Functional Expression of Odorant Receptors of Zebrafish, *Danio rerio*

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I. ABSTRACT

Olfaction is one of the important windows of our perception to the environment. The molecular mechanism underlying olfaction is largely unknown, in contrast to the advanced understanding of perception in other sensory systems. In the first step of olfaction, odors are detected by olfactory receptor neurons (ORNs) upon binding to the G-protein coupled olfactory receptors (ORs). It is believed that one ORN expresses one or a few OR types so that the response characteristics of an ORN should reflect that of the underlying OR. ORNs project to the olfactory bulb (OB), where a stereotyped and chemotopic “odor image” is found. Here different odors are displayed as different combinations of activated glomeruli - the functional modalities of the OB. Whereas mapping of olfactory information in neural space revealed some aspects of odors that are encoded by the olfactory system, for a thorough understanding of olfactory coding the discovery of receptive field of each single OR type is required. Although a decade has passed after the OR molecules have been found, the knowledge about the range of ligands of OR is still limited. Functional expression of ORs will result in direct evidence of these putative ORs functioning as odorant receptors, and it will also reveal the receptive field of these molecules. Unfortunately functional expression of OR is very difficult.

Compared to high vertebrates zebrafish, *Danio rerio*, exhibits a qualitatively similar but quantitatively much smaller olfactory system. Moreover its odorants are water soluble and well defined. Therefore zebrafish is used as a model system to study olfaction. This work starts with attempts of functional expression of zebrafish ORs *in vivo* in ORNs in their natural surroundings. However, with the paucity of genetic tools for zebrafish, no reproducible overexpression was observed in nose of adult animals, using Green Fluorescent Protein (GFP) as a marker. Effort was then put into the primary culture of olfactory epithelium, which retains some feature of the organ *in vivo* and where the genetic manipulation is easier. Due to the very limited size of the zebrafish epithelium, these studies had to be performed with mouse olfactory epitheliun. However, because of the rarity of mature neurons in the primary culture and low transfection efficiency, this system was not feasible for functional expression of ORs. In a third approach a non-neuronal cell line, the HEK293 cells, was employed as a functional expression system. Here Ca^{2+} increase in cells was analysed as response, which is measured by fura-2, a ratiometric Ca^{2+} indicator. It was found that the HEK293 cells are liable to give non-odor-, non-transfection-, -related responses. These responses appeared to depend on several factors which are difficult to control (e.g. confluency, stress from transfection), restricting the usefulness of the system to identify ligands for ORs. To improve this system, GFP was introduced as a marker of transfected cells. Since GFP did not alter the signal of fura-2, it is possible that the Ca^{2+} response in transfected cells which incorporate ORs be compared with that of the non-transfected cells, and thus the Ca^{2+} elevation related to OR activation can be found in such a system.

I. ZUSAMMENFASSUNG

Der Geruchssinn ist eines unserer wichtigsten Wahrnehmungsfenster zur Umwelt. Verglichen mit dem fortgeschrittenen Verständnis für die Wahrnehmungsmechanismen anderer Sinnessyteme, ist der exakte molekulare Mechanismus, auf dem die Olfaktion basiert, größtenteils unbekannt.

Im ersten Schritt der Geruchswahrnehmung werden Geruchsstoffe im olfaktorischen Epithel von Riechsinneszellen detektiert, indem sie an G-Protein gekoppelte olfaktorische Geruchsrezeptoren (OR) binden. Es wird angenommen, dass ein olfaktorisches Rezeptorneuron (ORN) nur einen oder wenige verschiedene OR-Typen exprimiert, so dass das Antwortverhalten eines ORN durch den exprimierten OR geprägt sein sollte. ORNs projizieren in den olfaktorischen Bulbus, wo man ein stereotypes und chemotypes "Geruchsabbild" findet. Hier werden verschiedene Geruchsstoffe durch unterschiedliche Kombinationen aktivierter funktionaler Einheiten des olfaktorischen Bulbus, den sogenannten Glomeruli, darstellt. Bisher wurden durch die Kartierung der olfaktorischen Information im Bulbus schon einige Aspekte der Geruchsstoffe identifiziert, die vom olfaktorischen System erfasst werden. Für das Verstehen der olfaktorischen Kodierung ist es jedoch notwendig, die Ligandenspektren für jeden einzelnen OR-Typ zu bestimmen. Obwohl seit der Entdeckung der ersten olfaktorischen Rezeptormoleküle etwa ein Jahrzehnt vergangen ist, ist das Verständnis für die Bindungseigenschaften der ORs noch immer begrenzt. Mit Hilfe der funktionalen Expression der ORs kann das Ligandenspektrum einzelner Rezeptoren bestimmt und direkt gezeigt werden, dass es sich bei den putativen ORs um Rezeptoren für Geruchsstoffe handelt. Unglücklicherweise ist die funktionale Expression der olfaktorischen Rezeptorproteine sehr schwierig.

Verglichen mit höheren Vertebraten besitzt der Zebrabärbling, *Danio rerio*, ein qualitativ vergleichbares, aber quantitativ reduziertes olfaktorisches System. Darüber hinaus sind seine Geruchsstoffe wasserlöslich und gut untersucht, was ihn zu einem attraktiven Modellsystem in der olfaktorischen Forschung macht. Diese Arbeit beginnt mit Versuchen zur funktionalen Expression der olfaktorischen Rezeptorproteine von *Danio rerio* in ihrer natürlichen Umgebung. Aufgrund der eingeschränkten genetischen Verfahren, die im Zebrabäbling anwendbar sind, wurde jedoch keine reproduzierbare Überexpression, die anhand der Expression des Markers *Green Fluorescent Proteins* (GFP) überprüft wurde, in der Nase von adulten Tieren erreicht. Bemühungen wurden anschließend in die primäre Kultur des olfaktorischen Epithels gelegt, das einige Eigenschaften des *in vivo* Organ beibehält, sich jedoch einfacher genetisch manipulieren lässt. Wegen der sehr geringen Größe des olfaktorischen Epithels des Zebrabärblings, mussten diese Studien an olfaktorischen Epithelien der Maus durchgeführt werden. Da die reifen Neuronen in der primären Kultur und der geringen Transfektionseffizienz selten waren, war das System jedoch nicht geeignet, um ORs funktional zu exprimieren. In einem dritten Ansatz wurde die nicht-neuronale Zelllinie HEK293 als ein funktionales Expressionssystem verwendet. Hier spiegelt der zelluläre Ca^{2+}-Spiegel die Antwort der Zellen auf Reize wider und

kann mit Hilfe von *fura-2*, einem ratiometrischen Ca^{2+}-Indikator, vermessen werden. Die Zellen erwiesen sich jedoch anfällig für Geruchsstoff- und Transfektions-unabhängige Antworten. Die Antworten scheinen von verschiedenen schwer kontrollierbaren Faktoren abhängig zu sein (z.B. Konfluenz, Transfektionsstress), die die Anwendbarkeit des Systems, Liganden für die ORs zu identifizieren, einschränken. Deswegen wurde das System verbessert, indem transfizierte Zellen mit GFP markiert wurden. Da GFP das Signal von *fura-2* nicht stört, ist es in einem solchen System möglich, das Ca^{2+}-Antwortverhalten von Zellen, die einen bestimmten olfaktorischen Rezeptor überexprimieren, mit nicht-transfizierten Zellen zu vergleichen und so Rückschlüsse auf das Ligandenspektrum des Rezeptors zu gewinnen.

II. INTRODUCTION

Perception of odors is one of the brain's dominant windows to sample the outside world. Olfaction, recognized as one of the five senses by ancient natural philosophers, has long been considered to belong more to the realm of artists and poets than to that of thorough neuroscientific investigation. Enormous progress has nonetheless been made in recent years at every step of the olfactory pathway - from the first contact of an odor molecule with a receptor in the olfactory epithelium, to the coding and processing of this information in the peripheral and central nervous system, to the arousal of feelings and storage of memories.

1. Biology of Olfaction

The molecular era in olfaction began in earnest in 1991 with the landmark discovery by Buck and Axel of a multigene family of ORs in rat (Buck and Axel, 1991). To have an overview of the molecular mechanism underlying olfactory perception, this part will be focused on mammalian models, where extensive investigation has been done in the past decade.

1.1 Overview of the Olfactory System

The first step of olfaction is the association of odorants with specific receptors on olfactory neurons. Odorants enter the nasal cavity and dissolve in the mucus that covers the luminal surface of the olfactory epithelium. They then bind to specific odorant receptors on the cilia of the dendrites of ORNs. After a several step signal transduction cascade action potentials are generated and propagate along the axons of ORN and thus the signals are transmitted to the olfactory bulb (Anholt 1993).

1.1.1 *Olfactory Epithelium*

The sense of smell consists of a series of neural structures in which olfactory sensory input is processed. In mammals, the first of these structures is the olfactory epithelium of the nose. Located in the dorsal recess of the nose, the mammalian olfactory neuroepithelium lies adjacent to respiratory epithelium covering the septum and turbinate cartilages. The two distinct compartments of olfactory epithelium are the highly vascular, glandular lamina propria or submucosa and the neuroepithelium proper which is separated by a basement membrane from the lamina propria, and which contains the ORNs, sustentacular cells (or supporting cells) and basal cells (Anholt 1993 and references therein).

Mucus of Olfactory Epithelium

The olfactory epithelium is covered by an ~30 µm thick layer of mucus. The mucus is a highly organized extracellular matrix, which has, in addition to providing stability to the ciliary network, two important functions: (1) protecting the neuroepithelium against damage by xenobiotics and invasion by microorganisms, and (2) providing a favorable milieu into which odorants can partition and gain access to and interact with odorant receptors at the chemosensory membrane. The mucus contains a vast array of glycoproteins and mucopeptides, which can be readily detected using a variety of lectins and antibodies (Anholt 1993 and references therein).

Like other body fluids, the olfactory mucus contains carrier proteins to accommodate hydrophobic molecules in an aqueous environment. Using radioactively labeled odorants, odorant-binding protein (OBP) has been identified in bovine and rat (Pevsner et al., 1990) olfactory tissue. These proteins belong to a family of hydrophobic ligand carrier proteins. OBP may help to promote the partitioning of odorants into mucus. Once bound, it may buffer the concentrations of odorants in the mucus layer and thus prevent nonspecific activation of receptor cells or deleterious effects by odorants at higher than physiological concentrations; it is also suggested that OBPs may play a role in odorant removal together with the detoxification enzymes (Schofield 1988).

Olfactory Sensory Neuron

ORNs (Fig. II.1) are bipolar cells forming part of a pseudostratified epithelium. ORNs project a dendrite toward the nasal lumen, where it terminates in a dilatation,
the “olfactory (or dendritic) knob”. From this knob a group of chemosensory cilia protrude, which contain classical 9+2 microtubular axonemal structures at their proximal ends. After a few micrometers they taper near their distal regions with a concomitant decline in the number of microtubular arrays (Anholt 1993 and references therein). The whole ciliary network provides a vastly expanded surface area for interactions with odorants. The cilia are regarded as highly specialized sensory organelles, responsible for the recognition and transduction of olfactory signals, since it is observed that deciliated olfactory neurons do not respond to odors (Kurahashi and Shibuya, 1990), and that putative ORs and key enzymes of the olfactory signaling cascades are primarily located in the cilia (Asanuma and Nomura, 1991; Bakalyar and Reed, 1990). Furthermore, fluorescence imaging experiments demonstrate that odorant-induced Ca^{2+} signals initially occur in the cilia before they can be detected in the knob (Leinders-Zufall et al., 1998).

A small unmyelinated axon projects from the opposite pole of the cell, penetrates through the basement membrane and, after making a 90° angle, forms together with other olfactory axons a nerve fascicle that is surrounded by processes of Schwann cells and traverses the lamina propria toward the olfactory bulb of the brain. These small olfactory fibers penetrate across the cribiform plate, a porous region of the

ethmoid bone, and synapse onto secondary neurons, the mitral or tufted cells in the olfactory bulb.

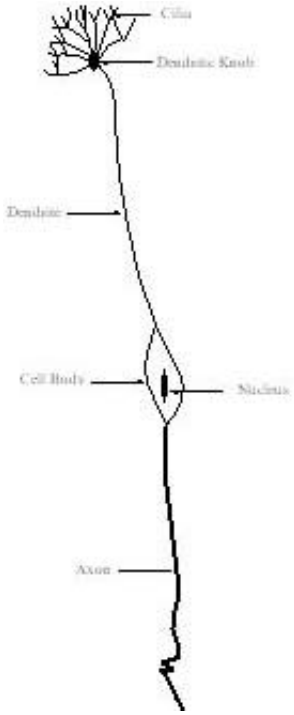

Figure II.1. An Olfactory Receptor Neuron (ORN).
A bipolar ORN sends dendrites distally which give rise to cilia, it also sends centrally an undiversified axon.

Sustentacular Cell

Olfactory receptor cells are surrounded by sustentacular cells. These are tall bottle-shaped cells that span the entire width of the neuroepithelium (approximately 150 μM) and contain microvilli at their luminal surface. They resemble glia and serve to electrically isolate neighbouring olfactory neurons. They are not excitable or electrically coupled, but undoubtedly affect interstitial ion concentration and influence consequentially the resting potential of olfactory neurons (Anholt 1993). Sustentacular cells of the olfactory system contain detoxification enzymes, including olfactory tissue-specific isoforms of cytochrome P-450 (Ding et al., 1991) and UDP-glucuronosyl transferase (Lazard et al., 1991). These enzymes also have been suggested to play a role in the metabolism of odorants, leading either to the removal of odorant or transformation of an odorant into a compound of different odor quality. Sustentacular cells also produce bacteriocidal proteins, including lactoferrin and lysozyme (Mellert et al., 1992; Dear et al., 1991).

Basal Cell

Basal cells are located near the basement membrane and constitute a neurogenic reservoir (Costanzo and Graziadei, 1983). Loss of olfactory neurons due to environmental damage, senescence, or experimental lesions stimulates divisions of

one population of basal cells, identified as “globose cells” (Schwartz Levey et al., 1991). Daughter cells from these divisions differentiate into mature bipolar olfactory neurons. This exceptional degree of neuroplasticity throughout adult life is a unique feature of the olfactory neuroepithelium (Costanzo and Graziadei, 1983).

1.1.2 *Olfactory Bulb*

The axons of olfactory neurons in each nasal cavity project to the ipsilateral olfactory bulb, which lies just above, and posterior to, the nasal cavity. The mammalian olfactory bulb consists of six layers: the olfactory nerve layer where the axon bundles of receptor neurons traverse the olfactory bulb; the glomerular layer where the olfactory processing unit – the glomerulus sits, and which harbors periglomerular interneurons (PG); the external plexiform layer where the tufted cells (T) sit; the mitral cell (M) layer; the internal plexiform layer and the granule cell (G) layer (Zigmond et al., *Fundamental Neuroscience, pp* 743).

Glomerulus

Both anatomical and physiological studies (Shepherd 1988; Scott 1991) have shown that the glomerulus is the nearly universal anatomical and functional unit

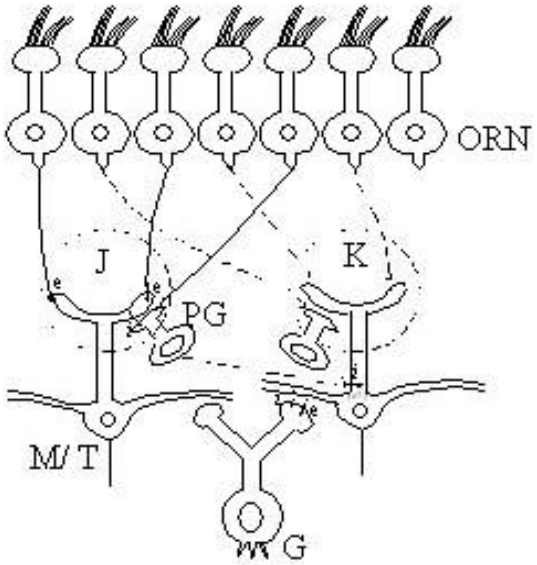

Figure II.2. Glomeruli and Their Related Synapses.
J and K are two glomeruli. Individual M/ T cells receive excitatory input from many ORNs; the neuronal signal is modified by e (excitatory) and i (inhibitory) synapses, intraglomerularly (PG) and interglomerulaly (GR, or G).

for olfactory processing found across phyla (Fig. II.2).

Mitral/ Tufted Cells

The two classes of primary output neurons in the main olfactory bulb, mitral and tufted (M/ T) cells, share several common features. Each receives sensory input from

olfactory nerve axons in the neuropil of the glomerular layer and in turn projects their axons to the piriform cortex. Within the bulb, dendrodendritic synapses form between interneurons and the primary and secondary dendrites of both M/ T cells. However, mitral cells are located in a compact layer referred to as the mitral cell layer, whereas tufted cells are dispersed throughout the external plexiform layer and the periglomerular region. Tufted cells are also considerably more diverse in their morphology (Wellis et al., 1991).

Our main insights in the understanding of olfactory bulb function are gained based on studies of mitral cells and the dendrodendritic synapses between mitral and granule cells. At this bi-directional synapse, release of glutamate from mitral cell dendrites drives GABA (γ-aminobutyric acid) release from granule cells that then leads to recurrent and lateral inhibition of mitral cells. Dendrodendritic inhibition of mitral cells can be quite prolonged and follows the slow kinetics of N-methyl-D-asparate (NMDA) receptors on granule cells (Schoppa and Westbrook, 2001). This provides the first step in the network processing of odorant responses that are mapped onto glomeruli in a highly ordered manner (Vassar et al., 1994). As a result, the duration and spatial extent of GABAergic inhibition is likely to have a major impact on sensory integration.

The role of tufted cells in olfactory processing is not well characterized. It is suggested that NMDA receptor-mediated excitation of interneurons drives inhibition of tufted cells at dendrodendritic synapses as it does in mitral cells. However, the spatial extent of this lateral inhibition is much more limited than that in mitral cells (Christie et al., 2001).

1.1.3 *Olfactory Cortex*

From the olfactory bulb, sensory information is transmitted to the olfactory cortex via the axons of M/T cells, which travel in the lateral olfactory tract (LOT) that extends along the olfactory cortex. The olfactory cortex has a three-layer structure that resembles the primitive structure of forebrain cortex found in fish, amphibia, and reptiles (Fig. II.3).

The principle neuron in the olfactory cortex is the pyramidal cell, with apical and basal dendrites bearing spines and recurrent collaterals connecting both to inhibitory interneurons and directly to other pyramidal cells. The M/ T axons make excitatory connections to the spine on the distal apical dendrites. Processing in the cortex takes place by means of the intrinsic excitatory and inhibitory synaptic circuits.

The olfactory cortex is divided into five areas: the main area is the piriform (sometimes called the pre-piriform) cortex. This area receives input from both mitral and tufted cells. It projects to the mediodorsal thalamus, which in turn projects to medial and lateral orbitofrontal areas of the neocortex. It is at this level that conscious perception of odors presumably takes place. The second area is the tubercle, which receives input mainly from tufted cells. The third is a group of amygdalar nuclei; these receive specific input from the accessory olfactory bulb and project to the

hypothalamus. Fourth is the lateral entorhinal area, which projects to the hippocampus. The fifth area is the anterior olfactory nucleus (AON), a sheet of cells just posterior to the olfactory bulb in primates, which appears to mediate communication among bilaterally symmetrical regions of the two olfactory bulbs (Buck 1996 and references therein).

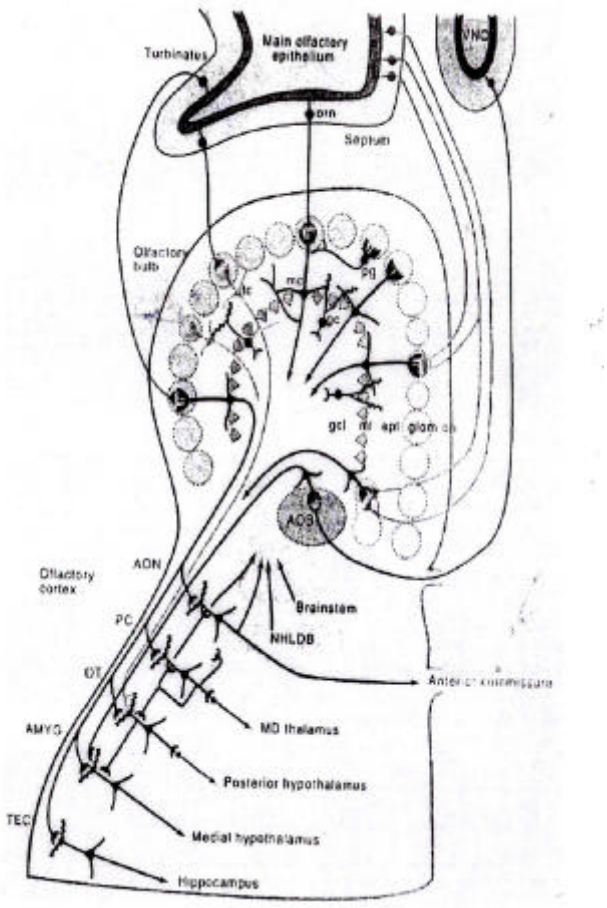

Figure II.3. Projection of M/ T cells in the Olfactory Cortex.
(taken from Zigmond et al. ***Fundamental Neuroscience***, *pp 755*)

In a very recent approach to gain insight into the connectivity within the olfactory system, transgenic mice were generated that coexpressed a transneuronal tracer with only one of the about 1,000 different odorant receptors (Zou et al., 2001). The tracer traveled from ORNs expressing that receptor to the olfactory bulb and then to the olfactory cortex, allowing visualization of cortical neurons that receive input from a particular odorant receptor. These studies revealed an invariant, stereotyped sensory map in the olfactory cortex in which signals from a particular receptor are targeted to

specific clusters of neurons, but this map in the cortex is more diffuse than that in the olfactory bulb. Signals from the same receptor are targeted to multiple olfactory cortical areas, permitting the parallel, and perhaps differential, processing of inputs from a single receptor before delivery to the neocortex and limbic system. These findings raise the interesting possibility that the organization of inputs to the olfactory bulb glomeruli from the olfactory epithelium is maintained to some extent in the projection to the cortex but is represented in multiple copies (Zou et al., 2001).

1.2 Odorant Receptor (OR) Genes

When odorants reach the chemosensory membrane after traversing the extracellular mucus, they trigger excitation of selective subsets of ORNs by binding to odorant receptors, which is followed by a cascade of signal transduction events. Ultimately, the accuracy of odor discrimination depends on the specificity with which odorants interact with appropriate ORs. Understanding the nature, diversity and specificity of odorant receptors is, therefore, crucial for understanding vertebrate chemoreception. How broad is the spectrum of an individual ORN? Which molecular features of an odor are detected and encoded by the olfactory system? Insight into the mechanisms underlying olfactory perception will require the isolation of odorant receptors, the characterization of their diversity, specificity, and patterns of expression, as well as the functional analysis in order to pair them with their ligands – odorants.

1.2.1 *Ligands for OR*

Many species have a sense of smell of enormous sophistication. A vast array of molecules can be detected, discriminated and identified, sometimes at extremely low concentrations. Odorants have different chemical structures, and subtle structural differences can lead to pronounced changes in odor quality. Odorous ligands are volatile for terrestrial animals and water-soluble for aquatic animals. For terrestrial animals, odorants are typically small hydrophobic organic molecules (Hildebrand and Shepherd, 1997).

1.2.2 *Cloning of OR Genes*

A major progress in olfaction was the 1991 discovery of a multigene family in rat (Buck and Axel, 1991). Their assumption was that OR genes should be members of a large gene family selectively expressed in ORNs, with a seven-transmembrane (7TM) spanning topology. This was based on biochemical evidence of G-proteins being involved in olfactory signal transduction. Using certain conserved amino acid motifs within other 7TM proteins, a large family of G-protein coupled receptors (GPCRs) selectively expressed in the olfactory epithelium was identified, and was referred to as odorant receptor (OR) genes.

OR Gene Sequences

Comparison of cDNA and genomic sequences indicated that OR genes have no introns within the coding sequence. The coding region of ORs is ˜ 1 kb long. Some of the conserved amino acids motifs distinguish ORs from other 7TM proteins (Fig. II.4, amino acid residues in white). Hypervariable regions can be discerned within TMs 3, 4 and 5 (Fig. II.4, amino acid residues in black; Buck and Axel, 1991; Pilpel and Lancet, 1999; Probst et al., 1992). The hypervariable regions may form the ligand-binding pockets. This fits to other 7TM proteins such as the β2-adrenergic receptor (Kobilka 1992), since structural diversity in ligand-binding domains is expected for receptors that interact with chemicals of vastly different structures.

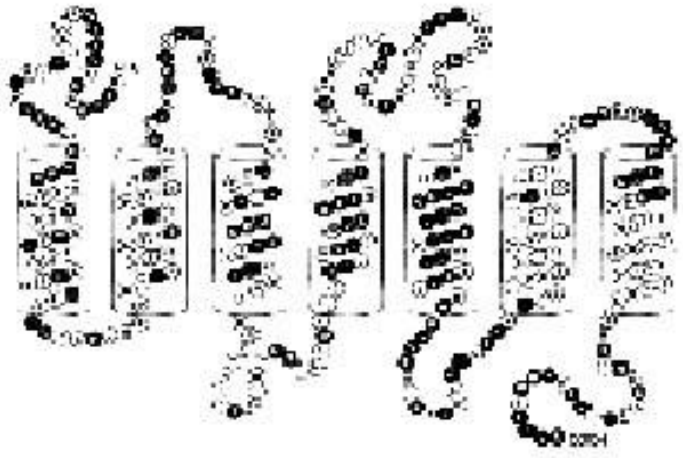

Figure II.4. An Odorant Receptor (taken from Buck & Axel 1991).
A typical OR (115) is shown schematically. Cylinders for hydrophobic transmembrane domains. N-terminal is extracellular.

Complexity of the OR repertoire is estimated to be 1000 genes in mouse and rat, 350 genes in human, and 100 genes in zebrafish and catfish (Axel 1995; Mombaerts 1999), thus the OR gene family is the largest family of the mammalian genome. A property is that there are very few examples of pseudogenes in the OR gene family, except for that of the humans, where pseudogenes are as many as functional genes. OR genes reside in clusters dispersed throughout the genome. Their sequences have been isolated from ˜ 20 vertebrate species ranging from lamprey to human (Cao 1998; Freitag 1999; Nef 1992; Ngai 1993; Ressler 1994; Weth 1996). In any species, only a small fraction of the genes has been cloned.

OR Gene Expression

The demonstration of mRNA for OR genes in ORNs is a necessary criterion for the claim that these genes encode receptors for odorants. Indeed, expression is restricted

to mature neurons. One feature of mammalian OR gene expression is that a given OR gene is expressed only within one of four stripes or "zones" of the olfactory epithelium (Ressler et al., 1993; Strotmann et al., 1994; Vassar et al., 1993). These zones are apparent from the earliest embryonic stages at which OR expression is detectable and do not require the presence of the olfactory bulb (Sullivan et al., 1995). Within a zone, a punctuate expression pattern is discerned, with ORNs expressing a given OR being interspersed with ORNs expressing other ORs in a mosaic fashion. The zonal expression is observed to be concomitant with the expression pattern of some glycoproteins (Nagano et al., 1998), which may help setting up the zones during development. The function of the zones is unclear. It is suggested that this compartmentalization is a primary determinant of the organization of the axonal projections. The wiring problem is rendered less complex by segregation of ORN expression into four subsets (Mombaerts 1999).

The commonly held belief that a single OR gene is expressed per neuron is derived from *in situ* hybridization analysis of the olfactory epithelium with probes for OR genes. Only a tiny percentage of neurons are labeled with a given probe, and overlap between different probes is not observed. Recent results from single cell RT-PCR also show that an ORN expresses one OR (Malnic et al., 1999). Thus it is fair to say that each neuron express only one or a small number of OR genes.

1.2.3 *Patterns of Axonal Projection and Glomerular Convergence Revealed by OR Transgenes*

The axonal projection pattern was exploited in an unconventional experiment in which *in situ* hybridization with OR probes was performed on sections of the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994). mRNA for a given OR probe could be detected at a few discrete sites per bulb, which appear to correspond to glomeruli. The location of these glomeruli is bilaterally symmetric and nearly invariant between different individuals. The interpretation is that small amounts of mRNA are present in axonal terminals of ORNs, and that the convergence of thousands of axons onto a glomerulus of 100 μm diameter provides enough mRNA for detection by ultrasensitive *in situ* hybridization methods.

This method, however, is not sensitive enough to allow visualization of small numbers of ORN terminals. Thus a genetic approach has been developed to visualize individual axons of ORNs expressing a given OR gene as they project from the epithelium to the bulb (Mombaerts et al., 1996). This strategy employed a transgenic mouse where the ORNs expressing olfactory receptor P2 also express a histochemical axonal marker taulacZ which was included as a second cistron after the P2 coding region via the linkage of an IRES (Internal Ribosome Entry Site) signal. Two labeled glomeruli were observed, with bilaterally and interindividually similar positions. No evidence for axons deviating from these common path were observed, indicating that all the axons from the same ORNs converge and project to discrete sites in the olfactory bulb.

1.3 Intracellular Signal Transduction

1.3.1 *Cloning of G α olf, AC III and OCNc*

It is believed that upon odorant binding to the putative ORs, a specific form of Gα protein, Gα olf (Jones and Reed, 1989), is activated, that leads to the activation of intracellular signal transduction molecules and finally causes the firing of ORN. In addition to Gα olf, other component molecules involved in this cascade, AC (adenylate cyclase) III and three OCNC (olfactory cyclic nucleotide channel) subunits have been cloned in the last decade.

Gα olf

The G-protein, which links activation of an odorant receptor to stimulation of adenylate cyclase, is enriched in olfactory cilia (Anholt, 1993). Cloning of cDNA encoding this protein showed that it had 88% sequence identity with G_S. It could be classified as a Gs-related but distinct G-protein, which was named " Golf " (Jones and Reed, 1989). This G-protein was able to couple to adenylate cyclase in a heterologous system and hence, is likely to present the G-protein responsible for regulating the elevation in cyclic AMP concentration in response to odorants (Jones and Reed 1989). However, Golf, despite its nomenclature, is not restricted to ORNs, but also is found in neuronal cell populations elsewhere in the nervous system (Wilkinson et al., 1999).

AC III

A new isoform of adenylate cyclase has been cloned from olfactory tissue (Bakalyar and Reed, 1990), and was designated "adenylate cyclase type III" (Bakalyar and Reed, 1990). Since the olfactory system expresses unique rather than conventional isoforms of the adenylate cyclase and G-protein, it has been suggested that these isoforms may possess kinetic properties to provide a particular sensitivity, and a high signal-to-noise ratio for signal amplification upon odorant detection (Bakalyar and Reed, 1990). It is also possible that the structural variations in these transduction components function as intracellular trafficking signals to direct transport of these macromolecules following their biosynthesis toward the dendritic rather than the axonal membrane (Anholt 1993).

OCNC

It is known that in sensory neurons, cyclic nucleotide-gated (CNG, or CNC) channels can be composed of several distinct subunits in unknown stochiometry (Zagotta and Siegelbaum, 1996). CNCα3 and CNCα4, as well CNCβ1b are expressed in sensory cilia of ORNs, the third being an alternative splice form of the rod photoreceptor

CNCβ1a subunit. Heterologous expression of these subunits in HEK293 cells with all possible combinations showed that with respect to gating kinetics, single-channel conductance, ion selectivity, and cAMP sensitivity, the combination that matches the olfactory-specific CNG channel best was observed with channels containing all three subunits. This suggested that the native CNG channel in mature olfactory neuron be built from CNCα3, CNCα4 and CNCβ1b subunits (Zagotta and Siegelbaum, 1996).

1.3.2 *Second Messengers Involved in Olfactory Signal Transduction*

Odorant-Mediated Generation of Cyclic AMP

Cyclic AMP was identified as a possible second messenger in olfaction in the 1970s, when several investigators noted that olfactory tissue contains adenylate cyclase activity and that membrane permeant analogs of cyclic AMP and phosphodiesterase inhibitors could modulate the electro-olfactogram (EOG). The EOG is an odorant evoked voltage transient that can be recorded from the surface of the olfactory neuroepithelium during application of odorants. It was not until the mid-1980s that partially purified preparations of olfactory cilia became available (Anholt 1993). The demonstration that these preparations were highly enriched in adenylate cyclase activity and its associated G-protein and that odorants could stimulate this enzyme in a GTP-dependent manner firmly consolidated the notion that cyclic AMP plays a role as a second messenger in olfaction (Shirley et al., 1986). Further support for a role for cyclic AMP in olfaction came in 1990 from rapid quench flow experiments (Breer et al., 1990). The involvement of a cyclic AMP-mediated transduction pathway in olfaction was further demonstrated by electrophysiological studies using the patch-clamp technique. Application of forskolin, analogs of cyclic AMP, or phosphodiesterase inhibitors to isolated olfactory neurons gave rise to electrical activity that mimicked the response to odorants (Firestein et al., 1991).

Odorant-Mediated Generation of Inositol Triphosphate

Soon after the identification of cyclic AMP as a key element in olfactory transduction, it was suspected that other second messengers must be involved in mediating and regulating the response to odorants. Some odorants, such as pyrazine, failed to elicit an increase in cyclic AMP levels, instead, they elicited a transient increase in inositol triphosphate (Breer et al., 1992). In the catfish, *Ictalurus punctatus*, inositol triphosphate-gated channels have been demonstrated in the ciliary plasma membrane (Restrepo et al., 1990). In this system, the response to odorants generates inositol triphosphate and is accompanied by influx of calcium, as monitored by the calcium-sensitive fluorescent dye, fura-2 (Restrepo et al., 1990).

However, these findings were not supported by the results from the mutant mice where Golfα (Belluscio et al., 1998) or OCNCα 3 (Brunet et al., 1996), believed to be involved in the cAMP specific cascade, were knocked out. In these mutants, no

EOG response was detected to all applied odors. These odors include the "IP3" odors. These results strongly supported the idea that at least for all the tested odors, cAMP is the exclusive signal transduction molecule.

It is not clear whether odorant-mediated generation of inositol triphosphate represents an independent parallel pathway or whether it is an intermediary step some odorants utilize that leads to the generation of cyclic AMP. The former hypothesis would imply two independent pathways that lead to the generation of action potentials in the olfactory neuron, whereas the latter scenario would funnel all odorant-elicited responses ultimately through the cyclic AMP pathway. Nonetheless, the collective evidence indicates that inositol triphosphate is generated in response to at least some odorants in some animals (Fig. II.5), and that this second messenger may mediate calcium influx in olfactory cilia.

Olfactory transduction is a tightly controlled process that involves a complex interplay of several second messengers. One important participant in this process is calcium, which may mediate crosstalk between these two transduction pathways (Anholt 1993).

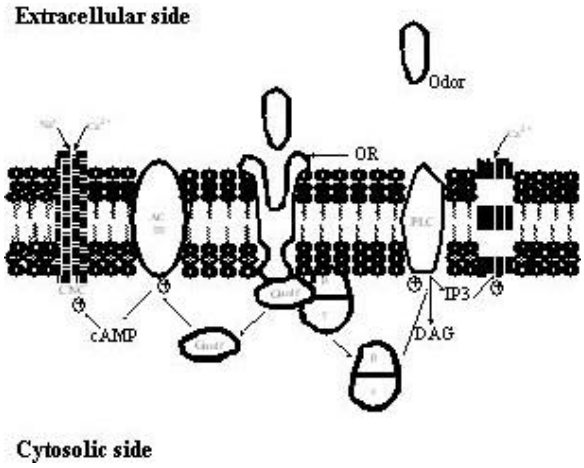

Figure II.5. Signal Transduction Components in an ORN.
Concentration of cAMP and possibly IP3 is increased upon binding of odorants to OR via activation of ACIII and PLC.

1.3.3 *Ion Conductances Underlying the Electrical Response of Olfactory Receptor Neuron*

Numerous studies over the last decade revealed that many odorants elicit the activation of a Ca^{2+}-permeable non-selective cation conductance (Frings et al., 1991) followed by a non-linear Ca^{2+}-dependent chloride conductance (Reuter et al., 1998). It has been assumed that the chloride conductance significantly amplifies the odor-induced depolarization of the cilia, and the non-linear Ca^{2+} sensitivity of this current is thought to introduce an excitation threshold to improve the signal-to-noise ratio of

the transduction process (Dzeja et al., 1999). The depolarization of the cilia propagates by passive electronic spread, and for a sufficiently large stimulus triggers the generation of action potentials at the initial segment of the axon (Zufall et al., 2000a).

Regulation of the Olfactory Response by Calcium: Amplification and Adaptation

A variety of electrophysiological and biochemical experiments implied that calcium may facilitate the response to odorants by providing a cellular amplification mechanism, which would be most important for the detection of low concentrations of odorants (Anholt 1993). Inositol triphosphate-mediated influx of calcium in response to some odorants (Restrepo et al., 1990) activates adenylate cyclase via calmodulin. Thus, calcium may mediate crosstalk between the two major olfactory transduction pathways and might present one mechanism that insures the ultimate generation of cyclic AMP in response to all odorants.

Signal Termination

Desensitization of the response to odorants during prolonged application of the stimulus likely involves phosphorylation of key components of the transduction machinery by protein kinase. Protein kinase A and C were implicated in the desensitization following odorant-mediated generation of cyclic AMP and inositol triphosphate, respectively (Breer et al., 1992). The biotransformation enzymes, P-450 and UDP-glucuronosyl transferase, may also play a role in signal termination, possibly by removal of odorants (Lazard et al., 1991; Nef et al., 1992).

1.4 Combinatorial Coding Strategy in the Olfactory System

1.4.1 *Odor Image in Neural Space*

2-deoxyglucose uptake in response to different odors revealed that different odors evoke different patterns of active glomeruli located in distinct domains within the olfactory bulb (Buck 1996 and references therein). For instance, the glomerular activity elicited by the odor of amyl acetate is localized in two broad zones, one medial and one lateral, within the olfactory bulb glomerular sheet. In contrast, the activity elicited by camphor is distributed in a curving line of smaller glomerular patches. Although the two domains overlap, their overall patterns are distinct and different. The result of this and other domains associated with other types of odor stimuli suggests that each type of odor elicit a characteristic pattern of glomerular activation in the olfactory bulb; these patterns may be considered to constitute **odor images** in neural space.

Other methods have confirmed and extended the 2-deoxyglucose uptake results. *In situ* hybridization of c-fos mRNA, voltage-sensitive dyes, calcium imaging in zebrafish and honeybees, have revealed similarly, that a combinatorial and chemotopical activity pattern exists in the glomeruli, to discern different odors.

1.4.2 *Synapses Involved in Modulation of Odor Processing by Glomerulus*

In addition to the excitatory synapses made by ORN axons onto dendrites of M/ T and PG cells, there is a wealth of synapses between the M/ T cell dendrites, the PG cell dendrites and the GR cell dendrites (Fig. II.2). These synapses are believed to provide for numerous types of interactions, including serial excitatory synapses (which spread excitation widely within a glomerulus), recurrent and lateral inhibitory synaptic circuits, and disinhibitory synaptic interactions (Zigmond et al., *Fundamental Neuroscience*, *pp* 743). Besides the *intra*glomerular processing, there is *inter*glomerular processing through the PG cells, which make synapses on PG cells related to neighbouring glomeruli, and through the GR cells. This may provide contrast enhancement between neighbouring glomeruli through inhibition. This action may also be excitatory by means of a network of presynaptic PG cell inhibitory actions and by GABA-activated synapses acting through a reversed chloride gradient (Lienders-Zufall et al., 1998; Treloar et al., 1996).

Like most other sensory pathways the olfactory system is also under control by intrinsic peptidergic and centrifugal inputs. Several studies suggested that dopamine exerts a neuromodulatory action on sensory input (Sallaz and Jourdan, 1992; Coopersmith et al., 1991). The noradrenergic centrifugal system arising in the brainstem is probably involved in modulating the olfactory system at both bulbar and olfactory cortical levels (Trombley and Shepherd, 1994).

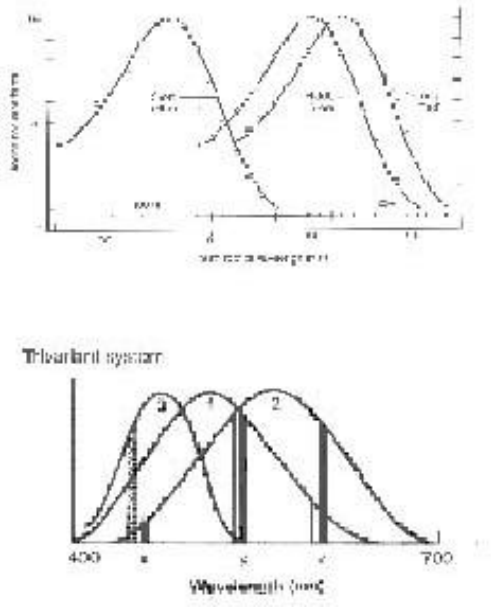

Figure II.6. Peripheral Mechanism of Color Vision.
Upper: absorption spectrum for three opsin receptors, which respond preferentially to short, middle and long wavelengths.
Lower: wavelength X, Y or Z is believed to be distinguished by the different combinations of the three kinds of activated receptors.

Despite several breakthroughs in the study of olfaction, the strategy employed by our nose to distinguish a rose from a sweat T-shirt remains enigmatic. Revealing the mechanism underlying odor detection in the peripheral olfactory system is the basis to discover how information of an olfactory stimulus is encoded. In color vision, the wavelength emitted by an object is perceived by the activation of different cone receptors preferentially detecting color of red, green or blue (Fig. II.6); in the auditory system, the physical property of sound-frequency is encoded by hair cells. However, in olfaction it is not yet clear which of the many molecular properties of an odorant are encoded by the ORNs.

We use zebrafish as a model system to address the question which stimulus features are detected by individual ORs (the receptive field of that receptor). The advantage of such an animal model and the feature of the fish olfactory system will be introduced in the coming part.

2. Olfactory System of Zebrafish

Zebrafish (*Danio rerio*, old name *Brachydanio rerio*) are amenable to genetic analysis due to large number of offspring frequently obtainable, ease of breeding and a short reproductive cycle. Moreover, the early embryonic development is rapid in zebrafish (hatching occurs about 72h postfertilization, pF) and morphological aspects can be studied by visual inspection in transparent embryos, which develop extramaternally. The repertoire of zebrafish odorant receptor is estimated at 100, one tenth of that of mammals; fish odors are water-soluble, some of which are well characterized, while odors for mammals are air-borne and thus more difficult to handle (Korsching et al., 1997).

These features led us to choose zebrafish as a model animal to study olfaction. The following part is focused on the characteristics of fish olfaction, with most of the findings from zebrafish, goldfish, salmonid fish or catfish.

2.1 Overview of Zebrafish Olfactory System

The zebrafish olfactory system consists of a neuroepithelium located in the nose, the olfactory bulb and the olfactory cortex (Fig. II.7).

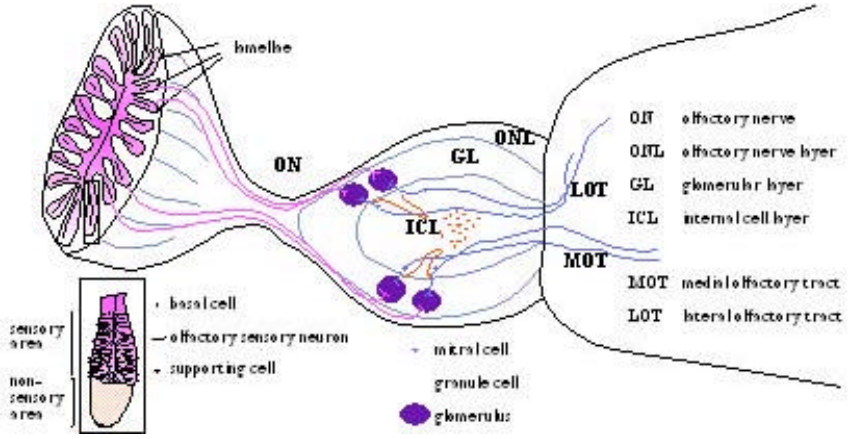

Figure II.7. Illustration of Zebrafish Olfactory System (from A. Çelik).
ORNs sit in the inner (sensory) part of nasal epithelium, which send their axons to make synapses with mitral cells in the olfactory bulb. Projection neurons (mitral/ ruffed) cells send their axons to make synapses with neurons in the telencephalon.

Peripheral Olfactory Organ

In zebrafish, two nostrils containing olfactory epithelium lie on the dorsal side of the head, close to each eye (Fig. II.8). Water conveying odorants enters the nasal cavity through the funnel-shaped inlet and exits through a posterior outlet. A single peripheral olfactory organ, varying between 250 µm and 1 mm in diameter, has the shape of a bilaterally symmetrical rosette whose size increases as the fish grows. The increase in size is slower but still present in fully mature fish. The center of the rosette is the midline raphe from which the lamellae project outward. The oldest and largest lamellae are arranged at the caudal end of the organ while young lamellae are formed rostrally at both sides of the midline raphe. The thickness of the sensory epithelium varies from ~15 µM in the valleys at the midline raphe to ~20 µM on the lamella (Hansen and Zeiske, 1998).

The nonsensory and sensory epithelium on one lamella are rather strictly segregated in zebrafish. The epithelium consists of two ultrastructurally distinct types of ORNs, the ciliated and microvillous ORNs; supporting cells and the small,

roundish basal cells, all of which are closely packed. The nonsensory epithelium immediately adjacent to the olfactory epithelium consists of ciliated cells, globlet cells and rodlet cells (Hansen and Zeiske, 1998). Very often, the ORNs with long slender dendrites bear cilia and ORNs with short thick dendrites bear microvilli. Supporting cells separate the dendrites of ORNs. Usually one supporting cell envelops several receptor dendrites. The small basal cells lie adjacent to the basal lamina, between the basal parts of the supporting cells and the axons of the receptor cells. The number of basal cells is not high while there seem to be more at the margins of the sensory area and valleys close to the midline raphe. Sometimes mitosis is recognizable (Hansen and Zeiske, 1998).

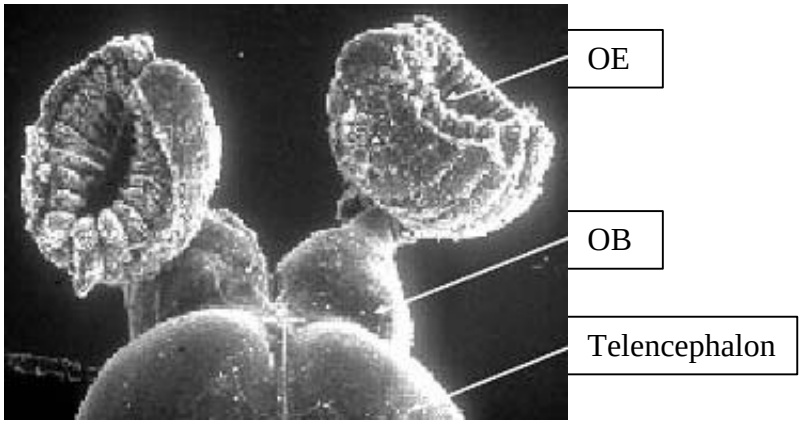

Figure II.8. Olfactory System of Zebrafish (from H. v. Campenhausen). An SEM micrograph of the zebrafish olfactory system, consisting of olfactory epithelium (OE), the peripheral olfactory organ; the olfactory bulb (OB), immediately posterior to the OE, connected through the short olfactory nerve; and the telencephalon containing several olfactory centers.

The olfactory axons (0.12-0.15 µM in diameter) accumulate within the sensory epithelium and penetrate the basal lamella in bundles. In the lamina propria these bundles form the fila olfactoria, which in turn aggregate and leave the olfactory organ. Axons of ORNs join together to form the olfactory nerve, the cranial nerve I. The olfactory nerve traverses through a single opening in the ethmoidal bone to the olfactory bulb (Hansen and Zeiske, 1998).

ORNs are replaced continuously, or following chemical or axonal lesions. One of the chemicals that are used widely to induce degeneration and regeneration of ORNs is $ZnSO_4$ (Cancalon, 1982). The ORN axons project directly to the olfactory bulb to synapse with dendrites of second-order olfactory neurons, the mitral cells. Zebrafish have sessile olfactory bulbs, which are in close contact to the telencephalon (Laberge and Hara 2001 and references therein).

Two Types of Fish ORNs

In many terrestrial vertebrates, the microvillous and ciliated ORNs are largely segregated in separate receptor organs. Microvillous cells constitute the large majority of ORNs in the vomeronasal organ, an olfactory organ that is believed to detect pheromonal odorants. Conversely, the main olfactory sensory epithelium contains mostly ciliated receptor neurons (Carr et al., 1991; Miller et al., 1995; Moran et al., 1982; Morrison and Costanzo, 1990). Because of this segregation, many investigators have postulated that vomeronasal, microvillous ORNs respond to nonvolatile, pheromonal components, whereas the ciliated ORNs respond to volatile components (Bargmann 1997). Microvillous ORNs, however are not always tuned to pheromones. In some species, e.g. garter snakes, microvillous ORNs of the vomeronasal organ respond to odorant cues that are used in tracking prey (Halpern et al., 1997).

In teleost fishes, two ontogenetically and morphologically different ORN types, ciliated and microvillar cells, are present but intermingled within the epithelium; no vomeronasal organ exists (Thommesen 1982). A segregated distribution of the two sensory cell types has been noted at the level of lamella in salmonid fishes and catfish, but there is no general agreement on which cell type serves which function. Thommesen (Thommesen 1982) described differences in responsiveness between areas of epithelium that are rich in microvillous ORN and areas that are rich in ciliated ORNs. Thommesen concludes that sensitivity to bile salts correlates with abundant ciliated receptor cells, whereas sensitivity to amino acids correlates with a high abundance of microvillous cells. However, comparative electrophysiological and anatomical studies on channel catfish failed to reveal functional differences between microvillous-rich and ciliated, ORN-rich areas (Morita and Finger 1998 and references therein), when EOG and multi-unit activity was recorded in the olfactory epithelium. In contrast, degeneration-regeneration studies in goldfish suggest that sensitivity to amino acids is correlated to the presence of ciliated ORNs (fast replacement), whereas sensitivity to steroid pheromones correlates with the presence of microvillous ORNs (slow replacement) (Zippel 2000). Thus the functional contribution of each type of ORN is controversial.

It is suggested that ORN morphology could be involved in odorant coding. However, because of the paucity of studies on this topic, generalizations can not be made. It seems that at least in some species the ciliated and microvillar ORNs might be specialized to detect different odorant classes.

Olfactory Bulb

In zebrafish, the olfactory bulb is a paired structure immediately adjacent to the telencephalon and connected to the olfactory rosettes by the olfactory nerve. The bulb can be divided into four principal laminae: the olfactory nerve, glomerular, mitral cell/ external plexiform, and granular cell layers (Byrd and Brunjes, 1995). The olfactory

nerve layer is a thin sheet of olfactory axons spreading over the surface of the bulb. The axons finally turn down to reach the glomerular layer where they intermingle with central processes. By Nissl staining the glomeruli do not appear as distinct spheres as those of mammals, most likely because of fewer juxtaglomerular cells and glial elements in this species. However the presence of glomerular structures composed of branches from axon layer and dendritic tufts from mitral cells are implied by labeling of axons of sensory neurons by DiI (Baier and Korsching, 1994).

The mitral/ plexiform layer deep to the glomerular layer is the most diffuse of the layers. It contains cells with large and small nuclei scattered throughout the neuropil. Mitral cells identified by their large pale nuclei are seen throughout the layer. They do not show the characteristic “mitre” shape found in mammals. Scattered all over in this layer are also darkly nucleated cells presumed to be glia. The innermost layer of the olfactory bulb is the granule cell layer (Laberge and Hara 2001 and references therein).

Telencephalon

The central neural pathways of the fish olfactory system are quite different from that of mammals. The existence of an anterior olfactory nucleus precursor in fish is controversial (Satou et al., 1990). It may correspond to a population of cells in the deep layer of the olfactory bulb that project to the telencephalon (Matz 1995). Also, the relay between the amygdala and the hypothalamus is not necessary in fish where olfactory fibers project directly to the preoptic area (POA) and diencephalon. The role of rich olfactory pallial projections in teleost fishes, or the homologous areas they would represent in other vertebrates are also not yet known (Laberge and Hara 2001).

Projection of Mitral Cells in Teleosts

The projections of the second-order neurons of the olfactory bulb form the bulk of the olfactory tract, which is divided into the medial (MOT) and lateral (LOT) olfactory tracts. The LOT is mainly formed by projection fibers from the lateral part of the olfactory bulb, while the MOT carries most fibers from the medial olfactory bulb. In agreement with the anatomical observations, neurons activated antidromically from the LOT are located mainly in the lateral part of the olfactory bulb, while those activated by MOT volleys are located mainly in the medial part of the bulb (Laberge and Hara 2001 and references therein). In addition, it has been demonstrated that lesions of the MOT prevent the effects of pheromone exposure in the African catfish and goldfish (Resink et al., 1989; Stacey and Kyle, 1983), while electrical stimulation or severing of the LOT affects feeding behavior (Stacey and Kyle, 1983), indicating the functional diversity conveyed by these two bundles of nerves.

The olfactory tract transmits odor information to the telencephalic hemispheres. Though there is some variation among fish species, the LOT usually projects to the dorsal part of the posterior telencephalon, with a smaller branch sometimes running

ventrally in the telencephalon (Matz 1995). The MOT has two major branches, the dorsolateral (DL) and ventromedial (VM) bundles. The VM exclusively innervates the ventral telencephalon, POA and the diencephalon. The DL terminates in various areas of the dorsal and ventral telencephalon as well as the POA and the diencephalon. A significant portion of the olfactory tract fibers project contralaterally through different commissures. Centrifugal connections from the telencephalon to the olfactory bulb also run in the medial part of the MOT.

Ontology

During the development of zebrafish, the olfactory placodes first appear at 14-16 hr postfertilization (6-10 somites) as bilaterally paired thickenings of subepidermal ectoderm at the rostral-ventral region of the head, between the developing forebrain and the optic vesicles. The epidermis splits around 30h pF, thereby allowing external access to the sensory surface, well before hatching (48-72 h pF). The subsequent morphological maturation is a slow process and takes several weeks (Hansen and Zeiske, 1993). By ~24 hr pF (20 somites), axons have emerged from the placodal cells and appear to make contact with the forebrain. The first terminals of ORNs in the future olfactory bulb are observed at 24 hr pF. Fasciculation of axons emerging from the olfactory placode is evident by 36 hr, and glomerular fields have been observed in the forebrain by 2-3 days of development (Wilson et al., 1990). The first recognizable glomerulus separates from the terminal plexus at day 2 pF. Subsequently the adult pattern is generated by ongoing addition of glomeruli over several weeks.

The developmental time course of glomerular formation is substantially different from the onset pattern of odorant receptor gene expression. Temporal waves of expression were observed in zebrafish embryos for subsets of receptor genes at 24-30 hr, 36-48 hr and 120 hr (Barth et al., 1996), not concomitant with the much slower increase in glomerular number. Thus, onsets of receptor gene expression and glomerulus formation appear to be regulated by at least partially independent mechanisms.

2.2 Molecular Biology of Fish Olfaction

The fish olfactory system is in many aspects similar to that of mammalians, but has its own distinctive feature as well. Fish live in a different environment and have different odors; they also occupy a different position in the phylogenetic tree. The following part will thus emphasize some special characteristics of fish olfaction, on the basis of the fragmentary knowledge obtained so far.

2.2.1 *Fish Odors*

Fish face enormous problems finding their way around their environment, which is dense, often dark and devoid of visual cues, but may be very spacious. Furthermore, the aquatic medium contains immense dissolved compounds. Notably, many species of related fish may be expected to frequently encounter each other so hybridization can be a problem. It is thus not surprising that teleost fish have evolved sophisticated and varied olfactory signaling systems. Olfaction was reported to be involved in the following behaviors:

1. the localization of food;
2. alarm behavior;
3. sex pheromone detection including the control of sexual behavior;
4. nonspecific arousal and specific recognition of chemical stimuli;
5. the formation of a chemical search image of prey and
6. recognition between conspecifics (Valenticic et al., 1994).

Substances identified as olfactory stimuli for fish are often classified on the basis of their chemical structure or the type of behavioral responses elicited. Some odorants, such as amino acids, sugars and nucleotides, mediate behaviors, such as feeding, common to all species. Other behaviors, such as mating, alarm responses, and predator recognition, are species-specific, unrelated to feeding, and are mediated by other odors, such as specific bile acids, steroids, prostaglandins.

Several major classes of compounds have been identified as specific olfactory stimuli and their stimulatory effectiveness have been characterized, these include: amino acids, bile acids/ salts, nucleotides, sexual steroids, and prostaglandins (Hara 1994).

Amino Acids

As chemicals related to food source, a wide range of amino acids are detected by the zebrafish olfactory organ, with threshold concentrations ranging from 10^{-9} to 10^{-7} M, approximating levels of free amino acids found in natural waters. Generally, L-α–amino acids containing unbranched and uncharged side chains are the most effective olfactory stimuli. In an approach to test zebrafish response to general fish odors, EOG pattern of relative stimulatory effectiveness established for the amino acid stimuli was neutral amino acids> basic amino acids >acidic amino acids> imino acids (Michel and Lubomudrov, 1995; Friedrich and Korsching, 1998). Except for certain basic amino acids, it is observed that ionically charged α–amino and α-carboxyl groups are essential for amino acids as olfactory stimulus, acetylation of the former or esterification of the latter results in reduced activity (Hara 1975; Fuss and Korsching, 2001).

The concentration-response relationship of amino acids exhibits a characteristic broad dynamic range, covering over 6-7 log units, which suggests the existence of a

multiple receptor system (Hara 1994). The olfactory spectrum of amino acids is generally similar across fish species examined, suggesting a function common to all fish species.

Bile Acids/ Salts

High olfactory sensitivity and specificity to bile acids/ salts have been shown in several fish species, with thresholds ranging between 10^{-12} and 10^{-10} M (Døving et al., 1980). In zebrafish, the general pattern of relative stimulatory effectiveness of 100 µM bile acid stimuli was taurine-conjugated bile acids > glycine conjugated bile acids ~ nonconjugated bile acids (Michel and Lubomudrov, 1995), as revealed by EOG. Naturally produced and released bile acids may provide a constant source of population- or species-specific chemical signals for kin recognition and homeward migration, at least in salmonid fish and sea lamprey. Behavioral studies in Arctic char and Atlantic salmon have shown that they were attracted to the intestinal contents of juveniles.

Though feces are a major source of bile acids released into water, the threshold for detection of bile acids by the lake char olfactory organ (nanomolar concentration) is far below the level of the one that is released (Zhang et al., 2001), revealing a high olfactory sensitivity to bile acids. Mammalian bile salts were also found to be stimulatory in the same experiment; among those tested, taurolithocholate, taurolithocholate 3-sulphate and taurodeoxycholate are the most stimulatory odors. Other species found to be also sensitive to bile acids include: sea lamprey (*Petromyzon marinus*), goldfish and suckers (*Catostomus commersoni* and *C. catostomus*) (Sorensen et al.,1987; Li et al., 1995; Cardwell et al., 1992).

Nucleotides

As ordinary odorants for fish and possible components of feeding stimuli, ATP, IMP and TTP were shown to elicit a specific activity pattern in the zebrafish olfactory bulb (Friedrich and Korsching, 1998) by optical imaging. Their corresponding foci are located at the posterior-lateral olfactory bulb, different from the response region for amino acids, which is located anterio-laterally, and that of bile acids, which is at the anterio-medial part.

In addition to these ordinary odors, fish can also detect vanishing concentrations of pheromones. Pheromones are involved in functions like recognition of injured conspecifics, kin recognition, synchronization of reproductive physiologies (Li et al., 1995; Dulka 1993), and synchronization of reproductive behavior patterns (Sorensen et al., 1988; Fujita et al., 1991).

Prostaglandins (PGs)

Most information about fish pheromones is documented from the goldfish, *Carassius auratus*, which is the only species where pheromone release, neural sensitivity, and behavioral responsiveness have all been well characterized. Using EOG, it was found that goldfish olfactory receptors detect PGF2α and 15-keto-PGF2α at a threshold of 10^{-10} M. PGF1α, PGF3α and PGE are also stimulatory, but at lesser degrees (Sorensen et al., 1988). 14 cyprinids, four catostomids, two salmonids, and one cobitid have been shown to have olfactory sensitivity to PGs (Cardwell et al., 1992; Moore and Scott, 1992) with difference in the response pattern, for instance, in Arctic char, *Salvelinus alpinus*, PGF2α, 16, 16-dimethyl-PGF2α and U-46619 are most stimulatory (threshold, 10^{-9} - 10^{-8} M) (Sveinsson and Hara, 2000). In zebrafish, optical imaging using Di8-ANEPPQ, a voltage-sensitive probe, has revealed a prominent glomerulus in the ventral olfactory bulb that is activated upon stimulation with 1 nM PGF2α, but not PGE2α (Friedrich and Korsching, 1998).

Sexual Steroids

The goldfish olfactory system detects a preovulatory pheromone, 17α, 20β-dihydroxy-progesterone (4-pregnen-17α, 20β-diol-3-one, 17, 20-β P), at a threshold of 10^{-12} M (Sorensen 1995). Further studies showed that of more than 20 closely related gonadal steroids, only sulfated 17,20βP and 17α,20β,21P are effective. Of other fish species, only 11 Cyprinidae, including common carp, *Cyprinus carpio*, and crucian carp, *Carassius carassius*, detect any of these steroids or their conjugates of glucuronide or sulphate (Cardwell et al., 1992). Precocious male Atlantic salmon, *Salmo salar*, exhibit high sensitivity (threshold, 10^{-14} M) to testosterone only during the month of October (Moore and Scott, 1992). Non-spermiating precocious male paar, non-precocious male paar, and immature female do not respond at any time. Furthermore, they become extremely sensitive to 17, 20βP-sulphate, but not to 17, 20βP, by exposure to the urine of ovulated females (Moore and Scott, 1992). No olfactory sensitivity to sex steroids is known in other salmonids. African catfish, *Clarias gariepinus*, detect certain reduced 21-carbon steroids including 5β-pregnen-3α, 17α-diol-20-one-3α-glucuronide, with a threshold concentration of 10^{-11} M (Resink et al., 1989). African catfish are insensitive to 17, 20βP, whereas goldfish are insensitive to steroid glucuronides. In zebrafish, 17, 20P-S was found to be a potent olfactory stimulus (Friedrich and Korsching, 1998).

Because of the paucity of studies in this topic, general conclusions about features of pheromones as fish olfactory stimuli can not be made. However, it appears plausible that in different fish species, different PGs and sexual steroids, or their mixtures, are potent olfactory stimuli.

Other Compounds

A number of compounds, odorous to human, are found to be non-stimulatory for the fish olfactory system, when tested electrophysiologically or by optical imaging. These include alcohols, carboxylic acids, amines and aromatic hydrocarbons (Hara 1994; Fuss and Korsching, 2001).

2.2.2 *Cloning of Fish OR*

Putative fish ORs have been cloned in zebrafish (Bath and Ngai, 1996; Weth et al. 1996), catfish (Ngai et al., 1993), puffer fish (*Fugu rubripes*) (Asano-Miyoshi et al., 2000), goldfish (Cao et al., 1998), and medaka fish (*Oryzias Iatipes*) (Yasuoka et al., 1999). The spatial and temporal expression pattern of catfish and zebrafish OR genes has been investigated in several studies.

Temporal and Spatial Pattern of Zebrafish OR Gene (ZOR) Expression

Nested expression domains of ZOR genes have been found in the zebrafish olfactory epithelium (Weth et al., 1996). Statistical analysis of the annular expression pattern visualized by *in situ* hybridization using several ZOR genes as probes revealed three expression domains. Such a zonal topology may have been overlooked in catfish (Ngai et al., 1993).

In an approach toward understanding the events that underlie the specification of ORNs, Barth et al. (1996) have examined the patterns of odorant receptor gene expression in the developing zebrafish. Surprisingly, with the six OR receptor genes investigated, the onset of specific odorant receptor expression occurs asynchronously in the developing olfactory placode, which suggests that odorant receptor expression is not strictly stochastic, but rather is governed by temporally regulated cues during development. Moreover, temporal waves of expression before 30 hr postfertilization (receptors 1, 2, 9 and 10), between 36 and 48 hr (including receptor 13) and until 120 hr (represented by receptor 6 and 10 subfamilies), may provide a mechanism for simplifying the regulation of the large odorant receptor gene family.

Regulation of ZOR Expression

The transcriptional activity of two 5'-upstream regions of the olfactory receptor genes was analyzed using zebrafish embryos using the microinjection technique (Mori et al., 2000). Constructs of 700 bp 5'-upstream of ZF 2A and 570 bp 5'-upstream of ZF 9A (ZF 2A and ZF 9A are two OR genes of zebrafish), drove expression of β-galactosidase reporter gene in the olfactory tissue. In addition, the results of deletion constructs of the ZF 9A 5'-upstream region indicated that the fragment from -370 bp to -570 bp of ZF 9A 5'-upstream region is necessary to elcit gene expression in the

olfactory tissue. However, it is still largely unclear how the expression of a single OR/ ORN is accomplished.

2.2.3 *Second Messenger Involved in Fish Olfactory Signal Transduction*

As that in mammals, cAMP is believed to be the intracellular second messenger in fish olfactory transduction. In addition, possible involvement of phosphoinositide-derived second messengers in olfaction was first described using isolated cilia from the freshwater channel catfish, *Ictalurus punctatus* (Bruch 1996). It was shown that amino acids elicited a rapid and transient increase in IP3 levels with no change in cyclic AMP levels during the same period. The IP3 involvement as second messenger in olfaction has been reported in several species including locust, cockroach and *Drosophila*. In Atlantic salmon, both amino acid and bile salt odorants elicited increased IP3 formation in a plasma membrane preparation from olfactory rosettes. The observation of an IP3-gated conductance suggested that the second messenger acted to increase intracellular calcium by binding to a receptor in the plasma membrane that was itself a calcium channel (Bruch 1996 and references therein).

The presence of different G-proteins in olfactory epithelium of channel catfish (*Ictalurus punctatus*) was investigated using antibodies against both the α and β subunits of G-proteins. Based on Western blotting and immunohistochemical data, the following G-protein subunits were identified in the olfactory epithelium: Gs/ Golf, Gi1, Gi2, Gq and Gβ. Immunohistochemical results indicated that all of these G-proteins, encompassing three G-protein subfamilies, were expressed in the dendrites and cilia of olfactory receptor neurons. These findings suggest that different G-protein subunits, which may mediate multiple signal transduction pathways, exist in the catfish olfactory epithelium, i.e., Golf/ Gs, may mediate odorant activation of adenylyl cyclase while Gi and Gβ may mediate odorant activation of phospholipase C (Abogadie et al., 1995). Thus, there possibly exist two intracellular signal transduction cascades, which is similar to the situation in mammals, although the IP3 pathway there has been discussed more controversially.

2.3 Physiological and Behavioral Studies in Fish Olfaction

Methods used for functional study of fish olfaction include: EOG (Electro-olfactogram), EEG (Electro-encephalogram), which are electrophysiological measurements that reflect the summed activity of many neurons; optical imaging, using calcium or voltage-sensitive dyes, to reveal the presynaptic glomerular pattern of activation upon odor stimulation, or “odor image”; functional labeling of ORNs using a cationic molecule with odor induced uptake, and behavioral studies.

2.3.1 *Electro-olfactogram (EOG)*

EOG to Amino Acid and Bile Acid

In an approach to test zebrafish responses to general fish odors, it was found that the responses to the most stimulatory bile acid odorants were up to 40% larger than the responses to the most stimulatory amino acid odorants. Females are significantly more sensitive to these odorants than males (Michel and Lubomudrov, 1995). Another work followed to test the overlap of amino acids and bile acids receptors. The results suggested that these groups of odorants interact with largely independent odorant receptors (Michel and Derbidge, 1997).

To date, all teleosts tested are sensitive to the majority of 20 L-α-amino acids. The consistency in olfactory sensitivity throughout many species suggests that amino acids may be involved in a function basic to all species; one such important function being feeding. In contrast, an extreme olfactory sensitivity to bile acids, coupled with their wide distribution and chemical variations, have been taken as evidence for their roles as pheromones. For instance, the finding that the olfactory system of sea lamprey (*Petromyzon marinus*) is specifically and acutely sensitive to unique bile acids released by conspecific larvae strongly support the hypothesis that these chemicals function as migratory pheromones in these species (Li et al., 1995).

EOG to Steroid Pheromones and Prostaglandins

Since the discovery that two fish hormonal pheromones, 17α,20β-dihydroxy-4-pregnen-3-one (17, 20P, or 17, 20 βP) and F-prostaglandin (PGF), control reproductive physiology and behavior in goldfish, a growing body of evidence from EOG supports the assumption that fish may commonly use hormonal pheromones. EOG recording showed that the goldfish is specifically and acutely sensitive to 17, 20P, 17, 20 β P-S and androstenedione, both as preovulatory pheromones. It is also sensitive to PGF2α and 15K-PGF2α, which are postovulatory pheromones (Hanson et al., 1998).

In one experiment for olfactory sensitivities of six salmonid species - rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmon salar*), brown trout (*Salmo trutta*), Arctic char (*Salvenilus alpinus*), lake char (*Salvenilus namaycush*) - it was shown that PGF2α and 15K-PGF2α were potent olfactory stimulants for all tested salmonids, except rainbow trout. None of the salmonids responded to 17, 20 β P. However, they were sensitive to etiocholan-3 α-ol-17-one glucuronide (Hara and Zhang, 1998). Thus, there exists some species specificity for pheromones, but the precise spectrum and function of the olfactory response to pheromones or pheromone mixtures (as those in insects) has yet to be determined.

2.3.2 *Electro-encephalogram (EEG)*

In salmonids, EEG to amino acids and taurocholic acid, applied singly or in combination, showed responses in two segregated regions, the lateroposterior and mid-olfactory bulb, respectively (Hanson et al., 1998). Surprisingly, neither PGF2α, 15K-PGF2α nor etiocholan-3α-ol-17-one glucuronide elicited EEGs, which is in contrast to the clear response with EOGs. This discrepancy might be explained by spatial segregation of amino acids and steroid response with the steroid response possibly being less accessible for EEG measurements.

2.3.3 *Behavioral Study and Functional Labeling of ORNs*

Behavioral studies are used to investigate the physiological response upon olfactory pheromone perception. Goldfish are one of the few vertebrate species for which pheromones have been identified. Reproductive synchrony in the goldfish is mediated by two hormonally derived pheromonal cues released by females. The preovulatory pheromone stimulates an increase in gonadotropin release in conspecific males and is known to comprise at least three sex steroids: 17, 20 β P, 17, 20 β P-S, and androstenedione. The postovulatory pheromone stimulates male reproductive behavior, and its main components are PGF2α and 15K-PGF2α (Sorensen et al., 1995).

Functional labeling of ORNs could also reveal the spatial pattern of a subpopulation of ORNs responding to a special odorant. Permeation of a cationic molecule, 1-amino-4-guanidobutane (=agmatine, AGB) through ion channels following odor stimulation, and its detection by an anti-AGB antibody, allow labeling of odor-stimulated ORNs. Activity-dependent AGB labeling was seen in the somata and axons of ORNs in the sensory epithelium of zebrafish. The majority of odor-stimulated AGB-immunoreactive cells after glutamine application were ciliated receptor neurons, although AGB immunoreactive microvillar receptor neurons and crypt receptor neurons were also identified (Michel et al., 1999). Thus the function of at least some ciliated ORNs appears to be to detect amino acids.

2.4 Coding Strategy of Fish Olfaction

In addition to these studies, an approach to directly display the "odor image" in the zebrafish olfactory bulb, optical imaging is carried out in our lab. Voltage- or Ca^{2+}-sensitive dyes are applied to the nose, which are then transported antereogradely into the ORN axons. Real-time optical imaging recorded foci that were activated pre-synaptically upon stimulation. By this means, the result directly displays an “odor map”. In accordance with morphological and other functional studies in the olfactory

epithelium, optical imaging results showed that a functional dimorphism exists at the level of zebrafish olfactory bulb (Friedrich and Korsching, 1998).

2.4.1 *Spatial Coding*

A particular odorant activates several glomeruli and this activation pattern varies with odorant concentration. The mapping of odor information by a glomerular code seems to be a common feature of the vertebrate and many invertebrate olfactory systems (Hildebrand and Shepherd, 1997). Our lab has first demonstrated the existence of a functional topography in the olfactory bulb in fish. Using a voltage-sensitive axon tracer, responses to different odorants were recorded optically in the zebrafish olfactory bulb (Friedrich and Korsching, 1998). The results showed that different classes of odorants elicit optical responses in different parts of the bulb. The responses to amino acids and bile salts were shown to activate a large array of small glomeruli, whereas the responses to putative pheromones were very restricted, e.g., the response to PGF2α was induced in a single ventral glomerulus. These results strongly suggest that in zebrafish, a spatial coding strategy be used to encode odorant quality. This strategy includes the presence of chemotopic glomerular modules, and the combinatorial and non-combinatorial representation of odors.

The importance of functional topography in the fish olfactory bulb had been suspected for some time. Anatomical, behavioral and electrophysiological experiments described a functional division between the lateral and medial parts of the teleost olfactory bulb (Satou 1990). EEG investigations of olfaction in salmonid fishes determined that the latero-posterior bulb is activated by amino acids while TCA, a bile acid, elicited activation in a small area in the middle of the dorsal olfactory bulb (Hara and Zhang, 1996). The same method applied to goldfish also demonstrated a spatial patterning of responses to the different classes of odorants (Hanson et al., 1998). EEG (Nikonov and Caprio, 2001) has also identified distinct functional zones activated by different biologically relevant odorants in the channel catfish olfactory bulb. The results in brown trout suggests that bile acids have more effects in the rostral half of the dorsal olfactory bulb, while the whole posterior bulb is preferentially responsive to amino acids. A lateral-medial division of the bulb was not obvious.

With regard to the response to pheromones, controversial results were obtained in different species. In lake whitefish, a large population of neurons at the posterior limit of the ventral olfactory bulb responded to PGF. In contrast, no neurons responsive to PGF could be found in the olfactory bulb in brown trout or other salmonids; whereas small medial areas of the goldfish bulb were shown to respond to reproductive pheromones (Fujita et al., 1991). In addition, the lake white catfish neurons with various excitatory or inhibitory response profiles seem to be very peculiar and restricted to PGF. The mass activation of this neuron population upon PGF stimulation, monitored by an audio output from the EEG electrode, was unusual since

it did not induce a synchronized oscillatory response, while other bulbar responses display oscillatory characteristics.

Thus, although it was only in zebrafish that optical imaging was employed to display the "odor image" in the olfactory bulb, the results are in accordance with those from electrophysiological methods in other fish species. It is thus possible to say that spatial coding, as found in mammals, is also a common principle in fish.

2.4.2 *Temporal Coding*

EEG recordings from the fish olfactory bulb surface demonstrated that the fish bulb is capable to generate oscillations upon stimulation with odorants (Laberge and Hara 2001 and references therein). It was shown in mammals that the projection neuron population discharges oscillate at the frequency of the associated EEG. In fish, analysis of temporal patterns of single neuron responses in the olfactory bulb led to the proposal that odor quality and intensity may be encoded by neuron populations using a temporal code. The rhythmic waves of the fish olfactory bulb induced by odor stimuli are rather slow (3-15 Hz) compared with other animals. Variations in the wave amplitude have commonly been used to differentiate responses between chemical stimuli, but a possible role for specific frequency patterns has also been hypothesized (Laberge and Hara 2001 and references therein). Using band-pass filters and spectral analysis to extract the frequency components from the EOG waveform, it is shown that some stimuli elicited a single dominant frequency in their response (Laberge and Hara 2001 and references therein). Moreover, a temporal change in frequency components was also observed.

However, a recent study found that the dominant frequencies of the EEG responses to different odorant encompassing many classes were similar (Hanson et al., 1998). Clearly, more experimentation, using precise methods of frequency component analysis, is required before a role for different oscillation frequencies in fish olfactory coding can be ascertained. Synchronized oscillations could have other functions; they might be involved in learning, in creating a periodic clock by which neurons measure the phase in which they fire, in filtering noise, in refining the coding space of a response, or they may be only epiphenomenal (Laurent 1999). It is also proposed that the encoding of the temporal variations in odor exposure could be relevant to fish navigations where odorant concentration gradients would be sensed by the olfactory system and help direct fish to their destination (Bronmark and Hansson, 2000).

In summary, since there are many analogous features between fish and mammals, principles of olfactory coding found in zebrafish might be generalized to vertebrates. Taking advantage of the qualitatively comparable but quantitatively much smaller zebrafish model to approach the question of how olfactory information is encoded and processed, it is essential to sort out the receptive field of zebrafish OR genes, that is, to establish the ligand spectrum of individual zebrafish ORs.

It has taken nearly a decade after the cloning of putative ORs until the first functional study for an OR has been performed (Zhao and Firestein, 1998). Rat OR I7 was expressed using a viral construct and a matching ligand was identified. A few systems have been set up to overexpress mammalian OR molecules. An introduction to these systems dealing with the daunting problem of functional expression of ORs is given in the coming part.

3. Functional Expression of Odorant Receptors

The principle function of ORs is to bind odorous molecules, to get activated and to transduce this signal to the downstream effector protein (the G protein) of the signal transduction cascade. Recent progress in functional expression of ORs allows us to begin exploring the function of OR proteins concerning the questions including: What is the relation between the protein sequence of an OR and its binding affinities for different odorants? Do ORs with similar sequences (members of the same subfamily) recognize related chemical ligands? How specific or nonspecific are these receptors for their diverse ligands? For odorants, what are the critical molecular structures, or “odotopes”, that determine binding affinities and selectivity? To answer these questions, information on the relation of ORs to their odorant ligands is an essential element (Zhao and Firestein 1999 and references therein).

In a screen of *C. elegans* mutants, it has been found that the odr-10 gene is likely to encode a receptor for the odorant diacetyl (Sengupta et al., 1996). Odr-10 mutants have a specific defect in chemotaxis to diacetyl, one of several odorants detected by the AWA olfactory neuron. Odr-10 encodes a predicted seven transmembrane domain receptor; its promoter drives expression of an odr-10-GFP fusion protein in AWA neurons being localized to the sensory cilia. The expression of odr-10 is regulated by odr-7, a transcription factor implicated in sensory differentiation of the AWA neuron. These results provide functional evidence for a specific interaction between an olfactory receptor protein and its odorant ligand.

Recently, a procedure that combined calcium imaging and single-cell RT-PCR (Malnic et al., 1999; Touhara et al., 1999) led to a breakthrough in linking an odorant with its receptor. In this procedure, the response profiles of isolated, individual olfactory neurons to a panel of odorants were first examined by calcium imaging, and the OR in each neuron was then identified by a two-step, single-cell RT-PCR. In the

study carried out by Malnic et al. (Malnic et al., 1999), 13 ORs from 14 olfactory neurons were identified as responding to some aliphatic odorants. The results showed that an OR from an individual olfactory neuron recognizes several odorants and that an odorant was recognized by more than one receptor. However, in these studies it could not be excluded that the ORN from which the OR was cloned might contain other ORs.

OR proteins have been purified through overexpression, solubilization and chromatography. By a nontraditional ligand-binding assay (Kiefer et al., 1996), a purified receptor, the rat OR 5 receptor, was shown to specifically bind the odorants lilial and lyral.

To establish the ligand binding spectrum of individual OR beyond doubt, a system for expression of ORs should be available, in which individual receptors can be expressed and tested for odorant binding specificities through measuring the odorant-induced responses (e.g. second-messenger generation or electrical activity of the cell). Functional expression, at the most elemental, serves to provide the functional evidence that the olfactory multigene family indeed encodes ORs. It also pairs individual ORs with their appropriate odorant ligands. A system for functional expression would further provide a tool to experimentally test the critical residues of receptors for odorant binding by conducting site-directed mutagenesis experiments. The functional expression of many GPCRs in heterologous systems has been routinely used to study the function of receptors. However, attempts to express functional ORs in heterologous systems, have encountered difficulties. The most prominent difficulty is that in heterologous systems, the expressed OR proteins usually fail to translocate efficiently to the cell membrane (McClintock et al., 1997).

3.1 Trafficking of ORs Needs Auxiliary Molecules

A screen for *C. elegans* mutants with defective olfactory behaviors led to the isolation of the odr-4, and odr-8 genes (Dwyer et al., 1998). Odr-4 mutants as well as odr-8 mutants are defective in a subset of AWA-mediated olfactory responses; they fail to respond to diacetyl and trimethylthiazole, but they respond normally to pyrazine. A comparison of effects of odr-4 and odr-8 on different signaling molecules, receptors, and in different cell types, indicates that receptor localization to the cilia is the critical function that is disrupted in these mutants. Thus odr-4 and odr-8 are required for localization of some odorant receptors to the cilia, including the diacetyl receptor odr-10. Odr-4 encodes a novel membrane protein expressed in chemosensory neurons that is localized to intracellular membranes in the cell body and dendrites and may function in the folding or transport of odorant receptors to mediate their efficient targeting to olfactory cilia.

In a recent study by the same group (Dwyer et al., 2001), protein sorting and transport in olfactory neurons were characterized *in vivo*, using a GFP-tagged odorant receptor protein, odr-10. Odr-10 is transported in rapidly moving dendritic vesicles

that shuttle between the cell body and the cilia. Anterograde and retrograde vesicles move at different speeds, suggesting that dendrites have polarized transport mechanisms. Residues immediately after the seventh membrane-spanning domain of odr-10 are required for localization, and are conserved in many G protein-coupled receptors. In unc-101 mutants, with a defective mu1 subunit of the AP-1 clathrin adaptor complex, in which dendritic vesicles are absent, the odr-10 receptor is evenly distributed over the plasma membrane. Other cilia membrane proteins are also mislocalized, implicating AP-1 in protein sorting to olfactory cilia.

Thus, the correct trafficking of ORs needs some auxiliary molecules, which might be lacking in the heterologous expression system. In addition, the receptors that do reach the membrane may be unable to efficiently couple with endogenous second messenger systems in heterologous systems, making a functional assay impossible. To complete the difficulties, the extremely large repertoire of odorants and the large number of receptors in mammals also make the likelihood of pairing a particular receptor with its odorant ligand(s) rather small. This last problem may be minimized in zebrafish with its tenfold smaller repertoire of ORs.

Taken together, proper membrane trafficking, efficient coupling to second messenger and appropriate receptor/ ligand pairing have to be achieved for functional expression of ORs, making it a daunting problem. Therefore, some authors have used *in vivo* expression systems where ORs were overexpressed in ORNs.

3.2 *In vivo* Functional Expression Systems

3.2.1 *In vivo Functional Expression in Rat Nasal Epithelium*

On the simple assumption that olfactory neurons themselves would be the most capable cells for expression, targeting, and coupling of odorant receptors, Firestein and colleagues (Zhao et al., 1998) have used the rat nasal epithelium as an expression system for ORs. The OR I7 has been included in a recombinant adenovirus that was used to infect rat nasal epithelium *in vivo*. Odorant responses were measured by EOG, the extracellular measurement of a transepithelial potential resulting from the summed activity of many olfactory neurons.

The vector used was a replication-incompetent bicistronic adenovirus vector, AdexCAG-I7-IRES-GFP. The GFP - green fluorescent protein serves to facilitate the EOG electrode placement. IRES is inserted in the expression cassette to produce the bicistronic transcript that would result in the expression of odorant receptor and GFP as separate proteins in the same cells (Zhao et al., 1998 and references therein). EOG recordings identified octyl aldehyde as ligand of the I7 receptor. Longer and shorter aliphatic aldehydes were less effective. However, no further receptor/ ligand pairing has been reported using this method.

3.2.2 *In vivo Functional Expression in Drosophila antenna*

Störtkuhl and Kettler overexpressed Or43a, a *Drosophila* odorant receptor gene in the fly antenna and tested for an increase in odor response *in vivo* (Störtkuhl and Kettler, 2001). Or43 is normally expressed in ~15 ORNs of the antenna, but the authors were able to drive its expression in a high fraction of the ~1,200 antennal neurons by using the GAL4/ UAS system. They then found a concomitant elevation in antennal response to a subset of chemically-related odors, as measured by electroantennograms (EAGs), which, like EOGs, are extracelluar recordings of the receptor potentials of a population of antennal neurons. The results showed that despite their low sequence homology to vertebrate ORs, fly ORs resemble vertebrate ORs in their ability to recognize multiple odorants, as shown by the analysis of Or43.

So far, these were the only successes reported. Although gene transfer methods are well established in both species, no further receptor/ ligand pairings have been achieved to date using these methods, apparently because of problems in finding the appropriate receptor/ ligand combinations. However, as an alternative, a few reports about functional expression of ORs *in vitro* have been documented (Krauwurst et al., 1998; Touhara et al., 1999; Wetzel et al., 2001). These experiments have revealed some ligand-receptor pairings, most of which reconfirm those found *in vivo*.

3.3 *In vitro* Functional Expression Systems

The very first attempt on heterologous expression utilized the baculovirus system to show that the expression of a rat odorant receptor OR5 in sf9 cells resulted in generation of the second messenger IP3, but not cAMP, in response to a mixture of lyral and lilial (Raming et al., 1993). In this study, however, responses were very broad and the signal to noise ratios were low. The odorants that increased IP3 production show no structural relationship. Expression of another receptor, OR12, that shares 75% identity with OR5, however, did not show a response to any of these odorants.

Functional expression of three fish ORs in HEK (human embryonic kidney) 293 cells has been reported (Wellerdieck et al., 1997). These receptors were expressed as fusion proteins with the N-terminal membrane import sequence of guinea-pig 5-HT3 receptor, and showed sensitivity to a commercial fish food mixture. However, they have not been paired with any specific ligand, leaving the exact nature of their ligand specificity open. In addition, our observations show that the same fish food extract elicited a response in non-transfected cells as well.

These two earlier *in vitro* functional expression results were somewhat at odds with the data from mitral cell recordings and with other recent functional expression results, but these discrepancies may be related to the expression system or the method of stimulus application.

3.3.1 *In vitro Functional Expression in HEK293 Cells*

A successful attempt at functional expression of ORs was reported recently using HEK293 cells as heterologous system (Krautwurst et al., 1998). In this study, all the expression constructs were generated having a 20 amino acid rhodopsin N-terminal extension. Rhodopsin shows a high level of expression in HEK293s cell as well as an efficient translocation to the plasma membrane. An N-terminal extension has been found empirically to facilitate the translocation of the synthesized proteins to the plasma membrane.

In these studies Gα15, 16 subunits from mouse and human that are known to promiscuously couple to different 7TM receptors including those that normally signal through other second messengers to the PIP2 pathway (Offermanns and Simon 1995), were coexpressed. This system was employed as a robust and sensitive reporter system to screen large numbers of ligands. Receptor activation leads to the generation of an IP3-mediated increase in intracellular Ca^{2+}, which can be measured at the single-cell level with high sensitivity and good temporal resolution using the dye fura-2 and ratiometric imaging.

The authors first generated an expression library containing a large and diverse repertoire of putative mouse olfactory receptor sequences (transmembrane II-VII region), to increase the likelihood of pairing a receptor with its odorant ligand(s), considering that there are hundreds of olfactory receptors and thousands of odorants. This library was joined in frame to the flanking regions of a particular OR, based on the assumption that the insertion of a cloned TM II-VII segment into an expression vector backbone might impart to the resulting chimeric protein the ligands specificity of the corresponding full-length receptor. Critical residues for ligand in other GPCRs are located within TM III to TM VI. Thus, the TM II-VII region of a significant fraction of the mouse olfactory receptor family was cloned by homology-based PCR approach and used for functional studies.

By testing 80 chimeric receptors against 26 odorants, three receptors were identified as responding to the odorants carvone, (-) citronellal and limonene, respectively. The authors further showed that the full-length I-C6 receptor retained the same stereo selectivity as the chimeric construct by preferring the (-) isomer of citronellal but was somewhat less sensitive. (-) Citronellyl bromide, with the substitution of bromine for the oxygen atom in the aldehyde function group, elicited a smaller response.

In this study, the different ligand specificity of rat receptor I7 and its homologue, mouse I7 was revealed, with rat I7 responding to odorant octanol at a lower concentration than to odorant heptanol, whereas the mouse I7 had the inverse preference. Remarkably, this difference in ligand specificity could apparently be attributed to a single-residue change in position 206 of the receptor sequence residing in TM5. Interestingly, the nature of these changes, valine versus isoleucine and octanal versus heptanal, is consistent with compensatory alterations in the structures

of ligand and receptor.

3.3.2 *In vitro Functional Expression in ORN or ORN-derived Cells*

Touhara et al. (1999) have analyzed the tuning properties involved in discriminating a variety of odorants at the receptor level, through a combination of Ca^{2+}-imaging and reverse transcription-coupled PCR analysis. They have thus achieved the functional identification of olfactory receptors for specific odorants from single olfactory neurons. First, a candidate odorant receptor was cloned form a single tissue-printed olfactory neuron that displayed odorant-induced Ca^{2+} increase. Next, recombinant adenovirus-mediated expression of the isolated receptor gene was established in the olfactory epithelium, using green fluorescent protein as a marker. The infected neurons elicited external Ca^{2+} entry when exposed to the odorant that originally was used to identify the receptor gene. Experiments performed to determine the ligand specificity revealed that the odorant receptor recognized specific structural motifs within odorant molecules.

Of ~3,500 tissue-printed viable olfactory neurons, a total of 226 positive cells could be identified that responded to some of the 11 odorants applied, including eurenol, ethylvanillin, geraniol, lilial, cresol and lyral. Each responsive cell was subsequently picked in a microcapillary tube and was subjected to single cell RT-PCR using degenerate oligonucleotides designed on the basis of the known conserved sequences among the olfactory receptor superfamily. Sequence analysis of the two-round PCR-amplified fragment obtained from one lyral-responsive cell revealed it to be identical to the mouse olfactory receptor MOR23. This receptor was chosen for further analysis because it was relatively well characterized.

A recombinant adenovirus vector containing a bicistronic expression unit for MOR23 and GFP was used to infect the mouse nasal epithelium. 16 of 22 picked green neurons responded to lyral (1 μM). In an attempt to identify other ligands for MOR23 based on two functional units in lyral molecules, i.e., the tertiary alcohol and aldehyde groups, a panel of chemicals with related structure were used. A rather narrow ligand specificity was found, which indicates that the functional group recognized by the MOR23 binding pocket is a tertiary alcohol moiety rather than a cyclohexenecarbaldehyde moiety. It should be noted that one has to control for the presence of an endogenous receptor for the odorant under study.

This work of the Touhara lab appears promising for studying OR ligand-receptor pairing using HEK293 cells as functional expression system.

ORNs die and are replenished throughout life (Farbman, 1990; Crews and Hunter, 1994), thus progenitor cells are present in the adult olfactory epithelium. Several groups have been working on the primary culture of ORNs or immortalized cell lines derived from ORN progenitors. One neuron cell line, Odora (for olfactory-derived, odorant-receptor activatable cells), retains some functional maturity (Murrell and Hunter, 1999). This cell line has been derived from the OE of perinatal rats. At this age, the OE has achieved a mature morphology, but is still undergoing massive

proliferation (Murrell and Hunter 1999 and references therein). Because of the high degree of division occurring in the basal cell populations at this age, a large number of dividing progenitors are present.

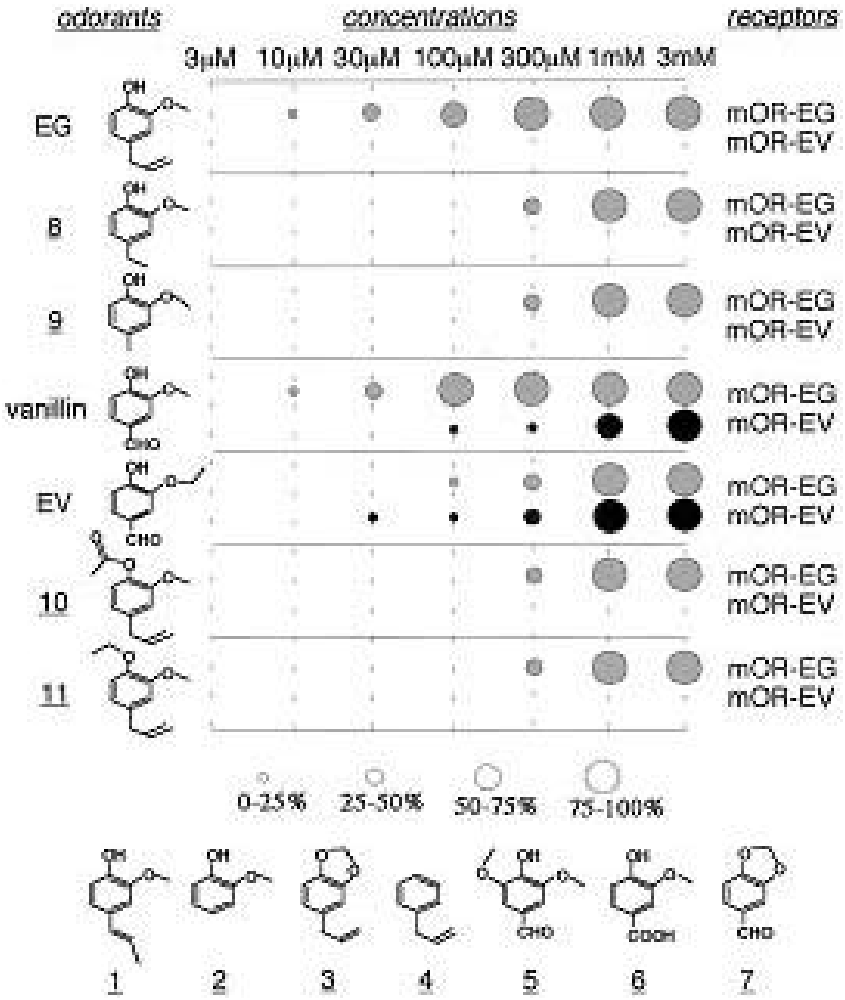

Figure II.9. mOR-EG and mOR-EV Recognize Overlapping Sets of Odors with Different Affinities and Specificities (taken from Kajiya et al., 2001).
1, Isoeugenol; 2, guaiacol; 3, safrol; 4, allybenzene; 5, syringic aldehyde; 6, vanillic acid; 7, heliotropyne; 8, 2-methoxy-4-ethylphenol; 9, 2-methoxy-4-methylphenol; 10, eugenol acetate; 11, eugenol ethyl ether.

An immortalizing oncogene is transmitted to these dividing populations via infection through a replication-incompetent retrovirus. A temperature-sensitive oncogene was chosen in the hope that cells that are proliferating at the permissive temperature of the oncogene would cease proliferation and differentiate when the cells were removed to a non-permissive temperature. Five of 18 colonies survived after freezing and replating, one of them was further studied and named *odora*. Characterization of differentiated *odora* cells showed that these cells possessed an

antigen profile similar to that for ORNs, including expression of NCAM, MAP5, GAP-43, Gαolf, ACIII, oCNCβ, as well as OMP.

Odora cells retained the ability to direct ORs to their surface, as shown by immunostaining against HA after being transiently transfected with an OR, U131, tagged with HA. Differentiated *odora* cells transiently transfected by U131 showed a calcium response to one of six odor mixtures, and displayed odorant induced desensitization, a phenomenon that has been described in ORNs (Bozza and Kazer, 1998). Further studies showed that only two compounds of the mixture, the seven-carbon saturated fatty acid (enanthic acid) and nine-carbon saturated fatty acid (pelargonic acid), elicited a response in U131-transfected *odora* cells. So far there are no further reports about successful expression of other ORs into *odora* cells. If these cells could be made more amenable to transfection, they would represent a good system for systematic functional expression for ORs.

In parallel, a few groups tested the Xenopus oocyte expression system, widely used for expression of ion channels, for functional expression of ORs. The following part is about the Xenopus oocyte expression system.

3.4 Xenopus Oocyte Expression System

Using the Xenopus oocyte system, an expression cloning strategy was employed to identify a goldfish odorant receptor that is activated by basic amino acids (Speca et al., 1999). RNA was synthesized from pools of goldfish olfactory cDNA clones, with pool containing ~1000 cDNA clones, and was injected into Xenopus oocytes together with synthetic RNAs encoding GIRK (G protein-gated inwardly rectifying potassium channels, as a reporter), and Gαolf. Iterative subdivision of pool 19, which was suggested to contain some receptor for amino aid cocktails, allowed the isolation of receptor 5.24, which was then shown to be a receptor for lysine and arginine. Binding studies done on the extracted membrane fraction of HEK293 cells expressing 5.24 reconfirmed the electrophysiological result. Sequence analysis of the receptor itself showed it to be a GPCR sharing sequence similarities with the calcium sensing, metabotropic glutamate, and V2R class of vomeronasal receptors, which possess a long N-terminal extracellular domain. *In situ* hybridization showed that this receptor, surprisingly, is expressed in a large fraction of cells in the apical part of the olfactory sensory epithelium, and also in non-olfactory chemosensory surfaces.

The lab of Hatt (Wetzel et al., 2001) has used the Xenopus oocyte system to overexpress the Or43 studied by Störtkuhl and Kettler (2001). The authors could confirm the receptor/ ligand pairings there.

In summary, there are some documented functional expression systems for mammalian and *Drosophila* ORs. The *in vivo* system works in some cases in animal models where genetic manipulation tools are available; *in vitro* and *in ovo* functional

expression systems have been successful in some cases. Still, the knowledge about odorant response properties is rather fragmentary.

4. Goal of the Study: Functional Expression of ZORs

The goal of this study is to identify ligands for zebrafish ORs by functional expression. Since odorants for fish are well characterized and are water-soluble, and since the OR repertoire in zebrafish is one tenth of that in mammals, we have employed zebrafish as a model to study olfaction. Our lab's previous work has resulted in cloning of several putative zebrafish OR genes. Functional expression of these cDNAs could confirm the OR nature, could lead to the identification of ligands for these ORs, to the establishment of the molecular feature of odorants that are recognized by these ORs and finally to the characterization of the molecular receptive field of each OR.

In order to achieve this, a stable expression system with efficient expression of ORs and efficient coupling to the second messengers has to be identified and characterized. In the present thesis, I will describe several approaches that have been taken for this goal and discuss their problems and advantages respecively. As no completely satisfactory solution could be achieved so far, I will also give a short outlook on possible future improvements.

III. MATERIALS AND METHODS

1. Equipment and Materials

1.1 Equipment

Balances	Sartorius L310, U4100 and H41 (Sartorius, Göttingen)
Centrifuges	Centrifuge 5415D (Eppendorf, Hamburg), Biofuge B (Heraeus, Hanau)
	Centrifuge 5417R (Eppendorf, Hamburg), Sigma 4K10 (B. Braun, Melsungen), Minifuge RF (Heraeus, Hanau)
	Sorvall® RC-5B Refrigerated Superspeed Centrifuge (Du Pont De Nemours GmbH, Bad Homburg)
CO_2 – Incubator	BBK 6220 (Heraeus, Hanau)
	CO_2-AUTO-ZERO (Heraeus, Hanau)
Computer	Power Macintosh 8500/ 120 (Apple Computer, Feldkirchen)
	Mac OS Computer (Apple Computer, Feldkirchen)
	CDD Compact Disc Recorder (Philips, Hamburg)
Cryostat	CM 19000 (Leica, Solms)
Electrophoresis Apparatus	Mini Sub Cell® GT, Wide Mini Sub Cell® GT, Sub Cell® GT and Power PAC 300 (Biorad, München)
	Powerpack P21, P23 (Biometra, Göttingen)
Electroporation System	Gene Pulser™, Pulse Controller and Capacitance extender (Biorad, München)
Fluorescence Microscopes	Zeiss Axioplan (Zeiss, Oberkochen)
	Atto Arc 2 HBO 100W light source (Zeiss, Oberkochen)
	UNIBLITZ® Model VMM-1 D1 Shutter Drive (Vincent Associates of Zeiss, Oberkochen)
	Axiovert S100 TV (Zeiss, Oberkochen)
	Polychrome II and IMAGO water-cooled S/ N 381 KL 0041 Digital Camera (Photonics, MA)
Fluorescence Photometer	LS–5B Luminescence Spectrometer (Perkin Elmer, Weiterstadt)
Fluorescence Stereomicroscope	SMZ-U (Nikon, Düsseldorf)
	Coolpix 950 Digital Camera (Nikon, Düsseldorf)
	Image Mate™ (SanDisk, Sunnyvale, CA, USA)
	SuperHigh Pressure Mercury Lamp Power Supply (Nikon, Düsseldorf)
Freezers	Bio-freezer (Forma Scientific)
	Freezers and refrigerators (Bosch, München)

	Freezers (Liebherr)
Gel Imaging System	ChemiDoc (Biorad, München)
Heating Block Thermomixer	Comfort (Eppendorf, Hamburg)
	Dri Block® OB-3 (Techne, Cambridge, UK)
Microwave	Bosch (München)
Magnetic Stirrer with Heater	IKAMAG® REO, IKAMAG® RET and IKA – COMBIMAG RCH (IKA-Labortechnik, Staufen i. Br.)
Objectives	Plan-Neofluar: 10xNA0.3; 20xNA0.5; 40xOil NA1,4
pH – Meter	pH Meter 766 Calimatic (Knick)
Printer	LaserJet 2100 TN (Hewlett Packard, Böblingen)
	Epson Stylus Color 760 (Epson Deutschland GmbH, Düsseldorf)
Puller	Flaming/ Brown Micropipette Puller Model LP – 97, (Sutter Instruments Co., USA)
Pumps	Peristaltic Pump P1 (Pharmacia Fine Chemical, Erlangen)
Scanner	Snap Scan 1236 (Agfa, Köln)
	Nikon LS-2000 (Nikon, Düsseldorf)
Sterile Hood	Laminar Air LFM 2448S (Heraeus, Hanau)
	Laminar Air HA 2448 GS (Heraeus, Hanau)
Thermocycler	Gene Amp PCR System 2400 (Perkin Elmer, Weiterstadt)
	T-Gradient (Biometra, Göttingen)
Vortex	Vortex Genie 2™ (Bender & Hobein AG, Melsungen)
Waterbath	Thermomix®M (Melsungen)
Injection Valve	Knauer (Berlin)

1.2 Chemicals and Suppliers

All chemicals, unless otherwise stated in the text, were purchased from Ambion (Austin, USA), Amersham Pharmacia Biotech (Freiburg), Applichem (Damstadt), Fisher Scientific (Schwerte), Biozym (Hessisch Oldendorf), Fluka (Seelze), Merck (Darmstadt), Molecular Probes (Leiden, NL), Roth (Karlsruhe), Serva (Heidelberg), or Sigma (Deisenhofen).

Dyes and Embedding Medium

Dyes

Name	Stock solution	Supplier	Excitation/ Emission (nm)
PicoGreen™	400x, -20°C	Mo Bi Tec (Göttingen)	480/ 520
FURA-2, AM ester	1 mM in DMSO, -20°C	Mo Bi Tec (Göttingen)	353/ 466
DiI C18 (3) "DiI"	5mg/ ml in DMSO, -20°C	Mo Bi Tec (Göttingen)	549/ 565
DAPI (nuclear marker)	5 mg/ ml in H_2O, -20°C	Sigma (Deisenhofen)	353/ 466
Cell Tracker Blue CMAC	5mg/ ml in DMSO, -20°C	Mo Bi Tec (Göttingen)	353/ 466
Trypan-Blue (viability stain)	0.4% in 0.85% saline, room temperature	Gibco BRL Life Technologies (Karlsruhe)	non-fluorescent

Embedding Medium

Name	Supplier
Mowiol	Polyscience, Niles, IL, USA
Vectashield	Vector Laboratories, Peterborough, UK
TissueTek®	Miles Laboratories, Elkhart, Indiana, USA

Enzymes

Enzymes used in molecular biology: restriction endonucleases were purchased either from Amersham Pharmacia Biotech (Freiburg) or New England Biolabs (Frankfurt).

T_4 DNA polymerase, Taq DNA polymerase, T_4 DNA ligase RNaseA and proteinase K were purchased from Roche Biochemicals (Mannheim).

Shrimp alkaline phosphotase (SAP) was from USB (Cleveland, OH, USA).

Trypsin 1:250 and pancreatin (from porcine pancreas) were purchased from GibcoBRL Life Technologies (Karlsruhe).

Films

Instant films of gel documentation (Polaroid 667, 31/4 x 41/4) were bought from Polaroid (Hertfordshire, U.K.). Diafilms (Ektachrome 160 T) were from Kodak (Kodak, Stuttgart).

Nucleotides

Nucleotides for PCR were purchased from Amersham Pharmacia Biotech (Freiburg).

Plastic and Glass- wares

Disposable plastic wares, e.g., Petri dishes with different sizes, plastic tubes, 6-, 24-, 96- well plates, were purchased from Castor supplied by Fisher Scientific (Schwerte). PCR tubes and sterile pipettes were from MßP supplied by Fisher Scientific (Schwerte). 96-well, 200 µl UniPCR™ used for colony-PCR were purchased from Whatman polyfiltronics (Clifton, NJ). Disposable pipette tips were supplied by Fisher Scientific (Schwerte).

Glassware (flasks, beakers etc.) are DURAN glass (Hirschmann® EM techcolor or Schott, Germany). Glasscoverslips used for cell culture were purchased from PLANO (Marburg).

Transfectional reagents

Transfectional Reagent Sample Pack and LipofectAMINE reagents were purchased from GibcoBRL Life Technologies (Eggenstein).

Calcium-phosphate precipitation transfection kit was purchased from Promega (Madison, USA).

1.3 Oligonucleotide Primers

Oligonucleotide primers were purchased from MWG Biotech AG (München), GibcoBRL Life Technologies (Karlsruhe) or Sigma-Ark (Deisenhofen). The list of oligonucleotide primers and their use are as follows:

To construct Rhodopsin N-terminal-Zebrafish odorant receptor fusion protein (Rho-ZOR, see Fig. III.3) expressing vectors, each receptor cDNA was amplified from its originally cloning vector pSPORT GibcoBRL Life Technologies (Karlsruhe) by a primer pair which adds 5'-prime an *EcoR I* (GAATTC) site immediately in front of the *ATG* codon and 3'-prime a *Not I* site (GCGGCCGC) (Fig. III.1).

The *EcoR I*-ORF-*Not I* fragment was then cloned into pGEM-T Vector (Promega, Mannheim), cut by *EcoR I*/ *Not I* digestion, and ligated into pCMV-Rho tag (Fig. III.2).

The plasmid pCMV-Rho tag is constructed on the basis of pEGFP-N1, a commer--cially available expression vector from Clontech (Heidelberg), taking advantage of its kanamycin/ neomycin resistance cassette (Kana/ Neo), which makes it possible to select for transfected cells.

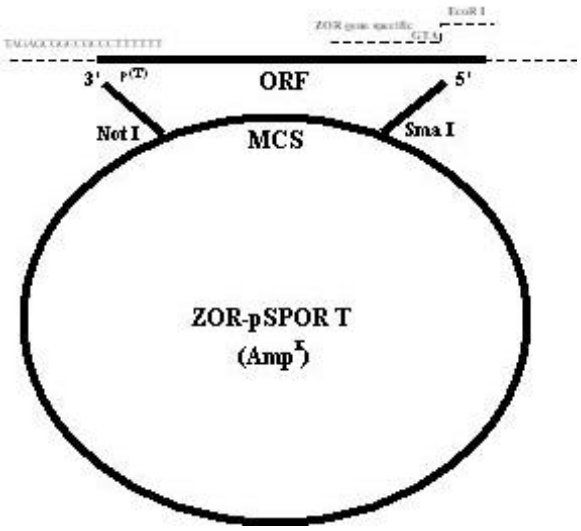

Figure. III.1. **Cloning Strategy of Rho-ZOR.**
Open reading frame (ORF) of ZOR, which was cloned into ZOR-pBS by *Sma I* (5') and *Not I* (3'), was amplified by a 5' primer (highlighted with dashed line) tagged with *EcoR I* and a 3' primer (strikethrough) tagged with *Not I.*

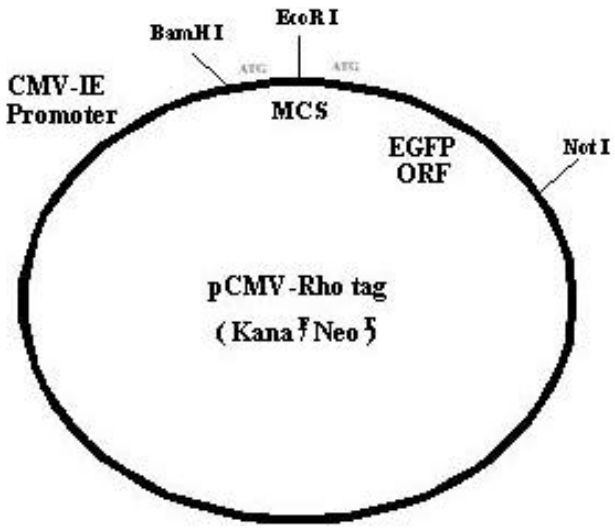

Figure III.2. Plasmid pCMV-Rho Tag.
Plasmid pCMV-Rho tag is derived from pEGFP-N1. The rhodopsin tag was inserted into the *BamH I*/ *EcoR I* sites of the multi-cloning-site (MCS), immediately followed by the ORF of EGFP.

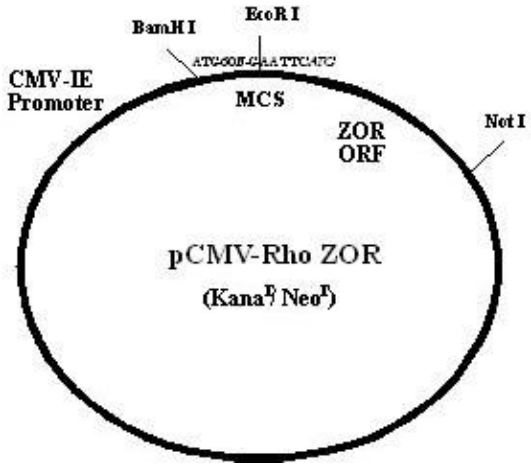

Figure III.3. Plasmid pCMV-Rho Tag.
Cloning strategy described in text. The pCMV-Rho-ZOR expression vector has a CMV-IE promoter, a rhodopsin N-terminal tag fused in frame to the ORF of ZORs (*ATG* codon of ZOR in italic), except for pCMV-Rho-ZOR5A where another cloning strategy was employed.

The resulting expression vector after ligation is shown in figure III.3.
The only exceptional expression vector was pCMV-Rho-ZOR5A. Here the ORF of ZOR5A was cut directly from ZOR5A-pBS by *EcoR I*/ *Not I* digestion and ligated into *EcoR I*/ *Not I* linearized pCMV-Rho tag, since ZOR5A has an *EcoR I* site at its 31st nucleotide 3' of ATG.

Primers used for this construction purpose are (restriction sites underlined, *ATG* coden in italic):

Name	Sequence	Length	Tm (°C)	Supplier	Purpose
934-ST.1	G<u>GAATTC</u>*ATG*TCACAGGAGG	20	57.3	MWG	Rho-ZOR10A
861-ST.1	GGGGGGG<u>GAATTC</u>*ATG*CTAA	20	59.4	MWG	Rho-ZOR9A
861-EN.2	TAGA<u>GCGGCCGC</u>CCTTTTTT	20	59.4	MWG	Rho-ZOR 3' common for all ZORs studied

813-ST.1	GGAATTC*ATG*GAAAACAATA	20	49.1	MWG	Rho-ZOR 3A
726-ST.1	GGAATTC*ATG*CTGTATTTTA	20	49.1	MWG	Rho-ZOR8C
613-ST.1	GGAATTC*ATG*GAAAATAATA	20	47.1	MWG	Rho-ZOR3C

To construct the Rho-ZOR5A-EGFP fusion protein, the Rho-ZOR5A fragment was amplified from the Rho-ZOR5A plasmid using primers tagged with a *BamH I* site immediately 5'-prime of the *ATG* codon and 3'-prime of the *TGA* codon. This *BamH I* Rho-ZOR5A *BamH I* was then ligated to *BamH I*-linearized pEGFP-N1. Primer pair used for this purpose (restriction site underlined):

Name	**Sequence**	**Length**	**Tm (°C)**	**Supplier**
Rho935-st.1.2	CGGGATCCCGAGCTTGGG	18	62.8	MWG
Rho935-en.2	CGGGATCCCGTTCTGAAGCAGT	22	64.0	MWG

Primers used for sequencing:

Name	**Sequence**	**Length**	**Tm (°C)**	**Supplier**
T7	CGACTCACTATAGGGCGAATTGGG	24	71.8	Gibco
SP6	TGATTACGCCAAGCTATTTAGGTGACACTATAG	33	76.8	Gibco
N1-P1	TTAGTGAACCGTCAGATC	18	50.4	MWG
N1-P2	CAAATGTGGTATGGCTGA	18	50.6	MWG
N1-3	GCCCAACAGAACAAACAT	18	51.0	MWG
N1-4	CATGTGCACTGATTTGGA	18	50.6	MWG
N1-5	GGTTTTGGCAGTACATCA	20	51.4	MWG
N1-6	ACGCTTACAATTTACGCC	20	51.4	MWG
M13-F	GTAAAACGACGGCCAGT	17	52.1	Gibco
M13-R	AACAGCTATGACCATG	16	51.6	Gibco

1.4 Plasmids and Vectors

List of cloning vectors:

Name	Supplier	Feature
pBluescript II KS (+)	Stratagene	2,96 kb; B/ W; ampicillin resistance
pGEM-T	Promega	3 kb; B/ W; T vector; ampicillin resistance
pSPORT1	Invitrogen	4.1 kb; B/ W; ampicillin resistance

B/ W: blue/ white selection possible.

List of eukaryotic expression vectors (all driven by a cytomegalovirus immediate-early promoter, pCMV):

Name	Supplier
pDsRed 1	Clontech
pEGFP-N1	Clontech
pIRES2-EGFP	Clontech

DsRed1: a red fluorescent protein (RFP) isolated from *Discosoma sp.*. Excitation maximum = 558 nm, emission maximum = 583 nm.

EGFP: a red-shifted and enhanced mutant of wild-type green fluorescence protein isolated from *Aequorea victoria*. Excitation maximum = 488 nm, emission maximum = 507 nm.

IRES2: a mutated version of IRES (internal ribosome entry site) from encephalomyocarditis virus which ensures 5'-cap free translation and which is widely used for construction of bicistronic expression vectors.

List of eukaryotic expression vectors for HEK293 cell transfection:

Name	Provided by	Feature
pRSV TAg	D. Krautwurst	encodes large T antigen under SV 40 promoter
pI7/ RK5	D. Krautwurst	encodes I7 under CMV promoter
pIC6/ RK5	D. Krautwurst	encodes IC6 under CMV promoter
pCISG? 15	D. Krautwurst	encodes G? 15 under CMV promoter
pCISG? 16	D. Krautwurst	encodes G? 16 under CMV promoter

TAg: SV 40 large T antigen, an oncogene widely used for transformation of cells *in vitro* (Krautwurst et al., 1998).

I7: mouse I7 odorant receptor, known to bind to octanol from a panel of 26 odorants tested (Krautwurst et al., 1998).

IC6: mouse IC6 odorant receptor, known to bind to citronellal from a panel of 26 odorants tested (Krautwurst et al., 1998).

Gα15: α-subunit of mouse G-protein 15, known to couple a lot of receptors promiscuously to IP3 cascade upon ligand binding (Wilkie et al., 1991).

Gα16: α-subunit of human G-protein 16, known to couple a lot of receptors promiscuously to IP3 cascade upon ligand binding (Amatruda et al., 1991).

1.5 Antibodies and Substrates

Monoclonal antibody against Rhodesian N-terminal peptide 3-14, B6-30 N, was a courtesy from the lab of J. Nathan's (Nathan et al., 1988), used at 1:1500 dilution.

Secondary antibodies (goat anti mouse, CY2 or CY3 coupled) were purchased from Dianova (Hamburg).

DAB substrate was purchased from Roche Diagnostics (Mannheim).

1.6 Culture Media and Reagents Used in Cell Culture

HEK 293 culture media (MEM 31095-029) were purchased from GibcoBRL Life Technologies (Karlsruhe), as well the additives of penicillin (10,000U/ ml, 100x)/ streptomycin (10,000 µg/ ml, 100x) and L-Glutamine (200 mM, 100x). Fetal calf sera were purchased from Sigma (Deisenhofen).

OPTIMEM (31095-047) used for LipofectAMINE-mediated transfection was purchased from GibcoBRL Life Technologies (Karlsruhe).

Calcium-free DME (DMEM 21068-028), Ham's F-12 (21765-029) were purchased from GibcoBRL Life Technologies (Karlsruhe), transferrin, progesterone, putrescine, sodium selenite, crystalline BSA were purchased from Sigma (Deisenhofen).

1.7 Antibiotics for Bacterial and Mammalian Cell Culture

Antibiotics used in prokaryotic work: ampicillin (Amp), tetracycline (Tet) and kanamycin/ neomycin (Kana/ Neo) were purchased from Sigma (Deisenhofen).

Stock solutions were prepared as in *Molecular Cloning* (Sambrook et al., 1989): Amp 50 mg/ ml in H_2O (1000x), Tet 5 mg/ ml in ethanol (333x), Kana/ Neo 10 mg/ ml in H_2O (400x), store at –20°C.

Antibiotics used in eukaryotic work: penicillin (100 U/ ml)/ streptomycin (100 µg/ ml) were generally supplemented in the growth medium. To select transfected HEK 293 cell line, G418 (Geneticin, GibcoBRL Life Technologies, Karlsruhe) is used at concentrations of 400, 600, 800 and 1200 mg/ L.

1.8 Preparation of Solutions

Solutions used in molecular biology: solutions were prepared with deionized filtered water (SeraPur). Solutions were autoclaved or filter sterilized as described (*Molecular Cloning, Sambrook et al., 1989*).

Solutions used in bacteria work: solutions used in bacteria work, including LB agar and LB medium for bacteria culture, SOB for electroporation of bacteria, were prepared as descrribed (*Molecular Cloning, Sambrook et al., 1989*).

Solutions and medium used in cell culture of HEK293 cells: basal medium for HEK293 culture is supplemented with of penicillin (100 U/ ml)/ streptomycin (100 µg/ ml), L-Glutamine (2 mM) and 10% fetal calf serum.

Media and Solutions Used in Primary Culture of Mouse Olfactory Epithelium:

Tissue culture PBS with serum: 25 ml bovine calf serum in 500 ml cold 1xPBS, supplemented with 2.5 ml penicillin/ streptomycin (10,000 U/ml/ 10,000 µg/ml).

Holding medium for turbinates: 575 ml of 1x PBS, 0.6 ml 0.5% phenol red, 3 ml penecillin/ streptomycin (10,000 U/ml/ 10,000 µg/ml), 6 ml 30% glucose dissolved in H_2O, 25 ml fetal bovine serum.

Post-trypsin rinse: 500 ml cold 1xPBS, 0.5 ml 0.5% phenol red, 2.5 ml penicillin/ streptomycin (10,000 U/ml/ 10,000 µg/ml), 5 ml 30% glucose dissolved in H_2O, 250 mg bovine albumin, crystalline, 125 mg trypsin inhibitor, type I-S from soybean.

Trituration medium: 500 ml cold 1xPBS, , 0.5 ml 0.5% phenol red, 2.5 ml penicillin/ streptomycin (10,000 U/ml/ 10,000 µg/ml), 5 ml 30% glucose dissolved in H_2O, 250 mg bovine albumin, crystalline.

Trypsin-pancreatin solution: in a 250 ml polypropylene centrifuge bottle add 1g pancreatin from porcine pancreas, 3g trypsin 1: 250, 0.5 ml 0.5% phenol red, 1 ml penecillin/ streptomycin (10,000 U/ml/ 10,000 µg/ml), 100 ml cold 1xPBS. Mix to dissolve at 4°C. Spin at 16,300 g for 20 minutes to remove insoluble material. Filter supernatant twice through Whatman No.1 filter paper.

Filter (0.2 µm) sterilizes all the solutions. Aliquot trypsin-pancreatin solutions (8 ml) and store at –80°C. Store all the other solutions at 4°C.

Neurobasal medium (21103-049) was purchased from GibcoBRL Life Technologies (Karlsruhe), as well as nerve growth factor 7S (NGF 7S, final concentration 100 ng/ µl) and B-27 supplement (50x).

Solutions Used in Calcium-phosphate Precipitation Transfection:

a) 2xHBS: 50 mM HEPES, pH 7.05; 10 mM KCl; 12 mM D(+) glucose, 280 mM NaCl; 1.5 mM Na_2HPO_4, final pH value of the solution should be 7.05+/- 0.05. Filter sterilize, aliquot and store at –20°C for up to one year.

b) 2M $CaCl_2$: prepare 2M $CaCl_2$, filter sterilize and store at –20°C.

It is observed that the efficiency of transfection is reduced dramatically with solutions older than 6 months.

Zebrafish E3 medium, (growth medium for embryos younger than five days) was prepared as described (*The Zebrafish Book*, Westerfield 1994).

HEK cell imaging medium (HEKIM, Krautwurst et al., 1998):

130 mM NaCl
2 mM $CaCl_2$
5 mM KCl
10 mM Glucose
10 mM NaHEPES (pH 7.4)

2. Isolation of Nucleic Acids

2.1 Quantification of Nucleic Acids

To estimate DNA concentration before further analysis or application, DNA samples were loaded on an agarose gel (0.8-2%, depending on the molecular weight of the DNA fragments) together with 1kb-ladder from GibcoBRL Life Technologies (Karlsruhe). After the gel ran for c.a. 3cm, the gel was stained by ethidium bromide (0.5 µg/ ml, *Molecular Cloning*, Sambrook et al., 1989) and DNA was visualized under UV light. The band of interest was compared with the 1031 bp band from the ladder, which is 10% of the total DNA in the ladder mixture. The DNA amount in the band was estimated by relative emission intensity under UV light.

Precise concentration of DNA samples was determined by fluorescence spectrometer. DNA samples (1-20 ng/ l) mixed 1:1 (v/v) with PicoGreen™ (Mo. Bi. Tec. , Göttingen) (1:200 diluted in buffer TE) were measured under 480/ 520 nm (Excitation/ Emission). The indicated fluorescence intensity was then calculated into DNA content by the formula below:

$$y = 0.924x - 1.0228$$

y: DNA content in ng/ ml; x: emission value of the solution as mentioned above.

2.2 Isolation of Plasmids from *E. coli*

The protocol for manually preparation of plasmid DNA is modified from alkaline lysis method (*Molecular Cloning*, Sambrook et al., 1989).

In brief, for small-scale isolation of plasmids, a single bacterial colony was picked with a sterile toothpick and put into 2-5 ml LB medium containing the proper antibiotics, and grown overnight at 37°C with vigorous shaking. Bacteria were harvested by centrifugation at 1000 rpm for 1 minute.

The pellet was resuspended in 200 µl resuspension buffer by vortexing. 200 µl lysis buffer was added and by gently inverting the tube, after the content in the tube was mixed thoroughly, the tube was stored on ice for 5 minutes.

The 300 µl neutralization buffer was added, the solutions were mixed by gently inverting, store on ice for 10 minutes. The mixture was then centrifuged for 10 minutes at 14,000 rpm. The supernatant was transferred into another tube and precipitated with isopropanol. In the following steps the pallet was washed twice with 70% ethanol, dried and resuspended in TE buffer or water. RRNA was removed by incubation in 0.1 mg/ ml RNase at 37°C for 30 minutes.

If plasmids are prepared for transfection and microinjection, where RNA and protein-free DNA is required, plasmid DNA preparation kits (mini-, midi-, maxi- or endotoxin-free maxi- preparation kits from Qiagen, mini- kit from Sigma) were used for DNA preparation of different scales, manipulation following supplier instructions.

Briefly, in these kits a silica-gel membrane for selective adsorption of plasmid DNA in high-salt buffer and elution in low-salt buffer is employed. This ensures that only DNA is adsorbed while RNA, cellular proteins and metabolites are not retained.

2.3 Agarose Gel Electrophoresis of DNA

The size of DNA used in this study ranged from 100 bp – 9 kb. So that native agarose gels at 0.8-2 % in TAE-buffer were used to separate the DNA bands under electrophoresis conditions of 3-5 V/ cm (*Molecular Cloning*, Sambrook et al., 1989).

2.4 Purification and Concentration of DNA

Plasmid DNA after digestion, blunt ending or dephosphorylation had to be separated from the enzyme for further use. Phenol-chloroform extraction (*Molecular Cloning*, Sambrook et al., 1989) was used for this purpose.

Add 100 µl Phenol: Chloroform: Isoamyl alcohol 25:24:1 into 200 µl DNA/ enzyme mixture after enzymatic treatment, mix thoroughly by vortexing, centrifuge 1 minute and transfer the upper aqueous phase into another clean tube. Add 100 µl H_2O in first tube and repeat vortex and centrifugation for a better yield. Purify the combined aqueous phase (approximately 300 µl) by adding 100 µl chloroform, mix and centrifuge. Water phase from this mixture was carefully transferred into another new tube. This extracted DNA with a rather low concentration was then concentrated by precipitation with ethanol.

2.5 DNA Extraction from Agarose Gel

After electrophoresis, a gel piece containing the DNA band of interest was isolated using “High Pure PCR purification Kit” (Roche Diagnostics, Mannheim).

A binding buffer containing the chaotropic salt guanidine thiocyanate was added at 300µl per 100 mg gel, mixed thoroughly and incubated at 56 °C for 10 minutes. The mixture was then centrifuged through the filter tube which is pre-packed with glass fibers. Wash the filter tube twice with washing buffer containing ethanol, elute the purified DNA in proper volume of elution buffer.

3. *In vitro* Work with Nucleic Acids

3.1 Digestion of Plasmid DNA with Restriction Endonucleases

For analytic digestion, each sample of approximately 300 ng DNA was digested in a total volume of 15 µl containing proper buffer, restriction endonuclease (5 U/ µg DNA), DNA and bovine serum albumin (if necessary) for at least 2 hours. The enzymes were inactivated by heating or adding electrophoresis loading buffer.

For preparative large scale digestion of fragments, 10 µg DNA was digested in a total volume of 50 µl containing proper buffer, restriction endonuclease (5 U/ µg DNA), DNA and bovine serum albumin (if necessary) for at least 2 hours. The enzymes were inactivated by heating or adding electrophoresis loading buffer.

3.2 Ligation of DNA Fragments

DNA mixture of small fragment (insert)/ large fragment (backbone) in a molar ratio of 3: 1 was incubated together with DNA dilution buffer and DNA incubation buffer. One unit of T_4 DNA ligase was added for each 1.0 pmol of total DNA. The reaction was first incubated at 37°C for 5 minutes before at 15°C overnight.

3.3 Blunt Ending of DNA Fragments

To ligate two fragments without appropriate adhesive ends, "blunt ending" treatment of DNA is necessary. T_4 DNA polymerase is used since it fills recessed 3'- termini and removes protruding 5'- termini in the presence of dNTPs. The reaction mix contains:

1.0 pmol DNA (4 μg of 5 kb plasmid) to be treated
0.1 mM dNTPs
1x T_4 DNA incubation buffer
1 μl T_4 DNA polymerase.

Incubation time 15 minutes at 12°C, inactivate the reaction at 75°C for 10 minutes.

3.4 Dephosphorylation of DNA Fragments

In order to insert a fragment into a linearized plasmid, religation of the linearized plasmid itself is minimized by dephosphorylation. This was accomplished by shrimp alkaline phosphotase (SAP, Amersham). Reaction mix contains:

DNA to be dephosphorylated
1x SAP buffer
1x Alkaline phosphotase dilution buffer and
enzyme used for a 1.0 pmol DNA (4 μg of 5 kb plasmid):

Terminus	Units of phosphatase
5'-Protruding	0.1 units
Blunt	0.2 units
5'-Recessed	0.5 units

The reaction was incubated at least 1 hour at 37°C, and inactivated by heating at 65°C for 15 minutes.

3.5. Fluorescence Labeling of Plasmid

To test if there is internalization of DNA molecules by ORNs when DNA is applied together with chemicals known to facilitate gene delivery, fluorescence-labeled plasmid DNA was prepared:

50 mM Tris-HCl (pH 7.5)
10 mM $MgSO_4$
0.1 mM DTT

50 µg/ ml	BSA
0.1 mM	dATP/ dGTP/ dCTP
0.05 mM	dUTP
0.05 mM	fluorescein-dUTP
20 U	DNA polymerase I

2 µl DNA polymerase I/ DNase I mixture
10 µg DNA (5kb) to be labeled

Total reaction volume was 100 µl, incubate the reaction in 37°C for at least 2 hours. Stop the reaction by adding 4 µl 0.5M EDTA, extract the labeled DNA by G50 column.

3.6 Amplification of DNA by Polymerase Chain Reaction (PCR)

PCRs were done in 100 µl PCR tubes in a volume of 20 µl, final concentration:

Primers (each 1 µM), dNTPs 1 mM, Mg2+ 1.5 mM, 1x Taq polymerase buffer (without Mg^{2+}), 2-4 ng template DNA, 1 U Taq polymerase.

Program:

Preheating	94°C, 3min (for a better efficiency, the Hot start PCR" is done by heating up the PCR mix without Taq polymerase till almost three minutes. Pause the reaction, add Taq polymerase and continue);
Cycle 34x	
	denature 94°C, 3 minutes
	anneal for 1 minute (when PCR was done with primers tagged with restriction sites thus do not fit perfectly to the template, a low annealing temperature calculated from the part that matches was used for 5-10 cycles, followed by a higher annealing temperature used for the cycles left, thus to ensure both an initial amplification and a high stringency)
	elongation 72°C for 1 minute (with increment of 3 seconds each cycle);
Final elongation	72°C, 5minutes

3.7 Sequencing of DNA

ABI Sequencing of DNA derived from the Sanger method of dideoxy-mediated chain temination (*Molecular Cloning*, Sambrook et al., 1989). ddNTPs labeled with different fluorophores were introduced by PCR.

PCR for sequencing was done by combining 1 µg template DNA, 0.4 µM (final concentration) primer and 2 µl terminator-ready Big-Dye reaction mix in a total volume of 10 µl. Programme: 96°C x 5 minutes, 25 cycles (96°C, 30 s.; 50°C, 15 s.; 60°C, 4 minutes). The PCR product is precipitated by adding 10 µl 3M NaAc (pH 5.2), 300 µl 100% EtOH, per 100 µl PCR product. Mix thoroughly and incubate for at least 30 minutes at -20°C. Centrifuge at 14,000 rpm 4°C for 30 minutes, discard supernatant, wash once with 70% EtOH, centrifuge again for 20 minutes, 14,000 rpm at 4°C, discard supernatant and dry.

Proceed for sequencing by the ABI PRISM™ 377 sequencer (Foster City, CA, USA).

4. Working with *E. coli*

XL1-Blue (*sup*E44*hsd*R17*rec*A1*end*A1*gyr*A46*thi rel*A1*lac*$^-$) and DH5α (*sup*E44Δ*lac*U169) strains of *E.coli* were used in this study. Plating of bacteria, and preparation of medium were done under sterile conditions.

4.1 Growth of Bacteria

Bacteria *E. coli* were grown on LB-agar plates with proper antibiotics in 37°C overnight. Blue–white selection was done in the presence of X-gal (80 µg/ ml) and IPTG (500 mM) in the agar plates.

A single colony picked was grown in 3-5 ml LB medium with proper antibiotics at 37°C overnight, this volume of culture can be used for plasmid mini-preparation.

For plasmid midi-, maxi- and mega- preparation, a 3-5 ml overnight culture was poured into a larger volume of LB medium (25 ml, 100 ml and 500 ml, respectively) and grown at 37°C overnight.

4.2 Conservation of Bacteria in Glycerol Stocks

Bacteria plated in LB-agar survive at 4°C for at least one month. For storage of bacteria for a longer period, medium containing growing bacteria was mixed with 90% glycerol, to a final concentration of 10 % glycerol and kept at -80°C.

4.3 Competent Cell Preparation

Plate the *E.coli* from glycerol stock in LB-agar plate with tetracycline (10 μg/ ml) and grow overnight at 37°C. Pick up single colony the next day and grow overnight in 5 ml LB medium (tetracycline 10 μg/ ml). Pour next morning this 5 ml into 1 L LB medium and grow the bacteria till OD_{600} of the culture reaches 0.5-0.6.

Incubate the flask on ice for 30 min, spinning down bacteria at 4000g, 4°C for 15 min. Discard supernatant, resuspend the pellet in 1 L ice-cold water. Repeat centrifugation, discarding and resuspending three times, but resuspending in 500 ml water, in 20 ml ice-cold 10% glycerol in water, and in 2-3 ml 10% glycerol respectively.

Aliquots of 50 μl bacteria in 1.5 ml eppendorf-tubes were immediately frozen in liquid nitrogen or dry ice with 70% ethanol. Electroporation-competent cells were stored at -80°C till use.

4.4 Transformation of *E.coli* by Electroporation

Parameters for electroporation of bacteria were: 1.8 V/ 200 ? / 25 μFD.

Thaw the electroporation-competent bacteria by storing on ice for 15 minutes. Incubate the electroporation cuvettes on ice. Prepare SOC medium (1 ml SOC contains 960 μl SOB, 20 μl 0.5 M $MgCl_2$, 20 μl 1 M glucose) and warm up to 37°C.

Mix DNA (for propagation of plasmid use 1-3 ng of plasmid DNA, from ligation mix usually 2 μl is used) with 40 μl competent cells, incubate on ice for 1 minute and transfer the mixture into the cuvette.

Dry the outside of the cuvette and distribute well the mixture in the slot of the cuvette. Press the pulse control, immediately after a slight beep indicating the work of the pulse, recover the bacteria by adding warm SOC medium into the cuvette and mixing. Transfer the bacteria with medium into a new tube and incubate for 1 hour at 37°C with shaking.

Plate up to 150 μl bacteria into an LB-agar plate and grow at 37°C overnight.

4.5 Colony PCR

To pick up the bacteria that had incorporated with desired plasmid construct, an efficient screening method - colony PCR - was employed.

96-well UniPCR™ plate with 200 µl volume for each well was used in combination with another 96-well culture plate.

Specific primer pairs matching the desired plasmid were used to detect the presence of insert. A single colony picked with a sterile toothpick from the LB-agarose plate served as template by dipping the toothpick into a well containing 10 µl PCR master mix. Lysis of bacterium and release of plasmid DNA serving as template was completed by the initial heating at 96°C. Dip the same toothpick into LB-medium in the corresponding well in another 96-well plate such that the colony of interest can be found back. Store the bacteria at 4°C until further use.

PCR programs were similar to general PCR but the annealing temperature varied according to the primer pairs.

5. Working with Zebrafish, *Danio rerio*

5.1 General care of zebrafish

Different strains of zebrafish were used in this study: partially inbred Ab/ Ab (University of Oregon), partially inbred TÜ/ TÜ (MPI, Tübingen), Ab/ TÜ, CO/ CO (an outbred stock from the group of Prof. Campos-Ortega, Institute for Development Biology, University of Cologne) and wild type fish from a local pet shop.

Adult zebrafish are kept in tanks with flowing water (tap water/ demineralized water 1/ 1 volume) at 28°C, day/ night rhythm is 14/ 10 hours. Fish are fed twice daily with dry fish food (TetraMarine, TETRA).

Fertilized zebrafish eggs are collected in the early morning, and kept in 10 cm diameter petri-dishes (40 eggs/ dish) filled with E3 buffer in a 28°C incubator. Embryos are not fed for the first five days until they are transferred into the 2 l tanks and fed with fish larvae food (TetraMin Mini, TETRA) and then with 1 day old living artemia.

5.2 Genomic DNA Isolation

To obtain genomic DNA of zebrafish, fish were killed by decapitation, and the head was put into a 1.5 ml tube then the tube into liquid nitrogen, a sterile pestle was used to help grind the sample into powder, from which DNA was extracted by the Qiagen genomic DNA extraction kit. Briefly, anion-exchange technology to purify DNA was employed by this kit. Samples are first lysed and proteins simultaneously denatured in the appropriate lysis buffer. Proteinase K is then added and after a suitable incubation period, lysates are loaded onto the QIAGEN Genomic-tip. Under the pH and low-salt condition, DNA binds to the column while other cell constituents pass through. Following a wash step to remove any remaining contaminants, pure, high-molecular-weight genomic DNA is eluted in high-salt buffer.

5.3 Dissection of Zebrafish Olfactory Epithelium

Adult zebrafish were killed by decapitation, and the head was transferred into ice-cold PBS. Under stereomicroscope the rossette-shaped olfactory epithelium inside a bony recess lying immediately lateral to the eye was cautiously taken out using fine forceps and scissors.

5.4 Cryosection of Zebrafish Embryo and Nasal Epithelium

Zebrafish embryos were killed in cold PBS, embryos were moved into TissueTek® and were frozen in –20°C. 15 μm sectioning was done in a Leica cryostat.

Nasal epithelium was moved into TissueTek® after dissection, it was orientated as such that the bottom of the bowl-like structure was down and the finger-like lamellae pointed upperward and outward. After freezing at -13°C, 15 μm sections were cut in a Leica cryostat.

5.5 Microinjection of Plasmid DNA in Zebrafish Embryos

The microinjection system consists of a stereomicroscope, a syringe (Cell Tram Oil 5176, Eppendorf-Netheier-Hinz GmbH, Hambug) filled with mineral oil, Teflon tubings, a needle-holder and borosilicate microinjection capillaries pulled by a pipette puller (Sutter, USA, Program: Heat 500, Pull 25, Vel. 50, Time 55).

Plasmid DNA at a concentration of 50-100 pg/ μl in Tris-HCl (pH 7.6) together with 0.25% phenol red was used for injection. 2-10 μl DNA solution was filled into the capillary. After fertilized zebrafish eggs were collected in E3 medium, the microinjection capillary was connected to the needle-holder and was broken carefully

for a small opening (0.05-0.15 mm diameter). Position the needle to cytoplasm of 1-2 cell stage embryos by hand controlling. About 5 nl DNA solution was injected into each embryo, until the color of embryo turned to pink.

Postmicroinjection embryos were kept at 28°C in E3 medium, they were checked several hours later to remove the dead eggs and to renew the E3 medium.

5.6 Transfection in the Zebrafish Nasal Epithelium

The apparatus to handle the fish for attempts of transfection in the adult nasal epithelium is shown in figure III.4. Adult zebrafish were anaesthetized by immersion for 2 minutes in a Tricaine solution (3-amino benzoic acidethylester in water, 0.16 mg/ ml, pH 7). They were then wrapped in a wet paper towel and positioned in a petri- dish with the dorsal side up, head to the manipulator.

Anaesthesia is maintained by continous perfusion through the fish mouth and gills of a less concentrated Tricaine solution (0.08 mg/ ml). The right nose is under constant perfusion of fresh water while the left nose is loaded with transfection cocktails containing DNA. Sometimes attempts for transfection were made by direct DNA injection in the olfactory bulb, which lies immediately posterior to the epithelium.

After the manipulation, which takes 20- 45 minutes, fish are put back into fresh water and recover quickly.

5.6.1 *Application of Plasmid DNA together with 0.1% Triton-X100*

Treatment with 0.1% Triton-X100 has been shown previously to cause a reversible degeneration of cilia and microvilla of zebrafish ORN, this could be used to introduce an anionic dye into these neurons. No cell death was observed and regeneration was accomplished in 48 hours (Friedrich and Korsching, 1997). Thus I investigated whether this Triton-X 100 treatment would allow internalization of DNA by the ORNs.

The cocktail for transfection contains:
plasmid DNA at 1, 2.5, 5, 10 µg/ µl final concentrations
0.1% Triton-X100
0.25% phenol red as indicator

The cocktail was applied to the nose through a gel-loader pipet tip, about 2-3 µl cocktail was loaded into each nose treated.

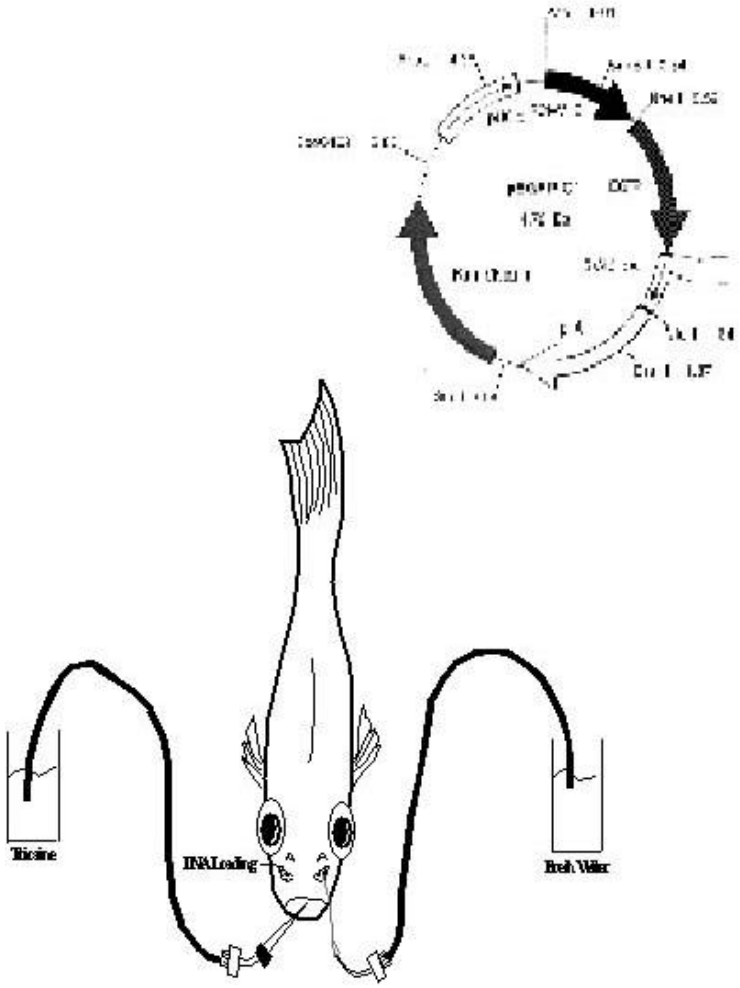

Figure III.4. Apparatus to Handle Fish during Attempts of Transfection.
Fish under anaesthesia is wrapped in wet paper towel, cocktail for transfection is loaded into one nostril by gel loader while the contralateral nostril is under constant perfusion by fresh water. In some cases (see 3.5.7), DNA is injected into the olfactory bulb lying next to the nose through a small window in the head.
Upper right, the plasmid pEGFP-N1 encoding green fluorescent protein is shown, which is used as a marker of transfection.

5.6.2 *Application of Plasmid DNA together with Transfectional Reagents*

Transfectional reagents are known to facilitate transfection *in vivo* or *in vitro* (Felgner et al., 1987; Felgner and Rringold, 1989; Ledley 1995 and references therein; Legendre and Szoka, 1993; Pinnaduwage et al., 1989), presumably because they are

positively charged and can form compacted particles together with the negatively charged DNA. These particles are relatively hydrophobic and can be taken up by the cell. The efficiency of transfectional reagents is rather variable and depends strongly on the cell type. Thus several transfectional reagents were investigated: Cellfectin, Lipofectin, DMRIE-C and LipofectAMINE (all Qiagen).

Cocktail for transfection:

1 µl transfectional reagent was incubated with 100-500 ng plasmid DNA at room temperature for 45 minutes, before 2–3 µl cocktail was applied as several aliquots to one nose in each fish. Sometimes 0.25% phenol red was included to supervise the load into the nose.

5.6.3 *Application of Adenovirus Derived Vector DNA*

Ad-5-EGFP, a replication-defective adenovirus with cDNA encoding enhanced green fluorescent protein (EGFP), is known to effectively infect the adult mouse and rat nasal epithelium, and cause expression of EGFP in the ORNs (Hermens and Verhaagen 1998 and references therein; Holtmatt et al., 1996; Holtmatt et al., 1997; Ivic et al., 2000; Zhao et al., 1998). In this attempt, Ad-5-EGFP was used briefly to check if it can also infect adult zebrafish nasal epithelium and cause the expression of EGFP there.

Each treated nose was loaded with 2–3 µl adenovirus ($6x10^6$ PFU/ µl) for up to 45 minutes. Sometimes 0.25% phenol red was included to supervise the load into the nose.

5.7 Injection of Plasmid DNA in Zebrafish Olfactory Bulb

Adult zebrafish was handled as shown in figure III.4. A small window (about 2x2 mm^2) was opened just neighbouring the midline of the dorsal scalp with the help of fine scissors and forceps. Through this window a blood vessel traversing the surface of the dorsal olfactory bulb is visible. Carefully avoid this blood vessel and inject 1 µl of plasmid DNA (500 ng/ µ) in artificial cerebral-spinal fluid (ACSF) with microinjection apparatus into the olfactory bulb. No further effort was taken to close the window. Recover fish by putting it into fresh water.

5.8 $ZnSO_4$-induced Degeneration of Olfactory Epithelium

3% $ZnSO_4$ is known to cause degeneration of zebrafish olfactory neuroepithelium and death of mature ORNs when injected into the nose. After lesion, neurogenesis increases and regeneration is completed at one month post lesion. Here, transfection is attempted during regeneration. DNA shall have better access in the nucleus in deviding cells.

Adult zebrafish was handled as shown in figure III.4. Three percent $ZnSO_4$ together with 0.25% phenol red was continously injected into the nasal epithelium by a pipette tip connecting to a syringe. The speed of flow was manually controlled such that in 10 minutes about 2-3 ml 3% $ZnSO_4$ was injected. The fish was recovered by putting it back to fresh water.

6. Cell Culture Methods

In this study a HEK 293 cell line is used (primary human embryonic kidney cell, originally transformed by sheared human adenovirus type 5 DNA (Graham et al., 1980), kindly provided by Dr. D. Krautwurst.

Cell culture hood and incubator were cleaned thoroughly with 70% ethanol weekly, and sprayed briefly with 70% ethanol each time when turned on or off. Cell culture work was performed under sterile conditions.

6.1 Cell Freezing and Thawing

Freezing: Cells prior to confluence were used for freezing to assure viability.

Culture medium was aspired and washed gently with 1x PBS (without Ca^{2+} or Mg^{2+}). The cells were trypsinized with 0.05% trypsin/ 0.53 mM EDTA in PBS until the cells easily detach and can be dissociated into a single cell suspension. Trypsinization is quenched with GM (growth medium). The cells were centrifuged at 500 g for 5 minutes. The GM was removed and 1 ml of freezing solution (90% FCS, 10% GM) per 10^6 cells was added, cells in freezing solution was transferred to 2 ml cryogenic vials. Let freeze slowly (overnight 4°C, one day -20°C, then transfer to -80°C and liquid nitrogen).

Thawing: Remove one vial from liquid nitrogen and thaw rapidly at 37°C.
1 ml GM was immediately added to the vial and transfer the mix to a 15 ml conical screw cap tube, add 2 ml GM and gently mix for osmotic equilibration. Add 10 ml GM, close the tube and spin cells at 500 g for 5 min. Remove the supernatant and resuspend in GM, transfer to culture dish.

6.2 Cell Growing and Passaging

Cells after thawing are grown and passaged until they are three months old. They are passaged when they are grown to 80% confluence (usually once every two weeks). To passage cells, wash, trypsinize and quench cells as done for freezing. If necessary, cells were counted by a hemocyte-counter. For viability tests, cells were stained by Trypan-blue in normal saline for 1 min. before counting. Cells were passaged onto new plastic culture dish or glass coverslips coated with 0.1 mg/ ml poly D-lysine.

6.3 LipofectAmine-mediated Transfection

Transfection was performed with cells around 50% confluence.

500 ng plasmid DNA/ 2 µl LipofectAMINE for each 250 µl OPTIMEM was used in a well of 24 well culture plates. Scale up for culture dishes with different sizes. Mix 125 µl opti-MEM and 2 µl LipofectAMINE for 30 minutes at room temperature. Plasmid DNA in 125 ml OPTIMEM was added and the mixture was incubated at room temperature for 15 minutes. Aspire the GM, substitute it with transfection cocktail, incubate for 5 hours, wash three times and substitute with fresh GM.

6.4 Calcium-phosphate Precipitation Transfection

Reagents: 1) HBS
2) $CaCl_2$

Transfection was done at 30%-70% confluent cells. Before transfection, change fresh GM for cells. Dosage was optimized for a 60 mm culture dish with 4 ml GM and can be scaled up or down.

Transfection cocktail: 6-10 µg plasmid DNA in water with final volume of 438 µl. Add 62 µl 2M $CaCl_2$ to the DNA/ H_2O. Add 500 µl 2x HBS (pH 7.05) by bubbling.

Immediately add the solution to the cells and slightly agitate for uniform mixing. Leave the cells in the incubator for 3-5 hours, afterwards change to fresh GM.

6.5 Immunostaining

30-96 hours after pCMV-Rho ZOR transfection, cells were immunostained by B6-30 monoclonal antibody against the N-terminal peptide 3-14 of rhodopsin. Except when otherwise stated, immunostaining was done on ice.

Wash cells 3x with PBS, block non-specific binding sites by 2% goat-serum in PBS (blocking solution) for 10 minutes. Wash 3x with blocking solution, incubate with 1:1500 primary antibody in blocking solution for 1 hour.

Wash 3x with blocking solution, incubate with 1:150 secondary antibody in blocking solution for 15 minutes, and wash 3x with PBS. The sample was soak with ice cold methanol (-20°C) for 10 minutes.

Mount with anti-quenching mounting solution (Mowiol, IL, USA) and seal with nail polisher.

6.6 Primary Culture of Mouse Olfactory Epithelium

Dissection and preparation methods of mouse olfactory neuron primary culture was performed according to Calof et al. (Calof and Chikaraishi, 1989; Calof and Lander, 1991; Calof et al., 1991). Newborn mice were used in this experiment.

1. Decapitate the newborn mice and place the heads in a 100 mm petri dish containing cold PBS with serum, distribute heads into 60 mm petri dish, with three to four heads per dish, dissection was performed on ice.

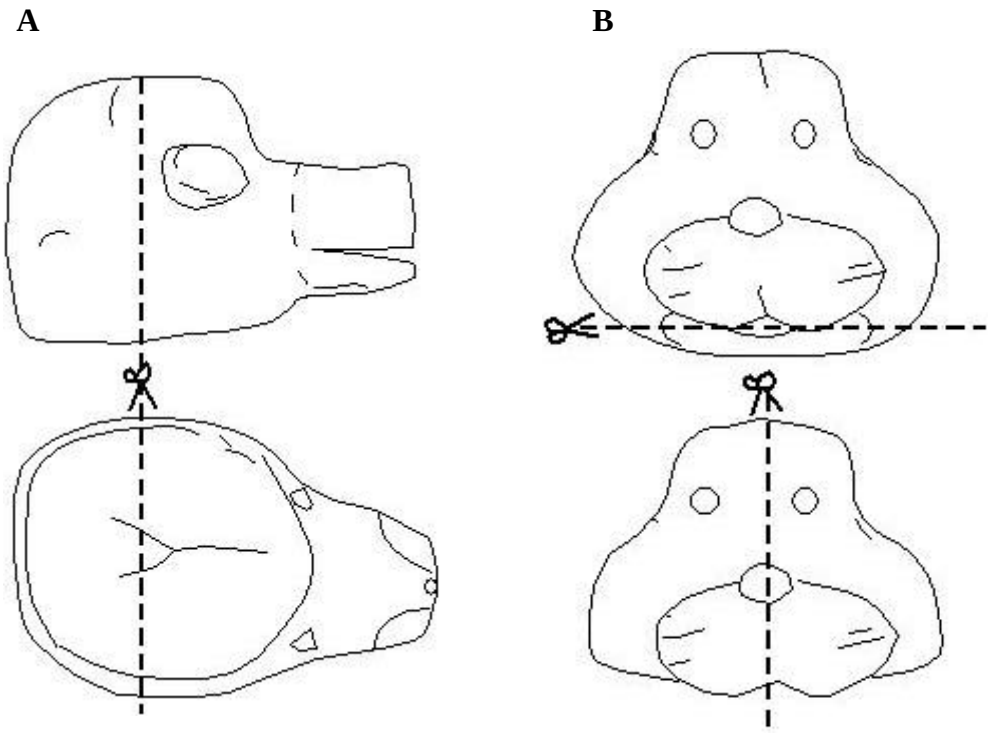

Figure III.5. Three Initial Incisions of the Newborn Mice Head.
After decapitation, a coronal incision separates the frontal part from the remainder of the head. The second, horizontal incision is made to remove the jaw and tongue, followed by a third, sagittal incision from the front of the snout through the back of the head that splits the nasal cavity into two halves.

1 After the first, coronal incision from the top to the base of the head (Fig. III.5A), make a second, horizontal incision to remove the lower jaw and tongue. A

third, sagittal incision from the front of the snout through the back of the head splits the nasal cavity from the rest (Fig. III.5B).

3. Lay the inside of nasal cavity up, pinch gently up to remove the oval buds of turbinates (Fig. III.6A). Transfer the turbinate into a 60 mm petri dish containing cold holding medium. Collect all the turbinates from one dissection (of 4-5 mice, Fig. III.6B). Remove the holding medium, rinse turbinates once in cold PBS, then substitute with 8 ml of trypsin-pancreatin solution.

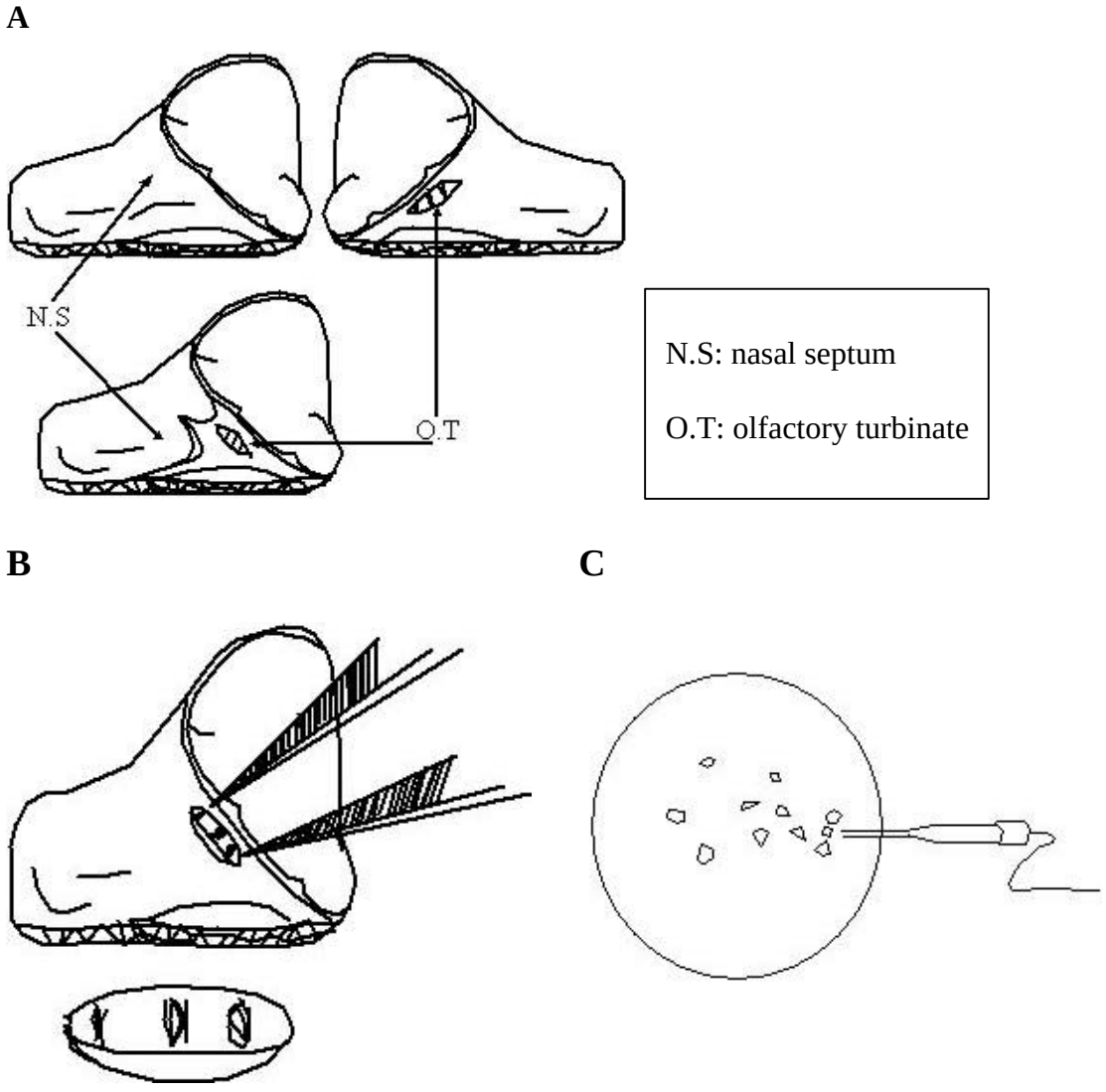

Figure III.6. Dissection and Collection of Olfactory Turbinates.
After splitting the nasal cavity into two halves, lay the inside of nasal cavity up, pinch gently up to remove the olfactory turbinates. Transfer the turbinate into cold holding medium. Collect all the turbinates. Rinse twice, then trypsinase and titurate the turbinates by a pasteur pipet to get sensory epithelium.

4. Incubate turbinates in the trypsin-pancreatin solution for one hour on ice, remove enzyme solution and add 8-10 ml post-trypsin rinse to the dish.
5. Distribute two turbinates in each 3.5 mm petri dish containing 2.5 ml cold trituration medium. Use a sterile Pasteur pipette to gently triturate the turbinates to dissociate the OE from its underlying stroma. It is necessary to continue trituration until there is no more stroma attached to the epithelium. The epithelium appears as shiny, translucent sheets whereas stroma appears as irregularly shaped, opaque masses (Fig. III.6C).
6. Transfer the pieces of epithelium through two successive rinses of cold low calcium culture medium.

Finally, mechanically dissociate the tissue with fine needles in the low calcium medium. Transfer the whole content of the dish into a nylon mesh of 10 μm, collect the filtered cell suspension into a 15 ml Falcon tube, spin for 10 minutes at 200 g. Aspire the low calcium medium and substitute with neuron cell growth medium. Finally, plate the cells in a 6 mm culture dish.

2 Optical Imaging

7.1 FURA-2 Staining

4 μM FURA-2 AM-ester was used to stain HEK 293 cells. Cells are washed three times in serum-free growth medium before staining. A stock solution of 10 mM FURA-2 in DMSO was diluted in serum-free DMEM and loaded by incubation in room temperature for an hour. Wash the cells three times with room temperature HEK cell imaging medium (HEKIM), wait for half an hour to allow the cleavage of the AM ester inside the cell. Proceed to optical imaging.

7.2 Preparation of Odors

Odors used in this experiment have been shown to elicit neuronal responses in the zebrafish olfactory bulb (Friedrich and Korsching 1998):

Amino acids (L-form): Alanine (A), Arginine (R), Asparagine (N), Aspartic acid (D), Cystine, Cysteine (C), Glutamine (Q), Glutamic acid (E), Glycine (G), Histidine (H), Isoleucine (I), Leucine (L), Lycine (K), Methionine (M), Phenylalnine (F), Proline (P), Serine (S), Threonine (T), Tryptophan (W), Tyrosine (Y), Valine (V).

Bile acids/ conjugated bile acids: Glycocholic acid (GCA), Lithocholic acid (LCA), Taurocholic acid (TCA), Taurolithocholic acid (TLCA), Chenodeoxycholic acid (CDCA), Taurodeoxycholic acid (TDCA), Taurochenodeoxycholic acid (TCDCA).

These odors were prepared at 10 mM in DMSO as stock solution and kept in freezer. They were freshly prepared every two weeks.

Nucleotides: Adenosine 5'-monophosphate (AMP), Adenosine 5'-triphosphate (ATP), Inosine 5'-monophosphate (IMP), Thymidine 5'-triphosphate (TTP).

These odors were prepared at 10 mM in water as stock solution and kept in freezer. They were freshly prepared every two weeks.

Prostaglandin: Prostaglandin 2α (PGF).

Sexual steroids: Androstenedione (AD), Progesterone (Pro), Testosterone (T).

Prostaglandin 2α and sexual steroids were prepared at 1 mM in DMSO as stock solution and kept in freezer. They were freshly prepared every two weeks.

To identify odorant – ligand pairs, odor mixtures were used as follows:
amino acid mix 1 (aa.1): A/ R/ N /D, each 2 mM;
amino acid mix 2 (aa.2): Cystine/ C/ Q/ E, each 2 mM;
amino acid mix 3 (aa.3): G/ H/ I/ L, each 2 mM;
amino acid mix 4 (aa.4): K/ M/ F/ P, each 2 mM;
amino acid mix 5 (aa.5): S/ T/ W/ Y/ V, each 2 mM;
bile acid mix 1 (ba.1): GCA/ LCA / TCA/ TLCA, each 25 μM, 250 μM or 2.5 mM;
bile acid mix 2 (ba.2): CDCA/ TDCA / TCDCA, each 25 μM, 250 μM or 2.5 mM;
Nucleotide mix: AMP/ ATP / IMP/ TTP, each 2 mM.;
Prostaglandin 2α and sexual steroids were used individually at 10 nM, 1 μM or 100 μM.

7.3 Application of Odorant Stimuli

Optic imaging was done in an open, elongated assay chamber (bath volume 200 μl). Cells after transfection with pCMV-Rho-ZOR or pCMV-Rho-I7 (positive control) and Gα 15/ Gα 16 were stimulated with test solutions.

Odorant stimuli were loaded through a carrier stream of HEKIM (1-2 ml/ minute) directed at one side of the recording well. Cells were under constant perfusion of image medium. An electrically operated injection valve switched the carrier physiological medium into odorant stimuli. With the tubing set-up used, the stimuli reach their peak concentration (1/ 5 of the applied concentration) at 50 seconds after switching the valve as monitored by the outflow of fluorescent rhodamine-dextran before beginning the actual experiments.

In some experiments, odorant was loaded manually through a carrier stream of HEK-IM directed at one side of the recording well carefully. By this means, cells immersed in the bath medium immediately have contact with stimulus.

Successive applications were separated by more than five minutes to exclude adaptation. 10 μM acetylcholine was applied at the end of the experiment to each well, to confirm the viability of the cells during the preceding experiments.

7.4 Image Acquisition and Data Processing

Imaging acquisition and data processing were done using T.I.L.L image software (version 3.3, T.I.L.L Photonics, Germany) running on a personal computer. Images (80 x 120 pixels) were acquired with a water-cooled 12-bit CCD camera at 0.05 – 2 Hz during a recording time of 90 to 300 seconds. Exposure time depends on the intensity of staining and was adjusted from 60 to 200 ms. Care was taken to choose the exposure time for each wavelength such that the intensity of emission was approximately the same for both wavelengths. Major components of image set-up are illustrated in figure III.7.

Ratiometric imaging was accomplished by sequentially illuminating the cells first at 340 nm and then 380 nm wavelengths, with 30 ms switching time. Fluorescence emission at 510 nm is monitored.

Two sequences of images were recorded by this means (seq. A at 340 nm and seq. B at 380 nm), and the calcium response was expressed as the F340/ F380 ratio. False-color images showing the relative ratio of F340/ F380 pixelwise were displayed in real time and monitored. About 20 areas of interest (AOI), corresponding to approximately 20 cells, were chosen in each sequence, and the calcium dynamics in these 20 AOIs was further analysed. A change of F340/ F380 more than 2 times SD of the noise level was considered as a cytoplasmic calcium rise.

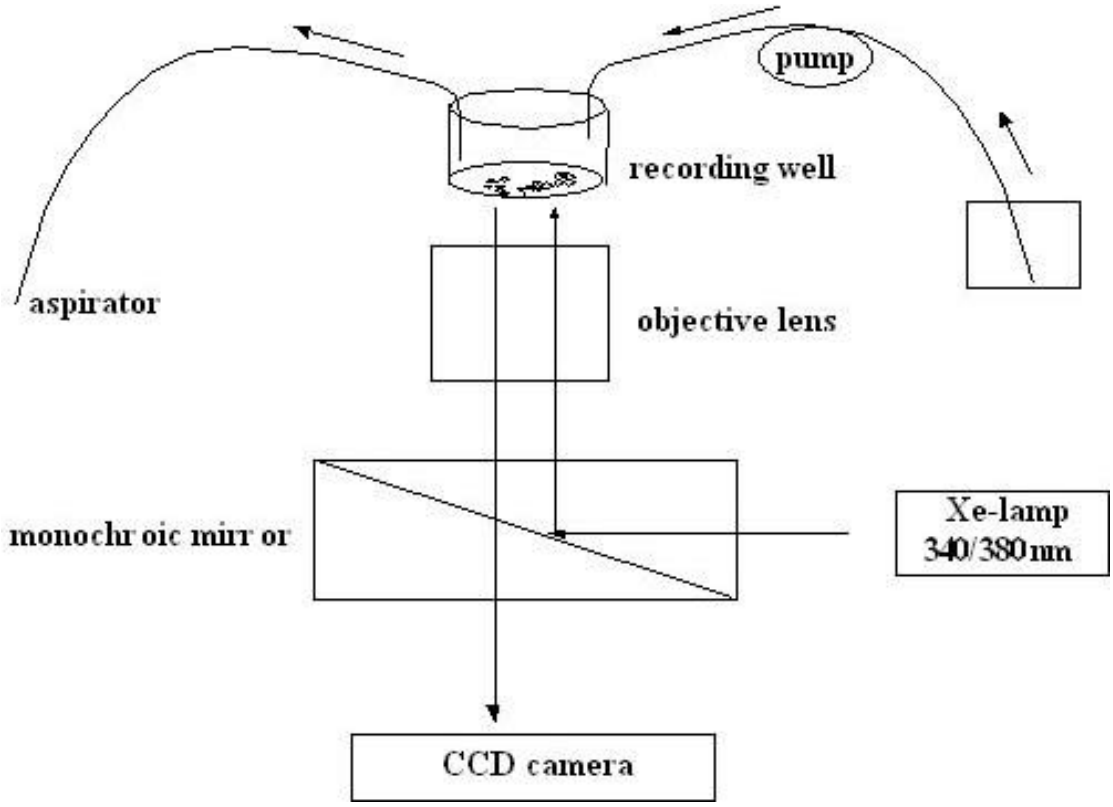

Figure III.7. Major components in Optical Imaging Set-up.
Optical imaging setup contains a monochromic mirror, a Xenon-lamp, and a CCD camera. Cells in the 24-well culture dishes were superfused by imaging medium and stimulated with odorants and the resulting fluorescence changes were recorded with the CCD camera.

IV. RESULTS

1. Attempts of *in vivo* Transfection of ORN in the Adult Zebrafish Olfactory Epithelium

To test various transfection methods, a eukaryotic expression vector, pEGFP-N1 was used. It appears prudent to establish first whether this vector could successfully drive expression in zebrafish. To this end, microinjection was used.

1.1 Microinjection of pEGFP-N1 in Zebrafish Embryos

Microinjection of zebrafish embryo is a well established technique and was employed in this experiment to check the expression of pEGFP-N1 in developing zebrafish.

Approximately 400 eggs were microinjected in several days, usually around 50% of injected embryos survived and 20%-60% of those surviving expressed visible GFP fluorescence in whole mount analysis. After microinjection of circular plasmid pEGFP-N1, GFP-positive cells started to been seen in embryos 12 hours after injection, and remained visible till five days, the longest time analysed. There were single GFP positive cells as well as clustered. Intense fluorescence was seen uniformly inside the cells (Fig. IV.1).

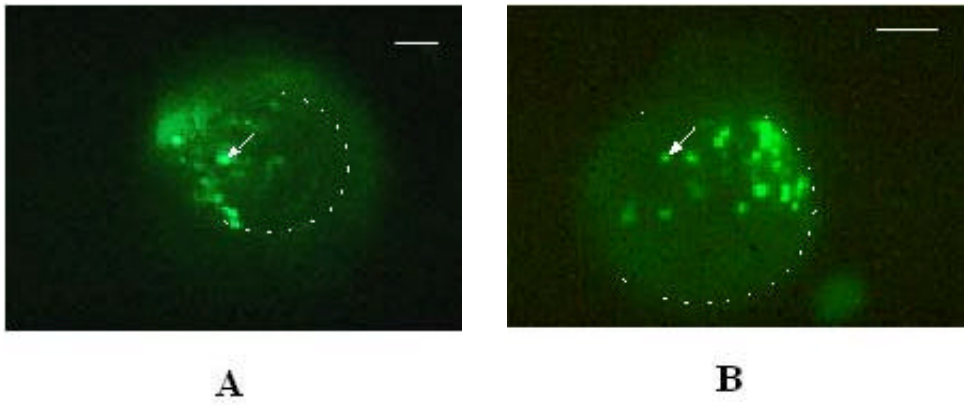

Figure IV.1. Two Embryos Expressing GFP.
Two embryos express GFP at 16 hours after fertilization (hPf) (A) and 18 hPf (B) after microinjection of circular pEGFP-N1. Microinjection was done at 1h pF. Dashed lines indicate the york sac. Clustered (arrow in A) and single (arrow in B) GFP positive cells were seen.
Scale bar: 100 μm.

There is a big variability in number of GFP positive cells among different injections, which might be attributed to the difference in quality of eggs from different parent strains, the slight difference in manipulation, or the difference in quality of DNA. However, such variability is commonly observed with zebrafish embryo microinjection. Thus the expression cassette appears suitable for zebrafish.

1.2 Application of Plasmid DNA into the Nasal Epithelium

Various transfection methods were tested using pEGFP-N1 and a bicistronic expression vector containing an OR and GFP. The construction of the latter vector is described below.

1.2.1 *Construction of the pCMV-ZOR5A-IRES-EGFP Expression Vector*

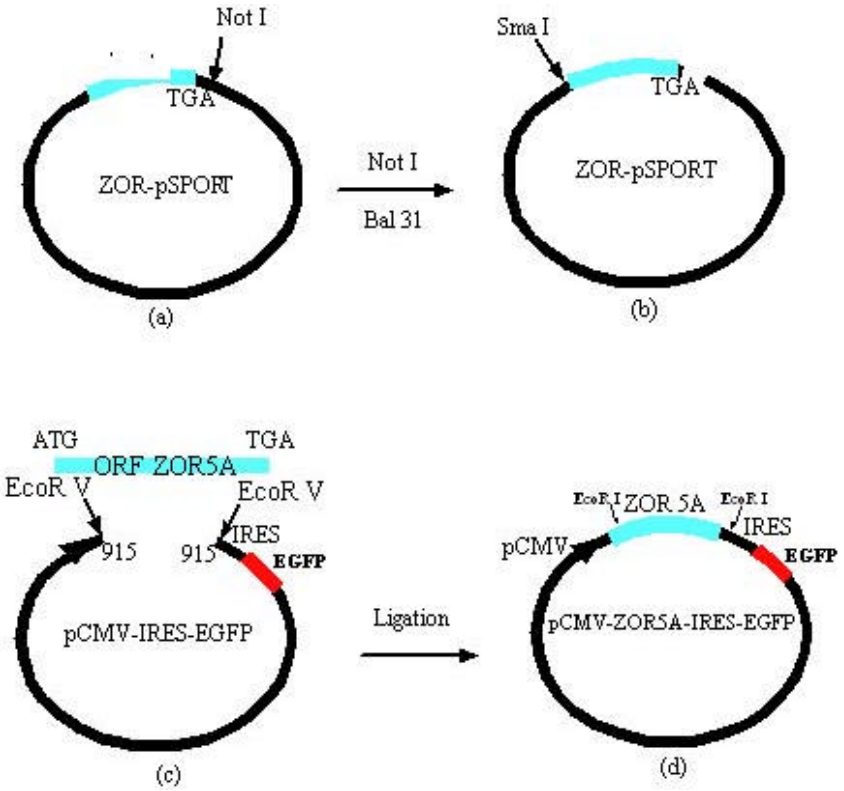

Figure IV.2. Construction of pCMV-ZOR5A-IRES-EGFP.
The ORF of ZOR5A was cut from the cloning vector pSPORT and ligated into the *EcoR V*-linearized pIRES-EGFP. The desired construct of 6.3 kb is shown in (d).

ORF of ZOR5A in pBluescript was linearized at the *Not I* (3'-prime) site, and modified by *Bal 31* exonuclease to cut off the surplus 3' sequences of about 200 bp. This procedure kept part of the 3'-sequence just neighboring the TGA stop codon (Fig.IV.2a and IV.2b).

ORF of ZOR5A was then cut out by *Sma I* (Fig. IV.2b). Digests were loaded on TAE-agarose gel, DNA fragment of ZOR5A ORF (ca. 1.2 kb) was cut and extracted from the gel. Backbone plasmid pCMV-IRES-EGFP was linearized at its *EcoR V* site of MCS and dephosphorylated (Fig. IV.2c).

The ligation mix was electroporated into E. *coli* (strain XL 1 Blue). And colonies were picked up for mini-preparation. Analytical digestion with *EcoR I* (Fig. IV.3) allows to distinguish the orientation of the insert (Cf. Fig. IV.2, the first *EcoR I* lies close to the *ATG* start codon while the second lies on the vector backbone). One out of 48 clones analyzed (clone 23), showed the desired insert in the desired direction. Results of partial sequencing, using primers N1-P1, N1-P2, N1-3, N1-4, N1-5 and N1-6, confirmed that clone 23 contains the desired construct, pCMV-ZOR5A-IRES-EGFP.

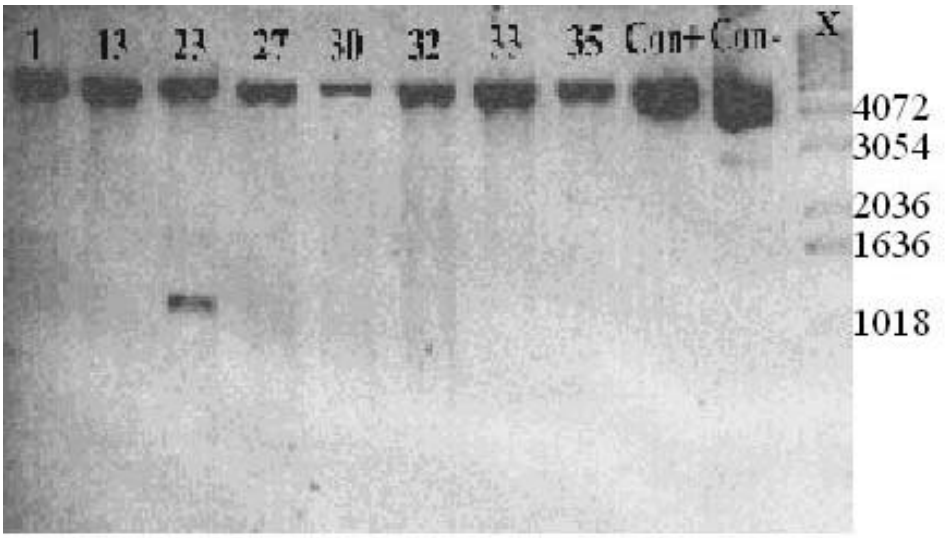

Figure IV.3. Analytical Gel of *EcoR I* Digestion.
Several clones were analysed, clone numbers are given on top of the lanes. Only clone 23 gave one band at 950 bp and one at 5.3 kb, indicating it has the right insert with right direction (Cf. Fig. IV.2d). Con+: digestion of control clone pEGFP-N1, Con-: control clone pEGFP-N1 without digestion. X: DNA marker X, length is given in bases, to the right.

1.2.2 *No GFP Expression in the OE after Administration of Expression Vector Encoding GFP together with 0.1% Triton-X 100*

Charged macromolecules may be taken up by ORN after Triton-X 100 treatment of nasal epithelium (Friedrich and Korsching, 1997). I therefore examined whether DNA could be taken up by the same way. Expression vector was applied to the nasal epithelium together with 0.1% Triton-X 100 by gel-loader pipet tips. In total 67 fishes were used. 4 fishes were treated with the construct of pCMV-ZOR5A-IRES-EGFP in the initial experiments. None of these showed any GFP expression. Since by then there was no report about the efficiency of this IRES sequence (from encephalomyocarditis virus) in fish, the lack of expression might be a consequence of insufficient translation of the second cistron. Thus in the subsequent experiments, all the other 63 fishes were treated with pEGFP-N1, where the expression of GFP is driven by the ubiquitous and robust CMV-IE promoter. The suitability of this promoter for expression in zebrafish was demonstrated (see 1.1).

Final concentrations of plasmid applied were: 1, 2.5, 5, and 10 µg/ µl (Tab. IV.1).. DNA with higher concentration was not used since it is too viscous to apply by gel-loader pipet tips.

A 1.5 µl cocktail containing plasmid and 0.1% Triton-X 100 in water was applied by gel-loader pipet tips. Fish were left in a wet papertowel after treatment for 5 to 30 minutes before being put back to the water tank.

After 2, 4, 7, 10 and 14 days treated fish were killed by decapitation, their epithelia were taken out and checked under the fluorescein filter of the stereomicroscope SMZ-U. No epithelia showed any green fluorescence in the whole mount analysis (Tab. IV.1). However whole mount analysis may not be sensitive enough to detect weak expression levels of GFP. Therefore cryosections (15 µm) of some randomly chosen epithelia were prepared and analysed for GFP fluorescence under fluorescence microscope Zeiss Axioplan. Again, no GFP expressing cells were visible.

1.2.3 *Fluorescence-labeled Plasmid Has No Access to ORNs When Applied with 0.1% Triton-X 100*

The absence of GFP fluorescence after plasmid applications could have two reasons. It could be a consequence either of insufficient uptake or of insufficient expression of the plasmids. After expression had been tested in zebrafish embryos (1.1), it is unlikely that adult nasal epithelium would behave differently. To test uptake and expression of DNA separately, fluorescein labeled plasmid (ca. 4 kb) was applied into the naris together with Triton X-100 under the same condition as used for

the expression plasmids. 9 fishes were treated, none of which showed fluorescence at 24, 36, 48 hours (3 fish for each time period) after treatment. To obtain an estimate for the threshold of fluorescein-labeled DNA detection, the visibility of dilutions of the application mix was examined. A 1/ 1000 dilution was still visible. Therefore it appears unlikely that sufficient DNA could get into the ORNs with this method.

Table IV.1. GFP Expression in ORNs after Nasal Application of Expression Vectors with 0.1% Triton-X 100.

Concentration of plasmid (µg/ µl)	1	2.5	5	10
Treated fish	17	16	18	16
Surviving fish	17	16	18	15
Numbers of fish expressing GFP	(3) 0 [2] (3) 0 [4] (4) 0 [7] (3) 0 [10] (4) 0 [14]	(3) 0 [2] (3) 0 [4] (3*) 0 [7] (3*) 0 [10] (4) 0 [14]	(3) 0 [2] (3) 0 [4] (3*) 0 [7] (3*) 0 [10] (3) 0 [14] (3) 0 [28]	(3) 0 [2] (3) 0 [4] (3) 0 [7] (3) 0 [10] (3) 0 [14]
GFP (+) ORNs after sectioning	(1) 0 [2] (2) 0 [4] (2) 0 [7] (2) 0 [10] (1) 0 [14]	(1) 0 [2] (2) 0 [4] (2) 0 [7] (1) 0 [10] (1) 0 [14]	(2) 0 [2] (1) 0 [4] (2) 0 [7] (1) 0 [10] (2) 0 [14] (1) 0 [28]	(1) 0 [2] (2) 0 [4] (2) 0 [7] (1) 0 [10] (1) 0 [14]

In rows 4 and 5, numbers in the first column (in parentheses) are the numbers of fishes analysed at the particular day after treatment (square brackets); numbers in the second column are the numbers of fishes expressing GFP.
*: 1 fish from each was treated with plasmid pCMV-ZOR5A-IRES-EGFP.

1.2.4 *Transfectional Reagents Do Not Improve Uptake of Exogenous DNA*

Since the direct uptake of plasmid was not successful, various transfectional reagents were examined in the next set of experiments. Transfectional reagents used in this experiment were: Cellfectin, LipofectAMINE, Lipofectin and DMRIE-C, all from GibcoBRL Life Technologies. All of these transfectional reagents are known to facilitate transfection *in vivo* or *in vitro*.

Plasmid pEGFP-N1 was incubated with transfectional reagents at room temperature for 45 minutes before administration of this mix into the fish nose. DNA/

transfectional reagent ratio was varied between 100 ng/ µl to 500 ng/ µl, as suggested by the supplier. 40 fishes were treated, 10 for each transfectional reagent. 38 out of 40 fish survived. The mortality observed seems to have been caused by too deep anaesthesia. After 20-45 minutes of treatment, fish were put back in fresh water and recovered quickly. After 4 days fish were killed by decapitation and whole mount epithelia were checked for expression of GFP using the fluorescein filter. No GFP positive epithelium was seen (Tab. IV.2).

To distinguish between uptake and expression of DNA (Cf. 1.2.3), fluorescein-labeled plasmid was administered in the nose instead of pEGFP-N1. After 24, 36 or 48 hours fish were killed by decapitation and whole mount epithelia were analysed using the fluorescein filter set. Only Lipofectin and Cellfectin, known to facilitate transfection in mouse brain *in vivo*, were chosen as transfectional reagents in this case. In total, 38 fishes were treated, 35 survived, among which 24 were treated with DNA/ Lipofectin and 11 with DNA/ Cellfectin.

After administration of transfectional reagent in the nose, sometimes noticeable fluorescence was seen in the treated epithelia. However, in all these cases the untreated contralateral epithelium showed the same increase in fluorescence. This autofluorescence made it difficult to evaluate treatment-related fluorescence. To overcome this problem, a whole mount epithelium that showed strong autofluorescence was washed for 30 min. in 0.1% sodium borohydride in phosphate-buffered saline before being compared to that of non-treated fish. This treatment is known to diminish the autofluorescence and is in fact suggested by the supplier of pEGFP-N1 (Instruction for plasmid pIRES-EGFP and references therein, Clontech, 1998). Afterwards both treated and contralateral epithelium had lost their autofluorescence and appeared indistinguishable from that of an untreated fish. The reason of this increased autofluorescence is unknown, but it is suggested by the supplier of GFP plasmids that autofluorescence in mammalian cells is principally due to flavin coenzymes (FAD and FFMN, absorption/ emission ~ 450/ 515 nm) and reduced pyridine nucleotides (NADH, absorption/ emission ~ 340/ 460 nm).

To determine whether tiny amounts of DNA below the threshold of the fluorescent detection might have been taken up by the ORN, PCR as a more sensitive method for detection of DNA was used. Nasal epithelia of 6 fish were treated with Lipofectin/ pBluescript (pBs) mix, 48 hours after application the 5 surviving fishes were killed by decapitation and total DNA from the nasal epithelium was extracted by Qiagen genomic DNA extraction kit. The extracted DNA served as template for PCR with the primer pair, T7 and T3. These primers should only be present in the plasmid, not in the fish genomic DNA. If plasmid is taken up by the nasal epithelium, PCR with these primers should result in a band at approximately 250 bp.

Table 2. GFP Expression in ORNs after Application of DNA/ Transfectional Reagent.

content of administered cocktail (DNA/ transfectional reagents)	number of fish treated	GFP(+)/ fish at day 4	GFP(+)/ fish at day 7
100 ng/ 1 ? l Cellfectin	4	(2) 0	(2) 0
300 ng/ 1 ? l Cellfectin	2	(2) 0	n.d
500 ng/ 1 ? l Cellfectin	4	(2) 0	(2) 0
100 ng/ 1 ? l LipofectAMINE	4	(2) 0	(2) 0
300 ng/ 1 ? l LipofectAMINE	2	(2) 0	n.d
500 ng/ 1 ? l LipofectAMINE	4*	(2) 0	(1) 0
100 ng/ 1 ? l Lipofectin	3	(3) 0	n.d
300 ng/ 1 ? l Lipofectin	4*	(2) 0	(1) 0
500 ng/ 1 ? l Lipofectin	3	(3) 0	n.d
100 ng/ 1 ? l DMRIE-C	3	(2) 0	n.d
300 ng/ 1 ? l DMRIE-C	4	(2) 0	(2) 0
500 ng/ 1 ? l DMRIE-C	3	(3) 0	n.d

In columns 3 and 4, numbers in parentheses are the numbers of fishes killed; numbers in the second column are the numbers of fishes expressing GFP.
n.d: not determined.
*: 1 fish died in each case.

Unexpectedly, the 250 bp band was observed in all samples tested, both from the treated and the contralateral side. The intensity of the band varied, but no correlation with the treatment is seen (Fig. IV.4). It is quite possible that the PCR product was the result of a contamination of the tissue, since the 250 bp band was not seen in a separate control PCR, where total DNA from the nose of non-treated fish was used as template (data not shown). Thus the PCR analysis could not be used to evaluate a potential low level of uptake of plasmid.

1.3 Application of Plasmid DNA during Regeneration of the Nasal Epithelium

A specific degeneration of neuroepithelium in the fish nose can be achieved by local administration of 3% $ZnSO_4$ (Cancalon 1982). Regeneration is completed in two weeks after lesion. During the regeneration, newly born ORN or precursors may take up plasmid DNA more efficiently. In addition, increased mitosis supplies more chances for the plasmid DNA to enter the cell nuclei. Thus the next approach took advantage of the chemically induced degeneration of nasal epithelium after 3% $ZnSO_4$-treatment, and the expression vector was applied during regeneration.

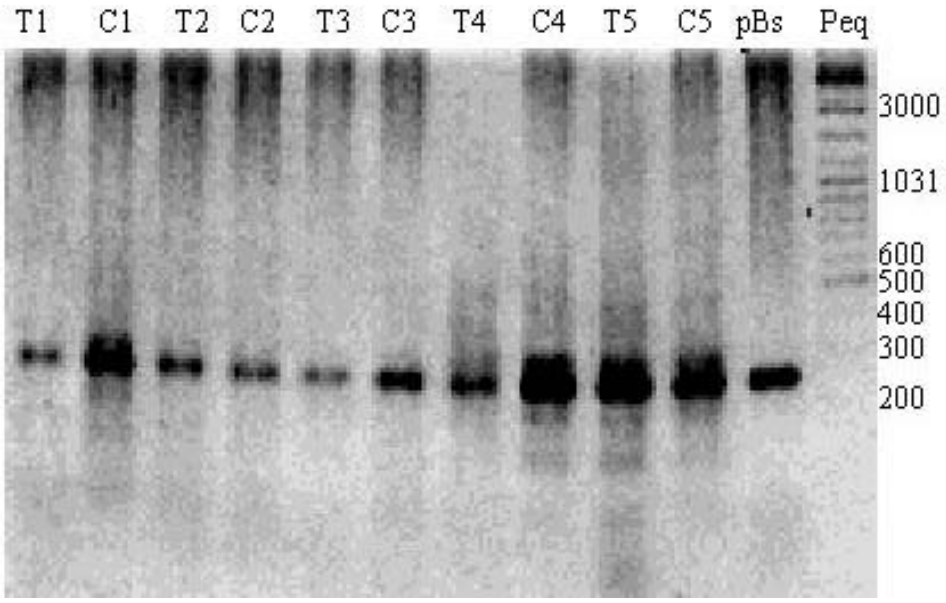

Figure IV.4. PCR Using Genomic DNA Extracted from Treated Side (T) or Control Side (C) Nose as Template.
Applied DNA, if incorporating in ORNs, shall give a band at 250 bp, as revealed by the band from control using the applied DNA (pBs) itself as a template. 1, 2, 3, 4, and 5: five fishes treated. Peq: DNA marker.

The time course of degeneration-regeneration in the zebrafish olfactory epithelium has been established by V. Oehlmann (doctorate thesis, 2001, Fig. IV.5). Axon terminals of ORNs in the olfactory bulb have been labeled by anterograde transport of DiI from the nose. Thus, the intensity of the fluorescence in the olfactory bulb is a direct measure of the destruction/ reconstruction of ORNs. From these results it is clear that the regeneration of neuroepithelium takes place in one week after administration of $ZnSO_4$. Hence the time chosen to load DNA was 1d, 3d, and 5d post $ZnSO_4$-treatment.

The expression vector pEGFP-N1, was administered into the nose either alone or together with one of the four transfectional reagents, Cellfectin, Lipofectin, DMRIE-C and LipofectAMINE. Fish were killed by decapitation at 4d or 7d after DNA loading, and whole mount epithelium was checked for green fluorescence under the fluorescein filter. Of the 43 fish treated, 40 fishes survived, but none of them showed any green fluorescence.

Thus administration of pEGFP-N1 during regeneration of nasal epithelium did not result in expression of GFP. It is conceivable that the mucus, which covers nasal epithelium, presents a mechanical barrier, which impedes DNA uptake drastically. This problem might be circumvented by application of DNA to the axonal terminal of ORNs in the olfactory bulb.

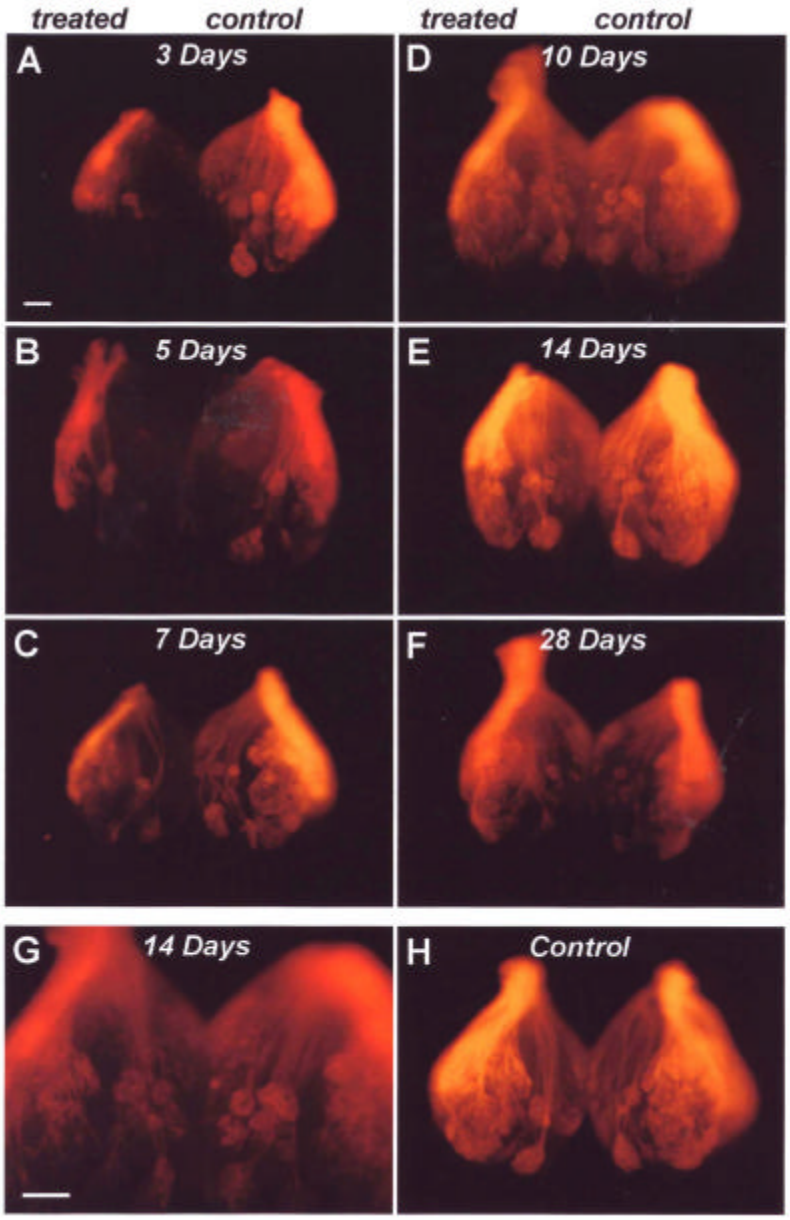

Figure IV.5. Pattern of DiI Staining in Olfactory Bulb after 3% $ZnSO_4$ Administration in the Nose.
A massive degeneration of ORNs was induced at 3 days after treatment, as shown by the strong reduction in DiI labeling in the tested side olfactory bulb. Regeneration was complete 10 days after lesion. Photograph kindly provided by V. Oehlmann (Doctorate thesis, 2001).
Scale bar: 100 µm.

1.4 Transfection by Injection of Plasmid DNA in Olfactory Bulb

One way of gene delivery in the nervous system takes advantage of the neuronal synapses. In the visual system, it was reported that injection of plasmid encoding reporter in the target area of retinal ganglion cells resulted in expression of the reporter in those neurons (Garcia-Valenzuela et al., 1998). This physical gene delivery method was adopted here. 1 µl of plasmid DNA (500 ng/ µ) in artificial cerebral-spinal fluid (ACSF) was injected into a small window opened in the dorsal scalp. Fish were decapitated 6 days after injection and epithelia proceeded for checking of GFP expression.

In initial experiments, 10 fishes were treated, of which 4 survived. Two epithelia showed GFP expression in whole mount analysis (Fig. IV.6, black arrrows). In epithelium 1, four of lamellae showed robust green fluorescence, while this was not seen in the control side epithelium of the same fish. The green fluorescence was seen in majority of the sensory epithelium may indicate that most axons have taken up plasmid DNA, presumably because of the injection at the initial site where all axons enter the bulb. In epithelium 2, two neighbouring lamellae showed robust green fluorescence, and the fluorescence was restricted to the inner 2/ 3 of the lamellae, the expected distribution for expression in ORNs. GFP may be retrieved to the two neighbouring lamellae because nerve fascicles corresponding to close lamellae have taken up the plasmid DNA. For technical reasons no further analysis of cryosection could be carried out in these two samples. Thus it was not possible to determine the cellular localization of the fluorescent structure. The green fluorescence seen in the nonsensory part of epithelium 2 (Fig. IV.6, white arrow) might be from ectopic expression.

After these promising initial experiments, 60 more fishes were treated. However, none of the 32 surviving fishes showed any green fluorescence. Thus injection of plasmid encoding GFP in the olfactory bulb resulted in expression of the reporter, probably in the ORNs of the nasal epithelium. However, the expression pattern is unpredictable, presumably because of the unpredictability of the exact locus of DNA injection - the whole olfactory bulb is only about 500 µm in diameter - and consequently the efficiency was very low. Therefore this method was not employed for gene delivery for functional expression studies.

1.5 Administration of Adenovirus Encoding EGFP

Adenovirus has been reported to infect mammalian neurons effectively. Adenovirus-mediated transfection of rat and mouse nasal neuroepithelium resulted in expression of reporter genes as well as functional odorant receptors in olfactory

receptor neurons (Ivic et al., 2000; Wim and Verhaagen 1998 and references therein; Zhao et al., 1998). To investigate the suitability of adenovirus-mediated gene transfer in zebrafish, an adenovirus containing a reporter gene was applied to the nasal epithelium.

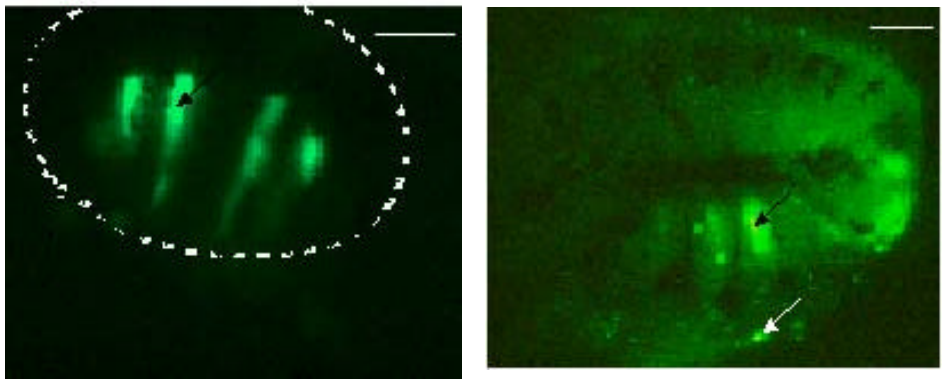

Figure IV.6. Epithelia 1 (left) and 2 (right): Two Epithelia from the Two Injected Fish that Showed Green Fluorescence under Whole Mount.
The green fluorescent, finger-like structure in epithelium 1 (black arrow, side view of the whole mount epithelium, dashed line indicates the outline of the nasal epithelium) are lamellae; in epithelium 2, two green fluorescent lamellae were visible.
Scale bar: 100 μm.

In this experiment adenovirus serotype 5, a self-replication-defective adenovirus encoding EGFP (Ad5-EGFP) was used as a vector (Kochanek et al., 1999 and references therein). The manipulation was done in the lab of Kochanek of Zentrum für Molekulare Medizin, Universität zu Köln, following the S2 lab instructions.

Virus was administered to the nasal epithelium of fishes under anaesthesia. Two to five μl Ad5-EGFP ($6x10^6$ PFU/ μl) was applied as several aliquots to the nose in a 20 - 30 minute time period. After treatment, fishes were killed at days 4, 7, or 14 and the reated epithelium was dissected. In whole mount analysis, no green fluorescence was seen in any of these fishes (Tab. IV.3). Cryosection was done in 6 fishes killed at different time after treatment. No fluorescence was seen after sectioning.

Table IV.3. GFP Expression in ORNs after Administration of Ad5-EGFP.

Days after treatment	Number of fluorescent epithelia / total number of fishes	
	Whole mount	15μm cryosection
4	0 / 5	0 / 2
7	0 / 5	0 / 2
10	0 / 6	0 / 2

Thus application of adenovirus Ad5-EGFP in zebrafish nose did not result in expression of GFP. The possible reason is that zebrafish is not a host for the adenovirus. It could not be excluded that the titer of virus may not high enough, however, this appears unlikely since the titer used was optimal for mouse ORN transfection, and when it is not optimal for zebrafish ORN, a less efficient GFP expression, rather than negative GFP expression, can be expected.

Most gene delivery methods tested in this experiment have been applied successfully for various mammalian neurons. Nevertheless, it was found that in zebrafish, the olfactory neuroepithelium is not amenable to traditional transfection methods. Thus the functional expression of OR in adult zebrafish ORN *in vivo* is not possible as long as no methods for efficient transfection in zebrafish ORN are available. Thus, as a next attempt, primary culture of ORNs was used as a candidate system for functional expression of ORs. Since not enough olfactory epithelium is available from zebrafish due to the small size of the organ and since the efficiency of obtaining and culturing dissociated ORN is very low in zebrafish (data not shown), I chose mouse ORN as model system. The dissociation and culture of mouse ORN is well-established (Calof et al., 1998 and references therein).

2. Establishing Primary Culture of Mouse Olfactory Epithelium

Published conditions for primary culture of dissociated mouse ORNs were followed (Calof et al. 1998 and references therein). Primary cultures of mouse olfactory epithelium obtained from new-born mice were established successfully and were kept for up to two weeks. Cultures were analysed daily using Nomarski microscopy. Results for cell morphology and cell number in primary culture are presented for day 2, 4 and 7. As the primary culture got older, non-neuronal cells gradually became the major population, although serum free medium was used to reduce proliferation.

2.1 Cell Type Identification by Morphology in the Primary Culture of Mouse Olfactory Epithelium

Epithelia from four to six new-born mice were dissected and dissociated as described (see III.6.6) using several mechanical and enzymatic steps. Dissociated ORNs were seeded onto poly-D-lysine coated 24-well culture dishes. Cell populations in the primary cultures up to two weeks were identified under light microscope. Dissociation of tissue was complete, aggregated cell masses were rarely visible. Sometimes, the cell suspension was filtered through 10 µm nylon mesh to enrich for ORNs, as ORNs are smaller in diameter than other cell types. This treatment enriched ORNs in primary culture, but did not ensure that cells in the primary culture are exclusively ORNs. The primary culture was always a mixed population with its component cell populations changing as cultures got older.

Immediately after dissociation, a few round, bright cells with distinct processes emanating from opposite sides of the cell, were seen. The processes, at this time, were often quite short, unless several of these cells were still in an agglomerate. These cells, with their small, round, bright cell bodies and short processes, were presumably a mixed population of both immature neuronal cells and mature ORNs whose axons had been broken during dissociation (Banker and Goslin, 1996). After one to two days in culture, cells adhered and grew on the bottom of the culture dish. At day 2, when most cells adhered well and started to grow in the culture dish, cells were sparsely dispersed on the dish surface. The bright, round bipolar cells, usually with short processes, were the major population (Fig.IV.7A, long black arrow). Sometimes mitosis of such cells was observed. These cells were referred to as INP (Immediate neuron precursor, see Calof et al., 1998), to distinguish them from non-neuronal cells and mature ORNs. Two other populations of cells were also present at this stage. One type was spindle-shaped cells, some with bright cell bodies but slender (Fig. IV.7B). The other type was flat and polygonal, with dark, big cell bodies. These were presumably fibroblastic cells (Fig IV.7B).

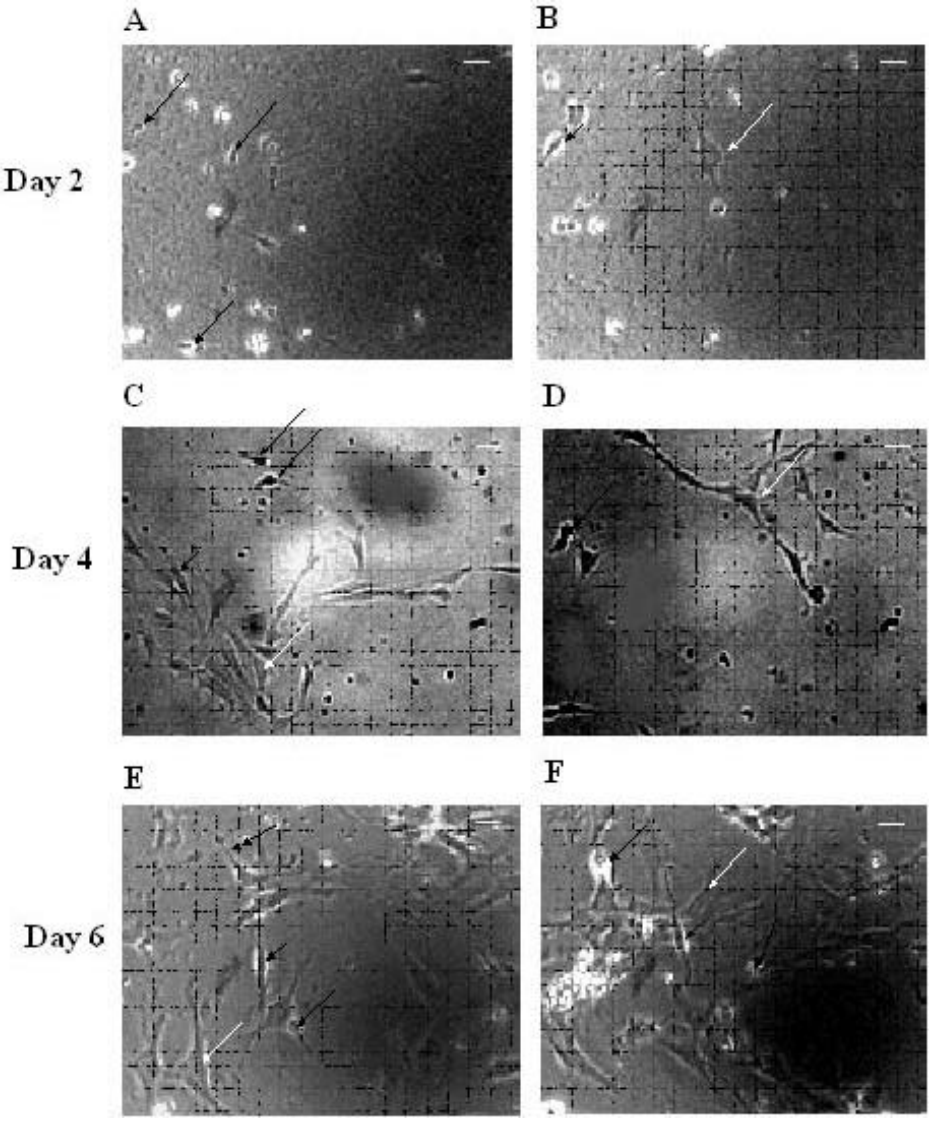

Figure IV.7. Cells in Primary Culture of Olfactory Epithelium from Newborn Mice.
From day 2 to day 6, cell numbers and cell composition in primary culture change. Shown are representative areas of the culture. Long black arrows for INPs, short black arrow for the spindle-shaped cells, and long white arrow for fibroblastic cells. Mature ORNs with long processes (long black arrow, double-headed) are rarely seen. Scale bar: 10 μm.

Cell density and composition varied from culture to culture. One possibility for variability is the range of development stages used. Since mice were checked for their labor twice a day, new-born encompasses a time difference of several hours after Neuronal cell differentiation and maturation is quite active at perinatal age (Murrell and Hunter, 1999 and references therein). In addition, non-neuronal cells tend to be a larger proportion of the whole population at later stages (Calof et al., 1998; for comparison see Gomez et al., 2000). Indeed, it was observed that for mice one day after birth, fibroblastic cells appeared as the major population in the primary culture already at day 2.

At day 4, neuronal cells were visible, but they constitute a minority of the total population. They were bright, small, round with short processes, dividing neuron-like cells were rarely seen (Fig. IV.7C). The other two populations of non-neuronal cells started to dominate the population (Fig. IV.7C and D). These cells grew larger cell bodies, and longer processes.

At day 7, the bright round ORNs were rarely seen. Very rarely, neuronal cells with long, slender, bipolar processes can been seen (Fig. IV.7E, long black arrow, double-headed). They were always in the immediate vicinity of other cells (Fig. IV.7E and F). Probably the other cell types could supply growth factors necessary for the survival of ORNs. Fibroblastic cells became dominant in the culture, intermingled with spindle-shaped cells. At this time, the cell number has massively increased and cells were densely grown, presumably because of the fast growth of fibroblastic cells. Thus it became difficult to distinguish single cells at this stage.

After 7 days, the cells grew even more dense, and fibroblastic cells remained the major component until 14 days, the longest period that cultures were kept (data not shown).

Judging from the morphology of cells existing in the primary culture of mouse olfactory epithelium, cell types and cell shape were reminiscent of those reported in the literatures (Calof et al., 1998; Gomez et al., 2000). The neuron like, spindle-shaped and fibroblastic cells were observed in other studies as well (Calof et al., 1998; Gomez et al., 2000).

Since the major purpose of primary culture in this experiment was to keep ORNs that might be utilized as an expression system, the interest here was the neuronal cells and their maturity. At day 2, neurons constitute the major population and at day 4, there were still a reasonable amount of neurons present, so that transfection of neurons at day 2 and analysis at day 4 would appear feasible. If this population of neuronal cells is both amenable to genetic manipulation and mature in function, they would fulfill the demands for being used as functional expression system. Therefore efforts were undertaken to determine the maturity of these cells, and to transfect them.

2.2 Maturity of ORN in Primary Culture of Olfactory Epithelium Was Determined Using OMP-GFP TransgenicMice

It is reported that the functional maturity of olfactory receptor neurons is hard to recapitulate *in vitro* (Gomez et al., 2000). To determine the maturity of ORNs in primary cultures, the expression of GFP was examined in cell culture derived from OMP-GFP transgenic mice (Mombaerts et al., 1996). In these mice, GFP is expressed in place of the endogenous olfactory marker protein (OMP) gene. Only mature ORNs express OMP (Walters et al., 1996). In the OMP-GFP transgenic mice, GFP expression is seen in all the mature ORNs *in vivo*, reminiscent of the expression pattern of that of OMP.

After the new-born transgenic mice were decapitated and their nasal epithelia dissected, the GFP fluorescence was very robust, as reported (Mombaerts et al., 1996). When pieces of olfactory epithelium were cut from the underlying connective tissues and checked under fluorescent microscope, GFP was strong in these pieces as well as in some clusters of two or three cells away from but attaching to the surrounding tissues (Fig. IV.8, arrow).

Surprisingly, when the pieces of olfactory epithelium were completely dissociated and a single cell suspension was seeded in the culture dish, no GFP+ cell was visible. In all five preparations, there was not a single cell visible under fluorescence stereomicroscope. Probably the dissociation procedure killed the mature ORNs expressing GFP by breaking their processes (Cunningham et al., 1999).

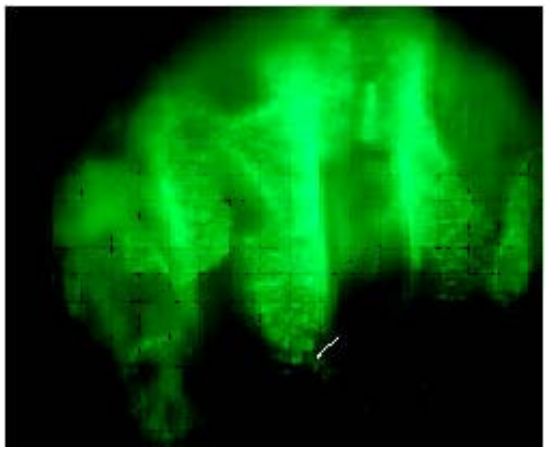

Figure IV.8. Freshly Prepared Olfactory Epithelium from OMP-GFP Transgenic Mice. GFP cells are overall seen in the epithelium as well as in some clusters of two or three cells away from but still attached to the surrounding tissues.

At day 3 and day 7 (Fig. IV.9), cell populations resembles those from the wild type mice. Round, bright, bipolar cells (Fig. IV.9) were present, with short processes, which were visually identified as neuronal cells. These cells, even in the rare cases where longer processes were visible (Fig. IV.10, long black arrowes, double-headed),

were always GFP negative. Except for the other two types of cells, namely, the spindle-shaped, slender cells (Fig. IV.9), and the flat and polygonal cells (Fig. IV.9), another type of glia-like cells with several thin processes are often encountered (Fig. IV.9). All these cells do not express GFP.

Therefore, there is no GFP positive cells in the primary culture of olfactory epithelium of OMP-GFP mice. It is possible that under *in vitro* conditions, the transcriptional factor(s) are not sufficient for the activation of OMP promoter and for a robust expression of GFP (Walters et al., 1996 and references therein; Kudrycki et al., 1993; Behrens et al., 1999; Behrens et al., 2000; Buiakova et al., 1994; Baumeister et al., 1999; Cunningham et al., 1999; see also discussion). Thus in contrast to what was expected, these transgenic mice are not more informative than the wild type to identify the maturity of ORNs, and it is not feasible to use these mice to determine the conditions to enrich ORNs in primary culture.

Although no OMP-GFP expressing neurons could be found in the culture, it is still conceivable that the morphologically identifiable neurons or INPs would be sufficiently mature to allow functional expression of ORs. Therefore, efforts were undertaken for the transfection of primary culture, using GFP as a reporter.

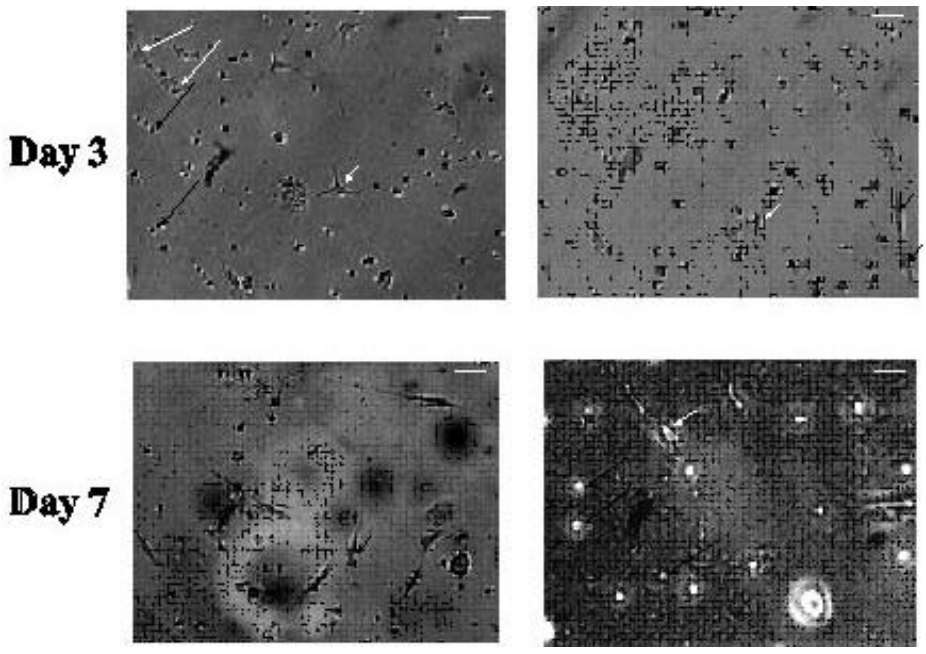

Figure IV.9. Cell Population in Day 3 and Day 7 Primary Culture of OMP-GFP Mouse Olfactory Epithelium.
Long black arrows for INPs, short black arrow for the spindle-shaped cells, long white arrow for fibroblastic cells, and the type of glia-like cells with several thin processes are indicated by short white arrows. Scale bar: 10 μm.

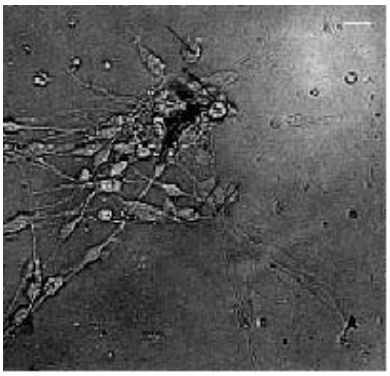

Figure IV.10. Neurons in 7 Day Primary Culture of OMP-GFP Mouse Olfactory Epithelium.
Rarely several neurons (long black arrow) are seen with long slender processes (long black arrow, double-headed), none of these is GFP positive. Scale bar: 10 μm.

2.3 Transfection of Primary Culture of Mouse Olfactory Epithelium

Although neurons are known to be refractory to genetic manipulation, some reports of successful transfection in primary culture of neurons or slice of mammalian brain with different methods are available (Banker and Goslin 1996 and references therein; see also discussion). Among these methods, calcium-phosphate precipitation was chosen since it is the classical chemically transfection methods for neurons and it is widely used.

Transfection of primary culture followed strictly protocols for neuron transfection (Banker and Goslin 1996 and references therein). Plasmid DNA was prepared from Endo-free maxiprep kit from Qiagen. All buffers used in this kit are made from endotoxin-free water, and an extra step in the preparation removes trace amount of endotoxin from the elute. Transfection incubation time is also known to be critical for neuron transfection. Usually the calcium-phosphate precipitate is left for 3-5 hours in cell lines for fully uptake of DNA, while in neuronal primary cultures, this incubation time ranges from 30 minutes to an hour, since longer exposure will destroy neurons. Indeed mortality was a problem in this experiment, whenever transfection cocktails were applied and left incubating with primary culture for more than two hours, all cells in the primary culture died.

Two days old primary culture were transfected with pEGFP-N1, and from 48 hours post-transfection on, GFP expression was analysed. At all time points analysed, between 48 hours and 72 hours post-transfection, GFP positive cells were seen. The transfectional efficiency was in general low, at most 5%, in 8 successful transfections. Most of the transfected cells were fibroblastic cells and the spindle-shaped cells (Fig.

IV.11). In some experiments the fibroblastic cells constituted the predominant transfected cell type, in others the spindle-shaped cells. These differences might be attributed to the slight difference in transfection incubation time, which was empirically judged by the size of precipitation particles. No effort was undertaken to determine the preferred transfection time for either of these two cell types. Sometimes glia-like cells (Fig. IV.11), another rare population, were seen transfected. In 8 successful transfections, less than 5 cells which were visually judged as INP, were green (Fig. IV.11).

At 72 hours post-transfection, cells were quite dense, so it was difficult to distinguish the type of cells transfected. However, under the microscope it could be seen that all the green cells that were individually discernable were non-neurons (Fig. IV.11).

Therefore, under the optimal transfection conditions when both primary cell viability and transfectional efficiency were considered, almost exclusively non-neuronal cells were transfected. Since such visually identified non-neurons never showed response to odorants (Gomez et al., 2000), they are unlikely to be useful for functional expression experiments. In very rare cases, INPs were seen to be transfected, but their numbers were less than 5% of transfected cells. Considering the low transfectional efficiency in primary culture, the low possibility of these transfected cells being neurons, and, the low excitability of neuronal cells in primary culture upon application of odors (presumably less than 10% in 5 days culture, Vargas and Lucero, 1999; Gomez et al., 2000; see also discussion), it is not feasible to use these transfected primary cultures for functional expression.

Taken together, the primary culture, although successfully established, can not be used as a candidate system for functional expression for ORs. Therefore in a third approach, a cell line, HEK293, which can be efficiently transfected, was investigated for its suitability as a functional expression system.

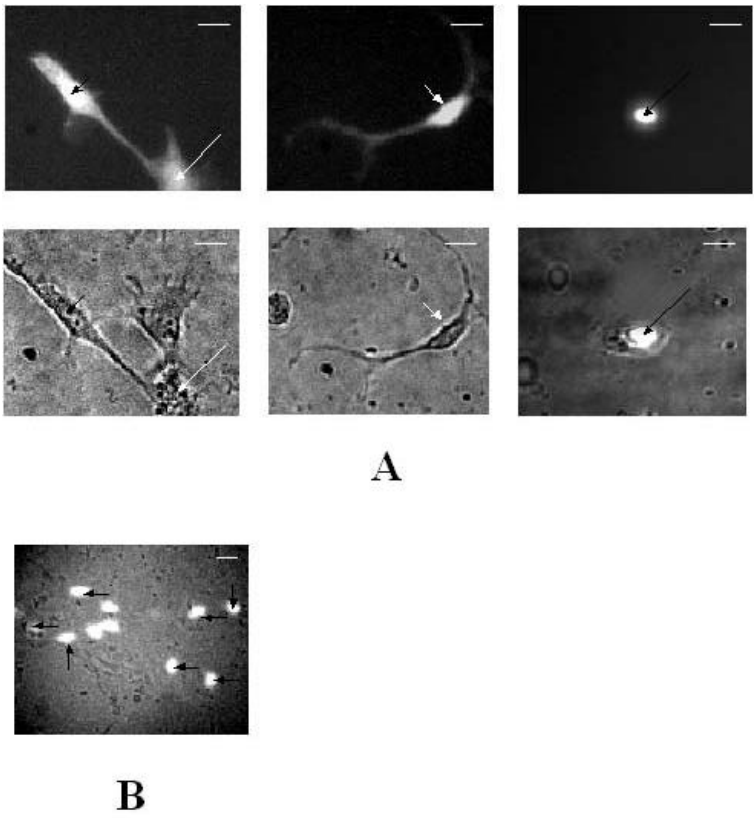

Figure IV.11. Different Cell Types in Primary Culture Expressing GFP after Transfection with pEGFP-N1.
In panel A, upper lane showes GFP expressing cells (50 h post-transfection) under fluorescein filter, lower lane are the same cells under normal light. Short black arrow for spindle-shaped cell, long white arrow for fibroblastic cell, short white arrow for glia-like cell, and long black arrow for INP.
Panel B shows several GFP expressing cells in an older culture (72 h post-transfection) where cells are densly grown.
Scale bar: 10 μm.

3. Functional Expression *in vitro* in HEK293 Cells

Heterologous expression of odorant recpetors in cell lines has encountered several problems, such as the incorrect membrane trafficking of receptor proteins, and signal transduction components to which ORs can couple are possibly missing. These problems can be overcome by: 1) using Rho-OR fusion proteins, where Rho- stands for rhodopsin N-terminal tag, empirically known to help membrane localization of fusion proteins in HEK293 cells; and 2) coexpression of Gα 15/ 16, known to promiscuously couple many GPCRs to the IP3 cascade. Increase of intracellular IP3 upon ligand binding of ORs will activate IP3 receptors located on the membrane of endoplasmic reticulum (ER), which results in the release of calcium from ER storage and thus an increase in cytoplasmic calcium concentration.

Fura-2 was employed as a calcium indicator. It is a fluorescent dye whose preferred excitation wavelength changes as calcium concentration changes. Optimal excitation wavelength for fura-2 is 340 nm when calcium is low, this excitation wavelength shifts to 380 nm when calcium concentration is high. In HEK293 cells stained with fura-2, and expressing Rho-OR and Gα 15/ 16, application of appropriate odorant ligands should activate the OR and subsequently the Gα, resulting in an increase of intracellular calcium, which is revealed as the increased ration of emission of F340 nm/ F380 nm (Fig. IV.12).

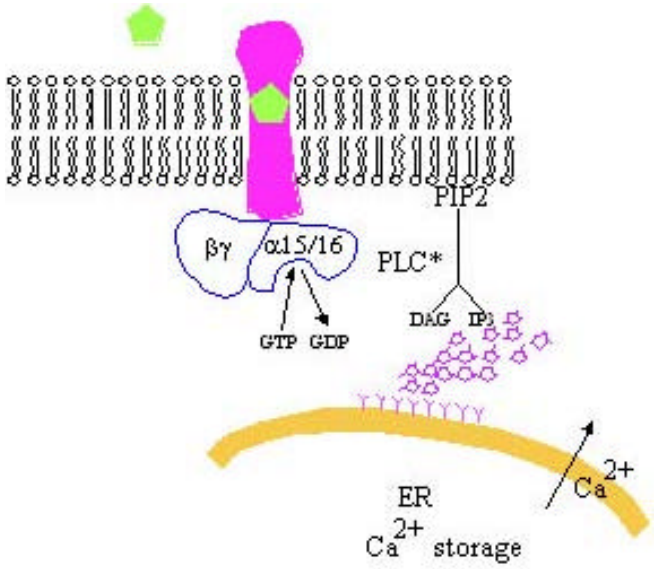

Figure IV.12. Schematic Illustration of How the HEK293 Functional Expression System Works.
Upon binding of OR to its ligand, activation of Gα 15/16 gives rise to hydrolysis of PIP2 by PLC, the subsequent increase in intracellular IP3 activates IP3 receptors on ER membrane, which leads to release of Ca^{2+} into cytoplasm.

3.1 Generation of pCMV-Rho-ZORs, Expression Constructs for HEK293 cells

The N-terminal sequence of rhodopsin is empirically known to help the membrane trafficking in HEK293 cells (Krautwurst et al., 1998). In this experiment, a rhodopsin N-terminal tag comprising 45 nucleotide 5' of *ATG* codon and extending 60 nucleotides downstream, provided by D. Krautwurst, was used as a leading sequence in the Rho-ZOR fusion protein.

PCR with primers introducing new restriction sites (*EcoR I* at 5' primer, and *Not I* at 3' primer) was employed to amplify ORFs of ZORs from the cloning vector pSPORT. PCR prodcuts were then digested by *EcoR I*/ *Not I*, purified and ligated to the *EcoR I*/ *Not* linearized pCMV-Rho. The fusion protein was constructed such that the ORF of ZOR was following in frame the rhodopsin N-terminal tag, leaving two additional amino acids (Glu/ Phe, from *EcoR I* restriction site, Fig. IV.13) in front of the start Met of ZOR. By this means, 5 expression vectors, pCMV-Rho-ZOR3A, pCMV-Rho-ZOR3C, pCMV-Rho-ZOR8C, pCMV-Rho-ZOR9A and pCMV-Rho-ZOR10A, were constructed. Expression vector of pCMV-Rho-ZOR5A was constructed differently, because ZOR5A has an internal *EcoR I* site at its 31st nucleotide. Digestion of ZOR5A directly from the backbone of pSPORT by *EcoR I*/ *Not* will thus ensure an in-frame ligation into pCMV-Rho.

All desired constructs were first picked out after restriction endonuclease digestion of minipreps, and reconfirmed by sequencing.

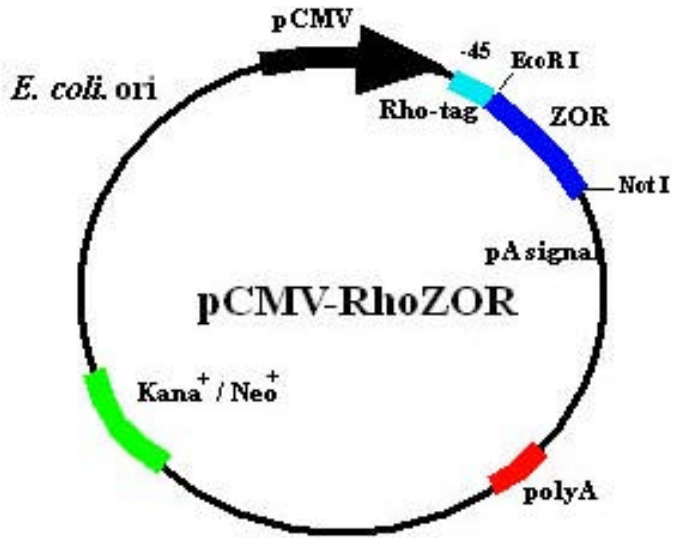

Figure IV.13. Expression Vector of pCMV-Rho-ZOR.
The expression vector of pCMV-Rho-ZOR includes a CMV-IE promoter, rhodopsin N-terminal leading sequence, ORF of ZOR, and a Kana/ Neo resistance cassettte, making it possible to select transfected cells.

3.2 Control Experiments Using the pCMV-Rho-I7 Construct

HEK293 cells were plated on poly-D-lysine coated glass coverslips, transfected with pCMV-Rho-I7 together with Gα 15/ 16, by LipofectAMINE-mediated transfection. B6-30, a monoclonal antibody against rhodopsin N-terminal amino acids 3-14, was used to analyse the transfection efficiency at protein level. Results of B6-30 antibody staining showed that part of the recombinant protein was localized to the periphery of the HEK293 cells (data not shown), as was reported (Krautwurst et al., 1998). Thus optical imaging was carried out on these pCMV-Rho-I7 transfected cells, where the ligand for I7 was known to be octanol.

Optical imaging were performed on 50 h post-transfected cells in 24-well dishes. 12 sec. after the recording started, stimuli were introduced, this 12 sec. delay kept constant for application of all stimuli except for acetylcholin, which was used as an indicator of the viability of cells and thus the response profile is known. In the I7 control experiments odorants stimuli (octanol and citronellal, 10 μM) were carried by flow of HEKIM and were pipeted carefully into the open, longitudinal well where the cells sit (see III.7.3). Carrier medium HEKIM was used as a control stimulus to which no response was seen (6 wells cells). When given 10 μM octanol, at 88 seconds after stimulation, several cells responded with an increase of intracellular Ca^{2+} (Fig. IV.14). The baseline before stimulation and at the areas devoid of cells during stimulation kept constant.

3.3 Immunohistochemical Detection of pCMV-Rho-ZOR5A Transfected HEK293 Cells by B6-30 Antibody Staining

HEK293 cells were plated on poly-D-lysine coated glass coverslips, transfected with pCMV-Rho-ZOR5A, alone or together with Gα 15/16, by either calcium-phosphate transfection or LipofectAMINE-mediated transfection. After transfection, cells were immunostained by B6-30. Immunostaining using Cy-3 coupled second antibody showed higher expression of recombinant protein at the periphery of the transfected cells (Fig. IV.15). Living cell immunostaining protocol, including immunostaining at 4°C and fixation as a final step (see 3.6.5), was used. Under these conditions, intracellular antigens are not expected to be stained strongly. The staining pattern appears as a mix of cytoplasmic and plasma membrane staining. Thus it is likely that a considerable amounts of Rho-ZOR have been translocated to the plasma membrrane. Therefore, such transfected cells were used to investigate the receptor-ligand pairing for some fish ORs.

Rho-ZOR expression was observed at 30 h post-transfection, the earliest time period analysed. Cells continue to express recombinant protein till 98 hours, the latest that was checked in the experiments. Judging from the pattern of immunostaining, the expression level reached maximal at around 50 hours post-transfection, and

transfection efficiency ranged from 20-50% in different transfections (data not shown). No apparent difference was found when the cells were transfected by either of the transfection methods, nor when cells were transfected by pCMV-Rho-ZOR alone or co-transfected with G proteins. Cells at about 50% confluence were chosen for transfection, when a high transfection efficiency, a high number of transfected cells, and a mono-layer growth at the time for optical imaging analysis were considered.

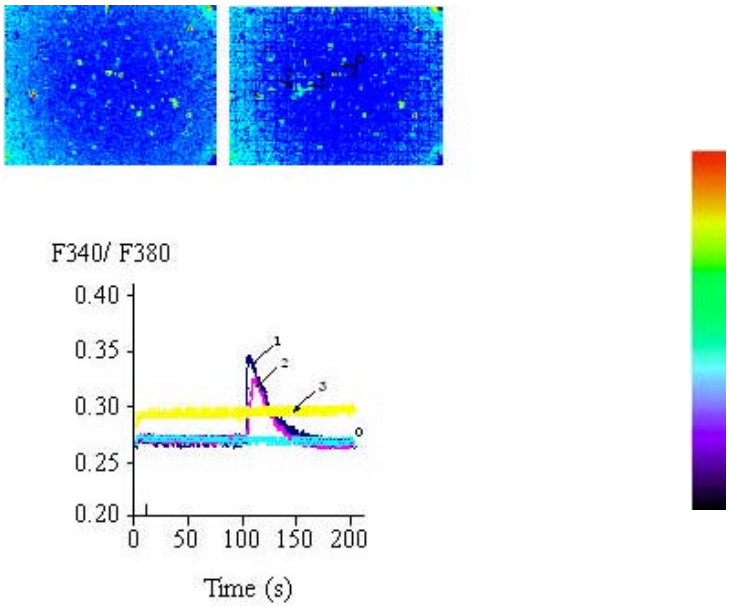

Figure IV.14. Response of I7-transfected HEK293 Cells to 10 mM octanol.
The visual field before stimulus (left) and at peak activity (right)was shown. Two responding cells (1 and 2), one non-responding cell (3) and one area devoid of cells (small circle) with their Ca^{2+} dynamics were shown. Small vertical bar at 12 sec. indicates the stimulation. Maximum/ minimum of scale bar: 0.45/ 0.17.

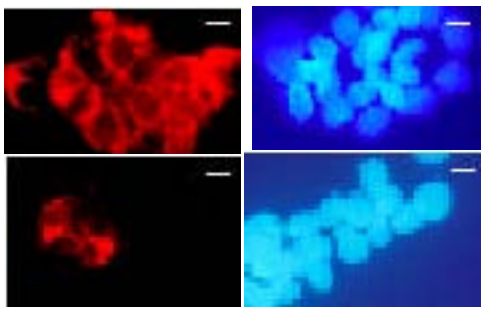

Figure IV.15. B6-30 Antibody Staining of HEK293 Cells Transfected with pCMV-Rho-ZOR5A.
Left: HEK293 cells stained with B6-30 antibody 50 h post-transfection, Cy-3 coupled 2nd antibody. Variability of transfection efficiency is seen in different experiments (top vs. bottom row).
Right: The same field of view, stained with DAPI.
Scale bar: 10 μm.

3.4 Optical Imaging of Calcium Levels in HEK293 Cells Transfected with ZORs

Optical imaging was carried out in HEK293 cells 50 h after transfection with Gα15/ Gα16 and different Rho-ZOR constructs respectively, to search for pairing of receptor-ligands, using fish odorants as stimuli.

Stimuli chosen for this experiment were those eliciting a response in electrophysiology experiments (Michel and Lubomudrov, 1995; Michel and Derbidge, 1997; Li et al., 1995; Hanson et al., 1998). They were also shown to elicit a calcium signal in presynaptic compartments in olfactory bulb of zebrafish once loaded in the nose, as shown by the work done by a former Ph.D. student in the lab (Friedrich and Korsching, 1998). These stimuli are classified as amino acids, bile acids, nucleotides prostaglandin (PG) and sexual steroids, which occur in the natural surrounding of fish.

3.4.1 *Nucleotides and moderate concentration of bile acids elicited Ca^{2+} response in non-transfected cells*

In the initial control experiment, nucleotides mixture, as well as the single compounds of this mixture, at concentration as low as 4 μM, were found to elicit a robust calcium increase in untransfected cells (6 wells tested). The calcium signal in non-transfected HEK293 cells elicited by nucleotides stimulation was rather complicated, sometimes composing several peaks (Fig. IV.16). It was reported that HEK293 cell has endogenous receptors for nucleotides, in the form of both GPCRs and ionotropic receptors (see discussion). The former works via G-proteins once activated, leading to the release of intracellular calcium store, whereas the later directly open ion channels leading to influx of extracellular calcium upon activation. Thus, depending on the different channels' status, one might expect variable Ca^{2+} peaks, as was observed here. Nucleotides were thus excluded from the panel of odor stimuli in the experiments done with transfected cells.

Mixture as well as simple compounds of two subsets of bile acids, namely, cholic and deoxycholic acids and their derivatives, were also shown to elicit a calcium change in the non-transfected cells, at concentrations as low as 2 μM, in the case of deoxycholic acid and its derivatives (data not shown), and at concentrations of 10 μM, in the case of cholic acid and its derivatives (Fig. IV.16).

Calcium change upon administration with bile acids may have resulted from their deteriorating effect for cytoplasmic membranes, since intracellular calcium does not come back to the original level, even with recording time as long as five minutes. In the following experiments, lower concentration (less than 1 μM) of these chemicals was used as stimuli, and these never elicited any Ca^{2+} elevation in the transfected as well as non-transfected HEK293 cells.

Thus, the odorant panel used for testing ZOR receptor-ligands pairing consisted of: amino acids, sexual steroids, prostaglandins and low concentrations of bile acids.

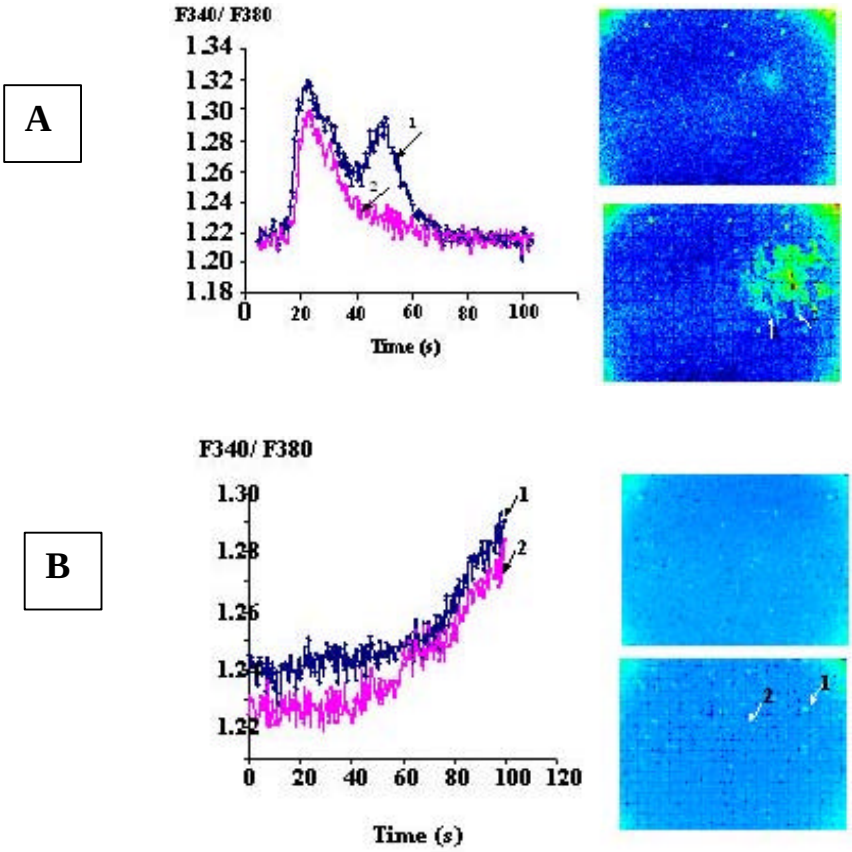

Figure IV.16. Responses of Non-transfected HEK293 Cells to Nucleotides and Some Bile Acids.
A: response to 4 μM ATP. B: to 10 μM mixture of cholic acid and its derivatives. Right panels showed F340/ F380 before stimulus (upper) and at peak activation (lower). In each case two responding cells were pointed out (arrow) and their responding profiles demonstrated. Maximum/ minimum for scale bar: 1.60/ 1.05 (A) and 2.80/ 1.00 for B.

3.4.2 *Variable Results for Stimulation with Amino Acids*

Concentration of amino acids in the growth medium for HEK293 cells are higher than that used as odorant stimuli (mM vs. μM). However, cells were deprived of amino acids for more than 1 h before experiments (see III.7.1), thus a response of ZOR transfected cells to amino acids can be expected. In initial experiments done with HEK293 cells transfected with Rho-ZOR5A and Gα proteins, rise of calcium upon stimulating with an amino acids mixture (A/ V/ G/ L, each 200 μM) was seen in one out of six wells of transfected cells (data not shown). In the same experiment using

another well of transfected cells to determine which compound(s) of the mixture led to the calcium rise, 10 µM alanine, as the second stimulus applied, was seen to elicit a calcium rise in several cells in the recording field (Fig. IV.17) in one of the four wells tested, while all the other compounds of the mixture did not elicit a response.

Encouraged by the initial results, more experiments were carried out for optical imaging upon odorant application in HEK293 cells transfected with Rho-ZOR5A and Gα proteins. Control experiments included cells transfected with either of the expression vectors, and non-transfected cells. Surprisingly, in the subsequent experiments, no calcium rise was seen in any of the transfected cells. This might result from poor transfectional efficiency. However, two out of the 12 wells with control cells, one transfected with only Gα15/16, as well as one non-transfected (Fig. IV.17), each in one out of six wells, showed calcium increase to the same stimulus.

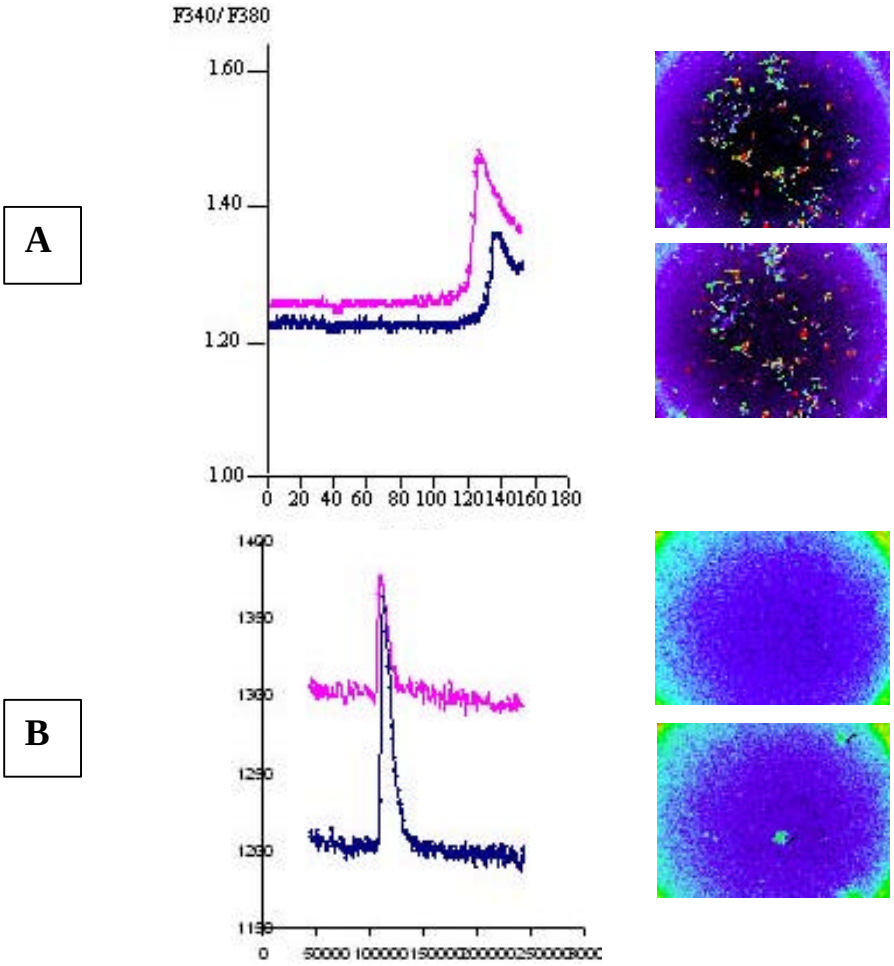

Figure IV.17. Apparently Specific Response of ZOR5A to 10 mM Ala Can Also Be Elicited in Non-transfected Cells.
A: responses of ZOR5A transfected cells to 10 µM Ala, B: similar responses to 10 µM Ala in non-transfected cells were seen. F340/ F380 at resting (upper) and activation (lower) are shown. The lower panel of dynamics has ms as unit for X-axis and 1000x F340/ F380 as unit for Y-axis, and for technichal reasons recording starts 50 s later than the data acquisation. Maximum/minimum for scale bar: 2.16/ 1.16 (A) and 1.87/ 1.06 (B).

With more cells checked, some of them being transfected by another Rho-ZOR construct (Rho-ZOR10A) and Gα proteins, some of them being transfected by only one of the expression vectors, some of them being non-transfected cells, similar phenomena were observed. Calcium increase was elicited occasionally in all kinds of cells upon application of different amino acids. Rho-ZOR10A/ Gα15/16 transfected HEK293 cells showed calcium elevation upon application of several different amino acids at higher concentration (10 mM), with different profiles. However, the same chemical also elicited calcium elevation in HEK293 cells transfected soly with Gα15/ 16 (Fig. IV.18).

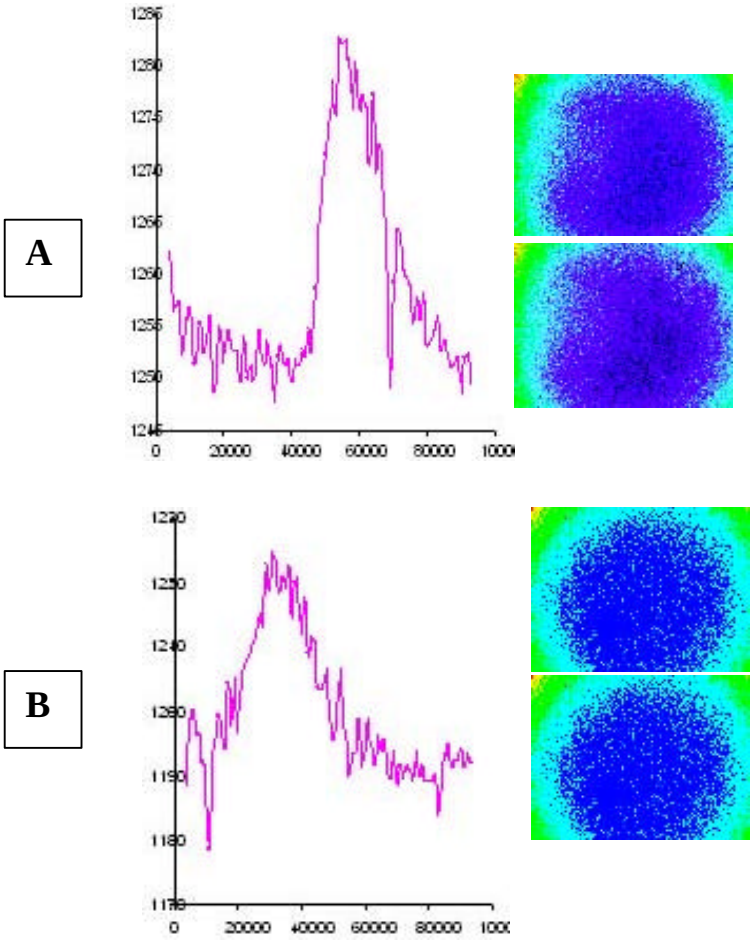

Figure IV.18. Apparently Specific Response of ZOR10A to 10 mM Asparrtic Acid Can Also Be Elicited in Soly Gα-transfected Cells.
A: representative responses of ZOR10A transfected cells to 10 mM aspartic acid. B: similar response to 10 mM aspartic acid in soly Gα-transfected cells. Both panels of dynamics have ms as unit for X-axis and 1000x F340/ F380 as unit for Y-axis. Cells at upper right in visual field of A and lower right of B were activated (see lower vs. upper picture) in both panels, same cells in A also responded to 10 mM valine (data not shown). Maximum/minimum for scale bar: 1.70/ 1.20 and 2.35/ 1.35.

No diffference in the frequency of calcium increase was seen in Rho-ZOR+/- cells, and no difference was found in the transfected/ non-transfected cells. The frequency of calcium increase was rather low, often took place one out every five to ten wells tested. In a positive well, about 5% of cells in a visual field responded, and the responding cells with a sharp, clear peak in Ca^{2+} dynamics were often clustered, whereas multiple, single cells usually responded with rather high noise and less steap slope(s) in Ca^{2+} dynamics. Responses to the same class of stimuli, namely, amino acids, had very different thresholds (alanine 10 μM, valine and aspartic acid 10 mM). The response onset after stimulus application can be very different, sometime a big latency was seen. The reason of such a heterogeneity of Ca^{2+} response is unknown. No response to sexual steroids, PGF2α or low concentration of bile acids was seen, although all the odorant stimuli were tested at same frequency.

Since no correlation between Rho-ZOR/ Gα transfection and calcium elevation upon application of specific chemicals was observed, and because of the low frequency of response, it is quite likely that the calcium increases seen in the experiments are an artefact.

HEK293 cell expression system is liable to show calcium elevation, as recently reported by another group working with mammalian OR functional expression (Kajiya et al., 2001; see also discussion). It appears that cells in a special growth and/ or metabolic stage may be particularly susceptible to Ca^{2+} peaks. Clear peak of calcium elevation always happened in clustered cells after a certain period after the experiments began. The artefact might also be caused by some manipulation which we were not able to determine.

However, it is difficult to evaluate this system until a real response of the receptor-expressing HEK293 cells upon stimulation by the proper ligand(s) is seen and compared with that of the non-transfected cells. In addition, this system could be improved by introducing a viable marker to visually distinguish the transfected and non-transfected cells. Once the transfected cells could be viewed and distinguished from the non-transfected visually, the cytoplasmic calcium dynamic profiles of these cells upon application of odors could be compared with those of non-transfected cells. The probability of finding a receptor/ ligand pair would be much higher under these circumstances.

Therefore, I next tested the feasibility to improve the HEK293 expression system by using GFP as a viable marker for transfected cells.

3.5 Introducing GFP as a Viable Marker for Transfected Cells

To test such a feasibility, HEK293 cells were first transfected with EGFP-N1. Cells expressing GFP were observed after transfection, and their calcium dynamics

upon stimulation with 10 µM acetylcholin were compared with those of the non-transfected cells in the same visual field.

3.5.1 *GFP Did Not Alter the Calcium Signal Measured by Fura-2*

HEK293 cells were transfected with pEGFP-N1. After 48 hours these cells were analysed. Expression of GFP was robust (Fig. IV.19). When F340/ F380 was displayed, the GFP+ cells appeared dark in the false colour display, this might be because the emission wavelength of fura-2 at 340 nm excitation is more preferred for the absorption by GFP than that from 380 nm excitation. Nevertheless, a dramatic calcium elevation upon 10 µM acetylcholine stimulation is observed (Fig. IV.19) in both transfected and non-transfected cells. Two cells that expressed GFP and were dark in F340/ F380 display, were chosen to compare their calcium dynamics with that of a non-transfected cell in the neighbourhood. The respond in these transfected cells, upon stimulation with 10 µM acetylcholin, is as dramatic as that of the non-transfected cells. Therefore, though the absolute value of F340/ F380 in GFP+ cells is lower, the change of value upon activation is as nearly high.

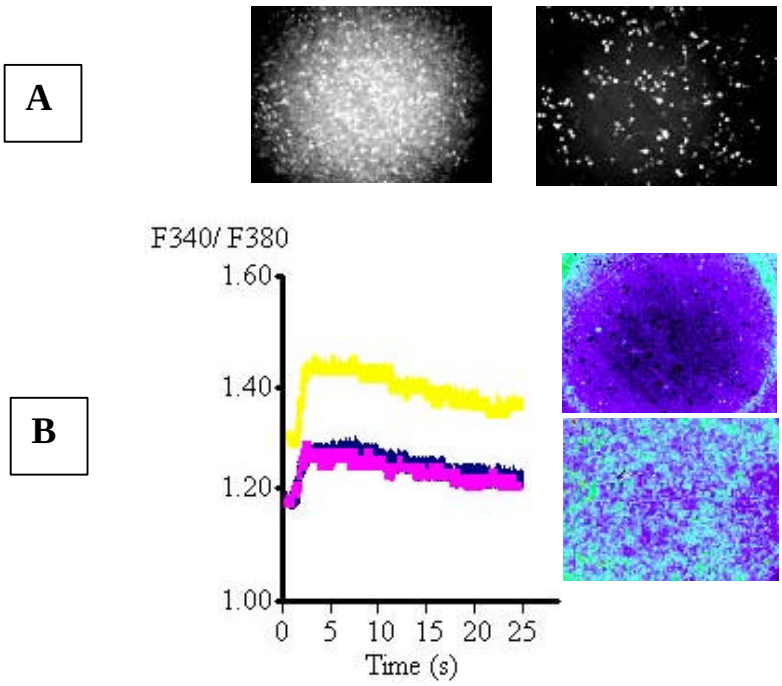

Figure IV.19. GFP+ cells showed similar response profiles to 10 mM acetylcholin as that of the non-transfected cells, as measured by fura-2.
A: GFP+ cells (right) were visible under both fura-2 (380 nm, left) and fluorescein filters.
B: When response profiles of two transfected cells (white arrrow) were compared with that of the non-transfected (black arrow, yellow in dynamics), they appeared similar.

Therefore, although GFP expressing cells absorb the emission light from fura-2, they showed similar patterns of calcium change upon activation by 10 μM acetylcholin, as measure by fura-2.

3.5.2 *GFP Expression from the Second Cistron is Suitable to Serve as a Viable Marker of Transfection in HEK293 cells*

GFP does not alter a dramatic calcium increase in HEK293 cells upon stimulation with acetylcholin, thus it can be used as a viable marker for transfected cell. However, if GFFP is introduced via a separate plasmid, a cell successfully transfected by the GFP plasmid is not necessarily transfected by another plasmid of cotransfection. Thus a GFP+ cell does not necessarily express Rho-ZORs. To overcome this problem, a bicistronic expression vector, with GFP as the second cistron, was used to transfected HEK293 cells, to test whether GFP expression driven by IRES is sufficient to function as a marker for transfected cells.

Transfection in HEK293 cells was carried out with pCMV-ZOR5A-IRES-EGFP (see IV.1.2.1), where GFP translation is realized by the 5'-prime cap free translation by IRES sequences, and for construct convenience, the first cistron was ZOR5A (without rhodopsin tag, and thus ZOR5A not expected to be functional in HEK293 cells). After transfection, it is observed that though GFP, as the second cistron, is weakly expressed, it is sufficient to let transfected cells be distinguished from non-transfected cells (Fig. IV.20).

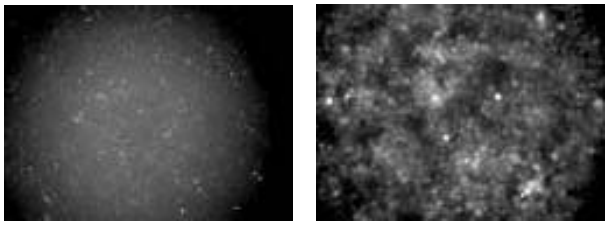

Figure IV.20. GFP Expression as the Second Cistron is Visible.
Left picture was taken under fluoroscein filter, right was taken under fura-2 filter (380 nm).

Unlike the situation when GFP expression is driven by CMV-promoter, in the transfection by bicistronic expression vector, when GFP is the second cistron, the GFP expression level is low, though sufficient. GFP+ cells did not appear dark under the F340/ F380 display, presumably because less light emitted by fura-2 is absorbed by GFP. The Ca^{2+} dynamics of GFP+ cells is similar to that of the non-transfected cells when stimulated with 10 μM acetylcholin (Fig. IV.21).

With these feasibility tests, it turned out that although GFP is fluorescent and its

excitation wavelength overlaps with the emission wavelength of fura-2, GFP expressing cells showed the similar calcium signal revealed by fura-2 measurement. In addition, as the second cistron driven by IRES, GFP positive cells could be discerned to identify a transfected cell. Thus, the HEK293 cell functional expression system, though it being labile to give calcium elevation under some circumstances which is difficult to contrrol, would be feasible for functional expression of ZORs, when the transfected cell incorporating ZORs can be distinguished from the non-transfected cells.

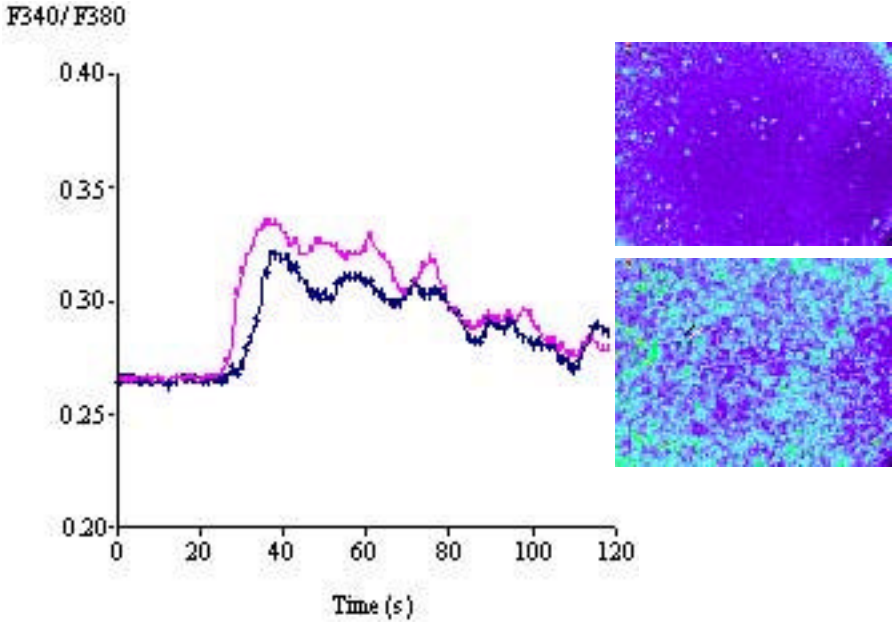

Figure IV.21. Weak GFP Expression Does Not Alter fura-2 Signals Elicited by 10 mM Acetylcholin.
A GFP expressing cell (white arrow) which could be discerned under microscopy has the very similar responding profiles when activated, compared to that of a non-transfected cell (black arrow, blue line in dynamics).

V. DISCUSSION

To find out ligands for zebrafish ORs, in this work, several functional expression systems have been searched and tested. However, none of them was completely satisfactory. In the following part, various approaches, together with their shortcomings and potential advantages will be discussed.

1. Attempts at Transfection of Zebrafish ORNs *in vivo*

The first approach for functional expression of zebrafish ORs was to overexpress cDNAs encoding ZOR proteins in adult olfactory epithelium *in vivo*. In principle, ORNs are advantageous since they themselves would be the most capable cells for expression, targeting, and coupling of odorant receptors. Under this assumption, adult zebrafish nasal epithelium was chosen as a target organ for transfection and several gene delivery methods were employed.

A successful gene delivery leading to the expression of recombinant protein in the target tissue requires: the amenability of genetic materials (here DNA) to the target cell membrane; the escape of DNA particles from the degradation by intracellular endosome-lysosome compartment; the access of DNA into the nucleus (Guy et al., 1995 and references therein). It is in general demanding to transfect neurons. The first two above-mentioned problems exist for all cell types while the third is extremely problematic for neurons since mitosis do not happen, leaving transfer of DNA into nucleus more difficult. Therefore, many transfection methods which work satisfactory in other cell types (e.g., calcium-precipitation, particle bombardment) are not reported reproducibly successful in transfection of neurons.

Even though several methods for gene delivery into neurons have been reported successfully, these include the virus-mediated gene transfer and microinjection of DNA into axon terminals, which were tested in this work as well.

1.1 Transfection by Adenovirus Did Not Result in Reporter Gene Expression in ORNs

Why Virus-mediated Gene Delivery

Viral vectors are becoming increasingly important tools to investigate the function of neural proteins and to explore the feasibility of gene therapy to treat diseases of nervous systems. This gene transfer technology is based on the use of a virus as a gene delivery vehicle. The principles of the use of viral vectors for genetic manipulation of the nervous system is based on the essential requirements that the

viral vector should be able to transfer non-dividing cells, excluding the use of classical retrovirus which demands a high degree of cell dividing in the target tissue.

To date, several viral vector systems are available for gene transfer in the nervous system. These include replication-defective adenovirus (adenovirus serotype 2 or 5, Karpati et al., 1996) and herpes simplex virus (HSV 1, Fink et al., 1996) to deliver genes to neurons both *in vivo* and *in vitro*. Other vectors, such as the adeno-associated virus (AAV) and Semliki forest virus (SFV), have also been developed to target genes to postmitotic cells (Karpati et al., 1996; Fink et al., 1996).

Why Adenovirus

Viral vectors can be applied to transfer genes to somatic, post-mitotic cells of fully developed animals. To date, among several viral vector systems, adenovirus is proven to effectively transfect rat and mouse ORNs (Holtmatt et al., 1996; Holtmatt et al., 1997; Ivic et al., 2000; Zhao et al., 1998). Initially found as natural host for airway epithelium, the 36 kb adenovirus does enjoy a broad range of potential hosts, including a wide variety of proliferating and terminally differentiated cells. The first demonstration that recombinant adenovirus could serve as an effective gene delivery system in the nervous system was in 1993 (La Salle et al., 1993). A recombinant adenovirus carrying the lacZ gene was shown to be able to target β-glycosidase expression to sympathetic neurons and glial cells *in vitro* and to macroglia, astrocytes and neurons *in vivo*.

Adenovirus binds to an as yet unidentified cell surface receptor through an interaction between the Ad fiber proteins, after internalization, the viral nucleocapsid is released from endosomes in the cytoplasm, and transported to the nucleus (Hermens and Verhaagen 1998). The membrane receptor system for the virus is still poorly understood, but shall be widely spread in its hosts since little cell type specificity is observed in the transfection of the hosts' central nervous system.

Possible Reasons for No GFP Expression in ORNs after Transfection Using Adenovirus

In this attempt, a self-replication defective adenovirus-derived expression vector, Ad5-EGFP, was administered in the adult zebrafish nose. There was no GFP expression in the nose after this treatment. It is possible that adenovirus has a narrow host spectrum and zebrafish is not among its hosts.

Adenovirus has been reported to successfully transfect central nervous system in rat and mouse, which are its non-permissive hosts. It can also infect human as its permissive host. There is no report of adenovirus being successfully used for transfection of any species except for rat, mouse, and human. In addition, in cotton rat, infection of adenovirus is reported to have much more deteriorating effects than that in the other species because of strong immune response (Rosenfeld et al., 1993).

However, this strong immune response is unlikely to be the reason of negative GFP expression since the epithelium after adenovirus application appeared normal.

From this work, it seems that adenovirus have a rather strict host species-specificity. It is quite probable that because zebrafish is not the host of adenovirus at all that no GFP was seen after administration of Ad in the nose. Several other possible critical factors affecting GFP expression are: dosage and titer of the adenovirus used for transfection; time window for GFP expression and the application time during transfection. Dosage of virus administrated in this experiment was scaled down from that of the dosage optimal for mouse nose infection (Holtmaat et al., 1996; Holtmaat et al., 1997); time point to check GFP expression was also referred to that reported for mouse nasal epithelium (Holtmaat et al., 1996; Holtmaat et al., 1997), such that it shall ensure enough time for recombinant protein expression - if there is any in zebrafish; administration time of Ad was up to 45 minutes, a quarter more than that used for irrigation in mouse nose (Holtmaat et al., 1996; Holtmaat et al., 1997). And these three factors, even if not optimal, shall affect the expression quantitatively but not qualitatively.

1.2 Transfection of ORNs by Retrograde Transport Was Occasionally Successful

Although physically gene transfer by microinjection of DNA is not widely used, reports indicated the possibility of using local injection to transfer genes into neurons (Lin et al., 1990; Acsadi et al., 1991). The presumed reason is that neuronal processes, particularly axons, which terminate at a considerable distance from perikarya, can take up transgenes. Thus transgenic constructs that are deposited at appropriate sites can be taken up by the axonal terminals and transported to perikarya, where transcription and translation take place (Lin et al., 1990; Acsadi et al., 1991). For example, transgenic constructs injected into motor-innervation zones of muscles are eventually transported into perikarya of motor neurons (Lin et al., 1990; Acsadi et al., 1991), where they are expressed. Even in the CNS, one case of successful physically gene transfer was reported (Garcia-Valenzuela et al., 1997), where the authors showed that application of high concentrations of plasmid DNA carrying reporter genes onto the proximal stump of axontomized retinal ganglion cells, or to their axon terminals in the superior colloculus, resulted in retrograde transport to the soma and in expression of the foreign genes. Addition of poly-L-lysine did not increase expression levels while did prolong the expression period, presumably it serves as a nuclear import signal, protects DNA from nuclease degradation and/ or facilitating its entry through specialized pores into the nucleus. The authors also noted that expression in individual cells did not reach the levels achieved by viral gene delivery methods (Garcia-Valenzuela et al., 1997).

In this work, the approach of microinjection of plasmid DNA in olfactory bulb came from the work of Garcia-Valenzuela et al. (1997). Here, administration of

plasmid DNA into olfactory bulb gave two green fluorescent epithelia under whole mount, out of 70 fish treated. In one of these two epithelia, green fluorescence is seen in most of lamellae while in another epithelium it is only seen in two neighbouring lamella. Though no cryosectioning was possible, there is reason to believe that the green fluorescence seen under whole mount is from the injected plasmid. First, the green fluorescence, especially that seen in the second epithelium, was not overall in the nose, but rather focal, in two neighbouring lamella, reminiscent of the converging pattern of fascicles of axons, and is in accordance with that of the pattern of retrograde-transported dye distribution after dye (Cell Tracke Blue CMAC) injection in the olfactory bulb (data not shown). Second, with all the epithelia checked so far (more than 100), there is never autofluorescence seen as intense to be comparable to the intensity of GFP shown in these two samples.

However, gene delivery by injection of plasmid DNA in the olfactory bulb was not adopted because of its low efficiency. The source of low efficiency is probably the unpredictability of exact locus of injection and the nerve bundles being labile to break in manipulation. It is critical, using this method, to inject plasmid DNA at the axon terminals in olfactory bulb, which means the superficial layer at antereolateral part of the olfactory bulb. The olfactory bulb in zebrafish is in the very vicinity of the ethmoid bone, where the nerve traverses and enters the olfactory bulb. In a small fish with very tender nerve bundle traversing through the tight hole of the ethmoid bone, a slightly heavier manipulation *in vivo* at this locus, or a slight movement of the fish during manipulation, would therefore cause the break of the nerve bundles. Such a possibility was confirmed by dye CMAC injection in this locus, when more than half of the treated fish showed breaking of nerve bundles (data not shown).

Thus, transfection of ORNs by microinjection of the transgene DNA in olfactory bulb is not a suitable method, since it did not result in the expression of transgene reliably.

1.3 Chemically Aided Transfection of ORNs Did Not Result in Reporter Gene Expression

In this work, access of DNA into ORNs by different means is reasoned. The possibility of DNA uptake by axon terminals, as well as directly from soma and cilia of ORNs are tested. Attempts for transfection of ORNs using chemically aided gene delivery included: administration of plasmid DNA with 0.1% Triton X-100, of plasmid DNA with transfectional reagents (Cellfectin, LipofectAMINE, Lipofectin, DMRIE-C), and chemically aided transfection after 3% $ZnSO_4$-mediated degeneration of ORNs; the reporter chosen is GFP, all of these methods did not work effectively.

Plasmid DNA with 0.1% Triton X-100

0.1% Triton-X 100 is known to specifically cause ablation of olfactory cilia and microvilla of zebrafish nose, followed by regeneration finished in 48 hours (Friedrich and Korsching 1997). This reagent is used successfully to help load an anionic dye of Calcium Green-dextran (Friedrich and Korsching 1997) into the nasal epithelium. Guided by this observation, plasmid DNA encoding reporter of GFP, or fluorescein-labeled plasmid DNA, was applied into the nose together with 0.1% Triton-X 100, there was no reporter gene expression by this gene delivery method, neither was fluorescence seen after application of fluorescent plasmid DNA, indicating that there was little DNA uptake, probably because of the thick mucus layer which lends possible penetration by dye but not DNA for its bigger size.

Plasmid DNA with Transfectional Reagents

Transfectional reagents are known to facilitate transfection *in vivo* and *in vitro*, though not as a routine transfection method for neuronal primary cultures, they were reported to work in mouse and rat brain *in vivo*. Formula of transfectional reagents used in this experiment is:

Lipofectin: DOTMA/ DOPE
LipofectAMINE: DOSPA/ DOPE
CELLFECTIN: M-TPS/ DOPE
DMRIE-C: DMRIE/ Cholesterol;

these are content of a transfectional reagent selection kit from Qiagen, which includes most of the commercially available and widely used transfectional reagent formulas.

However, plasmid DNA encoding GFP, or fluorescein-labeled plasmid, when applied with either of the four transfectional reagents, did not result in visible green fluorescent cells in the nose, indicating that there was little DNA uptake.

Since the DNA/ transfectional reagent ratio was always determined empirically, it is also possible that with the tested ratios, none of them is optimal for zebrafish ORNs. However, the ratio tried was suggested by the supplier and was thus widely used, and one would assume that in case that DNA/ transfectional reagent ratio was not optimal, a few fluorescent cells could be expected, but not little uptake at all. Another possibility, despite that the DNA/ transfectional reagent being quantitatively improper, is that the transfectional reagent used does not fit the targeted cell type. Support for this came from the observation that Lipofectin^{TM}, as the most extensively used of all commercially available liposome formulations, has a much less transfection efficiency in the same cell types when compared with a more recently developed transfectional reagent, DC-Chol, from the same lab developing both of them (Gao and Huang, 1995; Yang et al., 1997). Since the extensive studies on transfectional reagents were carried out for mammalian cells, these transfectional reagents might not be optimal for zebrafish ORNs.

Chemically Aided Transfection during Regeneration of ORNs

Supposing that when a small portion of DNA did enter the cells by endocytosis, lacking of dividing events in these postmitotic neurons lends no amenability of nucleus membrane to plasmid DNA, which might be a major reason for the failure in transfection. To test this hypothesis, 3% $ZnSO_4$ was administered in the fish nose, which leads to a massive degeneration followed by the regeneration of ORNs (Cancalon 1982), and transfection was attempted during the early regeneration time, under the assumption that a large population of progenitor cells would proliferate after degeneration, during which time plasmid DNA would have chances to enter the nucleus. However, this trial failed, indicating that little plasmid DNA had access to the ORNs, possibly because of the mucus and the basal localisation of the proliferating cells.

It is known that for non-viral gene transfer, one technical challenge is that DNA (commonly a plasmid) is a particulate material with a net negative charge, a negatively charged surface, and a hydrodynamic diameter of >100 nm. This particle must be introduced into the body and delivered to the target cell across various biological barriers, many of which are normally impenetrable by a particle with these characteristics. For example, because of its size, DNA can not effectively cross intact barriers represented by endothelium, keratinised epithelium, or blood-brain-barrier, which has porosity <100 nm. Thus it is possible that by chemically aided transfection, these chemicals do help dye to enter cells after dye diffuses through mucus and barriers covering the compacted pseudoepithelium but they fail to facilitate the effective cross of DNA through these membranes, because of the size of DNA. This was supported by the administration of fluorescence-labelled plasmid DNA into nose with Triton-X 100 or transfectional reagents, which resulted in no detectable fluorescence immediately after treatment, though the 1: 10^3 diluted loading cocktail is still visible.

Since using adult zebrafish nose as a target for overexpression of ZORs failed, the next attempt has turned to using the primary culture of mouse olfactory epithelium, as primary culture of neurons always retain some feature of the same cells *in vivo*, but it shall be more amenable to genetic manipulation.

ORNs are good candidates for primary culture, since an additional novel characteristic of mature olfactory neurons, that is distinct from virtually all other differentiated neurons, is their ability to be replaced in a process of continuous turnover throughout adult life. The globose basal cells undergo mitosis and subsequent migration in the apical direction and progressive differentiation to mature olfactory neurons, replacing those that degenerate and die. This process is associated with the induction of olfactory neuron-specific gene expression (Calof et al., 1998 and references therein).

Taking advantage of this feature of ORNs, primary culture was characterized and its feasibility of being used as a functional expression system for ORs was investigated. Primary culture was perforrmed using mice as a model animal, because

the method herein (and in general primary culture methods for mammalian cells) is well documented; because of the advantage of the availability of a transgenic mouse - the OMP- GFP mouse, where mature ORNs appear green fluorescent, and last but not least, because of the abundance of starting material using mice versus zebrafish.

2. Attempts for Functional Expression of Zebrafish OR in Primary Culture of ORN

2.1 Three Different Cell Types Present in Primary Culture

To investigate the suitability of primary culture for transfection of ORN, primary cultutre of mouse olfactory epithelium was established. Newborn mouse olfactory epithelium was dissociated and cultured as described by the lab of Calof (Mumm et al., 1998). Other labs have published methods for primary culture of dissociated ORNs (Murrel et al., 1996; Vannelli et al., 1995), but Calof's lab has given in most detailed protocols and there the culture conditions are optimised for neuron survival and growth (serum-free medium with neuron-specific additives). The authors have described the excistence of four types of cells (Fig. V.1) in the culture: A) medium-round cells (neuroblasts); B) round, small, bipolar phase-bright cells with neuritis (NCAM positive, presumably neuronal cells); C) spindle-shape cells (non-neuronal); and D) polygonal, flat big cells (fibroblastic-like).

In this work all four cell types were observed, however, progenitor cells were observed only once in 20 preparations. In this work newborn mice were used whereas Mumm et al. (1998) used embryonic mice (E14-15). Presumably the number of progenitor cells decreases with continuing development of the olfactory epithelium. In addition, culture in serum-free medium – as was done here to increase the percentage of neurons – is not optimal for progenitor cells (Banker and Goslin, 1996).

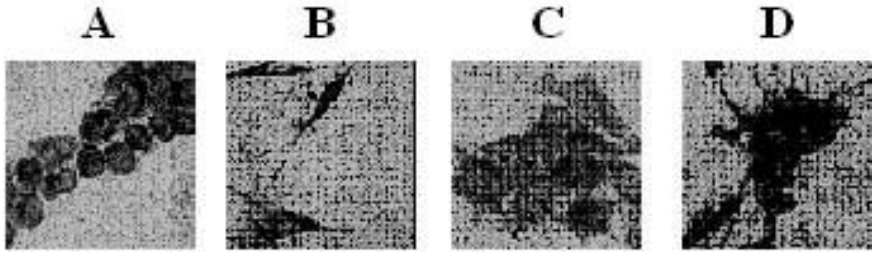

Figure V.1. Four Cell Types in Primary Culture of Mouse Olfactory Epithelium (taken from Mumm et al., 1998).

In this work all four cell types were observed, however, progenitor cells were observed only once in 20 preparations. In this work newborn mice were used whereas Mumm et al. (1998) used embryonic mice (E14-15). Presumably the number of progenitor cells decreases with continuing development of the olfactory epithelium. In addition, culture in serum-free medium – as was done here to increase the percentage of neurons – is not optimal for progenitor cells (Banker and Goslin, 1996).

Round, small, phase-bright cells were observed in every preparations and a minority of them had neuritis. By comparison with the work done by Mumm et al. (1998), these are supposed to be neuronal cells. Gomez et al. (2000) has shown by optical imaging that these cells can respond to odors, in contrast to the cells with other morphologies in the culture.

The results of my primary culture revealed that using newborn mice the cell population, cell morphology in primary culture of olfactory epithelium was similar to that documented, and the small, round, phase-bright cells are probably functional ORNs. To confirm that these cells visually identified as ORNs are the olfactory receptor neurons, I took advantage of a transgenic mouse line, in which the sequences encoding OMP are replaced by GFP (OMP-GFP mice, Mombaerts et al., 1996). In these mice, all the mature ORNs show green fluorescence, which otherwise express OMP. Thus the primary culture was repeated using the OMP-GFP mice.

No GFP Positive Neurons Were Observed in the Primary Culture Using OMP-GFP Mice

Primary culture from newborn OMP-GFP mice showed all the previously morphologically identified cells types, including neurons. However, none of these cells expressed GFP, including those cells visually identified as neurons. This was a surprising result. It can not be excluded that the OMP null genotype of the OMP-GFP mice may interfere with the expression from the OMP promoter, but this appears not very likely because *in vivo* the OMP promoter drives the expression of GFP.

It is believed that expression of OMP is a key characteristic of the mature olfactory receptor neuron phenotype. This 19 kd protein is phylogenetically conserved and expressed in olfactory neurons of vertebrate species from fish to humans. In primary culture, some authors have claimed that they could obtain abundant OMP-positive ORNs in primary culture (Feron et al., 1999) after stress-induction. However, a more detailed analysis showed only a few OMP-positive cells might be present in primary culture (Cunningham et al., 1999), there the authors also suggested that these are mature ORNs at time of dissection, and as being disrupted from their natural environment, as well as suffering from axonal damage or axotomy during the procedure, they follow a path of decline and senescence *in vivo* (Cunningham et al., 1999).

In this work, probably full mature, green fluorescent ORNs have a low survival probability due to axotomy and other damages during dissociation of the epithelium. As in the primary culture the cell/ cell interactions of the intact epithelium are absent,

and growth or other factory secreted by neighbouring cells may be too much diluted in the culture, less mature ORN and their progenitor cells may miss some influences necessary for full maturation under the conditions of primary culture. Indeed, as suggested by Calof et al. (1998), maturation may be impaired in the primary culture, and most of the neuronal like cells seen in the culture, namely, cells with small, round, phase-bright cell bodies, are actually not mature neurons, but INPs (Immediate Neuron Precursors), and these precursors remains INPs, until they die without being mature, under the *in vitro* conditions.

OMP Promoter May Not Be Activated in Primary Culture

It is also possible that the OMP promoter may not be activated in primary culture. Since in these mice, the GFP substitutes OMP, the complete regulatory region of OMP is available for the GFP expression. Looking closely at the OMP-promoter, the full 300 bp upstream contains an olf-1 binding motif. Olf-1 is a transcriptional factor that is important for olfactory-specific gene expression (Walters et al., 1996 and references therein; Behrens et al., 1999). The 5'-flanking regions of OMP, Golf, ACIII, and oCNC-1 genes all contain several similar sequence motifs, one of them being the Olf-1 site (Kudrycki et al., 1993) which binds to Olf-1/ EBF-like transcriptional factors (Behrens et al., 2000). Olf-1/ EBF-like proteins are indicated predominantly expressed in neurons in distinct brain regions, retina, and olfactory epithelium. Olf-1 is localized to the nuclei of immature and mature olfactory neurons (Behrens et al., 1999).

Another sequence motif common to OMP, ACIII, and oCNGC-1 is similar to binding sites for NFI (Nuclear factor I, Buiakova et al., 1994; Baumeister et al., 1999). It is demonstrated (Baumeister et al. 1999) that members of the NFI family of transcription factors are present in olfactory neurons and that they bind specific sites in promoters of olfactory specific genes. *In situ* hybridization results have revealed the presence of transcripts of four NFI genes, A, B, X and C in rat nasal mucosa, with the most abundant expression observed in nasal glandular tissue, the site of active protein synthesis and secretion (Baumeister et al., 1999).

Therefore, it is possible that without the natural surrounding which could supply enough transcriptional factors, OMP-promoter was not activated. However, Olf-1 may not be the essential factor here because mice with a partial deletion of this gene appeared to have normal olfactory-specific gene expression. However, it is conceivable that under culture conditions, not enough Olf-1 and/ or NFI are made in the ORNs to allow visible expression of GFP.

In vitro Culture Conditions and Null-OMP Might Also Effect the Maturity of ORNs

Function of OMP is largely unknown, the OMP-null mice have a reduced ability to respond to odor stimuli but are otherwise similar to that of the wild type (Ivic et al.,

2000 and references therein). The possibility of involvement of OMP itself for the maturity of ORNs is not excluded. OMP is reported to have mitogenic activity *in vitro*, while any other function of it is not documented.

Thus, lacking of sufficient transcriptional factors to robustly activate the OMP promoter, and probably because of many factors necessary for maturation of ORNs are missing, resulted in no visible GFP expressing cells in the primary culture of olfactory epithelium from OMP-GFP transgenic mice. In contrast to what was expected, primary culture from the transgenic mice is not more informative than that from the wild type mice.

Nevrethless, even partially mature neurons may be suitable for functional expression of OR if an efficient transfection method can be established. Thus effort was given for efficient transfection in primary culture of olfactory epithelium from newborn wild type mice.

2.3 Neuron-like Cells in Primary Culture of Mouse Olfactory Epithelium Are Refractory to Transfection

To transfect the neuron-like cells in primary culture of olfactory epithelium, calcium phosphate precipitation was chosen since it has been the most successful of the “fast and easy” transfection methods for neurons. Transfection efficiencies up to 20% and consistently in the range of 0.5% to 3% have been reported for neurons (Banker and Goslin, 1996; Wang et al., 1997), however, other groups have reported very poor transfection efficieny (20 cells/ dish, Watanabe et al., 1999). Neurons that have been transfected by the calcium phosphate method include cultured chick or rat neurons from cortex, hippocampus, spinal cord, dorsal root ganglion, and retina. In this work, optimal or optimised values were chosen for three parameters for transfection, the quality of the DNA, the time of transfection and the maturity of the cell culture.

It was found that the quality of DNA is critical. Plasmid DNA prepared from conventional kits did not work whereas endotoxin-free DNA prepared with Qiagen Endo-free kit resulted in albeit variable, transfection of cultured cells, similar results have been reported by Köhrmann et al. (1999). Consistent with recommendations from literatures it was found that the incubation time for transfection is critical, prolonged incubation resulted in bigger particles in the precipitation and drastically impaired viability, whereas shorter incubation periods (up to 1 hr) resulted in a “fine sandy” precipitate and good viability. Transfection by calcium precipitation is only reported to work after 2 days in culture (Craig 1994 and references therein). Therefore consider the population change in the culture, 3 days after plating was chosen for the time of transfection.

With all the reported conditions optimised, the transfection efficiency was still very low (less than 3%), as revealed by GFP reporter gene expression. Among them,

only few cells showed neuronal morphology. Transfection efficiency for neuron-like cells appeared not to be very different from that for no-neuronal cells.

Nevertheless, the combined low abundance of neurons in the culture and the low overall transfection efficiency make it impractical to use these cultures for functional expression of OR. Moreover, maximally 10% of neurons will respond to odors after a few days in culture (Gomez et al., 2000). Thus the primary culture appears not suitable for finding the pairing of a receptor with its ligand out of the large ligand repertoire .

Therefore, in a third approach, a heterologous expression system, the HEK293 cells (Krautwurst et al., 1998; Kajiya et al., 2001), was employed for functional expression of zebrafish ORs.

3. Functional Expression of ZOR in HEK 293 cells

3.1 Crosstalk of Endogenous Signalling System with Transfected Receptor/ G Proteins May Modify Ca^{2+} Responses

HEK293 cells express several ionotropic receptors and GPCRs, though these receptors are not directly related to the chemicals used as stimuli in the experiments. There might be crosstalks between several G proteins (Gαi, Gαq, Gαo, Gαi, which subsequently, through the activation of their Gβγ subunits) elicited by these receptors, which are rather complicated. Overexpression of proteins is known to alter the response profile in HEK293 by coupling of GPCRs to one subtype of Gα protein to another, as shown with overexpression of β2-adrenergic receptor, which is able to activate endogenous ERKs (Extracellular Signal-regulated Kinases) via Gαi/ Gαo-independent pathway, depending on the amount of β2-AR expressed (Schmitt and Stork, 2000). In the present experiment, the overexpression of Gα15/ 16 might make the situation of GPCRs' coupling to Gα protein more complicated.

It is also reported that in HEK293 cells, receptor-induced Ca^{2+} comprise at least two independent components - rapid Ca^{2+} release from Ca^{2+} stores in the ER and Ca^{2+} entry through slowly activating PM (plasma membrane) store-operated channels (SOCs), the trigger for SOC activation being decreased Ca^{2+} in the ER lumen (Putney and Bird, 1993). Thus, the HEK293 cell is probably very complicated with regard to its endogenous receptor expression, its G protein, and its intracellular Ca^{2+} dynamics. This system, is probably not as simple as a “test tube” where only the Gα protein and OR incorporated by overexpression would interact each other.

3.2 Some Fish Odorants Can Not Be Tested in HEK293 cells Because Endogenous Receptors Are Present

HEK293 cells express some endogenous receptors which respond to some of the fish odors.

Panel of Fish Odors that Can Be Used as Stimuli

HEK293 cells are commonly used to examine signalling pathways downstream of transfected receptors. The employment of this expression system in the experiment was initiated from the success of a few mammalian odorant-receptor pairings using these cells (Krautwurst et al. 1998). Whereas it is unusual that these mammalian odorants, being they air-borne, hydrophobic, small compounds, are present in the natural surrounding of HEK293 cells, neither are the receptors for these odors expressed as endogenous receptors of these cells, the situation with that of fishes is more complicated. As is mentioned, the well-defined fish odors are amino acids, bile acids, nucleotides, prostaglandins and sexual steroids, all of which are water-soluble and exist as components of metabolites or hormones in the blood stream.

HEK293 cells are derived from human embryonic kidney cells and it is thus not surprising that these cells might express, or might have the potential to express, endogenous receptor for chemicals of fish odors. In the initial experiments to sort out the panel of fish odors that can be used as stimuli for ZOR transfected HEK293 cells, some fish odors were excluded since they elicit calcium elevation when applied in the non-transfected HEK293 cells. Among them are nucleotides and some of bile acids.

Nucleotides

HEK293 cells express two kinds of purinergic receptors: P2x and P2y, the former being ionotropic receptor (ligand-gated ion channel), which, upon binding of ligand (ATP), open the gate of channel and cause extracellular calcium influx (Haines et al. 2001 and references therein); the later is activated by either ADP (P2y1) or ATP (P2y2) and subsequently cause an increase in intracellular calcium via the release from ER storage (Gq coupled pathway) (Mundell and Benovic 2000 and references therein). With both of ionotropic receptors and GPCRs, calcium respond upon nucleotides application is rather complicated, as revealed by the results. Thus, nucleotides were excluded from the panel for stimuli as odors in the following experiments.

Bile Acids

Some bile acids elicited calcium elevation upon application in the HEK293 cells (see results), and calcium responding differed from that with nucleotides. Here intracellular calcium did not come back to the pre-stimulation level, even when

recording time was as long as 5 minutes, indicating a non-reversible membrane deterioration. The control experiments done with non-transfected cells exclude the use of high concentration of bile acids as odorant stimuli.

Thus, panel of fish odors that can be applied as stimuli in the ZOR-transfected HEK293 cells did not include the whole spectrum of candidate fish odors.

3.3 Ca^{2+} Levels in HEK293 Cells Are Regulated Partially by Ill-defined Influences

Variable Ca^{2+} Responses May Be Elicited by Stress Related Signal or Depend on Growth Status of the Cells

Some amino acids and sexual seroids elicited Ca^{2+} elevation in HEK293 cells. Responses to some amino acids (including alanine, valine and aspartic acid) were seen in non-transfected cells. Non-odor, non-ZOR-transfection related responses were seen often, though not exclusively, in clustered cells. It is likely that the calcium increase seen in the experiments were caused by a special physiological status which several, but not all cells, in a visual field, possessed. There is no direct documentation about the expression of amino acid transport systems in HEK293 cell membranes, while several families of amino acid transporters or their activators were already found endogenously expressed in Xenopus oocytes. One of which is an anion exchanger, band 3, which may also transport glycine and taurine under some important physiological conditions such as hypoosmotic stress. Another family of transporters for cationic amino acids was found also serving as a viral receptor. These or other transporters were proposed to conceivably function in eggs as receptor for sperm and, more broadly, in cell-cell interactions as well as in amino acid transport (Van Winkle 1993). Thus, certain amino acid transporters might exist and be upregulated under certain circumstances in HEK293 cells.

Several sexual steroids were tested but no response was seen in transfected cells. However, once a response of non-transfected cells to PGF2α application was seen once (data not shown), which might be an outcome of stress of the cells, and shall be excluded, though the cells respond to 10 μM acetylcholin, indicating that they are physiologically healthy.

Ca^{2+} Levels in HEK293 Cells Are Regulated Partially by Ill-defined influences

In addition, the mechanical properties of eukaryotic cells are complex (Wang and Ingber, 1994). Forces applied to a cell are distributed over many components including the bilayer and cytoskeleton, with the later distributing forces within the cell cortex and as deep as nucleus. The cytoskeliton consists of many proteins that are dynamically linked and capable of causing complex dynamic responses to an applied force (Akinlaya and Sachs, 1998). In this experiment, the stimuli were loaded through

manually application or electrically controlled valve, no difference was seen by using these two means of stimuli loading. Though not likely, the possibility these Ca^{2+} increase being caused by mechanical stress during the loading of stimuli could not be excluded.

Limitation of HEK293 Cell as Functional Expression System

As described, the HEK293 expression system is not as ideal and reliable as a "test tube". These cells were liable to show calcium elevation, probably under some unknown physical, metabolically or pathological circumstances. Moreover, the more control experiments done with the Rho-I7 transfected cells showed that there was also a dramatic Ca^{2+} elevation upon application of citronellal, which is not a ligand of I7. However, probably one should not give up this expression system, until a real response of the receptor transfected HEK293 cells to the stimulation by the proper ligand(s) is seen and compared with that of the non-transfected cells. In addition, it is possible that ligands were missing with the experiments done, regarding that the major work was done with ZOR5A, composing approximately 1% of the OR repertoire of zebrafish, and the panel of odor stimuli in the experiment did not cover the complete spectrum of fish odors.

So far, no response to low concentration of bile acids was seen in the HEK293 cells transfected by Gα15/ 16 and Rho-ZORs. It is possible that these receptors used to construct expression vectors, are not the receptors for bile acids, it might also be that cells expressing receptors were missed in the visual field upon application of bile acids. With the experiments done so far, there was no response seen to some amino acids. Since a cystine transporter, xCT, was cloned and overexpressed in HEK293 cells to study the pharmacological profiles of this transporter, at least there is evidence that some amino acids would be paired to their receptors using this system (Ahih and Murphy, 2001).

The problem came up with these artefacts shall be testable by having a viable marker of the transfected cells and to compare the Ca^{2+} profile of these transfected cells versus those non-transfected.

3.4 Receptor/ ligand Pairing Might Be Feasible Once a Viable Marker for Transfected Cell Is Available

The feasibility of using GFP as a viable marker was tested in HEK293 cells, plasmid DNA encoding GFP weas used to transfect HEK293 cells. Responses of transfected and non-transfected cells to 10 µM acetylcholin were compared, and it turned out that GFP did not alter the signal measured by fura-2. This is in accordance with the work done by Billing-Marczak et al. (1999), which led to the conclusion that GFP is compatible with fura-2 measurements of intracellular Ca^{2+}. In their work, a fusion protein of calretinin-GFP is used to transfect glioma C6-cells, upon stimulation with

ionomycin, cells containing GFP were found to have the same fura-2 fluorescence change as untransfected cells. The observation of my work is thus in agreement with theirs. When the construct of pEGFP-N1, encoding GFP under a robust and ubiquitous promoter of pCMV-IE, was used to transfect HEK293 cells, GFP+ cells can be discerned. When the Ca^{2+}in the transfected cells was compared with that in the non-transfected cells, a similar pattern was seen.

As a next step, a bicistronic expression vector, where GFP expression is driven by IRES, was used to transfect HEK293 cells. IRES sequence ensures both cistrons being translated from the same transcript. GFP expression was much weaker as the second cistron, than that with the situation of the monocistronic vector, but it is possible to discern the GFP+ cells from that of the non-transfected, and the Ca^{2+} profile of these cells upon activation by 10 μM acetylcholin is very similar to that of the non-transfected cells. Thus it is feasible to have GFP and Rho-ZOR in the same plasmid to transfect HEK293 cells, and the transfected cells which incorporate Rho-ZOR will also appear GFP positive.

Therefore, GFP did not alter the dramatic Ca^{2+} signal caused by 10 μM acetylcholin, as measured by fura-2. So it is possible to discern the transfected cells, which were transfected by the construct incorporating ZOR as the first cistron and GFP as the second, from the non-transfected cells, and to compare Ca^{2+}signal in these two cells upon odorant stimulation. Use of such a system will reliably exclude the Ca^{2+}signals that are not related to transfection. Thus, ligand-receptor pairing can be accomplished in such a system.

3.5 Future outlook

From the work done so far, one can say that functional expression of fish ORs is a complex task which could not be solved satisfactory so far. However, recent advances have opened another prospect for functional expression of ORs. HEK293 cells expression system appeared not satisfactory for fish OR functional study, as lots of fish odors shall not be served as stimuli in HEK293 cells, although improvement of this system for functional expression, including the use of a bicistronic expression vector that will give rise for all the cells expressing OR also express a viable marker, is possible. Moreover, with the development of a constitutive Ca^{2+} indicator - the permutated, Ca^{2+} sensitive version of GFP - pericams, it is possible to directly monitor the Ca^{2+} change via the constitutively expressed pericams, which simultaneously serve as a vital marker for transfection.

Viral vectors have been and are extensively studied. Two very recent reports revealed the possibility of using olfactory bulb line variant mouse hepatitis virus to transfect the olfactory system (Schwob et al., 2001; Youngentob et al., 2001). However, the present study of this field is extensively done for mammalian, being its goal of gene therapy. Once a virus specifically infect fish olfactory epithelium is found and its viral vector available, an attractive functional expression system of fish ORs would be to use the adult nasal epithelium itself for target organ of overexpression, being it the natural surrounding for these ORs, and being it not a

system that one is vexed by possible functional development change if these ORs are constitutively expressed in the development - a scenario in the transgenic animal.

When *Odora* cells becomes more amenable to genetic manipulatione, this ORN-derived cell line may also become an attractive candidate model to study function of ORs. Since these cells are derived from neurons, the problem encountered in the HEK293 cells (endogenous receptor, fish odor being biogenic for these cells), might not be a problem to these neuronal cells, and they could well be suited for functional study of fish ORs.

The very recent work of cloning of zebrafish OMP gene, done by Çelik and Fuss in our lab, has turned it possible for a transgenic zebrafish line where an OR gene is overexpressed in a robust and tissue-specific way under the OMP promoter. These fish could become an ideal model to identify ligands.

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VII. ABBREVIATIONS

A	Adenosine
AAV	adeno-associated virus
AC	adenylate cyclase
AON	anterior olfactory nucleus
bp	base pairs
C	cytosine
cDNA	complementary DNA
2-DG	2-deoxyglucose
DIG	digoxigenin
DNA	desoxynucleic acid
dNTP	desoxynucleotide phosphate
DL	dorsolateral bundle
DMEM	Dulbecco's modified Eagle medium
DOPE	dioleoylphosphatidylethanolamine
DOTMA	1,2-dioleyoloxypropyl-3-trimethyl ammonium bromide
EEG	electro-encephalogram
EOG	electro-olfactogram
EtOH	ethanol
Fig.	figure
G	granule cell
G	guanine
GABA	γ-aminobutaric acid
GFP	green fluorescent protein
GM	growth medium
GPCR	G-protein coupled receptors
h	hour
HSV	herpes simplex virus
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
M/ T	mitral/ tufted cell
MOT	medial olfactory tracts
μ	micro
n	nano
NMDA	N-methyl-D-asparate
OB	olfactory bulb
OBP	odorant-binding protein
OCNC	olfactory cyclic nucleotide channel
OCNG	olfactory cyclic nucleotide gated channel
OE	olfactory epithelium
OMP	olfactory marrker prrotein
OR	odoraant receptor
ORN	olfactory receptor neuron
pF	postfertilization
PG	periglomerular interneuron
PG	Prostaglandin
POA	preoptic area
SFV	Semliki forest virus
T	thymidine
7TM	seven-transmembrane
UV	ultravoilet
VM	ventromedial bundle
ZOR	zebrafish odorant receptor gene

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