RESTRICTED AND COMPLEMENTARY EXPRESSION PATTERNS OF EPH RECEPTORS AND EPHRIN LIGANDS DEFINE POTENTIAL INTERACTION SITES IN THE EMBRYONIC AND ADULT OLFACTORY SYSTEM OF ZEBRAFISH, *DANIO RERIO*.

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I. ABSTRACT

The functioning of the central nervous system depends upon the organization of a very precise pattern of neural connectivity formed during development. Many CNS neurons are able to establish topologically ordered maps in their target regions. Mechanisms that govern this process on a cellular level include the coordinated action of guidance molecules that are specifically distributed on the axons and their target regions.

The enormous sensitivity and discriminative capacity of the olfactory system strongly depends on the establishment of specific connections. In the olfactory system the projection of olfactory receptor neurons to the olfactory bulb is non-topological. The characteristic feature is the convergence of same-function neurons into glomeruli, globular neuropil consisting of receptor neuron terminals and dendrites of mitral cells, the projection neurons. Glomerular position and function seem to be stereotyped (Baier and Korsching, 1994; Friedrich and Korsching, 1997, 1998). Little is known about the mechanism of how same-function olfactory sensory neurons find their target glomerulus. Surprisingly, olfactory receptors themselves have been implicated in the pathfinding of olfactory sensory neurons, but only an involvement in the positioning in the anteroposterior axis of the olfactory bulb could be shown (Wang *et al.*, 1998). Hence, other guidance molecules must be present in the target area to guide axons to their correct position.

The Eph family of receptor tyrosine kinases and their ligands represent one of the most interesting families of guidance molecules, since these molecules have been implicated in the establishment of neuronal maps in several sensory systems within the CNS. The aim of this study was to investigate the possible involvement of Eph receptors and its ligands in the guidance of olfactory sensory neurons. For this purpose the expression of most of the known members for zebrafish Eph/rin genes has been analyzed on the mRNA as well as the protein level during the establishment of initial connections in the embryo and in the adult olfactory system. This analysis shows that Eph receptors and ligands are differentially expressed in all main cell types of the olfactory system. Complementary expression of receptors to their corresponding ligands in synaptically connected regions suggests that activation could occur at the interface of their expression domains. Furthermore, a layer-specific expression of receptors and ligands in the olfactory bulb suggests a role of these genes in establishing and/or maintaining intrabulbar connectivity. Interestingly, a high degree of overlapping expression has also been observed.

For functional investigation of selected candidates a suitable expression system was constructed that allows for expression of the gene of interest in a particular cell type along with a reporter protein (EGFP). Cell type-specific expression was accomplished by using the olfactory marker protein promoter, that was isolated here for zebrafish and shown to drive reporter gene expression specifically in olfactory sensory neurons. The use of such a promoter is required since widespread expression of Eph/rins is observed during development. Experiments performed by overexpressing the full-length L5/ephrin-B2a gene in olfactory sensory neurons did not reveal any gross defects in pathfinding.

The characterization of the expression patterns of the Eph family of receptors and their ligands and the development of a suitable functional expression system is the basis for future work to study the function of some of these genes in the zebrafish olfactory system.

I. ZUSAMMENFASSUNG

Die Funktionstüchtigkeit des Zentralnervensystems hängt von der Organization eines präzisen Musters neuronaler Konnektivität ab, das während der Embryonalentwicklung etabliert wird. Viele der Neurone im zentralen Nervensystem etablieren in ihrem Zielgebiet topologische Karten. Zu den Mechanismen, die diesen Prozess auf zellulärer Ebene steuern, gehören die definierte Verteilung von Wegfindungsmolekülen, die spezifisch auf Axonen und ihren Zielgebieten verteilt sind.

Spezifische neuronale Verbindungen sind eine Voraussetzung für die enorme Sensitivität und die Diskriminierungskapazität des olfaktorischen Systems gegenüber verschiedenen Geruchsstoffen. Die axonale Projektion olfaktorischer Rezeptorneurone vom Epithel zum Bulbus ist nicht-topologisch. Ein charakteristisches Merkmal der olfaktorischen Projektion ist die Konvergenz 'Funktions-gleicher' Rezeptorneurone in Glomeruli. Dies sind globuläre Neuropilstrukturen, die aus den Terminalien der olfaktorischen Rezeptorneurone und den Dendriten der Projektionsneurone, den Mitralzellen, gebildet werden. Die Positionen und die Funktionen von Glomeruli sind stereotyp (Baier und Korsching, 1994; Friedrich und Korsching, 1997, 1998). Bisher ist nur wenig über die Mechanismen bekannt, mit deren Hilfe die Rezeptorneurone ihren Zielglomerulus finden. Überraschenderweise sind die olfaktorischen Rezeptorproteine an der Wegfindung der Rezeptorneurone beteiligt; jedoch konnte nur eine Rolle bei der Positionierung der Glomeruli in der anterio-posterioren Achse des olfaktorischen Bulbus gezeigt werden (Wang *et al*., 1998). Deshalb wird vermutet, dass es andere Wegfindungsmoleküle im Zielgebiet gibt, die den Axonen bei der Suche nach Ihrem Zielglomerulus helfen.

Die Eph-Familie der Rezeptortyrosinkinasen und ihrer Liganden ist eine der interessantesten Familien unter den Wegfindungsmolekülen, da sie in die Etablierung von neuronalen Karten in sensorischen Systemen des zentralen Nervensystems involviert sind. Das Ziel der vorliegenden Arbeit war es, die Funktion von Eph-Rezeptoren und ihren Liganden im olfaktorischen System zu untersuchen. Dafür wurde die Expression der meisten im Zebrabärbling bekannten Eph und Ephrin Gene während der Ontogenese des olfaktorischen Systems und im adulten olfaktorischen System auf mRNA- und Protein-Ebene untersucht. Diese Analyse zeigte, daß Eph-Rezeptoren und ihre Liganden in den drei Hauptzelltypen des olfaktorischen Systems, differentiell exprimiert werden. Komplementäre Expressionen von Rezeptoren und den dazugehörigen Liganden in synaptisch miteinander verbundenen Regionen lassen darauf schließen, daß die Rezeptor-Aktivierung entlang dieser Expressionsdomänen stattfindet. Darüber hinaus wird eine Schichten-spezifische Expression von Rezeptoren und Liganden im olfaktorischen Bulbus beobachtet, die auf eine Funktion in der Etablierung und Instandhaltung intrabulbärer Konnektivität schließen lässt. Interessanterweise wird ein hoher Grad an überlappender Expression von Rezeptoren mit Liganden, von Rezeptoren untereinander, und von Liganden untereinander beobachtet. Diese Ergebnisse deuten daraufhin, das Eph-Rezeptoren und Ephrine überlappende oder synergistische Funktionen haben.

Für die funktionelle Analyse ausgesuchter Kandidaten wurde ein geeignetes Expressionssytem etabliert, daß die gleichzeitige Expression eines Kandidatengens mit dem Reportergen EGFP in einem bestimmten Zelltyp erlaubt. Die spezifische Expression in olfaktorischen Rezeptorneuronen wurde durch den Promotor des olfaktorischen Marker-Proteins ermöglicht. Er wurde im Rahmen dieser Arbeit für den Zebrabärbling isoliert und seine Spezifität mit Hilfe von Reporterkonstrukten gezeigt. Spezifische Promoteren sind nötig, da eine weitreichende Expression von Eph-Rezeptoren und Ephrinen während der Entwicklung beobachtet wird. Experimente, in denen das L5/ephrin-B2a Gen in olfaktorischen Rezeptorneuronen überexprimiert wurde, zeigten keinen Einfluß auf das Wegfindungsverhalten der Rezeptorneuronaxone.

Diese Arbeit stellt mit der Charakterisierung der Expression von Eph-Rezeptoren und Ephrinen und der Isolierung Zelltyp-spezifischer Promotoren eine Grundlage für weitere Untersuchungen möglicher Funktionen dieser Proteine im olfaktorischen System des Zebrabärblings dar.

II. INTRODUCTION

Axon outgrowth is one aspect of the differentiation program of a neuron that occurs after its final cell divisions. As an axon grows from the neuronal cell body, its growth cone, a structure consisting of fingerlike filopodia and/or a ruffling membrane called lamellipodium, must choose among the various substrates it encounters along its course in order to reach its target area. Some of the cues along its pathway are provided by other cells, including other axons, having preceded later arriving neurites; the extracellular matrix provides additional cues. Once in the target tissue, the axon must recognize and synapse with the appropriate cells. Although the mechanisms by which axons accomplish the complex tasks of pathfinding and target recognition is far from being fully understood, in recent years there has been tremendous progress toward identifying the molecular basis for this remarkable feat of nervous system development. Most progress has been made in identifying guidance cues, that is, molecules expressed along the pathway and in the target tissue that influence the direction of axon outgrowth. Somewhat less is known about the relevant molecules expressed in and on growing axons; these would include receptors for guidance cues and elements of the signal transduction pathways that mediate changes in growth cone behavior.

Understanding how guidance decisions are made requires identifying the signals that guide growth cones to and at their choice points, the receptors for these cues, and the signaling mechanisms by which these cues influence motility and steering of the growth cone.

1. Pathfinding Mechanisms

Growth cones appear to be guided by at least four different mechanisms: contact attraction, chemoattraction, contact repulsion, and chemorepulsion. Guidance signals can be either positive (permissive or attractive) or negative (inhibitory or repulsive) (see Fig. II.1). Each of these signals can be attached to a physical cell surface of extracellular matrix (ECM), thus establishing a short-range step function of expression, or be diffusible, thereby establishing a long-range gradient. These definitions may imply distinct mechanisms, but in reality, the differences between these mechanisms are often blurred. For example, a secreted molecule may in certain contexts become immobilized by binding to the cell surface or ECM, and as a result form a sharp boundary (Goodmann, 1996). Evidence is accumulating that these mechanisms act simultaneously and in coordinated fashion to direct pathfinding, and that they are mediated by mechanistically and evolutionarily conserved ligand-receptor systems, which will be introduced in the next part.

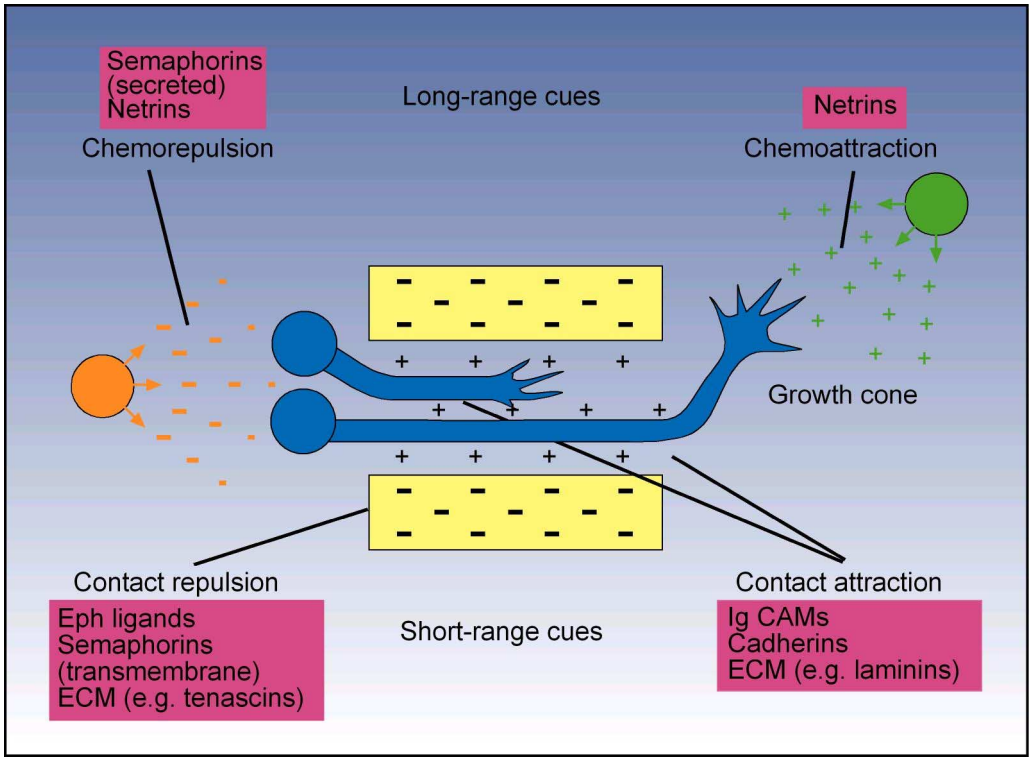

Fig. II.1.: Guidance Mechanisms. Four types of guidance mechanisms and some of the ligands implicated in mediating these mechanisms are depicted. There is not a one-to-one match between molecules and mechanisms because some guidance molecules are not exclusively attractive or repulsive. Individual growth cones might be 'pushed' behind by a chemorepellent (orange), 'pulled' from afar by a chemoattractant (green), and 'hemmed in' by attractive (gray) and repulsive (yellow) local cues (taken from Tessier-Lavigne and Goodman, 1996).

2. Pathfinding Molecules

Many mediators of axon guidance have been identified over the past decade using various approaches. The most important ones that show developmental effects comprise four conserved families of guidance cues, the netrins, Slits, semaphorins and ephrins. Netrins, Slits and some semaphorins are secreted molecules that associate with cells or extracellular matrix, whereas ephrins and other semaphorins are expressed on the cell surface. Netrins can act as attractants or repellents; Slits, semaphorins, and ephrins act primarily as repellents but can be attractive or adhesive in some contexts. For each of these molecules, one or more transmembrane receptors have been identified. For netrins two IgG superfamily members (UNC-40 and UNC-5) have been identified as receptors (reviewed in Culotti and Merz, 1998). Roundabout (Robo) proteins have been identified to be receptors for Slits (reviewed in Van Vactor and Flanagan, 1999), while neuropilin and plexins are receptors for semaphorins (reviewed in Raper, 2000). The sizes of the families varies substantially; while in the case of netrins and Slits, a small number of ligands interacts with a small number of receptors, in the case of semaphorins and ephrins a large number of related ligands interacts with a large number of receptor proteins.

All of these families of guidance molecules have been implicated in numerous axon guidance and targeting events. Moreover, functions outside the nervous system have also

been described. An extensive regulation and mechanisms that are able to integrate information from different families of guidance cues are necessary to generate the variety of structures in and outside the nervous system with so few guidance molecules. Some of these mechanisms have been uncovered and summarized recently (Yu and Bargmann, 2001).

Other molecules implicated in guidance are neurotrophins, hepatocyte growth factor/scatter factor and transforming growth factor β family members. Additional candidate guidance receptors include the procadherin family, immunoglobulin family cell adhesion molecules (Ig-CAMs) and neurexins. Although the list of guidance molecules is continuously growing, the number seems to be small compared to the high complexity of the nervous system.

3. Eph Receptors and Ephrin Ligands: Structure, Specificity and Signaling

The Eph receptor family is the largest subfamily of receptor tyrosine kinases. The receptors can be divided into 2 groups, EphA and EphB, based both on sequence similarities of their extracellular domains and their ability to bind to either the glycosyl-phosphatidyl-inositol (GPI)-linked ligands (ephrin-A ligands) or the transmembrane ligands (ephrin-B ligands), respectively (Eph Nomenclature Committee, 1997). There is a high promiscuity in binding; all receptors of one class can bind all the ligands of the same class, except for EphA1, which can only bind ephrin-A1. Interaction occuring between classes are also known, e.g. between EphA4 and ephrinB2/B3. The affinities of binding for each receptor-ligand pair is different, e.g. ephrin-A5 and ephrin-A2 bind with high affinity to the EphA3 receptor, while they bind only with low affinity to EphA4 and some interactions do not appear to trigger receptor activation (Brambrilla *et al*., 1995; Gale *et al*., 1996). It is of course conceivable that a higher specificity in binding exists *in vivo* than it is suggested by these studies done *in vitro* (see Fig. II.2.).

Binding of ephrins to Eph receptors typically involves a cell-cell interaction, since both proteins are membrane-associated. Such juxtacrine interaction appears to be required for Eph receptor activation even in cells that express both an Eph receptor and its ephrin ligand (Stein *et al.*, 1998). Ephrin binding causes Eph receptor autophosphorylation and increased kinase activity (Davis, 1994), thereby initiating a cascade of signals that eventually affect cell behavior. Recent evidence, however, suggests a more complicated mechanism of activation because different levels of Eph receptor oligomerization - caused by the binding of dimeric, tetrameric, or multimeric ephrins - appear to differentially regulate which signaling pathways become activated (Stein *et al.*, 1998).

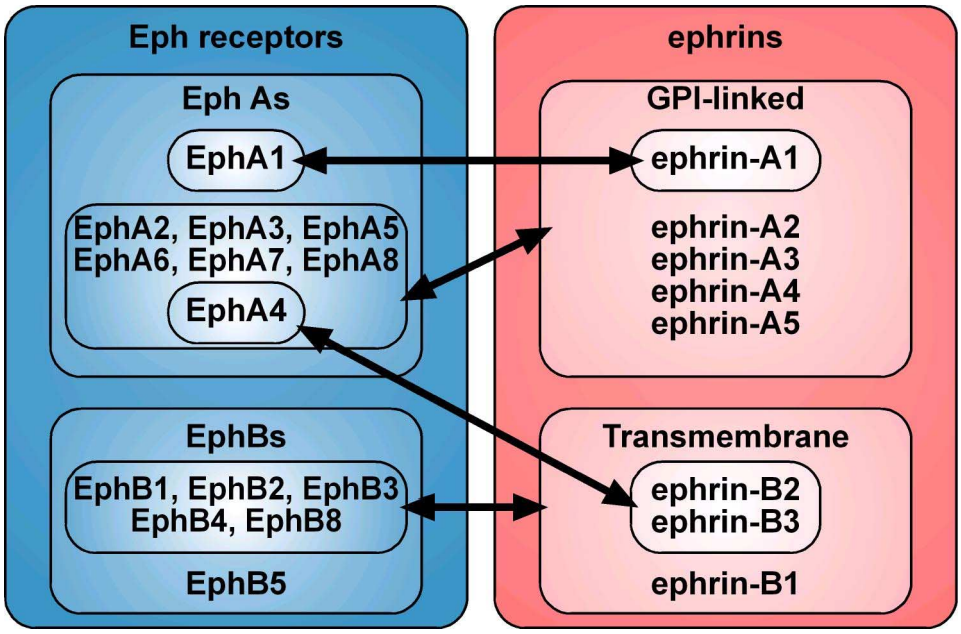

Fig. II.2.: Interactions of Eph Receptors and Ephrin Ligands (taken from Wilkinson, 2001). Eph receptors and ephrin ligands fall largely into two binding specificity classes, with the exception of EphA4, which interacts with ephrin-A and some ephrin-B proteins. Differences exist in the relative affinity of a receptor for different ephrins that may be functionally important. Additional ephrins probably exist, because EphB5 does not bind to any known ephrin.

Eph receptors have a standard structure (illustrated in Fig. II.3.). They have an uninterrupted intracellular catalytic domain and a cysteine-rich domain and two fibronectin type III repeats in the extracellular ligand-binding region. At the extracellular N terminus, there is a globular domain, which has recently been shown to be responsible for specificity of ligand binding (Labrador *et al*., 1997). The crystal structure of the N-terminal globular domain of EphB2 was solved (Himanen *et al*., 1998). The domain consists of two antiparallel β-sheets and has structural similarities with the carbohydrate-binding domain of lectins. Structure-based mutagenesis identified an extended loop packed against the concave β-sandwich surface as important for ligand-binding and subclass specificity.

Adjacent to the N-terminal domain is the cysteine-rich region of unknown function and two fibronectin type III repeats. Such fibronectin type III repeats appear in ectodomains of numerous cell adhesion molecules, RTKs and RTPs, and may be involved in dimerization. In fact, the presence of a dimerization motif has been demonstrated (Lackmann *et al*., 1998). It was suggested that Eph receptor activation occurs by a two-step mechanism, with distinct ligand binding to the globular domain followed by ligand-independent receptor-receptor oligomerization.

A transmembrane domain is followed by the C-terminal intracellular region of the protein that includes a kinase domain. A highly conserved motif containing two tyrosine residues is found in the juxtamembrane intracellular region of all Eph receptors (Ellis *et al*., 1996; Holland *et al*., 1997) that are likely to be important for intracellular signaling. A number of SH2 domain cytoplasmic proteins bind to the juxtamembrane region of the receptor when it is activated. These include the Src-like tyrosine kinases p59fyn and p60src,

in addition to the Ras GTPase-activating protein (RasGAP) and a 62-64 kD protein p60dok and the SH2/SH3 domain adaptor protein Nck.

C-terminal to the kinase domain is a **s**terile **a**lpha **m**otif (SAM) domain, a conserved region of 60-70 amino acids (Schultz *et al*., 1997). An invariant tyrosine located within the SAM domain of EphB1 is required for binding of the Grb10 adaptor (Stein *et al*., 1998).

Finally, a PDZ-binding motif (PDZ: **p**ostsynaptic density protein, **d**iscs large, **z**ona occludens; Sheng, 1996) that interacts with PDZ domain proteins is present at the C-terminal tail of Eph receptors. Some PDZ domain proteins were found to cluster and co-localize with Eph receptors and some PDZ proteins become autophosphorylated when complexed with Eph receptors (Torres *et al*., 1998).

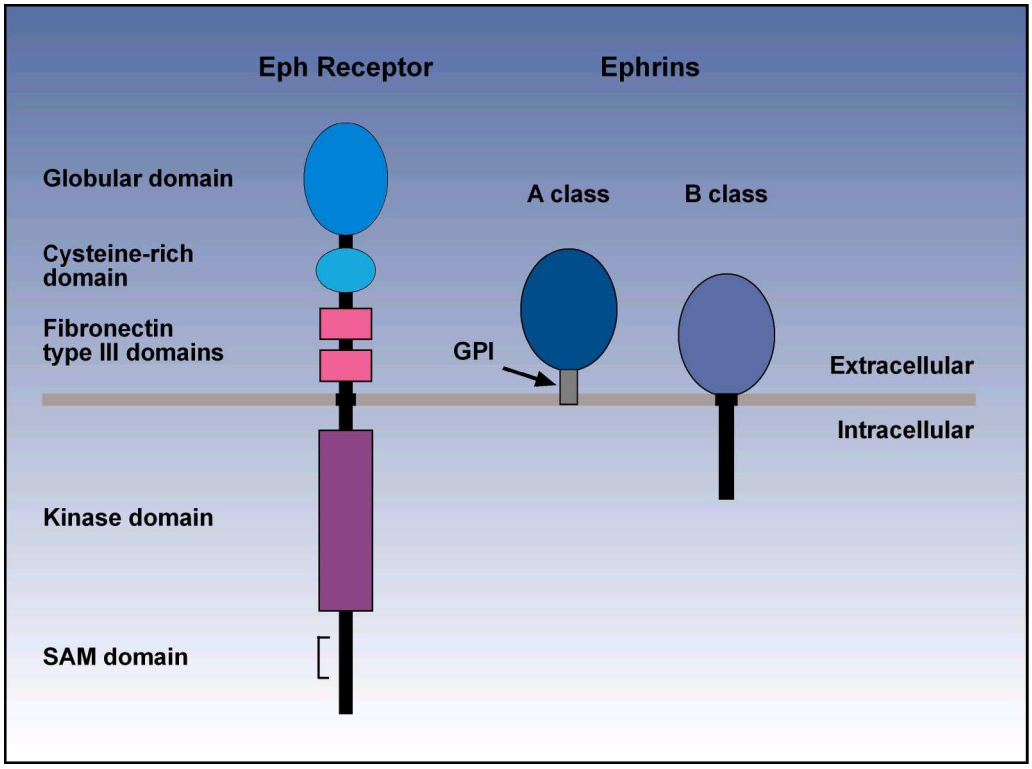

Fig. II.3.: Domain Structure of Eph Receptors and Ephrins. A and B class receptors have similar structures. The extracellular domain contains cysteine-rich and fibronectin type III domains. Ligands have similar extracellular domains. The A class ephrins are attached to the membrane via a GPI linkage, whereas the B class ephrins possess a cytoplasmic domain (taken from Holland *et al*., 1998).

The structure of the receptor varies in some cases and forms are generated that may function negatively in a signaling context. For example, the chicken EphB2 message exists in three forms, the full-length protein, a form in which an insertion of 48 nucleotides is made in the juxtamembrane region and a form that encodes a soluble protein consisting only of the extracellular region (Sajjadi and Pasquale, 1993; Connor and Pasquale, 1995). The function of these variants is not known. It is tempting to speculate that the kinase inactive forms could function as dominant negative proteins because engineered forms of receptors work in this way when overexpressed (e.g. Xu *et al*., 1995, 1996). Insertions in the juxtamembrane region could affect downstream signaling.

Interestingly, a functional PDZ-binding motif is also present at the C-terminus of transmembrane ephrinB proteins and at least one multi-PDZ domain protein, Glutamate-Receptor-Interacting Protein (GRIP) was shown to interact with both an EphB receptor and ephrinB ligands (Torres *et al*., 1998; Brückner *et al*., 1999). Moreover, ephrinB ligands are found in lipid-enriched raft microdomains, which are thought to function as platforms for the localized concentration and activation of signaling molecules (Brückner *et al*., 1999). Subsequent work has revealed that the transmembrane ephrin-B proteins may themselves transduce signals leading to the possibility that the interaction of Eph receptors and ephrin-B-expressing cells could lead to bidirectional signaling (Henkemeyer *et al*., 1996; Holland *et al*., 1998). This has been demonstrated in elegant experiments done in zebrafish, where it was shown that bidirectional signaling is required for the restriction of cell intermingling, and that unidirectional signaling in either direction is not sufficient (Xu *et al*., 1999; Mellitzer *et al*., 1999).

By analogy with other GPI-anchored molecules involved in signal transduction, one mechanism by which ephrinA proteins could transduce signals is via sequesteration to membrane raft microdomains at which other components are assembled, including a transmembrane co-receptor. Recent work has shown that ephrin-A5 is localized in specialized membrane microdomains, termed caveolae, and has provided direct evidence for a role in signal transduction (Davy *et al*., 1999).

3.1. Biological Functions of Eph Receptors and Ephrin Ligands

The first functional evidence of a role of Eph receptors in axon guidance came from the purification and cloning of ephrin-A5 (formerly known as AL-1/RAGS) as a tectal protein with the ability to collapse retinal axon growth cones (Drescher *et al.*, 1995). Multiple lines of evidence now argue for an important *in vivo* function for Eph receptors and ephrins. These include roles in directing axonal (Nakamoto *et al*., 1996; Orioli *et al*., 1996; Wang and Anderson, 1997) and neural crest cell (Wang and Anderson, 1997) migrations, regulating axonal bundling (fasciculation) (Orioli *et al*., 1996), and preventing the mixing of discrete cell populations during development and boundary formation (Mellitzer *et al.*, 1999; Xu *et al.*, 1999). *In vitro* assays have demonstated that ligand activation of Eph receptors in neuronal cells initiates anti-adhesive responses. These are characterized by repulsion of axons (Drescher *et al.*, 1995; Wang and Anderson, 1997) and neural crest cells (Wang and Anderson, 1997), and collapse of neuronal growth cones (Drescher *et al.*, 1995; Meima *et al*., 1997a, b).

In endothelial cells, different responses to Eph receptor activation are observed. Both transmembrane and GPI-linked ephrins stimulate cell adhesion and vascular network formation. The difference between these responses may depend on the cell type or alterations in ligand clustering. Recently, it was demonstrated that ephrin-B2 marks arterial but not venous endothelial cells from the onset of angiogenesis. Conversely, EphB4 marks veins but not arteries (Wang and Anderson, 1998).

3.2. Role of Eph Receptors and their Ligands in Topographic Mapping

Growth cones invade their target region, where they often form a topographic projection pattern, before they make their final synaptic connections. Topographic mapping in which the relationship of neighboring neurons is maintained in the target region occurs throughout the nervous system. The best-studied example of topographic mapping is the visual system of vertebrates. Neighboring retinal ganglion cells project to neighboring target regions in the tectum (or superior colliculus), thus projecting the retina's map as a topographic map across the tectum (see Fig II.4.). Classic experiments performed by Sperry and colleagues showed that after dissection of the optical nerve, retinal ganglion cells regenerated and established their initial connections. Thus, the positional information was encoded somehow in the tectum. In 1963, the theory of chemoaffinity was proposed; according to this theory molecular labels exist in gradients across the projecting and target areas and the axons find their correct location by matching up the labels (Sperry, 1963).

Evidences in favor of this theory came with the identification of gradients of repellent ligands for Eph receptors in the chick retinotectal system. In the last few years, ephrins and their Eph receptors have been identified as likely candidates for graded labels of the type predicted by Sperry. In chick, ephrin-A2 and ephrin-A5 are expressed in overlapping posterior > anterior gradients across the tectum (Cheng *et al*., 1995; Drescher *et al*., 1995), while the receptor EphA3 is expressed in a corresponding temporal > nasal gradient across the retina (Cheng *et al*., 1995). The ligands are sufficient to repel chick retinal axons with a topographically specific preference for temporal axons, as shown by assays *in vitro* (Nakamoto *et al*., 1996; Monschau *et al*., 1997; Feldheim *et al*., 1998) and gain-of-function experiments *in vivo* (Nakamoto *et al*., 1996). In addition to functions in the target, *in vitro* assays and gain-of-function experiments have led to the proposal that ephrin-A2 and ephrin-A5 may also act in the retina, downregulating functional receptors in nasal axons (Hornberger *et al*., 1999).

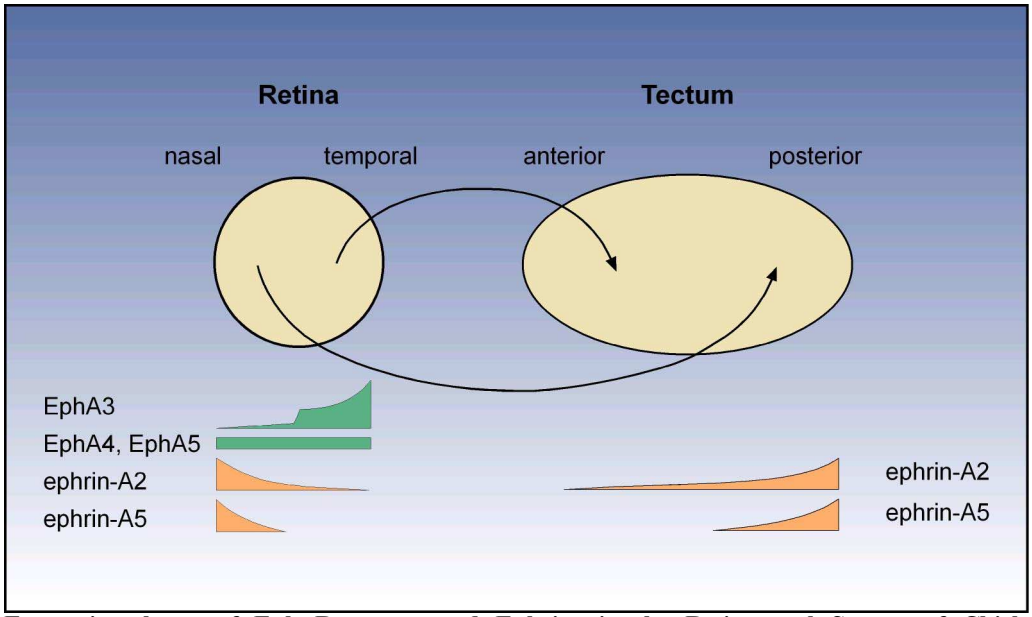

Fig. II.4.: Expressionscheme of Eph Receptors and Ephrins in the Retinotectal System of Chick (taken from Drescher, 2000).

Studies in the mouse support a similar function for ephrins. Graded expression patterns comparable to those in chick are seen in mouse (Cheng and Flanagan, 1994; Frisen *et al*., 1998). However, the gradient across the retinal ganglion cell layer is established by EphA5 rather than EphA3 (Feldheim *et al*., 1998). Loss-of-function studies by gene disruption show that ephrin-A5 is required for normal mapping, with temporal axons terminating more posteriorly than normal (Frisen *et al*., 1998). However map topography still appear largely intact, suggesting either that ephrins may not have a major role in mapping or that there could be partial redundancy of ephrin-A2, ephrin-A5, and perhaps, other ephrins.

As pointed out by Gierer (1981), it seems hard to account for topographic mapping in terms of only a single type of gradient per axis. If there were only a repellent gradient, presumably all axons would simply be repelled to one end of the target. However, if there were two opposing gradients, for example a repellent and an attractant, each axon could identify its correct place as the point where the opposing forces cancel out (Gierer, 1981). Such dual-gradient models have been considered a likely explanation of map formation (Drescher *et al*., 1997). However, alternative mechanisms, such as axon-axon competition for space in the target, could also explain the ability of axons to distribute throughout the map (Fraser and Hunt, 1980). Very recently, an axon competition mechanism for mapping with ephrins having a function not only in the specification of the anteroposterior but also the dorsoventral axis was demonstrated (Feldheim *et al.*, 2000).

Aside from the tectum, these receptor/ligand interactions have been implicated in axon growth and targeting in the septo-hippocampal system. Here, ephrin-A2 is expressed in a ventral (high) to dorsal (low) gradient in the septum, whereas EphA5 is expressed in the hippocampus in a medial (high) to lateral (low) gradient (Gao *et al*., 1996). Ephrin-A2 was shown to selectively induce pruning of topographically inappropriate medial hippocampal axons (Gao *et al*., 1996).

Nothing is known so far about the involvement of Eph receptors and ephrin ligands in the olfactory system. It would however be very interesting to see if they have a similar function in the guidance of olfactory sensory neurons.

4. The Olfactory System

The sense of smell is based on the ability of specialized receptor cells in the nasal cavity to respond to environmental chemicals by initiating a nerve impulse. These specialized cells are the olfactory sensory neurons (OSN) that make up the main olfactory epithelium (MOE) located in a complex series of folds in the nasal cavity. In the mammalian MOE, volatile odors bind to the olfactory receptors located on the cilia and dendritic knob of the sensory neurons. The olfactory receptors are members of the seven transmembrane domain receptor superfamily estimated to consist of around 1000 different genes, which activate heterotrimeric guanine nucleotide-binding proteins (G-proteins) (Buck and Axel, 1991). The olfactory system is precisely organized and highly dynamic. In higher vertebrates, each olfactory neuron expresses only one (or a few) of about 1000 receptor genes (Chess *et al*,

1994; Malnic *et al*., 1999). Additionally, olfactory neurons expressing a distinct odorant receptor are distributed in topographically distinct zones in the MOE (Ressler *et al*., 1993; Vassar *et al*., 1993; Strotmann *et al*., 1994). Within the four zones (I-IV), however, receptor distribution is random. Axons of OSNs expressing one particular receptor project to the same spatially defined regions in the main olfactory bulb (MOB) called glomeruli. Generally, axons converge onto one to three glomeruli per olfactory bulb. In rodents the number of receptor genes was estimated to be between 500-1000 and the number of glomeruli that they project to about 2000 (Royet *et al.*, 1998). *In situ* hybridization experiments in the olfactory epithelium showed that about 0.1% of OSNs are labeled with a particular olfactory receptor (Vassar *et al*., 1993; Ressler *et al*., 1993). This correspondence in receptor and glomerulus number suggests that one glomerulus is targeted by OSNs expressing one receptor type and was proven by *in situ* hybridization experiments with receptor probes on the olfactory bulb (Vassar *et al*., 1994; Ressler *et al*., 1994) and elegant gene targeting experiments (Mombaerts *et al*., 1996; Wang *et al*., 1998). Additionally, it could be shown that these OSNs respond to a small subset of related odorants (Zhao *et al*., 1998; Aradena *et al*., 2000). This led to the hypothesis that the olfactory receptors are involved in sensory axon guidance (Singer, 1995), a subject that will be discussed in more detail later. Recently, it could be shown that the position of individual glomeruli shows microheterogeneity, so that the position of glomeruli that are targeted by axons expressing a single receptor gene may vary between the two bulbs of one individual or between animals (Strotmann *et al*., 2000). Additionally, there is evidence that the position of glomeruli represents a chemotopic order, since OSNs that express structurally related receptor genes project to neighboring glomeruli (Tsuboi *et al*., 1999). Another outstanding feature of the olfactory system is that olfactory neurons are replaced continuously throughout life (Graziadei and Graziadei, 1979; Mackay-Sim and Kittel, 1991), but the neuronal population maintains a conserved connection pattern to the olfactory bulb.

5. The Wiring Problem

Taken together, the topographic organization of the olfactory system poses an enormously complicated wiring problem. Olfactory sensory neurons expressing one of about 1000 different receptor genes have to project to a specific position in the olfactory bulb. This has to happen during development and the attained projection pattern has to be retained somehow during adult life so that newly born neurons can find their correct target glomerulus and make correct synapses. *What are the cues that help the OSN axons to find their target?*

5.1. Pathfinding Cues in the Olfactory Epithelium

Findings of zones in the olfactory epithelium defined by expression patterns of subsets of olfactory receptor neurons correlate with anatomical tracing studies that subdivide the olfactory epithelium into broad regions that map onto corresponding regions in the olfactory

bulb (Saucier and Astic, 1986; Schoenfeld *et al*., 1994). Olfactory sensory neurons located ventrolaterally in the epithelium converge onto ventrolateral glomeruli within the bulb (Ressler *et al*., 1994; Vassar *et al*., 1994). It seems that the zonal organization in the MOE is preserved in the olfactory bulb. But, *what are the cues that define these zones in the epithelium?* The homophilic cell adhesion molecule OCAM is expressed by axons of olfactory sensory neurons that are located in zones II, III, and IV, but not in zone I (Yoshihara *et al*., 1997). Tracing studies of OCAM-expressing olfactory axons to their terminals in the glomeruli showed zonal segregated projections of olfactory axons. While OCAM-negative zone I axons project to glomeruli in the rostrodorsal *zone I* of the MOB, OCAM-positive zones II, III, and IV axons project selectively to caudoventral *zones II, III*, and *IV* of the MOB. A complementary pattern of expression was reported for the CC2 carbohydrate epitope, which is only positive for axons in zone I (Schwarting and Crandall, 1991). Molecular markers that distinguish among zones II, III and IV are still lacking. Besides OCAM and NCAM, a variety of other Ig superfamily molecules, including L1 (Miragall *et al*., 1989), TAG-1 (Yamamoto and Schwarting, 1991), Thy-1 (Terkelson *et al*., 1989), and BIG-2 (Yoshihara *et al*., 1995) are expressed by olfactory axons. The function of zone-specific axonal adhesion molecules might be a prerequisite for olfactory axon guidance, by broadly grouping zonally related OSNs together. Thus, the coordinated expression of cell adhesion molecules with odorant receptors might be required for the establishment of a functional projection of olfactory axons.

Olfactory sensory neurons projecting to different glomeruli lie together in the olfactory epithelium and the olfactory nerve. There must be some molecules that help them to segregate from one another. One candidate molecule is a lectin, *Dolichos biflorus* agglutinin (DBA). Lectins are molecules that bind sugar residues on proteins. This particular lectin binds to a subpopulation of OSNs, which are widely scattered in the nasal cavity (Key and Akeson, 1993). The DBA-reactive axons lying dispersed in the olfactory nerve sort out, selectively fasciculate after entering the olfactory bulb and terminate in glomeruli predominantly present in the dorsomedial part of the olfactory bulb (Key and Akeson, 1993). The importance of sugar-residues on proteins was shown in mice lacking the galectin-1 gene that codes for a lactose-binding protein, which forms homodimers. Mutants for galectin-1 show topographical errors in the projection of sensory neurons to specific areas of the olfactory bulb, with few DBA-reactive axons growing into caudal regions of the olfactory bulb, while the rostral projection appearing unaffected (Puche *et al*., 1996). Thus, galectin-1 may help olfactory axons to segregate from one another, despite its widespread expression.

The cell-surface proteins neuropilin-1 and neuropilin-2 (see above) are also expressed on OSNs. Olfactory neurons of *Xenopus* appear to express different levels of neuropilin-1. While being intermingled in the olfactory epithelium, OSNs expressing high levels of neuropilin-1 segregate from those expressing low levels (Satoda *et al*., 1995). However in mouse, neuropilin-2 is expressed heterogeneously in OSNs, and OSNs expressing high levels of neuropilin-2 are located more rostrally within the epithelium (Chen *et al*., 1997). Thus, similar to OCAM and galectin-1 neuropilins may function to coarsely segregate OSNs as they project to the olfactory bulb. In a quite recent report it was shown that spatial mapping of OCAM+/OCAM- glomeruli and NP1+/NP- glomeruli in the mouse olfactory bulb are

parceled into topographically distinct domains (Nagao *et al*., 2000). The topographic arrangement of these domains suggests that the olfactory bulb contains two mirror-image maps, one lying in the medial and one in the lateral hemisphere.

Although the olfactory receptors are good candidates for molecular tags (as proposed by Sperry) for olfactory sensory neurons, this theory would require the presence of as many molecular tags in the olfactory bulb that would recognize each particular receptor present on the OSNs. The presence of that many genes for this purpose is quite unlikely. It is also not conceivable how the olfactory receptors would serve two functions, odor recognition and axon guidance. Nevertheless, their role has been examined in experiments using targeted mice. In initial experiments it could be shown that an odorant receptor is required for convergence to specific glomeruli, since OSN axons lacking a receptor were able to leave the olfactory epithelium and enter the olfactory bulb, but failed to enter the glomerular layer. However, when the coding region of a receptor gene was replaced with the coding region (swapping) of another receptor axons converged, but the axons targeted to a different position that was neither the original nor the swapped receptor glomerulus (Mombaerts *et al*., 1996; Wang *et al*., 1998). In further swap experiments the target glomerulus of the swapped receptor could never be targeted, which indicates that additional factors are necessary for correct targeting and that the receptors themselves have only an instructive role in the guidance process (Wang *et al*., 1998). In the same experiments olfactory receptors were implied in guidance in the anteroposterior axis only. Thus, other guidance cues are needed in determining projection in the dorsoventral axis.

Is activity required for pathfinding of OSNs? The organization of neuronal systems is often dependent on activity and competition between cells. This has been shown clearly in visual system (Katz and Schatz, 1996), where the remodeling of cortical projections are driven by activity-dependent competition. The influence of odorant-evoked activity on the olfactory system has been investigated by surgical (naris occlusion) and genetic manipulation (genetic disruption of the *OCNC1* gene). The *OCNC1* gene, which encodes an essential subunit of the olfactory cyclic nucleotide-gated channel, is a key component in the olfactory signal transduction pathway. Its disruption blocks all odorant-evoked activity in embryonic and neonatal mice (Brunet *et al*., 1996; Lin *et al*., 2000), but the pattern of convergence was unaltered for olfactory sensory neurons expressing several odorant receptors (Lin *et al*., 2000; Zheng *et al*., 2000). Very elegant experiments that allow the direct visualization of *OCNC1*-deficient OSNs and their projections, suggest that odorant-evoked activity is crucial for olfactory neurons to survive in a competitive environment and implicate neuronal activity in the organization and maintenance of the olfactory system (Zhao and Reed, 2001).

5.2. Pathfinding Cues in the Olfactory Bulb

Relatively many differentially expressed guidance molecules have been identified in the olfactory epithelium. In contrast, the search for guidance molecules in the olfactory bulb has been less successful. *What are the cells that should express target molecules in the olfactory bulb?* Thinking conventionally, one would propose the M/T cells of being good

candidates, since they are the synaptic partners of olfactory sensory neurons. But, early experiments showed that this might not be the case. For example, when the olfactory bulb is removed, olfactory sensory neurons were able to make glomerulus-like structures in the telencephalon (Graziadei and Monti Graziadei, 1986). In the moth, ablation of mitral-like cells also does not prevent glomerulus formation (Oland and Tolbert, 1998). In the mouse, knock-out experiments of a T-boxed transcription factor, *Tbr1*, which is expressed in mitral cells, resulted in a loss of all mitral cells (Bulfone *et al*., 1998). However, the lacZ-targeted OSNs still converged to their proper glomerulus (Bulfone *et al*., 1998). In the same experiments periglomerular neurons were also knocked out (by genetic disruption of the *dlx2* gene), which also did not have any effect on proper targeting. These data indicate that mitral cells as well as periglomerular cells, the major cell types in the olfactory bulb, and even the olfactory bulb itself play a minimal role in establishing the spatial map of glomeruli.

What other cell types might be involved in targeting if not the M/T cells and periglomerular cells? There are only few cell-types present in the olfactory bulb, when convergence takes place (Mombaerts *et al*., 1996). The two major classes of glia that could be involved in early glomerulus formation are the ensheathing cell and radial glia. Radial glia are the cells that express pathfinding cues in gradients along the tectum in chick (Drescher *et al*., 1995). If these cells would express gradients of pathfinding cues in the olfactory bulb also, this situation would be quite analogous to the chick retino-tectal system. Both cell types have been investigated in rodents. Ensheathing cells (olfactory Schwann cells), migrate towards the olfactory bulb along with the growing OSN axons (Valverde *et al*., 1992), and initiate the formation of glomeruli. Radial glia processes in rat form 'glial glomeruli' at the same time that OSN axons coalesce into glomeruli, suggesting that they play a role in the formation and/or stabilization of glomeruli in the rat olfactory bulb (Bailey *et al*., 1999). Analysis of the development of radial glia in the mouse olfactory bulb showed that there is a tight spatiotemporal relationship between the glomerulization of radial glia processes and OSN axons during development, suggesting that radial glia processes could play a role in the formation and/or stabilization of mammalian glomeruli (Puche and Shipley, 2001).

What are the molecules that are differentially expressed in the olfactory bulb that could guide olfactory axons? One class of genes differentially expressed in the olfactory bulb, are the cadherin-like receptors, which are expressed in overlapping subsets of mitral, periglomerular, and granule cells (Kohmura *et al*., 1998). Semaphorin III, a ligand for neuropilin receptors is expressed in only a subset of mitral cells and periglomerular cells (Giger *et al*., 1998). Neuropilin-1-positive OSNs in mice lacking the semaphorin 3A gene are misrouted and terminate inappropriately in ventral glomeruli (Schwarting *et al*., 2000). A receptor of the Eph family, Bsk (EphA5) is expressed heterogeneously in mitral cells (Zhang *et al*., 1996). Finally, collapsin-1 is expressed at the posterior boundary of the olfactory bulb and may act as a stop signal, restricting olfactory axons to the bulb and preventing them from entering into the telencephalon (Kobayashi *et al*., 1997).

From the published data compiled here it is not clear if the expression pattern of these molecules is similarly restricted during development when the spatial map is being established. Also, molecules that show a ubiquitous distribution in the adult olfactory bulb might have a differential expression in the embryo. Search for guidance molecules in the

olfactory bulb involves a close examination of expression patterns to reveal even subtle differences.

Taken together, one can say that proper targeting in the olfactory system involves a series of distinct guidance decisions. First, OSNs have to find their correct zone within the olfactory epithelium, then they have to project their axons to a zone in the olfactory bulb that corresponds to the zone in which they are located in the olfactory epithelium. Finally, they have to find their correct position to make synaptic contacts with the second-order neurons and form their specific glomerulus. Although olfactory receptors play an important role in guidance of OSNs, their role has yet to be elucidated in detail. Concerning the olfactory bulb, which cells and what molecules provide positional cues is an intriguing question, which remains to be solved.

6. Organization of the Olfactory System in Teleosts

6.1. The Olfactory Epithelium

The primary interaction with odors takes place in the olfactory epithelium of zebrafish, which is composed of a rosette-like structure that can be divided into a sensory and a non-sensory area (Hansen and Zeiske, 1998). The sensory area contains mainly three different cell types, the olfactory sensory neurons, supporting cells (or sustentacular cells) and the basal cells, located adjacent to the basal lamina that separates the epithelium from the underlying *lamina propria*. As opposed to the differential pattern of distribution of cells within the mammalian olfactory epithelium the olfactory epithelium of teleosts is less organized. All cells are closely packed and cell bodies of receptor cells and supporting cells do not form distinguishable layers.

In teleost fishes, the olfactory sensory neurons can be divided into two morphologically distinct cell types: the ciliated and the microvillar receptor cells (Hansen and Zeiske, 1998). Both ciliated and microvillar receptor cells are intermingled within the epithelium and no vomeronasal organ exists (e.g. Yamamoto, 1982; Cancalon, 1983; Erickson and Caprio, 1984; Moran *et al*., 1992; Morita and Finger, 1998). The function of these different cell types has been proposed to be the detection of different odorant classes (Thomessen, 1982, 1983), but there is no general agreement on which cell type serves which function. While in electrophysiological studies in salmonids, the microvillar cells appeared to show a higher sensitivity to amino acids (Thomessen, 1982, 1983), degeneration-regeneration experiments performed in goldfish suggest that the sensitivity to amino acids is related to the presence of ciliated OSNs (Zippel *et al*., 1997a, b). Still other experiments where electrophysiological and anatomical studies were combined in channel catfish, no functional difference of these receptor types could be revealed (Erickson and Caprio, 1984). Thus, the relative functional contribution of each OSN type is controversial.

The olfactory sensory neurons are bipolar cells; ciliated cells have a well-pronounced olfactory knob that projects into the lumen of the nasal cavity, while the knobs of microvillar

cells is less pronounced. The olfactory knob of ciliated cells bears 3-7 cilia, while the microvillar knob bears 10-30 short microvilli. The axons of olfactory sensory neurons accumulate within the sensory epithelium and penetrate the basal lamina in bundles. Within the lamina propria these bundles gather together to form the *fila olfactoria*. These in turn converge to form the olfactory nerve that passes through a single opening in the ethmoidal bone and travels to the olfactory bulb. *The fila olfactoria* as well as the olfactory nerve are accompanied by Schwann cells and surrounded by a faint and often hardly visible basal lamina (Hansen and Zeiske, 1998).

Crypt cells are the fourth cell type in the sensory area of the olfactory epithelium. They occur at a low number, are located at the upper quarter of the epithelium and bear cilia as well as microvilli (Hansen and Zeiske, 1998). The existence of an axon that reaches the olfactory bulb supports the idea that this cell is a receptor neuron.

Supporting cells are cylindrical cells that have small irregular protrusions that reach into the lumen. They separate the dendrites of receptor cells (Hansen and Zeiske, 1998).

Basal cells lie adjacent to the basal lamina between the axons of OSNs and the basal parts of supporting cells. Their number is not high and slightly increased in the valleys close to the midline raphe (Hansen and Zeiske, 1998). These are also the areas where proliferation of basal cells is highest (Berger, 1998).

Cells in the non-sensory area are long and ciliated bearing up to 60 kinocilia, which can propel water and/or mucus over the lamellae. The mucus-secreting Bowmann's glands are missing, but numerous goblet cells, which fulfill the same function, are scattered throughout the epithelium (Byrd and Brunjes, 1995).

6.2. The Olfactory Bulb

The olfactory epithelium connects to the olfactory bulb through an olfactory nerve. The layering of the olfactory bulb of teleosts is not as obvious as that of mammals (Satou, 1990; Byrd and Brunjes, 1995). In the zebrafish, each olfactory bulb consists of four layers that are roughly concentrically arranged throughout most of the bulbs extent: the olfactory nerve layer (ONL), the glomerular layer (GL), and the internal cell layer (ICL) (see Fig.II.5). The most superficial layer is the olfactory nerve layer (ONL), which contains the axons of the olfactory sensory neurons and glial cells and their processes (Byrd and Brunjes, 1995). The glomerular layer lies right below the olfactory nerve layer and is readily distinguishable since it is composed of globular neuropil structures called glomeruli. A distinct secondary olfactory fiber layer present in many other teleosts is not apparent in the zebrafish (Wullimann *et al*., 1996). No periglomerular cells were identified, but in addition to the mitral/tufted cells an additional type of projection neurons was identified in goldfish, catfish and sea eel, the ruffed cells (Kosaka and Hama, 1979, 1980; Alonso *et al*., 1987). They have myelinated axons, make few synapses with other neurons, surround mitral cell dendrites, but receive no input from OSNs (Satou, 1990). The function of these cells is not known. In the zebrafish, the olfactory axons terminate in about 80 clearly defined and stereotyped glomeruli. In addition they also project to some plexus-like regions in the anterior and ventrolateral part of the

olfactory bulb, which are not clearly resolved (Baier and Korsching, 1994). One of the main differences to the MOB of mammals is that the M/T cell dendrites of lower vertebrates innervate many glomeruli. The M/T cell axons leave the olfactory bulb and enter the telencephalon by two tracts, the lateral (LOT) and the medial olfactory tract (MOT). While the MOT appears to carry information related to sexual behaviors, the LOT mediates feeding behavior and alerting responses (for review see Hara, 1992). After passing the telencephalon they terminate in three fields. These are the medial terminal field in the *area ventralis telencephali*, the lateral terminal field in the ventrolateral part of the *area dorsalis telencephali* and the posterior terminal field in the central part of the *area dorsalis telencephali* (Wullimann *et al*., 1996).

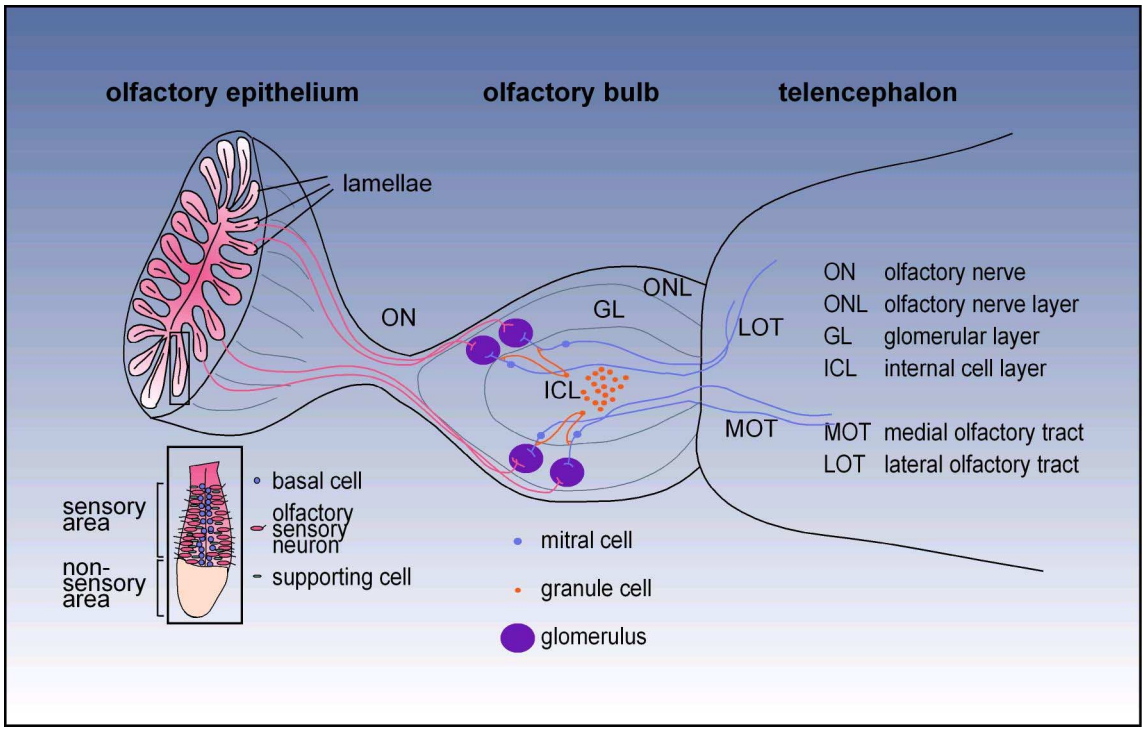

Fig. II.5.: Organization of the Zebrafish Olfactory System.

6.3. Molecular Biology of Fish Odorant Receptors

The characterization of the olfactory system in teleosts started with the cloning of genes for olfactory receptors. The first olfactory receptors to be cloned in fish were the catfish receptors (Ngai *et al*., 1993). Many others like receptor genes for zebrafish (Barth *et al*., 1996, 1997; Weth *et al*., 1996; Byrd *et al*., 1996; Mori *et al*., 2000), for goldfish (Cao *et al*., 1998; Freitag *et al*., 1998) and for medaka (Wendeler, 1999; Sun *et al*., 1999; Yasuoka *et al*., 1999) followed. In addition to this, vomeronasal receptors of the V2R-Type were cloned for goldfish (Cao *et al*., 1998; Speca *et al*., 1999), for fugu (Naito *et al*., 1998), and for zebrafish (J. Ngai, L. Buck, personal communication). *In situ* hybridization studies with the isolated receptor genes revealed a dispersed and punctate expression pattern in a subset of neurons in the sensory area of the olfactory epithelium. By quantitative analysis of receptor

expression in the adult zebrafish it could be shown that the receptors are expressed in 3 or possibly 4 nested expression domains (Weth *et al*., 1996). Olfactory receptor gene expression can be detected as early as 20 hours post fertilization (hpf) (Argo, 1995; Barth *et al*., 1996, 1997; Byrd *et al*., 1996). The onset of expression is asynchronous and can be as late as 120 hpf (Barth *et al*., 1996). It seems that genes that are tightly linked in the genome are activated at different times of development (Barth *et al.*, 1997). The biological significance of this non-coordinate expression of OR genes, which has not been reported in mammalia, remains unclear.

6.4. Topographic Organization

To date the topographic organization of the olfactory bulb was not shown for zebrafish or any other teleost. The only evidences for a (specific) kind of order in the olfactory bulb comes from anatomical and functional studies. For rainbow trout it was shown by examining their lectin-binding properties that subsets of olfactory sensory neurons that are distributed and intermingled with the other receptor neurons in the olfactory epithelium and nerve project to restricted regions of the glomerular layer (Riddle *et al*., 1993). Tracing studies in channel catfish revealed that a type of microvillar OSN with a short cell body projects preferentially to the dorsal quadrant of the posterior part of the olfactory bulb, and a type of tall and ciliated OSN projects preferentially to the ventral OB (Morita and Finger, 1998). In zebrafish, it could be shown using tracing experiments that the cell bodies of OSN whose axons terminate in a single glomerulus are distributed across the whole sensory area of the epithelium (Baier *et al*., 1994; Lieberoth, 1999). In some cases the labeled neurons appeared to be in domains that correspond to rings (Lieberoth, 1999). A correspondence between the neurons projecting into a single glomerulus and a single expressed receptor gene could not be demonstrated due to technical difficulties (Weth, 2001).

However, a hint towards a convergence of same-function OSNs comes from physiological studies in the zebrafish olfactory bulb. Using optical imaging, spatial patterns of neuronal responses to odorants in the olfactory bulb were shown to be similar between individuals. Responses segregate into different regions and subregions of the bulb depending on the given stimulus, i.e. the activated olfactory receptor (Friedrich and Korsching, 1997, 1998; Fuss and Korsching, 2001). Intriguingly, responses to specific odorants (pheromones PGF2α and 17-20βp) were localized to individual identifiable glomeruli (Friedrich and Korsching, 1998), suggesting that same-function OSNs converge in a single glomerulus.

6.5. Development

The olfactory placode of zebrafish forms by convergence of large cellular fields at anterior lateral edge of the neural plate as was shown using lineage tracing (Whitlock and Westerfield, 2000). In the same study the cells contributing to the telencephalon and the developing olfactory bulb were also identified as lying medial to the olfactory placode field along the anterior edge of the neural plate (Whitlock and Westerfield, 2000). This shows that

in zebrafish the olfactory placode and olfactory bulb develop in parallel from adjacent fields of cells in the neural plate. At about 17-18 hpf the placodes appear as thickenings of the ectoderm, which invaginate to form the naris at about 32 hpf. The first axons projecting to the presumptive olfactory bulb leave the olfactory placodes by 20 h (Hansen and Zeiske, 1993). Similarly, neurons with axons emanating from the olfactory placode have been identified using horseradish peroxidase labeling at 24 h (Wilson *et al*., 1990). The axons of so-called pioneer neurons that establish the initial pathway later followed by the axons of OSNs visualized by zns-2 exit the olfactory placode at the same time (Whitlock and Westerfield, 1998). The ablation of the pioneer neurons demonstrated that they are required for axon guidance of olfactory sensory neurons to the olfactory bulb (Whitlock and Westerfield, 1998). These neurons do not appear to express any of the previously cloned olfactory receptors, so it is not known if the receptors themselves play a role in the targeting of OSN during development (Whitlock and Westerfield, 1998). The axons show a directed growth to the olfactory bulb as described by transient transfection experiments performed by Dynes and Ngai where the growth of axons was observed in hourly intervals (1998). The stereotyped glomerular pattern present in the adult olfactory bulb is reached by the continuous addition of glomeruli during development as revealed by tracing studies and requires about 3 weeks (von Campenhausen, 1995; Lieberoth, 1999).

7. Aim of this Study

Basic to the investigation of olfactory functions is an understanding of the neuroanatomical and neurochemical organization of the olfactory system and its linkages to other parts of the brain.

The aim of this study was to investigate the expression patterns of a large number of Eph receptor and ephrin genes by *in situ* hybridization in the adult as well as in the embryonic olfactory system of zebrafish, in order to get insight into possible functions of this family of genes in OSN pathfinding. This study also involved the characterization of the main cell types of the olfactory system of zebrafish on the cellular and molecular level. This was done using neuroanatomical and molecular marker molecules. This work was supposed to be the basis for understanding the expression patterns and distributions of putative pathfinding molecules. To do functional studies with selected members of this family an expression vector was designed that allowed the simultaneous expression of a gene of interest and a fluorescent reporter (EGFP) specifically in olfactory sensory neurons. However, initial experiments performed by overexpressing the full-length L5/ephrin-B2a gene did not reveal any gross defects in axonal pathfinding.

III. MATERIALS AND METHODS

1. Biological Material

Animals

In this study wild-type zebrafish of different strains were used. These strains included the inbred Ab/Ab strain (University of Oregon), the inbred Tü/Tü strain (MPI, Tübingen), the Ab/Tü strain, the CO/CO strain (University of Cologne, Department of Developmental Biology, Campos-Ortega group) and wild-type zebrafish from a local pet shop. Adult zebrafish (*Danio rerio*) were kept in group tanks at a day/night rhythm of 14/10 h at a water temperature of 28°C and fed daily dry flake foods and brine shrimp (artemia; Brustmann, Oestrich-Winkel). The aquaria were filled with a one-to-one mixture of demineralized water and tap water.

Zebrafish embryos and larvae were kept in petridishes at a density of about 50 embryos/petridish in embryo medium (E3: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 $MgSO_4$, Methylenblue 5-10%) at 28°C without feeding for five days. Afterwards, they were raised in 2 l containers. Until an age of two weeks they were fed a special food for fish larvae (TetraMin Mini, TETRA), and afterwards with artemia.

In order to elicit controlled reproductive activity, selected females and males were put into a separate tank. Early in the following morning, fertilized eggs were collected and their age was determined using the staging criteria of Kimmel *et al.* (1995). The embryos were then raised and collected at 24h intervals for histological and immunohistochemical processing. Embryos fixed at a stage older than 24 h postfertilization (hpf) were raised in 2 mM 1-phenyl-2-thiourea (PTU) in embryo medium after the epiboly stage (about 12 h) to prevent pigmentation. The embryonic and larval stages used for all investigations reported here ranged between one and 21 days postfertilization (dpf).

Eukaryotic Cell Line

Two different cell lines were used in the framework of this study:

COS-7 cells (Kidney, SV40 transformed, African green monkey) fibroblast-like cell line established from CV-1 simian cells which were transformed by an origin-defective mutant of SV40 which encodes for wild-type T antigen (Gluzman, 1981). ATCC CRL 1651, kindly provided by K. Saijo.

Bacterial Strain

To amplify plasmid, cosmid and PAC DNA the following bacterial strains were used:

***Epicurian coli* XL1 Blue MRF'** Stratagene (Heidelberg)

Genotype: (mcrA)182, (mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac, [F', proAB, $lacI^qZ$, M15, $Tn10(tet^r)$]

2. Chemicals and Supplies

All chemicals used in this study were from Ambion (Austin, USA), Amersham Pharmacia Biotech (Freiburg), Applichem (Darmstadt), JTBaker supplied by Fisher Scientific (Schwerte), Biozym (Hessisch Oldendorf), Calbiochem (Darmstadt), Difco (Detroit, USA), Fluka (Neu-Ulm), Merck (Darmstadt), Molecular Probes (Leiden, NL), Roth (Karlsruhe), Serva (Heidelberg), or Sigma (Deisenhofen) unless stated otherwise in the text.

Enzymes

Restriction enzymes were used either from Amersham Pharmacia Biotech (Freiburg) or New England Biolabs (Schwalbach, Taunus). T4 DNA Polymerase, Taq DNA Polymerase, Expand High Fidelity Taq Polymerase, Expand Long Template Taq Polymerase, T4 DNA ligase, T3, T7, and SP6 RNA Polymerase and the Klenow enzymes were purchased from Roche Biochemicals (Mannheim). Shrimp alkaline phosphatase (SAP) was from USB (Cleveland, OH, USA). Reverse Transcriptase Superscript II was from Invitrogen Life Technologies (Karlsruhe). RNase-free DNase RQ1 was from Promega (Mannheim), RNaseA and Proteinase K were purchased from Sigma.

Nucleotides

Nucleotides for PCR and the radionucleotides [α-^{32}P]dCTP (3000 Ci/mmole on day of delivery) and [^{35}S]dCTP for labeling of probes were purchased from Amersham Pharmacia Biotech.

Membranes and Filters

Nylon and nitrocellulose membranes Hybond N™, Hybond were from Amersham Pharmacia Biotech. Nylon membrane positively charged for non-radioactive hybridization was from Roche Biochemicals. GB 003 Gel-Blotting paper and sterile filters were purchased from Schleicher&Schuell supplied by Fisher Scientific.

Films

Instant films for gel documentation was purchased from Polaroid. X-Ray films XLS-1, X-OMAT™AR, and X-OMAT™LS in various sizes and Diafilms (Ektachrome 160 T) were from Kodak (Stuttgart). X-Ray film cassettes IEL6040C with intensifier screens were from Rigo (Augsburg).

Plasticware

All disposable plasticware like 15 ml and 50 ml Falcon tubes, 6-, 24-, 48-, 96-well plates, petridishes in various sizes were from TPP or Castor, both purchased from Fisher Scientific. 96-well plates Polyfiltronics for colony PCR were from Whatman (supplied by Fisher Scientific), 0.2 ml PCR tubes and sterile pipette tips were from MβP supplied by Fisher Scientific. Non-sterile pipette tips were supplied by LaFontaine (Forst/Bruchsal) and Labomedic (Bonn).

3. Preparation of Solutions

Solutions were prepared with water from a SeralPur facility. Solutions were autoclaved for 20 min at 121 bar or filter sterilized (0.2-0.45 µm pore diameter). Glassware was autoclaved and oven baked for 2 h at 180°C. For RNA-work, solutions and water were treated with 0.1% diethylpyrocarbonate (DEPC), shaked vigorously and mixed for about 20 min on a magnetic stirrer to bring the DEPC into solution. The solutions were then autoclaved to remove any trace of DEPC. Tris buffers cannot be treated with DEPC because it reacts with primary amines. DEPC decomposes rapidly into CO_2 and ethanol in the presence of Tris buffers. Therefore, Tris buffers were prepared by using water that has been treated with DEPC first.

Most of the standard stock solutions like EDTA, Tris, TAE, TBE, TE, PBS, SDS, SSC, NaOAc, and culture media like LB and SOC were prepared as described in Sambrook *et al*., 1989. All solutions used are named in the text.

4. Plasmids and Vectors

Table III.1. List of Cloning Vectors

Name	Supplier	Description/Properties
pBluescript II KS(+)	Stratagene	2.96 kb; B/W; ampicillin resistance
pSPORT1	Invitrogen Life Technologies	4.1 kb; B/W; ampicillin resistance
pGEM-T	Promega	3 kb; B/W; T vector; ampicillin resistance
Lawrist7	RZPD	5.4 kb; cosmid vector; kanamycin resistance

B/W: blue/white selection possible

Table III.2. List of Expression Vectors

Name	Supplier	Description/Properties
pEYFP1	Clontech	4.2 kb; promoterless; kanamycin resistance
pDsRed1-N1	Clontech	4.7 kb; CMV promoter; kanamycin resistance
pIRES2-EGFP	Clontech	5.3 kb; IRES sequence; kanamycin resistance

EYFP-1: encodes an enhanced yellow-green variant of the *Aequorea victoria* green fluorescent protein (GFP). Excitation maximum = 513 nm; emission maximum = 527 nm.

DsRed1: encodes a novel red fluorescent protein (RFP) that was isolated from *Discosoma sp.* and has been optimized for high expression in mammalian cells. Excitation maximum = 558 nm; emission maximum = 583 nm.

IRES: internal ribosome entry site of the encephalomyocarditis virus (ECMV). This sequence allows the gene of interest and the EGFP gene to be translated from a single bicistronic mRNA.

EGFP: a red-shifted variant of wild-type GFP, which has been optimized for brighter fluorescence and higher expression in mammalian cells. Excitation maximum = 488 nm; emission maximum = 507 nm.

Table III.3. List of cDNA Clones

Name	Supplier	Description
rtk1	C. Brennan and N. Holder, King's College, U.K.	pBS containing 500 bp of the *rtk1* gene
rtk2	C. Brennan	pBS containing 1500 bp of the *rtk2* gene
rtk3	C. Brennan	pBS containing 400 bp of the *rtk3* gene
rtk4	C. Brennan	pBS containing 2640 bp of the *rtk4* gene
rtk5	C. Brennan	pBS containing 2616 bp of the *rtk5* gene
rtk6	C. Brennan	pBS containing 1155 bp of the *rtk6* gene
rtk7	C. Brennan	pBS containing 1473 bp of the *rtk7* gene
rtk8	C. Brennan	pBS containing 2046 bp of the *rtk8* gene
L1	C. Brennan	pBS containing 1903 bp of the *L1* gene
L2	C. Brennan	pBS containing 1500 bp of the *L2* gene
L3	C. Brennan	pBS containing 2200 bp of the *L3* gene
L4	C. Brennan	pBS containing 1800 bp of the *L4* gene
L5	C. Brennan	pBS containing 3500 bp of the *L5* gene
L6	C. Brennan	pBS containing 3000 bp of the *L6* gene
APV	C. Brennan	fusionprotein construct
L4-AP	C. Brennan	fusionprotein construct
L5-AP	C. Brennan	fusionprotein construct
R6-AP	C. Brennan	fusionprotein construct
R8-AP	C. Brennan	fusionprotein construct
dlx2	M. Westerfield, Oregon, USA	pBS containing 1668 bp of the *dlx2* gene
dlx3	M. Westerfield	pBS containing 1530 bp of the *dlx3* gene
dlx4	M. Westerfield	pBS containing 1120 bp of the *dlx4* gene
pax6	MPI Tübingen	pBS containing 441 bp of the *pax6* gene
otx1	MPI Tübingen	pBS containing 2083 bp of the *otx1* gene
otx2	MPI Tübingen	pBS containing 2161 bp of the *otx2* gene
six3	G. Begemann, Sheffield, U.K	pBS containing 1550 bp of the *six3* gene
mtbr3	A. Bulfone, TIGEM, Italy	PCR product of 1 kb of coding region of the mouse *Tbr3* gene

pBS: pBluescript

Table III.4. List of Genomic Clones

Name	Supplier	Description/Purpose
ICRFc70B1662Q2	RZPD	40 kb; *OMP* gene locus in Lawrist7 kanamycin resistance
BUSMP706N2039Q4	RZPD	115 kb; *dlx1* and *dlx2* gene locus in pCYPAC6; kanamycin resistance
BUSMP706H02207Q2	RZPD	120 kb; clone hybridizing to *ztbr1* 5' probe in pCYPAC6; kanamycin resistance
BUSMP706O09174Q2	RZPD	120 kb; clone hybridizing to *ztbr1* 5' probe in pCYPAC6; kanamycin resistance
BUSMP706N08248Q2	RZPD	120 kb; clone hybridizing to *ztbr1* 5' probe in pCYPAC6; kanamycin resistance
BUSMP706L15147Q2	RZPD	120 kb; clone hybridizing to *ztbr1* 5' probe in pCYPAC6; kanamycin resistance

5. Libraries

Zebrafish cDNA Libraries

Adult zebrafish olfactory epithelial cDNA library was prepared by Dr. Sigrun Korsching using about 125 adult olfactory rosettes (S. Korsching, 1995) and cloned into the pSPORT1 vector using *Not* I- and *Sal* I-adapters.

Adult zebrafish olfactory bulbal cDNA library was prepared by V. Oehlmann using olfactory bulbs of 32 adult zebrafish and cloned into the pGEM-T vector.

Adult zebrafish whole brain cDNA library was prepared by Dr. Sigrun Korsching using whole brains from adult zebrafish, and cloned into the pSPORT1 vector using *Not* I- and *Sal* I-adapters.

Zebrafish Cosmid and PAC Genomic Libraries

The zebrafish cosmid and PAC genomic libraries that were used in this study were kindly supplied of Hans Lehrach of the Human Primate Resource Center (RZPD), Berlin, Germany. The identified positive clones were ordered and provided by the Resource Center within a few weeks.

The cosmid library was prepared by Dr. C. Burgtorf (carola.burgtorf@embl-heidelberg.de) using *Danio rerio* (AB/AB) and cloned into the Lawrist7 vector. Insert sizes were in the range of 44 kb.

The PAC genomic library was prepared by Dr. C. Amemiya (camemiya@bu.edu) using *Danio rerio* and cloned into the pCYPAC6 vector. Insert sizes were in the range of 120 kb.

6. Oligonucleotide Primers

All oligonucleotide primers were purchased from MWG Biotech (München), Invitrogen Life Technologies or Sigma. The primers were delivered at a standard concentration of 100 mM. Working dilutions were prepared at a concentration of 10 mM and stored at -20°C.

Primers were used for different purposes like sequencing, cloning, addition of restriction enzymes to the ends for directional cloning and for preparation of probes, by addition of T3-, T7-, or SP6-RNA Polymerase binding sites. All used primers are listed in Table III.5., giving the annealing temperatures of primers based on the exact binding to the template.

Table III.5. List of Primers

Name	Sequence (5'→3')	T (°C)	Supplier	Purpose
ACSF-0	AGTGTTCAGGTTCTAGAGCTATG	58.9	MWG	C
ACSF-1	GACTCCAAGGACTCACCAGGG	63.7	MWG	C
ACSF-2	CTCCAGTGATTGGGATCCGCC	63.7	MWG	C
ACSF-4	GTAAAACCACCATCTGCCCATTG	60.6	MWG	C
OMP0	CAAGGACACACAGTAGACGC	59.4	MWG	S
OMP(-0)	GACGCATCATCTCCGTCAGC	61.4	MWG	S
OMP1	TGGACCCC(AGCT)GACCT(GC)ACCAAC(CT)T(AGCT)ATG	68.8	MWG	C, S
OMP2	AA(AG)TACAT(AGCT)AC(CT)TT(AGCT)C(GT)(AGT)AT(CT)TT	58.2	MWG	C, S
OMP3	GGAACAGACTGACCAGAAGAG	59.8	MWG	S
OMP4	CAGAAAAGCAGCCAAATTTGAG	56.5	MWG	S
prOMPnco	CATGCCATGGTGTTGTTTTTTTAACTT	58.9	MWG	M, C
prOMPndeco	GGAATTCCATATGGTTGTTTTTTTAACTTACCG	63.3	MWG	M, C
prOMP(-1)	GCTTCCAAAATCACCCCGGTG	61.8	MWG	S
prOMP(-2)	CAGGCTATTCTCACCCGGTC	61.4	MWG	S
prOMP(-5)	GCAATATTAGCTTAAGGATCTGAAGATGAA	52	ILT	S
dlx2-nco	CTCCAGCCATGGTTTTCATACCGCAAAAGCAC	78	MWG	M, C, S
dlx1/2iF	CCAGGACACTATGCAGCAGCC	63.7	MWG	C, S
dlx1/2iR	CGGCTTACTTGTAGAGACGGT	62.1	MWG	C, S
tau-nco	CATGCCATGGCCGCCTGCTCACCG	71.3	MWG	M, C, S
tau-ndeco	GGAATTCCATATGGCTGAGCCCCGCCAG	71	MWG	M, C, S
IRES-ndeco5'	GGAATTCCATATGGTTTAGTGAACCGTCAG	70	ILT	M, C
IRES2EGFP5	GCGGCCGCCCCTCTCCCTCC	81.4	MWG	M, C
IRESupstr	CGCACACCGGCCTTATTCCAAG	53	ILT	S
dsRed-ndeco	CGGAATTCCATATGGTGCGCTCCTCCAAGAACG	72	MWG	M, C
dsRed-not	GCAAGTAAAACCTCTACAAATGTGG	59.7	MWG	M, C
dsRed-upstr	GATCTCGAACTCGTGGCCGTTC	69	MWG	C, S
EYFP-ndeco	GGAATTCCATATGGTGAGCAAGGGCGAG	76	MWG	M, C
EYFP-not(nde)	GGAATTCCATATGCCTCTACAAATGTGGTATG	73	MWG	M, C

EYFP-upstr	CACGCTGAACTTGTGGCCGTTTAC	70.7	MWG	C, S
EYFP-5'	GTAAACGGCCACAAGTTCAG	55.4	Sigma-ARK	S, T
EYFP-3'	AACTCCAGCAGGACCATGTG	57.5	Sigma-ARK	S, T
M13-Fwd	GTAAAACGACGGCCAGT	52.8	MWG	B, S
M13-Rev	GGAAACAGCTATGACCATG	54.5	MWG	B, S
T3	AATTAACCCTCACTAAAGGG	53.2	MWG	B, S
T3-high	GCGCAATTAACCCTCACTAAAGGG	65.2	MWG	B, S
T7	GTAATACGACTCACTATAGGGC	58.4	MWG	B, S
T7-hi	GCGTAATACGACTCACTATAGGGC	62.7	MWG	B, S
T7-high	GCGCGTAATACGACTCACTATAGGGC	73.9	MWG	B, S
T7 T-Vector1	CGACTCACTATAGGGCGAATTGGG	70.7	MWG	B, S
T7 pSPORT1	TACGACTCACTATAGGGAAAGCTGG	70.2	MWG	B, S
SP6	CTATTTAGGTGACACTATAGAATAC	56.4	MWG	B, S
SP6T-Vector1	TGATTACGCCAAGCTATTTAGGTGACACTATAG	73.9	MWG	B, S
B338 T3	GGATCCATTAACCCTCACTAAAGGGAAGAG CTATGACGTCGCAT	74.1	MWG	CT3→T7
B338 T7	GGAAGCTCTAATACGACTACATATAGGGAA AGCTGGTACGCCTGCA	75	MWG	S
SV40	CAAATGTGGTATGGCTGA	50	MWG	B, S
SV40-hi	CCTCTACAAATGTGGTATGGCTGA	51	ILT	B, S
Ther	GATATGCGGTGACAGCAATG	47	Sigma-Ark	B, S
Thin	CCGGCGCGCACTGGATGCG	57	Sigma-Ark	B, S
GAPDH-1	GCATTACAGTAGCCTTTATACC	68	ILT	T
GAPDH-2	GCACTCCAAAGAGTAACTCC	68	ILT	T
L5ndeco	GAATTCCATATGGGCGACTCTTTGTG	55	ILT	R
L5bgl2	GAAGATCTCACACCTTGTAATAGATG	50	ILT	R
L5trunc	GAAGATCTGCGATGACGTCGTCG	54	ILT	R
rtk3T7	CGCGCGTAATACGACTCACTATAGGGCAAT TAACATGTGCTGTG	64	ILT	P
rtk4T7	CGCGCGTAATACGACTCACTATAGGGGGTG GTGAGAGTGAC	66	ILT	P
rtk5T/	CGCGCGTAATACGACTCACTATAGGGCTCT CCATATAATGACAG	64	ILT	P
rtk6T7	CGCGCGTAATACGACTCACTATAGGGATCC AAGGCCCCCAC	67	ILT	P
rtk7T7	CGCGCGTAATACGACTCACTATAGGGGCTG CGGTTCTTTTCC	66	ILT	P
rtk8T7	CGCGCGTAATACGACTCACTATAGGGAGGC GGTAGTCCTGC	67	ILT	P
L5T7	CGCGCGTAATACGACTCACTATAGGGCACA CCTTGTAATAGATG	64	ILT	P

The given T is the one calculated by the supplier. Before ordering, the T_m of a primer pair was calculated as T_m (°C) = (2 x (A, T) + 4 x (C, G)

B: Bacterial colony PCR
C: Cloning
M: RE-insertion
P: Promoter-addition
S: Sequencing
T: Transgene identification

7. Antibodies

Table III.6. List of Primary and Secondary Antibodies

Name	Origin of the Antibody	Supplier	Working dilution
Primary Antibodies			
γ–Aminobutyric Acid (GABA)	monoclonal mouse IgG1, clone GB-69	Sigma	1:100
Cytokeratine	polyclonal rabbit	Dako (CA, USA)	1:400
Growth Associated Protein - 43 (GAP-43)	monoclonal mouse IgG2a, clone GAP-7B10	Sigma	1:500
Globose Basal Cell (GBC-1)	monoclonal mouse	J E. Schwob, SUNY Health Sci. Center, Syracuse, NY, USA	1:20
Glial Fibrillary Acidic Protein (GFAP)	polyclonal rabbit	Biogenex (Hamburg)	undiluted
Green Fluorescent Protein (GFP)	rabbit IgG	Torrey Pines Biolabs, Inc. (Houston, TX, USA)	1:1000
Glutamate	monoclonal mouse IgG1, clone GLU-4	Sigma	1:100
Hu (Mab16A11)	mouse IgG2bκ	M. F. Marusich, U. of Oregon, Eugene	1:100
rOMP	rabbit	F. Margolis, U. of Maryland, Baltimore	1:500
S-100	polyclonal rabbit	Dako	1:200
β-III-Tubulin	mouse $F(ab')_2$ fragments	Promega	1:100
Tyrosine Hydroxylase	mouse monoclonal IgG1, clone TH-2	Sigma	1:500
Zns-2	mouse IgG1k	University of Oregon, Eugene	1:500
α-DIG $F(ab')_2$ fragments, alkaline phosphatase coupled	sheep	Roche Biochemicals	1:750-1:5000
α-DIG $F(ab')_2$ fragments, peroxidase coupled	sheep	Roche Biochemicals	1:150
α-DIG	mouse	Roche Biochemicals	1:5-1:200

Name	Supplier	Working dilution
Secondary Antibodies		
goat α mouse, CY2 coupled	Dianova (Hamburg)	1:100
goat α mouse, CY3 coupled	Dianova	1:100
goat α mouse, Alexa Fluor 594 coupled	Molecular Probes	1:200
goat α mouse F(ab')$_2$ fragments, peroxidase coupled	Dianova	1:100
goat α mouse, biotin	Biogenex	1:20
streptavidin (α biotin), peroxidase coupled	Biogenex	1:20
goat α rabbit, Alexa Fluor 488 coupled	Molecular Probes	1:200
goat α rabbit F(ab')$_2$ fragments, peroxidase coupled	Dianova	1:200

8. Dyes, Substrates, Embedding Media and Counterstains

Alkaline Phosphatase Substrates

CDP-Star™ (Roche Biochemicals)	chemiluminescent substrate
ELF™97 (Molecular Probes)	green fluorescent substrate
Fast Red (Biogenex)	red chromogen/fluorescent substrate
NBT/BCIP (Roche Biochemicals)	blue/violet chromogenic precipitate
NBT/BCIP Red (Biotium (Hayvard, CA, USA))	red chromogenic precipitate
PNPP (Sigma)	yellow substrate

Horseradish Peroxidase Substrate

Diaminobenzidine (DAB) (Roche Biochemicals)	brown chromogenic precipitate

Embedding Media

Mowiol (Polysciences Inc.)	embedding medium for chromogenic and fluorescent substrates
DABCO (Sigma)	bleaching retardant (2.5% in Mowiol)
Vectashield (Vector)	embedding medium for fluorescent substrates and dyes; good bleaching retardant

Dyes and Counterstains

DiI (Molecular Probes)	red fluorescent dye; 5 mg/ml in dimethylformamide (stock); working dilution 1:100
DAPI (Sigma)	blue fluorescent counterstain; 5 mg/ml in H_2O (stock); working dilution 1:1000
Hoechst 33342 (Molecular Probes)	blue fluorescent counterstain; 1 mg/ml in H_2O (stock); working dilution 1:1000
Phenol Red (Invitrogen Life Technologies)	red dye, 0.5% in H_2O; dye used as injection control

9. Equipment

Balances	Sartorius Laboratory, Sartorius, Heidelberg
	Sartorius Universal, Sartorius
	Sartorius Handy, Sartorius
Centrifuge	Speedvac Concentrator, Savant
	Vacuumpump, RD4, Vacubrand, Wertheim
	Cooling Device, MCI, UniEquip, Martinsried
	Biofuge 13, Heraeus Instruments, Hanau
	Eppendorf Centrifuge 5417 R, Eppendorf, Hamburg
	Eppendorf Centrifuge 5415D, Eppendorf
	Sorvall R5-BB, DuPont Instruments, Bad Homburg
	Sigma 4K 10, B. Braun, Melsungen
CO_2-Incubator	Kendro/Haereus, Hanau
Computer	G3, Macintosh, Apple
Cryostat	Cryostat CM 1900, Leica
Developing Machine	Curix 60, Agfa
Dot/Blot Apparatus	Biorad, München
Electrophoretic equipment	Mini-Sub®Cell GT, BioRad
	Sub-Cell®GT, BioRad
	Power Pac 300, BioRad
Electroporation	Gene Pulser™, Biorad
	Pulse Controller (BioRad, München)
	Capacitance Extender (BioRad, München)
Heating Block	Thermomixer Comfort 1.5 ml, Eppendorf
	Hybaid-Intelligent Heating Block, Biometra
	HB-130, Unitek
Heating Plate	Ikamag®Ret, Janke & Kunkel
Hybridization oven	Incubator 1000, Heidolph
	Techne, Cambridge, UK
Microinjector	PV830 Pneumatic PicoPump, WPI, Berlin
Microscopes	Axiophot (Photomicroscope Pol), Zeiss
	Axioplan, Zeiss
	AttoArc®2 HBO 100 W, Zeiss
	RT Slider Spot, Diagnostic Instruments Inc.
	Uniblitz® Model VMM-D1 Shutter Driver, Vincent Associates
	Binocular, Nikon, Düsseldorf
	Fluorescence Microscope, SMZU, Nikon, Düsseldorf
	Coolpix 950, Digital Camera, Nikon
	Visilux 150HL, Visitool®
MilliQ	Seralpur DELTA, NSF Seral
Objectives	Plan-Neofluar: 10x NA 0.3; 20x NA 0.5; 40x Oil NA 1,4

pH-Meter	pH Meter 766 Calimatic, Knick
Photometer	UNICAM 8625 UV/VIS Spectrometer
Printer	Laser Writer, PS, Apple
	Epson Stylus Color 760
Puller	Micropipette Puller Model P97, Sutter Instruments Co.
Scanner	Agfa SnapScan 1236
Shaker	IKA-VIBRAX-VXR, Janke & Kunkel
Slide-Scanner	Nikon LS2000, Nikon, Düsseldorf
Sterile Hood	Heraeus Lamin Air LFM 2448 S, Heraeus Instruments, Hanau
Transilluminator	UV-Transilluminator, Stratagene
	Gel-Doc System, Biorad
	Quantity One 4.2.1., BioRad
	Herolab UVT 2020 The Imager™
	High Performance CCD-Imager System, Biometra
Thermocycler	Gene Amp PCR System 2400, Perkin Elmer
	T Gradient, Biometra
UV Crosslinker	UV Stratalinker 1800, Stratagene
Vibratome	Ted Paella Incorporation
Vortex	Vortex Genie 2™, Bender & Hobein AG, Melsungen
Waterbath	Thermomix, B. Braun

10. Molecular Biological Techniques

All standard techniques like small and large scale plasmid DNA preparations, quantification of DNA and RNA, agarose gel electrophoresis, digestions, isolation of DNA-fragments, ethanol precipitations, filling up reactions of 3' and 5' overhangs, dephosphorylation of 5'ends, ligation of DNA fragments, preparation and transformation of competent cells were essentially performed as described in Sambrook *et al*., 1989.

10.1. Isolation, Purification and Quantitation of DNA and RNA

10.1.1. Isolation of Genomic DNA

Genomic DNA from whole fish was isolated according to Hogan *et al*., 1986. Adult zebrafish were decapitated and viscera were removed. The tissue was frozen in liquid nitrogen and pulverized. After addition of lysis buffer (0.1 M Tris/HCl, 0.2 M NaCl, 5 mM EDTA, 0.2% w/v SDS, pH 8.5) and proteinase K (150 µg/ml) the tissue was incubated under continuous rotation at 55°C overnight. Undissolved material was pelleted. After a phenol/chloroform extraction the DNA was precipitated using 1/10 volume of 3 M NaAc (pH 5.2) and 2 volumes of 100% ethanol. The DNA was washed two times with 70% ethanol, dried and dissolved in 100-500 µl H_2O overnight at 4°C.

10.1.2. Isolation of Genomic DNA from Zebrafish Embryos

For isolation of genomic DNA from zebrafish embryos, about 100 embryos were transferred to an Eppendorf tube containing 1 ml of DNA extraction buffer (10 mM Tris pH 8.2, 10 mM EDTA, 200 mM NaCl, 0.5% SDS, and 200 µg/ml pronase (or proteinase K)). They were vortexed briefly and incubated with gentle rotation at 55°C for 3-4 h. During this period the tube was vortexed briefly, once per hour. The samples were then centrifuged at 14000 rpm for 10 min at room temperature. Samples were pooled, by combining 20 µl of extraction solution from each sample, and the remaining DNA solution was stored at -20°C. The combined DNA mixtures were precipitated with 300 µl of absolute ethanol. Pellets were rinsed with 70% ethanol and dissolved in 100 µl of 1 x TE (pH 8.0).

10.1.3. Isolation of Plasmid DNA

Various methods are available for preparing high-quality plasmid DNA. To check the identity of a clone and for sequencing, plasmid DNA was prepared in small scale. For purposes like transfection of eukaryotic cells and microinjection, plasmid DNA was prepared in large scale.

10.1.3.1. Small Scale Plasmid DNA Preparation (Miniprep)

In cases where the recombinant *E. coli* clones had to be identified, plasmid DNA was isolated in small scale. Single colonies of interest were inoculated into LB-medium (10 g Tryptone, 5 g Yeast extract, 10 g NaCl; pH 7.0) containing the appropriate antibiotics (ampicillin [100 µg/ml], tetracycline HCl [50 µg/ml], or kanamycin [50 µg/ml]) in a volume of 3 ml and grown in an orbital shaker (~300 rpm) at 37°C overnight. In general, a miniprep kit (either from Qiagen or from Sigma) was used for this purpose. The plasmid purification protocols are based on a modified alkaline lysis procedure (Birnboim and Doly, 1979), followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. This method yields plasmid DNA in a range from 5-20 µg, which is enough for several test digestions and also for sequence analysis of likely recombinant clones.

10.1.3.2. Large Scale Plasmid DNA Preparation (Midiprep)

In cases where more DNA was required, like transfection of eukaryotic cell lines and microinjection of blastocysts, a midiprep was prepared. Here bacteria from a glycerol stock were streaked on a fresh selective plate and single colonies were inoculated into a small volume of LB medium and grown for several hours. This starter culture was then diluted 1/1000 into a volume of 100 ml LB-medium containing the appropriate antibiotics and grown on an orbital shaker (~300 rpm) at 37°C overnight.

10.1.4. Isolation of Cosmid and PAC DNA

Several methods were tested for isolation of cosmid and PAC DNA. These included the protocol for preparation of BAC DNA found in current protocols (modified alkaline lysis procedure, Birnboim and Doly, 1979), the large construct kit (Qiagen), the Midi Kit of Invitrogen Life Technologies. In my hands the following protocol gave the best time to yield and purity result. This protocol is a simple modification of the classical alkaline lysis protocol. Two milliliters of bacterial suspension were pelleted. The pellet was resuspended in 300 µl resuspension buffer (50 mM Tris-Cl, pH 8.0; 10 mM EDTA, 100 µg/ml RNase A). The bacteria were lysed by addition of 300 µl of lysis buffer (200 mM NaOH, 1% SDS), mixed gently and neutralized by addition of 300 µl neutralization buffer (3.0 M potassium acetate, pH 5.5). After mixing the tubes gently the white precipitate was pelleted by centrifugation for 10 min at 4°C. The supernatant was carefully pipetted to 800 µl of ice-cold isopropanol, after inverting the tube several times the DNA is pelleted and washed once with 70% ethanol. The pellet was air-dried and redissolved in 20 µl of 10 mM Tris-Cl, pH 8.5. For test digestions 30-40 µl of isolated DNA was used. For Southern analysis 60-100 µl DNA were digested per lane. For insert isolation and subcloning 200-300 µl of DNA was digested to get a reasonable amount of insert DNA.

10.1.5. Isolation of Total RNA

Total RNA was isolated using the RNA preparation kit of Qiagen. Olfactory epithelia or olfactory bulbs were isolated in calcium free buffer. The tissue was digested using 350 µl papain (1 mg/ml in calcium free ringer) for 50 min at room temperature and was disrupted and homogenized by pipetting up and down using a P1000. The dissociated tissue was centrifuged for 30 sec at 5000 rpm and the supernatant was discarded. The pellet was dissolved in 350 µl RLT^+ buffer and the solution was carefully pipetted onto a QIAshredder column (Qiagen). After centrifugation for 2 min at 13000 rpm the solution was precipitated using 350 µl 70% ethanol. For purification and concentration this mixture was loaded on a RNeasy column (Qiagen), centrifuged shortly (15 sec) at 10000 rpm, and washed with 700 µl RW1 buffer. After washing twice with 500 µl of RPE buffer the RNA was eluted from the column using 30 µl of DEPC-treated H_2O and centrifuging at 10000 rpm for 1 min. Genomic DNA was digested by treatment with 4.7 U of RNase-free DNase (Promega) for 30 min at 37°C.

10.1.6. Phenol/Chloroform Extraction

Reaction mixtures that had a smaller volume than 200 µl were adjusted to this volume using H_2O and phenol-chloroform extracted using an equal volume of phenol-chloroform-isoamylalcohol (PCI = 25:24:1). This mixture was vortexed and centrifuged for 1 min. The aqueous phase was carefully transferred to a new reaction tube and extracted again using 200 µl of PCI. The aqueous phase was then extracted using 200 µl chloroform. The aqueous phase was then ethanol precipitated as described in the next section.

10.1.7. Ethanol Precipitation

DNA was precipitated using 0.3 M sodium acetate and two to three volumes of ice-cold absolute ethanol. RNA was precipitated using 0.8 M lithium chloride and 2.5 volumes of ice-cold absolute ethanol. Precipitation was allowed at -20°C for 30 min or at -80°C for 10 min. After centrifugation at maximum speed for 30 min in case of DNA and 20 min in case of RNA at 4°C, the pellets were washed with 70% ethanol air-dried and resuspended in the appropriate buffer.

10.1.8. Quantitation of DNA and RNA

The concentration of DNA and RNA in solution was determined by UV spectrophotometry. The absorption of DNA samples was measured at 260 nm using a spectrophotometer, assuming that 1 OD of double-stranded DNA corresponds to approximately 50 µg/ml DNA and 40 µg/ml for single stranded DNA and RNA. To estimate

the purity of the DNA, ratios of readings between 260 nm and 280 nm were taken. A ratio of 1.8 for DNA and 2.0 for RNA indicates that the respective samples were pure.

Alternatively, the concentration of DNA and RNA was estimated using agarose gel electrophoresis by comparing the intensity of the bands of interest with the 1 kb band of a kb ladder (Marker X, Roche Biochemicals) of known concentration. RNA samples were denatured in 50% formamide for 3 min at 100°C before loading.

10.1.9. Agarose Gel Electrophoresis

DNA and RNA were loaded on 1% agarose gels containing 0.5 µg/ml ethidium bromide in 1 x TAE Buffer and run at 5-10 V/cm. Genomic DNA, cosmid and PAC DNAs were loaded on lower concentrated agarose gels (0.5%-0.7%) or on a SeaKem® Gold agarose gel and run slowly (1-2 V/cm) to ensure better separation and to avoid smearing of DNA. Various loading dyes were used, either Orange G or Bromophenol Blue (for preparation see Sambrook *et al*., 1989). Generally the DNA-ladder X (Roche Biochemicals) or PeqLab (PeqLab) were used. For cosmid and PAC digests the DNA-Marker XV (Roche Biochemicals) was used because it includes very large fragments.

10.2. Enzymatic Modifications of DNA

10.2.1. Digestion of DNA

Digestions for characterization of plasmid DNA were performed using about 200 ng of plasmid in 1 x restriction enzyme digestion buffer and 6 U of restriction enzyme in a total volume of 20 µl. Digestion mixtures were incubated for 1-2 h at the appropriate temperatures for each enzyme as suggested by the manufacturer. For Southern analyses about 10 µg of genomic DNA or 5-10 µg of cosmid or PAC DNA was digested in a volume of 300 µl and using the enzymes 5 times in excess.

10.2.2. Filling of Recessed 3' Termini and Removal of Protruding 3' Termini

To fill recessed 3' termini respective dNTPs were added to a final concentration of 1 mM to the digestion mixture. The reaction was incubated at 37°C for 15 min after addition of T4 DNA Polymerase.

In order to remove the 3' overhangs dNTPs were added to a final concentration of 2 mM to the digestion mixture. The reaction was incubated at 12°C for 30 min after addition of 1 U of T4 DNA Polymerase for each µg of DNA. The enzyme was heat inactivated at 70°C for 15 min. Reactions were purified before further use.

10.2.3. Dephosphorylation of Plasmid DNA

In order to avoid the religation of vector DNA during ligation reactions the 5' phosphate of linearized vector DNA was removed using shrimp alkaline phosphatase (Amersham) according to Sambrook *et al*., 1989 and the supplier's instructions.

10.2.4. Ligation of DNA Fragments and PCR Products

Ligation reactions were used to combine vector and insert DNA. For this purpose purified insert DNA was ligated to dephosphorylated vector DNA using T4 DNA ligase (Roche Biochemicals) according to Sambrook *et al*., 1989 and the suppliers instructions. PCR products (1-4 µl) were ligated directly after amplification into the pGEM-T vector (Promega) according to the manufacturer's instructions.

10.2.5. Isolation of DNA Fragments from Agarose Gels

DNA fragments were isolated from agarose gels according to the manufacturer's instructions. In general QIAquick Gel Extraction Kit (Qiagen), QIAEXII Gel Extraction Kit (Qiagen), Roche High Pure PCR Product Purification Kit (Roche Biochemicals) or GelElute (Sigma) were used. All these kits make use of a column filled with a silica-gel membrane. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities are washed away and the pure DNA is eluted with Tris buffer.

10.3. Labeling of DNA and RNA

10.3.1. Radioactive Labeling of DNA Using Random Priming

Radioactively labeled probes were prepared using the Prime-It II Random Primer Labeling Kit (Stratagene) and used to hybridize genomic or cDNA libraries. For this purpose 25 ng of double stranded purified insert DNA was denatured in a cooking waterbath together with 94.5 OD of an Oligo$(N)_9$-Primer-mix for 10 min. This mixture was cooled down to room temperature to allow primer annealing. Then a 5 x primer buffer (containing dATP, dGTP, dTTP, 0.1 mM each), [α–^{32}P]dCTP and Exo(-) Klenow enzyme (5 U/µl) were added according to the manufacturer's suggestions. The reaction was incubated for 1 h at 37°C, stopped by the addition of stop solution and purified using 'NICK™ Column Sephadex® G-50' purification columns (Amersham Pharmacia Biotech). The eluate contained the labeled DNA while the primers and unincorporated nucleotides remained in the column. The amount of radioactively labeled probe was estimated by measuring the probe before and after purification using a scintillation counter.

10.3.2. Random Primed DNA Labeling Using Digoxigenin

DIG-labeled probes were used to hybridize genomic or PAC Southern blots. The reaction was generally performed in a total volume of 20 µl. For this purpose 1 µg of phenol/chloroform extracted and ethanol precipitated insert DNA was used. After heat denaturation in a boiling water bath for 10 min, 1 x hexanucleotide mixture and 1 x labeling mixture were added to the tube on ice. After addition of 1 µl of Klenow enzyme (2 U/µl) the reaction was incubated at 37°C overnight. The reaction was stopped by addition of 2 µl of EDTA (200 mM, pH 8.0).

10.3.3. Labeling of RNA Using Digoxigenin or Fluorescein by *In Vitro* Transcription

Various kinds of probes can be used for the detection of mRNA in *in situ* hybridization experiments. However, *in vitro* transcribed riboprobes are the best choice on tissue sections (Cox, 1984). These probes are single-stranded and may span hundreds of nucleotides, which results in specific antisense probes with high detection sensitivity. Moreover, *in vitro* transcription allows the synthesis of ideal test antisense and control sense probes, both having a similar length and G + C content, defining similar properties of hybridization. After synthesis of the probes they were not hydrolyzed into smaller pieces, as this treatment leads to elevated background signals.

Sense and antisense RNA probes labeled with digoxigenin-labeled UTP (DIG-UTP) were generated by *in vitro* transcription according to the manufacturer's instructions (Roche Biochemicals). Before beginning the transcription reaction, the template DNA was linearized with an appropriate restriction enzyme lying downstream of the cloned insert. In order to avoid transcription of undesirable sequences restriction enzymes that leave 5' overhangs or blunt ends were used. Alternatively, template DNA was generated by PCR using vector specific primers or in some cases using insert specific primers that contained polymerase promoter sequences. In either case, the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The labeling reaction was performed in a total volume of 20 µl. Generally, about 1 µg (per kb insert DNA) was used for labeling. Transcription buffer and DIG-labeling mixture (or fluorescein-labeling mixture) were added to a final concentration of 1 x 4 U of RNA Polymerase (T3, T7 or SP6) and 20 U of RNAse inhibitor (RNAsin, Amersham Pharmacia Biotech) were also added. The reaction was incubated at 37°C for at least 2 hours and terminated by addition of 2 µl of EDTA (200 mM, pH 8.0). The RNA transcript was ethanol precipitated and analyzed for size and integrity using agarose gel electrophoresis. Labeling efficiency was estimated using either DIG quantification teststrips (Roche Biochemicals) or a spot test according to the instructions of the supplier.

10.4. Southern Blotting

10.4.1. Southern Transfer

Southern blotting was used for transfering DNA fragments from agarose gels to nylon membranes (Southern, 1975). After gel electrophoresis, the double stranded DNA in the gel was depurinated for 10 min in 0.25 M HCl. The gel was rinsed with water, denatured twice for 15 min in denaturation solution (0.5 M NaOH, 1.5 M NaCl), rinsed with water and neutralized two times for 15 min in 1 M ammonium acetate. Then the gel was put on an appropriately sized nylon membrane (Hybond N™ for radioactive hybridization, Amersham Pharmacia Biotech; Nylon for non-isotopic hybridization, Roche Biochemicals) to prepare a downward capillary transfer (Chomczynski, 1993), using 1 M ammonium acetate buffer (Allefs *et al*., 1990). DNA was blotted overnight to ensure complete transfer and cross-linked (Church and Gilbert, 1984) to the membrane using the UV-Stratalinker. The membrane was rinsed with water, air dried and used either immediately for prehybridization or stored dry at room temperature for future use.

10.4.2. Prehybridization and Hybridization

Prepared filters were prewetted in 2 x SSC and put DNA side facing to the lumen into a hybridization tube filled with 2 x SSC. When decanting the solution the membrane lays down on the tube avoiding air bubble formation between the membrane and the tube. Prehybridization was done using ULTRAhyb™ hybridization solution (Ambion) for 30 min at 42°C in enough solution to keep the membrane uniformly wet (about 6-10 ml depending on the size of the membrane and the size of the hybridization chamber).

DNA probes were denatured for 10 min in a boiling water bath before hybridization. Radiolabeled DNA probes were denatured by addition of NaOH to a final concentration of 25 mM and 10 min incubation at 56°C. Either 10^6 cpm/ml of radiolabeled probe or 1 pM of non-isotopic probe was used for hybridization. Hybridizations were carried out overnight at 42°C.

10.4.3. Posthybridization Washes

At the end of hybridization the hybridization solution containing the probe was discarded. In case of DIG-labeled probes the solution was stored at -20°C for reuse in future experiments. The blot was washed two times for 5 min in 2 x SSC, 0.1% SDS and then two times 15 min in 0.1 x SSC, 0.1% SDS at 42°C.

In cases where filters were hybridized with radioactive probes the filters were immediately sealed in a plastic bag and exposed to an X-OMAT film at -80°C overnight.

10.4.4. Chemiluminescent Detection

The membrane was equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% (v/v) Tween 20) for 1 min and then blocked in blocking solution (1% (w/v); blocking reagent dissolved in 100 mM maleic acid, 150 mM NaCl; pH 7.5) for 1 h. The blocking solution was poured of and replaced by the antibody solution (anti-DIG-AP antibody diluted 1:10000 in blocking solution) and the membrane was incubated with the antibody for 30 min. The antibody solution was discarded and the membrane was washed twice, 15 min per wash, in washing buffer. After the washing procedure, the membrane was equilibrated in detection buffer (100 mM Tris, 1 mM EDTA; pH 9.5) for two min. The substrate solution was prepared by diluting CDP-Star™ (Roche Biochemicals) 1:100 in detection buffer. The membrane was incubated with the substrate solution for 5 min, then sealed in a fresh plastic bag and exposed to a X-ray film for a few seconds up to a few minutes or detected using the ChemiDoc system (Biorad).

The membranes can be used repeatedly after removal of the probe by incubating the membrane twice in 0.2 M NaOH, 0.1% SDS for 20 min at 37°C.

Non-radioactive probes were stored at 4°C and used repeatedly.

10.5 Subcloning of DNA Fragments

10.5.1. Preparation of Electrocompetent Cells and Electroporation

E. coli of the XL1 Blue strain was used for preparation of electrocompetent cells. This strain of E. coli is particularly suited since it is recombination deficient. Electrocompetent cells were prepared according to the method of Dower (1988) by washing the pellet from a 1 l bacterial culture (XL1-BlueMRF' in LB/tet medium) several times in ice-cold water and after resuspension in 2 ml ice-cold 10% glycerol were stored as 50 µl aliquots at -80°C.

For electroporation the bacterial suspension was thawed on ice, mixed with 1-2 µl of ligation mixture and after a 1 min incubation at room temperature transformed using 1 mm cuvettes and the GenePulser from BioRad at E=18 kV/cm, C=25 µF, R=200 W. After transformation 1 ml of prewarmed SOC medium was added to the bacteria and they were incubated for 1 h at 37°C before plating on ampicillin (50 µg/ml) and tetracyclin (150 µg/ml) plates. In cases where blue-white selection was possible X-gal (800 µg/ml) and IPTG (0.5 mM) were added to the plates. The bacteria were grown on agar plates overnight at 37°C.

10.5.2. Colony PCR for Identification of Positive Clones and Determination of Insert Length

There are two main purposes for determining insert length. First, the information on insert length helps us to avoid sequencing small inserts or empty clones. Second, it is useful to evaluate whether the clone is full length and how much is missing from the 5' end (in case

of cDNAs). Single bacterial colonies were picked and inoculated in LB medium containing the appropriate antibiotic in 96 well multititer plates. The bacteria were grown for one to three hours in an orbital rotator at 37°C. Five µl of bacterial suspension was used as a template in a PCR reaction. All PCR reactions were carried out in a final volume of 20 µl containing 1 x PCR buffer, 1.25 mM $MgCl_2$, 10 pmoles of each primer, 0.1 mM of each dNTP, 1 U of Taq DNA polymerase (either from Roche Biochemicals or "home made" Taq polymerase prepared by V. Oehlmann). Generally M13 primers or other vector primers like T3 and T7 primers were used (see Table. III.5.).

Clones that were positive in the PCR were used to inoculate 3 ml of LB medium. DNA was extracted using the small-scale preparation of DNA protocol and digested to confirm the positive result of the PCR. Single clones were then subjected to sequence analysis.

10.5.3. Preparation of Glycerol Stocks

Glycerol stocks were prepared by adding 150 µl of sterile glycerol to 850 µl bacterial culture, vortexing to ensure even dispersion of the glycerol and freezing in liquid nitrogen. Afterwards, tubes were transferred to -80°C for long-term storage.

10.6. Working with cDNA Libraries

In the framework of this thesis three different libraries were plated for screening. These included a cDNA library from olfactory epithelia, one from olfactory bulbs and one from whole brain of zebrafish. S. Korsching prepared the olfactory epithelial cDNA library and the whole brain library, while V. Oehlmann prepared the olfactory bulb library. The preparation of filters for all three libraries was the same in general and will be described for the olfactory epithelial cDNA library, differences in preparation are indicated.

10.6.1. Plating of Bacterial cDNA Libraries on Nitrocellulose Membranes

Variable concentrations of cDNA were used for transformation of XL1 Blue competent cells. Three different dilutions of bacteria were plated on agar plates and the colony forming units were determined the next day. The transformed bacteria were kept at 4°C in the meantime. These bacteria were then plated on nitrocellulose membranes (Amersham) so that about 50000 clones, per membrane (round filters, Hybond N™ RPN 132N) were present. The membranes were laid on agar plates avoiding formation of air bubbles between the membrane and the plate and grown overnight at 37°C.

10.6.2. Replica-Plating

The next day a replica filter was prepared from these filters. For this purpose the filters were placed on slightly wet Whatman paper. A damp nylon membrane was put on top of the first membrane. Both membranes were numbered and marked at several points using a needle and covered with another Whatman paper. This sandwich was placed between two glass plates and applying uniform force to the glass plate transferred the colonies. Both membranes were placed on fresh agar plates and regrown overnight at 37°C. This procedure was repeated the next day to prepare a second replica from the master filter.

Placing them on a Whatman soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 10 min lysed the bacteria on the replica filters. The nylon filters were neutralized by floating on phosphate buffer (50 mM NaP, pH 6.5). Afterwards bacterial debris was removed from the filters mechanically with a wet Kleenex paper and the filters were rinsed with phosphate buffer. The DNA was then crosslinked to the membrane using an UV-crosslinker (Stratagene).

For long term storage a glycerol stock of the master filter was prepared by placing the master filters on Whatman paper soaked with 25% glycerol in LB for 1 h. The filter was then covered with a transparency on which millimeter paper was copied. The markings were copied to the transparency. This sandwich was wrapped in aluminum foil, frozen and stored at -80°C.

10.6.3. The cDNA Library of the Whole Brain

This library was first amplified on filters by transforming 1 µl of ligation mixture. 40 µl of bacterial suspension was mixed with 10 ml LB-medium and loaded on filters. After an overnight incubation the colonies on the filter were collected by aid of a cell scraper and LB-medium, pelleted and plasmid DNA was extracted by the midiprep method. In total 23 aliquots were obtained that were used as template in PCR reactions.

Additionally, the library was plated on filters and master and replica filters were prepared as described above. The 23 plasmid aliquots were diluted 1:100 and 10 µl of each dilution was combined. Different dilutions of this combined plasmid mix were used as template for transformation.

10.6.4. The cDNA Library of the Olfactory Bulb

Here, V. Oehlmann provided an adapter ligated cDNA pool that was prepared using the Smart Protocol (Clontech). This cDNA pool was amplified by PCR and aliquots of the reaction were removed every three cycles to determine the optimal cycle number. One of the products was then ligated into the T-vector (Promega); transformed and plated onto 10 filter sets as described above.

10.6.5. Screening and Identification of Positive cDNA Clones

Membranes were prehybridized and hybridized with radioactively labeled probes as described in 10.3. Generally both replica filters were hybridized and only those colonies present on both filters were considered as positive. The positions of the positive colonies were identified and marked on the transparency. A square of about 5 mm around the colony was cut out to make sure that the colony was not lost. The same region was cut out of the master filter using a sharp scalpel and the membrane harboring the bacterial colonies was immediately transferred to an reaction tube containing 500 µl LB medium with the appropriate antibiotics. The bacteria were equilibrated and grown at 37°C for 1 h. Then various dilutions of the bacterial suspension were plated on agar plates (82 mm) and grown at 37°C overnight. The remaining bacterial suspension was mixed with 100 µl of glycerol and stored at -80°C.

10.6.6. Rescreening

Plates with bacteria that were grown at a density that would allow the identification of colonies were transferred to nylon filters (Hybond N™, Amersham). For this purpose damp nylon membranes were carefully placed on the agar plate. The membrane and agar plates were marked with a needle for later identification of colonies. Then the membrane was removed, the bacteria were lysed and the DNA was fixed to the membrane as described above. The bacteria on the plates were regrown overnight at 37°C. The membranes were prehybridized and hybridized and membranes were exposed to X-ray films (X-OMAT, Sigma) overnight. Positive colonies were matched to their counterparts on agar plates. Positive colonies were picked, overnight cultures were inoculated, glycerol stocks were prepared, DNA was extracted using the miniprep method, and digestions to estimate the insert size and sequencing reactions were performed.

10.7. Synthesis of DNA

10.7.1. Polymerase Chain Reactions (PCR)

Polymerase chain reactions were performed for different purposes like subcloning of DNA fragments, identification of positive clones and determination of insert sizes in colony PCRs, preparing of a template for *in vitro* transcription, addition of restriction enzyme cutting sites to the ends of DNA fragments and sequencing.

Primers were designed according to standard methods using the DNASIS program. Annealing temperatures were adjusted and the sequences were selected so that no hairpins or primer dimers could form. Whenever possible, PCR was simulated using the Amplify 1.2 program.

Long template PCR was performed using the Expand™ Long Template PCR System (Roche Biochemicals).

10.7.2. Synthesis of cDNA by Reverse Transcription

The reaction mixture was prepared using 8 µl DNase-treated RNA, 15 µl DEPC-treated H_2O and 2.3 µl pdN_6 (100 ng/µl) and incubated 10 min at 70°C and then cooled on ice for 2 min. To start the reaction 9 µl of reverse transcriptase buffer (5x Superscript II, Invitrogen Life Technologies), 2.3 µl dNTP (10 mM, Amersham) and 4.5 µl dithiothreitol (0.1 M) were added and incubated first for 10 min at 25°C and then 2 min to 42°C. The reaction was started by addition of 2.3 µl (460 U) reverse transcriptase (Superscript II, Invitrogen Life Technologies) and synthesis was allowed for 50 min at 42°C. Inactivating the reverse transcriptase for 15 min at 70°C stopped the transcription. Two µl of this product was directly used for PCR reactions.

10.8. Sequencing of DNA

10.8.1. Sequencing Reaction

The cycle sequencing reaction was performed in 10 µl containing 2 µl terminator premix (BigDye, ABI Prism), 0.2-0.5 µg of purified plasmid DNA, 3.2 pmol primer. Amplification conditions were as follows:

96°C for 2 min

[96°C for 30 s, 50°C for 15 s, 60°C for 4 min] 25 cycles

The samples were ethanol precipitated and dried thoroughly. The dried samples were stored at -20°C in the dark until they were electrophoresed on a sequencing gel. Electrophoresis was carried out by Rita Lange on an ABI Prism™ 377 Sequencer (Applied Biosystems) in the core facility of the Institute of Genetics in Weyertal.

10.8.2. Sequence Analysis

Sequences were analyzed using online recources available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) which include several databases and search programs. Mainly, BLAST 2.0 (Gapped BLAST and Graphical Viewer) with the advanced BLAST option was used (Altschul *et al*, 1990) and the Wisconsin Sequence Analysis Package, Version 10 (Genetics Computer Group, Inc.).

10.8.3. Phylogenetic Analysis

Sequences were aligned with the help of the Genetics Computer Group Pileup program. Phylogenetic analysis was carried out using the program growtree. Sequences were compared at the amino acid level with the unweighted pair-group method using arithmetic averages (UPMGA) as implemented in the program package of the Genetics Computer Group, University of Wisconsin, WI, USA. Results were presented as dendrogram.

11. Histological Studies

11.1. Preparation of Material

11.1.1. Preparation of Slides and Coverslips

Glass slides were coated with a gelatin-chrome alum solution. For this purpose the slides were prewashed in an acid alcohol washing solution (1% HCl (v/v) in 70% alcohol) for 30 min with gentle agitation and then rinsed with deionized water (3-4 changes). The cleaned and drained slides were then put into the warm chrome alum solution (add 2 g gelatin to 500 ml stirring cold water, heat to 60°C, add 0.2 g chromium potassium sulfate, filter through fast filter paper) for 30 sec with gentle agitation. The slides were drained in a dust-free place overnight.

To inhibit binding of antibodies and probes to coverslips these were treated with Repel Silane (Amersham Pharmacia Biotech). Coverslips were oven baked, dipped into Repel Silane, acetone and absolute ethanol for 5 sec each and air-dried in a dust-free place on the back of a microtiter plate that was used as a rack.

11.1.2. Tissue Preparation and Sectioning

Animals were decapitated with a sharp scalpel. The head was put immediately in a petridish containing ice-cold PBS, pH 7.4. Both epithelia and bulbs with attached telencephali or whole brains were dissected out.

11.1.2.1. Cryosectioning

For cryostat sectioning, tissues were put in TissueTek (MILES, Elkhart, Indiana, USA), oriented and frozen at -20°C. Olfactory epithelia were sectioned at 10 µm and olfactory bulbs at 15-20 µm thickness. Sections were mounted on Vectabond (Alexis) coated slides, on coated Superfrost plus® slides and dried for 3 h at 60°C. Sections were used immediately or stored at -80°C.

11.1.2.2. Vibratome Sectioning

The olfactory epithelia or olfactory bulbs with attached telencephali fixed in 4% PFA directly after dissection for 1 h and embedded in 15% gelatin. After gelation the gelatin blocks were fixed in 4% PFA for 2 days and 50-100 µm sections were taken.

11.2. Immunohistochemistry (IHC)

The staining procedure for cryostat sectioned and vibratome sectioned material was essentially the same and will be described only for cryostat sections. In addition to this 'basic protocol' an alternative protocol for signal amplification based on the specific interaction between biotin and streptavidin was used. The procedure will be described for vibratome sections.

11.2.1. Antibody Staining on Fresh Frozen Cryostat Sections

Sections were fixed in 4% PFA for 10 min and washed three times for 10 min each in TBS (50 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Endogenous peroxidases were blocked with 3% H_2O_2 for 20 min and the slides were washed 2 x 10 min in TBS. Permeabilization was done with 0.5 M ammonium chloride and 0.25% Triton X-100 for 10 min and samples were subsequently washed 2 x 10 min in TBS. Blocking was done in 5% BSA and 3% normal goat serum (NGS) in TBS for at least one hour. The tissue was then incubated with the primary antibodies (as given in Table 6) in 0.8% BSA, 3% NGS in TBS overnight at 4°C. After extensive washing in TBS (4 x 10 min), the sections were incubated with horse-radish peroxidase coupled secondary antibodies (see Table 6) in 0.8% BSA, 3% NGS in TBS for 1 h. Detection was done using DAB (1:10 into peroxide buffer, Roche Biochemicals) as a substrate. To every staining, tissue was processed as a negative control by omitting the primary antibody. The sections were mounted on chrome alum coated slides and embedded in Mowiol (Polysciences INC.).

11.2.2. Alternative Staining Using Biotin Streptavidin Enhancement

This protocol describes a triple-step reaction technique that increases the sensitivity of the immunohistochemical reaction. This is achieved by using a biotin-secondary antibody conjugate followed by reaction of a peroxidase coupled streptavidin conjugate. Several steps were added to the 'basic protocol'. The modified protocol was as follows: After incubation of the tissue with the primary antibody it was washed extensively (4 x 15 min) in TBS to remove unbound antibody. To block endogenous biotin-binding sites the specimen were treated for 15 min with avidin (Biogenex), washed shortly with TBS and then treated for 15 min with biotin (Biogenex) to saturate endogenous avidin. After a short washing step, the samples were blocked with 3% NGS and 0.8% BSA in TBS for 20 min. Then a biotinylated-mouse-

antibody (Biogenex) was applied for 20 min. After two washings with TBS for 10 min each, horseradish peroxidase-coupled streptavidin was added for 20 min. After a thorough wash in TBS (3 x 10 min) detection was done using DAB as substrate (Roche Biochemicals). Color developed within a few minutes and was stopped by extensive washing with water. Sections were mounted on chrome alum coated slides and embedded in Mowiol.

11.2.3. Antibody Staining on Vibratome Sections

Free-floating vibratome sections were used in the subsequent procedure. Endogenous peroxidases were blocked in 3% H_2O_2, permeabilized with 0.5 M ammonium chloride and 0.25% Triton X-100. Blocking was done in 5% BSA and 3% normal goat serum. All washings were done with 50 mM TBS. The tissue was then incubated with the primary antibodies (as given in Table 3) overnight at 4°C. The antibodies were detected using horseradish peroxidase coupled secondary antibodies (Biogenex) and DAB as a substrate. To every staining, tissue was processed as a negative control by omitting the primary antibody to assess the specificity of immunological detection. The sections were mounted on chrome alum coated slides and embedded in Mowiol (Polysciences INC.).

11.2.4. Whole Mount Immunohistochemistry on Embryos

Dechorionated embryos were fixed in BT buffer (0.1 M $CaCl_2$, 4% sucrose in 0.1 M $NaPO_4$, pH 7.4) containing 4% PFA for 12 h at 4°C, and then rinsed in PBST (1 x PBS, 0.1% Triton X-100, pH 7.4). Embryos were frozen in -20°C acetone for 7 min, washed three times in PBST, and then incubated in PBS-DT blocking solution (1 x PBST, 1% BSA, 1% DMSO, 0.1% Triton X-100, 2% goat serum) for 1 h. Embryos were incubated with a primary antibody for 4 h at room temperature or 4°C overnight, washed 10 times for 2 h with PBS-DT, and incubated again with a secondary antibody at 4°C overnight. Embryos were rinsed 6 x in PBS-DT at room temperature and washed five times with PBS-DT followed by three times washing with 0.1 M $NaPO_4$, pH 7.4. Embryos were then incubated in 1 ml of DAB chromogenic solution (1% DMSO, 0.5 mg/ml diaminobenzidine, 0.0003% H_2O_2 in 0.05 M $NaPO_4$, pH 7.4) at room temperature. The color reaction was monitored for 5 to 10 min under the dissecting microscope, and extensive rinsing with 0.1 M $NaPO_4$, pH 7.4, stopped the chromogenic reaction.

11.3. *In Situ* Hybridization

In situ hybridization to cellular RNA was used to determine the cellular localization of specific messages within complex cell populations and tissues. Various methods were used for different purposes.

11.3.1. *In Situ* Hybridization on Sections of Olfactory Epithelia and Olfactory Bulbs

Hybridization with digoxigenin-labeled probes on fresh frozen sections of olfactory epithelia, olfactory bulbs, embryos and larvae were performed as previously described (Weth *et al.*, 1996).

11.3.2. Whole Mount *In Situ* Hybridization of Embryos

In situ hybridization to whole mounted embryos (24 h, 48 h, 72 h) was performed according to the protocol of Schulte-Merker *et al*. (1992). Embryos were fixed in 4% PFA overnight and stored in 100% methanol at -20°C. Specimens were washed twice with PBST (PBS plus 0.1% Tween 20) and then digested with 5 µg/ml proteinase K in PBST for several minutes depending on the stage of the embryos. They were washed and fixed again in 4% PFA for 20 min. After the proteinase K was washed off, embryos were transferred into HYB^+ solution (50% formamide, 5 x SSC, 0.1% Tween 20 (SSCT), 5 mg/ml torula RNA, 50 µg/ml heparin) and prehybridized overnight at 55°C. Approximately 5 ng/ml DIG-labeled RNA probe was added and hybridization was done overnight at 55°C. Embryos were washed at 55°C 2 x for 30 min in 50% formamide/2 x SSCT, 1 x for 15 min in 2 x SSCT, and 2 x for 30 min in 0.2 x SSCT. For detection, embryos were blocked at least for 1 h at room temperature in 150 mM NaCl (pH 7.5) plus blocking reagent (2% Blocking Reagent; Roche Biochemicals). Embryos were incubated overnight at 4°C in α-DIG-$F(ab')_2$-AP as supplied by Roche Biochemicals at a 5000-fold dilution in the solution described above. The following day specimens were washed 4 x 20 min in 150 mM maleic acid, 100 mM NaCl and 3 x 5 min in staining buffer (100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20). Embryos were then incubated in staining buffer with 4.5 µl NBT and 3.5 µl BCIP (Roche Biochemicals) per milliliter added. To stop the color reaction, PBST was added, followed by fixation in 4% PFA. Specimens were stored in 4% PFA. At every stage and per probe at least 20-40 embryos were analyzed. Older embryos were fresh frozen in TissueTek (Miles) and sections were hybridized according to Weth *et al*. (1996). Here, about 3-5 embryos were analyzed per stage and probe.

11.3.3. Whole Mount *In Situ* Hybridization of Olfactory Bulb and Telencephalon

To get a better impression of the spatial distribution of some transcripts whole mount *in situ* hybridizations were performed on the tissues of interest.

All incubations were carried out at room temperature unless otherwise indicated. The olfactory bulbs and telencephali were dissected out in PBS and the *meninx primitiva* was digested away with 15 µg/ml proteinase K (PK) in PBS for 10 min at 37°C. Fixation was done in 85% (4% PFA/ 0.1% glutaraldehyde (GA)) and 15% saturated picric acid for 5 min. The membranes were solubilized by washing them in PBSTx (PBS containing 0.15% Triton X-100) for 10 min. The tissues were dehydrated and rehydrated through an alcohol series (MeOH in PBS) as follows: 10% (5 min), 25% (5 min), 50% (3 x 10 min), 25% (5 min) and 10% (5 min). After rinsing and washing for 5 min with PBSTw (PBS containing 0.05% Tween 20) the membranes were permeabilized with 10 µg/ml PK in PBSTw for 15 min at 37°C. The bulbs were refixed in 4% PFA/ 0.1% GA for 5 min at 4°C before prehybridization in 50% formamide, 5 x SSC, 0.2% Tween 20, 0.5% Chaps, 100 µg/ml torula RNA, 100 µg/ml heparin for 2 hours at 60°C. Hybridization was carried out in hybridization buffer containing 1 ng/µl of a probe overnight at 60°C. Posthybridization washes were as follows: twice with hybridization buffer for 30 min at 60°C; a 1:1 mixture of hybridization buffer and MaBSTw (MaBS containing 0.05% Tween 20) for 20 min at 60°C; twice with MaBSTw for 30 min. To block non-specific binding, the samples were incubated with 2% blocking reagent (Blocking Reagent, Roche Biochemicals) in MaBSTw for 1 h. A second blocking step was done with 2% BR, 20% heat-treated sheep serum in MaBSTw (heat treatment of serum: 30 min at 56°C) for 2 hours at 4°C. The samples were immunoreacted with 1/2000 α-DIG-F(ab')$_2$-AP in 2% BR, 20% heat-treated sheep serum in MaBSTw overnight at 4°C. The bulbs were washed every hour (and o/n at 4°C) with MaBSTw. Then they were incubated twice with reaction buffer containing 0.05% Tween 20 for 10 min. Color detection was done with NBT/BCIP as described by the manufacturer (Roche Biochemicals). After sufficient color development samples were rinsed with PBSTw and refixed in 4% PFA overnight at 4°C. Then they were embedded in 12% gelatin, refixed again in 4% PFA overnight at 4°C. Serial 100 µm sections were taken with a vibratome (TPI, Ted Pella Incorporated, Redding, CA), put on gelatin-chrome alum coated slides, dried, mounted in Mowiol and photographed.

11.4. Double Labeling Experiments

11.4.1. Simultaneous Detection of Two Proteins

For simultaneous detection of two proteins the primary and secondary antibodies were applied together for overnight, given that the primary and secondary antibodies were from different species so that no cross-reaction could occur.

11.4.2. *In Situ* Hybridization and Antibody Staining

For simultaneous detection of a transcript and a protein, first the *in situ* hybridization was performed, since the RNA is very sensitive to degradation. The immunostaining procedure was performed on the same samples afterwards.

11.4.3. Double *In Situ* Hybridization

In some cases double *in situ* hybridization experiments were performed to detect transcripts of two different genes simultaneously. For this purpose, one of the RNA probes was labeled using digoxigenin and the other using fluorescein as described previously. Then *in situ* hybridization was performed by hybridizing the sample with both probes simultaneously. For detection, the α-Flu-AP antibody (1:750) was applied first and after several washing steps in PBST, the specimen were washed with 0.1 M Tris pH 8.2/ 0.1% Tween 20. During the washing steps one Fast-Red tablet (Biogenex, Hamburg) was dissolved in a vial of buffer (as supplied by the manufacturer). The reaction was started and after sufficient labeling it was stopped by washing 3 x 5 min in PBST. Free phosphatase activity was blocked by incubation for 10 min in 0.1 M Glycin-HCl pH 2.2/ 0.1% Tween 20. The specimens were then washed 4 x 5 min with PBST and incubated overnight in α-DIG-AP antibody. Detection of the second transcript was then performed as described above using NBT and BCIP as a substrate.

11.5. RAP (Receptor Alkaline Phosphatase) *In Situ* Assay

11.5.1. Cell Culture

Two different cell lines were used for cell culture experiments. In initial experiments COS-7 cells were used. These cells were not able to form 'hanging drops'. Therefore in later experiments HEK cells were used.

Human embryonic kidney (HEK) 293 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal calf serum (FCS) (Roche Biochemicals), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine in a humidified 5% CO_2-incubator at 37°C.

11.5.2. Constructs for Transient Transfection Experiments

The fusion protein constructs were prepared by cloning the coding sequence of receptors or ligands into the APtag-1 vector (Flanagan and Leder, 1990) containing a signal sequence. In this way it was provided that the synthesized proteins were secreted into the

medium and could be harvested simply by collecting the medium. Dr. C. Brennan kindly provided the fusion protein constructs used in this study.

11.5.3. Transient Transfection of Mammalian Cells Using the Calcium Phosphate Method

Transient transfection experiments were performed in the HEK293 and COS-7 cells. Cells were seeded 24 h before transfection at a density of 10^7 cells per 100 mm dish. Transfections were performed by the calcium phosphate precipitation procedure (Sambrook *et al*., 1989). A total of 10 µg of DNA per dish was used in each transfection. Precipitates were left on the cells for 16 hr, before changing the culture medium. The conditioned media containing the secreted fusion protein were harvested 72 hours after transfection and centrifuged to remove debris.

11.5.4. Assay for AP Activity

The SEAP (= **se**creted form of human placental **a**lkaline **p**hosphatase) reporter gene encodes a truncated form of the placental enzyme that lacks the membrane anchoring domain, thereby allowing the protein to be efficiently secreted from transfected cells. Levels of SEAP activity detected in the culture medium have been shown to be directly proportional to changes in intracellular concentrations of SEAP mRNA and protein (Berger *et al*., 1988; Cullen and Malim, 1992). SEAP has the unusual properties of being extremely heat stable and resistant to the inhibitor L-homoarginine (Cullen and Malim, 1992). Therefore, endogenous alkaline phosphatase activity can be eliminated by pretreatment of samples at 65°C and incubation with this inhibitor.

The supernatants collected at desired time points were subjected to a SEAP assay. For this purpose samples were heated to 65°C for 10 min to inactivate endogenous cellular phosphatase activity. AP activity was assayed by adding equal volumes of 2 x AP buffer (2 M diethanolamine [pH 9.8], 1 mM $MgCl_2$, 20 mM homoarginine, 12 mM p-nitro-phenyl phosphate) to conditioned supernatants. Incubation was carried out at room temperature (or 37°C, 45 min) and AP activity was determined at 405 nm.

11.5.5. Affinity Probe *In Situ*

Binding experiments with alkaline phosphatase (AP)-tagged receptors and ligands were performed essentially as described by Cheng and Flanagan (1994) with minor modifications. Embryos were used as whole mounts and were fixed for 30 min to one hour in 4% PFA, washed three times with PBSTx (PBS, 0.8% Triton X-100). Tissue specimens like olfactory bulbs and/or olfactory epithelia were either used as whole mounts, as 50 µm vibratome sections or as 15 µm cryostat sections. In case of whole mounts the tissues were incubated with the affinity probes unfixed. For tissue-sections, the tissue was prefixed with

4% PFA in PBS for 10-30 min at 4°C, then embedded in 15% gelatin, refixed overnight and 50 µm sections were prepared. Affinity probe *in situ* was then performed on free-floating sections.

After overnight incubation with the fusion protein conditioned media containing similar amounts of AP activity, the specimen were washed five to six times in HBHA buffer (Hank's balanced salt solution, 0.5 mg/ml BSA, 20 mM HEPES [pH 7.0]). Fixation for 30 s in freshly prepared acetone-formaldehyde fixative (60% acetone, 3% formaldehyde, 20 mM HEPES [pH 7.0]) was followed by three washes in HBS (150 mM NaCl, 20 mM HEPES [pH 7.0]). The specimen were then incubated in HEPES buffered saline at 65°C for 10 min to 1 h to inactivate endogenous cellular phosphatase activity. After rinsing once with AP buffer (100 mM Tris [pH 9.5], 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20) the specimen were stained in the same buffer containing 0.17 mg/ml BCIP and 0.33 mg/ml NBT to detect ephrin-A-L4-AP, ephrin-B-L5-AP, EphA-rtk6-AP, and EphB-rtk8-AP bound at the cell surfaces.

11.6. Tracing Using Lipophilic Dyes

11.6.1. Anterograde Tracing of Olfactory Receptor Neurons with DiI

Olfactory sensory neurons were traced with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) as described by Baier *et al*. (1994). Briefly, fish were anesthetized with 0.01% tricaine methanesulfonate (MS-222, Serva). Fishes were kept immobilized by placement on a paper towel drenched with ice-cold water. The DiI solution (fresh 1:100 dilution of a 5 mg/100 ml in dimethylformamide stock solution) was injected into each nasal cavity using fine, flexible pipette tips (GELoader tips, Eppendorf) and left in place for 5-10 min. Fish were put back into a water tank and kept in the dark for two days. Afterwards, fish were decapitated; the olfactory bulbs with the telencephalic hemispheres were removed and collected in chilled PBS. The staining was observed under a fluorescent binocular or microscope and eventually photographed for documentation. Successfully stained olfactory bulbs were fixed in 4% PFA in 0.1 M phosphate buffer (pH 7.4) overnight, embedded in 15% gelatin and postfixed for an additional 1-3 d. Then, 50-100 µm sections were taken on a vibratome (TPI) and mounted on gelatin-coated glass slides using Vectashield (Vector) and coverslips were secured by putting some nail polish on the edges.

11.6.2. Photoconversion of DiI

Photoconversion of DiI was generally performed as described in the Zebrafish-Book (Westerfield *et al*., 1995). After tracing, the olfactory bulbs were dissected out and kept in 1:3 diluted PBS. The bulbs were then put into 0.5 mg/ml diaminobenzidine (DAB) prepared in PBS/3 and incubated for at least 15 min at room temperature. The bulbs were then

mounted on a slide with height spacers and a coverslip and oriented. On a fluorescent microscope (Axiophot, Zeiss) the UV light was focused onto filled axons using the 20 x objective. The reaction was monitored once in a while and took about 30 min to 1 hour at each focal plane, in total about 5 h. Care was taken that the solution did not evaporate and the tissue did not dry out. The reaction product was then readily visible as a brown precipitate (see Fig. IV.2.). After photoconversion, the bulbs were rinsed in PBS/3, fixed in 4% PFA embedded in gelatin and fixed for two days. Sections were taken at 100 µm, mounted on gelatin-coated slides and photographed using the Axiophot and Nomarski optics.

11.7. Counterstaining and Mounting

In some cases like *in situ* hybridizations and antibody stainings counterstains appear helpful to differentiate the various cell types or subcellular structures seen in cell staining. In this study the most common counterstain used is DAPI (Sigma), which stains the nuclei of the cells. In some cases the Hoechst stain (Molecular Probes) was also used for no specific reason. DAPI was used after the staining procedures were complete. The tissues were incubated for 5 min at room temperature and then washed thoroughly with PBS.

Mounting media for histology must be compatible with the detection method used. Here, generally aqueous mounting media were used, most commonly Mowiol. Mowiol was directly added to the washed specimen. A coverslip was placed carefully on the drop of Mowiol avoiding air bubbles. Excess mount was removed using a paper towel. The slides were left overnight for setting the mount. For fluorescently detected specimen Mowiol with 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO) or Vectashield (Vectabond) was used for mounting. The coverslips were secured by putting some nail polish on the edges.

12. Experiments to Study Promoter Activity *In Vivo*

12.1. Construction of Expression Vectors

In our study it was necessary to construct a new expression vector rather than using one of the available ones, since we thought that an expression vector that contains an optimal translation initiation site in front of the reporter gene would be ideal. Furthermore, it appeared optimal that the putative promoter region should be directly ligated to this site without leaving additional restriction enzyme sites between the inserted fragment and the reporter gene. This is usually the case with all available expression vectors.

12.1.1. Construction of the Expression Vector pACSF-Y

A promoterless expression vector based on the common pBluescript was constructed by S. Fuss (2001). This vector has the following properties:

- it contains a multiple cloning site, allowing the cloning of promoters of interest
- it has an optimal Kozak consensus sequence (Kozak *et al*., 1999), provided by the *Nco* I cutting site around the ATG codon
- it contains the reporter gene EYFP, which is an enhanced variant of the green fluorescent protein (GFP)
- it has an SV40 polyadenylation signal

The sequence and map is given in the supplement. This vector was used for all following vector constructions.

12.1.2. Construction of the Expression Vector pACSF-dsRed

This vector was constructed by exchanging the EYFP reporter gene with a red fluorescing reporter, dsRed. The *dsRed* gene was amplified by PCR from the vector pDsRed1-N1 (Clontech) and using the primers dsRed-ndeco and dsRed-not. The PCR product was digested with the enzymes *Eco* RI and *Not* I and ligated to the pACSF vector lacking the EYFP gene that was removed by cutting with the enzymes *Eco* RI and *Not* I. It was necessary that the *Nco* I cutting site can be replaced with a *Nde* I site, since the *dsRed* gene contains a *Nco* I site within its open reading frame (ORF).

12.1.3. Construction of the Expression Vector pACSF-tau-Y

This vector contains an additional sequence from the microtubulin associated protein tau. The tau-protein simplifies/enhances the transport of the reporter gene in the axon terminals (Mombaerts *et al*., 1996). A coding fragment of 236 bp that appears to be functional was amplified by PCR from genomic DNA extracted from the liver of a I7-tau-GFP mouse (P. Mombaerts, unpublished) using the primers tau-nco and tau-ndeco. The tau-ndeco primer creates a change of the *Nco* I restriction site to a *Nde* I cutting site (as in dsRed case), while the tau-nco primer changes the *Not* I site of the tau sequence to a *Nco* I site. The PCR product was digested with *Eco* RI and *Nco* I and ligated into the basic vector pACSF-Y cut with the same enzymes.

12.2. Construction of Promoter Expression Constructs

Different putative promoters were identified (see IV.3.) and cloned into the pACSF-Y basis vector in order to test them *in vivo*. The construction of these expression constructs is briefly described here.

12.2.1. Construction of the Expression Vector prOMP$_{1.3}$-Y

The upstream region of the *OMP* gene was amplified using the prOMPnco and T7 primers and the clone L131 (pBluescript containing the 1.8 kb upstream *Eco* RI fragment of the *OMP* gene) as template. The amplified PCR product was digested with *Eco* RI and *Nco* I purified and ligated into the pACSF-Y vector that was processed with the same enzymes.

The additional constructs that were tested were generated by simple digestion of this vector with *Hind* III and *Sac* I to generate the prOMP$_{0.6}$-Y and prOMP$_{0.3}$-Y vectors, respectively.

12.2.2. Construction of the Expression Vector prOMP$_{1.3}$-dsRed

Here, the upstream region of the *OMP* gene was amplified using the primers prOMPndeco and T7 and L131 as template. The amplified PCR product was digested first with *Nde* I and then with *Eco* RI and ligated into the pACSF-Y vector that was previously digested with the same enzymes.

12.2.3. Construction of the Expression Vector prOMP$_{1.3}$-tau-Y

The OMP upstream region was amplified using the primers prOMPndeco and T7 and L131 was used as template for this amplification. The amplified PCR product was digested first with *Nde* I and then with *Eco* RI and ligated into the pACSF-tau-Y vector that was previously digested with the same enzymes.

12.2.4. Construction of the Expression Vector prdlx2$_{5.5}$-Y

The 5.5 kb upstream fragment was amplified using the primers dlx2nco and T7hi and the clone containing the 11 kb of the upstream region of *dlx2* as template. The amplified PCR product was cloned into pACSF-Y after both (PCR product and vector) had been digested with *Sac* I and *Nco* I.

The prdlx2$_{1.3}$-Y construct was prepared by simple digestion of the prdlx2$_{5.5}$-Y with the restriction enzyme *Afl* II.

12.2.5. Construction of the Expression Vector prdlx2$_{9.5}$-Y

This expression vector was constructed by removing the additional 4 kb of upstream region from the dlx2-11kb-upstream clone by simple *Sac* I digestion and ligation of this fragment into the prdlx2$_{5.5}$-Y vector that was treated previously with *Sac* I.

12.3. Generation of Transient Transgenic Zebrafish

12.3.1. Embryo Collection

Embryos were obtained by natural spawning between a pair of male and female fish that were kept in spawning tanks having a separator. Embryos were removed from the spawning tanks within 20 min of fertilization, cleaned in system water and embryo rearing medium, and transferred to the injection apparatus.

12.3.2. Microinjection

Linearized plasmid DNA at a concentration of 50 μg/ml in 0.1 M Tris-HCl (pH 7.6)/0.25% phenol red were injected into the cytoplasm of 1- or 2-cell stage embryos under a Nikon stereo microscope with a pressure-driven microinjector (World Precision Instruments) using a pulled capillary needle. Borosilicate glass capillaries (1 mm outer diameter) with filament were used as injection needles. Tips of appropriate length and thinness were pulled using a pipette puller (Sutter) and broken to give a small opening. The needle was positioned under the blastoderm in the region of cytoplasmic streaming. Successful injection was judged in the first instance by a visible red color in the cell. These embryos were discarded from further analysis. Optical control of the injections ensured that all embryos received similar amounts of dye and thereby similar volumes of the injected solution. With some experience, reproducible volumes could be injected even when needles with variable tip diameters were used. Dead embryos and unfertilized and damaged eggs were removed several hours after microinjection. Healthy embryos were reared in embryo rearing medium as previously described. Expression of EYFP was observed at daily intervals under a fluorescence dissecting microscope (SMZU, Nikon) and positive embryos were photographed under a Zeiss Axiovert 100 fluorescence microscope.

12.3.3. Test for Germline Transmission

To test if the raised fish had integrated the injected plasmid, injected adult zebrafish were mated to wild-type zebrafish and the embryos were analyzed for EYFP expression. Afterwards, embryos were collected and genomic DNA was extracted as described under II.10.1. This genomic DNA was used as a template in a standard PCR reaction using the primers EYFP-5' and EYFP-3' specific for EYFP. These primers are also able to bind to EGFP and are therefore useful in testing constructs containing one or the other fluorescent protein. To test for the integrity of genomic DNA primers for the endogenous gene GAPDH were used.

12.3.4. Mounting of Embryos in Agarose for Microscopic Analysis

Confocal recordings and other fluorescent microscopical analyses and photography require that the vibration of the embryo be minimized. In some cases living embryos were photographed, but even when fixed embryos were to be photographed these were embedded for optimal orientation into low melting point agarose. For this purpose a film of warm agarose was poured into a petridish, allowed to harden and then the embryo was positioned on this agarose film. Then, the embryo was overlaid with another film of agarose. After the agarose was hardened an agarose block was cut with a sharp scalpel so that not much agarose was left anterior to the embryo. The embryo was then placed anterior down on a coverslip attached to a small petridish. Several embryos were placed side by side and then overlaid again with a drop of agarose to prevent movement of the individual agarose blocks.

13. Experiments to Modify the Activity of Specific Gene Products

There are two major concepts to investigate the *in vivo* function of a gene product during development. A gene can be studied in gain-of-function experiments in which it is overexpressed, ectopically expressed, or presented in a more active or constitutively active form. In the reverse approach, the effect of a complete or partial loss of gene function is studied. The latter experiments reveal whether a gene is actually required for normal development. Both approaches should be applied, since often only one of the two gives a conclusive result. For instance, loss-of-function manipulations may have no effect due to the redundancy of genes with similar functions. On the other hand, gain-of-function manipulations, like ectopic gene expression, may give no effect due to the restricted competence of cells outside the natural expression domains.

Strategies for loss-of-function studies are to inactivate the products of the investigated gene by molecular means, applying dominant negative, antisense, or antibody strategies.

13.1. Test of the IRES Sequence in Zebrafish Embryos

In order to allow a reporter gene to be expressed at the same time with the gene of interest so called internal ribosome entry sites (IRES) can be used. These sequences generally found in viruses allow two genes that are connected by this sequence to be translated from a single bicistronic mRNA. It was not clear if IRES sequences also work in zebrafish; therefore two test constructs were prepared.

13.1.1. Construction of the Expression Vector prOMP$_{1.3}$-dsRed-IRES-EGFP

This vector contains two fluorescent proteins, dsRed and EGFP, that are connected by IRES. To prepare this vector the IRES-EGFP sequences were amplified from the vector pIRES2-EGFP using the primers IRES2-EGFP5 and SV40hi. The PCR product was digested with *Not* I and cloned into the *Not* I digested prOMP-dsRed vector. The proper orientation of the insert was determined by sequencing.

13.1.2. Construction of the Expression Vector prOMP$_{1.3}$-IRES-EGFP

This vector was constructed by amplification of the IRES-EGFP sequences using the primers IRES-ndeco5' and SV40hi, subsequent digestion with *Nde* I and *Not* I and ligation into the prOMP-dsRed vector, that was previously digested with the same enzymes.

13.2. Overexpression of L5/ephrin-B2a

To study the function of L5/ephrin-B2a a construct was prepared that leads to the overexpression of the full-length L5/ephrin-B2a gene in olfactory sensory neurons. The transgenic OSNs were monitored over the EGFP protein that is translated from the bicistronic message.

13.2.1. Construction of the Overexpression Construct prOMP$_{1.3}$-L5/ephrin-B2a-IRES-EGFP

The L5/ephrin-B2a sequence was amplified using the primers L5ndeco and L5bgl2 using the plasmid L5 that contains the full-length sequence of ephrin-B2a. The PCR product was digested using the restriction enzymes *Nde* I and *Bgl* II and ligated into the prOMP$_{1.3}$-IRES-EGFP vector that was previously digested with the same enzymes.

14. Microscopic Analysis and Photography

Whole mount specimens like *in situ* hybridized or immunohistochemically processed zebrafish embryos and olfactory bulbs were photographed using a stereomicroscope (SMZ-U, Nikon) and a digital camera (CoolPix 900, Nikon). Images were transferred to a Macintosh computer (G3) using the SmartCard (Viking) and its reader (SanDisk).

Documentation of sectioned material was performed with a light microscope (Axiophot, Zeiss). Photographic images were digitized on a slide scanner (Nikon-LS 2000, Nikon).

Fluorescent specimens were documented in either way, using the fluorescence stereomicroscope (SMZ-U, Nikon) or different types of fluorescence microscopes, including Axiovert S100 TV (Zeiss), Axiophot (Zeiss), or an automatic photomicroscope (Axioskop II, Zeiss). Confocal images were also taken in some cases, since confocal imaging is an excellent means of imaging fluorescent signals within zebrafish embryos. It provides a means of optically sectioning tissues that have been labeled with specific fluorescent probe molecules. Images were aquired either with a Leica TCS SP2 confocal microscope (Leica)

The digitized images were brightness, color, and contrast balanced, formatted for printing in Adobe Photoshop 5.0 (Adobe Systems Inc.) and printed on an Epson Stylus 760 printer (Epson).

IV. RESULTS

A key step in understanding the development and function of the olfactory system is characterizing the connections between the neurons and how they form. Although the zebrafish has become a model system to study olfaction, it is not as characterized at the cellular and molecular level as for example the olfactory system of rodents. All the knowledge gained so far comes from electromicroscopical, histological, and physiological studies. In the first part of this study it was intended to characterize the olfactory epithelium and the olfactory bulb on the cellular level. In the second part, expression patterns of putative guidance molecules were studied, putting weight on the Eph receptor and ephrin family of genes. In the third part, cell type specific genes and promoters were isolated for functional studies *in vivo*.

1. Characterization of the Olfactory System of Zebrafish by Establishing Markers for Specific Cell Types

In order to judge the staining of potential pathfinding molecules it was necessary to have convenient markers for the different cell types present in the olfactory epithelium like the olfactory sensory neurons, sustentacular and basal cells. The main cell types in the olfactory bulb to be defined were mitral cells, granule cells and glia. In mammalia many markers have been isolated to date that specifically label different cell populations. In the beginning of this study some of these markers, which are mainly antibodies, were tested for their usefulness in the zebrafish olfactory system.

1.1. General Considerations

Antibodies require optimal conditions to bind their antigen specifically. Therefore, it is important to find and optimize the conditions so that the most specific staining is obtained. Critical parameters include type of tissue embedding, as well as the methods for fixation, permeabilization and detection. The chosen method of tissue preparation must be suitable to answer the fundamental objective of the protocol; i.e. where the protein is localized within the system. If the protein is suspected to exist at the cell surface, it would be pointless to employ a protocol designed to look at cytoplasmic contents and which includes an intrinsic step that may disrupt the cell membrane. When cytoplasmic antigens are to be studied, the membranes must be permeabilized to allow antibodies into the cell. Furthermore, the choice of the labeling method is also important to optimize the sensitivity of antigen localization.

1.1.1. Embedding

Tissues can be prepared for sectioning either by fixing in paraformaldehyde, embedding in TissueTek (Miles) and preparing cryosections ('frozen sections'), or by embedding the tissue directly in TissueTek and cryosectioning ('fresh frozen'); additionally, fixed tissue can be embedded in gelatin and vibratome sections can be prepared subsequently. Many more methods are available, but in this study mainly these three methods were used.

Frozen sections require the fixation and cryopreservation of the tissue before embedding and sectioning. In many experiments where the concentration of the cryopreservative (sucrose) was varied from 5% → 30% in 4% PFA, and successive infiltration of the specimen with the cryopreservative (meaning the treatment of the tissue with increasing concentrations of sucrose), didn't give acceptable results. Although the tissue morphology was preserved quite well after sectioning with this method (similar to gelatin embedded and vibratome sectioned tissue) no staining could be obtained with several antibodies and also with *in situ* hybridizations. Therefore, this embedding method was not employed further.

The second method, embedding of fresh-frozen tissue in TissueTek and cryosectioning, was used because it is the method of choice for *in situ* hybridizations. Although, fresh frozen cryostat sections, in general, are less useful because morphology is adversely affected, it appeared as the method of choice for *in situ* hybridization on olfactory epithelia with olfactory receptor probes (Weth, 1993). Additionally, the antibodies were supposed to be used in double labeling experiments; therefore, it appeared important to know if they work on fresh-frozen sections. To make frozen sections the tissue has to be frozen properly. For this purpose, it is embedded in TissueTek and frozen directly on dry ice. Frozen tissue blocks are stored in a -70°C freezer until sectioning. They can also be sectioned directly by attaching the tissue block on the cryostat chuck. The block is adhered with a small amount of frozen tissue matrix and allowed to freeze. Routine sections were prepared at 10 µm for olfactory epithelia and 15 µm for olfactory bulbs with attached telencephali and picked up on glass slides. The sections were baked for 1 h at 60°C before fixation.

The third method employed included fixation, gelatin embedding and vibratome sectioning of the tissue. The sections will be referred to as 'vibratome sections' in the following. Tissue embedded in this way retains its morphology very well, but in contrast to cryosectioned tissue not so thin sections can be prepared. Vibratome sectioning was used when the antigen was apparently altered or destroyed by freezing. Fixed tissues sectioned using a vibratome do not require cryoprotection because this method of embedding does not involve freezing of the tissue.

1.1.2. Fixatives and Fixation Times

Optimal fixation time is that which gives good morphology as well as good signal-to-noise ratio after the staining. This has to be determined empirically for each tissue type and each antibody. For good and more homogenous fixation of individual organs, animals are

generally perfused, before harvesting of the tissue. This was not necessary here, because of the relatively small size of the tissues of interest.

The most commonly used fixative is paraformaldehyde (4% in PBS). PFA fixation is a continuous fixation, as the tissue will become increasingly fixed the longer it is left in the fixative. Therefore, the fixation time should be minimized, as overfixation will result in loss of reactivity for both *in situ* hybridization and immunohistochemistry.

Immobilization of the antigen varies depending on whether the antigen is considered to be associated with the cell surface membrane or contained within the cytoplasm. Low concentrations of PFA are used to fix surface antigens. This will generally not destroy antigenicity, and will preserve cell morphology. However, if fixation does remove antigenic activity, fixation can be omitted and incubations must than be performed at 4°C.

For antigens constituting small amino acids, like neurotransmitters, fresh frozen tissues are not usable since free amino acids will simply diffuse away. One must fix tissues to retain intracellular amino acids. However, even PFA alone is not particularly useful since it has been known for some 50 years that over 95% of all intracellular amino acids are lost in pure PFA fixation. Glutaraldehyde is a bifunctional linker with great capacity for trapping amino acids. Therefore, it is useful to use a mixture of these two fixatives for some antibodies.

1.1.3. Permeabilization

There are many methods that provide that the lipid bilayer is disrupted and the cytoplasmic protein is fixed, with generally good retention of antigenicity. The most commonly used one is the addition of Triton X-100 (or in some cases Tween 20) to all solutions after fixation. In some cases this is not enough and so other methods like the dehydration method (using methanol) and the cross-linking/permeabilization method (using a mixture of PFA and Triton X-100) can be used. Proteinase K treatment is not the best method of choice since it can adversely affect cellular morphology and the antigenicity of the protein to be detected.

1.1.4. Signal Intensification

Due to the amplifying potential of enzyme labels, immunolabelings, which utilize enzyme-conjugated antibodies, have become increasingly popular because of their high specificity and sensitivity. The most commonly used enzymes are horseradish peroxidase and alkaline phosphatase. In this study horseradish peroxidase coupled antibodies were used in general. However, if the signal was not intense enough, signal intensification using a biotin and streptavidin-based amplification was used. This system makes use of the fact that every avidin or streptavidin molecule contains four biotin-binding sites. So, the specimens are incubated with a biotin-conjugated secondary antibody conjugate that recognizes the primary antibody. In a second step, the horseradish peroxidase-coupled streptavidin conjugate is applied.

Taken together, the general strategy for immunohistochemical staining was as follows. First, stainings were performed on fresh frozen cryostat sections because of ease of preparation of the sections (and being the most useful method for double labeling). In cases where cryostat sections didn't give any or not acceptable staining results even after signal intensification, vibratome sections were tried. Usually, one particular protocol (see III.X.) was used and several parameters like type of fixative (4% PFA → 4% PFA/ 0.5% GA → 2.5% PFA/ 1% GA → 2.5% PFA/ 2.5% GA), permeabilization (0.5 M ammoniumchloride, 0.25% Triton X-100 → 0.5% Triton X-100), concentration of primary and secondary antibodies (1:20 → 1:500), incubation times with the antibodies (overnight for primary antibodies; 20 min → 2 h with secondary antibodies) were varied to obtain best staining results.

10.1. Detection of Olfactory Sensory Neurons

Several antibodies were tested for their potential to label olfactory sensory neurons in the adult as well as in the embryonic olfactory pathway of zebrafish.

1.2.1. Localization of the zns-2 Epitope in the Olfactory Epithelium and Olfactory Bulb

The zns-2 antibody was generated at the University of Oregon against embryonic zebrafish epitopes (Trevarrow *et al.*, 1990). It appeared to label several neural structures in zebrafish embryos, including neuropil structures, radial axons and outer and inner plexiform layers (Westerfield *et al*., 1995). The epitope for this antibody is not known, but it is speculated that it is a structure that is present in the plasma membrane. To investigate in detail, which structures of the olfactory system are labeled by this antibody, and if these are labeled throughout development and adulthood staining of adult olfactory epithelia and olfactory bulbs were undertaken.

When horizontal cryosections of olfactory epithelia are stained with this antibody fibrous structures within the lamellae, at the border of sensory and non-sensory area are labeled in apical sections. The staining extends throughout the basal lamina when looking at more basal sections and becomes more intense at the olfactory nerve (Fig. IV.1.A and B).

In whole mount preparations the staining is more apparent since the axons can be followed nicely within the lamellae (Fig. IV.1.C). There seems to be an ordered bundeling of axons from the apical to the basal part of the lamellae. About every 3-5 µm a small bundle of axons joins a large bundle to form the olfactory nerve in the very basal part of the epithelium (Fig. IV.1.D and E). When entering the olfactory bulb the axon bundles undergo a high defasciculation and reorganization (Fig. IV.2.B). Axons extend over the whole surface of the olfactory nerve layer and then turn into one deeper layer, the glomerular layer, to form the characteristic neuropil structures called glomeruli. The glomerular pattern observed with the

zns-2 antibody, is very similar to the staining of the glomeruli with the lipophilic dye DiI (Fig. IV.2.B).

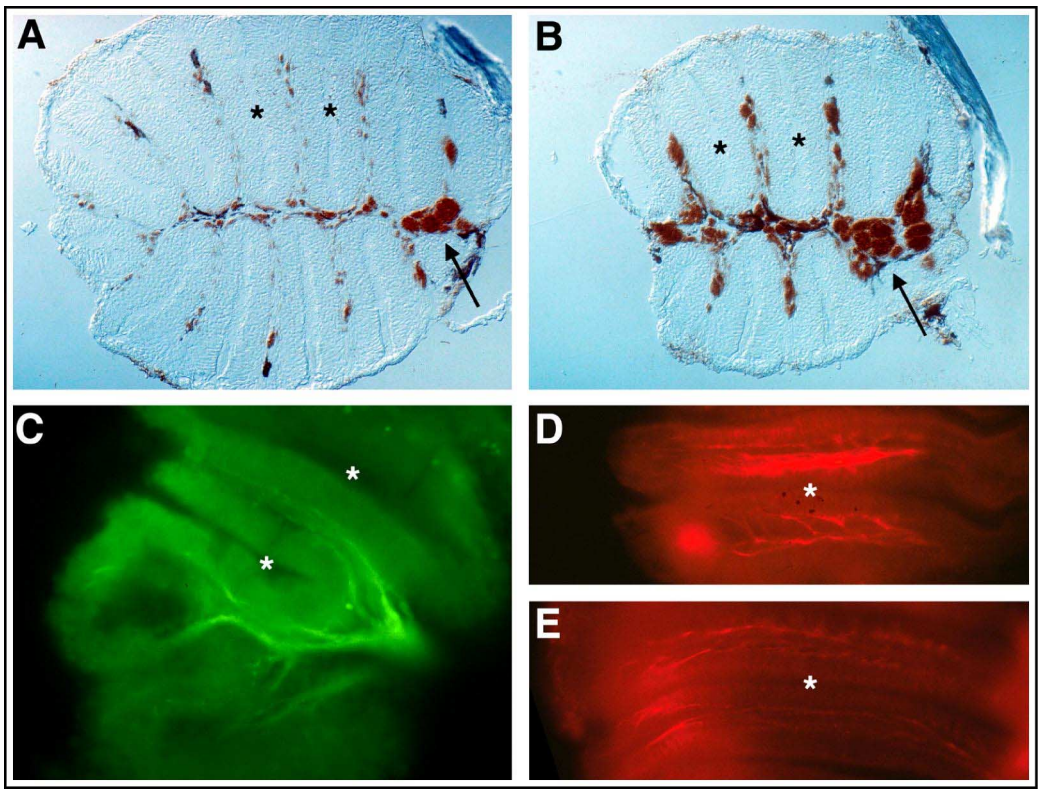

Fig. IV.1.: Immunohistochemical Detection of zns-2 Immunoreactivity in the Adult Olfactory Epithelium. A and B: Cryosections of an adult olfactory epithelium. A shows an apical section, whereas B is a more basal section. C, D, E: Vibratome sections of epithelia processed in whole mounts. C is a horizontal section, which is a bit tilted. D and E are vertical sections, parallel to the midline raphe. Arrows indicate the olfactory nerve, while asterisks show the lumen between the lamellae. Anterior is to the left in A and B. Apical is to the left in C, D and E. Scalebar in A, B and C corresponds to 50 µm, in D and E to 25 µm.

DiI is a highly fluorescent lipophilic tracer dye that when applied to the olfactory epithelium is taken up specifically by the olfactory sensory neurons. It diffuses within the membrane in both live and fixed tissue, effectively labeling the entire cell, including distant projections. Fig. IV.2. shows a comparison of both stainings.

The DiI staining was photoconverted and sections through this bulb are shown in images IV.2.D, F and H. Labeling of a bulb-telencephalon preparation with zns-2 is shown in Fig. IV.2.A, sections through the same preparation are shown in Fig. IV.2.C, E, and G. The staining obtained with the zns-2 antibody strongly corresponds to the stereotypic pattern of glomeruli that was extensively studied previously with DiI stainings (Baier and Korsching, 1994).

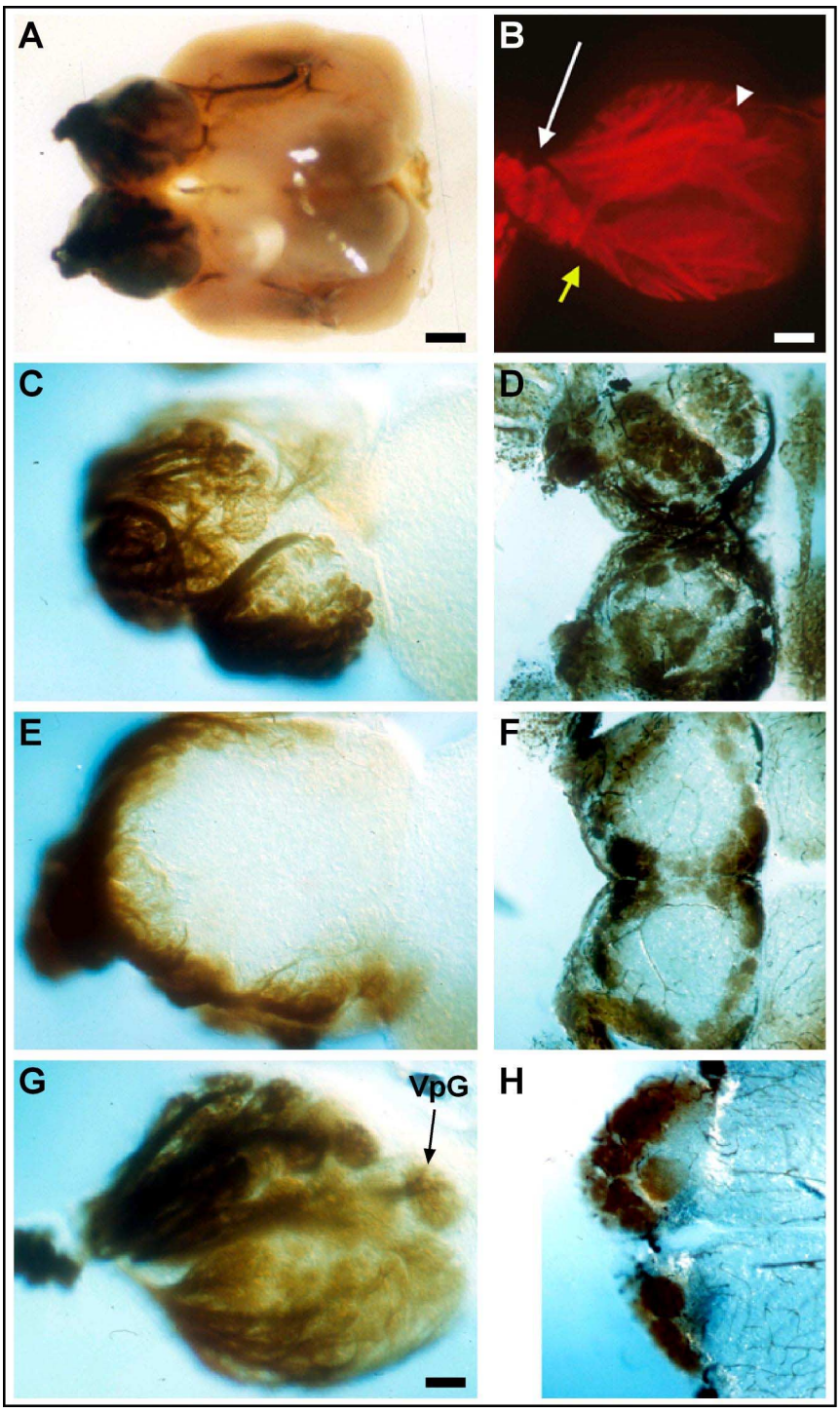

Fig. IV.2.: Visualization of the Glomerular Pattern Using zns-2 Immunohistochemistry, DiI Staining and Photoconversion. Vibratome sections of olfactory bulbs processed as whole mounts. C, E, and G: sections through the lower bulb shown in A after antibody staining with zns-2 and detection using an peroxidase-coupled secondary antibody. B. DiI staining of an olfactory bulb. The arrow indicates the olfactory nerve and the arrowhead points to a glomerulus; yellow arrow shows axons of olfactory sensory neurons. D, F, and H show sections through olfactory bulbs after DiI staining and photoconversion. Anterior is to the left in all cases. VpG: ventral posterior glomerulus. Scalebar in A corresponds to 150 µm, in B, D, F and H to 100 µm, in C, E, and G: 50 µm.

As a result it can be said that the zns-2 antibody can be used as a marker for olfactory sensory neuron axons, and can substitute the more tedious DiI stainings. Another advantage of having an antibody is the possibility of using it in double labeling experiments with *in situ* hybridizations. In initial trials where DiI was employed for double labeling experiments it turned out that this dye does not survive all the steps of the *in situ* hybridization procedure. The order of the staining procedures could not be reversed, since the dye has to be applied to the nose of the living fish, and so this staining has to be performed first. Alternative experiments were performed to turn the fluorescent DiI staining into a chromogenic precipitate using the photoconversion method. This was possible to a reasonable degree; the glomerular pattern could be displayed, but endogenous peroxidases presented a problem since they could not fully be eliminated (Fig. IV.2.). Furthermore, this method turned out to be very time-intensive and of low throughput, since only one bulb could be processed per four to five hours. A more serious problem was that the mRNA within the tissue was degraded during this treatment.

Development of the olfactory projection was analyzed at embryonic stages in whole mounts and also on cryostat sections of embryos, depending on the age of the embryo. Whole mount stainings were easily performed at 1-3 dpf; at later stages (7 d, 14 d (not shown) and 21 d) larvae were embedded in TissueTek and cryosectioned before staining.

Labeled axons arose from the basal ends of the placodal cells and coalesced to form the olfactory nerve. At 24 hpf, axons were traced onto the mid- to dorsal telencephalon (Fig. IV.3.A). This is the presumptive olfactory bulb, but it has not evaginated at this stage. The axons have multiple terminal branches. Many more axons are labeled at 48 hpf (Fig. IV.3.B-C). Groups of axons form glomerulus-like terminations on the dorsal telencephalon, but individual axonal terminations cannot be resolved. Staining of olfactory axons becomes more intense at 72 hpf, which is in accordance with the increase in number of mature olfactory neurons in the olfactory placode (compare to Fig. IV.27.). At 7 d particular glomeruli are identifiable, e.g. dorsal cluster in Fig. IV.3.D. This is in accordance with data obtained using anterograde tracing with DiI, where the first identifiable glomeruli are observable (von Campenhausen, 1995; Lieberoth, 1999). At 14 d the number of glomeruli increases further. At 21 d the glomerular pattern is probably complete (Fig. IV.3.E-H). Many glomeruli are readily identifiable, but the completeness of the pattern cannot be judged, because in cryostat sections the overall view of the pattern is lost.

As it is the case in the adult olfactory epithelium, no cell bodies are labeled. Some brown staining is observable in the epithelium, but this most probably arises from axons that leave individual OSNs to gather together to form the olfactory nerve. This is in discordance with a recent studie performed by Whitlock and Westerfield (1998), where zns-2 staining in the embryo was used to label placodal cells, the so-called pioneer neurons. According to the authors the zns-2 staining diminished at 42-44 h and was no longer detectable at 48 h in the cell bodies, while axonal labeling remained strong. As shown here the axonal zns-2 staining remains throughout development and is also present in the adult. Discrepancies may have arised from differing staining techniques.

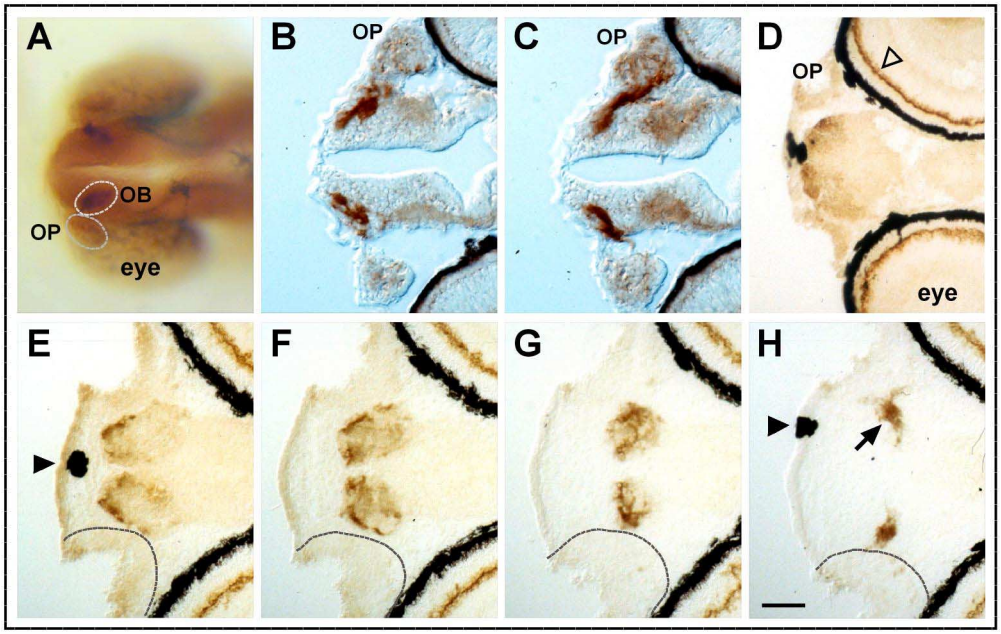

Fig. IV.3.: The Glomerular Pattern During Embryonic and Larval Development as Visualized by the zns-2 Antibody. A. Whole mount view of a 24 h old embryo after immunhistochemical staining with zns-2 and detection using a horseradish peroxidase coupled secondary antibody and DAB as substrate. B, C. Sections through a 48 h old embryo that was stained as whole mount. D. Cryosectioned 7 d old larval fish. Open arrowhead points to the labeled ganglion cell layer in the eye. E-H. Consecutive sections through a 21 d old larval fish. In all cases only the forebrain region including olfactory placodes and a part of the eyes are shown. The olfactory placode is outlined in A and E-H. The projection area of OSN axons (OB: olfactory bulb) is also outlined in A and lies directly adjacent to the olfactory placode (OP). Closed arrowhead in E and H point to a melanophore. Arrow in H points to the olfactory nerve. Anterior is to the left. Scalebar corresponds to 50 µm in all panels.

1.2.2. The Olfactory Marker Protein (OMP) Antibody Labels OSN Axons

The OMP-antibody used here was generated against a protein called olfactory marker protein (Margolis, 1980). This antibody recognizes a protein of yet unknown or uncertain function (Buiakova *et al*., 1996; Ivic *et al*., 2000; Youngentob *et al*., 2001), but it is the best-established marker for mature olfactory sensory neurons in mammals. It stains the cell bodies as well as the axons of the OSNs. This antibody was successfully employed in localizing the same structures in other species like *Xenopus laevis*, where it shows significant cross-reactivity. This led to the speculation that it could also show some reactivity in the zebrafish olfactory system.

All trials to use this antibody on cryosections did not succeed. A quite acceptable staining of axonal structures was obtained with vibratome sectioned olfactory bulbs (see Fig. IV.4.). Although the background is quite high (but homogenous), axonal structures, which terminate in glomerulus-like neuropil structures are clearly visible. However, a similar staining could not be obtained in the olfactory epithelium despite employing the amplification with biotin and streptavidin conjugates. Also, no staining was obtained in embryos.

In conclusion, this antibody can be used for visualizing the glomerular pattern, although it is not as potent as the zns-2 antibody.

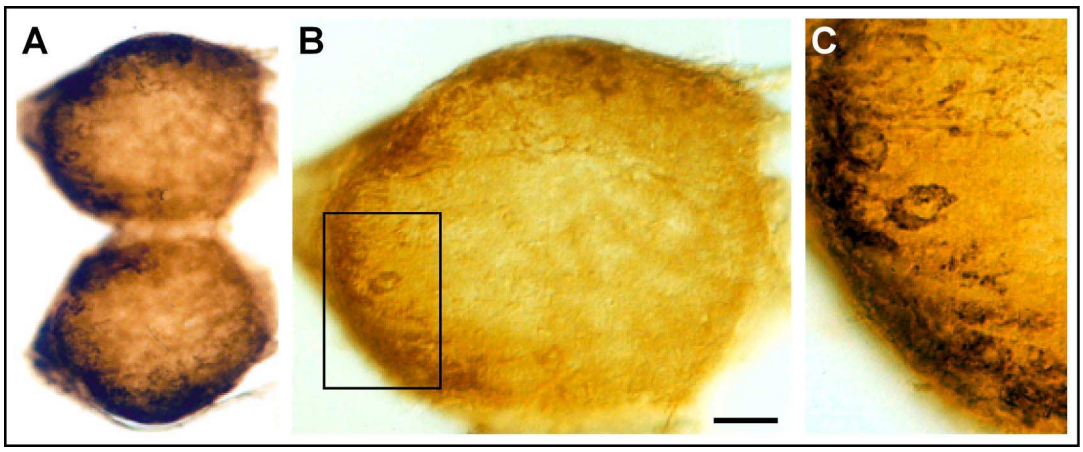

Fig. IV.4.: Visualization of the Glomerular Pattern Using the OMP Antibody. The immunolocalization of OMP was done on vibratome sections of the olfactory bulb with a horseradish peroxidase-conjugated secondary antibody and DAB as substrate. Box in B is shown in a higher magnification in C. Anterior is to the left and lateral is to the top. Scalebar in A: 100 µm, in B: 50 µm and in C: 25 µm.

1.2.3. The Hu (M16A11) Antibody Labels Neuronal Cells in the Olfactory Epithelium and Olfactory Bulb

The Hu antibody recognizes an epitope that is present in many neuronal cells (Marusich *et al*., 1994). Additionally, this antibody labels retinal ganglia and amacrine cells. Sandra Berger (1998) in our laboratory already demonstrated the staining of neurons in the adult olfactory epithelium with this antibody. It remained unclear if and which cells in the OE were labeled in addition to the OSNs.

Here, this study was extended to the adult olfactory bulb and to embryonic and larval stages of zebrafish. To investigate if OSNs are the sole cells labeled in the OE, stainings were performed on vibratome sections, in which the morphology of cells is better preserved as compared to cryostat sections.

The pattern of Hu labeling is shown in Fig. IV.5. In the olfactory epithelium staining is confined to the sensory area, labeling virtually all cells. Non-labeled cells are not or only rarely observable, so that it cannot be clearly distinguished if only particular cell types within the sensory area are labeled. Hence, this antibody can be used as a marker for staining the sensory area, but not to identify special cell types within this area.

Additionally, the Hu antibody was used to test, if a subset of neurons is labeled in the olfactory bulb. It turned out that here all important cell types are labeled (Fig. IV.5.C). The staining shows clearly the distribution of cell bodies throughout the olfactory bulb and displays its organization. A higher density of cells is seen in more central areas (corresponding to the granule cells) and sparse presence of large cells in the glomerular region (corresponding to the mitral cells), leaving large parts unlabeled. Hence, this antibody could be used to show the organization of olfactory bulb layers, but not for the purpose that was followed here, being the labeling of a subpopulation of neurons.

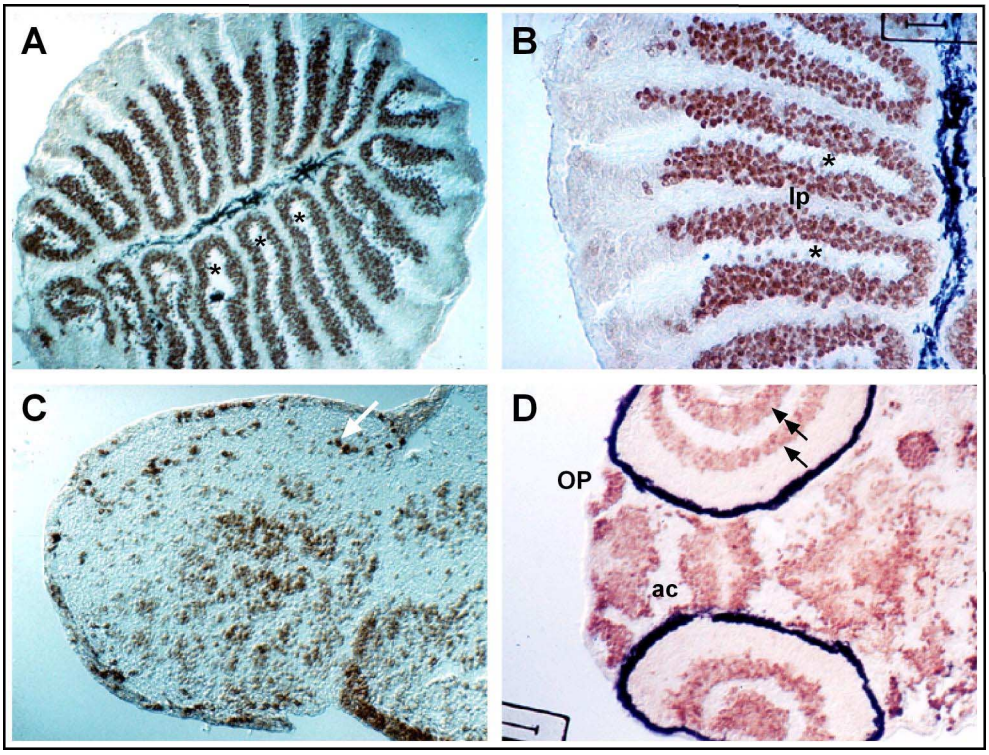

Fig. IV.5.: Immunohistochemical Localization of the Hu Epitope in the Olfactory Epithelium and the Olfactory Bulb of Adult and Embryonic Zebrafish. A and B show a cross section through the olfactory epithelium. Staining is readily seen in cells in the sensory area. Virtually no cells are left unlabeled. The higher magnification in B makes this clear. Asterisks indicate the lumen, lp stands for lamina propria. In the olfactory bulb (C) staining can be seen throughout all layers, with a strong staining of cells in the inner cell mass, reflecting the higher abundance of cells there. White arrow points to a labeled mitral cell. D shows a 3 d old embryo showing strong labeling of the olfactory placode (OP) as well as the olfactory bulb. Two cell layers (arrow and double arrow in B) in the eye are also stained in addition to many other neurons throughout the body. ac: anterior commissure. Anterior is to the left and lateral is to the top, except in B, where anterior is to the top. Scalebar corresponds to 50 µm in all panels.

In the embryos the placodal cells and cells in the forebrain were labeled at all stages examined. In addition retinal ganglion and amacrine cells as well as cells in the notochord were strongly labeled (see Fig. IV.5.D).

1.2.4. The β-III-Tubulin Antibody Identifies Olfactory Sensory Neurons

Tubulin is a major cytoskeleton component that has five distinct forms, designated α, β, γ, δ and ε tubulin. In the olfactory system β-III-Tubulin has been shown to label olfactory sensory neurons specifically. I have used an antibody against β-III-Tubulin (Promega) to test if this antibody labels similar structures in the olfactory epithelium of zebrafish. Different methods were tested and it turned out that only in vibratome sections a satisfactory staining could be obtained. Fig. IV.6. shows several lamellae of an olfactory epithelium stained with β-III-Tubulin.

It can be seen that the staining is mainly in the sensory area of the epithelium, but also that some cells in the non-sensory area are labeled. In the high power view shown in Fig. IV.6.B the labeled cells diplay the characteristical morphology of olfactory sensory neurons.

Most cells seen in this figure are lying apical and have only a short dendrite or no dendrite at all. These cells probably belong to the microvillar type of olfactory sensory neurons. But cells with long dendrites are also observable, indicating that this antibody labels both cell types of olfactory sensory neurons: the ciliated and microvillar ones. There are also large numbers of cells that are unlabeled most probably belonging to the supporting cells present in the olfactory epithelium.

β-III-Tubulin is an antibody that allows the labeling of OSNs, both the ciliated and microvillar type on vibratome sections of the adult olfactory epithelium. No staining was obtained using cryostat sections. Therefore, this antibody is not suitable for double labeling experiments with *in situ* hybridizations, which are generally performed on cryosections. Attempts to stain embryos in whole mounts did not give any result. A possibility to label embryos with this antibody would be to use vibratome-sectioned embryos. However, since these could also not be used in conjuction with *in situ* labelings this possibility was not tried out.

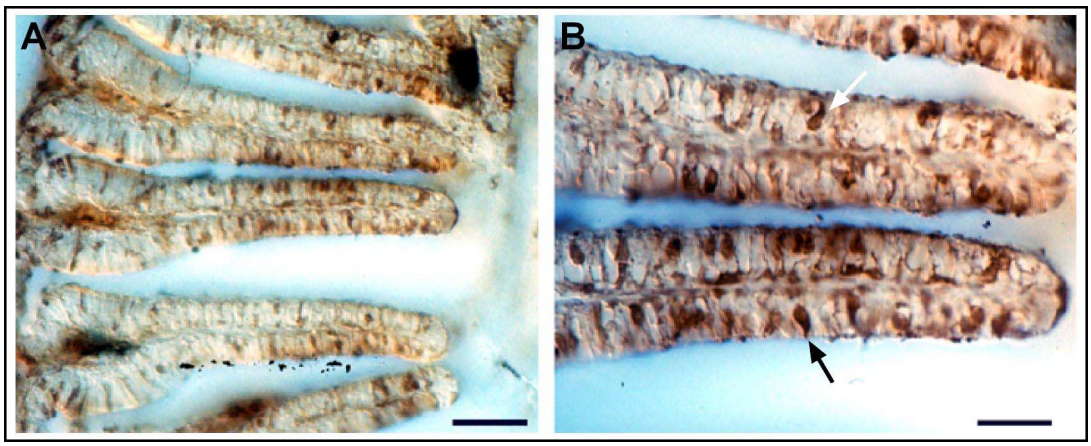

Fig. IV.6.: Localization of β-III-Tubulin-Positive Cells in the Olfactory Epithelium of Zebrafish. A and B: Labeled olfactory neurons are readily distinguishable in the sensory area of the olfactory epithelium. Labeled cells display characteristic morphology of olfactory sensory neurons. Occasionally stained cells are seen in the non-sensory area as well. B shows a higher magnification of two lamellae in A. Cells that are in focus here belong mainly to the microvillar cell type of OSNs (see black arrow in B). However, ciliated OSNs can also be observed (white arrow in B). Anterior is to the top. Scalebar in A: 50 µm, in B: 25 µm.

10.2. Identification of Olfactory Sensory Neuron Precursors

To identify the presence of OSN precursor types and differentiating neurons three different antibodies were used.

1.3.1. The Cytokeratin Antibody Visualizes Horizontal Basal Cells

Cytokeratins comprise a diverse group of intermediate filament proteins that are expressed as pairs in both keratinized and non-keratinized epithelial tissue. Cytokeratins play a critical role in differentiation and tissue specialization and function to maintain the overall

structural integrity of epithelial cells. In the olfactory epithelium cytokeratins have been a useful marker to label horizontal basal cells.

To date the presence and location of the two basal cell types known for other vertebrates, the globose basal and horizontal basal cells, was not demonstrated for the zebrafish. The α-cytokeratin antibody (Dako) was successfully employed in rodents to label horizontal but not globose basal cells. In my study I could show, that this antibody also labels a population of cells that lie directly on top of the basal lamina (see Fig. IV.7.). By analogy, one could assume that these cells also comprise the horizontal basal cell population. However, it cannot be ruled out that additionally globose basal cells or even only globose basal cells are labeled. Nevertheless, this antibody is a useful marker to demonstrate basal cells in the olfactory epithelium.

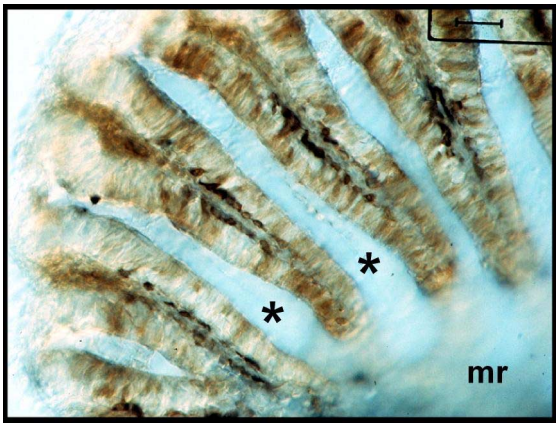

Fig. IV.7.: **Localization of Horizontal Basal Cells in the Olfactory Epithelium.** Dark labeled cells directly associated with the basal lamina correspond to horizontal basal cells. Asterisks label the lumen between the lamellae. mr: middle raphe. Scalebar corresponds to 25 μm.

1.3.2. Globose Basal Cell-1 (GBC-1)

In an effort to characterize the GBC compartment in zebrafish, I have tested the GBC-1 antibody, which was generated against epitopes of the rat olfactory epithelium 7 d after MeBr lesion and labels GBCs (Goldstein and Schwob, 1996). GBCs of rat were previously defined as cytokeratin (-)/NCAM (-)/BrdU (+) (Schwob *et al*., 1992).

Despite all efforts this antibody did not label any cells in the olfactory epithelium of zebrafish in my hands. This indicates that this antibody shows no cross-reactivity with zebrafish epitopes and is most probably because this antibody is a monoclonal antibody.

1.3.3. Growth-Associated Protein-43 (GAP-43)

Growth-associated protein-43, also designated neuromodulin, is an axonal membrane protein that is involved in the neuronal outgrowth and growth cone guidance (Skene, 1989) as well as in synaptic plasticity of developing and regenerating neurons associated with learning and memory (Fagnou and Tuchek, 1995; Benowitz and Routtenberg, 1997). GAP-43 binds to calmodulin in the absence of Ca^{2+} and functions as a substrate for PKC in the nerve growth factor (NGF) signaling pathway. In the olfactory system, GAP-43 has been successfully used

to label regenerating neurons in mouse and rat. Therefore, this antibody is a useful tool in the study of the role and function of GAP-43 during axogenesis and in the adult nervous system.

The GAP-43 antibody (Sigma) was tested using various immunohistochemical-staining protocols. All variations described in IV.1.1. were tried, but despite all these variations and optimization trials no staining was obtained with the antibody tested here.

1.4. Characterization of the Two Main Cell Types in the Olfactory Bulb, Mitral Cells and Granule Cells Using Antibodies Against Neurotransmitters

Several amino acids play a role as excitatory neurotransmitters in normal brain function. They are released from the terminals of one neuron and bind to receptors present on the surface of the adjacent neurons. Such amino acids exist at high concentrations in every part of the brain and play a vital role in almost all brain processes. At least five amino acids have been considered as neurotransmitters in the vertebrate brain: γ-amino-butyric acid (GABA), glutamate (GLU), aspartate (ASP), glycine and taurine. Compelling evidence has been presented for GLU and ASP as the quantitatively most important excitatory transmitters in the vertebrate central nervous system (CNS). GABA functions as the main inhibitory transmitter by increasing a Cl^- conductance that inhibits neuronal firing in the CNS. It has been shown to activate both ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors as well as a third class of receptors called $GABA_C$.

Antibodies against these five neurotransmitters were used as means of characterizing different cell types in the olfactory bulb of zebrafish. Unfortunately, no reaction product could be obtained with antibodies purchased from Signature Immunologics (USA), despite all efforts. In this case even the tedious paraffin embedding and sectioning was performed. These results are in contrast to results obtained with the same antibodies on zebrafish olfactory bulbs (M. Michel, personal communication). However, since the tests performed here were carried out on cryo- and vibratome sections, and those by M. Michel were carried out with plastic sections this could explain the difference in results obtained. The fixation of neurotransmitters is a critical step, because they are very small compared to other epitopes (see also III.1.1). On the other hand extensive fixation can mask the antigen and inhibit the proper binding of the antibody. The optimum between fixation and permeabilization might be obtained easier in plastic sections for some reason. For the purpose followed here it was however necessary to be able to obtain labeling on cryostat sections (for comparison reasons and double labeling purposes).

A second set of antibodies against GABA and glutamate were purchased from Sigma and tested for applicability. Results obtained with these antibodies are summarized below.

1.4.1. GABA

In the olfactory bulb GABA has been shown to be present in the granule cells (Ribak *et al*., 1977), which inhibits mitral and tufted cells (Jahr and Nicoll, 1982; Shepherd, 1972).

While no specific staining could be obtained with this antibody on cryostat sectioned tissue, a robust staining was seen on vibratome sectioned tissue. In Fig. IV.8.A, a typical result of a staining with α-GABA antibody on vibratome sections of the adult olfactory bulb is shown. Labeled cells are localized in the central area of the olfactory bulb, corresponding to the area where granule cells are mainly located.

In the olfactory epithelium a number of cells were also labeled using this antibody. These cells were located in the sensory area of the olfactory epithelium and displayed the characteristic morphology of OSNs. Generally, labeled OSNs have a short apical dendrite and lie apical within the lamellae. But neurons lying in deeper parts of the lamellae having long dendrites are also observed, indicating that both OSN types are labeled (Fig. IV.8.B, C). It seems that a subpopulation of neurons in the olfactory epithelium is GABAergic, however the significance of this staining is not clear.

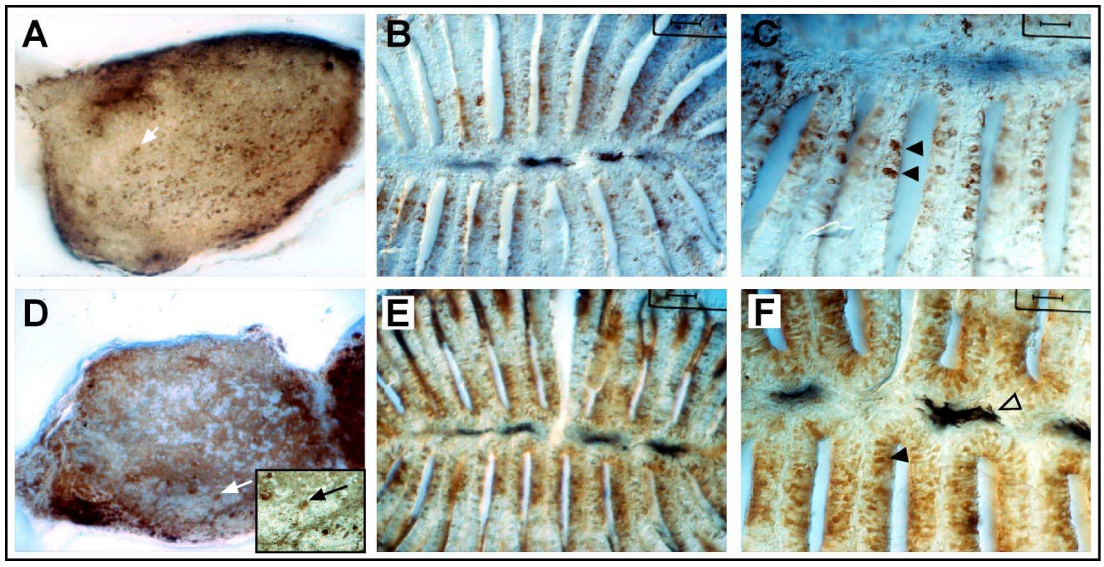

Fig. IV.8.: Localization of Neurotransmitters in the Olfactory System of Zebrafish. A-C: GABA; D-F: Glutamate immunohistochemistry on vibratome sections of the olfactory epithelium and olfactory bulb. A, and D show labeled cells in cross-sections of the olfactory bulb, while B, C, E, and F show cross-sections through the olfactory epithelium. Antibodies were detected with a horseradish peroxidase-conjugated secondary antibody and DAB as substrate. Anterior is to the left. Scalebar corresponds to 50 µm in A, B, D, and E, and to 25 µm in C and F. Arrow in A points to a labeled granule cell and arrows in D point to the identical labeled mitral cell. Arrowheads point to labeled cells in the olfactory epithelium, while open arrowhead indicates melanophores in the midline raphe.

1.4.2. Glutamate

Glutamate is the major excitatory neurotransmitter found on mitral cell synapses in the olfactory system (Liu *et al*., 1989).

Like the antibody against GABA the glutamate antibody worked only in vibratome-sectioned tissues and with fixation that included 1% glutaraldehyde. The background staining

is still slightly high, but readily distinguishable from specific staining. Fig. IV.8.D shows a representative example of a staining against glutamate. Few, but cells with large cell bodies are distinguishable in the periphery, which could correspond to mitral cell bodies. It is obvious that such few, labeled cells cannot represent the whole mitral cell population, although it is not clear why only a very small percentage of cells is labeled.

In the olfactory epithelium, a high number of neurons mainly located in the sensory area are stained (Fig. IV.8.E, F). What the significance of these glutamate-positive neurons could be is not clear at this point.

1.5. Detection of Glial Cell Types and Other Cell Types

1.5.1. Tyrosine Hydroxylase (TH)

The enzyme TH catalyzes the conversion of tyrosine to L-DOPA, which is the rate-limiting step in the biosynthesis of catecholamines such as dopamine, adrenalin and noradrenalin. Nerve cells with tyrosine hydroxylase content are referred to as being catecholaminergic. In mammals TH immunoreactivity is seen mainly in the glomerular layer in periglomerular and tufted cells (Halász, 1981). In the teleost olfactory system tyrosine hydroxylase positive neurons lie in deeper layers, mainly in the glomerular, and mitral cell layers (goldfish: Yoshida *et al*., 1983; zebrafish: Byrd and Brunjes, 1994).

An antibody against TH was used to display the mitral cell layer. Unfortunately, the antibody used in this study turned out to label not the expected structures, when tested on vibratome-sectioned olfactory epithelia and bulbs. The staining was confined to structures associated with the olfactory nerve and OSN axons. No labeled cell bodies within the olfactory bulb were observed. In the epithelium DAB precipitate was found to be associated with cilia in the non-sensory area (data not shown).

The reason for this untypical staining is not clear. No similar staining was observed in controls where this antibody was omitted.

1.5.2. S-100

It is suggested that the presence of glial-ensheathment along the olfactory pathway provides a suitable surrounding for the growing olfactory and vomeronasal axons, and thus plays an important role during the development of these systems. I studied the glia development within the sensory epithelia during adulthood by observing the staining with S-100, a Ca^{2+}-binding protein, which is mostly expressed in Schwann cells and astroglia as well as slightly in neurons and various extraneuronal tissues. The staining was performed on cryosections or vibratome sectioned olfactory epithelia and bulbs. Staining was only observed in vibratome-sectioned olfactory bulbs being localized to the olfactory nerve and the olfactory nerve layer of the olfactory bulb (Fig IV.9.A and B). This close association with the

OSN axons is consistent with data from the literature. A clear morphology of labeled structures could not be distinguished, even at high magnifications. Additionally, the olfactory nerve leaving the olfactory epithelium as well as the very apical part of the olfactory epithelium displayed some staining (Fig. IV.9.C and D). The latter staining might correspond to the cilia of OSNs. The meaning of this staining is not clear, however since no staining in similar location could be revealed when omitting the primary antibody, it cannot correspond to an unspecific labeling.

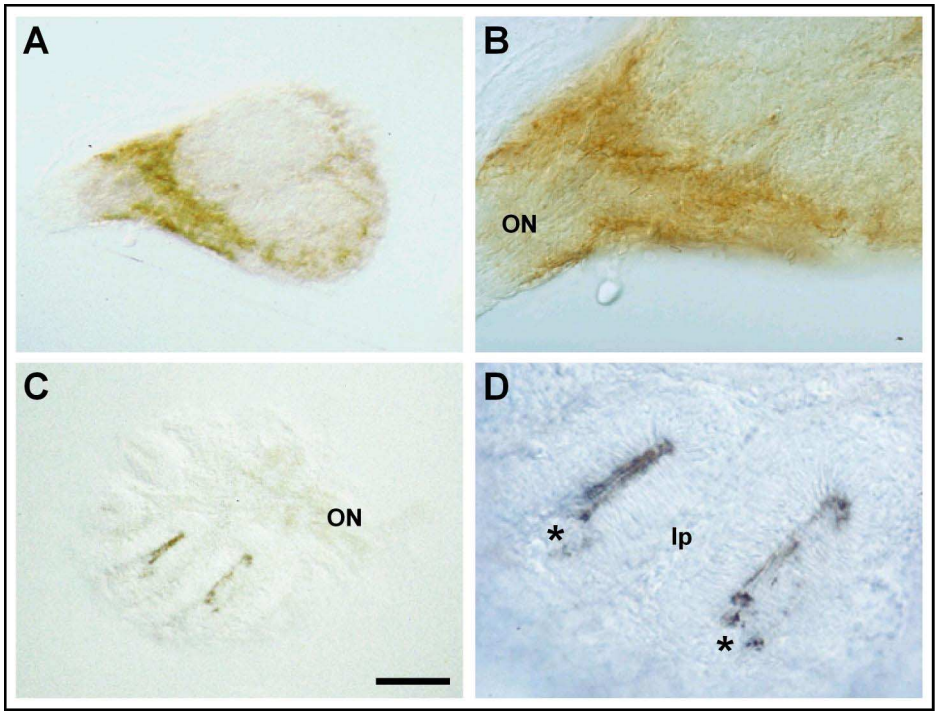

Fig. IV.9.: Distribution of S-100 Immunoreactivity in the Olfactory Epithelium and Olfactory Bulb. Immunoreactivity is localized to the olfactory nerve layer in the olfactory bulb (A, B). No specific labeled structure can be distinguished, even at higher magnification (B). In the olfactory epithelium (C, D) some immunoreactivity was observed in the most apical part of the lamellae, probably corresponding to the cilia of olfactory sensory neurons. ON. olfactory nerve; lp: lamina propria; Asterisks: lumen. Anterior is to the left. Scalebar corresponds to 100 µm in A and C and to 25 µm in B and D.

1.5.3. Glial Fibrillary Acidic Protein (GFAP)

GFAP is a 50 kD intermediate filament (IF) protein belonging to the type III subclass of IF proteins. GFAP is specifically found in astroglia and is widely used to label them. I have used an antibody against GFAP (Biogenex) to look for the presence and distribution of astroglia in the olfactory bulb. As shown in Fig. IV.10. no cell bodies but only the processes of glia could be detected. Glial processes were observed throughout the olfactory bulb with a higher density of labeled fibers in the olfactory nerve and the glomerular layer. Fig. IV.10.A, B show a counterstain with DAPI to display the distribution of cell bodies. In Fig. IV.10.E and F an overlay of both stainings is shown. It is apparent that the density of glial processes is higher in regions with low cell density.

In summary, this antibody can be used to display the presence and distribution of glial cell processes in the olfactory bulb.

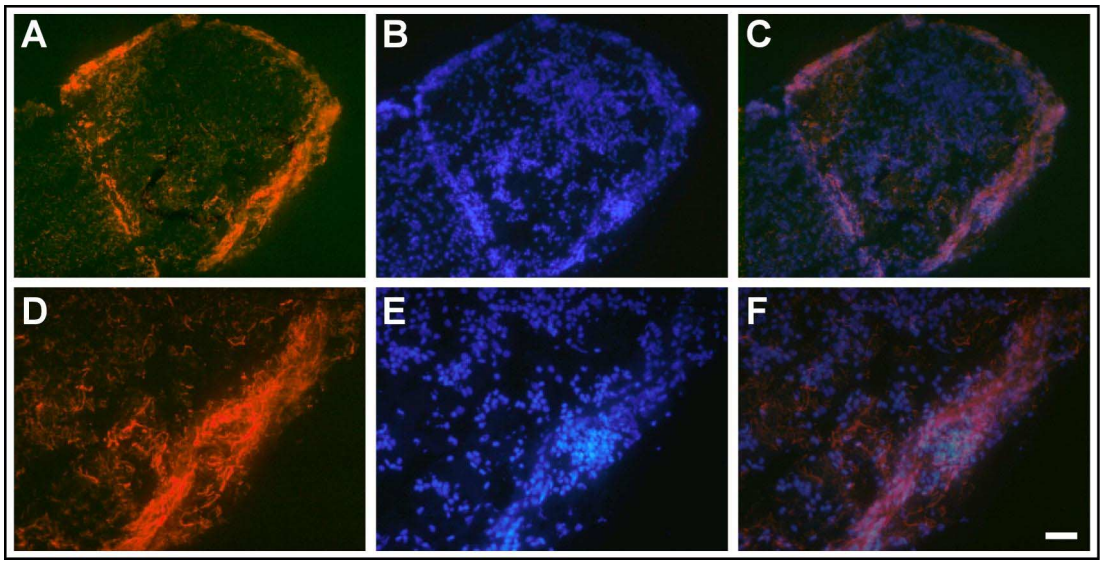

Fig. IV.10.: Distribution of Astroglia in the Olfactory Bulb. A and D show a staining with the GFAP antibody that was detected using a goat-a-mouse-CY3-coupled secondary antibody; staining is apparent throughout all layers, but is strongest in the olfactory nerve layer. No labeled cell bodies can be observed. B and E show the corresponding DAPI staining, where cell bodies are readily observable; empty spaces in this staining correspond to glomeruli. C and F represent an overlay of both stainings. Close association of both stainings is apparent in the olfactory nerve layer, but no double labeling can be seen. Anterior is to the right top corner. Scalebar corresponds to 50 µm in A, B, and Cand to 25 µm in D, E, and F.

In summary, the results of this study have revealed several markers for different cell types in the olfactory epithelium and olfactory bulb. Basal cells could be shown immunocytochemically for the first time in the zebrafish olfactory epithelium, taking not into account the labeling of proliferating basal cells and OSNs by detection of incorporated BrdU (Berger, 1998). A good alternative has been found for displaying the glomerular pattern in the olfactory bulb, the zns-2 antibody, which is applicable in the olfactory bulb and epithelium, processed in any desired way (cryo- or vibratome sectioned or whole mount). rOMP would be a very useful marker, especially if it would label the mature olfactory sensory neurons in zebrafish as well. It is still unclear if and which particular cell types in the olfactory epithelium the Hu antibody labels. However, it is one of the best markers known to date to label cell bodies of neurons located in the sensory area clearly, even in cryostat sections. β-III-Tubulin and cytokeratin appeared to work only in vibratome sections, which make them not so useful for further applications, unless double labeling is performed in vibratome sectioned tissue. The neurotransmitters GABA and glutamate, which were analyzed with very much effort, because of their strong feasibility as markers, did not fulfill the expectations. Since they were only applicable in vibratome sections further use in double labeling experiments with *in situ* hybridization were hindered. The staining obtained with TH is only explicable by unspecific or specific background problems. Specific background problems may arise from different sources, like the presence of contaminating antibodies with

spurious reactions, cross-reacting antibodies, and immunoglobulin binding proteins. Actually, these can probably be excluded since the antibody used here is a monoclonal antibody. Unspecific sticking can be encountered with particular detection methods and include autofluorescence (in case of fluorescent antibodies) and endogenous enzyme activities. Antigen binding does not cause non-specific background problems. They are due to either the primary or secondary reagents binding to the specimen that do not involve the antigen-binding site. S-100 and GFAP gave reasonable staining results. While S-100 worked only on vibratome-sectioned tissue, GFAP gave nice signals in cryosectioned tissue.

In general vibratome sectioning of the tissue gave better results than cryostat-sectioning. In some cases staining was evident only in vibratome-sectioned tissues, reflecting the better preservation of immunogenicity in tissues that are fixed as whole mounts and processed after embedding in gelatin and sectioning. Nevertheless, it cannot be ruled out that there is a possibility to obtain staining with the antibodies tested here presuming that the optimal conditions for processing can be revealed.

Table IV.1.: Summary of Immunohistochemical Stainings

Name of Antibody	Method I Cryosections	Method II Vibratome Sections	Whole-mount Embryos	Labeled Structures
zns-2	+	+	+	OSN axons
rOMP	-	+	-	OSN axons
M16A11 (Hu)	+	-	+	neuronal cells in sensory area of OE; all cells in OB
β-III-Tubulin	-	+	-	microvillar and ciliated OSNs in OE
cytokeratin	-	+	-	horizontal basal cells
GBC-1	-	-	n.d.	-
GAP-43	-	-	-	-
GABA	-	+	-	granule cells in OB
Glutamate	-	+	-	mitral cells in OB
Tyrosine Hydroxylase	-	+ (?)	-	(?)
S100	-	+	-	ensheathing cells in ON, ONL
GFAP	+	+/-	-	glial cell processes

- : no staining observed; + : staining observed; n.d. : not determined

2. Characterization of the Eph Receptor and Ephrin Ligand Families of Zebrafish and their Functional Implications in the Zebrafish Olfactory System

Eph receptors and ephrin ligands are an interesting family of genes playing roles in many different kinds of processes ranging from pattern formation and morphogenesis to cell migration and development of the vasculature. Moreover, they also appear to have a critical function in axon guidance and topographic map formation. For some members of this family, like EphA5 (formerly named Bsk), it was shown that it is expressed in a subset of cells in the olfactory epithelium as well as in the olfactory bulb (Zhang *et al*., 1996). To explore if similar distributions are present in the zebrafish olfactory system and if these distributions could account for a role in pathfinding, expression patterns of zebrafish Eph receptors and ephrins were analyzed by *in situ* hybridization. Additionally, the localization of receptor and ligand proteins was examined using affinity probes, fusion proteins of human placental alkaline phosphatase and the extracellular domains of receptors or ligands. These studies indicate that receptors and ligands are expressed in distinct but synaptically connected regions of the olfactory system, suggesting potential roles in neuron-target interactions.

In the beginning of this study only three Eph receptor genes (*rtk1*, *rtk2* and *rtk3*) were cloned partially for zebrafish (Xu *et al*., 1994). These were kindly provided by Dr. S. Schulte-Merker from the MPI in Tübingen. The other clones were obtained after their cloning and were all provided by Dr. C. Brennan and Dr. N. Holder from the University College, London.

In order to understand the distributions and their functional significance better and to allow comparisons to relationships in other species the Eph receptor and ligand genes were aligned and homology groups were assigned.

2.1. The Family of Eph Receptors and Ephrin Ligands in the Zebrafish

In the zebrafish 15 genes belonging to the Eph family of receptor tyrosine kinases and their ligands are known, four of them representing likely paralogues existing due to genome duplication events. Most recently, three new members have been added to this family, namely ephrin-A3, ephrin-B1 and ephrin-B3 (Hirate *et al*., 2001; Chan *et al*., 2001). With the addition of these genes the family of Eph receptors and ephrin genes has increased to 18 members. The ephrin-B gene repertoire for zebrafish appears to be complete as compared to the number of different genes identified in mammals so far. In case of the ephrin-A genes still one member seems to be missing (ephrin-A4) when compared with the number of genes known for mammals. However, the Eph receptor gene repertoire does not seem to be complete by far, with two and four members lacking for the EphA and EphB families, respectively. Since the ephrin genes seem to be at least as diverse as their mammalian

counterparts there are probably still some unidentified members of the corresponding receptors lurking in the genome.

The nomenclature for the mammalian homologues and their interactions were already shown in the introductory part in Fig. II.2. The homology of the zebrafish genes to their mammalian counterparts was determined using the GCG program to compare the extracellular domains of the receptors and ligands. The new genes have been included in the analysis that is shown in Fig. IV.11. The receptor rtk1 was excluded from the analysis because only partial sequence information was available, corresponding only to the kinase domain of the receptor. For comparative reasons all genes known from human were included; in cases where no homologue for human was available mouse or chicken genes were taken (e.g. mEphA6, cEphA9, cEphB5) to have a complete representation of all family members and a possibly more accurate assignment of homologies. The generated phylogenetic tree shows clearly the separation of the receptors into two groups, corresponding to the known A and B families. In contrast to previously determined orthologous relationships of several zebrafish Eph receptor and ephrin genes (Holder and Klein, 1999), some genes were assigned to different groups in this analysis. For example rtk2 seems to be a likely homologue of EphA4, rather than EphA3 and rtk7 (being identical to zek1) shows a higher homology to EphA4 than to EphA5 to which it was previously assigned. In the B family rtk3 (being very related to zek3) is most homologous to EphB3, and not EphB2. These differences may arise from various reasons. Firstly, full-length sequences including untranslated regions are required for proper assignments; however, these are not always available, even for published sequences. Secondly, it is still probable that there might be still members of the Eph-subfamily yet to be discovered and thirdly, there may not be a one-to-one relationship between Eph-related RTKs in different species. The use of different parameters in generation of phylogenetic trees could also result in slightly different assignment of orthology relationships. It is not stated how the homologies described by Holder and Klein have been determined; therefore, the actual reason for these discrepancies is not clear.

Comparing the extracellular domains of the ligands shows again a clear separation into two groups. In the A family homologues of ephrin-A1, ephrin-A2 and two likely homologues of ephrin-A5 have been identified, while recently all members of the ephrin-B family have been identified in zebrafish. Also in the B family of ephrins two likely homologues of ephrin-B2 were isolated. The presence of two genes in zebrafish that correspond to one gene in higher vertebrates is due to gene duplication events that have happened in the zebrafish genome (Poslethwait *et al*., 1998; Force *et al*., 1999).

To avoid confusion receptors and ligands will be named using the old nomenclature and the homology corresponding to the new nomenclature determined in this analysis will be indicated, e.g. *rtk3/EphB3*.

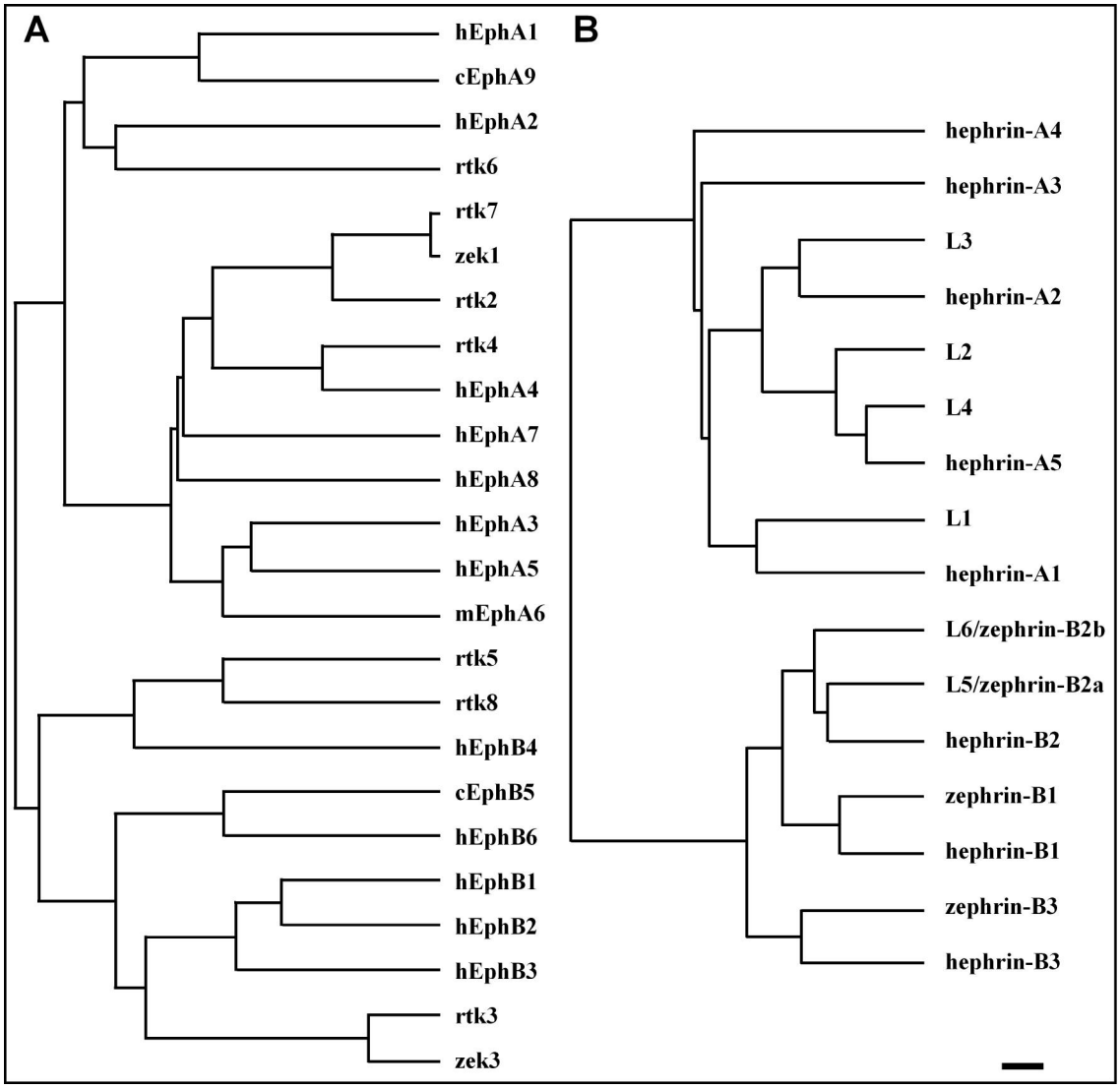

Fig. IV.11.: Phylogenetic Tree of Eph Receptors and Ephrin Ligands. The phylogenetic tree was generated using the Genetics Computer Group Pileup, Distances and Growtree modules. The dendrograms illustrate the relatedness of the zebrafish Eph receptors (A) and ephrin ligands (B) with other members of the Eph family members from human (mouse and chick). All sequences were obtained from GenBank databases. The scalebar corresponds to 10 substitutions per 100 residues.

2.2. Distribution of Eph Receptors and Ephrin Ligands in the Olfactory Pathway of Zebrafish

There is a high promiscuity in the interaction of Eph receptors and their ligands, such that within a subfamily each receptor can interact with each ligand and *vice versa* although with varying affinity. As a result, the functional characterization of the Eph family in a specific system requires an understanding of the expression pattern of all the members of the particular subclass. In order to establish whether members of the EphA and/or the EphB family might be involved in the formation of the projection of OSNs to the olfactory bulb, the expression patterns of all available members were studied at the protein and mRNA levels. This study was performed during the first three days of development, where most changes in the olfactory projection occurs and also in the adult where the established organization has to be maintained, during the continuous turnover of OSNs.

2.2.1. Expression in Embryonic Stages (24 h - 72 h)

Analysis was started at 24 h, a time when OSN axons are sent out from the olfactory placode. The next stage studied was 48 h, a stage when OSN axons have reached the presumptive olfactory bulb and glomerulus-like structures are observed as shown in stainings with the zns-2 antibody (see Fig. IV.3.). Finally, the 72 h stage was analyzed, when first identifiable glomeruli have formed as shown by DiI stainings of embryos (Dynes and Ngai, 1998; B. Lieberoth, 1999) and zns-2 staining (see Fig. IV.3.). The bulb has not evaginated yet, but its location is clearly identifiable.

First, whole mount embryos were analyzed with affinity probes. This *in situ* staining of embryos with receptor and ligand fusion protein probes was described in 1994 by Cheng and Flanagan, and is a widely used technique to study the ephrins and other molecular families (see III.11.4.). The fusion protein constructs used here were prepared and kindly provided by Dr. C. Brennan. Because of the promiscuity of binding shown by members of the Eph family, AP fusion proteins find, in principle, all unbound receptor or ligand molecules of the appropriate class (Gale *et al*., 1996). Hence, EphA-rtk6-AP and ephrin-A-L4-AP were used to detect the expression patterns of the entire ephrin-A ligand and EphA receptor class, respectively. Similarly, EphB-rtk8-AP was used to determine the expression pattern of the entire ephrin-B ligands and ephrin-B-L5-AP to determine the distribution of the EphB receptors. Two controls were included in these experiments; one being a construct consisting of the alkaline phosphatase-tag only (APV) and one without a construct at all, using simply the cell culture supernatant (DMEM) of untransfected cells.

In situ hybridizations were performed on whole mount embryos as well. To analyze the stainings at high magnifications horizontal cryosections of the embryos were performed after hybridization and photographed under Nomarski optics. In subsequent figures horizontal cryosections will be shown that were prepared as shown in Fig. IV.12.

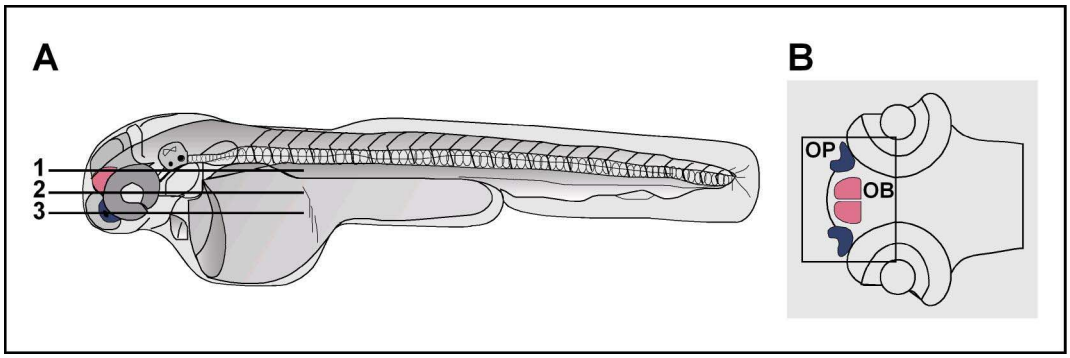

Fig. IV.12.: Schematic Drawing of an Embryo and Analysis. Zebrafish embryos were hybridized as whole mounts. For analysis, embryos were embedded horizontally in TissueTek before cryosectioning. Generally 3 consecutive cryosections depicted as 1, 2, and 3 in A are shown in subsequent figures. Panel B shows a schematic drawing of an embryo sectioned at level 2. The black box depicts the part containing the forebrain region with olfactory bulbs and olfactory placodes that is shown in subsequent figures. Olfactory placodes (OP) and olfactory bulbs (OB) are shown in blue and pink respectively.

2.2.1.1. Possible Interactions in the Class A Family of Receptors and Ligands

The class A receptor AP protein (EphA-rtk6-AP) shows no binding above background levels at any stage analyzed here. This might have several reasons. Protein levels could be relatively low leaving little free ligand once it has interacted with its endogenous receptor(s). However, no specific staining was obtained in any region of the embryo despite using comparable amounts of fusion protein supernatant with comparable amounts of AP activity in all cases, which speaks against this notion. It is not clear what the actual reason for the lack of staining is; it could also be that the fusion protein is less stable than the other ones for unknown reasons. A signal would be expected since ligands for the EphA family were shown to be present. In *in situ* hybridization experiments the antisense probe against *L4/ephrin-A5* showed a low expression in the olfactory bulb, in cells that might correspond to the granule cell population (data not shown). This localization was deduced from comparable stainings obtained with the granule cell-specific *dlx2* probe (for comparison, see Fig. IV.33.B). A more exact assignment awaits double labeling experiments using these two probes simultaneously.

The class A ligand AP protein (ephrin-A-L4-AP) gave a strong binding pattern within the anterior commissure and a weak staining in axon bundles within the olfactory bulb in 24 h to 72 h stage embryos (Fig. IV.13.A-C). These axon bundles most likely correspond to mitral cell projections. Looking at the expression pattern of EphA receptors that could account for this staining by *in situ* hybridization, *rtk2/EphA4b* and *rtk7/EphA4c* show a similar onset and spatial characteristics as ephrin-A-L4-AP expression (Fig. IV.13.D-F, and G-I, respectively).

This indicates that the ephrin-A-L4-AP staining represents one or all of these genes. In all analyzed embryos and at all three stages no staining in the olfactory placode was evident, while at the first two days of development a strong staining in the underlying mesenchyme and in few cells in the olfactory placode was observed for *rtk6/EphA2* (data not shown). It is not clear which cell type in the olfactory placode these very apically lying cells represent. They are too few to represent the whole OSN population. The staining is no longer evident at three days of development, pointing to an involvement in a process occuring during the first two days.

2.2.1.2. Possible Interactions in the Class B Family of Receptors and Ligands

The class B receptor AP protein (EphB-rtk8-AP) shows a strong expression pattern in the anterior forebrain (Fig. IV.13.J-L). Although at first sight this labeling seemed to correspond to the OSN axons, upon closer examination and comparison to *ztbr1* stainings (compare to Fig. IV.37.I-K) it turned out to be a staining of possibly mitral cell dendrites as well as their axons. *In situ* hybridizations with corresponding ligands *L5* and *L6* (*ephrin-B2a* and *ephrin-B2b*) showed a similar temporal and spatial characteristic of these probes as EphB-rtk8-AP expression (shown for *L5/ephrin-B2a* in Fig. IV.13.M-O). *L5/ephrin-B2a* and *L6/ephrin-B2b* show a homology of 52% on the nucleotide level at the 3'-end and can therefore be distinguished by *in situ* hybridization, since the antisense RNA probes were prepared from this part the genes. The hybridization patterns of *L5/ephrin-B2a* and *L6/ephrin-B2b* indicate that they could be the interacting partners of the fusion protein probe EphB-rtk8-AP.

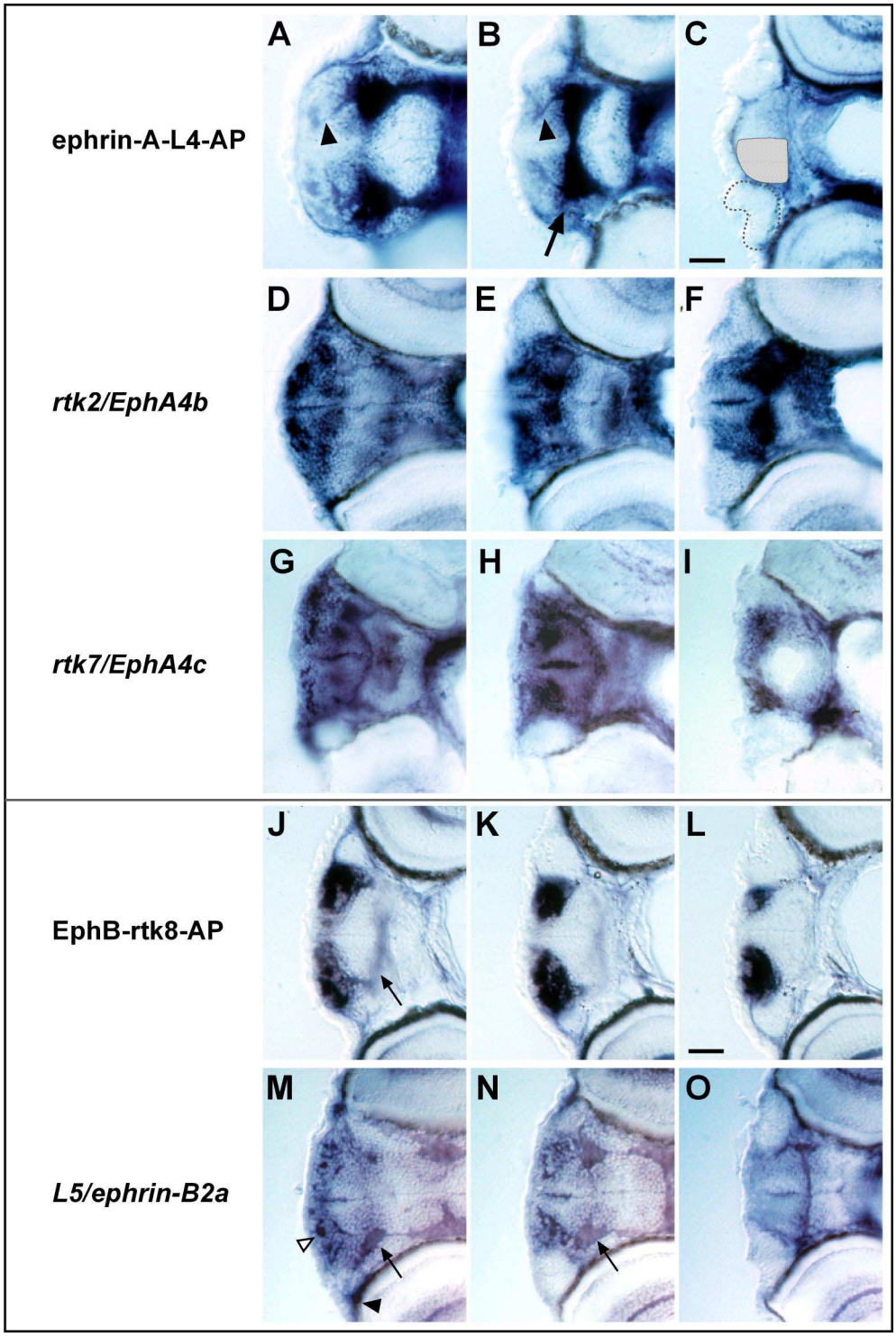

Fig. IV.13.: Possible Interactions in the Class A and Class B Family of Eph Receptors and Ligands. Panels A, B, and C show an affinity probe *in situ* hybridization in three consecutive horizontal sections through a 72 h embryo from dorsal to ventral (corresponding to positions 1, 2, and 3 in Fig. IV.12.A). Only the forebrain region, part of the eyes and the olfactory placodes are shown. Arrowheads in A and B show slightly labeled mitral cell projections. Arrow in B points to the heavily labeled anterior commissure; dashed line in C outlines the olfactory placode. The olfactory bulb is shown adjacent to the olfactory placode as textured area in C. Panel D, E, and F show consecutive sections through a same age embryo after an *in situ* hybridization with a corresponding receptor (*rtk2/EphAB4b*) probe. A strong staining in the mitral cells (D) and around the anterior commissure (E, F) can be seen. A similar pattern of expression is seen with the probe *rtk7/EphA4c* shown in panels G, H, and I. Panels J, K, and L show an affinity probe *in situ* with the probe EphB-rtk8-AP. An intense staining of the anterior forebrain and presumptive mitral cells can be seen. Panels M, N, and O show consecutive sections after *in situ* hybridization with a corresponding ligand RNA probe *L5/ephrin-B2a*. Staining can be seen in the mitral cell bodies (open arrowhead in M). Arrows in M and N point to the anterior commissure, while arrowhead points to the pigment layer of the eye. Scalebar corresponds to 25 µm in all panels.

In order to further underline the findings deduced by comparison to stainings with marker genes for granule and mitral cells, double labeling experiment will be necessary. They will help to clarify the level of co-expression of *L5/ephrin-B2a* and *L6/ephrin-B2b* as well as the co-expression with a mitral and/or granule cell-specific marker.

Analysis of the class B receptor proteins using the ephrin-B-L5-AP did not show any staining in the olfactory system at all, although strong labeling was obtained in other parts of the embryo including the eye and the brain. Respective *in situ* hybridizations with the B class receptors (*rtk3, rtk5 and rtk8*) indicate that these show an expression in the very apical part of the olfactory placodes (data not shown). It seems that this staining represents a particular stickyness of these probes to this part of the placode, since similar stainings were obtained with the sense probes. Even at high magnification of the cryostat sections no cellular structures could be distinguished. The receptor *rtk5/EphB4a* appeared to be expressed slightly in the mesenchyme underlying the olfactory placodes, but not in neighboring mesenchyme. This is quite interesting, since OSN axons leave the olfactory placode through a common exit point that lies in the basal part of the nasal placode and pass through the mesenchyme before entering the presumptive olfactory bulb.

In summary, it appears that the B class ligands are expressed in the mitral cells, as shown by *in situ* hybridization and affinity probe *in situ*'s. However, no expression of the corresponding receptors was seen in relevant structures of the primary olfactory projection. The staining in the apical part of the olfactory placode is not clear, but it probably represents an unspecific staining.

2.7.1.1. *Graded Expression of Receptors and Ligands in the Eye But Not in the Olfactory Pathway*

All stainings in the developing olfactory system did not reveal the presence of graded expression of Eph receptors and/or ligands. Such a distribution would however be of importance for a role in guidance. To see if gradients could be detected in general I have looked at athe retinotectal projection where the graded expression of eph receptors and ligands has been described previously in chicks, mice and zebrafish (Drescher *et al*., 1995; Feldheim *et al*., 1998; Brennan *et al*., 1997, respectively). Affinity probe *in situ* hybridizations in the embryo revealed the clear distribution of receptors and ligands as gradients along the retinal ganglion cell layer. A nice anterior low - posterior high gradient was observed with the ephrin-A-L4-AP probe (Fig. IV.14.A), which is consistent with data obtained previously (Brennan *et al*., 1997). A similar gradient although extended to all layers in the posterior half of the eye can be observed for the probe ephrin-B-L5-AP (Fig. IV.14.C). An opposite, anterior high - posterior low gradient can be observed with the probe EphB-rtk8-AP (see Fig. IV.14.B).

In addition, *in situ* hybridizations with the antisense RNA probe against the receptor *rtk2/EphA4b* displayed an anterior low – posterior high gradient in the eye of 24 h zebrafish embryos (data not shown). Additionally, a slight anterior high - posterior low gradient was observed with probes *L5/ephrin-B2a* and *L6/ephrin-B2b* (Fig. IV.14.D). These gradients nicely correspond to the gradients obtained with the affinity probes ephrin-A-L4-AP and EphB-rtk8-AP, respectively.

These results indicate that the fusion protein probes as well as antisense RNA probes are able to detect gradients in the tissue if they are present. Thus, one has to say that most probably there are no gradients in the olfactory system; otherwise they would have been detected.

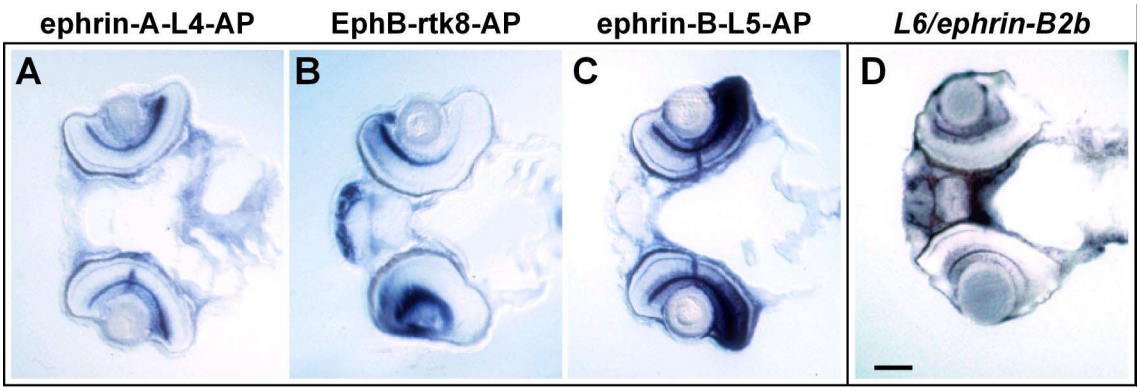

Fig. IV.14.: Graded Expression of Eph Receptors and Ligands in the Eye. Affinity probe *in situ* hybridizations (A-C) and *in situ* hybridizations (D) were performed on 72 h whole mount embryos. Horizontal cryostat sections through the embryo heads were prepared after staining. Anterior is to the left. Scalebar corresponds to 100 µm in all panels.

2.2.1.4. Eph Receptors and Ligands Are Potentially Involved in the Projection of Second Order Neurons During Development.

As the most important outcome of this study one can say that during the first three days of development, Eph receptors and ephrins of the A and the B class are mainly expressed in the mitral cells of the olfactory bulb as shown by *in situ* hybridization experiments. Affinity probe *in situ* hybridizations revealed the localization of receptor and ligand proteins either in the mitral cell dendrites and/or their axons. While the A class receptors appeared to be localized mainly in the axons of mitral cells thereby contributing to the strong labeling of the anterior commissure, receptors and ligands of the B class showed a strong localization to mitral cell bodies. In the strong staining obtained with the EphB-rtk8-AP probe it cannot be distinguished if mitral cell bodies along with their dendrites are stained because of the very dense packing of cells and the very intense labeling of structures in this region. It is of great importance to do double labeling experiments with Eph receptors and/or ligands to demonstrate the extent of co-localization. Additional experiments will help to unequivocally show which particular cell types are labeled. Although comparison of expression patterns with known genes is of great help for analysis, it is not an unambiguous method.

The findings obtained point towards the involvement of the Eph family of receptors and ligands in the projection of second order neurons to higher centers. While a role in the establishment of the primary olfactory pathway cannot be ruled out, no hints towards this direction were obtained in this study. It cannot be fully excluded that so far unidentified members of this family and the non-functioning EphA-rtk6-AP affinity probe would uncover such an involvement. For the time being it seems that these genes are not good candidates for the establishment of the primary olfactory connection of OSNs to the olfactory bulb during the first three days of development.

Strong labeling in several brain regions was observed with many of the analyzed probes. It would be interesting to reveal what role they play in the projection of secondary neurons. However, apparently nothing is known about the projection pattern of mitral cells in the embryonic zebrafish. The staining patterns obtained here were too complex to be resolved by simple observation. Unfortunately, none of the tested antibodies described in part one of this chapter labeled mitral cell projections. Anterograde or backtracing studies could be undertaken to get some insight, although this is not an easy task in such small animals. A good approach would be to use a cell-type specific promoter that could drive a reporter gene (lacZ or GFP) and thereby visualize axonal trajectories of mitral cells to their terminations in the brain. Experiments towards the elucidation of the projection pattern of mitral cells are described in part three of this chapter.

2.2.2. Expression of Eph Receptors and Ligands in the Adult Olfactory System

Similar to the experiments done in embryos affinity probe *in situ* hybridization and *in situ* hybridizations were performed on the adult olfactory epithelium and olfactory bulb, to investigate if a differential distribution of receptors and ligands can be found.

2.2.2.1. Methodological Considerations

Different approaches were undertaken to obtain a reasonable staining in the adult tissues. Initial experiments were performed in whole mount preparations to have a good view of the distribution pattern. However, for some constructs no obvious staining could be obtained. Because of the possibility that this might be due to low penetration of fusion proteins, in subsequent experiments, free-floating vibratome sections and cryostat sections were used. Both the olfactory epithelium and the olfactory bulb have a high amount of endogenous alkaline phosphatase activity. The most critical point in experiments with AP constructs, therefore, appeared to be the complete inactivation of this endogenous alkaline phosphatase activity without harming the activity of the alkaline phosphatase of the fusion proteins.

Generally the protocol described for embryos was used (see III.11.5.5.); however, whole mount preparations were not fixed and incubations were extended to over night. Heat inactivation was done at different temperatures ranging from 60°C to 65°C. It appeared that when heat inactivation was done at 60°C in all olfactory bulbs incubated with the fusion proteins APV (= alkaline phospahatase only), ephrin-A-L4-AP, EphB-rtk8-AP, and ephrin-B-L5-AP as well as the null control (DMEM medium) always a group of glomeruli were labeled in a ventrolateral position of the olfactory bulb (see Fig. IV.15.A, D). This indicates that the ventrolateral lying group of glomeruli has probably a very high endogenous alkaline phosphatase activity that remains active at 60°C, but is eliminated by heating to 62°C and 65°C. In addition, with the fusion protein construct ephrin-B-L5-AP a very distinct glomerulus, the ventral posteriorly positioned glomerulus VpG, was stained (Fig. IV.15.B, E). It is conceivable that this is due to a specific interaction of the ephrin-B-L5-AP fusion protein

with receptors in the VpG glomerulus or binding of another part of the fusion protein construct.

In whole mount experiments performed at 65°C signals were obtained only with the EphB-rtk8-AP probe, which showed clear staining in the olfactory nerve layer (see Fig. IV.15.C, F). Presumably all axons but not the glomeruli showed strong labeling for ephrin-B ligands. To exclude that the lack of staining of glomeruli was due to impaired penetration of the fusion protein probe, experiments with this probe were extended to vibratome sectioned tissue, which should obviate this problem. However, also here the staining remained in the olfactory nerve layer and did not clearly include glomerular structures.

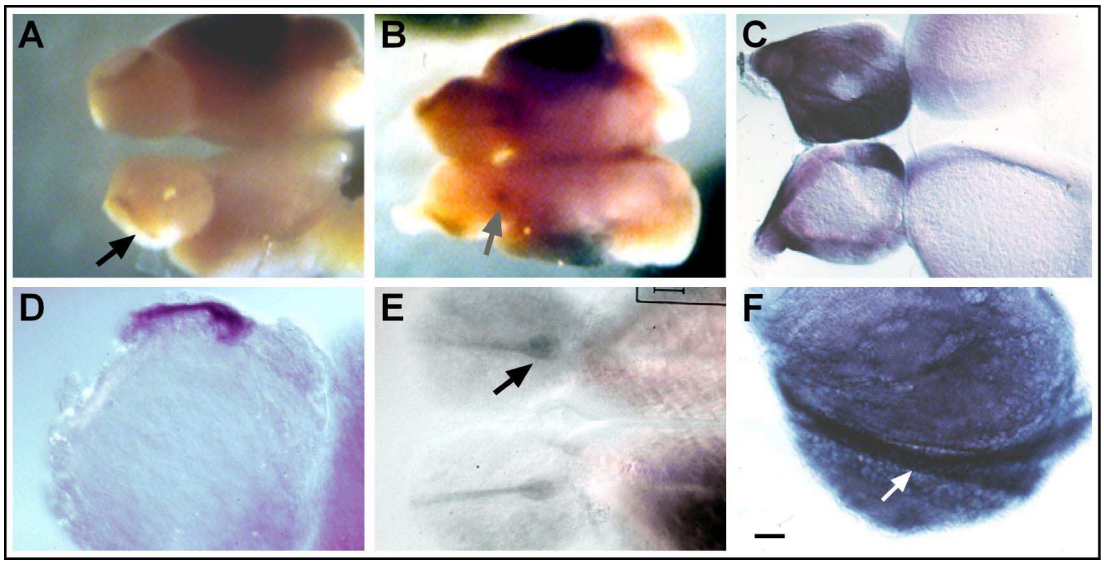

Fig. IV.15.: Affinity Probe *In Situ* Hybridization Patterns on Whole Mounts of Adult Olfactory Bulb-Telencephalon Preparations. A and D show a whole mount (ventral view) and a vibratome section through an olfactory bulb of an affinity probe *in situ* using the ephrin-A-L4-AP construct, respectively. Arrow in A points to a group of glomeruli in the ventrolateral part of the olfactory bulb that shows intense staining. B and E show a whole mount and a corresponding vibratome section of olfactory bulbs stained with the affinity probe ephrin-B-L5-AP. In addition to the lateral group of glomeruli the ventral posterior glomerulus (VpG, arrow) is also stained. Both probes show a strong staining in the dorsal and parts of the ventral telencephalon. Differences in labeling intensity between left and right telencephalon are due to differences in illumination while taking the pictures. C. A strong labeling of the olfactory nerve, olfactory sensory neuron axons and the whole olfactory nerve layer can be observed with the affinity probe EphB-rtk8-AP in vibratome sectioned olfactory bulbs. F. shows a high magnification of a vibratome sectioned olfactory bulb. A strongly labeled axon bundle is designated with an arrow. Anterior is to the left. Scalebar corresponds to 100 µm in all panels, except to 50 µm in panel D and 25 µm in panel F.

Affinity probe *in situ* hybridization experiments were performed on cryostat sectioned tissue to exclude the possibility of impaired penetration further. Fig. IV.16. shows a typical affinity probe *in situ* experiment performed on cryostat sections of the olfactory epithelium and olfactory bulb. The first column represents a section through the apical and the second column a section through the basal part of the olfactory epithelium, to show that background increases in basal direction. The last column shows a section through the olfactory bulb.

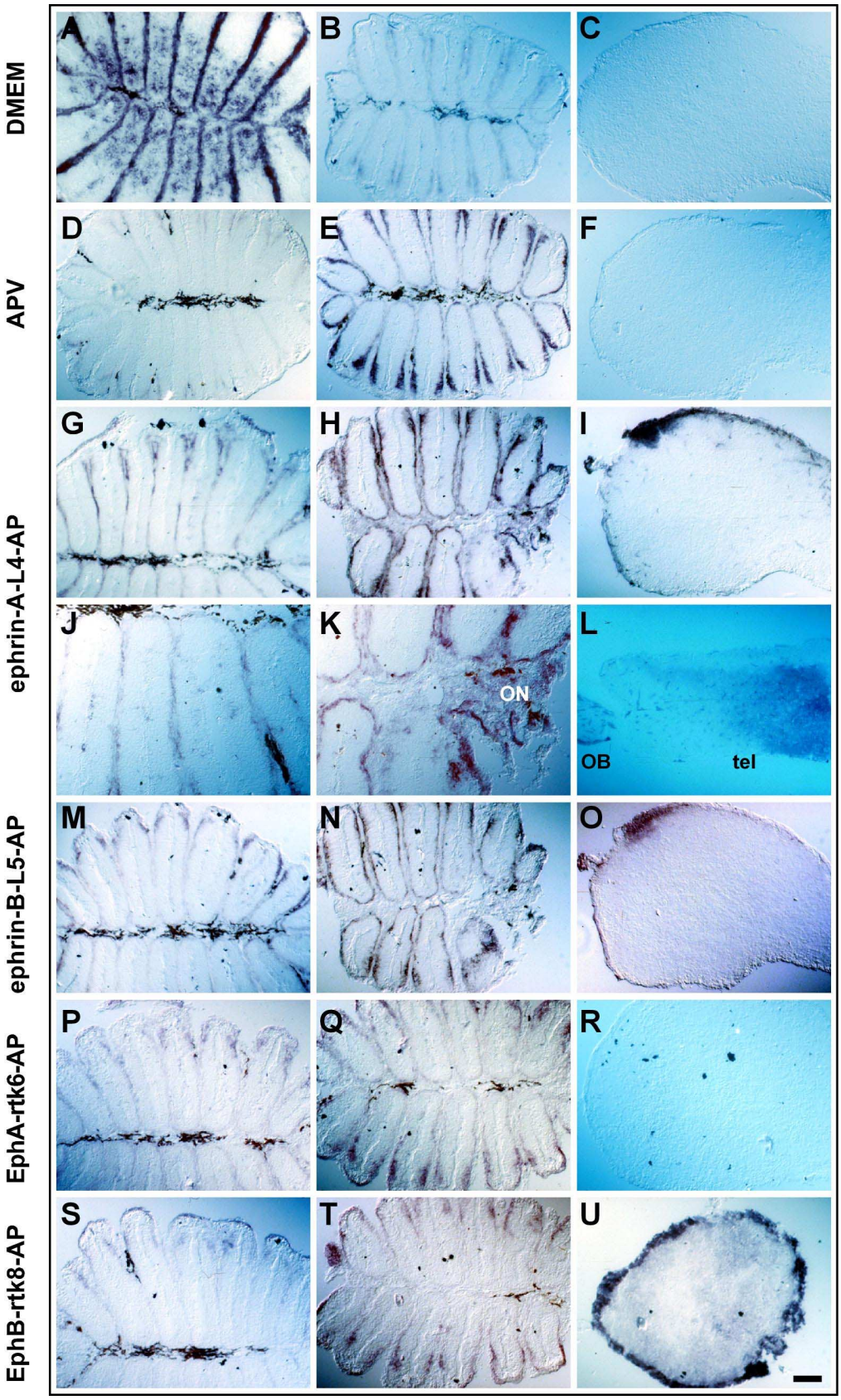

Fig. IV.16.: Affinity Probe *In Situ* Hybridization Patterns on Cryosections of the Adult Olfactory Epithelium and Olfactory Bulb. Affinity probe *in situ* hybridization was performed on cryosectioned tissue. No labeling above background was observed in the olfactory epithelium, except in panel A, which was performed at an unspecific temperature. Stainings of the lateral olfactory bulb in I and O were observed with probes ephrin-A-L4-AP and ephrin-B-L5-AP, respectively. The olfactory nerve layer was stained with the affinity probe EphB-rtk8-AP. ON = olfactory nerve, OB = olfactory bulb, tel = telencephalon. Anterior is to the left. Scalebar corresponds to 100 µm in L, to 25 µm in panels J and K and to 50 µm in all other panels.

Panel A in Fig. IV.16. shows an example of an unspecific staining, where the null control slide was incubated with cell culture medium that was heat inactivated at a lower temperature (62°C). Note that the unspecific staining is localized to the sensory area of the olfactory epithelium. In none of the other cases (AP only = APV and all fusion proteins) performed at the correct heat inactivation temperature (65°C) a signal above background could be obtained in the olfactory epithelium. For the probe EphA-rtk6-AP no signal was obtained in both tissues analyzed. With probe ephrin-A-L4-AP a strong signal in the lateral part of the olfactory bulb was observed (Fig. IV.16.I). In the telencephalon an anteroposterior (low - high) gradient was seen with the same probe (Fig. IV.16.L). A similar staining pattern was obtained with the ephrin-B-L5-AP probe in the olfactory bulb (Fig. IV.16.O; note that sections shown in I and O are alternating). The signal obtained for the A family ligand probe appeared to be broader and seemed to include staining of blood vessels as well, as compared to the B family ligand probe. With the EphB-rtk8-AP fusion protein a strong staining in the olfactory nerve layer of the olfactory bulb was evident (Fig. IV.16.U), labeling all OSN axons. However, no staining of glomeruli could be seen even with cryosections, indicating that the interacting ligand proteins are mainly localized on axons or that the proteins present in the axon terminals, which constitute the glomeruli, are already occupied.

While in the olfactory bulb-telecephalon preparations different stainings with the affinity probes could be obtained none of the used probes appeared to show a signal above background in the olfactory epithelium. The reason for this is not clear, but it might be due to a low level of protein that is not detectable with the fusion protein once the endogenous protein has interacted with its endogenous partners. Stainings in the bulb were consistent no matter what method was used (whole mount, vibratome sections, cryosections). The reason for the staining of the VpG with the ephrin-B-L5-AP probe at a lower inactivation temperature could not be resolved, but was not considered further.

To see which receptors and ligands underlie the stainings obtained with the fusion proteins on the mRNA level, *in situ* hybridizations were performed.

2.2.2.2. Possible Interactions within the Olfactory Epithelium

Of all the tested genes only two genes of the A class, the receptor *rtk7/EphA4c* and the ligand *L3/ephrin-A2* are expressed in the sensory area and presumably in the OSNs of the olfactory epithelium (see Fig. IV.17.A and D, respectively). Interestingly, a second receptor of the A family, *rtk6/EphA2*, was found to be expressed in the non-sensory area of the olfactory epithelium (Fig. IV.17.C). This receptor could in principle be able to interact with the ligand *L3/ephrin-A2* that is expressed in the OSNs. The interface between sensory and non-sensory area is an important turning point for OSN axons on their way to the olfactory bulb. Individual axons bundle together in the basal lamina of each individual lamella and form a large fascicle at this interface. There, this bundle turns downward to join the other fascicles formed within each lamella to combine to the olfactory nerve (compare to zns-2 stainings in Fig. IV.1.). The expression of the *rtk6/EphA2* receptor might involve a repulsive action on OSNs expressing the corresponding ligand *L3/ephrin-A2*, inhibiting them from growing outwards into the non-sensory area and forcing them to grow along this interface to form the olfactory nerve.

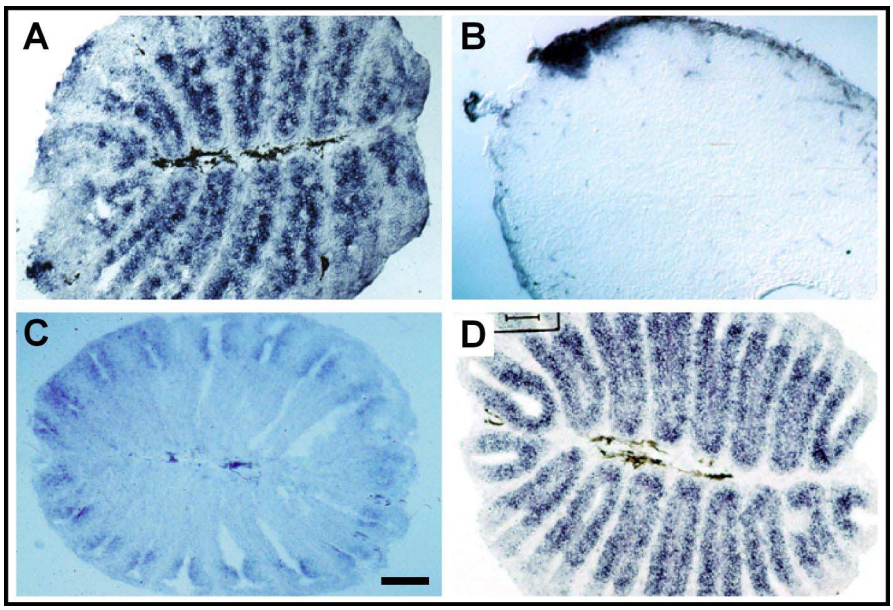

Fig. IV.17.: Possible Interactions within the Adult Olfactory Epithelium. *In situ* hybridization with the probes *rtk7/EphA4c* (A), *rtk6/EphA2* (C) and *L3/ephrin-A2* (D) on cryosections of adult olfactory epithelia. A. Signals are localized in a subset of cells in the sensory area of the olfactory epithelium. B. An affinity probe *in situ* with the ephrin-A-L4-AP fusion protein shows an intense staining in the lateral part of the olfactory bulb. The staining shown in (A) could account for this staining. C. Only cells in the non-sensory area are labeled. D. The sensory area is strongly labeled with this probe. Anterior is to the left and lateral is to the top. Scalebar corresponds to 50 µm in all panels.

The expression of the receptor *rtk7/EphA4c* appears to be not fully penetrant, meaning that only a subset of OSNs expresses this receptor. The distribution of the labeled cells does not allow making any suggestions about the type of labeled cells. However, it would be very interesting to find out if there is another quality that specifies these cells as being a subset. One could speculate that these cells are OSNs that express a specific subset of olfactory receptors. This would require them to be expressed in circular domains. However, the *rtk7/EphA4c*-positive cells do not appear to be expressed in a particular domain. Another intriguing possibility would be that there is a morphological distinction between cells expressing this particular Eph receptor and cells that do not express it. The olfactory epithelium contains two morphologically distinct types of OSNs, the ciliated and the microvillar ones. Neurons of the microvillar type have a different projection area in the olfactory bulb than ciliated ones. Affinity probe *in situ* data obtained with the probes ephrin-A-L4-AP and ephrin-B-L5-AP indicate that *rtk7/EphA4c* could be the underlying receptor for the staining obtained in the lateral part of the olfactory bulb (Fig. IV.16.I and O; see also IV.17.B). The expression of this receptor in only a subset of OSNs is in good accordance with the labeling of a subset of OSN axons in the olfactory bulb with the two affinity probes. The intriguing idea that only one of the two known receptor types is labeled by the receptor probe *rtk7/EphA4c* could not be analyzed further. The cryosections used for *in situ* hybridization are not good enough in preservation of morphology to allow clear statements about the morphology of *rtk7/EphA4c*-expressing cells. However, the stained cells lie in an apical position, which is consistent with the localization of microvillar cells. *In situ* hybridization experiments on whole mounts of olfactory epithelia or the use of paraffin

sections or a similar method that preserves the morphology good enough to distinguish ciliated and microvillar OSNs would help to analyze this observation further. I have tried whole mount *in situ* hybridizations of the olfactory epithelium, but these did not work in my hands. It appears that a lot of optimization would be needed to get reasonable results here. The difficulty lies mainly in the very compact nature of the olfactory epithelium, which makes cells not easily accessible for the RNA probes.

None of the other receptors or ligands of the A as well as the B family appeared to show a specific staining in the olfactory epithelium. With some probes a staining of the whole olfactory epithelium was observed, while some of these showed a slightly higher expression either in the sensory (*rtk5, rtk8, L5 and L6*) or non-sensory area (*rtk4*) (data not shown). All expression patterns are summarized in Table IV.2.

Table IV.2. Summary of Expression Patterns of Eph Receptors and Ephrins Obtained by *In Situ* Hybridization and Affinity Probe *In Situ* Hybridizations

Probe Name	*Family Name*	*Expression in the Olfactory Epithelium*	*Expression in the Olfactory Bulb*
rtk1	A	not detected	not detected
rtk2	A	not detected	mitral cells
rtk3	B	not detected	not detected
rtk4	A	whole epithelium, NS>S	mitral cells
rtk5	B	whole epithelium, S>NS	granule cells + mitral cells
rtk6	A	non-sensory area	not detected
rtk7	A	sensory area	mitral cells
rtk8	B	whole epithelium, S>NS	granule cells + mitral cells (?)
L1	A	not detected	not detected
L2	A	not detected	not detected
L3	A	sensory area	granule cells
L4	A	whole epithelium	granule cells
L5	B	whole epithelium, S>NS	granule cells + mitral cells
L6	B	whole epithelium, S>NS	granule cells + mitral cells
EphA-rtk6-AP	detects A family ligand protein	not detected	not detected
ephrin-A-L4-AP	detects A family receptor protein	not detected	lateral olfactory bulb
EphB-rtk8-AP	detects B family ligand protein	not detected	glomerular layer
ephrin-B-L5-AP	detects B family receptor protein	not detected	lateral olfactory bulb

S: sensory area; NS: non-sensory area

2.2.2.3. Possible Interactions within the Olfactory Bulb

Several receptors of the A family, namely *rtk4/EphA4a*, *rtk2/EphA4b* and *rtk7/EphA4c*, which are very related to each other or are paralogues of the same gene were found to be expressed in the mitral cells of the olfactory bulb (see Fig. IV.18.A, B, C, respectively). While *rtk2/EphA4b* showed a very strong expression the expression of *rtk4/EphA4a* and *rtk7/EphA4c* was rather moderate. Another possibility would be that the probes are not specific enough and that the *rtk2/EphA4b* probe recognizes the message of *rtk4/EphA4a* and *rtk7/EphA4c* as well. This possibility could be excluded, since the probes were prepared from the 3'-UTR, which is a highly divergent part of the mRNA sequence, in general. It is not clear if the difference observed in signal intensity results from substantial differences in the amount of the message present in the cells, because it is not possible to quantify signals obtained with RNA probes. Intensities of staining may vary largely with the length of the probe and the composition of the mRNA leading to different labeling efficiencies. Although I tried to account for these kinds of differences one cannot ensure that always absolutely equal amounts of probes were used. Alternatively, *rtk4/EphA4a* and *rtk7/EphA4c* may be expressed only in a subpopulation of mitral cells.

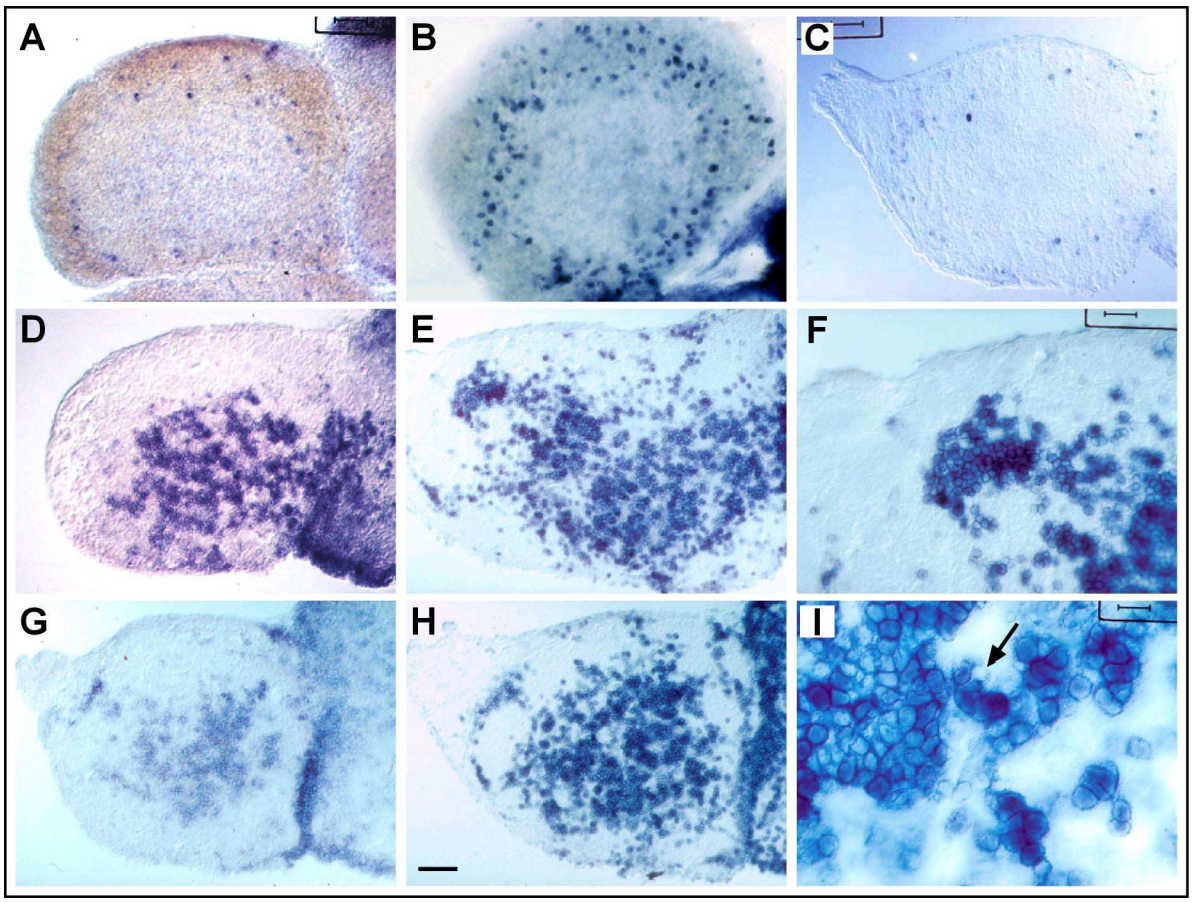

Fig. IV.18.: Posssible Interactions within the Adult Olfactory Bulb. *In situ* hybridizations on cryosections of olfactory bulbs with RNA probes for *rtk4/EphA4a* (A), *rtk2/EphA4b* (B), *rtk7/EphA4c* (C), *L4/ephrin-A5* (D), *L3/ephrin-A2* (E, higher magnification shown in F), *L5/ephrin-B2a* (G), *L6/ephrin-B2b* (H, higher magnification shown in I) are shown. Arrow in I points to an unlabeled cell. Sections hybridized with sense probes were always free of staining. Scalebar corresponds to 10 µm in I, to 25 µm in F and to 50 µm in all other panels.

The ligands corresponding to these receptors, *L3/ephrin-A2* and *L4/ephrin-A5* were expressed mainly in the granule cells (Fig. IV.18.E and D, respectively). The signal seen with the ligand *L3/ephrin-A2* appeared to be much broader and might include mitral cells. However, it is difficult to say if and to what extent mitral cells are labeled. Double labeling experiments with a mitral cell-specific marker would be helpful to solve this question. The labeling of the granule cell population does not seem to be homogenous. The staining appears very patchy and cells displaying varying levels of labeling (unlabeled to strongly labeled) can be clearly distinguished at higher magnifications (Fig. IV.18.F). The significance of this differential staining is not clear. Interestingly, a paralogue of the *L4/ephrin-A5a* gene, *L2/ephrin-A5b*, did not show any expression in the olfactory bulb.

When analyzing members of the B family, stainings were obtained with the receptors *rtk5/EphB4a* and *rtk8/EphB4b*, which seem to be paralogues of the same gene, while no staining was observed with the receptor probe *rtk3/EphB3*. Using cryostat sections the probes *rtk5/EphB4a* and *rtk8/EphB4b* gave very high background stainings. Despite using gene specific primers that were targeted to the 3'-UTR for preparing the probes this problem could not be obviated. Whole mount *in situ* hybridizations performed on the olfactory bulb appeared to be less prone than cryostat sections to generate a high background. It appears that *rtk8/EphB4b* is mainly expressed in the granule cells of the olfactory bulb (Fig. IV.19.F), while *rtk5/EphB4a* was mainly expressed in the mitral cells as well as granule cells.

The ligands *L5/ephrin-B2a* and *L6/ephrin-B2b* showed an overlapping expression to their receptors. While *L5/ephrin-B2a* was mainly expressed in the granule cells, its paralogue *L6/ephrin-B2b* was expressed in granule cells as well as in mitral cells of the olfactory bulb (Fig.IV.18.G and H, respectively). Again the expression levels as well as the pattern of expression of paralogous genes appears to be different from each other. As was seen with A family ligands a differential labeling of cells within the granule cell population can be observed.

2.2.2.4. Possible Interactions between the Olfactory Epithelium and Olfactory Bulb

The expression patterns obtained at the mRNA and protein level suggest some interactions between the olfactory epithelium and the olfactory bulb. One can imagine that a receptor or ligand expressed in the sensory neurons within the olfactory epithelium might interact with projection neurons, or interneurons within the olfactory bulb expressing the corresponding ligand or receptor. This might be possible in the case of *L3/ephrin-A2* that is expressed on OSNs and could interact with *rtk4/EphA4a*, *rtk2/EphA4b* and *rtk7/EphA4c* that are expressed on mitral cells (Fig. IV.19.A, B). A similar interaction might occur between *rtk7/EphA4c*-expressing OSNs and *L3/ephrin-A2* and *L4/ephrin-A5*-expressing granule cells (Fig. IV.19.C, D). The affinity probe EphB-rtk8-AP displayed a strong labeling of OSN axons indicating the presence of a B family ligand in OSNs that might interact with granule cells expressing *rtk8/EphB4b* (Fig. IV.19.E, F). Further experiments are necessary to confirm such an interaction. Functional tests are required to elucidate the involvement of one or the other gene in the establishment or maintenance of the primary olfactory pathway.

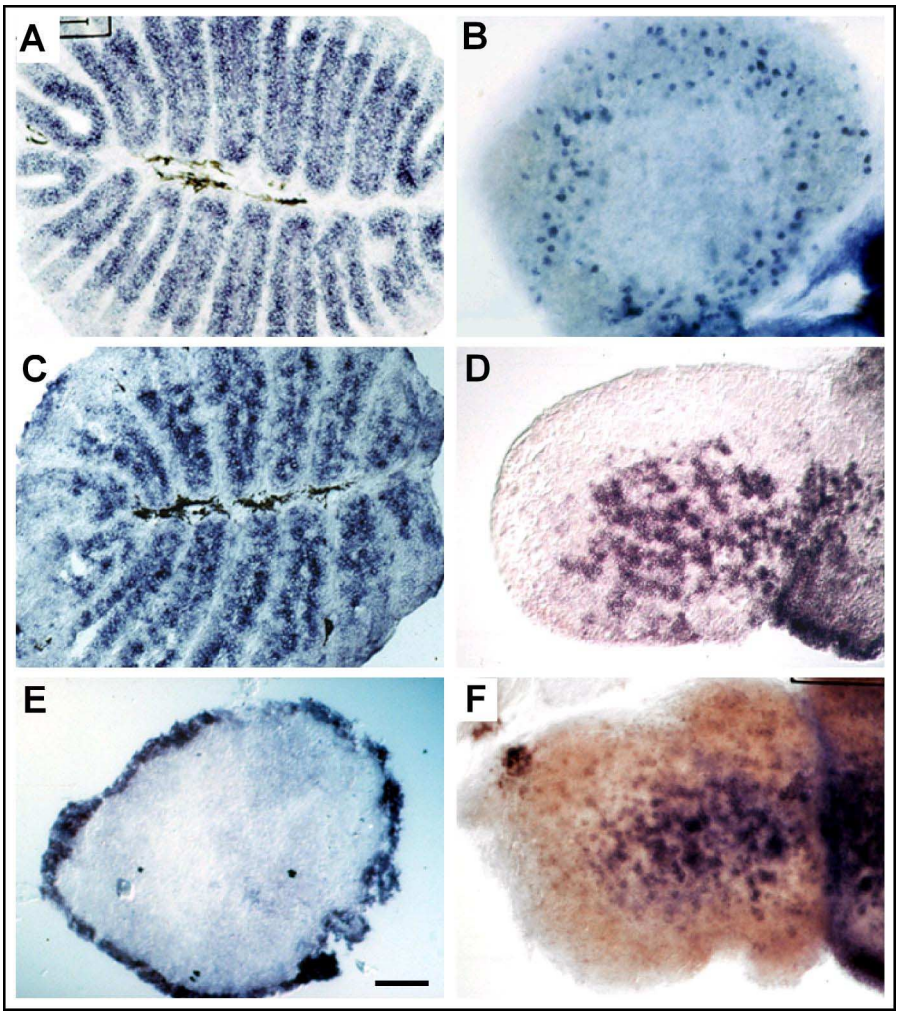

Fig. IV.19.: Possible Interactions Between the Adult Olfactory Epithelium and Olfactory Bulb. A ligand *(L3)* expressed in the olfactory epithelium (A) could interact with a receptor *(rtk2)* expressed in the mitral cells of the olfactory bulb (B). A receptor *(rtk7)* expressed in the olfactory sensory neurons (C) might interact with a ligand *(L4)* expressed in the granule cells (D). A ligand expressed in the olfactory sensory neurons as visualized by affinity probe *in situ* with EphB-rtk8-AP on the olfactory bulb (E) might interact with a receptor *(rtk8)* in the granule cells of the olfactory bulb (F). Scalebar corresponds to 50 µm in all panels.

2.2.2.5. Concluding Remarks

Looking at the results obtained in the adult olfactory pathway several features are notable. Expressions can be complementary within an organ or between two organs, e.g. *L3/ephrin-A2* and *rtk6/EphA2* within the olfactory epithelium and *L3/ephrin-A2* and *rtk2/EphA4b* in the mitral cells of the olfactory bulb. Expressions appear to be selective to particular cell types, e.g. *rtk2/EphA4b* only in mitral cells, while others despite being specific, can be found in two cell types of the primary olfactory pathway, e.g. *rtk7/EphA4c* in OSNs and mitral cells. There is a high number of overlapping expression of receptors as well as ligands in a particular cell type, being either the OSNs, the mitral cells and/or the granule cells.

It is astonishing to note how much expression patterns can vary between the embryonic versus the adult animal. Especially in cases where a clear expression was seen in the adult olfactory epithelium no expression was detected in the embryo. This means that

these genes have probably been turned on at a time point that lies between the ones analyzed here. To find out at what time point expression starts and so get some insight into the process these genes might be involved, I performed *in situ* hybridizations on cryosections of zebrafish larvae at the stages 7 d, 14 d and 21 d postfertilization. Unfortunately, due to a high background on the sections nothing can be said about the onset of expression at this moment. Further experiments will be needed to optimize the protocol for cryosections of larval fish.

In situ hybridizations with digoxigenin-labeled antisense RNA probes of Eph receptors and its cognate ligands revealed that only a subset of the tested genes were expressed in the primary olfactory pathway of zebrafish. Several interesting features were evident. While in some cases complementary expression of receptors and ligands were observed from the olfactory epithelium to the olfactory bulb, complementary expression was observed also within the epithelium, between sensory and non-sensory area and within the layers of the olfactory bulb, namely the glomerular and granule cell layers. Another interesting feature was the overlapping expression of receptors and ligands within the same cells, which was previously described in other systems like the retinotectal system as well. Additionally, in most cases a differential expression of paralogous genes was observed in the olfactory bulb.

2.2.3. Double Labeling Experiments

In situ hybridizations with Eph receptor and ligand probes showed that there is a high degree of overlapping as well as complementary expression. It appeared desirable to get an estimate about the extent of colocalization of two mRNAs in labeled cells and to demonstrate complementary expression in a single specimen. In addition to that it was challenging to compare the location of the transcript of a gene localized in a specific cell type, e.g. mitral cells, to that of a protein that specifically labels OSN terminals (zns-2). For these purposes double labeling experiments were performed. In the first case an antibody staining followed an *in situ* hybridization. In the second case a two-color *in situ* experiment was performed using two differently labeled probes.

2.2.3.1. Simultaneous Localization of a Protein (zns-2) and a mRNA

This method requires an antisense RNA probe to localize the mRNA and an antibody to recognize the protein product. First of all it was important to determine the sequence of the protocol. It appeared better to perform the *in situ* hybridization first, since that reduces the chance of the target mRNA to be degraded by RNases. The detection of the protein requires a 'robust' antibody that will still recognize the antigen after it has gone through the rigorous treatments of the *in situ* hybridization procedure.

Various protocols were tested on whole mount preparations of the adult olfactory bulb. In all experiments single labelings for the antibody and RNA probe were included as controls.

Detection of the RNA probe and the primary antibody against the protein of interest can be done in various ways. In initial experiments zns-2 was detected using a red fluorescent

secondary antibody (CY3), while the mRNA was detected with the classical alkaline phosphatase-coupled antibody, using NBT/BCIP as substrate. A typical double labeling experiment with the zns-2 antibody and *rtk2*/*EphA4b* as RNA probe is shown in Fig. IV.20. Single images of each staining (A, B) are shown. What is apparent is that the blue chromogenic substrate NBT/BCIP (A) can be seen also in the fluorescent image (B). It quenches the fluorescent signal at the positions it is present. It is not possible to get an image of the fluorescent signal only. In any case, a close apposition of mitral cells and glomeruli is apparent. Individual mitral cells are found localized in between glomeruli. Spaces between mitral cell bodies and axonal structures could correspond to mitral cell dendrites, which are not labeled by the *in situ* hybridization procedure. These are the structures that actually interact with the ORN axons to form the glomeruli.

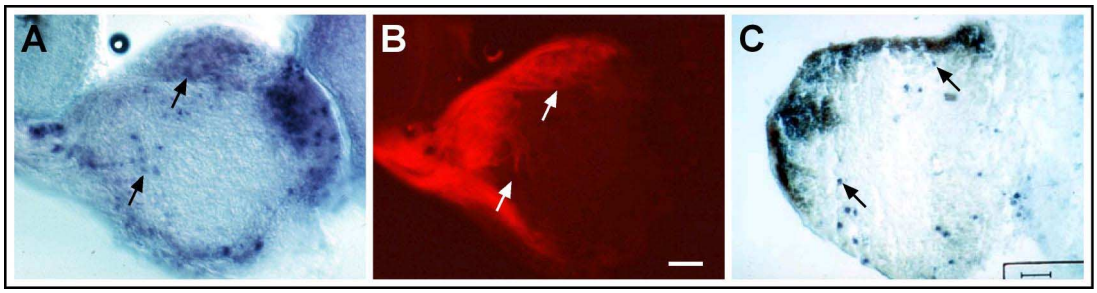

Fig. IV.20.: Simultaneous Detection of a Protein (zns-2) and a mRNA (*rtk2*). A. Whole mount *in situ* hybridization with *rtk2*/*EphA4b* on an olfactory bulb to visualize mitral cells. B. The same bulb shown in A after antibody staining with zns-2 performed after *in situ* hybridization. The DIG-labeled probe was detected using an α-DIG-AP antibody and NBT/BCIP as substrate (blue). The zns-2 antibody was detected using a CY3-coupled secondary antibody (red fluorescence). Arrows in A and B point to cells at identical positions in panels A and B. C. Double labeling of *rtk2/EphA4b* and zns-2 using chromogenic substrates on cryosections of the olfactory bulb. The DIG-labeled probe was detected as in A and is shown in blue. The zns-2 epitope was detected with a horseradish peroxidase-coupled secondary antibody and DAB as substrate (brown). Arrows point to labeled mitral cell bodies. Anterior is to the left and lateral is to the top. Scalebar corresponds to 50 µm in all panels.

In whole mount experiments the very stable NBT/BCIP precipitate appeared to lose its localization, giving a 'blurry' signal after the antibody staining procedure was finished (data not shown). This was most probably due to the extensive washes that were needed to permeabilize the tissue for the antibodies with Triton X-100.

In another set of experiments a horseradish peroxidase-coupled secondary antibody and the chromogenic substrate DAB were used to detect the protein and NBT/BCIP was used to detect the mRNA signal. Although initial experiments were started with whole mounts these experiments were continued on cryostat sections of the olfactory bulb for simplicity. A double labeling experiment done in this way is shown in Fig. IV.20.C. The blue-violet staining corresponds to the *in situ* signals of the mRNA probe (*rtk2*) while the brown staining represents the zns-2 staining of the axons. Again the distance between the cells and labeled axons is apparent. Not very well distinguishable in this figure is the light brown staining of axons in the medial part of the olfactory bulb (lower part of the bulb in this figure). For this reason the distance between cells and glomeruli appears even larger and rarely mitral cells are found intermingled with glomeruli. This is in some part due to the relatively thin sections

prepared with the cryostat. While these are 15 µm, sections prepared with the vibratome after whole mount staining are 100 µm. In addition, the sensitivity of the horseradish peroxidase-antibody to label single or small groups of axons seems to be insufficient.

In conclusion one can say that the method appears to work in whole mounts as well as for cryostat sectioned tissue, although results have to be interpreted with caution, especially when axonal staining is used in conjunction with horseradish peroxidase-coupled antibodies. In principle, alkaline phosphatase-coupled antibodies are more sensitive (see next section).

2.2.3.2. Simultaneous Detection of Two mRNAs

The availability of differentially labeled probes, antibodies to the different haptens used for labeling, and alternative chromogenic substrates allow two-color *in situ* hybridization. The antisense RNA probes can be labeled with digoxigenin and fluorescein. These are then identified with antibodies that were raised against digoxigenin and fluorescein, respectively, which are conjugated with alkaline phosphatase or horseradish peroxidase. The most common peroxidase substrate used is diaminobenzidine (DAB), which produces a highly insoluble brown precipitate. It can be readily distinguished from the blue/violet precipitate of NBT/BCIP, especially when they are used in experiments where signals are not expected to be overlapping (see previous section).

Prior to doing the actual double labeling experiment, probes were tested separately, to check their effectiveness, especially paying attention to the time required to obtain the final signal. The quantities of used probe in the double label experiment were then adjusted accordingly. There are several alternative ways in which the probes may be visualized, but some rules should be obeyed. For example, the probes should be applied together in the hybridization solution. When the antibodies are conjugated to different enzymes they also can be applied together to the specimen. However, it appears that stronger signals are obtained if the antibodies are applied sequentially. The fluorescein-labeled probe should be visualized first because it is less stable as compared to the digoxigenin-labeled RNA probe. Since the alkaline phosphatase along with NBT/BCIP gives the better visualization reaction, this detection should be used for the weaker probe, while the horseradish peroxidase along with DAB, being considerably less sensitive, should be used for the stronger probe.

Another possibility of detecting two differentially labeled probes simultaneously is the sequential incubation in antibodies conjugated with alkaline phosphatase and staining for each antibody with different substrates. When two alkaline phosphatase-based antibodies are used the antibodies cannot be added simultaneously. They are added sequentially with an enzyme-deactivating step (100 mM glycine-HCl, pH 2.2, for 10 min) after the development of the first signal. This step is very important since a failure to inactivate the first alkaline phosphatase will lead to false signals with the second substrate.

These kinds of two-color double label *in situ* hybridization experiments with chromogens give good results for non-overlapping signals. Best contrasts are obtained with a Fast red substrate and NBT/BCIP. Care must be taken since the blue stain can be very intense and easily hide the red stain if the signals are colocalized (see Fig. IV.21.A and B). This problem could probably be overcome, when the reactions are monitored carefully and the blue

staining is stopped before it totally masks the red one. An easier solution would be the use of fluorochromes to visualize the signals.

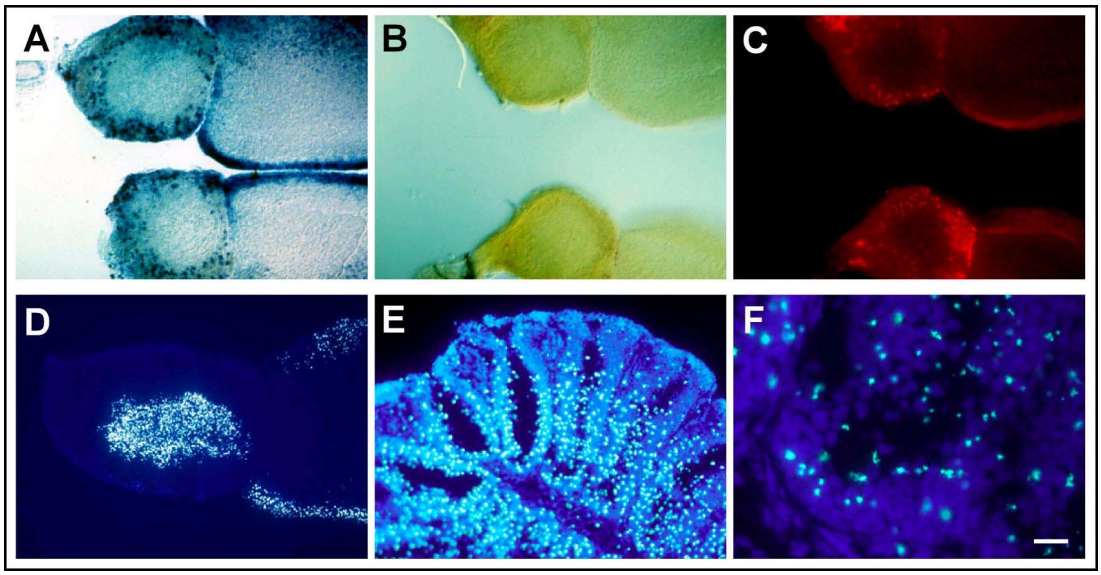

Fig. IV.21.: Test of Different Substrates for Alkaline Phosphatase. Whole mount *in situ* hybridizations with *rtk2/EphA4b* on olfactory bulb-telencephalon preparations are shown in panels A, B and C. Substrates NBT/BCIP (A) and Fast red (B, C) were used for detection. *In situ* hybridizations on cryostat sections of an olfactory bulb (D) and an olfactory epithelium (E and F) hybridized with antisense RNA probes against *dlx2* (D) and *L3/ephrin-A2* (E and F) and detected with the ELF™-97 (yellow-green) substrate are shown. Sections in E and F were counterstained with the nuclear stain Hoechst 33342 (blue). Tissue probed with sense probes was always free of staining. Anterior is to the left and lateral to the top. Scalebar corresponds to 100 µm in panels A, B and C, to 50 µm in D and E, and to 25 µm in F.

The most sensitive method of fluorescent *in situ* hybridization is to use enzyme substrates that produce insoluble fluorescent precipitates (Jowett and Yan, 1996). For example, the red precipitates produced by the Fast red substrate (Biogenex) fluoresce strongly when viewed by epifluorescence with a rhodamine filter set. The ELF™-97 (Molecular Probes) alkaline phosphatase substrate is initially non-fluorescent, but when activated by the enzyme, produces a crystalline precipitate that fluoresces yellow-green with a DAPI filter set. The use of alkaline phosphatase substrates increases the sensitivity of the method, because there is an amplification of the signal.

Both of these substrates were first tried in single-labeling experiments. In Fig. IV.21.B and C *rtk2/EphA4b* was detected with an alkaline phosphatase-coupled antibody, using Fast red as substrate. Under the rhodamine filter (Fig. IV.21.C), bright fluorescing cells in the glomerular layers of the olfactory bulbs, the mitral cells, can be seen. For comparison see the same section under bright-field illumination (Fig. IV.21.B). The second probe was detected again with an alkaline-phosphatase antibody using ELF™-97 substrate in a single-labeling experiment. The detection of the *dlx2* probe in a single labeling experiment is shown in Fig. IV.21.D. A strong yellow-green staining can be seen in the central region of the olfactory bulb, which corresponds to the localization of granule cells that can be detected with this probe. Parallel staining performed in the olfactory epithelium with the *L3/ephrin-A2* probe showed a strong labeling of the sensory area with some staining in the non-sensory area

(Fig. IV.21.E). In a higher magnification (Fig. IV.21.F), and counter-staining with the nuclear stain Hoechst 33342 (in blue) the small ELF™-97 precipitates are more apparent. It is very difficult to assign these small precipitates to particular cells. The staining is real, since no staining was obtained in control hybridizations performed with sense probes. The label is localized to the correct region as judged by detection with NBT/BCIP substrate. However, the precipitates are distributed very broadly in this region since labeling is also observed in the midline raphe and outside the lamellae. Thus, although identification of labeled cell type is not possible with this substrate it could be useful for broader applications, when a particular region has to be stained.

All substrates worked in control experiments where single *in situ* labeling was performed. However, no second staining could be obtained in all trials to obtain a double labeling. The reason for this is not known definitely, but could lie in the critical step to inactivate the first alkaline phosphatase-coupled antibody. Further experiments should go into the optimization of this step.

In conclusion I can say that a two-color *in situ* hybridization could not be established, while the simultaneous detection of a mRNA and a protein product appears to be possible in whole mounts as well as on cryostat sections. A little more effort would probably lead to the establishment of a suitable protocol for two-color *in situ* hybridizations, which would be useful for many applications.

As a next step I thought about the establishment of a suitable technique to interfere with the function of one or some of the Eph receptors or ligands. Several methods seem to be available to do so. These will be described in the next section (IV.2.3.). The method I have chosen involved the isolation and testing of several components. Because this is a very large part I will describe it in a separate chapter. However, parts of the isolated components will be mentioned already in the following functional analysis part.

2.3. Functional Analysis

2.3.1. General Introduction

The zebrafish has provided embryologists and developmental geneticists with an attractive system for studying the growth and organization of vertebrates, largely due to the accessibility of the embryo and the ability to isolate developmental mutations that disrupt various processes. Overexpression of mRNA encoding wild type, activated, and dominant negative alleles has provided some information about the function of particular genes, but the construction of these variant mRNAs requires extensive knowledge of the biochemical properties of the gene product. One limitation of the zebrafish system, therefore, is the inability to disrupt the function of a gene based on sequence alone, as is possible in mice through homologous recombination in embryonic stem cells. This technology in zebrafish was hampered by the absence of ES cells that are a necessary requirement for this technology.

Although recently it was shown that short-term cell cultures derived from gastrula stage embryos were able to generate germ-line chimeras after the introduction into a host embryo (Ma *et al*., 2001), the production of real homologous recombinants is still in its infancy.

Nevertheless, some methods are used in zebrafish to do functional studies. One candidate technology is the use of double-stranded RNA (dsRNA) to silence gene expression. First noted in *Caenorhabditis elegans*, this dsRNA "interference" (RNAi) relies on dsRNA homologous to a target gene as a specific means of dramatically decreasing endogenous gene expression. In practice, RNAi relies on the introduction of double-stranded RNA corresponding to a portion of a particular mRNA into the parental germ cells or the early embryo. Subsequently, the expression of the endogenous gene is perturbed. Steady state mRNA levels diminish, resulting in a concomitant decrease in the amount of encoded protein. As a result the animal expresses a complete partial phenocopy of a null mutation of the gene in question. Recently, claims of successful RNAi in zebrafish (Wargelius *et al.*, 1999; Li *et al.*, 2000) and mouse (Wianny and Zernicka-Goertz, 2000) have been published. However, a recent set of 'blind' experiments indicated that the frequency of non-specific effects consequent to RNAi application renders the method impracticable for investigating early zebrafish development (Oates *et al*., 2000).

Very recently, another 'knock-down' technology has been developed. Here so-called antisense morpholinos are used to facilitate direct assignment of function to specific genes. Morpolinos are chemically modified oligonucleotides with base-stacking abilities similar to those of natural genetic material. They have been shown to bind and block translation of mRNA effectively and specifically in zebrafish embryos (Nasevicius and Ekker, 2000).

Another powerful approach for studying gene function is gain-of-function by ectopic expression of genes in a temporally and spatially specific way. However, there is still no simple and effective system for the temporal and/or spatial control of gene expression in zebrafish. This kind of method would be necessary to study the function of genes like Eph receptors and their ligands that are widely expressed during embryonic development and required for many different processes. I tried to use such a gain-of-function approach to study the role of L5/ephrin-B2a in the primary olfactory pathway of zebrafish.

2.3.2. Strategy to Interfere with L5/ephrin-B2a Function

As described in this chapter, ligands of the ephrin-B family appeared to be interesting candidates for having a role in growth cone guidance. As revealed by *in situ* experiments the *L5/ephrin-B2a* and *L6/ephrin-B2b* genes appeared to be expressed in the mitral cells of the olfactory bulb. On the protein level the presence of B family ligands was shown by affinity probe *in situ* hybridization. However, none of the tested receptors were expressed in the OSNs. If one presumes that an unidentified receptor is present on these cells, an overexpression of a ligand for this receptor should be able to interfere with its function. The neurons that expect to get in contact with their ligand in the target area should be misrouted if they are exposed to the ligand much earlier, e.g. already in the olfactory epithelium.

Taking advantage of the properties of ephrin ligands, which is the requirement for clustering as homodimers or heterodimers with other family members before a signal is send to the cell. Hence, overexpression of one ligand, in this particular case L5/ephrin-B2a could help. In a second approach the function of L5/ephrin-B2a was investigated by taking a dominant negative approach analogous to that used to study the role of signaling through the FGF, activin and BMP4 receptors in the *Xenopus* embryo (Amaya *et al*., 1991; Hemmati-Brivanlou and Melton, 1992; Graff *et al*., 1994). Receptor kinases are activated by a ligand-induced dimerization of receptor leading to trans-phosphorylation and activation of the intracellular catalytic domain (Ullrich and Schlessinger, 1990). Activation can therefore be disrupted by the overexpression of truncated receptor, comprising the extracellular and transmembrane domains, but lacking kinase function. Upon binding of the ligand this truncated receptor is capable of dimerizing with the endogenous receptor to form a complex that, because of the absence of kinase function, cannot activate the catalytic domain of the endogenous protein (Amaya *et al*., 1991; Ueno *et al*., 1991, 1992).

To interfere with L5/ephrin-B2a function, sequences encoding the wild-type form of this gene were cloned into the prOMP$_{1.3}$-IRES-EGFP vector. This vector allows the simultaneous transcription of the gene of interest and the reporter gene EGFP due to the presence of an internal ribosome entry site (IRES). The OMP promoter (see next chapter) is able to specifically drive reporter gene expression in olfactory sensory neurons. The vector alone was used to provide a negative control for any non-specific effects of microinjection or vector sequences only. The constructs are depicted in Fig. IV.22.A.

In initial experiments the full-length construct was used to evaluate the feasibility of the approach. EGFP fluorescence was observed in individual OSNs as early as 24 hpf, which is consistent with the onset of expression of the reporter from the prOMP$_{1.3}$-Y vector alone and the bicistronic vector prOMP$_{1.3}$-dsRed-IRES-EGFP (for details see IV.3.1.10.). As already observed for the bicistronic vector prOMP$_{1.3}$-dsRed-IRES-EGFP, the fluorescent signal is not so intense for the second cistron. This is in accordance with previous studies performed in other species where IRES sequences were used successfully. Transgene expressing OSNs could, although hardly, be visualized under the epifluorescence microscope, but generally no axons were observed. For an example of direct visualization of L5/ephrin-B2a-overexpressing cells see Fig. IV.22.B. Even under high power magnification using a fluorescence microscope no axons were revealed (Fig. IV.22.D). The lack of labeling in the axons is probably due to the lack of sufficient amounts of EGFP protein. To confirm this assumption the EGFP signal was enhanced using an antibody staining against GFP. Using this antibody the number of observable positive neurons increased 2-4fold, and in many cases axons could be visualized. Some examples of embryos microinjected with the overexpression construct and observed after antibody staining using an α-GFP antibody and an Alexa-488-coupled secondary antibody are shown Fig. IV.22.C-F. For these two neighboring cells axon terminals and even dendrites could be visualized (Fig. IV.22.F, black and white arrows, respectively). Panel G shows the same embryo as in F counter-stained with the nuclear stain DAPI.

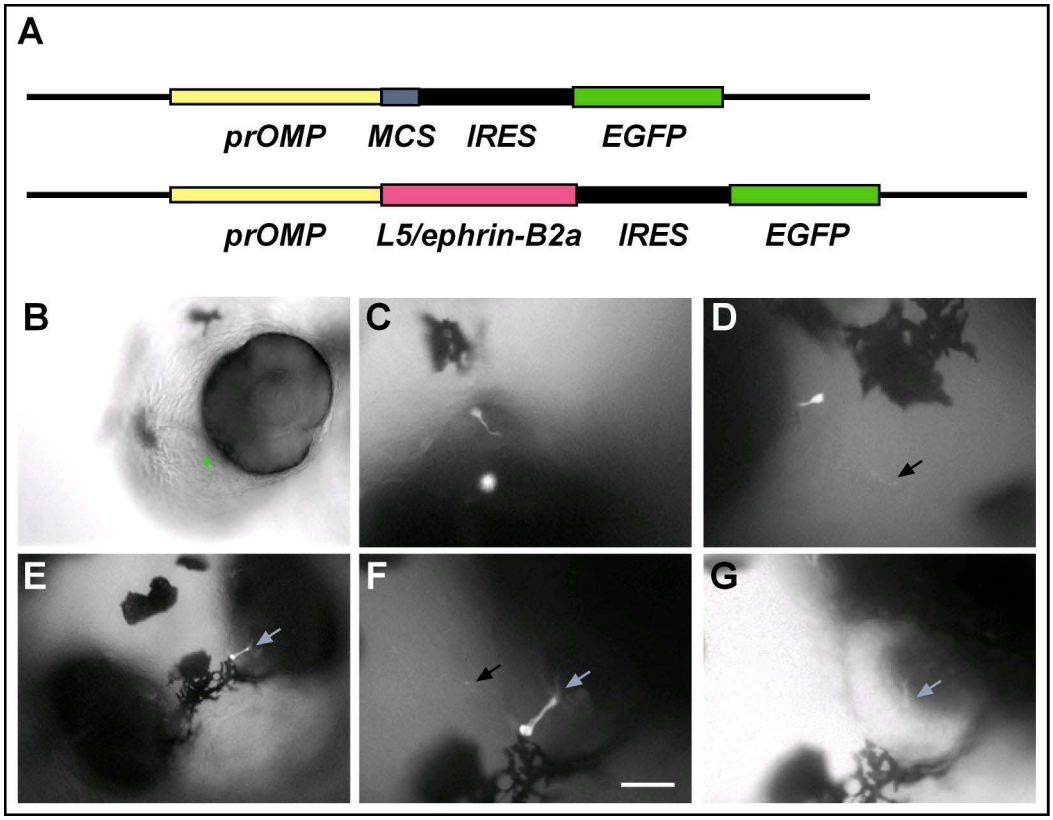

Fig. IV.22.: Overexpression of the B Family Ligand L5/ephrin-B2a in Olfactory Sensory Neurons. The basic vector and the overexpression construct are depicted in A. The coding region of L5 was cloned into the multiple cloning site (MCS) of the basic vector and is linked over an internal ribosome entry site (IRES) sequence to the reporter gene EGFP. Both genes are under the control of the OSN-specific OMP promotor. B. Overlay of a brightfield and fluorescent image of a 48 h embryo expressing the overexpression construct shown in A. C shows a neuron expressing the construct at higher magnification. No labeling in the axon can be observed. D. Transgene-expressing neuron and its axon (arrow) after antibody staining with the α-GFP antibody and an Alexa Fluor-488-coupled secondary antibody as visualized by fluorescence microscopy. Frontal views of an 72 h embryo at low (E) and high power (F) magnification displaying two neighboring transgene-positive neurons after antibody staining. Note the staining of the cilia (gray arrow) and the axon terminals (black arrow). G. Same image as in F as observed under the DAPI filter. DAPI-staining of the same embryo to show the cup-shaped olfactory placode more clearly. Gray arrows in E-G point to identical positions. Scalebar corresponds to 50 µm in B and E, and to 28 µm in all other panels.

These results show that the vector is able to direct expression of the bicistronic message. However, the amount of reporter protein produced is quite low, so that the simple injection of the construct and *in vivo* observation of the axons was not possible to as desired. An antibody staining against the reporter enhanced the number of positive neurons and allowed the visualization of their axons, which are the structures of interest. Hence, subsequent studies included an antibody staining using this α-GFP antibody after microinjection.

For final analysis 730 one-cell stage embryos were injected with the overexpression construct prOMP$_{1.3}$-L5/ephrin-B2a-IRES-EGFP that was predicted to interfere with Eph signaling. Control experiments included the injection of the prOMP$_{1.3}$-IRES-EGFP vector. Surviving embryos for the prOMP$_{1.3}$-L5/ephrin-B2a-IRES-EGFP construct (202) were stained 72 h after microinjection and double labeled using the α-GFP antibody to detect the transgene expressing neurons and the zns-2 antibody to visualize the whole population of OSN axons.

This was done to make it easier to judge the projection of the L5/ephrin-B2a-overexpressing neurons compared to the wild-type neurons. 27 of the embryos appeared to be positive and had varying numbers of transgene-expressing neurons. Nine embryos had one positive neuron, seven embryos had two positive neurons, 3 had 3, 3 had 5, 2 had 6, 1 had 7, 1 had 8 and 1 had 10 positive neurons.

As in wild-type embryos, embryos injected with the control vector (prOMP$_{1.3}$-IRES-EGFP) and the prOMP$_{1.3}$-Y construct, axons of transgenic OSNs converged to form the olfactory nerve and then grew directly to the presumptive olfactory bulb. Double labeling experiments using the zns-2 antibody revealed that transgenic OSN axons, positive for the overexpression prOMP$_{1.3}$-L5/ephrin-B2a-IRES-EGFP construct and wild-type axons were intermingled and projected to the same target region. A typical confocal analysis of the projection pattern is shown in Fig. IV.23. What is apparent is that although axon terminals project to the same target region, there is no 100% overlap of red and green fibers within the target region, although this would be expected. Green (transgenic) fibers look in same cases somewhat displaced from the red (wild-type) ones. This effect might me due to an incomplete staining with the zns-2 antibody for unknown reasons. What still needs to be shown is that *L5/ephrin-B2a* is expressed in the olfactory placode at all. Unfortunately, no functional antibody against L5/ephrin-B2a that works in zebrafish is available. Alternatively, *in situ* hybridization experiments along with an antibody staining would be necessary to clearly show that the EGFP expressing neurons also express L5/ephrin-B2a, although, the expression of the second cistron (EGFP) is a strong hint that L5/ephrin-B2a is expressed as well.

Since a 100% overlap for L5/ephrin-B2a expressing and EGFP expressing neurons was expected the detection ideally should be performed using two fluorescent secondary antibodies (see IV.2.2.3. for considerations). The DIG-labeled antisense RNA of *L5/ephrin-B2a* was detected first using a primary mouse-α-DIG-antibody and an Alexa Fluor-594-coupled secondary antibody. The EGFP expressing cells were labeled as previously using the rabbit-α-GFP antibody and an Alexa Fluor-488-coupled secondary antibody. Since I knew already that the detection of the EGFP works, only a test of the *in situ* hybridization conditions with the α-DIG-antibody was necessary. This antibody was tested in various concentrations using the *zOMP* gene as probe, which is an abundantly expressed gene in mature olfactory sensory neurons (see Fig. IV.26.). However, in several experiments using varying concentrations of probe and varying concentrations of the primary antibody no signals could be obtained. The integrity of the probe was tested using an α-DIG-AP antibody, while the functionality of the secondary antibody was tested with the zns-2 antibody. These controls showed that for some reason the primary α-DIG-antibody does not work. The use of another antibody to detect the DIG-labeled probe or probably even the use of the α-DIG-AP antibody in conjunction with the Fast red substrate will help to give this final proof.

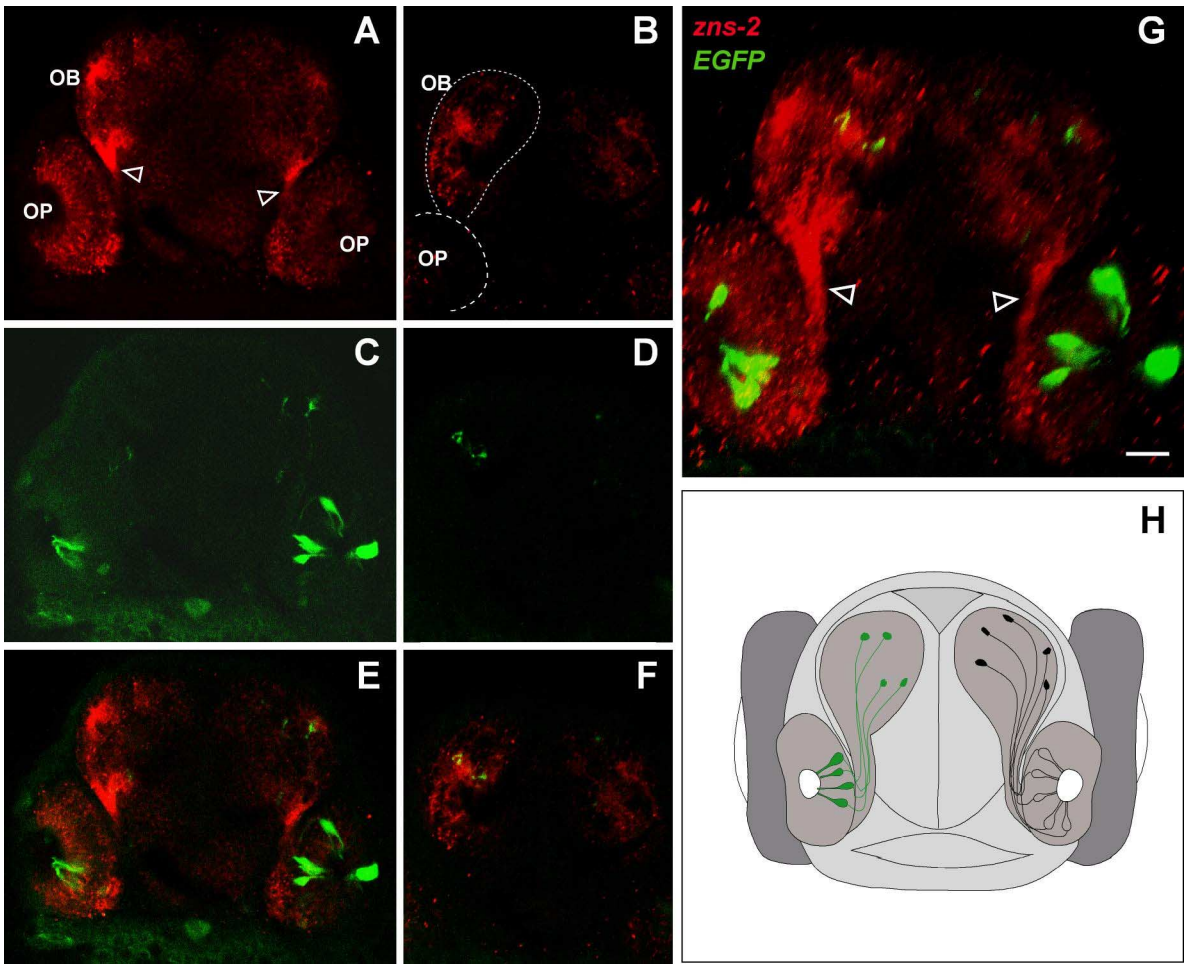

Fig. IV.23.: Double Labeling of OSNs Overexpressing the B Family Ligand L5/ephrin-B2a with zns-2. Frontal views of the same 3 d old zebrafish embryo are shown. The orientation of the embryo is shown schematically in H. The glomerular pattern was visualized using the zns-2 antibody and an Alexa Fluor-594-coupled secondary antibody (red), while the EGFP expressing neurons were visualized with an α-GFP antibody and an Alexa Fluor-488-coupled secondary antibody (green). Images were taken using a confocal microscope. A, C, and E, show the zns-2 staining, the GFP staining, and an overlay of both stainings, respectively. A number of green neurons expressing the transgene and their axonal terminations can be observed. Terminal arborizations in the olfactory bulb do not overlap 100%. B, D, and F show a similar set of images that were aquired at a different focal plane. Here, OSN cell bodies cannot be seen, since lie in a different focal plane. Transgene-expressing axonal terminations in the olfactory bulb do overlap in about 50% of the cases. G shows an overlay of all aquired images where an overlay of all aquired images is shown. H. Schematic representation of the analyzed embryo. Green cells represent the transgene-expressing cells, while uncolored represent wild-type ones. Dorsal is to the top. Open arrowheads point to the olfactory nerve. OP: olfactory placode, OB: olfactory bulb. Scalebar corresponds to 25 µm.

3. Isolation and Characterization of Cell-Type Specific Genes and Promoter Elements

A critical factor for successful transgenic research is the design of a DNA construct, which allows proper expression of the transgene and consists of a gene promoter, a reporter gene or a gene of interest, and a transcription termination signal. Among the three components, the promoter is the most important in directing the structural gene to be activated at a correct stage and in a proper tissue. However, because of the lack of zebrafish gene promoters, most of the early work on transgenic zebrafish used heterologous promoters from viruses or from other species of animals (for review see Iyengar *et al.*, 1996). Expression of a transgene under a heterologous gene promoter often was unreliable, and highly mosaic. Thus, until recently, it was unclear whether transgenic zebrafish is capable of expressing the transgene faithfully. Recently, several groups have successful reported the faithful expression of transgenes using zebrafish gene promoters (Meng *et al.*, 1997; Long *et al*., 1997; Higashijima *et al*., 1997). These studies made clear that the use of endogenous gene promoters gives better and more predictable results.

In this study it became necessary to have specific promoters for the three most important cell types in the olfactory system, the OSNs, the mitral cells and the granule cells to do anatomical and functional studies. Steps were undertaken to isolate suitable promoters for each of these cell types. In one case the gene of interest was available (*dlx2*), in other cases the genes had to be cloned first (*OMP* and *tbr1*). First, I will focus on the isolation and characterization of the promoters and then I will describe the other necessary elements for the construct to do functional expression *in vivo*.

3.1. The Olfactory Marker Protein (OMP)

The OMP gene is a protein that is expressed primarily in mature olfactory sensory neurons. First identified more than 10 years ago (Margolis, 1980) it has been used repeatedly as a marker for these neurons in several mammalian species.

3.1.1. Cloning of the Zebrafish OMP Gene

To identify the zebrafish *OMP* gene, amplification of a 170 nucleotide fragment was attempted from different templates. Zebrafish olfactory epithelial, bulbal and brain cDNA was used in a PCR with degenerate primers directed against a highly conserved *OMP* sequence motif (see Fig. IV.24.). PCR products could only be generated in the PCR using olfactory epithelial and bulbal cDNA as template. The products obtained from the olfactory epithelial cDNA were cloned into the T-vector and individual colonies (n = 12-17) were checked for correct insert size using a colony PCR with vector primers. Clones displaying the expected insert size (n = 12) were sequenced.

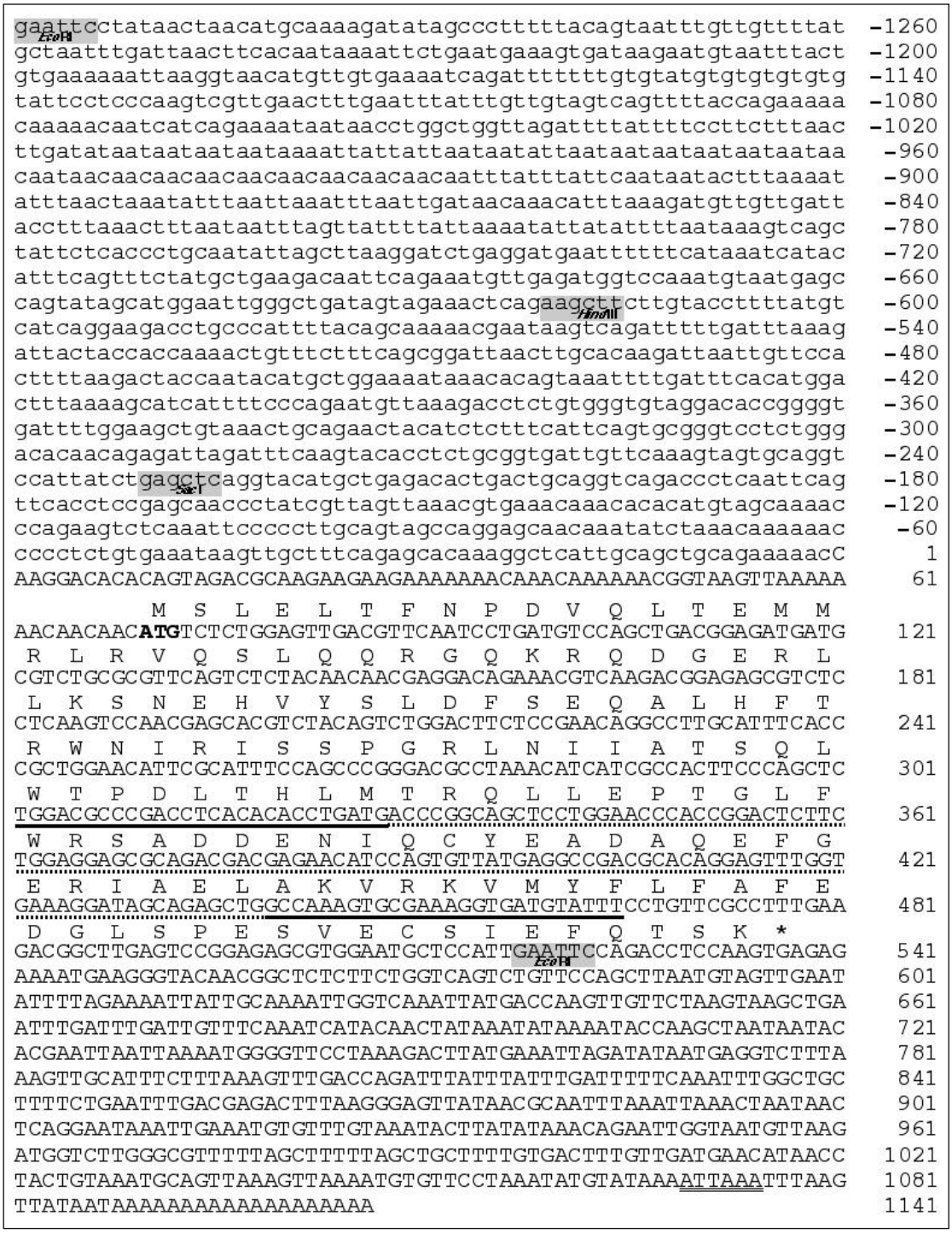

Fig. IV.24.: Nucleotide and Deduced Amino Acid Sequence of the Zebrafish OMP cDNA and Upstream Region. Numbering of nucleotide and amino acid sequences are given to the right. Amino acids are given using the one letter code. The start codon is shown in bold and the stop codon is marked with an asterisk. Nucleotide sequences of the cDNA is given in uppercase letters, while genomic sequences are given in lowercase letters. Gray boxes indicate relevant restriction enzyme cutting sites. The dotted line represents a region conserved between species and sequences flanking the dotted line correspond to the sequences where the degenerate primers are located. The double underlined sequence shows a putative polyadenylation signal.

Two of these clones designated (E56-B4 and E56-E3) showed homology to OMP over the entire length in Blast searches. Both clones appeared identical in sequence; in subsequent experiments clone E56-B4 was used. To obtain a full-length sequence of this gene, the amplification product (E56-B4) was radioactively labeled with the PrimeIt II DNA Labeling Kit (Stratagene) and used to screen a full-length cDNA library of olfactory epithelium (see

III.10.3 and III.10.6.). Positive clones were obtained at a frequency of 0.07%, in accordance with the expectation for a moderately abundant mRNA. Five of the colonies giving the strongest hybridization signals were rescreened. Eigtheen clones were isolated from the rescreen and insert sizes were checked by colony PCR. Six of these clones were then subjected to sequencing. Obtained sequences showed homology to OMP genes in four cases. These four independent clones were analyzed in more detail. They were sequenced in both directions by primer walking (using vector primers and primers OMP-0, OMP-1, OMP-2, OMP-3 and OMP-4, that are located at about 300 bp from each other). The clones all contained poly A tails (i.e. were 3' complete) of different length and thus presumably were derived from different mRNA molecules. A single sequence was obtained from all clones and thus the sequence appears to be 5' full-length as well.

3.1.2. Characterization of the Isolated Zebrafish *OMP* Gene

A 1088 nucleotide cDNA contained the complete coding region of the zebrafish homologue of the *OMP* gene (Fig. IV.24.), as well as the complete 5'- and 3'-UTRs. The open-reading frame is 465 nucleotides in length and encodes a putative protein of 155 amino acid residues with a predicted molecular weight of around 14 kD. The putative initiation codon (ATG) is located at position 71. However, the surrounding sequence shows only weak homology to the Kozak consensus sequence for initiation of translation (Kozak, 1999). The putative stop codon (TGA) begins at nucleotide position 536, followed by 550 bases of 3'-UTR. Canonical polyadenylation motives (AATAAA) only were found in atypical positions (3 and 120 bases from the poly A tail). However, a less frequent but similarly effective polyadenylation motive (ATTAAA) was found at position 1070, which is in the expected range from the end of the poly A tail to be functional, which is 10-30 bp from the poly(A) cleavage site (Zhao *et al.*, 1999).

The sequence of the *OMP* gene does not allow many predictions about the structure of the protein. In hydrophobicity plots no highly hydrophobic regions were predicted (Kyte and Doolittle, DNASIS v2.0, data not shown).

Sequence comparisons of the complete amino acid sequence with known *OMP* genes revealed that zOMP is most similar to one of the two frog OMPs. Homologies ranged from 55 to 52 and 51% (frog, human and rodents, respectively, see Fig. IV.25.B). A comparison with the medakafish sequences was only possible for a 37 amino acid fragment (indicated by a dotted line in Fig. IV.24.), since full length information is not available for these proteins (Yasuoka *et al*., 1999). Sequence comparisons for this fragment showed highest homology of zOMP to the medaka OMPs (68% homology for medaka OMP2 and 65% homology for medaka OMP1 as opposed to 62, 59 and 54% for frog, human and rodents, respectively, see Fig. IV.25.B).

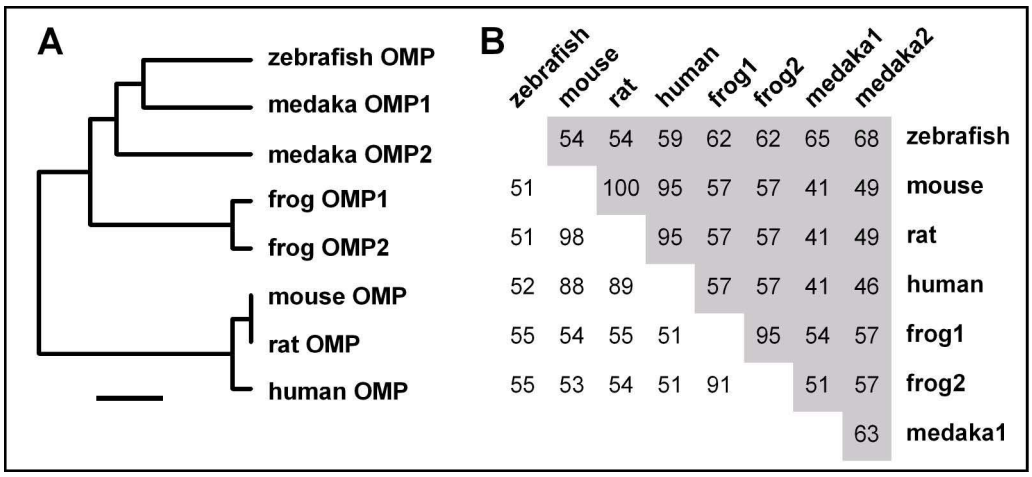

Fig. IV.25.: Structural Comparison of Zebrafish *OMP* and Other *OMP* Genes. A. A phylogenetic tree was constructed based on the amino acid sequence of medaka, frog, mouse, rat, human OMPs over a region of 37 amino acids using the ClustalW program. Zebrafish OMP is more related to medaka and frog OMPs as compared to the mammalian counterparts. Scale bar indicates an evolutionary distance of 0.1%. B. Direct comparison of the genes on the amino acid level based on the amino acid level of the whole sequence and 37 amino acid residues (gray boxes) available.

3.1.3. *zOMP* Is Expressed Specifically in Olfactory Sensory Neurons

To see whether *zOMP* expression is selectively in OSNs, *in situ* hybridizations were performed on the olfactory epithelium of adult animals using digoxigenin-labeled RNA probes derived from the 3'-UTR of the *OMP* gene. Control experiments with sense probes did not reveal any specific hybridization signal. Probes containing the coding region tended to give a strong but homogenous staining throughout the whole epithelium, which could also be observed with sense probes, making this part of the sequence unsuitable for use as a probe in *in situ* hybridization experiments. On cross-sections of the olfactory rosette hybridization signals were found exclusively in the inner region of the epithelium (Fig. IV.26.A). The location of these signals corresponds exactly to the position of the OSNs, which occupy the central region of the nasal epithelium, while non-sensory cells are located in the peripheral region. Within the sensory region, OMP-positive cells were located primarily in the apical part of the lamellae, while only few signals were detected basally (Fig. IV.26.A, B). A reduced abundancy of OMP-positive cells was observed for the inner curves of the lamellae, regions with intense proliferative activity, i.e. predominantly immature cells (Berger, 1998). Thus, all facets of the OMP spatial distribution pattern correspond exactly to the distribution of OSNs known from neuroanatomical tracing studies (Baier *et al*., 1994; Lieberoth, 1999), *in situ* hybridization with odorant receptor genes (Weth *et al*., 1996) and another OSN-specific gene, the CNG-channel (Berger, 1998), as well as stainings with the neuronal marker Hu (Berger, 1998 and this work).

To analyze expression in all tissues, whole mount *in situ* hybridization was performed in embryos at different developmental stages, using the same probe as above. Expression was restricted to the olfactory placode and other tissues of the embryo were not labeled (Fig. IV.26.C, D). Hence, it can be concluded that *zOMP* is a reliable marker for OSNs throughout development and adulthood.

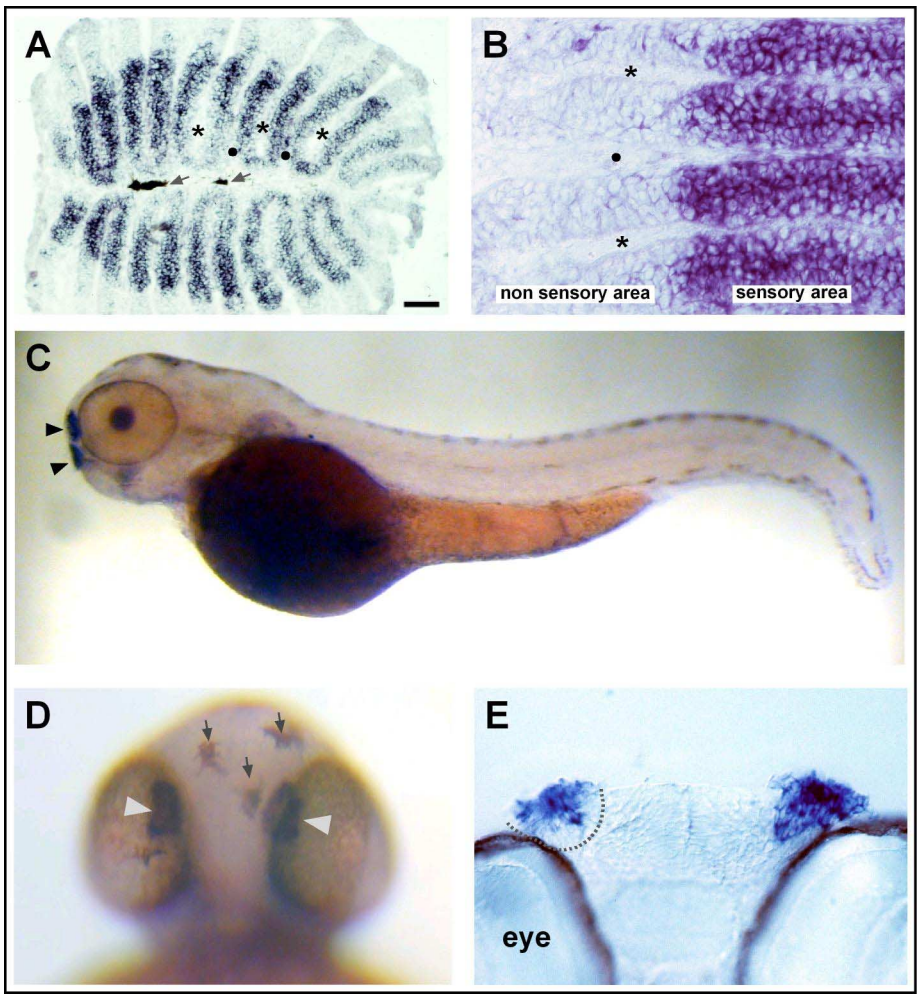

Fig. IV.26.: Localization of the OMP Transcript in Adult Olfactory Epithelia and Zebrafish Embryos. A. Cryostat sections were hybridized *in situ* with digoxigenin-labeled sense or antisense RNA probes. Bright-field image of olfactory epithelia showing specific hybridization to the sensory area. The basal lamina is indicated by dots, whereas the lumen between the lamellae is indicated by asterisks. B. High power view of epithelium shown in A. C. Lateral view of a 72 h old zebrafish embryo shown after whole mount *in situ* hybridization. The two labeled placodes (arrowheads) can be seen just anterior to the eye. D. Frontal view of a 48 h whole mount embryo. Placodes are indicated by arrowheads. E. Horizontal section of a 96 h old zebrafish larva after hybridzation as whole mount. The olfactory placode (dashed line) lies adjacent to the eye. Scalebar corresponds to 100µm in panel C, to 50 µm in panels A and D, and to 25 µm in panels B and E.

3.1.4. Ontogenesis of *zOMP* Expression

zOMP expression in the olfactory placode was detected already at 24 hpf, the earliest time point investigated. This is consistent with the first occurrence of mature OSN reported around 20 hpf (Hansen & Zeiske, 1993). The expression of olfactory receptors is also evident around the same time point (Barth *et al*., 1995; Argo, 1995; Byrd *et al*., 1996).

zOMP expressing cells are mainly present at the outer, apical part of the placode, while cells at the basal part are not labeled (Fig. IV.26.E). Those could be precursor cells, which would not be expected to express *OMP*. To obtain an estimate for the growth of the OSN population during early development *OMP* positive neurons were quantitated at several embryonic and larval stages (Fig. IV.27.). OSN numbers increase massively during the first 48 hours of development. At 24 hpf 25±2.5 cells were labeled per placode while 92±7 of labeled cells were observed at 48 hpf. At later developmental stages OSN maturation slowed

down. Between 48 and 96 hpf no significant increase in the number of mature OSNs was detectable. So, in 72 hpf embryos on average 120±4.5 cells were counted per placode, while at 96 hpf 130±4 cells were counted. This slow growth continued accounting for 140±8 *zOMP*-positive cells per placode at 120 hpf. A similar growth curve was observed for OSNs expressing a particular receptor gene (Argo, 1995; Whitlock and Westerfield, 1998).

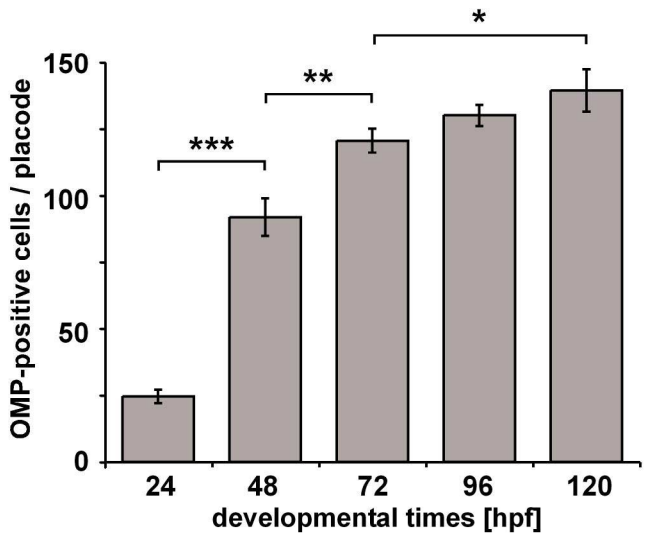

Fig. IV.27.: **Expressivity of *zOMP* Expression as Determined by *In Situ* Hybridization.** Numbers of zOMP-positive cells per olfactory placode were counted for five developmental stages (24, 48, 72, 96 and 120 hpf). Mean +/- SEM, for 96 h mean +/- range is given (n = 8, 10, 6, 2, and 10 placodes, respectively). Significance of increases is evaluated by an unpaired Student's t test, ***, P<0.0001; **, P<0.005; *, P<0.03.

3.1.5. Characterization of the zOMP Locus

It was interesting to investigate if the isolated *OMP* gene has close family members, since in other species family sizes of 1, 2 and 4 genes have been found (rat, medaka and *Xenopus*, respectively, Danciger *et al*., 1989; Yasuoka *et al*., 1999; Rössler *et al*., 1998).

No cross-hybridization to any other gene was found in high as well as low stringency Southern Blots using DIG-labeled full-length cDNA as a probe. Only one band was detected in *Hind* III, *Pst* I, *Sac* I, and *Bam* HI digests (Fig. IV.28.A). The 2 bands detected in the *Eco* RI digest are consistent with the internal *Eco* RI site found at position 518 within the coding sequence of the *OMP* gene. Hence, a single *OMP* gene occurs in the zebrafish genome.

A genomic cosmid clone was isolated from a zebrafish genomic DNA library (RZPD, Berlin) using the radiolabeled 170 bp PCR product as a probe. The cosmid clone itself was hybridized using a full-length as well as an upstream *Eco* RI fragment of the cDNA as probe. Sizes of the fragments hybridizing with the *OMP* probe were quite similar to those obtained in the genomic Southern Blot, indicating that the cosmid clone represented the native DNA configuration. The map of the OMP locus, shown in Fig. IV.28.B, was established using the informations gained from both Southern hybridizations. To identify a fragment containing 5' flanking regions of the *zOMP* gene the Southern where the whole cDNA was used as probe was not informative enough, since usually more than one fragment hybridized. Therefore, the cosmid clone was hybridized with the upstream *Eco* RI fragment of the *OMP* cDNA as a probe. A 1.8 kb *Eco* RI fragment was chosen as having the most suitable length. It was

subcloned into the pBluescript vector and sequenced from both sides. Sequence analysis with vector primers revealed that this fragment contains 1.3 kb of the 5' flanking region, the 5' untranslated region, and 447 bases of the open reading frame (sequence shown in Fig. IV.24.).

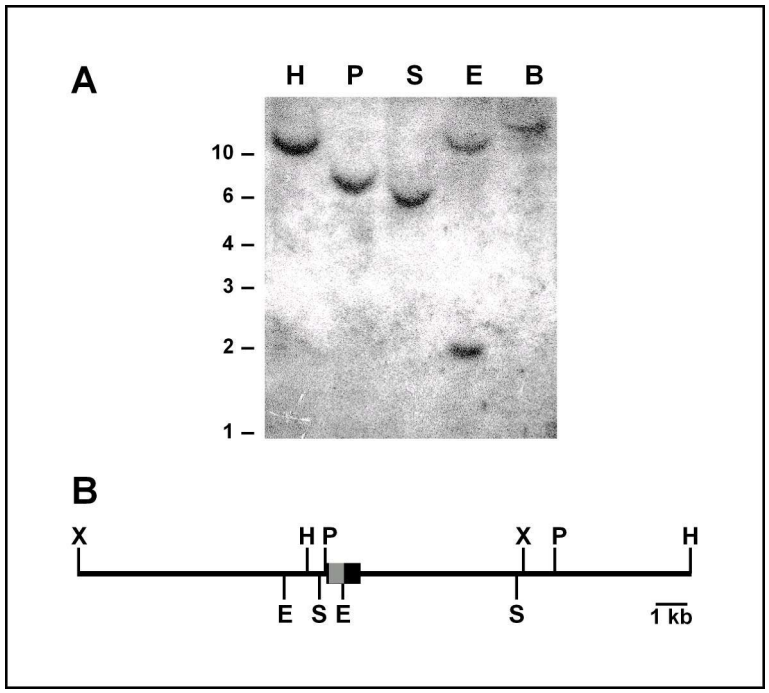

Fig. IV.28.: Southern Blot Analysis of the Zebrafish *OMP* Gene and Mapping of the Genomic Region of *zOMP*. A. The genomic DNA was digested with *Hind* III (H), *Pst* I (P), *Sac* I (S), *Eco* RI (E) and *Bam* HI (B) and probed with the zebrafish *OMP* cDNA. The length of the DNA fragments in kilobases is shown to the left. There are two bands in the *Eco* RI digest resulting from the presence of an *Eco* RI site within the gene. B. The genomic surrounding of the *zOMP* gene was mapped using various endonucleases. E: *Eco* RI, H: *Hind* III, P: *Pst* I, S: *Sac* I, X: *Xho* I. The cDNA is indicated with a black box. The gray segment corresponds to the open reading frame.

3.1.6. Characterization of the 5' Upstream Region

The 1.8 kb fragment was sequenced from both strands to get full sequence information that would give hints towards the presence of relevant regulatory sequences. The sequencing was done in a combination of primer walking and sequencing of smaller fragments of this *Eco* RI fragment. Smaller fragments were generated by taking advantage of the available restriction sites *Sac* I and *Hind* III, digestion and religation of the fragment of interest. Problems were encountered in sequencing of the upstream region. These were mainly due to the presence of highly repetitive sequences within this region, where primers tended to bind unspecifically as revealed by PCR with sequence specific primers. Generally, in addition to the expected fragment many smaller bands were observed. The design of primers with a higher annealing T helped to obviate this problem, only in some cases. Therefore, fragments were subcloned and sequenced using vector primers like T7hi (see Table III.5.), which have a high annealing T.

Various sequence analysis programs were used to analyze the upstream region of the *zOMP* gene. However, none of these could reveal any obvious TATA box or CAAT box as is

the case in *OMP* genes from several other species (Buiakova *et al*., 1994). Olf-1 and NF-I are two transcription factor binding sites that are present in the promoter regions of neuron-specific and particularly in olfactory-specific genes (Bakalyar & Reed, 1990; Dhallan *et al*., 1990; Jones & Reed, 1989). They are generally located in a proximal and distal position to the genes. Despite a strong effort in analyzing the upstream region of the *zOMP* gene for the presence of these transcription factor-binding sites, they could not be found. The presence of these two binding sites is not crucial for olfactory-specific expression, however a role in the regulation of olfactory-specific genes was shown. It is conceivable that these transcription factors do not exist in zebrafish, or that the binding sites are significantly different from that of higher vertebrates. An additional large set of genes expressed in OSNs is comprised by the olfactory receptors. It is tempting to speculate that these genes use the same transcription factors, which would require the presence of these binding sites in the upstream regions. Recently the sequences of a large genomic locus comprising 25 olfactory receptor genes were released to the database (Dugas and Ngai, 1999). Additional genomic information was known from sequences submitted by Mori *et al*. (2000). Extensive comparative analysis of upstream regions of all known OSN receptor genes with each other and the *OMP* upstream region did not reveal a conserved motive that could correspond to a transcription factor binding site (see S. Fuss, 2001).

3.1.7. Construction of A Suitable Expression Vector

A pBluescript based promoterless expression vector was constructed that carried the EYFP reporter gene (Clontech, Heidelberg, Germany) followed by an SV40 polyadenylation signal. It appeared necessary to do so, since all the conventionally available vectors do not ensure the optimal localization of the upstream region to the reporter gene, that is cloning of the upstream region, directly adjacent to the ATG codon. This is made possible in this vector where the upstream region is cloned using a *Nco* I site which covers the ATG codon. The vector is depicted in the supplement. Details of the construction of this vector (pACSF) are described elsewhere (S. Fuss, 2001).

3.1.8. The *OMP* Upstream Region Restricts Reporter Gene Expression to Olfactory Sensory Neurons *In Vivo*

To determine if the cloned OMP upstream region contains sequences that direct expression to OSNs *in vivo*, despite the absence of typical regulatory regions, the 1.3 kb upstream of the translational start codon was reamplified by PCR and cloned into the pACSF vector in front of a sequence coding for the EYFP reporter protein (for details see III.13.2.). Sequencing of positive clones revealed the exact insertion of the fragment. The positive clone 446-2 was named prOMP$_{1.3}$-Y subsequently, and used in all experiments thereof and for variations of the vector.

The prOMP$_{1.3}$-Y was linearized and injected into developing zebrafish embryos. These initial experiments revealed that in fact reporter gene expression could be observed in

the olfactory placode as early as 24 h post injection. As shown in Fig. IV.29.B-E bright cells could be detected, which displayed the characteristic morphology of olfactory sensory neurons. Round and oval shaped somata were located in a ring surrounding the olfactory naris. Long and thin as well as shorter and thicker dendrites, corresponding to the ciliated and microvillar receptor types, pointed towards the naris of the developing epithelium. Round shaped cells without dendrites that could correspond to pioneer neurons (Whitlock and Westerfield, 1998) were not observed. Individual axons extended into the developing olfactory bulb through a common exit point on the medial side of the placode, the presumptive olfactory nerve, and into the adjacent forebrain. Directly after the entrance point the paths of the olfactory sensory neuron axons diverged as they grew towards their targets. From all the axons analyzed, the growth cones appeared to grow directly to their target glomerulus, without making substantial changes in their direction.

Although expression is most prominent in the olfactory sensory neurons (Fig. IV.29.A, B), occasionally ectopic expression did also occur. Its occurence was rare and irreproducible and limited to epidermal cells mostly covering the yolk, but also the surface of the embryo. Higher concentrations of injected plasmid (100-200 ng/µl) than those used for analysis drastically increased ectopic reporter expression in epidermal cells (data not shown). The pACSF-Y vector without a promoter was used as control. Linearized plasmids were injected and showed no expression at all at standard concentrations. At higher concentrations some expression was seen in muscle but never in epidermal cells or neurons. In contrast, prOMP$_{1.3}$-Y, even at high concentrations, never drove expression in muscle cells.

3.1.9. Modifications of the Basic Expression Vector

One variation of the basic expression vector prOMP$_{1.3}$-Y included the addition of a tau fragment between the upstream region and the reporter gene resulting in the prOMP$_{1.3}$tau-Y (see III.12.2.3.). For analysis of the pathfinding of OSN axons it was necessary to be able to observe the axons and their terminals. The tau protein is a microtubule-associated protein present in brain and other neuronal tissues (Binder *et al*., 1985; Drubin *et al*., 1986). It is found in the axonal microtubules of mature neurons (Binder *et al*., 1985) and in the axon-like elongated neurite processes synthesized by differentiating neurons in culture (Drubin *et al*., 1986). The tau fragment used here was previously shown to be effective in transporting a reporter (lacZ) to the axon terminals (Mombaerts, 1996). It was isolated from genomic DNA of a knock-in mouse, which has GFP under the control of an olfactory receptor gene promoter (I7) that was kindly supplied by P. Mombaerts (for details see S. Fuss, 2001). However, as the experiments above show this fragment is not necessary for the labeling of the axon terminals. Transfection experiments using linearized prOMP$_{1.3}$tau-Y did not enhance the staining of the axons as judged by visual inspection (data not shown). This difference to the lacZ data may be explained by the size of the reporter protein. EYFP is a small protein that can diffuse along the axons to the terminals thereby labeling these, while lacZ is a large protein that needs to be actively transported, presumably by aid of the microtubule associated protein tau.

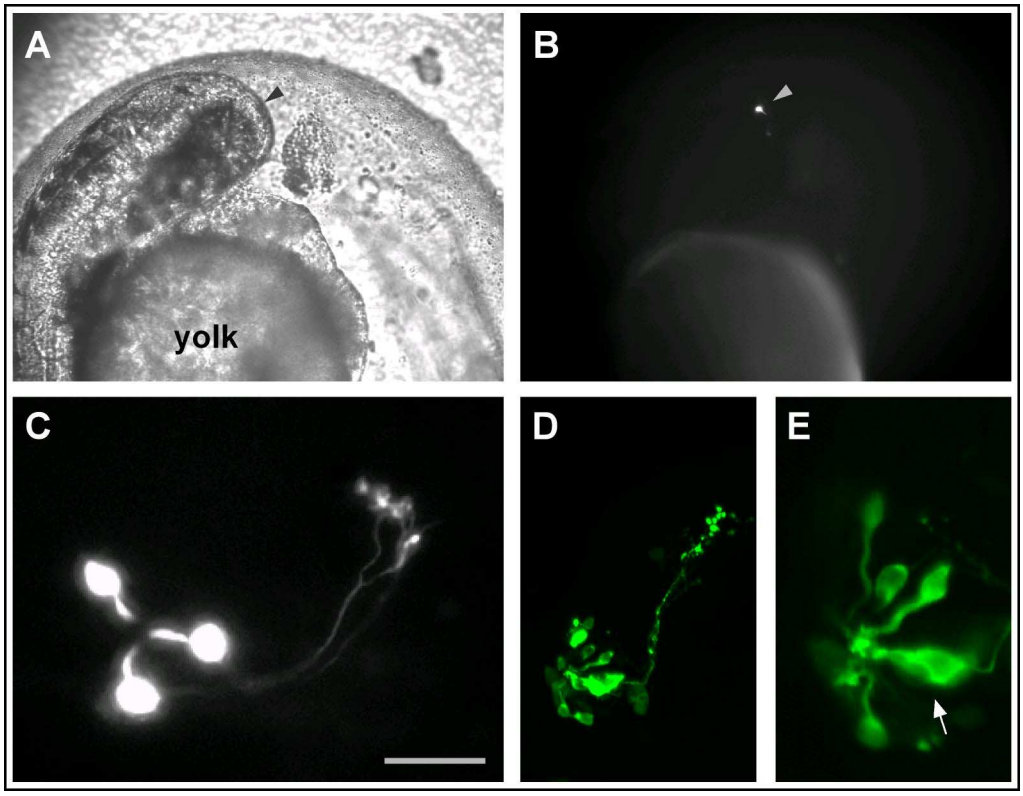

Fig. IV.29.: ***In Vivo*** **Reporter Gene Expression in Zebrafish Embryos.** Bright-field (A) and fluorescence image (B) of a 48 h embryo showing EYFP expression in a single neuron (arrowhead). C. High power view of an olfactory placode and the prospective olfactory bulb in a 72 h embryo showing three fluorescent neurons. Note the typical morphology of ciliated olfactory sensory neurons, a long dendrite and axons that arborize in the terminal field, the olfactory bulb. D. Reconstruction of a confocal image series (maximum projection mode) of many fluorescent olfactory sensory neurons in a 72 h embryo visualized with an α-GFP antibody. E. Projection of selected layers of the confocal series shown in D. A microvillar olfactory sensory neuron (arrow) is intermingled with ciliated cells in the same olfactory placode. The scale bar corresponds to 200 µm in panels A and B, to 100 µm in panel D, and to 25 µm in panels C and E.

An analogous vector containing DsRed as reporter gene was constructed. DsRed is very suitable for expression analysis because of its particularly high signal-to-noise ratio due to reduced autofluorescence at longer wavelength. However, when injected as linearized plasmid it showed to be less effective as compared to prOMP$_{1.3}$-Y. Although the localization of labeling was comparable and also very intense, strong differences in the percentage of positive fish were observed. While generally about 20-50% of embryos injected with prOMP$_{1.3}$-Y were positive only 10-30% of embryos were positive with prOMP$_{1.3}$-dsRed. A reason for this difference may lie in the construction of the prOMP$_{1.3}$-dsRed vector (for details of construction see III.12.2.2.). Due to the presence of an internal *Nco* I restriction site within the *dsRed* gene the 1.3 kb fragment had to be added to the pACSF-dsRed vector using the *Nde* I cutting site. The *Nde* I restriction site provides a less optimal Kozak sequence than the *Nco* I cutting site used for the construction of the previous vectors. This may be reflected in the less effective translation of the reporter gene. Furthermore, DsRed expression was detected significantly later than EYFP, which is consistent with reports in the literature (Finley *et al*., 2001).

3.1.10. Test of the Internal Ribosome Entry Site (IRES) in Zebrafish Embryos

Internal ribosome entry sites (IRESs) are short DNA sequences that initiate internal translation of RNA. IRES elements have been widely used in many different expression vectors to produce polycistronic transcripts for various purposes (including gene therapy, transgenesis, gene trapping, etc.). I wished to test and make use of an IRES element in a bicistronic mRNA construct with EGFP as reporter to perform functional studies in developing zebrafish embryos. The mRNA construct with IRES and EGFP was used to analyze the function of L5/ephrin-B2a (see IV.2.3.). But before that it was necessary to test the approach and the vector for feasibility. For this purpose a reporter construct prOMP$_{1.3}$-dsRed-IRES-EGFP was prepared as described in III.14.1. This vector would allow the visualization of the DsRed and EGFP proteins simultaneously in a cell that has received the DNA. This approach was also compared to the co-injection method of the two vectors prOMP$_{1.3}$-dsRed and prOMP$_{1.3}$-Y.

It appeared that only about 10% of embryos injected with the plasmid prOMP$_{1.3}$-dsRed-IRES-EGFP (see III.13.1.1.) displayed DsRed fluorescence when observed under the fluorescence stereomicroscope. The filter sets available at this microscope did not allow to distinguish between the red and the green signal, because intense signals could always be observed in both filter sets (seen for injections with either prOMP$_{1.3}$-dsRed or prOMP$_{1.3}$-Y plasmids). Therefore, analysis was conducted under the fluorescent microscope that has appropriate filters. Here, neurons that displayed DsRed fluorescence also displayed EGFP fluorescence although at a very low level in OSN somata and dendrites, as observed under a fluorescence microscope (Axiovert). In comparison, the co-injection experiment with prOMP$_{1.3}$-dsRed and prOMP$_{1.3}$-Y plasmids showed that the co-injection approach is less effective than the bicistronic approach, because in only two of 31 cases a co-expression could be observed. This is probably because the prOMP$_{1.3}$-dsRed and the prOMP$_{1.3}$-Y plasmids are not always co-segregated and translated in the same cell.

To unambigously demonstrate that the IRES element works in zebrafish embryos, I constructed another vector, prOMP$_{1.3}$-IRES-EGFP, which lacks the first cistron (see III.13.1.2.). Microinjection of this vector into one-cell stage embryos showed that EGFP fluorescence is observable and that this construct could be used to drive the expression of a gene of interest and a reporter gene that are connected via an IRES element.

3.1.11. Finding the Minimal Promoter

To determine which sequences of the 1.3 kb upstream region contributed to the observed directed expression, this fragment was consecutively shortened at its 5' end by the use of suitable restriction sites (*Hind* III and *Sac* I). The resulting EYFP expression in the olfactory epithelium was analyzed at 24, 48, 72, 96 and 120 hpf. Three different lengths of the upstream region were used, 1.3 kb, 0.6 kb, and 0.3 kb (Fig. IV.30.A). Around 800-900 embryos per construct, in total 2548 embryos were used for the analysis. 20% of embryos survived after 24 hpf. This high mortality rate cannot be due to the injection only, since similar amounts of uninjected embryos died, but is rather due to the low quality of eggs. It

was observed that fish lay bad quality eggs when they are not mated routinely. The effect of microinjection is however seen in the increased rate of malformed embryos. These were not included in the analysis. As a result 145 embryos were analyzed for prOMP$_{1.3}$-Y, while 155 and 182 embryos were included in the analysis for prOMP$_{0.6}$-Y and prOMP$_{0.3}$-Y, respectively.

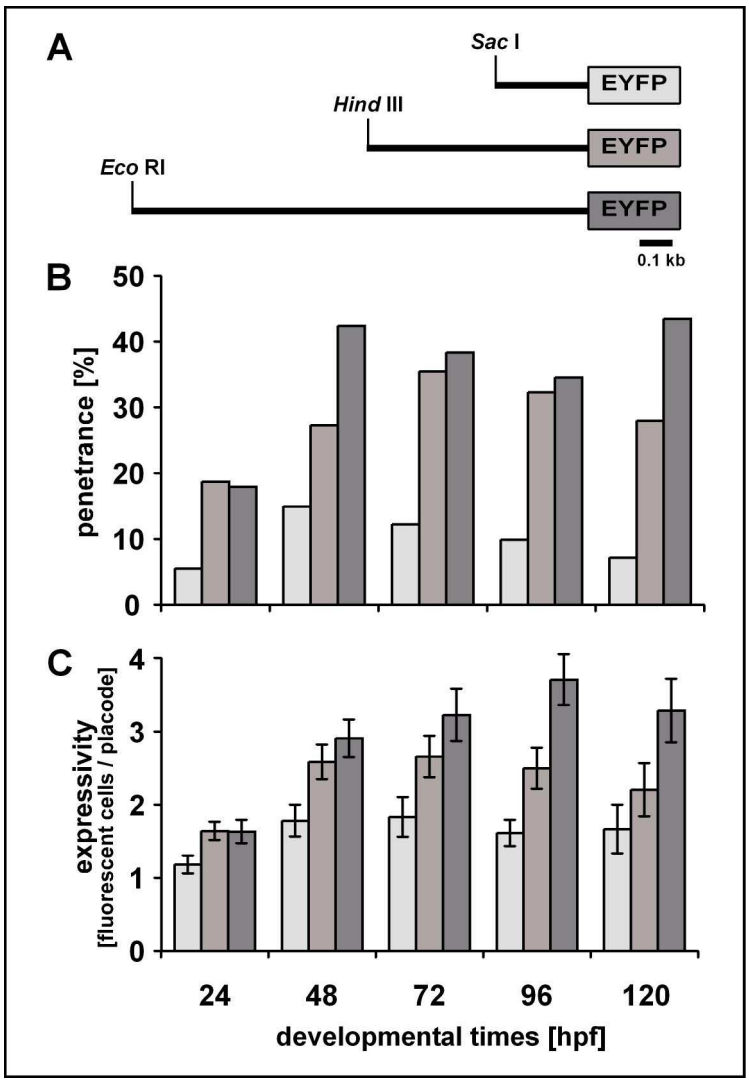

Fig. IV.30.: Penetrance and Expressivity of the EYFP Transgene in Three Reporter Constructs. A. Unilateral deletion analysis of the subcloned 1.4 kb of 5'-flanking sequence and 5'-UTR. Three fragment lengths were analyzed (0.3 kb, 0.6 kb, 1.3 kb promoter regions, 915, 819, 814 embryos were injected, respectively). B. Percentage of surviving fish (182, 155, 145 embryos, respectively) with at least one fluorescent olfactory sensory neuron (penetrance) given as function of developmental stage. The percentages increase up to 48 hpf, followed by a slight decrease, because mortality among the EYFP-expressing animals was somewhat higher than that of non-expressing fish. C. Number of fluorescent olfactory sensory neurons per positive placode (expressivity (mean +/- SEM) given as function of developmental stage. Note the continuous increase over time for the longest promoter region.

All fragments directed expression to OSNs at all developmental stages, but with differing frequencies. Generally longer fragments generated a higher percentage of positive fish (penetrance) and a larger number of positive OSN (expressivity) per olfactory placode (Fig. IV.30.B, C). At 24 hpf effects of the two longer regions, prOMP$_{1.3}$-Y and prOMP$_{0.6}$-Y, were undistinguishable, showing a penetrance of 18% and 19%, respectively (Fig. IV.27.B). This is much higher than the penetrance of the shortest fragment, prOMP$_{0.3}$-Y, being only 5.5%. Penetrance increased in all three cases and maximal values (43.5, 35.5, and 15%) were reached at 120 h, 72 h and 48 h, for prOMP$_{1.3}$-Y, prOMP$_{0.6}$-Y and prOMP$_{0.3}$-Y, respectively.

The number of labeled OSN per placode increases steadily with development for all the constructs, however maximums were reached at different stages (Fig. IV.30.C). At 120 h on average 3.3 cells were detected in the embryos injected with the prOMP$_{1.3}$-Y construct, but only 2.2 cells in fish injected with the prOMP$_{0.6}$-Y, and 1.7 in fish injected with the prOMP$_{0.3}$-Y construct. During the analyzed period prOMP$_{1.3}$-Y reached its maximal expressivity at 96 h with 3.7 cells, prOMP$_{0.6}$-Y at 72 h with 2.7 cells and prOMP$_{0.3}$-Y at 72 h with 1.8 cells. The maximal value obtained was 13 cells within a single olfactory placode for the prOMP$_{1.3}$-Y promoter region.

These numbers represent the overall increase in expressivity, but not necessarily the expressivity within a single individual fish, especially since not all the embryos included in the analysis survived the observation period. To get a more accurate representation of the expressivity, the expressivity of those fish surviving the whole period were plotted for prOMP$_{1.3}$-Y separately. This evaluation included 14 embryos and showed that there is a continuous increase in the number of positive neurons per placode reaching a maximum of around 5 positive cells per positive placode at 120 h (see Fig. IV.31.A). This is in good correlation with the growth of the olfactory placode as quantified by the number of positive cells in the *zOMP in situ* hybridizations (Fig. IV.31.B and see Fig. IV.27.).

Thus, a 0.3 kb fragment of the 5' upstream region of the OMP gene is already sufficient to drive directed expression in olfactory sensory neurons. However, the penetrance of reporter gene-expressing fish, as well as the average expressivity can be significantly increased by the addition of 1 kb of upstream region, that was present in the longest construct. Therefore, the prOMP$_{1.3}$-Y vector is the vector of choice for further experiments.

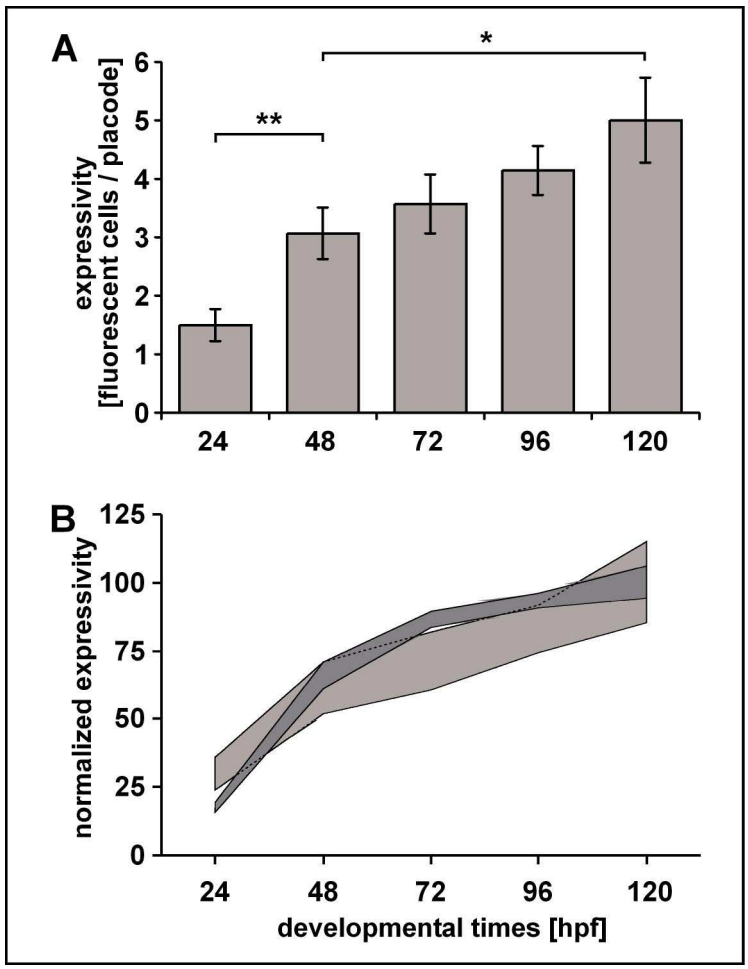

Fig. IV.31.: Comparison of the Endogenous OMP Expressivity and the prOMP$_{1.3}$-Y Transgene During Early Development. A. Longitudinal study. A defined set of 14 individually followed embryos was used to determine numbers (mean +/- SEM) of fluorescent cells per olfactory placode for five developmental stages. Significance of increases is evaluated by a paired Student's t test. **, P<0.005; *, P<0.03. B. Values depicted in panel A are normalized to maximal expressivity and their confidence interval (+/- one SEM) is shown (light grey). Values for the expressivity of endogenous OMP are taken from Fig. IV.27. and normalized correspondingly. Their confidence interval is shown in dark grey. Note the close correspondence of developmental time course for endogenous OMP and the transgene.

3.1.12. Search for Germline Transgenic Founder Zebrafish

It would be interesting to have germline transgenic zebrafish rather than transient transgenic ones. The expression pattern of a transgene reflects usually that of the endogenous gene from which the promoter of the endogenous gene is derived (for review, see MacDonald and Swift, 1998). The penetrance and expressivity of the genes is much higher than that of transient transgenic fish. However, it is not easy to obtain integration and germline transmission of the injected fragment. In general, only 2-5% of injected positive fish integrate the exogenous DNA and transmit the transgene (Stuart *et al*., 1990), although sometimes, higher integration efficiencies (up to 50%) can be obtained (Higashijima *et al*., 1997). Founder fish display mosaic germlines and the levels of GFP expression vary (Stuart *et al*., 1990; Higashijima *et al*., 1997).

In this study linearized plasmids were used to allow for the integration of the plasmid DNA. Embryos were raised to sexual maturity and screened for germline-transmitting

founders. For this purpose, injected fish were mated to each other or wild-type fish and the embryos were analyzed for EYFP expression. In total, only 12 fish could be analyzed, due to a very bad survival rate of fish in our facility and in particular to an increased mortality rate of injected embryos as compared to normal ones. None of the analyzed embryos (in total 1486 embryos were observed) displayed EYFP fluorescence. To exclude the possibility that the fluorescence was not observed because of a too low expression of the reporter gene, PCR was performed on genomic DNA extracted from the pooled progeny embryos using specific primers against the EYFP gene. Control experiments included genomic DNA that was extracted from embryos where one and four transient transgenic embryos were mixed with 99 and 96 wild-type embryos, respectively. A specific band of expected size was observed in these controls (see Fig. IV.32.; lanes 7-10), indicating the sensitivity of the PCR approach. Additionaly, the injected plasmid DNA was used as control, which was positive in the PCR reaction with EYFP primers and negative for the endogenous gene (lane 11). Wild-type genomic DNA was used as negative control (lanes 5 and 6). Another control included an endogenous gene (GAPDH) to check for the integrity of genomic DNA. As shown in Fig. IV.32. (bottom row) in all cases a specific band was obtained, except in the plasmid DNA (lane 11) and null control (lane 12). Genomic DNA was used at two different volumes, 1 or 2 µl. This appeared useful, because the extracted DNA is not very clean and in some cases better results could be obtained with one or two µl of genomic DNA. In total the genomic DNA of 9 fish injected with the prOMP$_{1.3}$-Y construct, two fish injected with the prOMP$_{1.3}$-dsRed and 1 fish injected with the prOMP$_{1.3}$-dsRed-IRES-EGFP construct were analyzed by PCR. In only one case the PCR appeared to be positive with the EYFP primers. The positive fish was the prOMP$_{1.3}$-dsRed-IRES-EGFP fish (lanes 3 and 4).

Recently, 7 further embryos that displayed EYFP fluorescence after injection survived to adulthood. However, these fish could not be tested yet, since they have not reached sexual maturity.

The transgenic founder (F0) fish can now be bred to generate homozygous transgenic populations of fish that can be maintained for generations without loss of the integrated transgene. For this purpose F0 fish are crossed with wild-type fish, and their progeny (F1) are grown to sexual maturity. The presence of the transgene can then be detected by analyzing GFP expression in F1 fish. Alternatively, live transgenic F1 fish can be identified by performing PCR on genomic DNA isolated from caudal fin clips. Positive F1 fish are then mated, and an F1 pair should normally produce 25% transgenic homozygote positives, 50% heterozygotes and 25% wild-type homozygotes among their progeny. After the F2 embryos are grown to sexual maturity, and individual F2 fish are mated to wild-type fish, the progeny can be analyzed as before. An F2 fish homozygous for the transgene should be able to pass the transgene to 100% of its offspring when mated to a wild-type fish. Homozygous F2 fish can be mated to each other to produce a large population of homozygous fish.

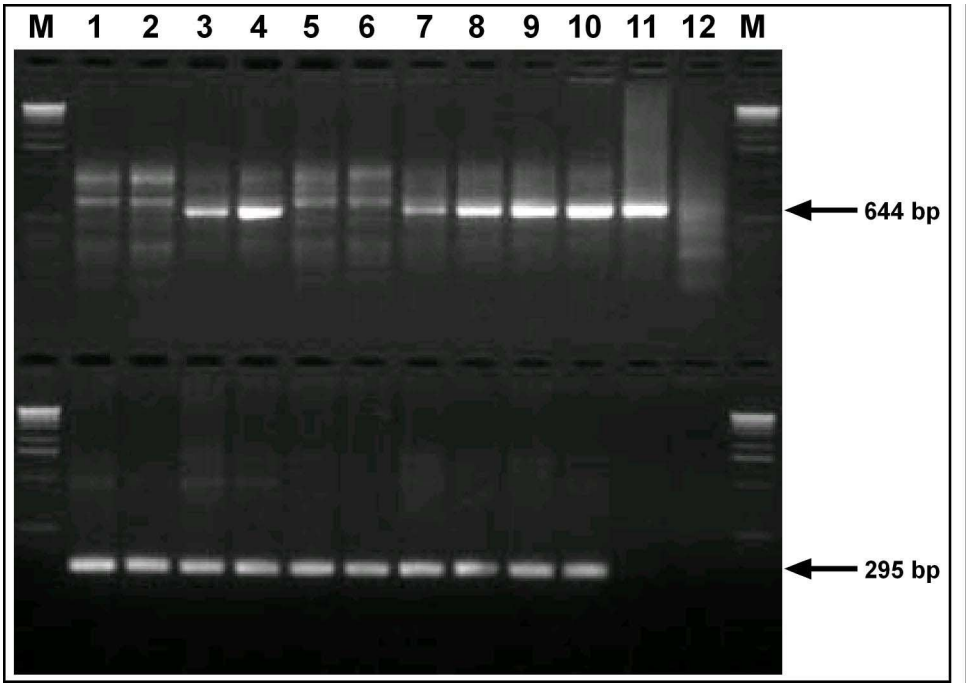

Fig. IV.32.: PCR Analysis to Detect Transgenes in the Genome of Microinjected Zebrafish Embryos. An ethidium bromide stained gel that shows a PCR that was performed on genomic DNA extracted from 48-72 h zebrafish embryos using EYFP-specific primers (top row) and primers against the endogenous gene GAPDH (bottom row) as control. PCR with EYFP primers generate a band of 644 bp while the PCR with GAPDH generate a band of 300 bp. Lanes 1 and 2 (1 and 2 µl DNA, respectively) represent embryos of a non-transgenic founder fish as indicated by the lack of the EYFP PCR product. Odd numbered lanes correspond to 1 µl of used genomic DNA, while even numbered lanes correspond to 2 µl. Lanes 3 and 4 represent embryos of a transgenic founder fish as indicated by a PCR product with both primer sets. Lanes 5 and 6 represent wild-type embryos, giving no PCR product with the EYFP primers as expected. Lanes 7 and 8 represent a control population of embryos where one transgenic embryo was mixed with 99 wild-type embryos. As expected bands were observed with both primers, indicating the sensitivity of the PCR approach. A second control is shown in lanes 9 and 10 of a population of embryos where four transgenic embryos were mixed with 96 non-transgenic embryos. Again in both cases bands are seen with both primer sets. In lane 11 plasmid DNA of the injected plasmid was amplified with both primer sets. As expected only a band in the EYFP PCR was observed. Lane 12 represents a negative control, where no DNA was included.

3.2. The *dlx2* Gene - A Marker for Granule Cells

The *dlx2* gene belongs to the distal-less family of homeobox genes that specify positional information in the head. In mice mutant for this gene differentiation within the forebrain is abnormal and the fate of a subset of cranial neural crest cells is respecified (Qiu *et al*., 1995). Mice lacking Dlx-1 and Dlx-2 show no detectable cell migration from the subcortical telencephalon to the neocortex and also have few GABA-expressing cells in the neocortex. Moreover, they lack all interneurons in the olfactory bulb (Anderson *et al*., 1997). The zebrafish homologue of this gene was described in 1994 (Akimenko *et al*., 1994). There it was shown that the *dlx2* gene is expressed in the forebrain of embryonic zebrafish. For the *dlx2* homologue in mouse it is known that this gene is expressed in granule cells of the olfactory bulb (Anderson *et al*., 1997; Bulfone *et al.*, 1998). A cDNA clone containing the full-length sequence of this gene was kindly provided by M. Westerfield and analyzed in the

olfactory bulb of zebrafish. Similar to the approach employed for the *OMP* gene the aim was to identify a region that drives specific expression of a reporter to the granule cells.

3.2.1. The *dlx2* Gene Labels Granule Cells in the Adult Olfactory Bulb

The distribution of *dlx2* transcripts was studied in the adult olfactory bulb by using digoxigenein-labeled antisense RNA probes. Labeled cells are found in the central part of the olfactory bulb, a region corresponding to the inner cell layer. Double labeling experiments with zns-2 underline the non-overlapping distribution of the zns-2 epitope and the *dlx2* transcripts (Fig. IV.33.A). The expression pattern is consistent with the expression pattern known for rodents (Anderson *et al*., 1997). Thus, *dlx2* appears to be a useful marker for granule cells in the adult olfactory bulb of zebrafish. To investigate its tissue-specificity and its developmental expression pattern *in situ* hybridization was performed using whole mount embryos.

2. Ontogenesis of Expression of the Zebrafish *dlx2* Gene

Expression of *dlx2* was studied at the first three days of embryonic and larval development. A massive expression of *dlx2* transcript is observable in the forebrain region already at 24 hpf (Fig. IV.33.B). Also very apparent is the expression in the visceral arches (mandibular and hyoid, first and second arrow in Fig. IV.33.B, respectively) and gill arches (arrowheads in Fig. IV.33.B). At 48 hpf a rostral-to-caudal loss of *dlx2* expression from the gill arches is seen, while expression in the forebrain persists (not shown). Strong expression of *dlx2* is apparent in the forebrain and presumptive olfactory bulb at 72 hpf. Serial sections through a 72 h embryo are shown in Fig. IV.33.C-H. The expression pattern in the forebrain can be described as being very central, very close to the midline within the brain. This appears to be a complementary expression to that of *ztbr1* (Fig. IV.37.I-K), which appears to give a more peripheral staining, as it would be expected from the adult distribution of mitral cells and granule cells. Additional expression is seen in pectoral fin buds (open arrowhead) and in visceral and branchial arches (arrowheads in Fig. IV.33.C and D) during the whole period analyzed.

As a result, *dlx2* is expressed strongly in the forebrain and the presumptive olfactory bulb. Since it is a good marker for granule cells in the adult olfactory bulb of zebrafish, it is presumable that the labeled cells in the forebrain region of embryos constitute the same cell population, although it cannot be excluded that additional cell populations are labeled. This would mean that granule cells are present already as early as 24 hpf, the earliest time point investigated here.

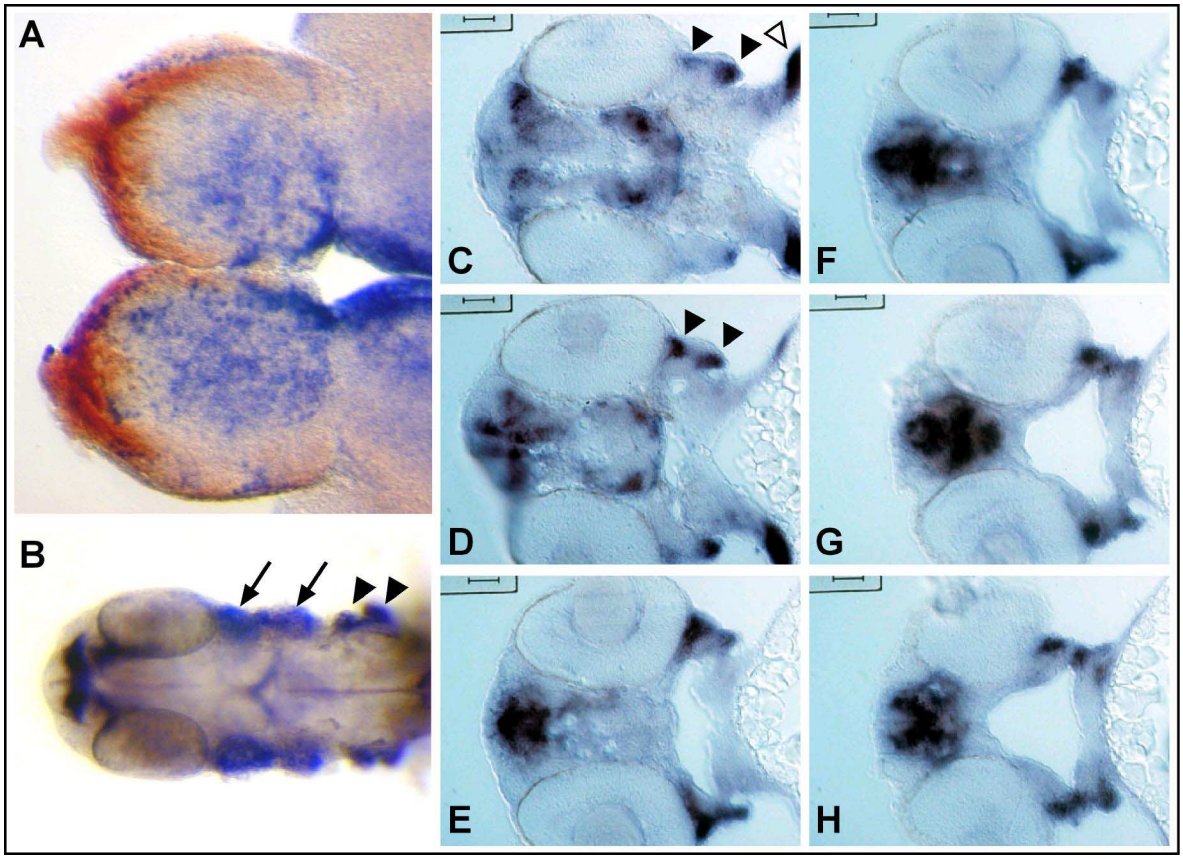

Fig. IV.33.: Expression of the *dlx2* Gene During Development and Adulthood. A. Double labeling of granule cells (*in situ* hybridization with an antisense probe against *dlx2*) shown in blue and glomeruli (antibody staining against zns-2, detected with a peroxidase-coupled secondary antibody and DAB as substrate) shown in brown. B. Dorsal view of an *in situ* hybridization of a 24 h zebrafish embryo, showing high expression in a band of cells in the forebrain area, the visceral (mandibular and hyoid, first and second arrow, respectively) and gill arches (arrowheads). C-H. Serial cross-sections through a 3 d old zebrafish embryo hybridized with the *dlx2* probe. Note that the staining in the forebrain area is very central, apposed to the *ztbr1* staining which is more rostral, indicating the separation in localization of cell bodies already at this early stage. Arrowheads point to branchial arches, while open arrowhead points to the pectoral fin. Scalebar corresponds to 50 µm in all panels.

3. Cloning of the *dl x2* Upstream Region

Using an *Eco* RI probe from the *dlx2* cDNA clone (shown in Fig. IV.34.A) a zebrafish genomic cosmid library (RZPD) was screened. Unfortunately, this clone could not be recovered from the RZPD. Therefore, a zebrafish genomic PAC library was screened with the same fragment. The strongest hybridizing clone (BUSMP706N2039Q4) was ordered and analyzed further.

Restriction fragment analysis of this clone indicated that the insert has a size of about 115 kb (Fig. IV.34.B). Southern analysis revealed hybridizing bands from 300 bp to about 48 kb in size. The genomic surrounding of the *dlx2* gene was analyzed using three different probes (shown in Fig. IV.34.A). The *Eco* RI 5' probe was not useful in revealing potential upstream fragments since generally more than one fragment hybridized. Therefore the shorter *Pst* I probe was used, which helped to identify the upstream lying fragments. A *Pst* I fragment of 11 kb was chosen for further analyses. This fragment was successfully cloned into the pBluescript vector. Sequence analysis revealed the presence of 450 bp of the *dlx2*

gene and the upstream region (see Fig. IV.35.A). Using many restriction enzymes this fragment was digested and subjected to Southern analyses to reveal smaller fragments that could be subcloned into the pACSF-Y expression vector for testing (for details see Fuss, 2001). Resulting fragments were either relatively long or very short. For first experiments a 5.5 kb *Sac* I fragment was chosen. This fragment was amplified using a sequence-specific primer to introduce a *Nco* I site to subclone the fragment directly upstream of the ATG codon and a vector primer (for details see III.13.2.). This cloning resulted in the vector prdlx2$_{5.5}$-Y. Another fragment was obtained by digesting this one using the enzyme *Afl* II that cuts about 1.3 kb upstream of the ATG codon, resulting in the construct prdlx2$_{1.3}$-Y. Attempts were made to reamplify the whole fragment by long template PCR with prdlx2nco and vector primers, but were not successful. However, using a subcloning strategy additional 4 kb of upstream sequence was added by digesting the original *Pst* I clone with *Sac* I and cloning this fragment into the *Sac* I site of the prdlx2$_{5.5}$-Y vector. This resulted in the prdlx2$_{9.5}$-Y construct. The proper orientation of the *Sac* I fragment within the construct was confirmed by sequencing.

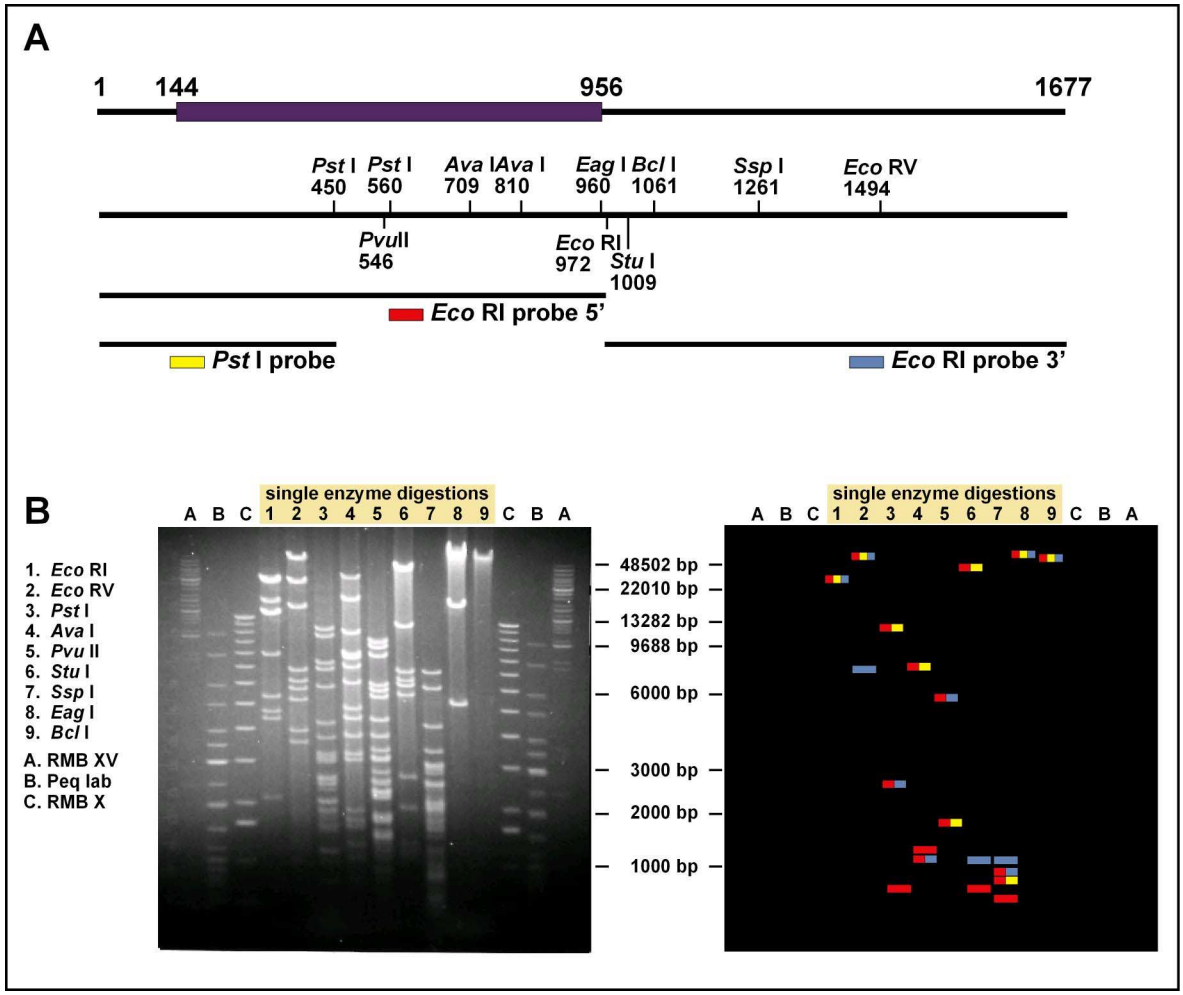

Fig. IV.34.: Southern Analysis of the *dlx2*-PAC Clone. A. *dlx2*-cDNA, restriction sites and three probes that were used for hybridization of the *dlx2*-PAC clone. The coding region is shown in violet. B. Restriction analysis of the *dlx2*-PAC clone with various enzymes and demonstration of the hybridizing fragments using the three different probes shown in A. Hybridizing fragments are color-coded with colors specified in A. Some fragments hybridized with two, others with all three probes. Length of fragments are given in base pairs.

3.2.4. Potential of Upstream Regions to Drive Reporter Gene Expression

To test the potential of *dlx2* upstream regions to drive reporter gene expression in granule cells three constructs prdlx2$_{1.3}$-Y, prdlx2$_{5.5}$-Y and prdlx2$_{9.5}$-Y were linearized and injected into one-cell stage embryos. Reporter expression was observed starting at 24 h and continued in daily intervals. The shortest construct did not reveal any (specific) expression in 1542 injected embryos analyzed. The medium sized construct prdlx2$_{9.5}$-Y showed unexpectedly expression in the olfactory placodes but not in the olfactory bulb of injected embryos (see Fig. IV.35.B-D). Expression started quite late and was first observed at 4 d after injection. The morphology of labeled cells was analyzed in higher magnifications (Fig. IV.35.D). The fluorescent signal displayed by the cells is lower than that of cells labeled with prOMP$_{1.3}$-Y, but the percentage of positive embryos was quite high (32%). In total 1212 embryos were injected of which 128 embryos survived and were analyzed at 4 d after injection. Cells displaying the morphology of OSNs were clearly distinguishable by their labeled cell bodies and dendrites projecting towards the naris. It is not clear however, if this is the only cell type that is labeled, since the number of positive neurons is so high that non-labeled cells cannot be clearly distinguished. No staining in any other cells in the entire embryo were observed. The reason for this untypical but specific labeling of cells in the placodes is not clear so far. A labeling of axons was not observed in any case. The longest construct was expected to elicit a specific signal in granule cells. 1056 embryos were injected with the longest construct prdlx2$_{9.5}$-Y of which 382 embryos survived. Of these 78 were scored to be positive giving a percentage of about 20% of transgenic embryos. The expression started as early as 24 hpf and the expression pattern of the transgene was highly diverse, with many cells labeled over the body. Generally there was an apparent labeling of neurons in the hindbrain (see Fig. IV.35.H, I), in the eye and in motor neurons. Expression in non-neuronal tissue like muscle was also evident in rare cases (see Fig. IV.35.J). Occasionally, expression was observed in the forebrain region and the presumptive olfactory bulbs (see Fig. IV.35.E-G). The cell bodies of EYFP-expressing cells were round and small and they did not have any axon projecting outward of the olfactory bulb region, which indicates that these could be local interneurons. In some rare cases positive cells in the forebrain displayed the morphology of mitral cells, being large cells with dendritic arbors and thin axons that project through the anterior commissure (see Fig. IV.35.K-M).

As a result it can be said that the longest upstream fragment tested here, being 9.5 kb in length, seems to be the most potent region to drive reporter gene expression. Expression occurs mainly in neurons and occasionally also in granule cells of the olfactory bulb. The number and frequency of labeled cells is quite low. For some purposes, a low number of expression is desirable, however for functional overexpression a higher penetrance and expressivity of the transgene would be desirable. This makes this region not the best candidate for being used in subsequent functional experiments.

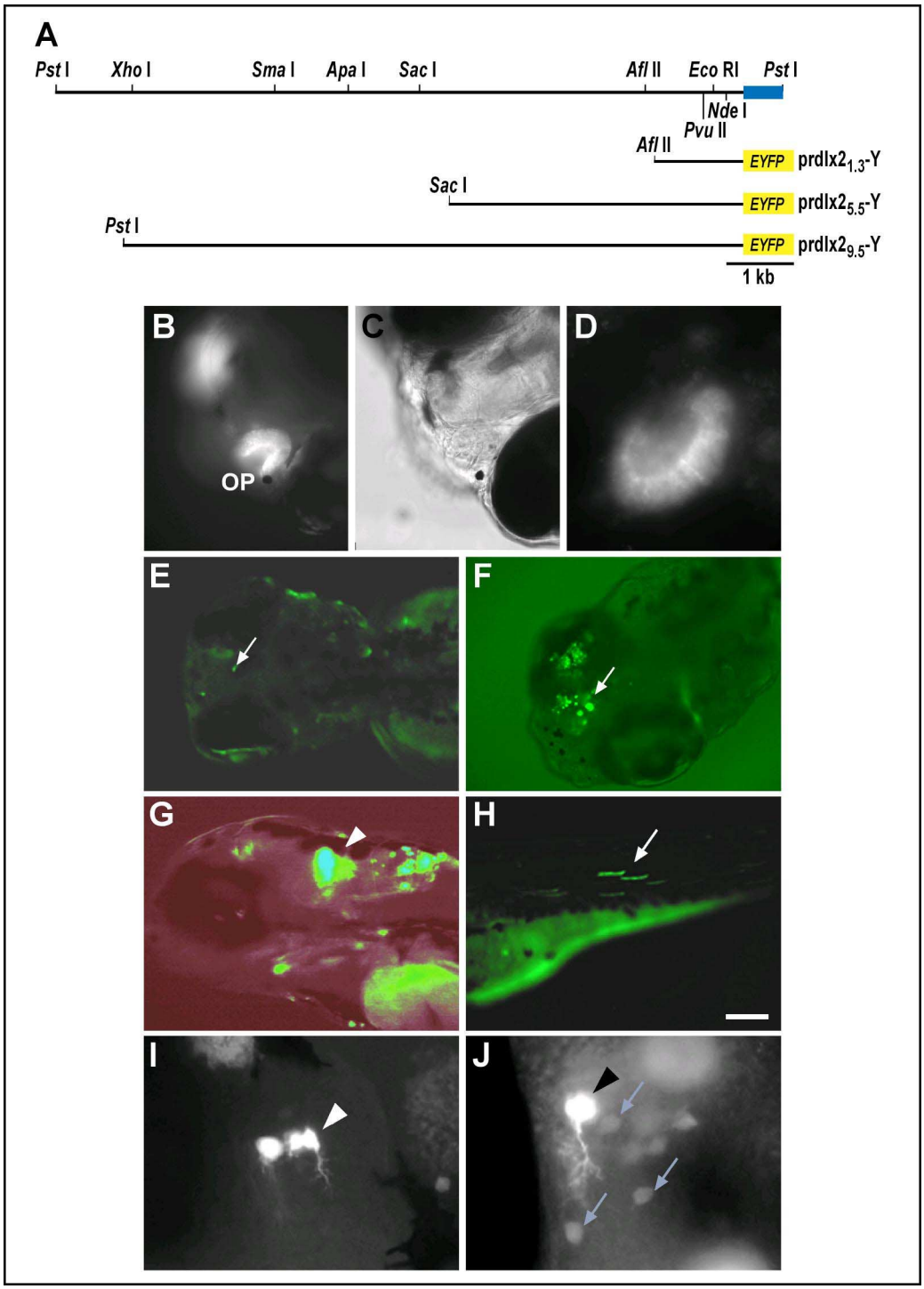

Fig. IV.35.: ***In Vivo*** **Reporter Gene Expression of Various Upstream Fragments of the** ***dlx2*** **Gene.** A. An 11 kb genomic *Pst* I fragment containing 450 bp of the *dlx2* gene was mapped using various restriction endonucleases. Always the first restriction site with respect to the *Pst* I site within the *dlx2* gene are shown. Three different sizes of the upstream fragment were subcloned into the expression vector pACSF-Y. B-J. One-cell stage embryos were injected with the prdlx$2_{5.5}$-Y or the prdlx$2_{9.5}$-Y vector and analyzed several days later under fluorescent light. A fluorescent (B) and bright-field (C) image of an embryo 4 days after injection with the construct prdlx$2_{5.5}$-Y are shown. Olfactory placodes (OP) display bright fluorescence. D. High magnification of one placode shown in (A). Apparently all cells within the placode display bright fluorescence. (E-J) Images of embryos 3 days after injection with the prdlx$2_{9.5}$-Y construct. E. Only two granule cells (arrow) are labeled, while in (F) many granule cells (arrow) display fluorescence. Other parts of the embryo are free of labeling. G. Cells in the hindbrain area display bright fluorescence (arrowhead). Here, no granule cells are labeled in the olfactory bulb. H. Ectopic expression in muscle cells (arrow). (I-J). Bright-fluorescing mitral cells (arrowheads) with their dendrites and granule cells (arrows) in 3 day old embryos after antibody staining with α-GFP antibody and detection with a secondary antibody coupled with Alexa Fluor-488. Scalebar corresponds to 100 µm in D-I, and to 50 µm in A and B and to 28 µm in all other panels.

In fact quite recently, for mouse and zebrafish *dlx* genes, it was demonstrated that two highly conserved regions constituting an enhancer are located in the intergenic region between *dlx4/dlx6* genes (corresponding *to Dlx5/Dlx6* genes in mouse) (Zerucha *et al.*, 2000). These sequences extend over a few hundred base pairs and are the potential site of action of a vast number of regulatory factors (Zerucha *et al.*, 2000). Reporter transgenes containing these two sequences and a basal β-actin promoter are expressed in the forebrain of transgenic mice and zebrafish with patterns highly similar to the endogenous genes. The similar genomic organization of the *dlx1/dlx2* gene pair suggested that an analogous enhancer region could be present in the *dlx1/dlx2* intergenic region and that this region could function as an enhancer for granule cell specific expression of the reporter genes.

3.2.5. Cloning of the *dlx1-dlx2* Intergenic Region

Southern analysis of the PAC clone we had in hand using a probe prepared from the 3'-end of the *dlx2* cDNA (probe *Eco* RI 3' shown in Fig. IV.34.A), revealed that it contains not only the *dlx2* gene, but the intergenic region as well (see Fig. IV.34.B). Long template PCR with primers designed to the 3' ends of *dlx1* and *dlx2* genes (dlx1/2iF and dlx1/2iR) were used to amplify this genomic region; however, this approach was not fruitful. Therefore, a hybridizing 7.5 kb *Eco* RV fragment, which had the appropriate size to contain the whole intergenic region, was chosen for subcloning into the pBluescript vector. Subcloned fragments having the expected size were sequenced and in two cases positive inserts could be revealed. The clones D4 and D10 showed homology to the 3'-UTR of the *dlx1* gene and on the other end homology to the 3'-UTR of the *dlx2* gene, as expected.

The next step was to clone this fragment into the expression vectors, prdlx$2_{9.5}$-Y and prdlx$2_{5.5}$-Y, downstream of the EYFP gene, a position that would reflect the genomic organization of the *dlx2* gene. Unfortunately, it appeared difficult to subclone the intergenic region into either of the previously constructed vectors that contain varying lengths of upstream regions of the *dlx2* gene. None of the many different approaches appeared to be successful. In the first case one can speculate that the pBluescript vector behaves refractory to take up an additional 7.5 kb of insert (it already has an insert of about 9 kb). But the unsuccessful trials to subclone this fragment into the prdlx$2_{5.5}$-Y vector, which has 4 kb less insert, argues against this notion. Here, at least it should not be a big problem. The actual reasons for these subcloning problems remain unsolved so far.

In conclusion it can be said that a relatively large region (9.5 kb) lying upstream of the *dlx2* gene displays the potential to drive expression in the granule cells of the olfactory bulb. However, the specificity, penetrance and expressivity of this fragment do not lie in the expected range. A higher specificity would be desired in order to be able to observe the granule cells, without being disturbed by the fluorescence of other labeled cells. A high penetrance and expressivity would be desirable to increase the probability of generating an effect with an overexpression construct. The contribution of the dlx1/2 intergenic region could not be tested so far. However, it is still intriguing to think that the intergenic region could confer the required specificity and increase expressivity of the transgene, probably even when using short upstream fragments of the *dlx2* gene as a minimal promoter region.

3.3. The *tbr1* Gene – A Marker for Mitral Cells

T-box genes are a family of transcription factors that have a highly conserved DNA binding domain named the T-domain (Papaioannou and Silver, 1998). They have important developmental functions during gastrulation (e.g. Brachyury, Wilkinson *et al*., 1990), mesodermal specification (*Tbx6*, Chapman and Papaioannou, 1998), limb patterning (*Tbx2-Tbx5*, Gibson-Brown *et al*., 1998; Logan *et al*., 1998), and brain development (*Tbr1*, Bulfone *et al*., 1995, 1998).

Here the isolation and the expression pattern of zebrafish *tbr1* are described. The *ztbr1* gene has a highly restricted pattern of central nervous system expression, which is largely limited to some regions in the telencephalon and mitral cells in the olfactory bulb.

1.1.1. Cloning of the Mitral Cell-Specific Gene *ztbr1*

Two approaches were employed to clone the *tbr1* gene for zebrafish. First, degenerate primers (ACSF-0, ACSF-1, ACSF-2, and ACSF-4) were designed to highly homologous regions of the known *tbr* genes from human and mouse. PCR reactions were performed on cDNAs reverse transcribed from RNAs isolated from olfactory epithelia, olfactory bulbs or total brain. Products of expected size were obtained only in the reactions using olfactory bulb and whole brain cDNA as template. Subcloning and analysis of several clones by sequencing did not reveal any positive clone (Fuss, 2001). As a second approach a zebrafish brain cDNA library was screened at low stringency with a mouse cDNA clone for the *Tbr3* (*mTbr3*) gene. The *mTbr3* gene seems to have a more restricted expression pattern in the brain than *Tbr1*, being only expressed in mitral cell bodies (A. Bulfone, personal communication). A zebrafish brain cDNA library (prepared as described in III.10.6.) was screened with a radiolabeled probe prepared from the *mTbr3* gene at low stringency (37°C, ULTRA Hyb™ Hybridization Solution, Ambion). About 39 positive clones of varying intensity were obtained on both replicas when screening 5 x 10^5 clones. The strongest hybridizing 12 clones were rescreened. Five of them appeared to be still positive after the second screen. Single clones (n = 27) were obtained and sequenced. Two of them corresponded to the same cDNA and showed homology to *Tbr* genes and mainly to *Tbr1* in Blast searches. These clones coded for a 5'-truncated protein. Both of the isolated cDNAs seem to represent most likely the zebrafish homologue of *Tbr1*. Since our interest was to get the zebrafish homologue for *Tbr3* additional attempts were undertaken by screening a zebrafish bulb cDNA library in which the amount of DNA molecules representing the mitral cell-specific genes should be higher as compared to the brain library. When 2 x 10^5 clones were screened with the *mTbr3* probe, nine positive clones were obtained. Eight clones were rescreened and from seven of these clones good sequences could be obtained. However, none of these clones showed homology to any T-box or T-box-related gene. It seemed that we were less successful in finding positives in the olfactory bulb library than the brain library, for unknown reasons. Having the zebrafish *tbr1* clone in hand we now concentrated on this clone.

Efforts were taken to get the full-length sequence of the partial clone obtained in the first screen. For this purpose, the olfactory bulb and brain cDNA libraries were screened again, using the 5'-part of the isolated *ztbr1* cDNA. However, no longer clone than we had in hand could be isolated. In a second approach RACE PCR was performed with primers designed to the very 5'-part of the known *ztbr1* cDNA. The template DNA for the RACE PCR consisted of olfactory bulb and brain cDNA. However, all subcloned and sequenced PCR fragments did not contain any additional sequence information when compared to the previously identified *ztbr1* cDNA clone (Fuss, 2001). All attempts to get the full-length sequence of the *ztbr1* clone failed.

3.3.2. Structure of the Partial *ztbr1* cDNA

So far we could identify two cDNA clones representing a single mRNA, that have a length of about 2.5 kb. These clones were sequenced from both ends. The sequence revealed that the cDNA is complete at the 3'-end, since a long poly A-tail was present. Using the sequences we have obtained in this study, amino acid sequences were aligned and a phylogenetic analysis which is shown in Fig. IV.36 was performed. The phylogenetic tree shows that *ztbr1* is most homologous to the mouse and human *Tbr1* genes.

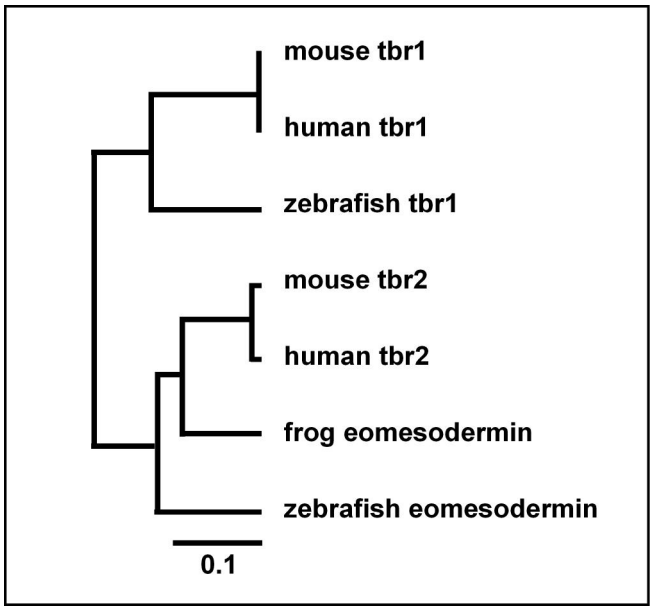

Fig. IV.36.: Structural Comparison of the *ztbr1* Gene and Other *Tbr* Genes. The phylogenetic tree was constructed based on the amino acid sequence of mouse, human and frog *Tbr1* and *Tbr2* genes (using a sequence of 218 amino acids). Zebrafish *tbr1* is more related to human and mouse *Tbr1* genes, as compared to zebrafish or frog *tbr2* (eomesodermin). Scalebar indicates an evolutionary distance of 0.1%.

Independent from our efforts, another group recently published the sequence of zebrafish *tbr1* and *tbr2* (*eomesodermin*) genes (Mione *et al*., 2001). The clone identified for *tbr1* in their study, which was the longest of three clones that corresponded to different length of the same cDNA was about 150 bp longer than the clones isolated here. It is interesting that in both cases, despite screening different libraries, only partial clones could be isolated. This could reflect a difficulty of the reverse transcriptase to transcribe beyond a particular region, giving rise to truncated cDNAs.

3.3.3. The *ztbr1* Gene Is Expressed in Mitral Cells in the Adult Olfactory Bulb

To confirm the assumption that the *ztbr1* gene isolated here is expressed in the mitral cells of the olfactory bulb like its homologues in other species (Bulfone *et al*., 1998), *in situ* hybridizations were performed. DIG-labeled antisense RNA probes prepared from the 3'-UTR of the *ztbr1* cDNA and hybridized to cryosections of the olfactory bulb as well as to whole mounts of the brain. Specific staining was obtained in large cells of the olfactory bulb located within the glomerular layer (see Fig. IV.37.A). At high magnification of vibratome-sectioned olfactory bulbs, the mitre shape of the labeled cells was apparent (Fig. IV.37.B, arrows), indicating that the labeled cells are mitral cells. Additionally, staining was observed in dorsal parts of the telencephalon, but other brain regions were not labeled. This expression pattern is in accordance with the expression of the mammalian homologues (Bulfone *et al*., 1998). Interestingly, the *ztbr1* gene was also expressed in a small number of cells in the sensory area of adult olfactory epithelia (Fig. IV.37.C). The identity of these cells is not clear. They lie dispersed throughout the sensory area and are not localized to a particular expression domain that could correspond to a zone of OR expression. Within a lamella they appear to have an intermediate to apical localization; almost never a basal localization was observed. This is consistent with findings that indicate that the mouse homologue, *mTbr1*, is only expressed in post-mitotic cells (Bulfone *et al*., 1995).

The number of the *ztbr1*-positive cells in the olfactory bulb was determined as being 1200 / olfactory bulb (Fuss, 2001). This number of mitral cells / olfactory bulb is much higher than the number estimated by Dr. W. Michel (in Friedrich and Laurent, 2001). There the number was determined to be about 300. It is not stated in that publication how this number was determined, therefore it cannot be said, what the reason for this large difference in numbers is. In a very pessimistic scenario one would have to postulate that the *ztbr1* gene is not expressed exclusively in mitral cells. Nevertheless, its restricted expression makes it still an interesting gene to study.

3.3.4. Expression of *ztbr1* During the First Three Days of Development

To study the spatial and temporal expression of the isolated *ztbr1* gene in the embryo, whole mount *in situ* hybridizations were performed. At 24 hpf *ztbr1* transcripts are readily detectable in the dorsal telencephalon and are also expressed in a strip of cells in the diencephalon adjacent to the dorsal telencephalon (Fig. IV.37.D-F, arrow in D and in E, respectively). By 48 hpf most dorsal telencephalic areas, including the presumptive olfactory bulb, express *ztbr1* (Fig. IV.37.G-I). The strip of cells in the dorsal diencephalon continues to express *ztbr1* (Fig. IV. 37.H, arrow). This expression persists also at 72 hpf (Fig. IV.37.L, arrow). At 72 hpf cells in the optic stalk (arrow in Fig. IV.37.J) and some cells in the eye start to express *ztbr1*. The main staining is located in the rostral part of the telencephalon and olfactory bulb, in a cell population that could represent the mitral cells (Fig. IV.37.J-L).

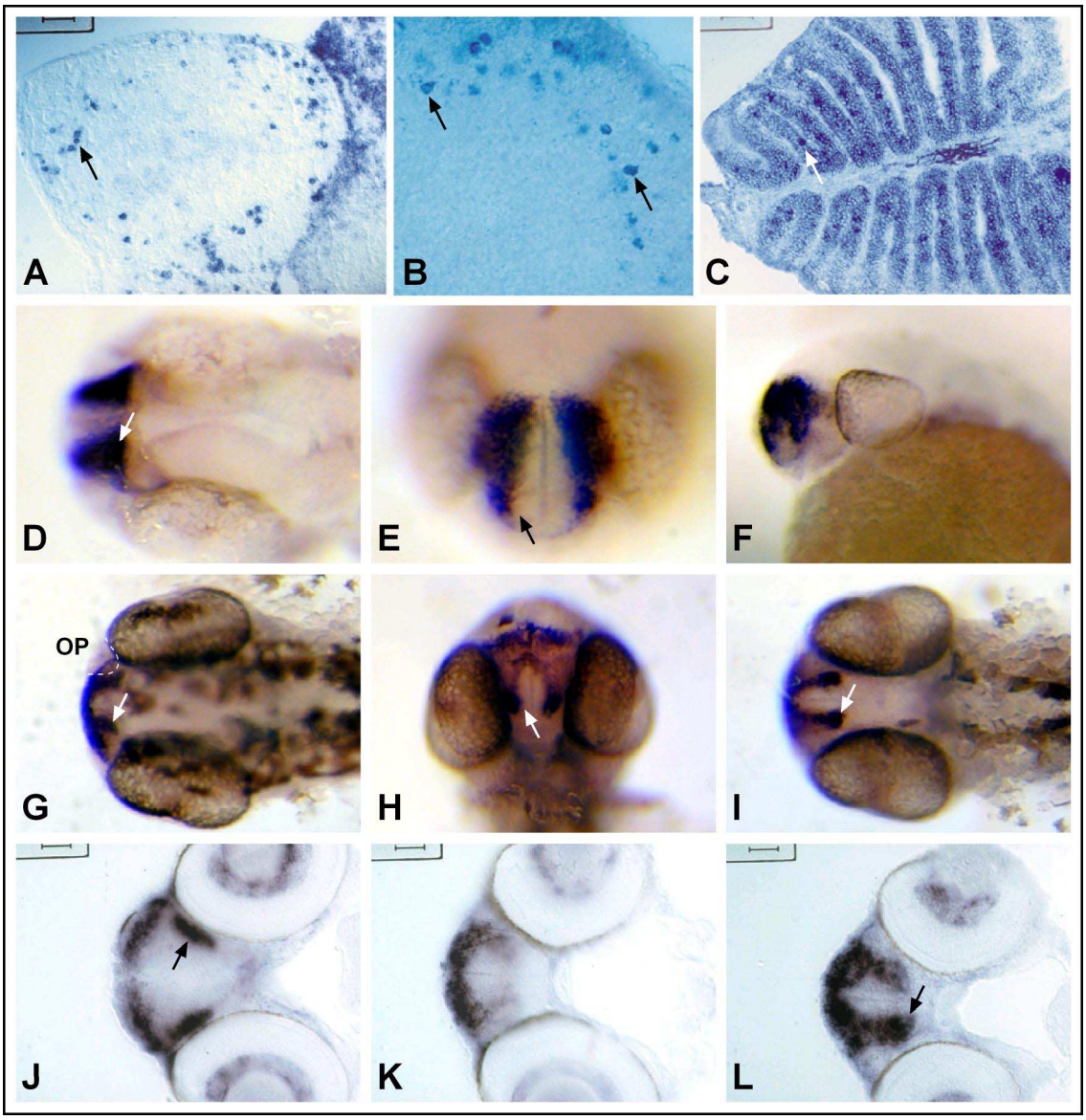

Fig. IV.37.: Expression of the *ztbr1* Gene in the Adult and Embryonic Olfactory System. *In situ* hybridization on cryostat sections (A) or vibratome sections prepared after whole mount hybridization of the olfactory bulb (B) and cryostat sections of the olfactory epithelium (B). Antisense RNA probes were prepared from the 3' untranslated region of the *ztbr1* gene. Intense staining can be seen in the glomerular layer of the olfactory bulb (A). At higher magnification the mitre shape of the labeled cells becomes evident. Thus the labeled cells correspond to mitral cells. In the olfactory epithelium a number of cells in the sensory area are intensely stained. The cell type of labeled cells is unclear. One, two and 3 d old embryos were hybridized as whole mount's and 3 d old embryos were cryosectioned subsequently. Dorsal (D), frontal (E) and lateral (F) view of a 24 h embryo showing strong expression in the forebrain region (arrow in D), and the diencephalon (arrow in E). Dorsal (G), frontal (H) and ventral (I) view of a 48 h embryo showing expression in dorsal telencephalon (arrow in G) and diencephalon (arrow in H and I). Consecutive sections through a 3 d old embryo are shown in J-L. Note the strong expression in the rostral part of the forebrain corresponding to the localization of mitral cells in the olfactory bulb. Expression in the optic stalk (arrow in J), in cells in the diencephalon (arrow in L) and in some cells in the eye can also be seen. Scalebar corresponds to 50 µm in all panels, except for D and E being 70 µm.

3.3.5. Larval Expression of *ztbr1*

Studies of the expression of *ztbr1* at later stages by *in situ* hybridization were performed on cryostat sections. Several stages (7 d, 14 d, 21 d) are interesting in terms of the developing glomerular pattern (Lieberoth, 1999). At around 21 d the glomerular pattern appears to be complete, since all stereotypic glomeruli known from the adult pattern are already visible (Lieberoth, 1999). To see if the localization of mitral cells correlates with glomerular development 21 d old zebrafish were analyzed at first instance. *ztbr1* is strongly expressed in cells within the olfactory bulb as well as in the optic stalk. Moreover, *ztbr1* transcripts are also present in a small population of cells within the olfactory epithelium (Fig. IV.38.). Cells lie dispersed in the olfactory epithelium in all layers of the apical-basal extension. The identity of these cells is not clear.

The localization of labeled cells within the olfactory bulb already resembles very much the organization of the glomerular pattern as visualized by DiI staining of OSN axons and terminals (shown in Fig. IV.38.F). The labeling in the very dorsal sections of the zebrafish (Fig. IV.38.A and B), for example, strongly resembles the localization of the dorsal lying 'dorsal cluster' (Baier and Korsching, 1994). These data indicate that the mitral cell bodies are located close to the OSN axon terminals, which is in accordance with the adult localization (see Fig. IV.20., and also, Fuss, 2001).

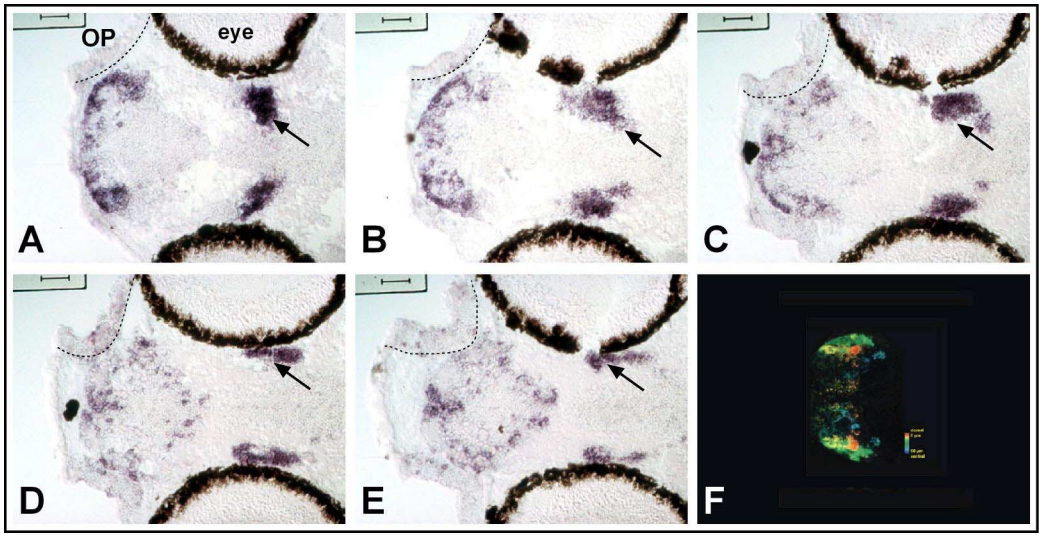

Fig. IV.38.: Larval Expression of the *ztbr1* Gene. *In situ* hybridization on consecutive sections (A-E, dorsal→ventral) of a 21 d old zebrafish hybridized with a *ztbr1* probe prepared from the 3' untranslated region of the *ztbr1* cDNA. Many cells in the olfactory bulb are stained. A number of cells in the olfactory placode (sections C-E) and the optic stalk (see arrows in A-E) are also labeled. F. DiI labeling of a 21 d old zebrafish. The olfactory bulb was dissected out and analyzed as a whole mount by confocal imaging. The glomerular pattern is color coded with warmer colors lying more dorsal and colder colors lying more ventral. Note the close correspondance of position of labeled cells in A-E as compared to F. The image shown in F was kindly provided by B. Lieberoth. Scalebar corresponds to 25 µm in all panels.

3.3.6. Number of *tbr* Genes in the Zebrafish

Attempts were undertaken to determine the family size of the isolated *ztbr1* gene. This was necessary, since the initial intension was to find the *tbr3* gene for zebrafish. *mTbr3* has a more specific expression pattern than *mTbr1*, being only expressed in mitral cells of the olfactory bulb (A. Bulfone, personal communication). Southern blot analyses were performed by digesting genomic DNA with various restriction enzymes that was isolated from individual zebrafish; hybridization was done with a probe derived from the 5' part of the *ztbr1* gene (Fuss, 2001). In all digest two bands were observed, while four bands were evident in the *Pst* I digest. The four bands in the *Pst* I digest are due to a *Pst* I cutting site within the gene. In summary, these data indicate the presence of two *ztbr1*-related genes, making the presence of a *mTbr3* homologue in zebrafish improbable.

3.3.7. Isolation of A Genomic Clone Containing the T-box

The *ztbr1* cDNA sequence that was isolated here is not full-length. The actual aim of this study was to find a functional promoter that can drive expression of reporter genes to mitral cells. Despite no available full-length cDNA clone that would help in the characterization of the genomic organization of the *ztbr1* gene, a genomic PAC library was screened. At first instance a probe corresponding to *mTbr3* was used for screening. 43 moderately hybridizing clones, but no strong hybridization signal were detected at low stringency (ULTRA Hyb™, 37°C). This approach did not give the desired outcome, because too many only slightly positive clones were obtained. The same library was screened using a 5'-fragment (254 bp) generated from the partial *ztbr1* cDNA clone. Four strongly hybridizing clones were obtained and ordered to ensure that at least one of them contains *ztbr1*. These clones were also positive in the screen performed with the *mTbr3* probe. All four clones were analyzed by Southern analysis. One of them appeared negative in the rescreening, while the other three remained to be positive. Southern analysis of restriction fragments generated by single and double digestions with a probe corresponding to the most upstream fragment revealed several positive fragments (shown for clone 4 in Fig. IV.39.A). The 12 kb *Eco* RI and the 8 kb *Eco* RI / *Eco* RV fragments were chosen for further analysis and isolated from PAC clone 4. These rather large fragments were chosen, because no full-length cDNA clone exists and no sequence information for zebrafish *tbr1* is available on the cDNA level for the 5'-end. Looking at the organization of the mouse *Tbr1* gene it is obvious that the exon-intron structure is quite complex. Assuming that a similar organization might be present in zebrafish too, it appeared important to isolate a quite large fragment that would contain the lacking 5'-parts of the gene and hopefully some upstream sequences. Despite many attempts to subclone these two fragments none was successful for unknown reasons. Recently, a smaller fragment, a 2 kb *Hinc* II fragment, that was positive in the Southern hybridization (Fig. IV.39.A), was subcloned into the pBluescript vector from PAC clone 4. Several colonies of the subcloned fragment were sequenced from both directions and revealed homology to *Tbr*-family genes on one end, indicating that the other end lies within an intron. Interestingly, the fragment showed highest homology to the zebrafish *eomesodermin* gene, which corresponds

to *Tbr-2* in mouse and human, although identity was not 100%. This means that the PAC 4 clone represents a clone of the family of T-box genes, but does not necessarily correspond to the *ztbr1* gene.

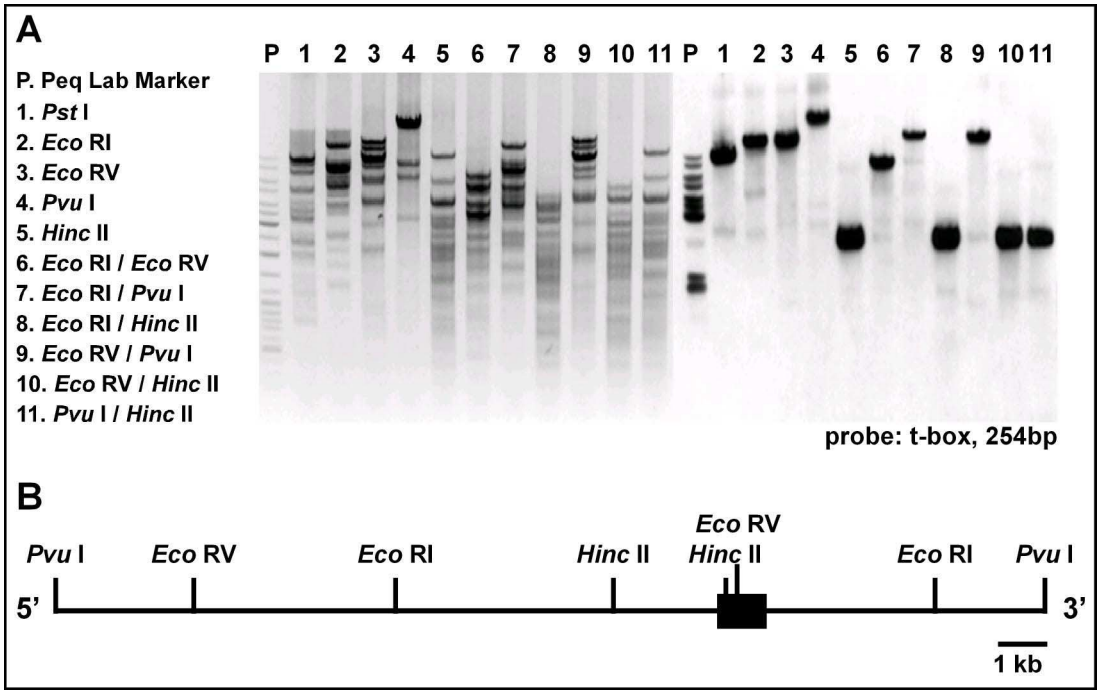

Fig. IV.39.: Genomic Organization of A *ztbr1*-Related Gene Present on PAC Clone 4. A. Restriction analysis of the PAC clone 4 using single and double digests with various enzymes (left). Southern hybridization was performed using a probe generated from the t-box region of the isolated cDNA clone, which is the 5'-most fragment available. B. Genomic organization of the *ztbr1*-related gene as elucidated from the Southern analysis shown in A.

In conclusion, one can say that a suitable marker for mitral cells, *ztbr1*, has been identified. Its expression corresponds closely the expression of *Tbr1* genes in other species. A quite large, although not full-length cDNA clone, was isolated and used for searching the upstream region of this gene. While three genomic clones have been isolated and analyzed by Southern hybridization, it turned out that in one of the closer examined cases (PAC 4) the genomic clone did not correspond to the *ztbr1* gene. This unexpected result led us to screen the genomic library with a probe generated from the 3'-end of the *ztbr1* gene. It turned out that again 4 strongly hybridizing clones could be identified, which are different from the previously identified ones. This means that probably the 5'-part of the *ztbr1* gene that lies in the T-box was not the best probe to be used for screening the genomic library. It seems that some more effort is needed to isolate a correct genomic clone and a suitable upstream fragment of this very interesting gene.

V. DISCUSSION

Understanding the mechanism of how olfactory sensory neurons find their target glomerulus is a very interesting problem in olfaction. Although olfactory receptors themselves have been implicated to play a role in pathfinding, only an involvement in the positioning in the anteroposterior axis of the olfactory bulb could be shown (Wang *et al*., 1998). Hence, other guidance molecules appear to be necessary within the target region to determine the correct position of the glomerulus. A plethora of guidance molecules has been identified to date in various systems and the Eph family of receptor tyrosine kinases constitutes one of the most interesting families among these. A very important function of these molecules in the guidance of retinal ganglion cells to the tectum has been demonstrated, recently (Drescher *et al*., 1995; Feldheim *et al*., 1998, 2000). In this study the expression of Eph receptors and their ligands in the developing and adult olfactory system of zebrafish was examined to get a better understanding if and how these molecules could play a role in axonal pathfinding of olfactory sensory neurons. Novel spatially and temporally restricted patterns of expression of receptors and ligands have been uncovered in the olfactory system of zebrafish. Eph receptors and their ligands appeared to be expressed in all main cell types of the olfactory system, being the olfactory sensory neurons, the mitral cells and the granule cells. In some cases, a complementary expression of interacting Eph receptors and ephrins was observed, such that activation could occur at the interface of their expression domains. Moreover, sites of overlapping expression of binding partners or of ligands or receptors only were evident. The functional implications of such overlapping expressions will be discussed.

Functional studies require the ability to access the cells or tissues of interest specifically. This is of great importance if the gene of interest has a widespread expression and many functions. This description holds true for many of the Eph receptors and ephrins. Thus, before doing functional experiments it became necessary to establish a system that will allow studying the function of an Eph/rin in a particular cell type. For this purpose, in the second part of this work, cell type-specific genes and promoters were isolated and characterized for the three main cell types of the olfactory system where Eph/rin genes were expressed. So far, the isolation of a specific promoter has been accomplished for olfactory sensory neurons and this promoter has been used to investigate the function of a particular ephrin (L5/ephrin-B2a) in OSNs. No obvious changes in the termination of the transgene-expressing neurons were observed. In the case of the granule cells several upstream fragments were tested in reporter constructs. It turned out that some of these regions have the potential to drive reporter gene expression to granule cells, but also to many other neurons throughout the whole embryo. Additional work is needed to enhance the specificity of this upstream region, i.e. by addition of a downstream region. Such a fragment has already been isolated, but its contribution needs to be tested yet. For the mitral cells a gene that is highly expressed in these cells was isolated. Although no upstream region of this gene could be isolated and tested so far, it is of great interest to do so. Many of the Eph/rins are expressed in mitral cells, and a promoter for mitral cells will be an ideal tool to investigate their functions in these cells.

1. Eph Receptors and Ephrin Ligands in the Olfactory System of Zebrafish

1.1. Zebrafish Eph Receptors and Ligands Show High Homology to their Mammalian Counterparts

Comparison of the extracellular domains of zebrafish Eph receptors and ligands with their mammalian counterparts shows that in zebrafish this family of receptor tyrosine kinases is as diverse as in higher vertebrates. However, compared to mammals the repertoire of Eph receptors and ligands is far from complete. It is known that in zebrafish many genes have been duplicated during evolution (Postlethwait *et al*., 1998); thus, some genes exist as two paralogues of a single gene in mammals. This is also true for some genes of the Eph receptor and ephrin family (e.g. *L5/ephrin-B2a* and *L6/ephrin-B2b*). In most of the cases both genes were expressed and expression domains appeared to be largely overlapping, although distinct sites of expression existed. The overlap of expression domains was not due to cross-hybridization of probes, since genes could be distinguished from each other. Probes were prepared from the 3'-UTR of the cDNAs and in case of *L5/ephrin-B2a* and *L6/ephrin-B2b* only a homology of 12% could be determined in this region. The preservation of expression of both homologues of the same gene suggests that each of them has developed an important role during zebrafish embryogenesis (Postlethwait *et al*., 1998; Force *et al*., 1999). Their differential expression patterns would allow the interaction with different Eph receptors. In addition, subtle changes in their predicted amino acid sequences could also allow the two proteins to exhibit differential receptor-binding affinities or to initiate alternative signaling cascades.

1.2. During Development Eph Receptors and Ephrin Ligands Are Mainly Localized to Mitral Cells and the Anterior Commissure.

Analysis of most of the Eph/rin genes in the zebrafish olfactory system revealed that many of the receptors and ligands appear to be expressed in the projection neurons (mitral cells) that are located within the olfactory bulb. This was shown both by *in situ* hybridization and affinity probe *in situ* assays. Both are important techniques that give a general picture of expression patterns. The latter one involves the use of fusions of the Eph receptor or ephrin extracellular domain with alkaline phosphatase as affinity reagents to stain whole mount embryos. As a result of the promiscuous interaction of receptors and ligands within a binding specificity class, each reagent will detect an entire class of Eph receptors or ephrins.

Using the fusion protein probe EphA-rtk6-AP no expression could be detected in any tissue of the zebrafish embryo as well as in the adult olfactory pathway. A possible explanation for the failure of fusion proteins to detect all sites of expression is that co-expression of Eph receptor and ephrin ligand can lead to sequestering of the less abundant

component into receptor-ligand complexes that are not accessible to the soluble detection reagent (Flenniken *et al*., 1996). Indeed, direct evidence for such masking has been obtained by analysis of transgenic mice that overexpress ephrin-A5 (Sobiezczuk and Wilkinson, 1999).

By *in situ* hybridization analysis on 1-3 d wild-type zebrafish embryos I could show that *rtk2/EphA4b* and *rtk7/EphA4c* are expressed in the majority of cells in the anterior forebrain including mitral cells. Of potential importance was the finding that cells strongly positive for the EphA4 probe were located immediately dorsal to axons that pass through the anterior commissure. In addition to these receptors mitral cells express *L5/ephrin-B2a* and *L6/ephrin-B2b* ligands specifically. These data show that mitral cells express both the EphA4 receptor as well as ephrin-B2 ligands. Consistent with the mRNA expression patterns, EphA proteins were localized at a low level on mitral cell axons and strongly on AC axons, which include many other axons as well. Moreover, ephrin-B proteins were expressed strongly in mitral cell bodies and eventually their dendrites and at low levels on their projections (for a summary see Fig. V.1.). These findings may suggest a potential interaction between EphA4-positive cells adjacent to AC axons and ephrin-B-expressing AC axons. EphA4, although being an A family receptor, binds to ephrin-B2 with high affinity, and the transmembrane ephrin ligands have been shown to induce signaling upon receptor binding (Gale *et al*., 1996; Holland *et al*., 1996). The stronger expression of ephrin-B proteins on mitral cell bodies and dendrites as compared to their axons also indicate the possibility of an interaction with a receptor that is expressed on OSNs. Although no expression of EphA and/or EphB proteins was detected this possibility cannot be excluded and will be discussed in the next section.

Role in Anterior Commissure Formation?

Taken together these data suggest that AC axons appear to migrate preferentially along a pathway defined by *EphA4* expression in the basal forebrain, in such a fashion that these axons do not migrate into the *EphA4* expression domain. This is consistent with the notion that EphA4 acts as a signal for mitral cell axons bearing ephrin-B ligands to be guided appropriately. Also consistent with this model, *ephrin-B2a* and *ephrin-B2b* mRNA was detected within the mitral cell bodies, which suggests that these transmembrane ligands are expressed on mitral cell axons as they extend through the anterior commissure.

Consistent with these data obtained in zebrafish, a previous report indicated the requirement for EphA4 in the formation of the AC. The AC is a major forebrain axon tract that consists of an anterior (aAC) and posterior branch (pAC), which connect both lobes of olfactory bulbs and temporal cortex, respectively in mice (Dottori *et al*., 1998).

Both *in vitro* and *in vivo* studies have suggested that the Eph receptor family regulates axon guidance through mechanisms of contact repulsion rather than attraction (Drescher *et al*., 1995; Nakamoto *et al*., 1996; Wang and Anderson, 1997). For example, EphB2 is normally expressed in areas ventral to the commissure, and the commissural axons express a ligand for EphB2, ephrin-B1. This suggested that EphB2 repels AC axons from entering this ventral area via ephrin-mediated signals (Holland *et al*., 1996; Brückner *et al*., 1997). Direct analysis of the function of EphB2 by targeted mutation in mice has revealed a profound defect in the pathfinding of commissural axons. Here, the posterior tract of the AC innervates the floor of the brain aberrantly (Henkemeyer *et al*., 1996).

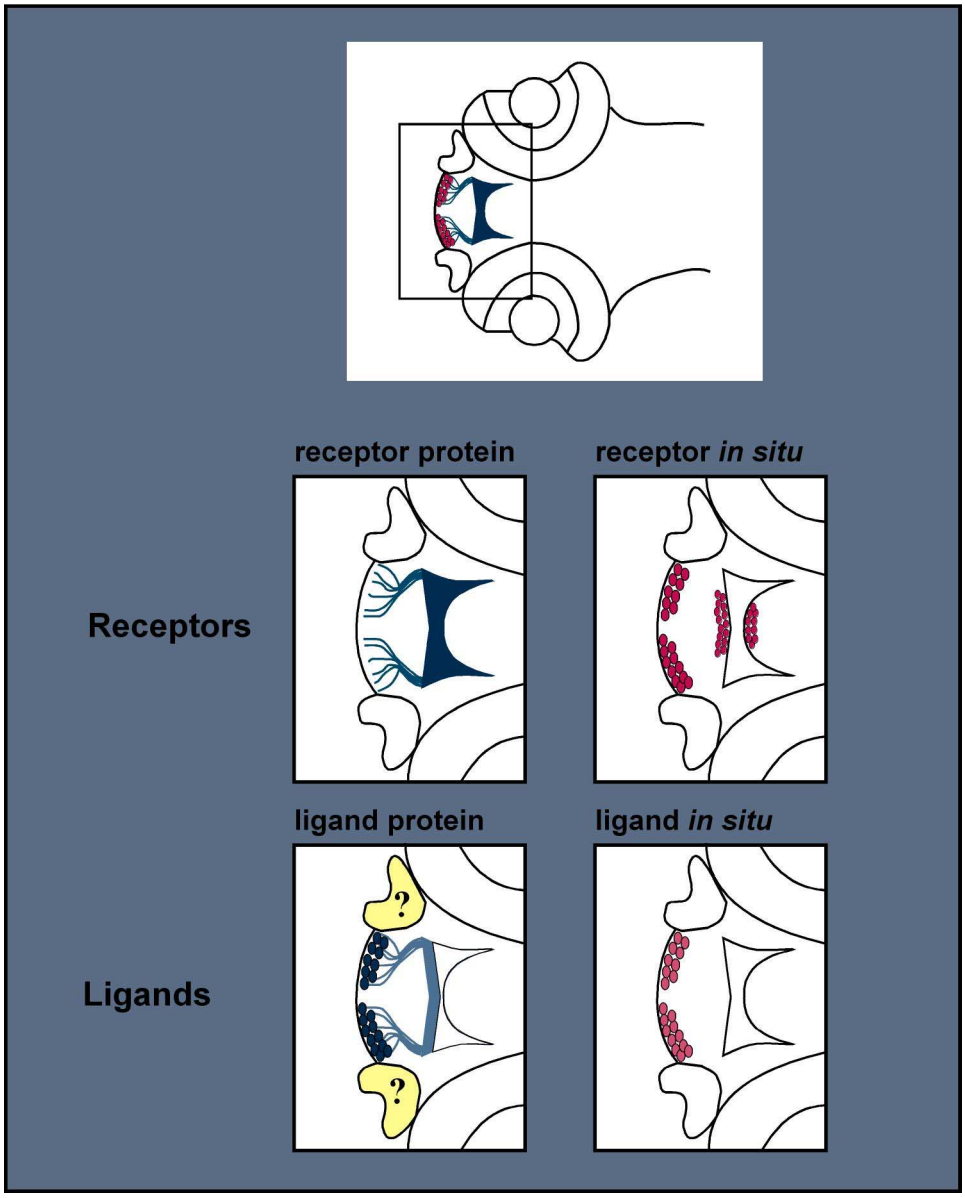

Fig. V.1.: Schematic Representation of Dorsal Views of Gene Expression Domains in Mitral Cells and the Anterior Commissure in P3 Wild-Type Zebrafish Embryos. In the A family, the ligand fusion protein (ephrin-A-L*4*-AP) shows strong expression in the anterior commissure and a low level in mitral cell projections. Corresponding *in situ* hybridizations indicate a strong expression of receptor mRNA (*rtk2/EphA4b*) in mitral cell bodies and cells located dorsal to the anterior commissure. The B family receptor fusion protein EphB-rtk8-AP shows a strong expression of ligand in the mitral cell bodies/dendrites, while only a weak expression in axonal projections was detected. Consistent with this expression ligand mRNA (*L5/ephrin-B2a*) was detected in mitral cell bodies. EphA4 is the only receptor of the Eph family known to interact with both ligand families. Questionmarks indicate the possible presence of a yet unknown receptor in the olfactory sensory neurons.

Eph receptors and their ligands are not the only molecules involved in the pathfinding of commissural axons. Several studies have examined the mechanisms of axonal guidance using the central olfactory projection as model system (Schwob and Price, 1984; Brunjes and Frazier, 1986). In rodents, the major output neurons of the olfactory bulb, the mitral cells, project long axons caudolaterally into the telencephalon and form the lateral olfactory tract (LOT). Although the projection is simple, two types of guidance cues are proposed in this system. First, before the first mitral cell axons grow out of the olfactory bulb, the future

pathway of these axons is already marked with a specific array of early-generated neurons called lot cells, which are recognized by monoclonal antibody lot1 (Sato *et al*., 1998). Growing mitral cell axons strictly follow this cellular array, and pharmacological ablation of lot cells stalls the growth of mitral cell axons (Sato *et al*., 1998). Co-culture experiments have shown that mitral cell axons must contact lot cells to grow (Sugisaki *et al*., 1996; Sato *et al*., 1998), suggesting a short-range guidance cue from lot cells. Mitral cell axons are also guided by a long-range cue. When the olfactory bulb is co-cultured in collagen gel with the septum that positions caudomedially next to the olfactory bulb, mitral cell axons are repelled by a factor released from the septum (Pini, 1993). This repulsive activity was recently identified as Slit (Li *et al*., 1999; Tuyen *et al*., 1999). These studies together suggest that the septum secretes Slit, which directs mitral cell axons growing along the natural pathway, seemingly by overriding the LOT cue (Li *et al*., 1999).

Role in Axon Growth, Sorting and Glomerulus Formation?

None of the analyzed molecules appeared to exhibit restricted spatial expression patterns that would coincide with major periods of axon growth, axon sorting and glomerulus formation in the primary olfactory pathway.

Recently, it was shown that EphB2 and its two ligands are expressed on the perikarya of mitral cells in the early developing bulb of rats (St John *et al.*, 2001). During that stage mitral cell perikarya condense from a multicellular layer to a discrete single cell layer. Thus, subpopulations of cells must sort out and segregate from each other to establish the adult-like cytoarchitecture of the olfactory bulb. The expression pattern of EphB2 and its ligands in the olfactory bulb is consistent with a role in this process. During the early post-natal period there is a shift in the distribution of both EphB2 and ephrin-B1 from the mitral cell perikarya to their dendritic processes within glomeruli. Initially, the apical dendrite of a mitral cell branches and enters several glomeruli but it is later pruned so that by the end of the first post-natal week each mitral cell has an apical dendritic tree that arborizes in a single glomerulus (Brunjes *et al*., 1982). It was suggested that EphB2-ephrin-B1 interactions in the olfactory glomeruli could somehow contribute to the pruning and reorganization of mitral cell dendritic trees (St John *et al.*, 2001).

Various shifts in the distribution of receptor and ligand proteins within particular cells were observed in immunohistochemical studies performed for one Eph receptor and its ligands in the olfactory system of rats (St John *et al*., 2001). In contrast no striking shifts in the spatial distribution of receptor and ligands on cells of the primary olfactory pathway were observed in zebrafish using mRNA *in situ* hybridization and fusion protein constructs. These methods give information about the presence of a particular transcript and the presence and localization of interacting partner(s), respectively. It turns out that the fusion proteins that are able to detect the receptor or ligand at the protein level, are not potent enough compared to antibodies to give detailed information about the localization of receptor and ligand proteins. This however would be important considering the possibility that the compartmentalization of the protein to different parts of the cells (dendrites, axon terminals) during different processes could have a functional significance. Such a mechanism could provide a means of using the same molecule in different processes that involve cellular interactions during the establishment of the neural circuitry.

1.3. Targeting Eph/rin Function by Gain-of-Function in the Embryonic Olfactory System of Zebrafish: Effectiveness, Limitations, and Essential Controls

There are essentially two ways to tackle the problem of ascribing physiological functions to gene products - to overexpress the gene or cDNA, or to inactivate it. Each strategy has its limitations; when expressed at higher-than-physiological levels, proteins can have widespread and non-specific effects, whereas the loss of one gene product can sometimes be compensated for by another gene product, causing an 'artefactual' absence of phenotype. Consequently, rigorous gene-function analyses now use a combination of these two techniques, along with the generation of site-directed mutations and the development of conditional knockout animals to avoid problems such as early lethality of a null mutation.

Different strategies have been developed to specifically inactivate genes, each with their own advantages and drawbacks in terms of speed, specificity and cost, and with their limitations in terms of applicability to a given gene or organism.

Gene targeting by homologous recombination involves replacing one, or in the case of diploid species possibly both, allele(s) of a gene at the endogenous locus with a mutant allele. This technology is now used effectively in haploid eukaryotes such as yeast and *Dictyostelium discoideum* (in which targeting of one allele is sufficient to achieve complete gene inactivation). It is also a routinely used technique in mice. Until recently, similar strategies had proven unsuccessful in the worm, fly, frog, and zebrafish. However, a way has now be found to adapt homologous-recombination-mediated gene targeting for use in flies (Rong and Golic, 2000). Although a similar strategy could not be established for zebrafish so far, the recent identification of ES-like cells have laid the ground for further developments (Ma *et al*., 2001).

An alternative, less time-consuming and cheaper technique to study loss of gene function is RNA interference (RNAi). This was initially carried out by injecting antisense RNA, but further studies revealed that sense RNA and double-stranded RNA (dsRNA) are also effective, and dsRNA is now most commonly used. The injected dsRNA interferes with gene expression by an unknown mechanism, possibly involving specific degradation or translation inhibition of the corresponding mRNA (see Bosher and Labouesse, 2000 for review). RNAi was developed in *C. elegans*, where it holds great sway. There have also been reports for effective RNAi in vertebrates, such as the mouse (Wianny and Zernicka-Goertz, 2000), frog and zebrafish (Wargelius *et al.*, 1999; Li *et al.*, 2000). Other laboratories, however, have failed to obtain effective, specific gene inactivation in these species, and it is unclear whether these discrepancies are the result of differences in the stage of development of the embryos used or in the particular RNA sequences that were injected (Oates *et al*., 2000). Many laboratories working with vertebrates are returning to the original principle of RNAi, using antisense single-stranded RNA or oligonucleotide fragments of antisense RNA. The new trick, however, is to incorporate modified bases (phosphorothioates or morpholinos) into the oligonucleotides to increase their stability, an approach that seems to be effective in both the frog and zebrafish (Nasevicius and Ekker, 2000).

Another strategy combines the transgenic and RNAi approaches, using vector DNAs encoding palindromic 'foldback' sequences to express dsRNA (Tavernarakis *et al*., 2000). This method allows tissue- and stage-specific gene targeting and is expected to be much less labourius than traditional gene inactivation by homologous recombination. However, it does not result in complete gene inactivation, as the level of the mRNA thought to be reduced only three-to-fivefold. Depending on the gene, this modest change may be either a drawback (if insufficient to affect the function of the gene product) or an advantage (for dosage-sensitive genes, in cases in which null alleles are lethal, or for screening for suppressors and/or enhancers of a phenotype in the same pathway).

A powerful approach for studying gene function in zebrafish *in vivo* is gain-of-function by ectopic expression of genes in a temporally and spatially specific way. However, there is still no simple and effective system for the temporal and/or spatial control of gene expression in zebrafish. To allow for such restricted gene expression it is necessary to have a suitable promoter that is expressed in the cells or tissue of interest. This kind of method would however be necessary to study the function of genes like Eph receptors and their ligands that are widely expressed during embryonic development and have many functions. A suitable promoter for olfactory sensory neurons could be identified for zebrafish. The effectiveness of this promoter will be discussed later (V.2.1.). This promoter was used in a gain-of-function approach to study the role of L5/ephrin-B2a in the primary olfactory pathway of zebrafish.

As discussed previously *in situ* hybridization experiments revealed that the *L5/ephrin-B2a* and *L6/ephrin-B2b* genes are expressed in the projection neurons within the olfactory bulb. On the protein level the presence of B family ligands was shown by affinity probe *in situ* hybridization with the fusion protein Eph-B-rtk8-AP. However, none of the tested receptors appeared to be expressed in the OSNs (Fig. V.1.). If one presumes that a yet unidentified B family receptor is present on these cells, an overexpression of a ligand for this receptor, should be able to interfere with receptor function. Neurons expecting to get in contact with their ligand in the target area should be misrouted if they are exposed to the ligand much earlier, e.g. already in the olfactory placode.

For this purpose an overexpression construct was prepared that allowed the simultaneous expression of a gene of interest and a fluorescent reporter gene in OSNs, to easily identify the cells where misexpression has taken place. Using this vector the *L5/ephrin-B2a* gene has been expressed in OSNs and their projections were analyzed in double labeling experiments with zns-2 that visualizes the entire OSN projections. The zns-2 labeling was used to visualize the 'wild-type' projections, while an antibody against GFP visualized the transgene-expressing neurons and their projections. Confocal analysis of the projections revealed that the transgene-expressing OSN axons project normally to the olfactory bulb. Although it would be expected, in some cases the no 100% overlap of red and green fibers was observed, which could be explained by an incomplete staining with the zns-2 antibody. An actual mistargeting of axons could have been explained with an adhesive role of L5/ephrin-B2a. In the wild-type situation OSN axons expressing a putative Eph receptor contact L5/ephrin-B2a expressing mitral cells in the olfactory bulb. Since in the gain-of-

function experiment OSN axons do not reach their target exactly, because the receptor in the axons is activated prematurely, resulting in the loss of 'adhesiveness'. Although, these experiments suggest L5/ephrin-B2a to have some function in the primary olfactory projection, clearly more work is required to determine its exact role. For example, one could take advantage of the properties of Eph receptors, which is the requirement for clustering as homodimers or heterodimers with other family members before signaling. A co-expression of a truncated receptor in OSNs in a dominant-negative approach would inactivate the signaling pathway of OSNs, thereby making the axons unresponsive to the ligand in the target cells. This experiment would probably give a similar result as the experiment performed here. Moreover, one could also think of the expression of a soluble form of the ligand by OSNs that would bind to the receptor on OSN axons prematurely. Since, clustering of ligands is required for receptor activation ligand binding would not activate the receptor, but would inhibit the OSN axons to interact with the ligand on mitral cells, because no binding sites are available.

Although Eph receptors and their ligands have been implicated in mainly having repulsive activities, adhesive properties have also been shown. What determines whether there is a repulsion or adhesion response to Eph receptor activation is not clear exactly. One possibility is that the nature of the cellular response is due to the presence of distinct mediators of signal transduction in different cell types. In favour of this, the same ephrin cue promotes neurite outgrowth of some neuronal cell types, and inhibits outgrowth in others (Gao *et al*., 2000). As overlapping Eph receptor and ephrin expression occurs in some neuronal cell populations, it will be important to analyze whether co-activation of Eph receptors and ephrins within the same cell modulates the nature of the cellular response. Alternatively, quantitative or qualitative differences in Eph receptor activation could lead to the assembly of distinct signal transduction complexes that regulate repulsion or adhesion.

The upregulation of adhesion might be concomitant with the suppression of repulsion or de-adhesion, and three mechanisms have been found that can affect this. One mechanism is the desensitization of growth-cone repulsion by overlapping Eph/ephrin expression that drives sustained receptor activation (Hornberger *et al.*, 1999). In the second mechanisms, co-expression of truncated EphA7 lacking the tyrosine kinase domain (encoded by alternative spliced mRNA) with full-length EphA7 promotes adhesion to cells expressing ephrin-A5 (Holmberg *et al*., 2000). Truncated EphA7 acts in a dominant-negative manner and presumably suppresses repulsion (Holmberg *et al*., 2000).

The third possible mechanism for the suppression of de-adhesion is through an inhibitory effect of other classes of RTKs. Activation of some RTKs leads to tyrosine phosphorylation of ephrin-B proteins independently of binding to Eph receptors (Brückner *et al.*, 1997; Chong *et al*., 2000). Surprisingly, phosphorylation of ephrin by the FGF receptor suppresses de-adhesion of cells that normally occurs upon clustering and activation of ephrin-B proteins (Chong *et al*., 2000). The mechanism of this inhibition is not understood, but possibly different tyrosine residues are phosphorylated, thereby triggering distinct downstream pathways.

Methodological Considerations

To understand gene function in developing vertebrate embryos, co-injection of a mRNA/DNA for a testing factor and a mRNA/DNA for a reporter protein is widely used. However, because of the mosaic segregation of injected nucleic acids during early embryogenesis, whether both mRNAs are translated in the same cell remains uncertain. In the present study, I have tested a new system of tracing the expression of a testing gene in zebrafish using an internal ribosome entry site (IRES) to express two proteins from the same mRNA template, thus eliminating the problem of independent translation observed in co-injection assays. A DNA construct was made for synthesizing a bicistronic mRNA for *L5/ephrin-B2a* and the *enhanced green fluorescent protein (EGFP)* reporter under the control of an olfactory sensory neuron-specific promoter. When the bicistronic construct was injected into one-cell stage zebrafish embryos, 10% of embryos displayed EGFP expression. To date, the usefulness of IRES has been tested only in mammal and avian systems (Martinez-Salas, 1999). This present study as well as another study published recently (Wang *et al*., 2000) shows that IRES can also be applied to a lower vertebrate such as zebrafish, indicating that the IRES element is likely to be functional in all vertebrates. Thus, the IRES element-containing construct might be a convincing means of analyzing gene function in developing zebrafish embryos.

The first IRES element was found in picornavirus RNAs, and subsequently many more have been identified in the 5'-UTR of other viruses as well as eukaryotic organisms (Kieft *et al.*, 1999; Mountford and Smith, 1995). These IRESes have sizes of about 450 nucleotides, although recently, elements of 9 nucleotides were found to have IRES activity (Chappell *et al*., 2000). In this study, I have used a commercially available IRES element that was originally isolated from encephalomyocarditis virus (ECMV). However, although this IRES element appears to function quite well in mammalian OSNs (Mombaerts *et al.*, 1996), it does not seem to function optimally in zebrafish olfactory sensory neurons. The level of expression of the second cistron consisting of the reporter protein EGFP did not allow for easy selection of transgenic embryos and for live analysis of transgenic OSNs and their axons. However, live visualization of OSNs and their axons would allow for a dynamic analysis of the effects of the molecular manipulation (overexpression of a gene of interest) on pathfinding by growth cones. The ability to assay effects dynamically would increase the detectability of phenotypes because the effect of some molecular manipulations may be most apparent and informative in a dynamic analysis. The use of more potent IRES elements could help to enhance the level of expression of the reporter protein and thereby improve the functionality of the approach presented here.

Here, I have presented one example of expression of a gene in the zebrafish embryo in a spatially restricted manner. Other genetic strategies involve the targeted gene expression using the GAL4-UAS system. This was accomplished in zebrafish by generating and crossing two stable transgenic lines, one carrying a construct with a given promoter to drive GAL4 expression, and the other carrying the effector gene downstream of UAS (Sheer *et al.*, 1999). A simpler method in that it does not require generation of any transgenic lines is the photo-mediated gene activation using caged RNA and/or DNA (Ando *et al*., 2001), which has recently been shown in being an effective gene knockdown strategy for zebrafish. This method appears to be very potent since it allows genes to be activated in a spatially and temporally restricted manner.

1.4. In the Adult Olfactory System Eph Receptors and Ephrin Ligands Are Expressed Complementarily and Have Overlapping Expression Domains

Some of the Eph receptors and ephrin ligands analyzed here are expressed in distinct but synaptically connected regions in the olfactory system of zebrafish. This kind of complementary expression pattern would be in accordance with a role of these receptors in growth cone guidance. *In situ* hybridization experiments showed that *rtk7/EphA4c* and *L3/ephrin-A2* are expressed selectively in OSNs. The receptor *rtk7/EphA4c* and the ligand *L3/ephrin-A2* are in the same specificity class and therefore might interact with each other. Similar overlapping expression was observed for ligands of the A (e.g. *L3* and *L4*) as well as the B family of ligands (*L5* and *L6*) in the granule cells of the olfactory bulb (see Fig. V.2.), and for the receptors *rtk4/EphA4a*, *rtk2/EphA4b*, and *rtk7/EphA4c* in the glomerular layer. Expression within a specific layer, i.e. a particular cell type, occurred at variable levels. It is not clear however if this reflects true differences in the level of expression.

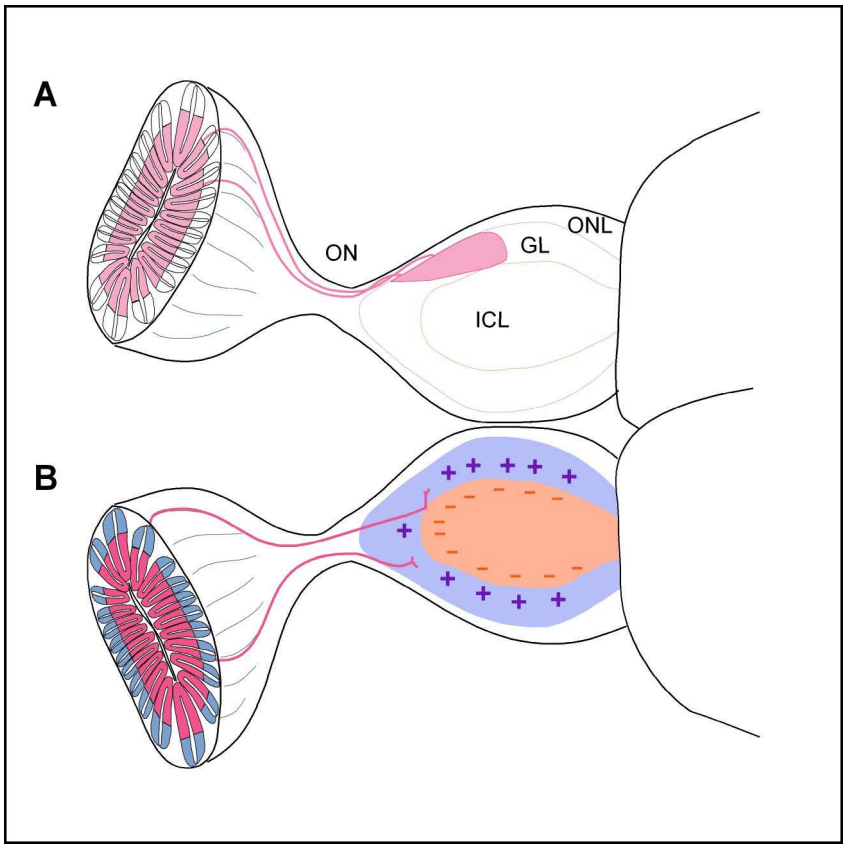

Fig. V.2.: Schematic Representation of Possible Functions of Eph Receptor and Ephrin Ligands in the Adult Olfactory System of Zebrafish. A. Olfactory sensory neurons expressing the receptor *rtk7/EphA4c* project to the lateral olfactory bulb. This region is also stained with affinity probes ephrin-A-L4-AP and ephrin-B-L5-AP, which both can interact with this receptor protein. Some circumstantial evidence suggests that this part of the bulb represents the vomeronasal organ of zebrafish. B. Olfactory receptors and/or ligands are expressed in overlapping as well as complementary domains. Repulsive activities between receptors and ligands in the olfactory epithelium may repel OSN axons from entering the non-sensory area and/or help to define the boundary of sensory and non-sensory area. A similar function in the olfactory bulb to define layers by restricting cell intermingling is also plausible. Differential expression of receptors and ligands might also help OSN axons to stay in the glomerular layer (lower neuron) and/or to be repelled from the granule cell layer (upper neuron).

Role of Complementary Expression - Boundary Formation

Until recently, Eph receptors and their ligands were considered to be expressed in mutually exclusive complementary spatial domains. In this way, the chemorepulsive properties of these molecules were assumed both to prevent mixing of different cell populations, and to provide distinct guidance cues for migrating cells or growth cones. Complementary expression of interacting Eph receptors and ephrins can lead to bidirectional activation at the interface, whereas overlaps in expression lead to persistent activation within the expression domain. One important role of reciprocal expression of Eph receptors and ephrin-B proteins is in unidirectional or bidirectional repulsion at boundaries, preventing cells or axons from entering inappropriate territory. In the nervous system, this mechanism is involved in stabilizing the organization of hindbrain segments, and in the guidance of migrating neural crest cells and neuronal growth cones (Xu *et al*., 1999; Mellitzer *et al*., 1999).

Role of Overlapping Expression

In this study support could be provided for a complementary expression of interacting Eph receptors and ephrins in the adult olfactory system of zebrafish. However, in contrast to the results of affinity probe *in situ* experiments, *in situ* hybridization has revealed that overlaps occur in the expression of interacting Eph receptors and ephrins. This raises the possibility that in the olfactory system as well as in many other tissues, Eph receptor or ephrin family members could have overlapping ('redundant') or synergistic roles. For example, EphA receptors are co-localized on retinal ganglion cells with ephrin-A ligands and it appears that this co-expression plays an important role in modulating the activity of the receptor (Connor *et al*., 1998; Hornberger *et al*., 1999). This overlapping expression persistently activates the receptor (Connor *et al*., 1998), presumably through cell-cell or axon-axon interactions, raising the possibility that this modulates axonal pathfinding. Indeed, the effects of ectopically expressing or removing ephrin-A ligand show that axons expressing both EphA receptor and ligand are no longer able to respond to gradients of ephrin-A ligands in the target zone (Hornberger *et al*., 1999). Very recently, it was shown that EphA5 and ephrin-A ligands are co-localized in the embryonic olfactory nerve where they may modulate axon adhesion and hence optimize conditions for axon outgrowth (St John *et al*., 2000). Co-localization of EphA receptors and ephrin ligands also occurs outside of the nervous system in somites, branchial arches and mesoderm in the developing mouse (Flenniken *et al*., 1996). Recently, it has been demonstrated that the EphB receptors, EphB3 and EphB4, are co-expressed with the ligands ephrin-B1 and ephrin-B2 on endothelial and mesenchymal cells (Adams *et al*., 1999). This co-expression of receptors and ligands during the development of the vasculature may provide a mechanism for cell-cell signaling associated with morphogenesis and sprouting (Adams *et al*., 1999).

1.5. Olfactory Versus Retinotectal Projection

Both the visual and olfactory systems represent sensory information within the brain through the use of sensory maps. The projection of sensory axons to the brain forms these maps through the spatial segregation and orderly termination of their axonal connections in specific target tissues. However, the visual map is fundamentally different from the olfactory map in that it is strictly topographic: a two-dimensional sheet of retinal ganglion cells (RGCs) is re-represented in the brain as more or less the same two-dimensional sheet through the retinotopic terminations of RGC axons. In contrast, the olfactory map is formed by the convergence of axonal projections of a specific set of functionally similar OSNs that are randomly distributed in the olfactory epithelium onto specific glomeruli.

The development of ordered axonal projections in the retinotectal and olfactory systems has clear distinctions, which relate in part to differences in their functional requirements. In the retinotectal system, the main objective is to represent the visual world in the brain, that is, to reconstruct a topographic representation of the world that projects onto the retina and is remapped in the tectum. To carry out this function requires the maintenance of a precise spatial ordering of axonal connections within the tectum that reflects the origins in the retina. In contrast, in the olfactory system, since odors have no relevant spatial component, there is no overriding need to maintain spatial continuity, either between cells expressing a given OR, or between glomeruli in the OB. This functional difference relates to differences in the mapping strategies employed in the first relay in the brain of the two systems.

Classically, the criteria for a topographic guidance molecule are that RGC axons exhibit a differential response to it and that it is expressed in a graded or restricted manner in the tectum. Molecules that meet both these criteria are limited to topographic mapping of the temporal-nasal retinal axis along the A-P tectal axis.

The retinotectal projection is controlled to a large extent by the reciprocal expression of members of the EphA family in projecting and target area, such that retinal axons with a high EphA receptor concentration project to regions of the tectum with a low ligand concentration and *vice versa* (Drescher *et al.*, 1997; Flanagan and Vanderhaeghen, 1998).

Expression data obtained in this study suggest that in contrast to the retinotectal system, Eph receptors and their ligands in the olfactory system of zebrafish do not display gradients of expression. As described above they are mainly expressed in different layers, i.e. different cell types. This cannot account for the direct targeting of OSN growth cones to their topographically correct target glomerulus in the OB. However, it could help the growth cones to distinguish between different layers.

1.6. Lateral Olfactory Bulb - Vomeronasal Organ of Zebrafish?

Affinity probe *in situ*'s using ephrin-A or ephrin-B fusion protein probes show a differential staining of the lateral olfactory bulb indicating the presence of an EphA or of an additional EphB receptor on olfactory sensory neurons. *In situ* hybridizations with receptor probes revealed the presence of the *rtk7/EphA4c* receptor in a subset of cells located in the sensory area of the olfactory epithelium (see Fig. V.2.). Both of the affinity probes are in principle able to recognize this receptor, since the EphA4 is the only receptor able to interact with ligands from both families (Gale *et al*., 1996).

There is a functional and anatomical dichotomy in the olfactory system in rodents. While neurons in the main olfactory epithelium detect volatile odorants, neurons in the vomeronasal organ mediate mainly the detection of nonvolatile compounds (Buck, 1996; Dulac, 2000). OSNs in the MOE of rodents are ciliated while the vomeronasal organ contains only microvillar OSNs that project only to the accessory olfactory bulb, which is situated on the dorsoposterior part of the main olfactory bulb. There are about 1800 glomeruli in the main olfactory bulb (Royet *et al*., 1998), while glomeruli in the accessory olfactory bulb are loosely defined, precluding precise counting (Rodriguez *et al*., 1999). This dichotomy also exists at the molecular level. In the accessory olfactory system, two families of genes encoding seven-transmembrane proteins have been proposed to encode pheromone receptors. The first family of genes are expressed selectively in the vomeronasal sensory neurons (VSNs) of the apical part of the vomeronasal organ (Dulac and Axel, 1995; Saito *et al*., 1998). The second family in expressed in the basal zone of the VNO (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Neurons in the apical and basal zones express different G protein subunits, $G_{\alpha i2}$ and $G_{\alpha o}$, (Halpern *et al*., 1995; Berghard and Buck, 1996), and project their axons to rostral or caudal regions in the mouse AOB, respectively. These proteins serve as useful molecular markers whose expression tends to correlate with that of the two families of vomeronasal receptor genes. Axons of VSNs expressing a single receptor project to multiple glomeruli, about 15 small ones) in the AOB as shown by genetically labeling axons of a particular receptor (Rodriguez *et al*., 1999; Belluscio *et al*., 1999). Again, in contrast to the MOB second-order neurons in the AOB, the mitral/tufted cells, project their dendrites to several, often distant glomeruli of the AOB (Takami and Graziadei, 1991). It remains to be shown if a given mitral cell in the AOB sends dendrites to glomeruli that receive input from neurons expressing a single or different VRs.

Two morphologically distinct OSN types, ciliated and microvillar cells, exist in the olfactory epithelium of fish (Hara, 1993). A segregated distribution of these cell types has been noted at the level of the lamellae in salmonid fishes and catfish. Using backtracing studies from the olfactory bulb of catfish it was shown that different cell types were labeled depending on where the injection site was. A set of microvillar receptor cells was shown to project to the dorsal posterior and lateral part of the olfactory bulb (Morita and Finger, 1998). Suprisingly, within the epithelium labeled OSNs were not concentrated in a particular area. In goldfish, two different OR gene families have been cloned. Goldfish cells that are probably equivalent to the ciliated OSNs express receptor genes related to the ORs of fish and

mammals, whereas the cells that look like microvillar sensory neurons express OR genes similar to the pheromone receptors found in the vomeronasal organ of mammals (Cao *et al*., 1998). Recently, for goldfish it could be shown that an odorant receptor that shares sequence similarity to vomeronasal receptors of the V2R class is preferentially tuned to recognize basic amino acids (Speca *et al*., 1999). Amino acids are potent odorants for fish and amino acid-responsive glomeruli are located in the lateral part of the olfactory bulb (Friedrich and Korsching, 1997; Fuss and Korsching, 2001). Glomeruli located in this region are small and their boundaries are difficult to define (Baier and Korsching, 1994), which is in accordance with the appearance of glomeruli in the AOB of rodents. In addition, it appears that in zebrafish there is a segregation of neurons expressing particular G-proteins. While the G-protein subunit $G_{\alpha olf}$ labels the majority of OSNs, the G-protein subunit $G_{\alpha o}$ is localized only in the lateral olfactory bulb (L. Buck, personal communication). Thus, several lines of evidence suggest that the lateral part of the olfactory bulb is distinct from the other parts of the olfactory bulb and might represent the vomeronasal organ of the zebrafish. A critical requirement along this line would be to show that the *rtk7/EphA4c*-expressing cells belong to the microvillar type of olfactory sensory neurons. The *in situ* hybridization studies performed on cryosections of olfactory epithelia do not allow making statements in this direction. So, further experiments are needed to elucidate if the lateral part of the olfactory bulb could represent the vomeronasal organ of zebrafish. It would therefore be of great interest to find the interacting partner of the EphA4 receptor and to see if it is involved in the specification of this organ.

The involvement of the EphA family during development of the mouse vomeronasal projection has been demonstrated recently (Knöll *et al*., 2001). It was shown that the ligand ephrin-A5 is expressed in the apical part of the vomeronasal organ, while ephrin-A3 is distributed uniformly. In the AOB a differential expression of a receptor (EphA6) was observed with a high level of expression in the anterior part of the AOB and a low level in the posterior part. In mice mutant for the ephrin-A5-ligand the differential expression of ephrin-A molecules on vomeronasal axons is lost, resulting in the uniform expression of ephrin-A3. This loss seems to result in the misprojection of apical vomeronasal axons into anterior and posterior parts of the AOB, while wild-type axons project only into the anterior part of the AOB. These experiments argue for a role of the higher expression of ephrin-A5 on apical VNO neurons in providing the corresponding ingrowing vomeronasal axons with the ability to discriminate between AOB regions with high and low levels of EphA6 (Knöll *et al*., 2001).

1.7. Role of Projection Neurons and Interneurons in Establishment of the Topographic Map.

The involvement of the projection neurons and interneurons in the establishment of the olfactory projection has been investigated using genetic means, taking advantage of two transcription factors that are expressed in subpopulations of neurons in the developing forebrain and olfactory bulb (Bulfone *et al*., 1998). Mice with a homozygous deficiency in the *Tbr1* gene fail to form normal mitral and tufted cells, while mice lacking both *Dlx1* and *Dlx2* genes lack mature GABAergic olfactory bulb interneurons, namely the periglomerular and granule cells (Bulfone *et al*., 1995; Anderson *et al*., 1997, respectively). Analysis of the olfactory projection in mice lacking one or the other gene, i.e. projection neurons or interneurons, revealed that OSNs expressing a particular receptor (P2) still converge to glomeruli at positions analogous to those in wild-type mice. These observations suggested that the establishment of the topographic map is not dependent upon cues provided by the major neuronal types in the olfactory bulb (Bulfone *et al*., 1998). However, it might be possible that both of these cell types express redundant spatial cues, such that the presence of either neuronal population is adequate to guide sensory axons to their appropriate glomerular target. This possibility has also been addressed by generating double mutant mice that lack both neuronal cell types of the olfactory bulb. However, it appeared to be difficult to analyze the bulbs of these mice, since they were markedly hypoplastic and anatomically deformed, making interpretations impossible (Bulfone *et al*., 1998).

It was suggested that alternatively a transient population of cells present in the olfactory bulb, the radial glia, could provide the necessary guidance information for OSN axons. Radial glia have been implicated in providing the graded expression of guidance cues in the tectum and superior colliculus in visual map formation (Wu *et al*., 1995; Davenport *et al*., 1996). Recently, the development of radial glia in the mouse olfactory bulb has been analyzed. This analysis showed that there is a tight spatiotemporal relationship between the glomerulization of radial glia processes and OSN axons during development and suggested that radial glia processes could play a role in the formation and/or stabilization of mammalian glomeruli (Puche and Shipley, 2001).

In the framework of this study I have analyzed the presence and distribution of glia in the olfactory bulb of zebrafish making use of an antibody (GFAP) against astrocytes. I could show that glial processes are present throughout the olfactory bulb, although enrichment in the glomerular layer could be observed, consistent with a role in the formation and/or stabilization of glomeruli. However, the timing of appearance of glia could not be studied, since a protocol for analyzing embryonic stages could not be established. It will be interesting to see if the appearance of glial cells correlates with stages of OSN axon outgrowth and pathfinding in order to get insights into the possible involvement of these cells in glomerular map formation.

1.8. Multiple Cues - Concluding Remarks

It is well known that in many systems multiple cues, both repulsive and attractive guide neuronal growth cones to their targets and that these can have cooperative or overlapping functions (Goodman, 1996). The complexity of the neural circuitry in the olfactory system is such that it is likely to require a multitude of different guidance signals that act in parallel with ephrins during its establishment as well as its maintenance. One class of molecules that have likely roles in the olfactory system includes the semaphorins. It was shown that OSNs and their axons express neuropilin-1, which serves as the ligand-binding subunit in the semaphorin 3A receptor. In chick embryos, Sema3A is expressed in the developing telencephalon and prevents premature entry of olfactory axons (Kobayashi *et al*., 1997). The laminar expression of Sema3A in olfactory bulb neurons is thought to prevent axon growth beyond the glomerular layer (Pasterkamp *et al*., 1999). In addition to mitral and tufted cells in the OB and the mesenchyme of the cribriform plate, $p75^{+}$ ensheathing cells in the nerve layer of the OB differentially express Sema3A. In mice lacking the Sema3A gene, neuropilin-1 positive axons are misrouted throughout the embryonic nerve layer and terminate inappropriately in ventral glomeruli of neonatal mutant mice (Schwarting *et al*., 2000). Similarly, chick OSNs transfected with a truncated form of the neuropilin-1 receptor enter the brain prematurely and overshoot the area that will become their appropriate target (Renzi *et al*., 2000). These results suggest that class 3 semaphorins are involved in the guidance process of OSNs and act as repellents *in vivo* preventing axons from entering their target prematurely.

In addition, cell adhesion molecules may act in parallel with repulsive cues, and evidence that Eph receptors may regulate the function of adhesion molecules suggests that these two types of molecules could interact.

Many data indicate that in the main olfactory system olfactory receptors are crucially involved in the guidance of olfactory axons to their target areas, suggesting that they might function as both odorant as well as guidance receptors (Mombaerts *et al*., 1996; Wang *et al*., 1998; Bulfone *et al*., 1998). However, these data also indicated that olfactory receptors couldn't be the sole determinant of the position of a particular glomerulus. It will be of interest to analyze a possible interplay between Eph family and olfactory receptors, for example, at the level of similar intracellular signaling pathways, and the contribution of each of these families to the guidance of OSN axons.

2. Cell-Type Specific Genes and Promoters

In order to be able to do functional expression genetically in a cell type-specific manner, the use of specific promoters for the cell type of interest is inevitable. In zebrafish not many promoters have been identified to date. In particular, promoters for olfactory cell types are not known. For this purpose, attempts have been made to find and isolate cell type-specific promoters for olfactory sensory neurons, mitral cells and granule cells.

2.1. The *zOMP* Gene and Its Promoter Are Valuable Tools to Visualize Mature Olfactory Sensory Neurons

In the present study the most likely homologue for the olfactory marker protein in zebrafish, *zOMP*, was identified with complete sequence. Sequence comparison demonstrated that the overall homology of the protein is moderate, but the consensus regions exhibit high homology to OMPs from other species. It appears to be more homologous to another fish *OMP* (which is a short fragment from medaka) than to frog or mammalian *OMPs*. In contrast to the presence of four *OMP* genes in frog (Rössler *et al*., 1998) and two *OMP*-gene fragments in medaka (Yasuoka *et al*., 2000) only one gene could be detected for zebrafish using genomic Southern analysis, even at low stringency hybridizations. It is suggested that four different OMP genes exist in *Xenopus*, possibly as a consequence of genome duplication as a result of tetraploidization (Rössler *et al.*, 1998). The zebrafish and teleost fishes in general have undergone massive gene duplication events (Postlethwait *et al*., 2000). It is quite surprising that possibly such a gene duplication event resulted in the presence of two *OMP* genes in medaka, but not in zebrafish.

Analysis of it expression indicated that *zOMP* is a reliable marker of OSNs during embryonic development as well as in adulthood. This is consistent with the specific expression pattern described for other species (Buiakova *et al*., 1994; Rössler *et al.*, 1998; Yasuoka *et al*., 2000) and may indicate that the function of OMP is conserved throughout vertebrate evolution. Expression appeared to be localized in the sensory area and mainly to apical parts of the lamellae where mature olfactory neurons are found. Since it is not possible to distinguish cellular morphology in *situ* hybridization experiments performed on cryostat sections, it cannot be said if expression is confined to one of the two OSN types. However, since no particular lack of expression in apical parts of the lamellae were observed, there is no reason to believe that only one of the two types might be labeled. Basal areas containing immature neurons are mainly free of labeling. This is consistent with α-cytokeratin stainings performed in this study and with studies where two proliferation zones have been identified. Proliferation zones are located in the 'curves' of the lamellae adjacent to the middle raphe and in the region between sensory and non-sensory area (Berger, 1998). In the embryo, expression was very specific and confined to the olfactory placodes. This indicated that the

zOMP gene is a specific marker for OSNs in the adult zebrafish as well as in the embryo, like its other vertebrate counterparts. Expression was first observed at 24 hpf, which correlates with the first appearance of olfactory receptor expression (Argo, 1995; Barth *et al*., 1996). The number of cells increased continuously over time with a boost between 24 and 48 hpf, as determined by counting OMP-positive cells. This increase in number of cells is not surprising for a growing placode, although it is not clear what kind of event is represented by the high increase in cell number between 24 and 48 hpf. Comparable growth curves were also obtained when counting cells expressing a particular OR gene.

Analysis of upstream regions that were cloned into an expression vector showed that all tested fragments are able to drive the expression of a fluorescent reporter gene. Consistent with the expression pattern of the endogenous gene, the upstream region directed the EYFP transgene expression predominantly in ciliated as well as microvillar olfactory sensory neurons. Even a short upstream fragment of 300 bp was able to drive reporter gene expression, while longer fragments of 600 bp and 1.3 kb turned out to increase the penetrance as well as the expressivity. Transgene expression was strong in all cases and a high penetrance could be obtained using the longest fragment, reaching levels of about 50%. The temporal activation of the *OMP* gene promotor is in good agreement with the timing of the activation of the endogenous gene during normal development, as shown by *in situ* hybridization. The *zOMP* mRNA can be detected as early as 24 hpf. Consistent with this, the EYFP expression under the zOMP promoter was observed quite early at about 24 hpf.

The characterization of the 5'-flanking region of the *zOMP* gene by sequencing revealed that it is somewhat unusual in that it lacks an obvious TATA and CAAT motif. However, it has been shown that *OMP* genes from several other species lack these motives as well (Buiakova *et al*., 1994). Absence of a TATA box is not unusual as it also has been reported for other zebrafish promoters, e. g. HuC promoter (Park *et al*., 2000).

The transcription factors Olf-1 and NF-I have been implicated in the regulation of several OSN-specific genes in rodents like OMP, adenylyl cyclase type III, G_{olf} and the cyclic nucleotide-gated channel. Functional binding sites for these factors are present in the promoter regions of all of these genes, including the OMP promoter (Jones and Reed, 1989; Dhallan *et al*., 1990; Bakalyar and Reed, 1990; Buiakova *et al*., 1994; Baumeister *et al.*, 1999). Additionally, these binding sites were found to be present in the promoter regions of rodent and human olfactory receptor genes (Glusman *et al.*, 2000). Interestingly, no putative binding sites for these factors were found in the upstream region of the *zOMP* gene or the olfactory receptor promoters (intergenic regions). Other OSN-specific gene promoters have not been identified so far, so that a comparison with these sequences in order to find a putative binding site is not possible. It is probable that in zebrafish the Olf-1 and NF-I binding sites are altered beyond recognition or that the regulation of these genes is completely different (cf. Mori *et al*., 2000; Dugas and Ngai, 2001).

It is worth mentioning that, in all cases, the transient transgenic expression was mosaic and highly variable among the embryos injected with the same construct; i.e. not all OSNs or mitral cells or granule cells expressed the transgene, and not every embryo showed an identical pattern of expression. These phenomena are primarily attributable to the differential segregation of the injected DNA during embryogenesis, as documented in early transgenic

fish research (for review see Iyengar *et al.*, 1996). Nevertheless, the transient transgenic system remains an effective and reliable system to investigate the pattern of gene expression by analysis of a large number of individuals. It also provides a rapid, convenient assay with which to dissect the cis-elements controlling the temporal and spatial patterns of expression.

Exploiting Different Reporters for Multi-Labeling Purposes

In the zebrafish system, use of an *in vivo* fluorescent reporter has been limited to green fluorescent protein. I have analyzed an additional fluorescent protein alone and in conjunction with EGFP to investigate their efficacy as markers for multi-labeling purposes in live zebrafish. By injecting plasmid DNA containing yellow fluorescent protein (EYFP) and red fluorescent protein (dsRed) under the control of the OMP promoter, transient transgenic embryos were generated and analyzed under fluorescent stereomicroscope. Observation demonstrated that EYFP and DsRed are highly stable proteins, exhibiting no detectable photoinstability. Both have a high signal-to-noise ratio, although the signal-to-noise ratio of DsRed appeared to be higher. Under appropriate filter sets, both fluorescent proteins can be independently detected even when simultaneously expressed in the same cell (as has been investigated using the polycistronic construct prOMP$_{1.3}$-dsRed-IRES-EGFP construct). The preparation of creative expression vectors and insightful experiments using multiple fluorescent proteins will help elucidate signaling pathways and regulatory elements and simplify insertional mutagenesis studies. In the olfactory system, in particular, the use of a multiple-labeling approach would help to elucidate the wiring of the olfactory pathway from the olfactory sensory neurons to higher centers, given that suitable promoters could be identified for the granule and mitral cells.

Future Prospects - Establishment of a Transgenic Line and its Use

The establishment of a transgenic line with fluorescent olfactory sensory neurons and their axons will be a useful tool for various studies. A zOMP-EYFP line would be useful for cellular manipulations such as laser ablation experiments. One of the classic strategies to study the *in vivo* functions of selected neuronal populations or distinct neuroanatomical structures is cell ablation. Cell ablation can be achieved using a variety of techniques, ranging from physical to pharmacological lesioning (Shah and Jay, 1993), however, such techniques remain relatively crude. The greatest selectivity could be potentially achieved using a genetic approach by directing the expression of a transgene encoding a cytotoxin under the control of a neuron-specific promoter. For example, in mice two types of toxin have been used in transgenic mice, those that are constitutively toxic, such as diptheria toxin or ricin (Landel *et al*., 1988; Palmiter *et al*., 1987), and those that can be controlled conditionally by the administration of a prodrug (Borrelli *et al*., 1988). More recently this kind of conditional ablation was used in olfactory sensory neurons of mice, where cells expressing a particular receptor were ablated by driving DT expression under the control of an olfactory receptor-specific promoter (Gogos *et al*., 2000). The results of these kinds of experiments could be compared with alternative, crude methods of ablation such as $ZnSO_4$ irrigation of the naries (Oehlmann, 2001) and olfactory bulbectomy (Liebenauer and Slotnick, 1996).

Furthermore, the transgenic fish might be also useful for electrophysiological or optical recording studies because it would allow researchers to record the activity of a EYFP-labeled neuronal type reproducibly. The use of genetically encoded calcium-sensors, that are variants of GFP, would add another dimension to the analysis (Fuss, 2001).

Targeted expression of EYFP allows for the isolation of live OSNs for studies at the cellular level and *in vitro* studies, by taking the EYFP expressing cells into culture. Isolation of a pure OSN population would enable experiments involving the search for particular genes expressed in these neurons and the subtraction from the same cell population treated differently or from different cell populations.

Because the zebrafish is genetically manipulable, the zOMP-EYFP zebrafish provides an opportunity for genetic analyses of the development of neurons such as the OSNs. Recent large-scale mutant screens have identified many mutations that cause defects in CNS. The zOMP-EYFP fish will enable further examination of these mutations after introduction of the transgene into the mutant background by crossing mutants with EYFP lines. Finally, the zOMP-EYFP line can be used as a starting strain for mutational analysis of GFP-positive neurons. Furthermore, the fact that axon outgrowth can be so readily assayed in these embryos should make it possible to screen directly for mutations that affect outgrowth, pathfinding, and synapse formation by the GFP-positive neurons.

2.2. The *dlx2* Gene Is a Good Marker for Granule Cells in Zebrafish

The second gene investigated for its usefulness as marker is the *dlx2* gene. Previous studies have indicated that *dlx2* is a useful marker for granule cells in rodents (Bulfone *et al*., 1998). In this study I could show that the zebrafish *dlx2* gene is expressed similarly in zebrafish, labeling granule cells in the inner cell layer within the olfactory bulb. During development it is expressed in a number of other tissues like the pectoral fin buds. However, since this additional expression would not interfere with the desired use, I continued with the isolation of upstream regions of this gene. An 11 kb upstream fragment was subcloned and various fragments were analyzed for their potential to drive reporter gene expression in granule cells. While a 1.5 kb upstream fragment was not able to drive any expression, interestingly, a 4.5 kb fragment of the upstream region resulted in reporter gene expression in the olfactory placodes. The reason for this is not known but may be due to the presence of an enhancer in this region that directs expression to OSNs. An additional 4 kb fragment that was added to the 4.5 kb fragment showed a widespread expression in many different kinds of neurons, that included granule cells as well as mitral cells, and occasionally occurred in muscle cells also. It seems that this fragment is able to drive neuron-specific expression. What is not clear is, if granule cells are labeled because they are neurons also, or if this region has the potential to drive granule cell-specific expression and just lacks an additional element like an enhancer.

Dlx homeobox genes of vertebrates are often organized as physically linked pairs in which the two genes are transcribed convergently (tail-to-tail arrangement). Three such *Dlx* pairs have been found in mouse, human, and zebrafish and are thought to have originated from the duplication of an ancestral gene pair. These pairs include *Dlx1*/*Dlx2*, *Dlx7*/*Dlx3,* and *Dlx6*/*Dlx5* (the zebrafish orthologue of *Dlx5* is named *dlx4*). Expression patterns of physically linked *Dlx* genes overlap extensively. Furthermore, orthologous *Dlx* genes often show highly similar expression patterns. Detailed analysis of expression in mouse and zebrafish have shown that *Dlx* expression patterns at early stages are better conserved between paralogous pairs of physically linked genes than between orthologous pairs. This suggests that early expression of *Dlx* genes existed prior to the duplications that led to the multiple pairs of physically linked genes but was differentially conserved in different paralogues in zebrafish and mice.

The similarities between the mouse and zebrafish Dlx orthologues in their genomic organization, the relative short distances that separate the two genes in such pairs and the similarities in their expression patterns (Ellies *et al*., 1997) suggest the presence of shared cis acting regulatory elements in the intergenic region. And indeed, recently highly conserved sequences in the intergenic region between *Dlx5/Dlx6* (*dlx4/dlx6* in zebrafish) that extend over a few hundred base pairs have been identified (Zerucha *et al*., 2000).

This finding prompted us to look for the presence of such an enhancer element in the *dlx1/dlx2* intergenic region. The genomic clone that contained the *dlx2* gene appeared to contain the *dlx1* gene also. A genomic clone of 8 kb containing the whole intergenic region and parts of the 3'-UTRs of the *dlx1* and *dlx2* genes was subcloned. Unfortunately, all the attempts to subclone this fragment into the expression vector that contains parts of the upstream region failed. Therefore, nothing can be said about the contribution of this region to the expression of the reporter gene so far.

2.3. The *ztbr1* Gene Is a Potent Marker for Mitral Cells

The *Tbr1* gene was shown to be expressed specifically in mitral cells of the olfactory bulb in mice (Bulfone *et al*., 1998). It is a transcriptional regulator, and it has been implicated in the control of differentiation. It belongs to the T-box gene family and was the first member that is extensively expressed in the vertebrate CNS. Therefore, it was suggested that *Tbr1* defines a new subfamily of T-box containing genes. In this study, only a partial cDNA clone of about 2.5 kb could be isolated despite many efforts to get the full-length sequence. Independent from this work a partial clone for the zebrafish *tbr1* gene was published recently that was about 150 bp longer in 5' direction (Mione *et al*., 2001). It is not clear how much of the sequence is missing, but it is stunning that in two independent approaches no full-length sequence could be isolated. This finding might indicate the difficulty of the reverse transcriptase to transcribe over a particular region in the sequence that gives rise to these truncated cDNAs. The size of known *Tbr1* genes is large in general; cDNAs of mouse and human *Tbr1* have a size of 3.8 and 2.9 kb, respectively. The *Tbr1* gene in mouse has a

complex exon-intron structure, consisting of six exons and extending over a region of about 10 kb on the genomic level. To isolate a genomic clone a genomic PAC library was screened using three different probes. While no strongly positive clone could be identified in a screen with the mouse *Tbr3* gene (that was used initially to isolate a homologue in zebrafish), several clones were obtained when using the *ztbr1* T-box fragment as a probe. One of four clones was analyzed in more detail and used to isolate an upstream fragment that could contain sequences corresponding to the cDNA and more upstream sequences. Efforts to clone a large fragment were not successful and sequencing of a subcloned shorter fragment indicated that the analyzed clone does not correspond to *ztbr1*. Interestingly, the obtained sequences also do not show a high homology to zebrafish *eomesodermin* (*ztbr2*), while in comparisons with known mouse sequences homology is quite low but seems to be higher to *mTbr2* sequence. The reason for not getting a genomic clone for *ztbr1* might lie in the fact that the probe lies in the T-box that is highly homologous between species. However, it does not seem to be so diverse, since only four hybridizing clones could be revealed in the genomic library screen. In a third attempt, to be more specific a probe from the 3'-end of the gene was used and again four hybridizing clones were obtained. Interestingly, none of the previously hybridizing clones were positive in this screen. Clearly, more work is required to obtain genomic upstream sequences of the *ztbr1* gene.

In situ hybridization experiments showed that *ztbr1* is expressed in a regionally restricted pattern in post-mitotic cells of the developing and adult telencephalon and olfactory bulb. The expression of *ztbr1* in the forebrains seems to be identical to mouse *Tbr1* (Bulfone *et al*., 1995) but different from *Xenopus Eomesodermin*, which is closely related to mouse *Tbr1* (Ryan *et al*., 1996). Interestingly, expression could be detected in a small population of neurons located in the olfactory epithelium. The identity of labeled cells in the olfactory epithelium is not known. Expression in the olfactory placode could be detected as early as 3d after fertilization and expression persisted until adulthood.

The restricted expression pattern of the *ztbr1* gene makes it a very good marker for mitral cells throughout early and larval development as well as in the adult olfactory bulb. Thus, it is worth putting more effort into the isolation of genomic upstream sequences that could contain a functional promoter. Making mitral cells accessible by a genetic means will open the road to answer many interesting questions.

3. Concluding Remarks

In this study I could show that the zebrafish Eph genes resemble each other not only in the sequences of their predicted protein products, but also in some but not all aspects of their embryonic and adult patterns of expression. These patterns suggest that distinct combinations of functionally active Eph/rin genes participate in the development of the primary and possible the secondary olfactory projection, in the zebrafish embryo. The novel spatially restricted expression patterns of members of the Eph receptor family support a role for these molecules also in the maintenance of the adult olfactory system. Using a gain-of-function

approach no changes in the projection of L5/ephrin-B2a ligand-overexpressing OSN axons were observed. Although it is not yet clear what particular role(s) Eph-ephrin interactions play in the olfactory system of zebrafish, it will now be possible to examine the functional consequences of perturbations to interactions between some of these members in the developing olfactory system. The basis for further work has been laid with this study.

In the second part of this thesis specific genes for the main cell types in the olfactory system of zebrafish were isolated. These genes are expressed in highly specific, largely exclusive domains in the developing olfactory placode and forebrain of zebrafish embryos and in mature olfactory sensory neurons, mitral cells and granule cells of adult animals, as is observed in other vertebrates. Thus, they represent useful molecular tools to study the zebrafish olfactory system. For one of these genes a very potent promoter could be identified, that drives reporter gene expression exclusively in olfactory sensory neurons. This promoter was then used in the overexpression of a particular gene along with a reporter gene in a bicistronic message using an IRES sequence. The usefulness of the IRES sequence could be demonstrated, although one has to admit that the expression of the second cistron is not good enough with the IRES sequence tested here. Other IRES elements may need to be used to make the system more effective. For the interneurons a large upstream fragment could be identified that drives neuron-specific expression, occasionally including granule cells and mitral cells. The assumption that an intergenic fragment contains an enhancer element that could help to restrict the expression to granule cells needs still to be tested. For the mitral cells the cloning of an upstream fragment is pending. The isolation and characterization of three promoters for the most important cell types in the olfactory system will add to the usefulness of zebrafish as a model system in olfactory research.

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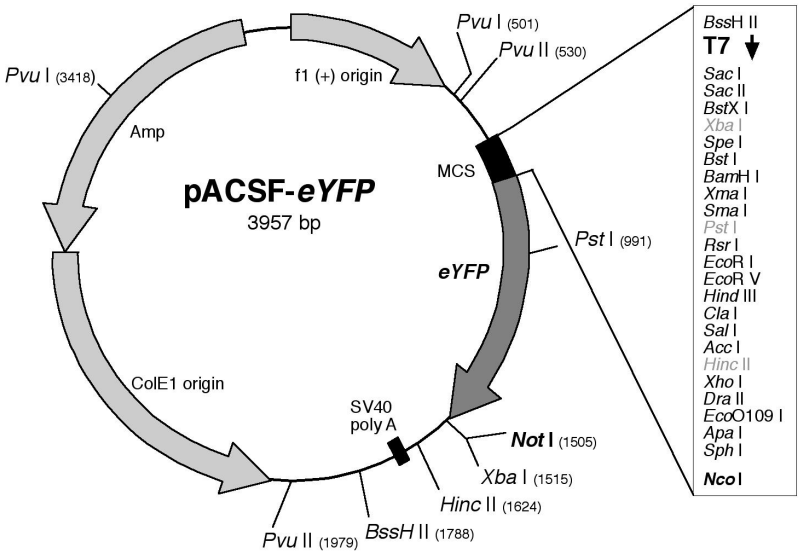
VII. SUPPLEMENT

1. Abbreviations

A	adenosine
AC	anterior commissure
AP	alkaline phosphatase
A-P	anteroposterior
AOB	accessory olfactory bulb
bp	base pairs
BSA	bovine serum albumine
C	cytosine
cDNA	complementary DNA
Ci	Curie
CNS	central nervous system
cpm	counts per minute
DAB	diaminobenzidine
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	desoxynucleic acid
dNTP	desoxynucleotide phosphate
DMEM	Dulbecco's modified Eagle medium
dsRNA	double-stranded RNA
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
EYFP	enhanced yellow fluorescent protein
FCS	fetal calf serum
FGF	fibroblast growth factor
Fig.	figure
G	guanine
GABA	gamma aminobutyric acid
GAP	growth-associated protein
GAPDH	glycerinaldehydephosphate dehydrogenase
GBC-1	globose basal cell-1
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GL	glomerular layer
h	hour
hpf	hours post fertilization
HRP	horseradish peroxidase
ICL	internal cell layer
IPTG	isopropyl-β-D-1-thiogalactopyranoside
IRES	internal ribosome entry site
kb	kilo base
lacZ	β-galactosidase gene
LOT	lateral olfactory tract
M	molar

MCS	multiple cloning site
µg	microgram
min	minutes
MOB	main olfactory bulb
MOE	main olfactory epithelium
MOT	medial olfactory tract
ng	nanogram
NGS	normal goat serum
OE	olfactory epithelium
OB	olfactory bulb
ONL	olfactory nerve layer
OMP	olfactory marker protein
OP	olfactory placode
OR	olfactory receptor
OSN	olfactory sensory neuron
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
RGC	retinal ganglion cell
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RTK	receptor tyrosine kinase
ss	single strand
SSC	sodium citrate
T	thymidine
TBS	tris buffered saline
TE	tris-EDTA
tel	telencephalon
TH	tyrosine hydroxylase
U	unit
UTR	untranslated region
UV	ultraviolet
VNO	vomeronasal organ
VR	vomeronasal receptor
VSN	vomeronasal sensory neurons
v/v	volule per volume
v/w	volume per weight
X-Gal	5-Bromo-4-chlor-3-indoyl-β-D-galactopyranosid

2. Vectors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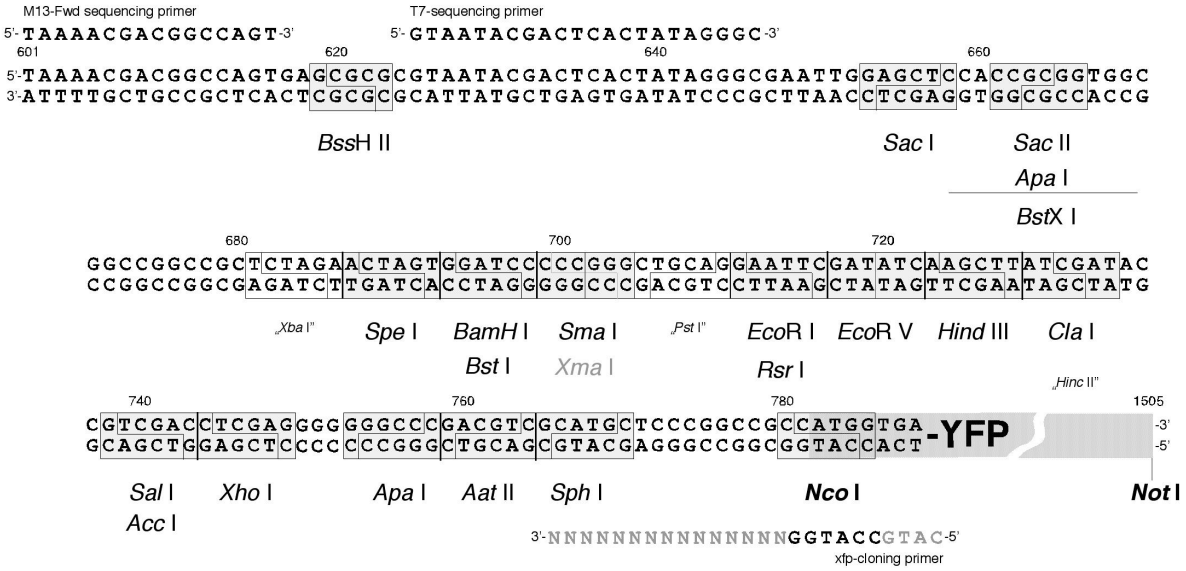
Pvu II (530)
f1 (+) origin
Pvu I (3418)
Amp
MCS
eYFP
Pst I (991)
ColE1 origin
SV40
poly A
Not I (1505)
Xba I (1515)
Hinc II (1624)
Pvu II (1979)
BssH II (1788)
BssH II
T7
Sac I
Sac II
BstX I
Xba I
Spe I
Bst I
BamH I
Xma I
Sma I
Pst I
Rsr I
EcoR I
EcoR V
Hind III
Cla I
Sal I
Acc I
Hinc II
Xho I
Dra II
EcoO109 I
Apa I
Sph I
Nco I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T7-sequencing primer
5'- GTAATACGACTCACTATAGGGC -3'
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620
640
660
5'-TAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGTGGC
3'-ATTTTGCTGCCGCTCACTCGCGCGCATTATGCTGAGTGATATCCCGCTTAACCTCGAGGTGGCGCCACCG
BssH II
Sac I
Sac II
Apa I
BstX I
680
700
720
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„Xba I"
Spe I
BamH I
Sma I
„Pst I"
EcoR I
EcoR V
Hind III
Cla I
Bst I
Xma I
Rsr I
„Hinc II"
740
760
780
1505
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-3'
-5'
Sal I
Acc I
Xho I
Apa I
Aat II
Sph I
Nco I
Not I
3'-NNNNNNNNNNNNNNNGGTACCGTAC-5'
xfp-cloning primer

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Köln, den 26. November, 2001