Effects of retinoic acid in
the mouse olfactory sensory systems

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Front cover picture:
A heterogenously innervated glomerulus in an OMP-dnRAR transgenic mouse, with odorant receptor staining in green and omp staining in red. Blue cells are periglomerular cells visualized by nuclear staining.

Back cover picture:
Hjalmar (postnatal day 671) experiencing the advantages of a functional olfactory system
Ju mer man tänker, ju mer inser man att det inte finns något enkelt svar
/Nalle Puh (A.A Milne)
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ABSTRACT

A common characteristic in neurodegenerative diseases of the brain is death of specific neuronal populations. The lack of neuron proliferation and axon extension in most parts of the central nervous system leads to chronic loss of neurons in the case of injury or disease. Therefore it is essential to identify signals involved in neurogenesis and neuronal survival. A favorable model in which to study these events is the olfactory sensory neurons in the main olfactory epithelium and their target in the glomeruli of the olfactory bulb.

In spite of constant regeneration, each olfactory sensory neuron maintain expression of one particular odorant receptor and the specificity of their axonal projections to the glomeruli. Most mammals also have an accessory olfactory system consisting of the sensory neurons in the vomeronasal epithelium and their target area the accessory olfactory bulb. Differential expression of receptors and other genes divides the olfactory and vomeronasal epithelium into zones, but the function and mechanisms underlying the establishment of these zones are still elusive.

We identified four genes with graded expression patterns that correlated with the zones of the olfactory epithelium. One of the identified genes encodes a retinoic acid synthesizing enzyme, RALDH-2. We showed that RALDH-2 was expressed in a gradient in cells of the lamina propria underneath the olfactory epithelium, suggesting a possible retinoic acid regulation of zonally expressed genes in the olfactory epithelium.

To investigate the role of retinoic acid in the olfactory systems, we generated a transgenic mouse strain that selectively expressed a dominant negative retinoic acid receptor in mature olfactory and vomeronasal neurons. We found that subsequent to the establishment of axonal projections, the neurons of both olfactory systems died prematurely by retrograde caspase-3 activation. In the main olfactory system the onset of apoptosis was associated with the appearance of incorrect heterogeneous glomeruli with axons of more than one OR identity. Additionally, the activity regulated cell adhesion molecule kirrel-2 was down regulated suggesting an additional regulation of this gene by retinoic acid. Deficient retinoic acid signaling in olfactory sensory neurons could thus induce apoptosis by changing the parameters for axonal competition by neural activity and kirrel-2 expression.

We found evidence for a selective neuronal death in the accessory olfactory system of the dnRAR mice, where only vomeronasal sensory neurons belonging to the basal zone died by retrograde caspase-3 activation. This implies that the two populations of sensory neurons in the vomeronasal epithelium differently depend on retinoic acid for their survival.
SVENSK SAMMANFATTNING

Sjukdomar och skador på det vuxna centrala nervsystemet (CNS), som utgörs av hjärna och ryggmärg, är idag ofta obotliga. Detta beror på att nervceller i ett vuxet CNS i princip inte nybildas, och att det är svårt för skadade nervcellers utskott (axoner) att växa tillbaka och kontakta sina korrekta målceller. För att hitta sätt att bota, lindra eller förebygga skador och sjukdomar som drabbar CNS, är det viktigt att ta reda på vilka signaler som är krävs för en nervcells överlevnad och dess förmåga att skicka ut axoner till rätt målområde. Hjärnan är svår att studera p.g.a att dess nervceller inte delar sig och nybildas. Nervceller i näsan, som utgör basen för våra luktsinne, befinner sig i en utsatt miljö eftersom de är i direkt kontakt med den luft vi andas in vilket innebär att de skadas och dör relativt lätt. De har dock utvecklat stor kapacitet för nybildning, och luktnervceller klarar även att skicka ut nya axoner till rätt målområde i hjärnan hos vuxna. Denna kontinuerliga process av död och nybildning pågår under individens hela livstid. Luktsinnet är därför ett utmärkt system för att studera vilka signaler som är viktiga för en nervcells förmåga till överlevnad och nybildning.

This thesis is based on the following articles and manuscripts, which will be referred to in the text by their Roman numerals (I-IV)


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* These authors contributed equally.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC III</td>
<td>adenylate cyclase III</td>
</tr>
<tr>
<td>AOB</td>
<td>accessory olfactory bulb</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine 3’, 5’-monophosphate</td>
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<td>BG</td>
<td>bowmans gland</td>
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<td>CaM</td>
<td>calmodulin</td>
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<tr>
<td>CaMKII</td>
<td>calmodulin kinase II</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CNG</td>
<td>cyclic nucleotide gated</td>
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<td>dnRAR</td>
<td>dominant negative retinoic acid receptor</td>
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<tr>
<td>GBC</td>
<td>globose basal cell</td>
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<tr>
<td>HBC</td>
<td>horizontal basal cell</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5- triphosphate</td>
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<tr>
<td>IRES</td>
<td>internal ribosomal entry site</td>
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<tr>
<td>MASH-1</td>
<td>mammalian achaete-scute homologue 1</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NQO1</td>
<td>NADPH: quinone oxidoreductase 1</td>
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<tr>
<td>OB</td>
<td>olfactory bulb</td>
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<td>OE</td>
<td>olfactory epithelium</td>
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<td>OEC</td>
<td>olfactory ensheathing cell</td>
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<tr>
<td>OMP</td>
<td>olfactory marker protein</td>
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<td>OR</td>
<td>odorant receptor</td>
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<tr>
<td>OSN</td>
<td>olfactory sensory neuron</td>
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<tr>
<td>PD</td>
<td>postnatal day</td>
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<tr>
<td>RA</td>
<td>retinoic acid</td>
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<tr>
<td>RALDH</td>
<td>retinaldehyde dehydrogenase</td>
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<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
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<td>RARE</td>
<td>retinoic acid response element</td>
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<tr>
<td>RGS</td>
<td>regulator of G-protein signaling</td>
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<tr>
<td>NCAM2</td>
<td>neural cell adhesion molecule 2</td>
</tr>
<tr>
<td>Np</td>
<td>Neuropilin</td>
</tr>
<tr>
<td>TRP2</td>
<td>transient receptor potential channel 2</td>
</tr>
<tr>
<td>VN</td>
<td>vomeronasal</td>
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<tr>
<td>VSN</td>
<td>vomeronasal sensory neuron</td>
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INTRODUCTION

The central nervous system (CNS) regenerates poorly after injury and disease, and neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease are thereby, with today’s knowledge, chronic and incurable. A common feature in most adult neurological disorders is an excessive neuronal degeneration, leading to a permanent loss of neurons. Gaining a deeper understanding of the mechanisms behind neuronal death and survival will aid in the search of new therapeutic tools.

Neurons of the primary olfactory system have advantages compared to other neuronal systems in studies of neuronal death and survival, since they have the unusual capacity to regenerate, extend axons and make new synaptic contacts throughout life. In this thesis I have focused on the role of retinoic acid signaling in neuronal survival, death, and axonal targeting in the mouse primary olfactory systems. An understanding of the signals involved in death and survival of these neurons could give information of how to stimulate damaged neurons in the brain and spinal cord to extend axons and make new contacts.

The olfactory systems

Olfaction is one of our most ancient and primal senses, and the ability to detect odorants is critical for the survival in most species. Discrimination among thousands of different odorants is necessary to find food, identify mating partners, and to avoid danger. In order to transmit information about our odorous environment to the brain, where it must be processed to create an internal perception of the external world, most mammals have evolved different types of olfactory systems. For the purposes of this thesis, I will focus on the two major systems in mouse: the main olfactory system which primarily is responsible for detecting most volatile odors in our environment and the accessory olfactory system, generally believed to be limited to responding to non-volatile pheromones from individuals of the same species.
Figure 1. A schematic representation of the mouse main and accessory olfactory systems. In the middle panel, the position of the different structures of the olfactory systems is visualized. The VNE is located in the anterior part of the nose, while the OE lines part of the nasal cavity. The OB and AOB are situated at the most anterior part of the forebrain. In the left panel, the top and bottom pictures represent schematic views of coronal sections through the OE and VNE, respectively, at positions marked by the hatched lines. The left panel is a schematic view of the OB, position marked by the hatched line. The glomerular (Gl) layer, mitral/tufted (M/T) layer, and granule cell layer (GC) are depicted.

The olfactory epithelium

The amazing capability of odor discrimination is made possible by the expression of around 1000 different odorant receptor genes by the olfactory sensory neurons (OSNs) located in the olfactory epithelium (OE) in the nasal cavity. This epithelial structure is highly convoluted in order to maximize the surface of the OE, and when volatile molecules, the odors, are carried through the air they easily reach the epithelium when breathing (fig 1).

Cell types

The OE is a pseudostratified neuroepithelium, with three major cell types that have their cell bodies located at different levels in the epithelium: the basal cells close to the basal lamina, the OSNs in the middle, and the sustentacular cells in the most apical position. The underlying lamina propria contains connective tissue, Bowman's glands, blood vessels and the OSN axons surrounded by olfactory ensheathing cells (fig. 2).
The basal cells are divided into two populations, the horizontal basal cells and the globose basal cells. The globose basal cells are proliferating cells with the purpose of replacing damaged neurons and sustentacular cells. Thus the OE will at all times contain OSNs at different stages of maturation. The non-dividing immature neurons, which have not yet extended cilia, reside in a basal position compared to the most mature neurons. The mature OSNs extend cilia at the surface of the OE and express the olfactory marker protein (OMP), a general marker for mature OSNs (Keller and Margolis, 1976). The sustentacular cells are supporting cells with their cell soma at the most apical position in the OE. They have a single basal process that span the whole epithelium and terminate in end feet positioned along the basal lamina. These cells are not only mechanically supporting the OSNs, but in similarity to resident macrophages also involved in the phagocytosis of dead neurons (Suzuki et al., 1995). The Bowman’s glands reside in the lamina propria, with their ducts extending through the epithelium to the surface. These glands produce the thin protective layer of mucus that covers the OE.

Figure 2. The different cell types of the OE. Left panel represents a schematic view of the OE, visualizing the different cell types of the OE with the horizontal basal cells (HBCs) and globose basal cells (GBCs) in the most basal position, the immature non-dividing OSNs and the mature OSNs in the middle, and the soma of the sustentacular (SUS) cells in the most apical location. Bowman’s glands (BG), with ducts extending through the epithelium, produce the mucus layer that covers the epithelium. Olfactory ensheathing cells (OEC) surround the bundles of axons as they exit the OE. Right panel show an OE double stained with an antibody against the OMP protein (red), which labels the mature OSN population and an antibody against the SCG10 protein (green), which labels the non-dividing, immature OSN population. Hoechst is used as a histological nuclear staining (blue), revealing the nuclei of the sustentacular cells in the most apical position.
Regenerative capacity

The OSNs are the only neurons projecting into the CNS that have their cell bodies in contact with the external environment. They are thus easily damaged by inhaled toxins, infectious agents, and mechanical trauma. As a consequence of this exposed position, a remarkable capacity to regenerate as a response to injury or after natural loss of neurons has evolved as a necessity to maintain the olfactory sensory function (Graziadei and Graziadei, 1979) and reviewed in (Schwob, 2002). Several experimental models introducing lesions to the OE have proven its regenerative capacity. The models include surgical transection of the olfactory nerve as well as inhalation or injection of various substances such as zinc sulphate or methyl bromide (Schwob et al., 1995). In order to repopulate the OE with all cell types after damage, there has to be a population of proliferating stem-cell like cells. The globose basal cells constitute the major proliferating population in the OE, and are clearly neuronal progenitors (Caggiano et al., 1994; Huard et al., 1998). This cell type has been shown to harbor at least two populations of progenitor cells, MASH-1 positive cells believed to serve as transit amplifying cells and neurogenin-1 positive cells that are immediate precursors to olfactory neurons (Calof et al., 2002). The identity of a true OE stem cell however remains unclear. Some reports have proposed that the globose basal cell population also contains the stem cells (Jang et al., 2003), and others have identified the horizontal basal cell population as having stem cell properties in vitro (Carter et al., 2004) (fig 3). A recent study has proposed the horizontal basal cell population as the progenitors to the globose basal cell population (Leung et al., 2007). In addition, this group provided evidence that these cells can give rise to both neuronal and non-neuronal cells after methyl bromide-induced lesion (Leung et al., 2007). It has also been shown that differentiated OSNs produce a signal that mediates negative feed-back to inhibit proliferation of the progenitor cells (Mumm et al., 1996). Loss of mature neurons will thus lead to an increase of proliferating basal cells in order to replenish the OE.

Figure 3. Regeneration of OSNs. Horizontal basal cells (HBC) and globose basal cells (GBC) proliferate with the capacity of self-renewal. The neurogenin-1 population of GBCs will divide asymmetrically and give rise to cells that mature into OSNs.
Odorant receptors and receptor zones

In 1991, the olfactory research was revolutionized when Linda Buck and Richard Axel cloned the odorant receptors in rat, for which they later received the Nobel prize in Physiology or Medicine (Buck and Axel, 1991). The odorant receptors belong to the family of seven-transmembrane, G-protein coupled receptors. The odorant receptor genes (around 1300 in mouse, of which around 20% are pseudo genes) make up the largest known gene family to date in mammals (Zhang and Firestein, 2002). In contrast to the mouse genome, the human genome has retained only around one third of the odorant receptor genes (Malnic et al., 2004). The OSNs express the odorant receptor protein on their cilia to enable responses to different odors. With a few exceptions, each neuron expresses only one specific odorant receptor gene and furthermore, only one allele of the expressed gene is transcribed (Chess et al., 1994; Ishii et al., 2001). Neurons expressing a given receptor project their axons to spatially invariant synaptic structures in the olfactory bulb called glomeruli (Graziadei and Graziadei, 1979). Different odors will thus elicit defined patterns of glomerular activation, creating an odorant map in the olfactory bulb. The stable expression of one single receptor in a certain olfactory neuron is therefore an essential feature for correct odorant perception. Studies have proposed that monoallelic odorant receptor expression is dependent on a cis-acting DNA region, termed the H-region, which activate the expression of only one odorant receptor gene in a specific receptor gene cluster (Fuss et al., 2007; Lomvardas et al., 2006; Serizawa et al., 2003). The functional expression of a particular odorant receptor gene product will inhibit the transcription of other receptor genes in order to ensure the expression of only one odorant receptor gene in a certain OSN (Lewcock and Reed, 2004; Serizawa et al., 2003). It has also been shown that in immature OSNs there is a low frequency of odorant receptor gene switching, which can occur at higher frequency if a non-functional odorant receptor is expressed (Shykind et al., 2004). This is likely a mechanism to ensure the ultimate expression of a functional receptor in all neurons.

The OE is classically divided into four topographically distinct zones based on the expression patterns of different odorant receptors. A certain receptor is expressed only in one particular zone, and cells expressing this receptor are stochastically distributed within that zone (Ressler et al., 1993; Vassar et al., 1993). The four zones are positioned in a dorsomedial to ventrolateral manner, with zone 1 being the most dorsomedial zone and zone 4 the most ventrolateral one (fig. 4). The
odorant receptor genes are divided into two classes, class I and class II (Ngai et al., 1993; Zhang and Firestein, 2002), with distinct zonal expression patterns. Class I odorant receptors are expressed mainly in zone 1 (Tsuboi et al., 2006), while class II odorant receptors are expressed in all four zones (Zhang et al., 2004). A zonal OE expression pattern is evident also for several non-odorant receptor genes. Expression of the cell-adhesion molecule NCAM2 is restricted to zones 2-4 (Alenius and Bohm, 1997), as well as one member of the regulators of G-protein signaling (RGS), RGSZ1 (Norlin and Berghard, 2001). Another RGS member, RGS9, was instead shown to be heavily expressed in zone 1 with only weak expression in zone 2-4 (Norlin and Berghard, 2001). In addition, the enzyme NADPH:quinone
oxidoreductase (NQO) shows selective expression in zone 1 (Gussing and Bohm, 2004). Although the zones are evident when analyzing for odorant receptor expression in the OE already at embryonic day E13 (Conzelmann et al., 2001; Sullivan, 1995) and they persist throughout life of the animal, the actual function of these zones is still not clear.

**Signal transduction**

Binding of an odorous molecule to the transmembrane odorant receptors on the cilia of the OSNs will trigger a signal transduction pathway which leads to depolarization of the membrane, a subsequent action potential along the axon, and finally release of neurotransmitters at the synapse in the olfactory bulb. When a particular odorant receptor gets activated by binding its cognate ligand, the transduction pathway starts with activation of the heterotrimeric G-protein Golf, which is associated with the odorant receptor (Jones and Reed, 1989). The $\alpha$-subunit will stimulate adenylate cyclase III to produce cAMP (Bakalyar and Reed, 1990). The elevated levels of cAMP in the cell will then trigger depolarization by opening of cyclic nucleotide-gated cation channels that allow influx of Na$^+$ and Ca$^{2+}$ in the cilia. The intracellular Ca$^{2+}$ concentration will rise, which will lead to further depolarization by opening of Ca$^{2+}$-activated Cl$^-$-channels (reviewed in (Matthews and Reisert, 2003; Menini, 1999; Schild and Restrepo, 1998)) (fig 5). The olfactory signal transduction pathway can rapidly adapt its sensitivity to stimulation. This adaptation is Ca$^{2+}$-dependent and involves negative regulation of the signaling components (Kurahashi and Shibuya, 1990; Zufall et al., 1991). Several studies have proven that rapid odorant adaptation occurs through a complex between Ca$^{2+}$ and calmodulin which modulates the cyclic nucleotide-gated cation channel, leading to a reduced affinity for cAMP (Bradley et al., 2004; Bradley et al., 2001; Chen and Yau, 1994; Kurahashi and Menini, 1997; Liu et al., 1994). Moreover, the Ca$^{2+}$-calmodulin complex will activate calmodulin dependent protein kinase II which phosphorylates adenylate cyclase III (Leinders-Zufall et al., 1999; Wei et al., 1996; Wei et al., 1998), thus reducing the amount of intracellular cAMP. Ca$^{2+}$-calmodulin can also mediate adaptation by activating phosphodiesterase which hydrolyzes cAMP to AMP (Borisy et al., 1992). However, it has been shown that activity of ciliary phosphodiesterase is not required for fast adaptation of the signal transduction pathway (Boccaccio et al., 2006). The odorant receptors are also directly desensitised via phosphorylation by G protein-coupled receptor kinase 3 (Boekhoff et al., 1992; Peppel et al., 1997).
Figure 5. Signal transduction in the OSNs. When odorant binds to the OR, Golf is activated and the Gα-subunit will stimulate ACIII to produce cAMP. The levels of cAMP rises, triggering the opening of the cyclic nucleotide gated (CNG) channel leading to an increase of intracellular Ca$^{2+}$. This in turn will lead to opening of Ca$^{2+}$ activated Cl$^{-}$ channels, further depolarizing the cell. Adaptation is Ca$^{2+}$-dependent and is mediated by negative feed-back on several components of the transduction pathway.

The main olfactory bulb and axonal projection map

The main olfactory bulb (OB) is a bilateral structure that is a part of the forebrain (fig.1). It is the sole synaptic target of the OSN axons. The axons leave the OE through the basal lamina, forming bundles that are surrounded by glial cells that are specific for the olfactory system: the olfactory ensheathing cells. These axon bundles contain over hundreds of axons, and after crossing the cribriform plate they will form the nerve layer surrounding the OB. Upon reaching the OB, the axons will defasciculate and target specific regions, making contact with the second order neurons mitral/tufted cells in neuropil structures called glomeruli (Graziadei and Graziadei, 1979). The information is modulated by interneurons surrounding the glomerular structure, the periglomerular cells, and by granule cells. The mitral/tufted cells will in turn project to the primary olfactory cortex where the information is further relayed to higher cortical areas and limbic areas for conscious and emotional perception of odor (fig 6).

Of the over 1800 glomeruli in the mouse (Royet et al., 1988), neurons expressing a given odorant receptor will project to 1-3 topographically fixed glomeruli in each hemi-bulb (Ressler et al., 1994; Treloar et al., 2002;
Since the position of each individual glomerulus is spatially defined, activation of certain odorant receptors in the olfactory epithelium will lead to a specific pattern of activated glomeruli in the OB, which is part of the topographic activity map (Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1994). This spatial map of axonal projections is maintained throughout the lifespan of the animal, in spite of the fact that there is a continuous neuronal turnover in the OE (fig. 6).

**Figure 6. OSN projections to the OB.** OSNs expressing a particular OR (black or grey) will project their axons to the same glomeruli in the OB. In the glomeruli the OSN axons make synaptic contact with mitral/tufted (M/T) cells, which project to the olfactory cortex.

**OB zones and glomerular positioning**

Much effort has been made to clarify how the OE axons are guided to their correct position in the OB. There is evidence that the zonal organization of the OE is also represented in the OB, since OSNs that reside in a certain zone, but express different odorant receptors, tend to project their axons to glomeruli in the same region of the OB (Strotmann et al., 2000; Tsuboi et al., 1999) (fig. 7). In addition, the cell adhesion molecule NCAM2, which is expressed in OE zones 2-4, has an expression pattern in the OB which only excludes the dorsomedial part (Alenius and Bohm, 1997; Yoshihara et al., 1997). This dorsomedial part of the OB is on the other hand exclusively innervated by axons from zone 1, which are visualized by the expression of the marker NQO1 (Alenius and Bohm, 2003; Dellacorte et al., 1995; Gussing and Bohm, 2004). NCAM2 has been proposed to be involved in separating projections from zone 2-4 from entering the area in the OB.
corresponding to zone 1 (Alenius and Bohm, 1997; Yoshihara et al., 1997). However, neither ectopic expression of NCAM2 in zone 1 nor a knock out of the NCAM2 gene has influenced the general zonal projection pattern (Alenius and Bohm, 2003; Walz et al., 2006). There is evidence that the dorsal/ventral arrangement of glomeruli is roughly correlated with the zonal expression in the OE of the corresponding odorant receptor (Alenius and Bohm, 1997; Miyamichi et al., 2005; Ressler et al., 1994; Vassar et al., 1994). A recent report has proposed the axon guidance molecules slit-1 and its receptor Robo-2 to be determinants of the dorsal/ventral positioning of glomeruli. In mice lacking slit-1 and robo-2 by gene targeting mutations, som axons mistarget and form glomeruli in a more ventral position (Cho et al., 2007). A study by Sakano and co-workers show that the use of genetic engineering of odorant receptors and different signaling components could change the levels of cAMP in OSNs expressing one specific odorant receptor. These studies provided evidence that the levels of cAMP are important in dictating the anterior-posterior location of glomeruli. The authors show that a higher level of cAMP will direct the axons to a more posterior glomerulus position. This finding is also supported by the fact that the expression level of the guidance molecule neuropilin (np)-1 correlates to that of cAMP, as np-1 tends to be highly expressed in axons in a more posterior position (Imai et al., 2006). The importance of cAMP levels is also demonstrated when the levels of cAMP are decreased by disruption of the adenylate cyclase III gene, resulting in dramatically lower levels of np-1 and ectopic glomeruli in more anterior positions (Col et al., 2007).

Figure 7. Zone-specific projections from the OE to the OB. Left panel shows a schematic overview of the axonal projections from zone-1 and zones 2-4 in the OE. Axons from zone-1 will project to the dorsal area of the OB, while axons from zones 2-4 project more ventrally. The right panel shows a schematic 3D picture of the OB, visualizing the different axes of glomerular position.
**Odorant receptors and axon convergence**

Numerous studies have addressed the possibility of a role for the odorant receptors in axonal convergence. The discovery of odorant receptor proteins in the axonal termini further supports this hypothesis (Barnea et al., 2004; Strotmann et al., 2004). A significant breakthrough in this field was made over a decade ago, when Mombaerts and collaborators generated a strain of knock-in animals where the odorant receptor P2 was co-expressed with the marker protein LacZ and tau, a microtubule-associated protein which enables localization of LacZ to the axons. Such experiments enabled visualization of OSNs expressing the P2 receptor in the OE and their axonal projections to the OB and confirmed the notion that neurons expressing the same receptor converge to a few spatially invariant glomeruli (Mombaerts et al., 1996). Following this, a number of receptor substitution experiments have been done where one specific odorant receptor is expressed from the locus of another odorant receptor (Feinstein et al., 2004; Mombaerts, 2004; Mombaerts et al., 1996; Wang et al., 1998). The consensus of these studies is that expression of an odorant receptor from the wrong receptor locus will lead to axon convergence and projection to a glomerulus at a position separate from that of both the original odorant receptor and the glomerulus of the substitute receptor locus. This implies that the odorant receptor is indeed involved in guiding the axons to their glomeruli, but that it cannot be the sole determinant. Another piece of evidence pointing towards the importance of the odorant receptor in axonal convergence is the observation that genetic disruption of a particular receptor will prevent the axons expressing the mutant allele from converging (Wang et al., 1998). The amino acid sequence of the odorant receptor has been shown to be important for determining the axonal projections, and also the level of receptor protein expressed (Mombaerts, 2004). However, additional mechanisms involved in the precise axonal convergence remain to be identified.

**Activity and axonal convergence**

Today, mounting evidence points to a role of neuronal activity in establishment and maintenance of the projection map. The cellular activity in the primary olfactory system is often referred to as either odor-evoked activity or spontaneous activity. The odor-evoked activity, i.e. the odorant-induced activation of odorant receptors, can be inhibited by closing one of the nostrils (“naris closure”), leaving the other one open as a control. A hallmark of a mature glomerulus is the homogenous innervation of axons expressing the same odorant receptor. During early postnatal days, axons of
a particular odorant receptor identity often project to more than the normal 1-3 glomeruli and these glomeruli can also be innervated by axons of more than one odorant receptor identity. Depending on the particular receptor, such atypical heterogenous glomeruli will disappear after two weeks to one month, leaving only the 1-3 normal stereotypical and homogenous glomeruli (Conzelmann et al., 2001; Kerr and Belluscio, 2006; Zou et al., 2004). Experiments using naris closure have shown that this procedure results in an inappropriate persistence of the extra glomeruli and heterogenous glomeruli into adulthood, suggesting a need for odor-evoked activity for proper maturation and refinement of glomeruli (Zou et al., 2004). However, the spatial map of glomeruli is not affected by naris closure since the heterogenous glomeruli are roughly in their normal position (Zou et al., 2004). Spontaneous activity is a term used to define the intrinsic depolarization of neurons, independently of odorant stimuli. The role of spontaneous activity, together with odorant- induced activity, in the formation and maintenance of the glomerular map was elegantly shown by Yu et al. (2004), where they used a genetic approach to overexpress the inward rectifying potassium channel kir2.1 in either the entire population of OSNs or a subpopulation. Overexpression of kir2.1 blocked the neurons ability to depolarize, and hence blocked all neuronal activity in the OSNs overexpressing kir2.1. The authors have concluded that in the absence of neural activity in all OSNs, the glomerular map is grossly perturbed with delayed axon entry into the OB and disorganized, heterogenous glomeruli. If only one OR population of neurons expressed kir2.1, these axons failed to pass through the cribriform plate and eventually disappeared after a few weeks (Yu et al., 2004). This implies that spontaneous activity and activity-driven axonal competition is necessary for proper glomerular formation.

The major signaling components of the odorant receptor transduction cascade have been analyzed in knock-out mice. While the knock-out mice of the cyclic nucleotide gated channel and Gαolf show virtually no perturbations of the glomerular map (Belluscio et al., 1998; Brunet et al., 1996), disruption of the adenylate cyclase III gene leads to severe defects in axonal projections (Chesler et al., 2007; Col et al., 2007; Trinh and Storm, 2003; Zou et al., 2007). It was recently shown that odorant receptors can couple to the stimulatory G-protein Gs during development, and overexpression of Gs under the control of a particular odorant receptor promoter leads to convergence and targeting to glomeruli in axons lacking a functional receptor (Imai et al., 2006). This may explain the lack of a glomerular phenotype in Gαolf mutant mice, and it could also be the explanation to why OSNs expressing a β-adrenergic receptor converge to a
specific glomerulus, since it is known that these receptors can couple to Gαs (Feinstein et al., 2004). Another report from the Sakano group identified the adhesion molecules kirrel-2 and kirrel-3 as genes with expression levels differentially determined by different odorant receptors, and more specifically by the level of activity of the particular odorant receptor (Serizawa et al., 2006). In addition, these two molecules have complementary expression patterns; kirrel-2 being highly expressed in axons with high activity, and kirrel-3 in axons having less receptor activity. It was earlier shown that the levels of ephrinA in neurons vary dependent on the odorant receptor expressed (Cutforth et al., 2003), and Serisawa et al. further confirmed this and also demonstrated that its receptor, EphA, has a complementary expression to ephrinA in both the OSN cell bodies in the OE and in the synaptic targets. Together, these molecules that are regulated by the activity of a particular odorant receptor, and likely also other still unknown proteins, may constitute a neural identity code for convergence of axons expressing the same OR.
The accessory olfactory system

The vomeronasal epithelium

Pheromones are odorants that provide information about the social and sexual status of other individuals within the species. Many animals have evolved a specialized olfactory system for detecting and responding to pheromones in the immediate environment, which is called the primary accessory olfactory system. The accessory system consists of the vomeronasal epithelium (VNE) located at the vomer bone at the base of the nasal septum, and the accessory olfactory bulb (AOB) located in the dorsal part of the main olfactory bulb (fig.1). The VNE consists of a pseudostratified epithelium much like the OE, with sensory neurons, basal cells and sustentacular cells as the main cell types. The vomeronasal sensory neurons (VSNs) are divided into two subpopulations distinguished for instance by the localization of the cell bodies within the VNE, expression of vomeronasal receptor class, and the G protein subunit used in signal transduction (Berghard and Buck, 1996; Halpern et al., 1995). Similar to that of the OE, the VNE neurons also have the capacity to regenerate throughout the life of the animal but proliferation occurs to a lesser extent.

Vomeronasal receptors and receptor zones

Two multigene families of G protein-linked vomeronasal receptors have been identified, V1R and V2R, consisting of around 150 genes each (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). When the first class of vomeronasal receptors, the V1R family, was identified it was discovered that this gene family has no homology to the OR gene family except that it is a seven transmembrane receptor with a short N-terminus (Dulac and Axel, 1995). The subsequent cloning of the V2R receptor family a few years later showed yet another receptor family with no homology to the two previously known receptor families of the olfactory systems (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). The V2R receptor family differs in that the N-terminus is large, with amino acid similarities to calcium sensing and metabotropic glutamate receptors. In analogy with the main olfactory epithelium there is a zonal division of the VNE, where areas of expression of V1Rs and V2Rs divide the VNE into an apical and a basal zone,
respectively. These zones are also defined by differential expression of G protein subunits, where the Gai2 protein subunit is expressed in the apical zone and Goα is expressed in the basal zone of the VNE (Berghard and Buck, 1996; Halpern et al., 1995). The V1Rs are co-expressed with and likely signal via Gai2 while the V2Rs are co-expressed with and likely signal via Goα. The different localization, structure, and use of G protein variant indicate that the vomeronasal receptors of the separate parts of the VNE recognize different types of pheromone ligands and therefore may mediate distinct behaviors of the animal. Another feature indicating differences between the two zones in the VNE, is that the V2R family of receptors has been shown to be co-expressed with the non-classical MHC class 1b molecules and β2 microglobulin, possibly resulting in an altered specificity of pheromone detection by these receptors (Ishii et al., 2003; Loconto et al., 2003).

Signal transduction

The signaling mechanism downstream of vomeronasal receptor activation is still not fully understood. However it differs from that in the OSNs since the major components of that signaling cascade, Goαolf, adenylate cyclase III, and OCNC1, are not expressed by VSNs (Berghard and Buck, 1996). Instead, V1R and V2R signaling seem to rely on the activation of phospholipase C which results in generation of phosphatidylinositol-3-phosphate and diacylglycerol. This will in most VSNs lead to the activation of the TRPC2 cation channel, localized in the microvilli of VSNs, which has been proven necessary for proper vomeronasal function (Leypold et al., 2002; Stowers et al., 2002). However, there is a population of V2Rs responsive to MHC peptides that has been shown to be unaffected by deletion of the trpc2 gene, indicating that a different mechanism of signal transduction may be used in cells expressing this particular V2R population (Kelliher et al., 2006).

The accessory olfactory bulb and axonal projection map

Axons from the VNE travel along the medial surface of the OB in a separate nerve from the OE axons. The vomeronasal axons project to glomeruli in the AOB where they form synapses together with mitral/tufted cells. The AOB is organized into two different areas corresponding to the VNE zones, in that VSNs expressing receptors from the V1R family in the apical part of
the VNE will project to the anterior AOB while axons expressing the V2R family are in the basal part of the VNE and project posteriorly (fig. 8).

**Figure 8. VSN projections to the AOB.** VSNs of the basal population in the VNE will project their axons to the posterior part of the AOB, while axons of the apical VSN population will project to the anterior part of the AOB.

The pattern of convergence in the AOB glomeruli is somewhat different from that in the OB, since axons from VSNs expressing the same vomeronasal receptor converge onto several (6-30) glomeruli in the AOB (Belluscio et al., 1999; Del Punta et al., 2002; Rodriguez et al., 1999). Moreover, the projections differ from the OB in that the location of glomeruli of a certain receptor identity is not precisely conserved between animals or even between the two bulbs. Instead a broader area in the AOB seems preserved for a given vomeronasal receptor. (Belluscio et al., 1999; Del Punta et al., 2002; Rodriguez et al., 1999). A recent study by Wagner et al. further clarify the organization of the glomerular map of the AOB, when they reveal that axons from closely related receptor subfamilies of the V1R receptor family project to the same general area, or “projection domain” (Wagner et al., 2006). Different receptor subfamilies thus create multiple non-overlapping projection domains which are conserved between individuals, making up a receptor subfamily-based glomerular map rather than a map based on individual receptors. Using single-cell fluorescent labeling they also very elegantly show how the second-order neurons, the mitral/tufted cells, connect to several different glomeruli within the same subfamily and hence within the same projection domain. This allows the brain to bring together information from highly related receptors, which probably is the ultimate way to detect pheromonal blends.
Behavior

The accessory olfactory system is generally believed to be responsible for innate behaviors and neuroendocrine responses in the animal elicited by pheromonal cues that provide information about gender, dominance or reproduction status. I will here give a selective summary to some of the behavioral effects mediated by vomeronasal sensing. Several studies have been done by surgical removal of the VNE, resulting in impaired sexual responses and reduced mating frequency in male rodents, (Clancy et al., 1984; Meredith, 1986) and also dramatically reduced aggressive behaviors in males (Bean, 1982; Clancy et al., 1984). Removal of the VNE in females can inhibit puberty and estrus in the presence of males (Lomas and Keverne, 1982). Genetic ablation of trpc2 abolishes male-male aggression and dominance behavior and leads to a lack of gender discrimination in males (Leypold et al., 2002; Stowers et al., 2002). In addition, trpc2-/- female mice display markedly reduced female behaviors, and instead show typical male-specific mating behaviors (Kimchi et al., 2007). However, it has been shown that a population of V2R expressing VSNs are still functional in the trpc2 -/- mice, and that a physiological response leading to termination of pregnancy, named the Bruce effect, is intact in these mice (Kelliher et al., 2006). This may imply that at least some of the behavioral defects reported in the ablation studies are mediated mainly by the apical part of the VNE, by neurons expressing receptors of the V1R family. The initial evidence for different functions of the two VNE populations came from a study using mutant mice where the Gαi2 gene is disrupted, where it was evident that the apical population is necessary for maternal aggression and proper male aggression. Moreover, these mutant mice showed normal sexual behavior and preference (Norlin et al., 2003), which may indicate that these particular animal behaviors are primarily dependent on a functional basal VSN population. Another possibility would be that both VSN populations mediate the sexual behaviors, and one functional population is enough to ensure proper exhibition of the behavior.
Retinoic acid

Vitamin A is well recognized as an essential dietary component, since postnatal vitamin A deprivation leads to symptoms including blindness, reduced immune function and keratinization of epithelia (reviewed in (Maden, 2002)). The consequences to embryos of females deprived of vitamin A are even more pronounced, and will lead to severe malformations of many organs such as the eye and the brain. The effects of vitamin A are exerted by its biologically most active metabolite, retinoic acid (RA), through transcriptional activation of a number of genes. Retinoic acid has been shown to play a major role in the fetal development of the nervous system, including the olfactory system.

Metabolic pathways of vitamin A

Vitamin A, or retinol, is converted to RA by two oxidative reactions, involving different groups of enzymes. Retinol is first oxidized to retinaldehyde in a reaction which is catalyzed by alcohol dehydrogenases (ADH). ADHs have overlapping expression patterns and act in a redundant manner as is evident by the lack of significant phenotypes in null mutants for these genes (reviewed in (Duester et al., 2003)). Retinaldehyde is then further oxidized into RA, by retinaldehyde dehydrogenases (RALDH). The RALDHs are a group of enzymes with strictly localized expression patterns that determine regions of RA synthesis. There are four known members in this enzyme family, of which RALDH-1, -2, and-3 are the most important for the developing embryo. A null mutation of RALDH-2 will lead to embryonic death in utero by day 10.5 with severe morphological defects in the trunk region and the forebrain (Niederreither et al., 1999), demonstrating the essential nature of RA synthesis in early embryos.

The two reactions for converting retinol into RA take place in the cytoplasm. Retinoic acid is then transported to the nucleus either of the same cell or of another cell, where it will exert its effects on transcription by binding to one of the retinoic acid receptors (RARs), specific nuclear receptors of the steroid/thyroid hormone superfamily. These receptors exist in the isoforms RAR-α, -β, and –γ, which are all activated by the RA variant all-trans RA. RAR heterodimerizes with RXR, which can bind the 9-cis RA metabolite (Levin et al., 1992). RXR can also function as partners for RAR
and other nuclear receptors in the absence of ligand (Rowe, 1997). The RAR-RXR heterodimers bind to RA response elements (RARE), in enhancer regions of RA target genes (fig.9). Retinol and RA do not exist as free molecules in the cytoplasm, but are bound to cellular retinol binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs), respectively. The functions of these binding proteins are not fully understood. CRBP has been suggested to aid in the metabolism of retinol to retinaldehyde (reviewed in (Napoli, 1993)). CRABP-II may assist in association of RA to the receptors in the nucleus (Delva et al., 1999) and CRABP-I promote RA catabolism (Boylan and Gudas, 1992). Retinoic acid is catabolized to what presumably are non-active products by the action of three cytochrome P450 enzymes (CYP), i.e. CYP26A1, CYP26B1 and CYP26C1 (Fujii et al., 1997; White et al., 1997; White et al., 2000).

**Figure 9. Metabolic pathway of RA synthesis.** Retinol is converted to retinaldehyde by ADH. Retinaldehyde is further oxidized into the active metabolite RA by the rate-limiting enzyme RALDH. RA binds to its nuclear receptor, RAR, which is heterodimerized with RXR. The complex binds to specific regions of the DNA, RARE, and controls transcription of the target genes.
Retinoic acid signaling in the olfactory system

The formation of the olfactory epithelium and olfactory bulb during embryonic development is dependent on proper RA signaling. The use of RA inducible transgenes have shown sites of RA production in the olfactory placode, which gives rise to the OE, and in the ventrolateral forebrain, which gives rise to the OB (LaMantia et al., 1993). The expression of RARs, RALDHs, and binding proteins in olfactory placodal epithelium and underlying mesenchyme has provided further proof for the role of RA in early development (Anchan et al., 1997; Bhasin et al., 2003), and it has been shown that RA is involved in mesenchymal/epithelial induction which contribute to development of the olfactory pathway (LaMantia et al., 2000). Molecules involved in RA metabolism and function are also present in the later stages of the developing OE and OB (Niederreither et al., 2002; Thompson Haskell et al., 2002; Whitesides et al., 1998). Additional examples of the importance of intact RA signaling during olfactory development is evident by the fact that the small eye Pax 6 mutation that leads to abnormal olfactory morphogenesis is accompanied by a disrupted RA signaling (Anchan et al., 1997), and a null mutation of RALDH-2 disrupts early olfactory development (Niederreither et al., 1999). Most interesting from the point of view of this thesis, is that RA signaling is maintained in the adult olfactory system. Production of RA in postnatal OE is evident by the localization of RALDH-1 protein in sustentacular cells and olfactory ensheathing cells and that RALDH-2 positive cells surround the nerve bundles in the lamina propria underneath the OE (Asson-Batres and Smith, 2006). Further strengthening the evidence for postnatal RA signaling, is the localization of the proteins and mRNAs for RARs and RXRs in the postnatal OE and OB (Krezel et al., 1999; Zetterstrom et al., 1999; Zhang, 1999). The expression of the CRABP- I and CRABP-II can be viewed as indicators that RA is present in a tissue. CRABP-I has been shown to be localized in immature OSNs and also in axon bundles in rat, while CRABP-II is expressed in the basal cell layer of the OE (Asson-Batres et al., 2003a; Gustafson et al., 1999). Since the OE contains cells that are proliferating and differentiating, it is plausible that local RA synthesis and binding proteins may reflect a role for RA in these events, given the known function of RA in regeneration of other tissues (reviewed in (Maden and Hind, 2003)). This view is supported by the fact that the number of mature OSNs decrease while the proliferation and number of immature OSNs increase in vitamin A deficient rats (Asson-Batres et al., 2003b). There is evidence that that RA accelerates olfactory recovery after surgical transection of the olfactory nerve in the mouse (Yee and Rawson, 2005).
2000), and that RARα protein levels increase in the OE and the lamina propria underneath the OE as a response to OSN axon transection (Yee and Rawson, 2005). The OB is one of the sites in the adult forebrain with highest RA synthesis, with RALDH-1 and -2 being expressed in the meninges surrounding the OB and RALDH-3 localized to the periglomerular cells (Wagner et al., 2002). The granule cells and periglomerular cells of the OB are continuously being renewed throughout life, from a population of precursors residing in the subventricular zone of the lateral ventricles. Interestingly, there are indications of ongoing RA signaling in the adult subventricular zone (Thompson Haskell et al., 2002). RA signaling has been shown in a slowly dividing population of granule cells in the adult OB, and also in a distinct subset of glial cells in the adult subventricular zone, suggesting a role for the glia cells in maintainance of proliferative properties of the OB precursors (Haskell and LaMantia, 2005).
AIMS

The sensory neurons of the olfactory system are easily accessible at their location in the nose. This accessibility, together with ongoing regeneration, axon elongation, and synaptic targeting make these neurons a perfect model to study the processes of neuronal survival and axon targeting in adult animals. In this thesis I have used the mouse olfactory systems to study the role of retinoic acid signaling in these important events.

The specific aims were to:

- identify expression patterns of genes involved in the synthesis and degradation of retinoic acid in the olfactory epithelium
- investigate the role of retinoic acid receptor signaling specifically in mature olfactory and vomeronasal neurons
- study the consequence of retinoic acid receptor activation on glomerular formation and maintenance
RESULTS AND DISCUSSION

In this section, I will update the discussion, where necessary, of the results from the papers and discuss papers and manuscripts that are included in this thesis in a broader context. Details are found in the papers, as well as the methodology.

Graded patterns of gene expression correlating with odorant receptor zones.

(Paper I)

An essential feature of neural connections is the organization of axonal projections into maps. For topographically organized maps, the location of a certain neuron may be important for projecting its axon to the correct target area. The formation of a neuronal map requires signals to specify the identity and location of each neuron in a certain neuronal system. In the olfactory system each olfactory sensory neuron is specified to express one odorant receptor in a zonally restricted manner, as described in the introduction. This zonal topography in the OE is maintained throughout life of the animal, even though there is a continuous generation of new OSNs. Exactly how this is achieved at a molecular level is not yet fully understood. As the zonality of OR expression has been shown to be independent of the target (Fan and Ngai, 2001; Sullivan et al., 1995) it is likely that signals within or in close proximity to the OE might contribute to the regulation of receptor expression. In paper I we have provided evidence that the spatial extent of expression of zone 1 and zone 2 odorant receptors do not overlap, thus creating a sharp border between these two zones. The sharp boundary between zone 1 and zones 2-4 is also evident from analyses of NCAM2 which is expressed solely in zone 2-4 (Alenius and Bohm, 1997; Yoshihara et al., 1997) and NQO1 which is exclusively expressed in zone 1 (Gussing and Bohm, 2004). We also show that the odorant receptor expression borders between zones 2 to 3 and 3 to 4 are not distinct, but instead display an overlapping pattern (paper I), a finding that has also been confirmed by others (Iwema et al., 2004; Miyamichi et al., 2005).

Aiming at identifying genes with a possible involvement in zonal organization, we screened for genes with an expression pattern correlating with the zonal topography of the odorant receptor expression. Retinoic acid is a molecule known to regulate morphogenesis and provide positional
information to cells during development of for instance the retina (Wagner et al., 2000), and during regeneration of adult epithelial tissues (reviewed in (Maden and Hind, 2003)). Interestingly, we found that the RA synthesizing enzyme RALDH-2 was expressed in the lamina propria underneath the OE, in a gradient corresponding to the odorant receptor expression zones, with the highest levels in zone 4 and lowest in zone 1 (paper I, fig.10). This gradient-like expression was even more pronounced when analyzing for the expression of RALDH-3 in cells in the immediate vicinity to the OE basal cells (paper IV). Targeted disruption of RALDH-2 in mice results in embryonic lethality and severe morphological defects including a truncated frontonasal region, suggesting a role for RA signaling in development of the olfactory system (Niederreither et al., 1999). RA signaling is also involved in specification of motorneurons (Novitch et al., 2003;Sockanathan and Jessell, 1998). Moreover, manipulations of the level of retinoic acid signaling via its receptor has identified a role for RA in the determining the positional identity of motorneurons, along the rostrocaudal axis of the spinal cord (Sockanathan et al., 2003). Our observation that RALDH-2 is expressed in cells directly underneath the OE therefore implies that RA signaling may play a role in neuronal specification and positioning also in the olfactory system.

Retinoic acid exerts its effects through transcriptional regulation of target genes. Therefore, it was interesting when we found that the graded expression of RALDH-2 correlated with the expression pattern of the homeobox gene msx-1, which was expressed in a population of basal cells in the OE (paper I). Msx-1 is involved in specifying exact locations of neural induction in the developing CNS and it is, in accordance with its expression in the OE, generally expressed in proliferating cells (reviewed in (Bendall and Abate-Shen, 2000)). Expression of the Msx-1 gene is also necessary for proper dorsal-ventral patterning of the Drosophila nervous system, and for tooth-development in mouse (reviewed in (Ramos and Robert, 2005)). It is possible that the similarities in the graded expression patterns could reflect a direct RA regulation of Msx-1 expression in the OE since RA is known to activate msx-1 transcription in other systems, including cell culture and during embryonic development (Shen et al., 1994; Wang and Sassoon, 1995). Additionally, Msx-1 and Msx-2 have been suggested to be involved in mediating retinoid effects on facial morphogenesis in chick (Brown et al., 1997). Msx-1 expression is also known to be regulated by bone morphogenetic proteins (BMPs) (Bei and Maas, 1998; Suzuki et al., 1997). We found that one BMP receptor, Alk-6, was expressed in the OE in a spatially restricted manner. However, Alk-6
was expressed in an opposite gradient to that of msx-1 and the expression was localized to a different cell type, the sustentacular cells (paper I, fig.10). Therefore we found it unlikely that msx-1 expression is regulated by Alk-6 in this system.

Figure 10. Gradients of gene expression in the OE. Four genes with a graded expression pattern correlating to the OR expression zones were identified. RALDH-2 is expressed in the lamina propria, with a high expression in zone 4 that gradually declines towards zone 1. Msx-1 in the basal cells and np-2 in the OSNs had a similar gradient as RALDH-2, while Alk-6 was expressed in a counter-gradient in sustentacular cells.

If RA, possibly through regulation of msx-1 in basal cells, has a role in specifying the zonal expression of odorant receptors, it would be reasonable to assume a patterned expression of genes involved in specifying neuronal identities also in the more mature OSNs. Indeed, we found the axon guidance molecule neuropilin (np)-2 to have a graded expression pattern corresponding to that of RALDH-2 and msx-1, and its expression was confined to mature OSNs in the OE (paper I, fig.10). It is important to note that not only are the OSNs zonally positioned in the OE, according to expression of a certain receptor, but the axonal projections to the OB also maintain this zonal division. Np-2 is, together with plexins, a co-receptor for class 3 secreted semaphorins (Sema 3). Sema 3F signaling via np-2 has been shown to be involved in directing spatial and temporal coordination of axon extensions and target innervation in spinal motor axons (Huber et al., 2005).
Interestingly, another family of the semaphorins, Sema 1A, was recently shown to be necessary for proper axon-axon interactions and correct axon projections in the *Drosophila* olfactory system (Lattemann et al., 2007; Sweeney et al., 2007). Even though the expression pattern of np-2 in the OE made it an interesting candidate for RA regulated axon guidance, we have found that it is unlikely that np-2 expression is directly regulated by RA (unpublished data). The function of np-2 in the olfactory system is so far unclear. Targeted disruption of np-2 show a low percentage of axons overshooting their targets, but the spatial projections to the olfactory bulb remain unperturbed (Walz et al., 2002).

**Retinoic acid signaling is necessary for postnatal survival of mature olfactory and vomeronasal sensory neurons.**

(*papers II - IV*)

Taken together, the results presented in paper I indicate a possible role for RA in zonal specification of olfactory sensory neurons by regulation of different target genes. There are however alternative, not necessarily mutually exclusive, explanations for the zonal expression of the RA synthesizing enzymes. There is no documented evidence of zonal regulation of proliferation and tissue homeostasis, but there are indications of a lower proliferation rate in zone 1 of the adult mouse (Vedin et al, unpublished data). During embryogenesis, RA closely regulates formation and differentiation of neurons, such as motoneurons in the spinal cord (Sockanathan and Jessell, 1998). The RA-mediated effects continue to be important in the adult CNS. Two events where RA has been shown to be necessary are in early stages of adult neurogenesis and survival in the hippocampus (Jacobs et al., 2006), and in regulation of cell proliferation in a population of astrocytes in the adult subventricular zone (Haskell and LaMantia, 2005). These reports support a possible function for RA in regulation of proliferation also in the OE. It is plausible that RA could be involved in regulating axonal targeting to the correct areas of the OB by regulation of genes involved in axon guidance or cell adhesion. Even though np-2 did not appear to be regulated by RA (unpublished data), there are other candidates. The axon guidance molecule Robo-2 was recently shown to be expressed in the OE in a gradient opposite to that of RALDH-2 and RALDH-3 (Cho et al., 2007), opening for the possibility of a negative RA regulation of this gene. As will be further discussed, there is evidence for
RA regulation of the cell adhesion molecule kirrel-2, known to be involved in proper axon targeting in the OB (Serizawa et al., 2006).

In order to further characterize the involvement of RA signaling in the different events in the olfactory systems, we generated transgenic mice expressing a dominant negative RAR (dnRAR) under transcriptional control of the OMP promoter (papers II-IV). The OMP promoter ensured expression of the dnRAR in all non-dividing OSNs and in both populations of VSNs (fig.11) and thus allowed us to study the effects of a disrupted RA signaling specifically in these neurons.

**Figure 11. Expression of the OMP-dnRAR transgenic construct.** Upper panel: dnRAR was expressed under transcriptional control of the OMP promoter. Lower panel: *In situ* hybridization showing strong expression of the transgenic construct in the entire OE and VNE (positive signal in white).
Postnatal neurodegeneration of mature OSNs and VSNs in dnRAR transgenic mice

When we analyzed the OMP-dnRAR transgenic mice we found that there was a significant loss of mature neurons in both the OE and the VNE which occurred postnatally, subsequent to formation of axonal projections to the OB and AOB, respectively (papers II and III, fig.12). The loss of neurons appeared to be due to an increased death induced by caspase-3 activation. Surprisingly, this neurodegeneration was not accompanied by a compensatory increase in proliferation and thus resulted in a reduced number of OSNs and VSNs in adult OMP-dnRAR mice. In accordance with our results, rats depleted of vitamin A also display a loss of mature OSNs. In the rats however, the total number of OSNs remains normal due to a compensatory increase of proliferation (Asson-Batres et al., 2003b). This disparity between the vitamin A deficient animals and the OMP-dnRAR mice may be due to an effect on the proliferating cells in the vitamin A deficient rats, suggesting that RA regulates progenitor cell proliferation.

Figure 12. Loss of mature OSNs and VSNs in OMP-dnRAR mice. Upper panel: Immunohistochemistry analyses with an antibody directed against OMP (white) reveals a reduction of mature neurons in the transgenic mice. Lower panel: *In situ* hybridization with a cRNA probe specific for Gαo shows that this population of VSNs is reduced in the OMP-dnRAR mice (signal shown in dark grey).
This hypothesis is supported by studies showing localization of CRABPII in a population of basal cells in the OE (Asson-Batres et al., 2003a). In addition, we found β-gal positive basal cells in the OE in the RA responsive reporter mouse RARE-Hsp-lacZ which indicated that RA mediated transcription occur in the basal cells (paper II). An interesting finding in the VNE of the OMP-dnRAR mice was that only cells of the basal, Gαo expressing, population was affected by the RA signaling defect, even though the dnRAR is strongly expressed in both the apical and basal population of VSNs (paper III). This suggested that the two populations are differentially regulated by RA signaling. The fact that dnRAR affected one particular population in the VNE raised the question whether a specific population in the OE was affected as well. This possibility remains to be explored since we have not been able to find a marker to show this. The negative effect of dnRAR on cell survival in the adult olfactory systems is in agreement with the normal role for RA signaling in survival of adult hippocampal neurons (Jacobs et al., 2006). In addition, RA is suggested to have an anti-apoptotic role in noise-exposed cochlear hair cells (Ahn et al., 2005). We also noted a down regulation of the RA catabolic enzyme Cyp26B1 in a subpopulation of mature OSNs, likely due to a feedback mechanism of RA signaling (paper II). This may be a possible mechanism for normal cell survival regulation of OSNs, where the mature neurons expressing Cyp26B1 would be more prone to die as a result of less RA and thus a decrease in RA signaling.

In summary, proper RA signaling seems to be necessary in the postnatal mouse for survival of mature OSNs and VSNs that have made synaptic contacts to their respective targets. Moreover, the neurodegeneration of VSNs is selective to the basal population. Possible mechanisms for this neuronal death will be discussed in more detail below.

**Selective degeneration of the Gαo population of VSNs in RA signaling-deficient mice**

In accordance with the specific loss of basal VSNs due to caspase-3 mediated apoptosis, a significant increase of activated caspase-3 was evident in the posterior part of the AOB (paper III). The specific neurodegeneration led to a reduction of the posterior AOB which was not compensated for by an expansion of the anterior population (paper III). Mice with a targeted deletion of Ga o show a cellular phenotype very similar to that of OMP-dnRAR, with an increase of postnatal apoptosis of the Gao
population of VSNs (Tanaka et al., 1999). Additionally, studies on Gαi2 knock out mice show a diminished area of the anterior AOB population (Norlin et al., 2003). Taken together, these data imply a requirement for G-protein signaling in cell survival of both VSN populations, while our results suggested that the Gao population also requires functional RA signaling in order to survive after axonal projections are established. One possibility for the different sensitivities to RA signaling in the apical and basal populations could be a different availability of the molecules involved in the RA signaling pathway. RA is produced by RALDH-2 in cells surrounding the vomeronasal nerve all the way from the VNE to the AOB, thereby providing a source of RA for the VSN axons (paper III). RA produced in the immediate vicinity to the vomeronasal axons could thus function as a survival factor for one population, possibly by regulation of neurotrophic factors. However, the axons from both the apical and basal populations are intermingled in the vomeronasal nerve bundles and both populations express endogenous RARα, suggesting that there is no difference in the availability of RA between the two populations of VSNs. Therefore it is plausible that there are separate mechanisms for regulating the survival of the apical and basal populations of postnatal VSNs, not including differential expression of molecules involved in RA signaling.

A crucial event for the survival of mature neurons, is the formation and refinement of functional synapses. In the AOB, synaptogenesis is initiated postnatally between day 1 and day 8 (Horowitz et al., 1999). The regulation of synaptic plasticity is normally associated with activated caspase-3 (reviewed in (McLaughlin, 2004). We found a transient increase of activated caspase-3 in the posterior AOB of control mice around postnatal day 4-7, which could be in accordance with a normal process of synaptic refinement at that time point (paper III). Since we only found activated caspase-3 in the posterior AOB, this suggested that the mechanism of postnatal synaptic refinement and neuronal survival could differ between the Gao and the Gai2 populations. This notion was further supported by the selective degeneration of the basal population observed in the RA-signaling deficient mice (paper III). Additionally, while the degeneration of the Gao population in the dnRAR transgenic mice was associated with an increase of activated caspase-3, the apoptosis occurring in the anterior population in the Gai2 knock out mice was not accompanied by elevated caspase-3 levels (paper III). Since the increased levels of activated caspase-3 in the posterior AOB of the OMP-dnRAR mice occur around the time point for postnatal synaptogenesis and refinement, a defect in these processes in the dnRAR mice is possible. Aberrant synaptogenesis could in turn result in impaired
signaling from the synapse to the nucleus, which is important for neuronal survival (reviewed in (Deisseroth et al., 2003)). A possible role for RA in synaptic refinement is supported by studies showing that RA signaling is involved in synaptic plasticity in the adult hippocampus, where vitamin A deprivation as well as null mutations targeting RARβ and RARγ will result in a loss of synaptic plasticity (Chiang et al., 1998; Misner et al., 2001). The differential regulation of synaptogenesis and neuronal survival in the two VSN populations may be an indication of possible separate functions of the two populations.

Another piece of evidence that pointed towards different regulatory mechanisms between the two vomeronasal populations was the expression profile of the cell adhesion molecule kirrel-2. Kirrel-2 has been shown to be differentially expressed between OSNs with different odorant receptor identities, and is presumably regulated by the level of activity of each particular odorant receptor (Serizawa et al., 2006). We found kirrel-2 to be expressed in a fraction of both apical and basal VSNs in control mice (paper III). An interesting observation was that kirrel-2 was down regulated in both populations of VSNs in the OMP-dnRAR mice before a loss of basal VSNs was apparent. However, while the Gαo population continued to express low levels of kirrel-2 in the dnRAR mice, the levels of kirrel-2 in the Gαi2 population gradually increased. Interestingly, Kirrel-2 expression was also reduced in the anterior population in Gαi2 mutant mice, implying that it is regulated by G-protein mediated activity also in the vomeronasal system (paper III). However, the down regulation of kirrel-2 in all VSNs before the loss of basal VSNs clearly suggests an additional, RA mediated, mechanism of regulation. Considering the fact that the levels of kirrel-2 expression in the Gαi2 population increases with time in OMP-dnRAR mice, the regulation of kirrel-2 in this population may depend less on RA signaling and more on neuronal activity after synapse formation. Since kirrel-2 is known to be involved in the precise formation of glomeruli in the main olfactory system, it is possible that the down regulated kirrel-2 in the Gαo population may interfere with specific axonal targeting to the AOB, which in turn could lead to an increase of apoptosis in this population.
Heterogenous glomeruli in OMP-dnRAR mice

During the early postnatal days, several glomeruli in the OB are heterogeneously innervated by axons of more than one odorant receptor. The axonal projections are subsequently refined, so that in the adult mouse a given glomerulus will become homogenous, containing only axons of the same odorant receptor identity. This glomerular refinement is dependent on neuronal activity (Conzelmann et al., 2001; Kerr and Belluscio, 2006; Zou et al., 2004). An intriguing finding in adult OMP-dnRAR mice, was that glomeruli of the odorant receptors MOL2.3 in zone 1 and P2 in zone 2 appeared heterogeneously innervated, suggesting an inhibition of postnatal refinement in these mice (fig. 13). However, we did not find evidence for a postnatal refinement of the P2 glomerulus in the control mice, while the ratio between heterogenous and homogenous P2 glomeruli in the OMP-dnRAR mice increased with age (paper IV). This result indicated that heterogeneous glomeruli increased at the expense of homogeneous glomeruli, and may imply that the mechanism that prevents excessive plasticity during glomerular refinement is perturbed in dnRAR transgenic mice.

Figure 13. Heterogenous glomeruli in adult OMP-dnRAR mice. Double immunohistochemistry with α-OMP (grey) and histochemistry for β-gal staining (white) in a P2 glomerulus of control and OMP-dnRAR expressing P2-IREStauLacZ. Left panel shows a P2 glomerulus in a control mouse, where the entire glomerulus is filled with β-gal. Right panel shows a heterogeneously innervated glomerulus (demarcated by dotted line), with OMP positive axons that are β-gal negative (arrow)
The degeneration of mature OSNs by activated caspase-3 in the OMP-dnRAR mice was preceded by enhanced levels of activated caspase-3 in the glomeruli of the OB (paper II). This suggested that the OSNs died by a retrograde mechanism of apoptosis. Loss of synaptic target is known to induce retrograde apoptosis in OSNs, which is associated with activated caspase-3 (Cowan and Roskams, 2004; Cowan et al., 2001). We found it unlikely that a target deprivation was the cause of the retrograde apoptosis observed in the OMP-dnRAR mice, since the activity markers c-fos and tyrosine hydroxylase were unchanged (paper II). In addition, the semaphorin receptor np-1 was upregulated in the OMP-dnRAR mice (paper IV). Neuropilin-1 expression has recently been shown to be dependent on odorant receptor signaling and the cAMP levels (Imai et al., 2006). In adenylate cyclase III knock-out mice, where np-1 expression is abolished, heterogenous glomeruli are formed (Chesler et al., 2007; Col et al., 2007; Zou et al., 2007). It is therefore interesting to note that heterogenous glomeruli appear also where np-1 expression is upregulated.

Kirrel-2 expression is down regulated in response to sensory deprivation by naris closure, or by blocking the signal transduction pathway (Serizawa et al., 2006). Given that there is no apparent effect on the level of activity in the OMP-dnRAR mice, it was an interesting finding that kirrel-2 was down regulated in the transgenic mice (papers III and IV). Thus the level of kirrel-2 in OSNs appears to be regulated by RA as well as by neural activity. The expression levels of kirrel-2 has been shown to be important for segregation of glomeruli, since gain of kirrel-2 expression results in the segregation of axons with the same odorant receptor into two separate glomeruli (Serizawa et al., 2006). One possible explanation for the appearance of heterogenous glomeruli in the RA signaling deficient mice could be that the inhibited kirrel-2 expression might disrupt already formed homogenous glomeruli due to diminished cell adhesion.

Kirrel-2 has a heterogenous expression pattern in the glomeruli of the OMP-dnRAR mice, with compartments of high versus low levels of kirrel-2 (paper IV). The expression levels of kirrel-2 were associated with the levels of activated caspase-3, i.e. where kirrel-2 levels were low, activated caspase-3 was high (paper IV). This indicates that inhibition of RAR signaling affect various subpopulations differently. Moreover, the result provides evidence that OSNs expressing low levels of kirrel-2 might die by a mechanism that involves competition between axons innervating the same or a neighboring glomerulus. Since OMP promoter-driven expression of dnRAR is presumably equal in all OSNs, the different sensitivity to dnRAR
may reflect intrinsic properties of individual OSN populations or environmental factors. Kirrel-2 is regulated by both RA and activity, which opens for the possibility that deficient RA signaling increases the contribution of neuronal activity on kirrel-2 expression. Thus axon terminals with low levels of kirrel-2 and high levels of caspase-3 would represent an OSN population that fail in competition because they are relative silent. An OSN could be silent as a result of lower levels of spontaneous activity from its particular odorant receptor, or due to lack of ligands for the odorant receptor in the environment. In this scenario, the function of RA would be to decrease the contribution neuronal activity has on gene expression, and thereby inhibit neuronal death by activity-dependent competition.

Kirrel-2 was expressed in a gradient in the OE, corresponding to the graded expressions of RALDH-2 and RALDH-3. This is in accordance with an RA regulation of kirrel-2. The high levels of RA in zone 4, and thus high levels of kirrel-2, most likely underlies the relative minor effects of dnRAR in the OSNs expressing the zone 4 receptor OR256-17. Since RA may inhibit the negative effect activity-dependent competition has on neuronal survival, these findings have the interesting implication that OSNs in zone 1 could depend more on odorant-evoked activity whereas OSNs in zone 2 could depend more on genetically determined RA synthesis for survival, maintenance of precise connections, and structural plasticity.

In conclusion, a disrupted RAR signaling leads to retrograde neurodegeneration of a subpopulation of postnatal neurons that have established axonal projections. This is an important finding in light of neurodegenerative diseases of the brain, where a selective death of neurons is a common characteristic. Defective RA signaling has been implicated in the onset of Alzheimer’s disease and amyotrophic lateral sclerosis (Goodman and Pardee, 2003). In addition, knock out studies of RARs and RXRs have implicated a role for retinoids in controlling the function of the dopaminergic mesolimbic pathway which is affected in Parkinson’s disease and schizophrenia (Krezel et al., 1998). An understanding of the mechanisms involved in pathological degeneration of neurons and in survival of neurons during normal circumstances, is of high priority considering the possible implications in therapeutics for these chronic diseases.
CONCLUSIONS

The findings described in this thesis can be summarized as follows:

- The graded expression pattern of the retinoic acid synthesising enzyme RALDH-2 in the lamina propria underneath the olfactory epithelium correlates with the odorant receptor expression zones. This implicates a role for retinoic acid signaling in regulation of genes involved in neuronal specification and precise axonal projections in the main olfactory system.

- Inhibition of retinoic acid signaling in non-dividing olfactory and vomeronasal sensory neurons, by a dominant negative retinoic acid receptor, results in postnatal, premature death of mature sensory neurons of the main and accessory olfactory systems by caspase-3 activation. This neurodegeneration is most likely due to a retrograde mechanism of apoptosis.

- In the vomeronasal epithelium, the neurodegeneration due to inhibited retinoic acid signaling exclusively affects the Gαo population, suggesting differential mechanisms of neuronal survival in the two vomeronasal populations.

- The cell adhesion molecule kirrel-2 is down regulated in the retinoic acid-signaling deficient mice, suggesting a dual regulation of this gene by neural activity and retinoic acid. The decreased levels of kirrel-2 correlates with an appearance of heterogeneously innervated glomeruli in the main olfactory system of the transgenic mice. Moreover, a low level of kirrel-2 was associated with high levels of activated caspase-3. A possible role for retinoic acid could be to decrease the contribution of neuronal activity on gene expression and thereby diminish neuronal death due to neural activity-driven competition.
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REFERENCES


