BBB, after intravenous injections.

MATERIALS AND METHODS

ANIMALS AND ANIMAL EXPERIMENTS

Tissue preparation

Male young adult (150-200g) Sprague-Dawley rats or Wistar rats were used for surgical experiments and studies on normal adult animals. Pregnant Sprague-Dawley rats were used to obtain embryos and young pups. All rats were delivered by an established local breeder (B&K Universal, Stockholm, Sweden). Rats were kept under standardized barrier-breeding conditions, with free access to water and food pellets. Fixed tissue was obtained by the following procedure. Adult rats were anesthetized with chloral hydrate (300 mg/ kg) and perfused with Tyrode's solution followed by 4% formaldehyde and 0.4% picric acid in phosphate buffer. Olfactory bulbs and epithelium were dissected out in one piece, post-fixed in the fixative for 2 hours and rinsed in phosphate buffer over night. To obtain unfixed tissue adult rats were killed by CO^2 overdose. The thoracic cage was opened and an incision made in the heart to reduce the amount of blood in the tissue. The olfactory tissue was subsequently removed, covered with Tissue Tek® and frozen on dry ice.

Embryonic specimens were obtained by killing pregnant female rats by CO_2 overdose and collecting the embryos by cesarean section. The embryos were decapitated and the heads were rapidly frozen as described above. Newborn rats were anesthetized by hypothermia and killed by decapitation at postnatal day 0.5 (P 0.5) or 4.5 (P 4.5). Noon the day of delivery was considered as P0.5. After rapid removal of most of the skin and most of the cartilage the heads were fresh frozen as described for embryonic tissue above.

The tissue was cut into 14 µm thin sections, coronal or sagittal, with a cryostat and thaw mounted on chrome-alum-gelatin-coated slides for immunohistochemistry. For in situ hybridization, tissue sections were collected on Probe On® or Superfrost Plus® glass slides. Care was taken to cut through the nasal-cavity and olfactory bulb in the same section.

Animal experiments

Cell cultures

Young adult rats were killed by an overdose of chloral hydrate and olfactory epithelium carefully dissected out, cut into small pieces and dissociated with papain (0,5 mg/ ml) in 0.01M PBS for 30 min at 37°C. Dissociated cells were collected by centrifugation, re-suspended in Dulbecos Modified Eagles Medium (Gibco) with 10% fetal calf serum (Gibco), 50 µg/ml streptomycin and 50 U/ml penicillin (Gibco) and plated in plastic tissue culture dishes (Costar). The cultures were maintained in 37°C in 95% air/ 5% CO2. The cells were fixed with the fixative described above and immunohistochemistry was performed as above.

Surgery

Adult male Wistar rats (150g, ALAB, Sweden) were used for olfactory nerve axotomy experiments (paper II). The animals were deeply anesthetized with chloral hydrate (0.3mg/kg). An instrument for transection of the olfactory nerves was made by cutting a sheath of 100 µm acetate foil into 1-2 mm thin strips. The bone over the left olfactory bulb was removed and the acetate strip was inserted between the cribiform plate and the olfactory bulb. The olfactory axons were cut by deliberate sideward movements of the acetate foil strip. By this procedure the axons were cut without causing any gross damage to the olfactory bulb. A similar technique for olfactory nerve axotomy has previously been described (Costanzo 1991). The survival times were 1.5, 4 or 12 days.

Evans Blue staining

To obtain a blood vessel staining Sprague-Dawley rats (body weight 200-250 g) were intracardially perfused as described above with the addition of 0,02 g/ml of Evans Blue to the Tyrode´s solution. The olfactory tissue was rapidly removed, covered with Tissue Tek® and frozen on dry ice. The tissue was cut into 14 µm thin cryostat sections and kept at –20°C until use. To prevent diffusion of the water-soluble Evans blue staining during the documentation, one slide at a time was thawed, mounted with glycerol and examined a Leitz DM RBE microscope equipped with a rhodamine filter. The results were rapidly documented on a Kodak T-max 400

film. For each image the micrometer scale co-ordinates at the microscope stage were noted.

After documentation of the Evans Blue localization the slides were transferred to 0,01M PBS, to remove the cover slips, and then further rinsed 3 x 30 minutes in the same solution to remove the Evans Blue staining. The sections were then incubated with combinations of primary antibodies against BBB or a combination of laminin and Glut1 antiserum followed by Cy2 or Cy3-conjugated secondary antiserum according to the immunohistochemical protocol (see section immunohistochemistry). Images were recorded at the previously noted microscope stage co-ordinates.

Intra-vascular injections of HRP and alpha-toxin

Adult (200g) white Wistar rats (ALAB, Sweden) were used for injection experiments. Horseradish peroxidase (HRP, Sigma type II, 200 mg/kg b.wt.) was injected into the femoral vein of rats deeply anesthetized with chloral hydrate (0,3 mg/kg) and allowed to circulate for 30 min(Brightman et al. 1973). Subsequently, the rats were briefly perfused with Tyrode´s solution followed by 4% glutaraldehyde in a 300 mOsm phosphate buffer. The olfactory bulb were dissected out and placed in fixative for 2 h. Sections were cut at 70 µm with an Oxford Vibratome. The sections were incubated for HRP reaction product according to Hanker et al.(Hanker et al. 1977). Sections were osmicated, dehydrated by in graded steps of acetone and section-embedded in Vestopal W and examined in a bright-field microscope. The result was documented on Kodak T-max 100 film.

For studies of alpha-toxin distribution, rats were deeply anesthetized with chloral hydrate (0,3 mg/kg) intraperitoneally and approximately 2 mg/kg of alpha-toxin was injected into the jugular vein. The toxin caused immediate death and the rats were briefly intracardially perfused with Tyrode´s solution followed by Lana´s fixation as described above. Cryostat sections (14 µm) from the olfactory bulbs were incubated at 22°C for 1 h with monoclonal antibodies against alpha-toxin (Blomqvist and Sjogren 1988) from *staphylococcus aureus* (strain Wood 46), coupled to 5 nm colloidal gold particles (Roth et al. 1978). The sections were rinsed and the gold staining was visualized for light microscopy using a silver enhancement reaction (Intense M, Jansen Biotech, Belgium). The result was viewed in episcopic illumination and an IGS filter block and documented on Kodak T-max 400 film.

Ethical approval

All animal experiments were approved by the local ethical committee (Stockholms Norra Djurförsöks-etiska Nämnd; project no. A 384/88, A 385/88, A 386/88, N41/91, N49/95, N56/96, N5/97, N5/99, N6/99).

LABORATORY PROTOCOLS

Immunohistochemistry

Prior to incubation the sections were placed in a humid plastic chamber and covered with 0,01M phosphate buffered saline (PBS) for at least 10 minutes. The sections were then incubated with the desired primary antibody, or combination of primary antibodies, over night at 4°C. For information about used primary antibodies, see table 1. All primary antibodies were diluted in 0.01M PBS with 5% bovine serum albumin (BSA), 0.3% triton X-100 and 0.1% sodium azide. After rinsing in 0.01M PBS, the sections were incubated with secondary antibodies for 45 min at room temperature. For information about used primary antibodies, see table 2. Secondary antisera were diluted in 0.01M PBS with 0.3% triton X-100 and 0.1% sodium azide. When needed, secondary antibodies were preincubated 30-60 minutes with 1% rat serum before application to reduce unspecific binding. After a last rinse in 0.01M PBS, the sections were mounted with glycerol/ PBS (3:1).

The specificity of the antibodies from Santa Cruz Biotechnology was controlled by preabsorbing the antiserum with a 10 fold concentration of the peptide it was raised against. As a negative control one slide for each secondary antibody was incubated as described in immunohistochemical procedures, but omitting the primary antiserum.

Sections were viewed in a Leitz DM RBE microscope equipped with a DMRD photomodule and a Kappa CF1/8 FMCC cooled CCD camera (Mikroskop-System AB, Näsviken, Sweden).

Table 1.
Primary antibodies

Antibody	raised in	dilution	source	tissue preparation
mAb SMI71	mouse	1:200	Sternberger Monocl.Inc.	fixed/fresh
digoxigenin	mouse	1:100	Boehringer-Mannh.Bioch.	fixed
flk-1	rabbit	1:100	Santa Cruz Biotech.	fresh
flt-1	rabbit	1:100	Santa Cruz Biotech.	fresh
GAP43	mouse	1:2000	Boehringer-Mannh.Bioch.	fixed
GFAP	goat	1:100	Santa Cruz Biotech.	fixed/fresh
Glut-1	goat	1:100	Santa Cruz Biotech.	fixed/fresh
integrin $\alpha 6/\beta 1$	mouse	1:100	Chemicon	fresh
keratin	rabbit	1:1000	Dako	fixed/fresh
laminin	rabbit	1:200	Sigma Immunochemicals	fixed/fresh
N-CAM	mouse	1:100	Sigma Immunochemicals	fixed
occludin	rabbit	1.200	Zymed lab.Inc.	fresh
OMP	goat	1:1000	gift; dr. F.L.Margolis	fixed
pan-neurofilament	mouse	1:200	Zymed lab.Inc.	fresh
trkB	rabbit	1:1-200	Santa Cruz Biotech.	fixed
VEGF	rabbit	1:100	Santa Cruz Biotech.	fresh
vimentin	mouse	1:50	Dako	fixed

Table 2.
Secondary antibodies

raised against	conjugation	raised in	dilution	source
mouse	rhodamine	goat	1:100	Boehringer Mannh.Bioch.
rabbit	rhodamine	swine	1:10	Dakopatts
rabbit	FITC	swine	1:10	Dakopatts
mouse	Cy2	donkey	7,5 µg/ml	Jackson ImmunoRes.Inc.
rabbit	Cy2	donkey	7,5 µg/ml	Jackson ImmunoRes.Inc.
goat	Cy2	donkey	7,5 µg/ml	Jackson ImmunoRes.Inc.
mouse	Cy3	donkey	3 µg/ml	Jackson ImmunoRes.Inc.
rabbit	Cy3	donkey	3 µg/ml	Jackson ImmunoRes.Inc.
goat	Cy3	donkey	3 µg/ml	Jackson ImmunoRes.Inc.

***In situ* hybridization**

Oligonucleotide probes were labeled at their 3'-ends with deoxyadenosine-alpha(thio)triphosphate(35 S) (NEN, Boston, MA) by using terminal deoxynucleotidyl transferase (Amersham Pharmacia biotec, Sweden) and hybridized to the sections without pretreatment for 16-18 hours at 42°C. The hybridization mixture contained: 50% formamide (Fluka, Sigma-Aldrich, Sweden), 4 x SSC (1 x SSC 0.15 M NaCl and 0.015 M sodium citrate), 1 x Denhardt's solution (0.02% each of polyvinyl-pyrrolidone, BSA and Ficoll), 1% sarcosyl (N-lauroylsarcosine; Sigma-Aldrich, Sweden), 0.02 M phosphate buffer (pH 7.0), 10% dextran sulfate (Amersham Pharmacia biotec, Sweden), 500 µg/ml sheared and heat denatured salmon sperm DNA (Sigma), and 4% dithiothreitol (DTT, Sigma-Aldrich, Sweden). Following hybridization, the sections were washed 5 times in 1 x SSC for 15 minutes at 60°C, adapted to room temperature, briefly rinsed in destilled water followed by ascending concentrations of ethanol. For detection by emulsion autoradiography the sections were then coated with NTB2 nuclear track emulsion (Kodak, KEBO lab AB, Sweden). After 5-6 weeks, the sections were developed in D-19 developer (Kodak, KEBO lab AB, Sweden) for 3-4 minutes at room temperature and fixed in high speed fixer (Stena Miljö AB, Sweden) for 3 minutes. Sections were counter stained with cresylviolett (Sigma-Aldrich, Sweden), mounted in Entellan® or Mountex® and coverslipped.

Electron Microscopy and bright-field morphology studies

Sprague Dawley rats were perfused with Tyrode´s solution followed by 4% glutaraldehyde in a 300 mOsm phosphate buffer. The olfactory tissue was dissected out, post-fixed for 2 hours in the fixate and rinsed in 300 mOsm phosphate buffer. The tissue was osmicated, acetone dehydrated and embedded in Vestopal W. For light microscopy 100 film. For electron microscopy studies thin sections were cut on an LKB Ultramicrotome and contrasted with uranyl acetate and lead citrate. The sections were examined in a Philips EM 201 electron microscope and the result was recorded on Kodak KS-1870 film.

Terminal deoxynucleotidyl Transferase mediated dUTP-digoxigenin Nick End Labeling (TUNEL)

Terminal deoxynucleotidyl transferase (TdT) can be used to incorporate digoxigenin-labeled deoxyuridine at sites of 3'-OH ends of DNA breaks, enabling detection of cells with fragmented DNA in tissue sections with antiserum to digoxigenin.

TUNEL-protocol

Cryostat sections (14 µm) collected from formalin fixed tissue and were post-fixed in ethanol/acetic acid (2:1) for 5 min at -20°C and rinsed in 0.01 M PBS. The sections were then placed in a humidified chamber and incubated for 5 min with equilibration buffer (ONCOR, s7110-1), 60 min in TdT solution (ONCOR, s7110-2 + 3) and 30 min in STOP/WASH solution (ONCOR, s 7110-4), at 37°C. After rinsing in PBS, the sections were incubated with monoclonal anti-digoxigenin antibodies (see table 1) followed by incubation with Cy3-conjugated donkey-anti-mouse antiserum (see table 2) according to the immunohistochemical protocol.

Procedures for EM detection of TUNEL labeled cells

Rats were deeply anesthetized and intracardially perfused with Tyrode's solution (37°C) followed by ice-cold 4% formaldehyde with 0.4% picric acid in PBS. The nasal cavity and olfactory bulbs were carefully dissected out in one piece. Thereafter the tissue was incubated for 30 min in 0.3 % triton X-100 in 0.01 M PBS in order to increase the permeability. Subsequently the epithelium was cut in pieces and transferred to microcentrifuge tubes and TUNEL was performed as described elsewhere. After the incubation with mouse anti-digoxigenin antibodies the tissue was rinsed and then incubated for 2 hours at 20°C with 1nm gold particle-conjugated goat anti-mouse antiserum (diluted 1:50, Auroprobe One, Amersham). All antisera were diluted in 0.01 M PBS containing 0.5% IGS gelatin (Amersham). 1% goat serum was added to the secondary antiserum. After rinsing, the sections were osmicated for 30 min and gold labeling was intensified with a silver enhancement reaction (Intense M, Amersham, UK). The sections were then dehydrated in

graded steps of acetone, embedded in Vestopal W, thin-sectioned by the use of an Ultrotome III (LKB) and contrasted with uranyl acetate and lead citrate. A Philips CM12 electron microscope was used for evaluation.

Cell counting procedures

The number of animals were three at each survival time. TUNEL labeled cells on the lesioned side of the olfactory epithelium overlying the ossified part of the nasal septum were counted on horizontal sections through the olfactory bulb and epithelium. Four sections from each rat were used and the total length of epithelium analyzed in each rat varied between 5-7 mm. Counting were performed at 400 times magnification in a Leitz DM RBE microscope. This view-field covered a length of 500µm epithelium. Only heavily labeled cells with small round nuclei were considered apoptotic. Every counted view-field was documented on a videoprint using a Kappa FMCC cooled CCD camera and Mitsubishi video copy processor P67E.

Illustrations

Dark-field images were recorded on Kodak T-max 400 or Tri-X pan 400 film and bright-field on T-max 100 film for paper I, II, IV and V. The images for paper III were sampled directly from the microscope, using a Nikon CP950 digital camera or a Kappa CF1/8 FMCC cooled CCD camera and a Macintosh computer equipped with a Perceptics PixelBuffer Imagegrabber card.

RESULTS AND DISCUSSION

Paper I

Transection of the olfactory nerve leads to cell death in the olfactory epithelium (Carr and Farbman 1992). This cell death is accompanied by the characteristic DNA fragmentation associated with apoptosis (Michel et al. 1994). To see if the new TUNEL-technique would confirm these initial studies, we investigated if TUNEL-labeled cells could be detected in the normal and injured olfactory epithelium. To relate the DNA-fragmentation detected by the TUNEL-technique to ultra-structural characteristics of apoptosis we modified the protocol to enable detection of TUNEL-labeled cells by electron microscopy. The TUNEL protocol was also combined with immunohistochemistry to get a more detailed description of which cells where undergoing apoptosis.

By use of TUNEL-technique combined with immunohistochemistry the occurrence of apoptotic cell death of both immature and mature olfactory receptor neurons in the adult, uninjured rat olfactory epithelium could be demonstrated. The number of TUNEL-labeled neurons was significantly increased, compared to control levels, 1.5 days after transection of the olfactory axons. The number of TUNEL-labeled neurons in the olfactory epithelium had declined to control level 4 days after the lesion and so remained up to 12 days after the injury. The ultractructural study confirmed that the TUNEL-labeled neurons showed morphologic characteristics of apoptosis. These data provide evidence for apoptotic death of neurons in the normal and injured adult mammalian nervous system.

It has been shown that more olfactory receptor neurons than required are formed and that some of these cells die at early stages (Buttke and Sandstrom 1994; Caggiano et al. 1994) and that one of the two generated daughter neurons generated by mitosis of a globose basal cell is destined to die at an early stage (Buck 1996). If this assumption is correct, a massive increase in the number of TUNEL-labeled neurons in the basal part of the olfactory epithelium could be expected during the phase of active regeneration 12 days after olfactory axotomy. Since the number of TUNEL-positive cells 12 days after the lesion did not exceed control levels, we suggested that the proportion of immature olfactory receptor neurons undergoing apoptosis may

be finely tuned to the actual need for new neurons. This assumption has later been supported by *in vitro* studies (Calof et al. 1998).

Paper II

The olfactory receptor neurons are dependent on the olfactory bulb for survival and maturation (Carr and Farbman 1992; Schwob et al. 1992) and they die by apoptosis if their contact with the olfactory bulb is interrupted by axotomy (see paper I). Which specific factors, released by the bulb, the olfactory receptor neurons depend on is not fully known. Apoptotic cell death in neurons can be triggered by withdrawal of neurotrophic factors (McConkey et al. 1996). The neurotrophins NGF and BNDF are potent neurotrophic factors expressed in the olfactory bulb (Guthrie and Gall 1991). It can therefore be hypothesized that the apoptotic cell death seen after olfactory axotomy is induced by lack of neurotrophic support.

To exert their actions, neurotrophins bind to tyrosine kinase receptors designated trkA, trkB and trkC. To clarify if olfactory receptor neurons can bind and be influenced by neurotrophins we studied the expression of trkA, trkB and trkC mRNA and trkB protein in the primary olfactory system. We found that olfactory receptor neurons express tyrosine kinase trkB receptors, while BDNF mRNA is expressed in their target cells in the olfactory bulb. These data indicate that BDNF might be a target derived factor involved in the differentiation and/or survival of olfactory receptor neurons. This study also shows that the non-catalytic form of trkB receptor is constitutively expressed by glial cells in the olfactory nerve layer of the olfactory bulb.

Paper III

The members of the tenascin family are involved in a number of developmental processes, mainly by their ability to regulate cell adhesion. Lesions in the peripheral and central nervous systems are generally followed by up-regulation of tenascin gene expression (Martini et al. 1990; McKeon et al. 1991; Fruttiger et al. 1995; Zhang et al. 1995; Zhang et al. 1997). The implication of a lesion-induced tenascin expression is however still unclear, since the tenascin family embodies both stimulatory and anti-adhesive or inhibitory properties for axon growth (Faissner 1997).

In order to gain further insight in the relation between tenascin and neuronal regeneration, the expression of mRNAs for tenascin-C, tenascin-X, tenascin-R and tenascin/J1

was analyzed after lesions in the ventral funiculus of the spinal cord as well as their expression in the rat olfactory system during the later stages of development. This type of cut lesion in the ventral part of the spinal cord is followed by regeneration of motoneurons (Cullheim et al. 1999). Both in this specific spinal cord lesion model and in the olfactory system some degree of axonal growth can be expected to occur also in the adult CNS (Risling et al. 1983; Cullheim et al. 1999; Murray and Calof 1999).

In this comparative study we have found similarities in tenascin expression in the normal spinal cord and olfactory bulb. Transcripts encoding Tenascin-C, tenascin-R and tenascin/J1, but not tenascin-X, are expressed in the spinal cord and olfactory bulb during development. Transcripts for tenascin/J1, the only extensively expressed tenascin in the adult rat, were found in neuronal as well as non-neuronal cells in both regions. Even if axonal outgrowth can be assumed to occur in both the primary olfactory system and in the scar tissue after this type of spinal cord lesion, the expression patterns of the tenascin forms are very different in these two situations. The study not only supports previous findings indicating that tenascins are involved in cell migration, axon guidance and formation of the glomerular layer, but also points out which of the tenascin family members might be involved in these processes.

Paper IV

Successful neuronal regeneration after nerve injury is much more likely to occur in the PNS than in the CNS. It has been suggested that this phenomenon could in part be explained by differences in the vascular supply in these two regions of the nervous system after lesions (Mellick and Cavanagh 1968; Risling et al. 1989; Seitz et al. 1989). The olfactory axons have a remarkable capacity to cross the border between the PNS and CNS to establish synapses with their target cells in the olfactory bulb (Doucette 1991). While the glial cells in the olfactory nerve layer, which are believed to be of utmost importance for this process, have been intensively studied (for reviews see (Doucette 1993; Doucette 1995; Ramon-Cueto and Avila 1998)), the characteristics of the vascular supply in the same region has not been thoroughly described.

The data collected in this study indicate that VEGF signalling, via both VEGFR-1 and VEGFR-2, is involved in the development of the vascular structure in the primary olfactory system. The robust expression of VEGFR-1 in the adult olfactory bulb indicates that VEGF acts on VEGFR-1-positive endothelial cells. The function for VEGF signalling via VEGFR-1 in the

adult olfactory bulb blood vessels remains to be elucidated, but might possibly involve regulation of blood vessel permeability.

Paper V

Studies on penetrating lesions in other parts of the CNS have shown a correlation in time between disruption of the BBB in the scar tissue and neurite extension in the lesioned area. These findings have led to the suggestion that leakage of blood-borne molecules could directly or indirectly stimulate neurite growth. This study explores if axonal growth in the primary olfactory pathway occurs in an environment protected from blood-borne macromolecules or not. The results of intra vascular injections of substances that do not pass the intact BBB, immunohistochemistry with antibodies against molecular markers of blood-brain barrier, bright field and electron microscopy studies of vascular morphology were compiled. The data show that the primary olfactory pathway is exposed to blood-borne molecules that cannot pass an intact BBB and that axonal growth takes place in an environment that largely differs from the normal CNS. This implies that trophic molecules in the blood might leak out and directly or indirectly stimulate the growth of olfactory axons. Furthermore, this paper also shows that alpha-toxin from *Staphylococcus aureus* in the blood can gain access to the olfactory bulb indicating that noxious agents in the blood may reach the CNS via the primary olfactory system.

GENERAL DISCUSSION

The data on vascular permeability presented in this thesis show that axonal outgrowth of olfactory axons takes place in an environment that largely differs from the normal CNS. Olfactory axons appear to be exposed to certain blood-borne macromolecules that do not pass the BBB. This indicates that olfactory axon growth could be stimulated by blood-borne nerve growth factors that leak out from the blood vessels into the tissue. Neurite extension could also be affected indirectly by leakage of blood-borne factors that stimulate glial cells to produce molecules that promote axon growth. Is it tempting to hypothesize that BDNF produced by blood cells (Besser and Wank 1999) could leak out from the blood, diffuse into the bundles of olfactory axons and bind to truncated trkB receptors expressed on the surface of ensheathing cells. The bound BDNF

molecules could then serve to stimulate neurite extension of immature olfactory receptor neurons expressing the full-length trkB receptor (Deckner et al. 1993; Roskams et al. 1996). On the other hand our data show that the primary olfactory pathway may serve as a route of entry for noxious agents circulating in the blood. That finding that olfactory nerve ensheathing cells do not seem to interact with blood vessels to induce the formation of a BBB in the olfactory nerve layer is intriguing since it might give rise to the doubts about the capacity of ensheathing cells to restore the BBB when transplanted into other parts of the CNS. This issue remains to be elucidated.

In the adult rat tenascin/J1 was the only extensively expressed tenascin form in the rat olfactory bulb, while all tenascin forms were up-regulated in the scar tissue after penetrating lesions in the ventral funiculus of the spinal cord. Thus, the expression of the tenascin molecules in the adult primary olfactory system could be not be correlated to tenascin expression in the lesion area after this type of spinal cord injury. However, this study shows that similarities can be seen, in the scar tissue after penetrating lesions in the ventral funiculus of the spinal cord and the primary olfactory pathway, with regards to vascular permeability and trkB expression in glial cells

CONCLUSIONS

Paper I
This study provides evidence for apoptotic death of neurons in the intact and injured adult nervous system. Both immature and mature olfactory receptor neurons show DNA-fragmentation characteristic for apoptotic cell death in the uninjured adult rat. The massive death of olfactory receptor neurons seen 1.5 days after axotomy is mainly caused by apoptosis.

Paper II
The data of this study show that olfactory receptor neurons express trkB, the high affinity receptor for BDNF, while BDNF mRNA is expressed in their target neurons in the olfactory bulb. These finding indicate that BDNF might be a target derived factor for olfactory receptor neurons.

Paper III
This study shows that tenascin-C, tenascin-R and tenascin/J1, but not tenascin-X, are differentially expressed in the primary olfactory system during the perinatal period and in the adult rat. Tenascin/J1 was the only extensively expressed tenascin in the adult rat
While similarities in the general tenascin expression patterns were seen in the perinatal primary olfactory system and spinal cord, the tenascin expression in the adult primary olfactory system could not be correlated to tenascin expression after lesions in the ventral funiculus of the spinal cord. The data suggest specific functions for the different tenascin molecules during the development of the olfactory epithelium and bulb.

Paper IV
The results from this study on the distribution of VEGF and VEGF-receptors in the perinatal and adult rat olfactory epithelium and bulb indicate that VEGF signalling via VEGF receptor-1 and receptor-2 is of importance for angiogenesis during development. A persistent expression of VEGF receptor-1 was seen associated with blood vessels also in the adult olfactory bulb.

Paper V
This study shows that the primary olfactory pathway is exposed to blood-borne molecules that do not pass an intact BBB and that axonal growth takes place in an environment that largely differs

from the normal CNS. This implies that trophic molecules in the blood might leak out and directly or indirectly stimulate axonal outgrowth from olfactory sensory neurons. Additional data showing that alpha-toxin in the blood can gain access to the olfactory bulb show that noxious agents in the blood may reach the CNS via the primary olfactory system.

GENERAL CONCLUSIONS

i) *Are olfactory receptor neurons dependent on neurotrophins produced by the olfactory bulb?*
The results obtained in paper I and II indicate that olfactory receptor neurons might depend on BDNF for survival and/or maturation.

ii) *Which of the tenascin family members influence the development and the adult neuronal turnover in the primary olfactory system?*
The results obtained in paper III indicate that tenascin-C, tenascin-R and tenascin/J1, but not tenascin-X, may influence the development and neuronal turnover in the perinatal primary olfactory system. Tenascin/J1 was found to be extensively expressed in the adult rat olfactory bulb.

iii) *Are olfactory axons in the olfactory nerve layer exposed to blood-borne molecules that cannot pass an intact BBB?*
Olfactory axons in the olfactory nerve layer appear to be exposed to blood-borne molecules that cannot pass an intact BBB.

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