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Mechanisms of sensory neuron diversification during development and in the adult *Drosophila*

How to make a difference

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Nihil est in intellectu quod non fuerit prius insensu

(Nothing reaches the intellect before making its appearance in the senses)

Latin saying

Man vill inte vara den man är.

Man vill vara den man inbillar sig att man är.

Hjalmar Söderberg

Abstract

The nervous system contains a vast number of neurons and displays a great diversity in cell types and classes. Even though this has been known for a long time, the exact mechanism of cell specification is still poorly understood. How does a cell know what type of neuron to which it should be specified? It is important to understand cellular specification, not only for our general understanding of biological processes, but also to allow us to develop treatments for patients with destructive diseases, such as Alzheimer's, Parkinson, cancer or stroke. To address how neuronal specification and thereby diversification is evolved, we have chosen to study a complex but defined set of neurons, the *Drosophila* olfactory system. Olfactory sensory neurons (OSNs) detect an enormous variety of small volatile molecules with extremely high specificity and sensitivity. The adult *Drosophila* olfactory system contains 34 OSN classes each defined by their expression of a specific odorant receptor (OR). In both insects and vertebrates, each OSN expresses only one OR. In mouse there are approximately 1200 and in *Drosophila* 60 different OR genes. Despite the range of mechanisms known to determine cell identity and that the olfactory system is remarkably conserved across the phyla, it is still unclear how an OSN chooses to express a particular OR from a large genomic repertoire. In this thesis, the specification and diversification of the final steps establishing an OSN identity is addressed. We find seven transcription factors that are continuously required in different combinations for the expression of all ORs. The TFs can in different gene context both activate and repress OR expression, making the regulation more economical and indicating that repression is crucial for correct gene expression. We further identified a repressor complex that is able to segregate OR expression between OSN classes and propose a mechanism on how one single co-repressor can specify a large number of neuron classes. Exploring the OSN we found the developmental Hh signalling pathway is expressed in the postmitotic neuron. We show several fundamental similarities between the canonical Drosophila Hh pathway and the cilia mediated Hh transduction in component function. Further investigation revealed a function of cilia mediated Hh signalling in sensory neuron modulator. The results generated here will create a greater in vivo understanding of how postmitotic processes generate neurons with different fates and contribute to the maintaining of neuron function.

Contents

List of papers

This thesis is based on the following papers, which will be referred to by their Roman numerals.

Paper I

Combinatorial Activation and Repression by seven Transcription Factors Specify *Drosophila* Odorant Receptor Expression. Shadi Jafari, Liza Alkhori, Alexander Schleiffer, Anna Brochtrup, Thomas Hummel and Mattias Alenius

Plos Biology (Vol. 10) March 2012

Paper II

The corepressor Atrophin specifies odorant receptor expression in *Drosophila*. Liza Alkhori, Anita Öst and Mattias Alenius

FASEB J. (Vol. 28) March 2014

Paper III

Cilia-mediated Hedgehog signaling in *Drosophila.* Anujaianthi Kuzhandaivel, Sebastian W. Schultz, Liza Alkhori and Mattias Alenius

Under revision in Cell Report

Paper IV

Hh signalling regulates odorant receptor cilia localization in *Drosophila*. Liza Alkhori, Gonzalo M. Sanchez, Sebastian Schultz, Anujaianthi Kuzhandaivel, Björn Granseth and Mattias Alenius

Manuscript.

Abbreviations

OR, Olfactory receptor

OSN, Olfactory sensory neuron

AL, Antennal lobe

OE, Olfactory epithelium

OB, Olfactory bulb

TF, Transcription factor

RNAi, RNA interference

Introduction

Neuron diversification

The recognition that neurons are distinct functional entities was the first great contribution of neurobiology's founding father, Santiago Ramon y Cajal, who was able to make that discovery because he had a method, the Golgi stain, which showed individual neurons in spectacular structure from their neighbours. It was immediately apparent that neurons come in a florid variety of shapes and the identification of neuronal types was possible. Since the work of Ramón Y Cajal at the turn of the century the work of Mountcastle, Hubel and Wiesel (ca 1950) stands as the most fundamental early advance in our understanding of the organization of the brain. They identified the functional significance of theses neuron networks, and showed that the connections filter and convert sensory information on the way to the cortex. Further they revealed that the cortex is organized into functional compartments and that these can be modulated by experience (Lopez-Munoz et al., 2006).

Although the morphological diversity of neuronal cell types had been recognized over a century ago, we are still only beginning to recognize the underlying molecular diversity of neuron types. The key limiting factors have been difficulties in isolating specific types of neurons and getting sufficient amounts from complex neuronal circuits. Some significant progress has been made in the last years, both in vertebrates and invertebrate models, and has led to the understanding of the complexity in the terminally differentiated neuron and how little we know.

> Terminally differentiated neuron is in permanent mitotic quiescence, thus has reach maturity and can perform its main function for the rest of the organism's life.

The questions of how cells are precisely generated, specified and integrated in the CNS are some of the most challenging issues in research and in developmental neurobiology. How are neuronal types distinguished and why do neurobiologists care so much about them?

The importance of such an understanding goes much beyond basic biology. For instance, a better understanding generated here will create a greater in vivo understanding of the processes involved to generate neurons with different fates and in the long run, facilitate the development of therapeutics for example neurodegenerative diseases.

The nervous system

The human brain has somewhere around 85 billion neurons (Herculano-Houzel, 2009). Furthermore, it is estimated that the human central nervous system holds at least 10 000 different types of neural and glia cells and within these types there are subtypes or classes. They display an enormous variety in identity i.e. morphology, function, types of neurotransmitter used and connectivity (McConnell, 1991; McKay and Hockfield, 1982; Polleux, 2005).

Specification is the process by which cells acquire identities or fates that they and their progeny will adopt (de-Leon and Davidson, 2010).

Neuron types such as dopamine, gustatory, nociceptive, or olfactory neurons are distinguished on the basis of their unique location, wiring pattern or molecular properties. Furthermore they are part of complex networks that are extremely plastic and adaptable to changes in the environment and in response to experiences, an aspect that is fundamental for behavioral functions such as memory and learning (Figure 1) (Masland, 2004)

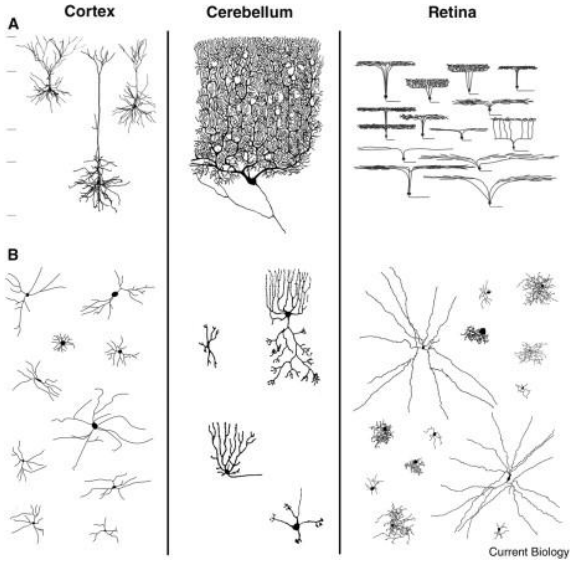


Figure 1. Selected types of neuron in three different CNS structures in humans. (Masland, 2004)

The neural stem cells are the precursors from which this tremendous cell diversity is generated. Stem cells are multipotent. Differential gene expression and morphogenesis modulate the characteristics and connectivity of the neurons. Embryonic neural development

begins with the birth and differentiation of neurons from stem cell precursors, then the migration of immature neurons from their birthplaces to their final destination. While still migrating, the neurons start the mechanisms underlying synapse formation and axons outgrowth towards their postsynaptic partners. The generation of synapses and outgrowth of axons between the neurons and the lifelong modifications in these are what underlies learning and memory (Jessell and Sanes, 2000).

I will discuss here some theoretical concepts about how one can envision the specification of individual, mature and terminally differentiated neurons look like and through what regulatory mechanisms such gene expression could be brought about. I will examine available research data and assess how these concepts correlate to our work.

Before going into describing our model system and gene regulatory networks involved in the terminal differentiation processes, I will briefly describe some key signaling pathways generally considered to be crucial throughout the development of an organism.

Developmental signaling pathways

In animal development there are some key signalling pathways such as the Wnt, Notch and Hedgehog that are important for controlling cell fate determination and tissue organization/homeostasis (Pires-daSilva and Sommer, 2003).

The Hedgehog (Hh) gene is broadly found across the metazoan. It has a central role in development both in flies and humans and has been implicated in cancer research. Hh was first described in Drosophila and was identified as one of segment polarity gene products, establishing a difference between the anterior and posterior parts of the body axis (Nusslein-Volhard and Wieschaus, 1980).

Hh encodes a secreted morphogen that in a dose-dependent manner (as a gradient) directs distinct cell fates within a target field. Hh also works as mitogens regulating cell proliferation. The Hh signaling pathway has been extensively investigated in the Drosophila imaginal disc, a monolayer epithelium that grows inside the larva and averts during metamorphosis to give rise to adult body parts such as wing and antenna. Hh transduces its signal by promoting the cell surface localization of Smoothened (Smo), a seven transmembrane protein, belonging to the G-protein-coupled receptor (GPCR) super family and is related to the Frizzled family of Wnt receptors (Dann et al. 2001). In the absence of Hh, its receptor Patched (Ptc), a cell surface transmembrane protein, destabilize and prevents membrane localization of Smo. In

the presence of Hh, Ptc is inhibited and an accumulation of Smo to the cell surface is allowed as well as the modification of a large microtubule-associated complex consisting of Suppressor of Fused (SuFu), Costal-2 (Cos2), Fused (Fu) and Cubitus interruptus (Ci). Ci is a zinc- finger transcription factor and crucial downstream effector of Hh. In absence of Hh, Ci is processed to its repressor form and as a result of Hh signalling, Ci is stabilized and are able to induce transcription of Hh target genes. One of the target genes is the inhibitor Ptc, which will convey negative feedback of the pathway (Varjosalo and Taipale, 2008).

The primary cilium is an evolutionarily conserved, microtubule-based organelle that can serve as a signalling centre. Defects in the cilium, have been shown to cause a broad range of diseases, from developmental defects to cancer, obesity and diabetes (Goetz and Anderson, 2010) .

A similar but more complex pathway has been described in vertebrates though the Hh signalling is suggested to be associated with the primary cilia for basically two reasons. First, in development, cilia function appears to have an essential role for normal patterning of tissues. Second, the IFT (intraflagellar transport) system mutations in mouse suggest the developmental defects are associated with the abnormalities in the Hh signaling pathway. There are three Hh homologs (Shh, Ihh, Dhh), two Patched homologs, one Smoothened, and three Ci homologs (Gli1, Gli2, and Gli3). The immediate steps following Smo activation in mammals remain unclear, but similar to that in Drosophila, the final step is that the Gli proteins are activated and the repressor form is inhibited (Goetz et al., 2009).

Intraflagellar transport system, IFT, is two protein complexes, IFT A and B. A regulate the retrograde cilia transport and B the anterograde transport of cilia proteins. The processes are required for construction of the primary cilia (Goetz and Anderson, 2010).

The usage of gradients to set up an order during initial development is a common and efficient mechanism to divide a large set of cells to specific classes. Gradients have the advantage of being economic, as a small number of molecules can direct position and direction across a broad field. Though, the drawback of gradients is the imprecise differentiation between nearby points and therefore the diversification process needs additional mechanisms for the fine-tuning.

If Hh controls cell fate specification and diversification over a broad target field, Notch signaling plays fundamental roles during lineage formation resulting in different cell fates through so called asymmetric divisions. Notch is a membrane bound transcription factor, which binds Notch ligands, Delta and Serrate, on an adjacent cell triggering the cleavage of Notch intracellular domain (NICD) and activating Notch signalling cascade (Notch on). Numb protein is asymmetrically segregated to the cell where Notch is not activated and represses Notch signalling in that particular cell (Notch off). The binary difference in Notch activity gives rise to two different cell fates by transcription of Notch target genes. In cells in which the Notch ligand is absent the sequence-specific DNA-binding protein Suppressor of Hairless (Su(H)) recruits a co-repressor complex; Hairless, CtBP, Groucho and suppress target gene expression. In Notch active cells, the NICD translocates to the nuclei where it binds to Su(H), which no longer can bind co-repressors, and recruits the co activator Mastermind, leading to transcription of Notch target genes (Louvi and Artavanis-Tsakonas, 2006; Pires-daSilva and Sommer, 2003)

The identification of many of the developmental signaling pathways has led to the general conclusion that there is a remarkable conservation in the use of members of the same signaling families to regulate development across different species.

Gene regulation in neuronal diversity

Whole genome mapping and comparative genome analysis of vertebrate and invertebrate species have revealed surprising facts. The *C. elegans*, a simple nematode has close to 20 000 genes whereas the Drosophila possesses 14 000 genes in spite of its greater range in cell types and tissue. Even more surprising is that the human genome only displays 30 000 protein coding genes and that the increase is only a consequence of gene duplication rather than new genes. These discoveries argue for more complex gene regulation in more complex organism, additional from alternative splicing and posttranscriptional regulation that traditionally been suggested (Levine and Tjian, 2003). Thus, the transcriptional networks are crucial in developmental biology and physiology and underlie mechanisms of specification as well as diversification.

The regulatory machinery during development contains two complementary components. The regulatory genes, i.e., the transcription factors, in concert with their networks and the

signaling molecules. Transcription factors (TFs) bind to specific sequences in the DNA and activate or repress the transcription of a gene. Signaling molecules carry out the communication between the cells and initiate the activation of certain transcription factors in the cells that receive the signal. While a significant amount of signaling pathways has been mapped, the gene regulatory networks are just starting to be appraised.

Transcription factors (TFs), sequence-specific DNA binding proteins that bind cofactors or the polymerase holocomplex and mediate gene-selective transcriptional activation or repression.

Components of the gene regulatory networks.

A small part of the metazoan genome is protein coding sequences and 5 – 10 % of these coding sequences are proteins that regulate gene transcription. Among these proteins are first the TFs, second, the diverse but general protein family in complex with the RNA polymerase machinery and third, cofactors and the chromatin remodeling and modifying factors (Levine and Tjian, 2003). I will briefly go through the actors in the gene regulatory network.

TFs were initially employed as markers for cell types and distinct anatomical domains but studies on their function revealed an essential role in controlling the expression of genes that eventually state the identity and function of cell types. TFs bind to 5-20bp sequences, or motifs that has a core pattern for each TF though the sequence can vary due to some positions is not essential for binding. TFs control spatiotemporally the level of gene expression by binding to motifs in regions far upstream or downstream the gene called enhancers or promoter region. The promoter region are typically located in the sequence region upstream of the gene they are regulating, towards the 5´region, the length is thought to vary between 100 to 1000 bp. Core promoters are the regions that bind the RNA polymerase II and contain the transcription start site (TSS) (TATA box in many cases) and general TF binding sites. The general TFs include the TATA-binding protein (TBP) and its associated factors (TAFs), together referred to as TFIID. Their function is to recognize the promoter and stabilize the binding of the RNA polymerase II to the core promoter. RNA polymerase II is the enzyme that catalyzes the synthesis of mRNA from the DNA strand. The core promoter is considered to be the minimal promoter region that is able to initiate gene transcription, and most of the times comprised of only 40bp. Many genes have a proximal promoter just upstream the 5´of the core promoter containing more specific TF binding sites (Butler and Kadonaga, 2002).

The TATA box is typically located about 25–30 nt upstream of the transcription start site. The consensus sequence for the TATA box is TATAAA but this can vary. TATA boxes are found in a subset of core promoters and bind RNA polymerase II (Butler and Kadonaga, 2002).

Promoters regions control gene transcription in concert with other cis regulatory regions such as enhancers to control transcription of a particular gene in a specific tissue or cell type. Enhancers can work over distances of 100 kb (10 kb in flies) or more and can be located 5´ and 3´ in relation to the gene as well as in introns. A gene may have several enhancers and they are typically thought to be 500 bp in length containing numerous binding sites for TF with an activator or repressor function (Levine and Tjian, 2003). For example the expression of even-skipped in Drosophila embryo is controlled by 5 separate enhancers (Fujioka et al., 1999). There are several examples of long-range gene regulation by enhancers in Drosophila and vertebrates. In Drosophila, the cut gene is regulated by a wing margin enhancer located 100kb upstream the TSS (Dorsett, 1999). On the other hand, the decapentaplegic gene (Dpp) and its vertebrate orthologous genes are controlled by enhancers lying downstream of the coding sequence, as far as 270 kb from the promoter for the vertebrate genes (DiLeone et al., 1998; Merli et al., 1996). Long range enhancers are not found in yeast and might be a mechanism used by more diverse systems.

Studies in drosophila already a decade ago suggested that transcriptional repression is as important as transcriptional activation for cell type specification during development. The repressors can be divided into short range and long range repressors acting either locally to inhibit neighbouring activators or broadly to silence an entire locus (Gray and Levine, 1996; Mannervik et al., 1999). Hairy (enhancer of split, HES) is characterized as a long-range repressor and can inhibit activators located over 1kbp away. While knirps, a short range repressor, interferes with activators located only 100 bp away. Short- and long-range repressors recruit common corepressors, CtBP and Groucho (discussed below), thus, they will in a distinct way influence the chromatin depending on the gene context (Courey and Jia, 2001; Li and Arnosti, 2011; Martinez and Arnosti, 2008). An additional ability of repression is that it can be fast and reversible via interactions of the transcriptional machinery as a response of short-term changes in the milieu or more prolonged repressed state by the epigenetic machinery (Courey and Jia, 2001; Li and Arnosti, 2011).

The bridge between the sequence-specific activators and repressors at the enhancers and the core promoters is the cofactors which can activate, called coactivators, or repress, called corepressors, gene transcription. The TFs interact with the cofactors that either hold enzymatic activities themselves or that in turn recruit enzymes that will remodel the chromatin (histones and nucleosomes) along with the recruitment or inhibition of the RNA polymerase II transcription complex at the promoter (Mannervik, 2014). Most co-activators are associated with histone acetylation and co-repressors with histone deacetylation giving rise to an open or closed state, respectively. This process is discussed further in the next section.

Epigenetic mechanisms

The name epigenetics refers to potential heritable changes in gene expression caused by mechanisms other than changes in the DNA sequence itself. The changes remain throughout cell divisions as a memory during the cell's life and may also last for multiple generations, not to be confused with the direct regulation carried out by TFs (Chambon, 1978; Holliday and Pugh, 1975; Jaenisch and Bird, 2003). Although post-mitotic neurons lack the heritable aspect of classical epigenetic regulation, accumulating evidence argues that the mechanisms used in mitotic cells can be reused in maintenance and plasticity of neurons (Borrelli et al., 2008).

In a simple view eukaryotic genomes can generally be divided in two distinct chromatin environments; active euchromatin, where the DNA is more accessible (open) for transcription, and silent heterochromatin, where the DNA is not accessible (closed) for transcription. These two states are associated with a discrete chart of modification, the so called histone code (Jenuwein and Allis, 2001). In order to regulate gene transcription, the epigenetic machinery needs to regulate the chromatin conformation. Chromatin consists of DNA and all of its associated proteins. Nucleosomes are the core of chromatin and consist of four core histones, H3, H4, H2A and H2B which are wrapped by 147 base pairs of DNA. The histones N-terminal ends are where the posttranslational modifications occur, often acetylation or methylation. The N-terminal tails can be modified on several sites; while one acetylgroup is put on each site, the methylation can be mono-, di- or trimethyl for lysines and mono- or dimethyl for arganine adding to the complexity (Kouzarides, 2007).

The modifications have several functions: first, to either link or disrupt the connection between the nucleosomes and thereby opening or closing the chromatin, respectively. Second, to recruit proteins involved in the gene regulation. The recruitment is often ordered in series and the proteins bind via specific domains. Chromo-like domains and nonrelated PHD domains bind methylated histonse residues, the bromodomains bind acetylated histone residues. The SANT binds unmodified histone tails (de la Cruz et al., 2005). Heterochromatin protein 1, HP1, has a chromodomain and bind methylated H3K9 (H3K9me) which is associated with deacetylase and metyltransferase activity. The fact that the histone tails have multiple residues for modifications suggests the occurrence of crosstalk. For example, the binding of one protein can disrupt the binding of a protein on the adjacent residue (Fischle et al., 2005) or the binding will be more effective (Clements et al., 2003).

The acetylation modification has the best capacity to open the chromatin as it neutralizes the basic charge in lysine and thereby destabilizes the chromatin architecture. Actively transcribed genes in yeast show enrichment of acetylation marks on the promoter and the 5´ end of the coding sequence. These characteristics are also found in mouse and humans and thus appear to be conserved (Bernstein et al., 2005). Additionally of high levels of acetylation, euchromatin and actively transcribed genes typically have trimethylated H3K4, H3K36 and H3K79. Euchromatin is though more dynamic, thus, the histone modification patterns are more diverse and can change rapidly. The chromatin opening can be locally such as in the transcription of a single gene or more genome wide such as in DNA replication (Kouzarides, 2007).

Heterochromatin is defined by low levels of acetylation and high levels of methylated H3K9, H3K27 and H4K20. H3K9me recruit HP1 which play an important role in pericentric heterochromatin whereas polycomb protein PC2 is recruited by the H3K27me mark and is thought to be involved in the imprinting of the X chromosome (Ebert et al., 2006; Greil et al., 2007). Recent studies reveal bivalent regions/promotors that will have both activating and repressive marks at the same time (Bernstein et al., 2006; Voigt et al., 2013). Thus, it is more likely that context is important, a given mark can be activating or repressing depending on its position. Methylated H3K9 on the promoter is potentially repressive while on the coding region is, activating.

During the last decade the enzymes that directly modify histones have been found and most histone modifications if not all have at least one designated enzyme (Kouzarides, 2007). The

most studied histone modifying enzymes include methylases, demethylases, acetylases and deacetylases, and will be the ones that will be discussed here. The enzymes that acetylate lysines, histone acetyltransferases (HATs), represent three major families; GNAT, MYST and CBP/p300 (nejre in Drosophila) (Sterner and Berger, 2000). CBP (CREB bindning protein) turned out to interact with over 400 proteins, thus, is considered to be a general co activator that has HAT activity. p300 binding has been shown, both in mouse and Drosophila, to overlap with H3K4 monomethylation on distal gene regions associated with enhancer activity (Negre et al., 2011; Visel et al., 2009). Histone deacetylases (HDACs), removes lysine acetylation, and are associated with transcriptional repression, though they are also found on active genes (Wang et al., 2009). In fact most co repressors interact with HDACs which show no great specificity for a single acetyl group or histone. The HDAC family is divided into 3 classes, termed class I, II and III. Class I HDACs, HDAC1, 2, 3 and 8 have been shown to be involved in general gene expression, the other are thought to be involved in developmental and differentiation processes (Figure 2) (de Ruijter et al., 2003). HDAC1/2 (Rpd3 in Drosophila) is part of many co repressor complexes such as CtBP, CoRest and Groucho. HDAC3 interact with co-repressor SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and NCoR (nuclear receptor corepressor). The co repressor Atrophin (Atro also known as Grunge) interacts with class I HDACs and recruits as well the H3K9 histone methyltranferase, G9a. The co repressor binding is necessary for the enzyme activity for all class I HDACs (Guenther et al., 2001; Wang et al., 2008).

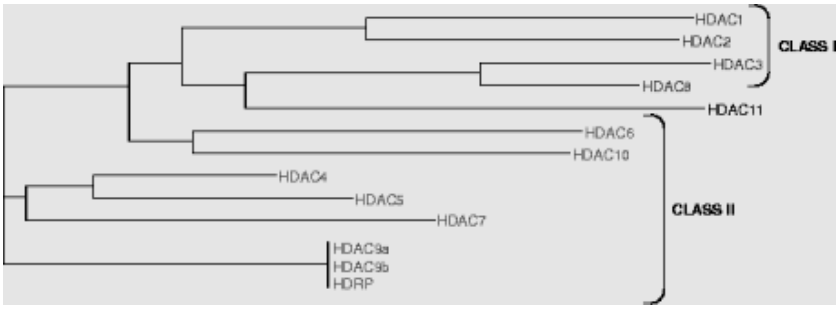


Figure 2. Evolutionary relationship between the HDACs.
Biochem J. 2003 Mar 15;370(Pt 3):737-49

The histone methyltransferases (HMTs) identified are mostly lysine methyltransferases and they display a high specificity. The demethylases are selective for mono-, di- or trimethylation, one single lysine residue is methylated on a single histone by each enzyme. Unlike acetylation, histone methylation does not alter the charge of the histones (Bannister and Kouzarides, 2005). The first HMT to be identified was SUV39H1 that targets H3K9 (Rea et al., 2000). The HMTs that methylate N-terminal lysines contain a so-called SET domain that holds the enzymatic activity. The first histone demethylase identified was LSD1 (lysine-specific demethylase 1, *Kdm1a*) (Shi et al., 2004). LSD1 demethylates H3K4me1/2 (mono-dimethylation) in complex with the co repressor CoRest thereby repressing transcription however in other complex, together with androgen receptor, it demehylates H3K9 which activate transcription. Thus the enzymes are influenced by the protein and substrate they bind and are able to switch from a repressor function to that of an activator (Klose and Zhang, 2007).

Nuclear chromatin organization

In most cell type the nuclear lamina will locate the heterochromatin to the periphery of the nucleus and the euchromatin will predominantly locate to the nuclear center (Peric-Hupkes and van Steensel, 2010). Allowing for transcription in limited areas and placing the genes in other compartments where silencing is ensured after differentiation (Pickersgill et al., 2006).

The classical division of the genome by heterochromatin and euchromatin is now changing as new studies are revealing a finer classification. A genome-wide analysis of 53 chromatin-associated proteins in an embryonic cell line identified 5 types of chromatin, that each got a color name (Filion et al., 2010). The GREEN and BLUE represents what is known as heterochromatin and includes HP1 and polycomp group (PcG) proteins respectively. Also the deacetylase Rpd3 occur in the GREEN and BLUE chromatin. Though most proteins are not limited to a single chromatin type, it's rather unique combinations of proteins. Around 50% of the genome was the BLACK class defining a new highly repressive chromatin with large domains (>100 kb) tightly coupled to the lamina and relatively limited gene content. These genes did express in other tissue and were considered to be developmentally regulated. YELLOW and RED chromatin are typical transcriptionally active domains with high levels of mRNA and RNA polymerase. H3K9me2 and H3K27me3 marks are low while levels of H3K4me2 and H3K79me3 are typically high. There are some differences though; YELLOW chromatin contains H3K36me3 marks that is absent in RED chromatin. Furthermore yellow

chromatin hold broadly expressed genes with housekeeping functions while in RED chromatin genes with specific processes and more complex regulation are found.

Another player that in the recent years have been recognized to regulate chromatin and post-transcription levels is non coding RNA, including microRNA and small interfering RNA (siRNA), and contribute to the generation of neuronal cell diversity. Small RNAs has been shown to bring histone modifiers to the chromatin (Mattick and Makunin, 2005; Verdel et al., 2004).

Selector and terminal selector genes

The final step of the integrated mature nervous system is the terminal differentiation of the post-mitotic neurons. A step that needs the activation of distinct set of terminal differentiation genes and provides the post mitotic neuron type its unique properties; such as receptors, ion channels, expression of neurotransmitters. There are several models for how this is achieved and I will shortly describe some here.

> Terminal differentiation genes are expressed in a mature, non-dividing neuron throughout the life of a neuron and determining its functional properties, such as ion channels, neurotransmitter-synthesizing enzymes, etc (Hobert et al., 2010)

The term *terminal selector gene* was first proposed by Oliver Hobert, and referred to TFs that are directly involved in the gene expression of the terminal differentiation genes and act through the cis regulatory region of these genes (Hobert, 2008). The term is an extension of Garcia-Bellidos concept of selector genes that control developmental pathways and determine the identity of a specific tissue (Garcia-Bellido, 1975). The selector genes included earlier developmental pathways and were required throughout the development to maintain the specific pathway. The concept included no specific mechanisms. Selectors, such as Eyeless, Vestigial (Vg), or Distalless (Dll), are required for the growth of their respective tissue (Mann and Carroll, 2002). Terminal selector genes are required for the identity and maintenance of a neuron type, but furthermore they need to directly regulate the expression of terminal differentiation genes, by binding to the cis-regulatory regions of those. These regulatory

regions are named terminal selector motif and are found at the promoter of terminal selector genes. The differentiated identity is maintained by positive autoregulation, i.e. the terminal selector genes bind their own terminal selector motifs and regulate their own expression. Another feature of the terminal selector gene is the feed forward loop where a terminal selector gene activate the expression of the another TF to jointly activation the terminal differentiation genes (Hobert, 2008). Examples are found in vertebrate and include *Crx* a terminal selector gene that control photoreceptor fate and acting through a small cis-regulatory motif, activates the Nrl TF and jointly specify rod photoreceptor. Photoreceptors lacking Nrl switch to become cone (Hsiau et al., 2007).

Combinatorial regulation

Studies in Drosophila and vertebrates have identified combinatorial regulation as a central mechanism for neuronal type specification in diverse cell types such as the motor neuron. This means that the properties of an individual fully differentiated neurons are not defined by a single gene rather by a unique combination of genes. It is thought that early developmental cues, such as Hh and Notch signaling, lead to the different spatial and temporal expression of early regulators, typically patterning genes or proneural TFs that act on terminal differentiation factors (post mitotic regulators) which in turn act in combination. In the motor neuron the combinatorial expression of various genes such (LIM-HD factors) Lim1, Lim3, Isl and Hb9 has been found to responsible for the fate determination (Lee and Pfaff, 2001; Sharma et al., 1998; Tsuchida et al., 1994) Binary genetic switches that operate in early post-mitotic neurons represent also combinatorial regulation. In the dorsal spinal cord, three post-mitotic TFs are involved in the choice of a GABAergic versus a glutamatergic cell fate. The homeobox gene Lbx1 defines the basal GABAergic cell fate, the Lbx1 is then antagonized by homeobox genes Tlx1 and Tlx3 that promote the glutamatergic cell fate (Cheng et al., 2005) “Combinatorial coding” allows, at least in theory, for the building of almost infinite number of combinatorial gene expression profiles. The developmental economics of this model is definite especially in higher animals. The so-called TF combinatorial control mode (Lee and Pfaff, 2001) have been challenged by the findings that TFs are rarely expressed in a restricted pattern i.e. one type of cell instead they are often expressed in multiple region in the brain (Gray et al., 2004). Rather, it is suggested that combinatorial regulation by TFs is able to define the basic pattern of the nervous system, and other context-dependent control

mechanisms argue for additional layers of regulation, such as stochastic and epigenetic regulation of gene expression, contribute to the generation neuron diversity.

Olfactory system

The olfactory system is, on the whole, an accessible part of the central nervous system and represents unique advantages for studying neuronal diversity. In contrast to most CNS structures, we know precisely what the olfactory system is designed to do. The olfaction is crucial for most animals, from insects to humans, to detect chemical signals from the environment, such as presence of food or predators. Olfactory cues are used in many social and sexual interactions between animals. Our nose can discriminate between at least 10000 different odors; theoretically based on the possible combinations of 1000 receptors it could be billions of odors

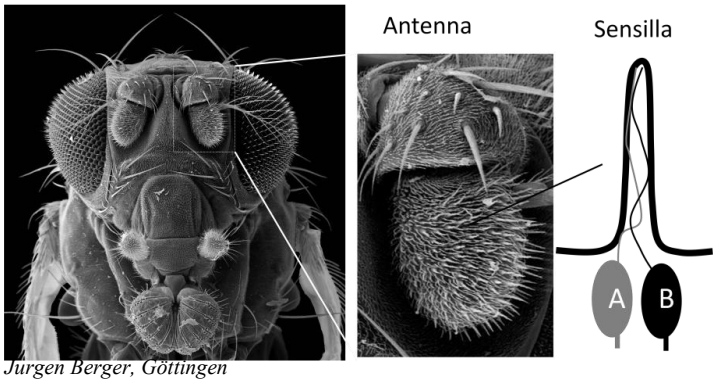
Since the initial discovery of odorant receptor (OR) genes encoding G protein-coupled receptors in rat (Buck and Axel, 1991) the molecular mechanisms governing our sense of smell have made enormous progress. Researchers have revealed a system with a precise organization that translates a chemical structure to a topographic fingerprint of activated neurons. This generates maps of chemical properties of an odour that are converted into meaningful neural information, bringing forth proper behavioural response (Laissue and Vosshall, 2008; Mori et al., 2006). Mammals and insects have solved this complex sensory perception problem in remarkably similar ways (Hildebrand and Shepherd, 1997; Vosshall, 2001). In both systems, odours are detected by large families of highly divergent ORs. Each olfactory sensory neuron (OSNs) expresses only one, or exceptionally a few, OR genes and OR proteins are located to the surface of the ciliated endings of OSN dendrites where they sense the surroundings. ORs are the main determinant of the odour-response profile of OSNs and are activated or inhibited by overlapping subsets of odours. The axons of OSNs expressing the same OR converge to specific glomeruli within the olfactory bulb (OB) in mammals or the antennal lobe (AL) in insects, and synapse with second-order neurons. Together, these findings have led to a model in which an odour is identified by the combination of ORs that recognize it, and interpreted in distinct spatial patterns of glomerular activity in the brain. A strategy that is conserved or remarkably analogous in insects and mammals.

Vertebrate olfactory system

The largest known subdivision is in the mouse olfactory system, where 1296 OR genes are identified, of which they about 1000 are functional (Zhang and Firestein, 2002). The actual detection of odours occurs in the olfactory epithelium (OE) where the OSNs are harboured. The OSNs are bipolar neurons with a dendrite ending in a knob that elaborates 20-30 cilia spaying out over the mucus covered epithelial surface with the OR proteins detecting odorants and on the other end, an axon projecting to higher brain regions. The OSNs are believed to express only a single OR gene from a large genomic repertoire (consequently 1200 x 2 OR genes). Although the neurons expressing a given OR are scattered throughout the OE the axons of the OSNs expressing the same OR gene, i.e. same OSN class, converge to a (spheroidal module) called glomeruli in the OB of the brain. The mammalian olfactory system, similarly as the invertebrates, uses a combinatorial strategy to encode chemical diversity (Malnic B et al 1999). The odours bind their cognate receptor a cascade of intracellular events is initiated that transforms the chemical energy of binding into a neural signal (that is, a change in the membrane potential of the OSN). Although still obscure in invertebrates, this process is now generally well understood in mammals and other vertebrates (Buck, 2000; Firestein, 2001).

Drosophila olfactory system

The fruit fly *Drosophila melanogaster* has two bilaterally symmetrical pairs of olfactory organs: the antennae and the maxillary palps (Figure 3).



Jurgen Berger, Göttingen

Figure 3. Olfactory system anatomy in *Drosophila.*

Odors are detected by 38 OR types expressed in 1300 OSNs compared to the mouse 1000 ORs in $>10^6$ OSNs (Vosshall et al., 1999). The insect OSNs project their cilia into hair like structures, sensilla, and here the OR interacts with the odors. A total of 410 olfactory sensilla cover the antenna, divided into three distinct morphological and functional types: basicoconic, club-shaped sensilla containing OSNs tuned to food odorants, trichoid sensilla, which are long and needle shaped and contain the OSNs tuned to pheromones and coeloconic, fin-shaped sensilla sensitive to organic acids, aldehydes and humidity levels. The sensillas fall into three subtypes and the different sensilla types are distributed in a stereotyped manner over the surface of the antenna. Basiconic sensilla are concentrated in the proximomedial region of the antenna, trichoid sensilla are predominantly in the distolateral region and coeloconic sensilla are spread broadly on the antennal surface as well as in a sensory pit known as the sacculus. Their relative position is well-conserved, as are the numbers of neurons innervating a given sensilla (Figure 4) (Couto et al., 2005; Shanbhag et al., 2001; Shanbhag et al., 2000).

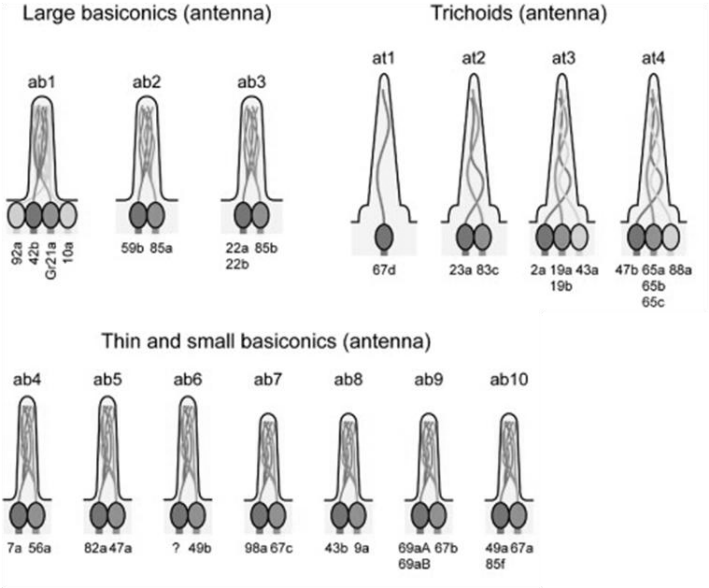


Figure 4. Organization of sensillum and OSN classes.
Curr Biol. 2005 Sep 6;15(17):1535-47

As in vertebrate olfactory system the OSNs express a single type of OR that localize to the sensory cilia, and the OSN expressing similar ORs, defined hereafter as belonging to the same OSN class, converge their axon projections to the same glomeruli in AL of the brain. In

contrast to mouse olfactory system, *Drosophila* OSNs first send their axons to the AL before the onset of OR expression (Komiyama and Luo, 2006). These around 50 glomeruli are stereotypically organized in distinct units to form a spatial map (Figure 5) (Couto et al., 2005; Fishilevich and Vosshall, 2005). These units represent different OSN classes detecting different odors from the periphery. Each glomerulus has also the dendrites of a single projection neuron, the second-order neurons that carry the information to higher olfactory centers.

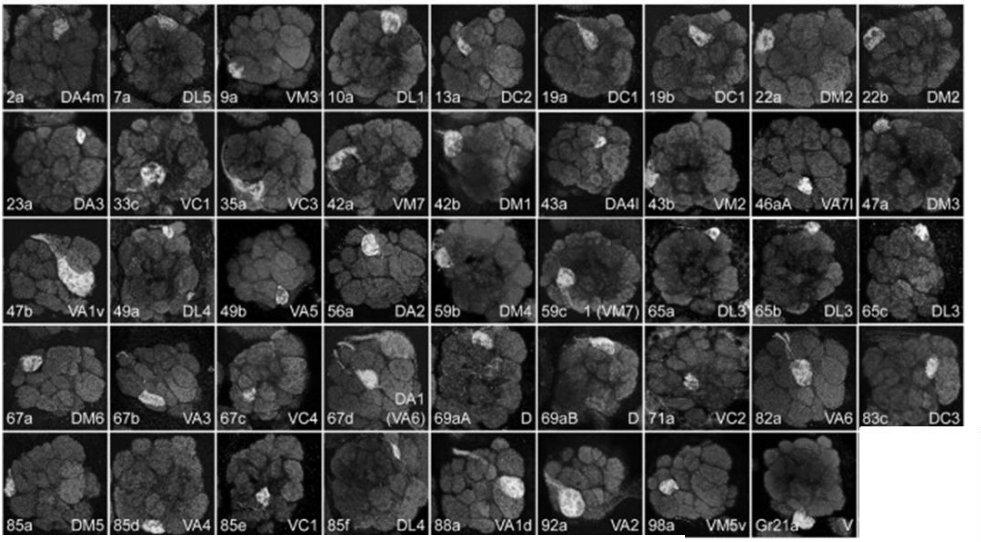


Figure 5. Map of the OSN projections in the antennal lobe.
Curr Biol. 2005 Sep 6;15(17):1535-47

The olfactory map depends on positional cues already in the antennal imaginal disc. The main peripheral part of the olfactory circuit, the third antennal segment, arises from the eye antennal imaginal disc in three temporal waves giving rise to the different 3 sensilla types (Gupta and Rodrigues, 1997; Rodrigues and Hummel, 2008) A typical sensillum holds two OSNs and three non-neuronal support cells that are generated from the sensory organ precursors (SOP) within a fixed lineage. The SOP are diversified by the output of three iterative Notch-mediated asymmetric cell fate decisions to give rise to two different OSN classes in the same sensilla (Guo et al., 1996). In the first division the SOP cells generates two different intermediate precursor cells, termed pIIa and pIIb, with Notch high and Notch low, resulting in non-neuronal and neuronal precursors, respectively, the neuronal precursor cell divides again asymmetrically giving rise to a second generation of intermediate neuronal

precursors termed pNa and pNb. The pNa and pNb go through a third round Notch-dependent asymmetric division giving rise to four different postmitotic neurons, Naa, Nab, Nba and Nbb (Endo et al., 2007). Furthermore, Endo and colleagues discovered *Hamlet*, a downstream effector of Notch which in a context-dependent manner erases the Notch state, enabling for the subsequent rounds of notch signaling in the sensillum lineage. Hamlet acts directly on chromatin modification to repress Notch targets. Loss of *Hamlet* causes a switch in OSN differentiation Nab to Nba (Endo et al., 2012). As mentioned, in most cases a sensilla harbor two paired OSNs, the Nab and Nba, consequently the Naa and Nbb is subjected to cell death, apoptosis.

Additionally, mapping of Notch off and Notch on OSNs revealed that OSN targeting to distinct glomeruli in the AL was also influenced by Notch signaling. If Notch signalling is disrupted the targeting of the OSNs in the same sensilla to different glomeruli is lost and both OSNs target the same glomeruli, the Notch off glomeruli (Endo et al., 2007). Thus, Notch signalling provides the OSNs different guiding molecules to allow for the correct targeting within the lineage. Another early player in the OSN targeting is Hh signalling. Already in the imaginal disc the epithelium divides in two compartment, one with high Hh signaling levels and one with low Hh signaling levels and the SOPs specified will give rise to OSNs that project to distinct parts of the AL, Ptc-positive and Ptc-negative domains (Chou et al., 2010). Thus, the Al exhibit 4 overlapping domains with different levels of the early developmental signals, Notch and Hh. Furthermore, in most species the glomeruli with comparable receptors seem to be clustered in the AL, which is not the case in the sensory epithelia where individual OSN classes have their cell bodies intermingled with other OSN classes. A dispersed organization in the periphery and clustering centrally could enhance odor detection sensitivity and accuracy (Couto et al., 2005; Mori et al., 2006).

Odorant receptors

Mammalian ORs are members of the Class A rhodopsin-like G protein–coupled receptor (GPCR) superfamily. The OR gene families in vertebrates are highly divergent and encoded proteins display not more than 20 % amino acid identity. They are classified into two classes, which converge to two distinct domains in the OB (Bozza et al., 2009). GPCR signaling as the name implies via G proteins and second messengers as cAMP and Calcium influx. The

Drosophila ORs bear no homology to vertebrate ORs, although both have a predicted seven-transmembrane (TM) domain structure (Mombaerts, 1999). They are classified as ORs since they express in the olfactory system and have somewhat conserved region in the seventh transmembrane domain. Insect ORs adopt an inverse membrane topology compared to GPCRs, with their N-termini located intracellularly (Benton et al., 2006), indicating a possibility of a different odour signal transduction than vertebrate.

In vertebrates, the OR proteins have been detected at the OSN axon terminal and experiments suggest that they are involved in OSN axon targeting to the specific glomeruli (Barnea et al., 2004; Strotmann et al., 2004). Homotypic interactions between the neurons expressing the same OR are thought to promote the convergence of the OSNs. This is not the case in insects, in *Drosophila* axon guidance is prior to the onset of *OR* gene expression that are concentrated exclusively in the dendritic endings of the OSNs (Dobritsa et al., 2003). *OR* gene expression takes place in the second half of OSN development and as a final step in OSN differentiation. The involvement of ORs in vertebrate axon guidance is likely an additional mechanism to assure the correct targeting and perhaps a result of a larger olfactory system to instruct.

The first functional study of an odorant receptor was the characterization of the *C. elegans* receptor odr-10, for which the identified ligand was diacetyl (Sengupta et al., 1996). Further dissection in mechanisms of odorant receptor progressed poorly due to the difficulty of expressing the ORs in heterologous systems suitable for mutational analysis. The OR protein, while produced in transfected cells was trapped in endoplasmic reticulum, Golgi and endosomal compartments and no OR was transported to the cell membrane. This observation and the fact that the OSNs are able to express cloned ORs on the surface led to the assumption that olfactory-specific chaperone or co-factor is necessary for functional receptor expression. The identification of the ubiquitous expression of the conserved co-receptor Orco (also known as 83b) solved the issue at least in insects (Benton et al., 2006). Orco has a similar structure as GPCR but with an inverted positioning in the cell membrane with the N-terminal intracellularly, like the specific OR in insects. Most if not all ORs are expressed together with Orco and the two proteins associate via their cytoplasmic domains. In fact, the formation of the ORX/Orco complex is critical for the localization to and maintenance in the sensory cilia. Homologous of Orco have been identified in other insect species but not in mice. Flies lacking Orco exhibit severe defects in there olfactory behavior and physiological response to odors (Larsson et al., 2004). Thus, the insect specific OR/Orco complex is interesting as a target for highly selective insect repellents and interference of insect disease vector.

The principles of insect odor recognition and transduction are still on debate. The question is if there is any G protein coupled signaling. Benton et al have suggested that *Drosophila* and other insect ORs are heteromeric ionotropic receptors that exhibit odor-dependent ion channel activity without any need of cyclic nucleotide second messengers (Benton et al., 2006; Sato et al., 2008). This hypothesis is in contrast to vertebrate where it is second messenger pathways that transduce the signal and is thought to be for the reason that the ligand-gated ion channels can respond faster to odor concentration changes in air when the animal is flying. In contrast, others have put forward that odor recognition is composed of both a specific G-protein coupled OR sensitive to the odor as well as a common co receptor, which is the essential subunit of an odorant-gated ion channel that results into a rapid response. This will ensure the distinction between high odor concentration recognition by the ionotropic pathway and a slower but prolonged more sensitive response by the G-protein mediated amplification and synthesis of cAMP (Wicher et al., 2008).

In *Drosophila*, ORs is one of two major receptor types expressed in OSNs in the antenna, the second type, ionotrophic receptors (IRs) have similar modular organization to ionotropic glutamate receptor-like genes (iGluRs), but are highly divergent just like the ORs. The IRs are expressed in the OSNs in coeloconic sensilla and the do not co express Orco, instead the broadly expressed *IR8a* and *IR25a*, which encode closely related receptors functions as coreceptors. The OSNs expressing IRs respond to defined odour classes as water, acids, and amines (Benton et al., 2009).

OR gene choice; the one receptor – one neuron hypothesis

The *OR* gene choice can be considered from two perspectives: how an individual neuron selects which *OR* gene to express? How the sequences flanking an individual *OR* gene dictate its expression in particular neurons?

Two regulatory models are often proposed to explain the problem of receptor gene choice - a deterministic model and a stochastic model; The deterministic model include a combinatorial code of transcription factors and regulatory elements, an individual gene is selected in the neurons to produce a precise organization such exist in *Drosophila*. The stochastic model includes an individual gene selection by a singular process that only can act at one gene at a time. A stereotyped receptor-to-neuron map has not been found in mammals and though the

OR that can be expressed in a given OE zone can be specified by a deterministic choice, a stochastic mechanism of the final receptor gene choice appear to govern in the mouse. These two regulatory mechanisms are further described in the next section.

Stochastic OR choice in mammals

The stochastic choice is known to be accomplished via several mechanisms operating together in concerted fashion. In the vertebrate immune system, DNA rearrangement generates an enormous diverse set of T cell receptors on T cells and immunoglobulins on B cells (Market and Papavasiliou, 2003). Alternative splicing, such as the Down Syndrome Cell Adhesion Molecule (DSCAM) gene that displays over 30 000 isoforms and expresses different subset in each neuron (Neves and Chess, 2004).

In mammal olfactory system it is thought that transcriptional regulatory mechanisms direct expression of OR genes in constrained zones of the olfactory epithelium, but within a zone *OR* gene choice is based on a stochastic selection mechanism which is followed by negative-feedback inhibition. Only a single allele of an *OR* gene is expressed in an OSN in the OE. How exactly the monoallelic OR expression is achieved is not known but involves a mechanism in which OR protein functions in olfactory neurons abrogate expression of other OR genes (Serizawa et al., 2000; Serizawa et al., 2003) . Two general TFs have been identified, Lhx2 and Emx2, which have binding sites (homodomain sites) upstream the mouse *OR* genes and are required for complete development of one OSN class and *OR* expression (Hirota and Mombaerts, 2004; Kolterud et al., 2004; McIntyre et al., 2008). Little is though known about the regulatory genes that directly specify mouse ORs.

Mammalian *OR* gene expression depend on both short-range control exerting its effect within ∼160bp (Rothman et al., 2005; Vassalli et al., 2002) and long-range control by sequence elements as far away as ∼200 kb from the *TSS* (Plessy et al., 2012; Serizawa et al., 2000) or even on another chromosome (Lomvardas et al., 2006). The model proposed by Lomvardas and coworkers suggested that the *H* enhancer associates in cis and trans with active OR genes, although the deletion of the *H* turned out later not to effect most ORs.

The short cis regulatory sequence upstream the *OR* gene *TSS* produce expression in the OSN but not as restricted as the endogenous OR. This studies together with the fact that the short elements have conserved feature, such as homodomains and O/E-like sites, the EBF-binding motifs which are strongly enriched argue for a compact *OR* gene promoter (Clowney et al.,

2011; Hoppe et al., 2006; Plessy et al., 2012; Vassalli et al., 2002). The promoter studies also revealed an extremely AT-rich sequence, a feature suggested for genes with high evolutionary plasticity and compatible with non-deterministic gene choice.

In mammals, as mentioned above, the ORs take part to secure the singular expression of ORs in OSNs and a stable repression of the rest of ORs is crucial and still not very well understood, though proposed to involve the epigenetic machinery. As the OSN is maturing, the *OR* genes are marked with H3K9me3 and H4K20me3, hallmarks for constitutive heterochromatin, silencing the chromatin. At the time for OR choice the silencing is reversed for only one *OR* gene to allow single OR expression. The active OR allele is marked with H3K4me3 (Lewcock and Reed, 2004; Magklara et al., 2011). Thus, perhaps the feedback mechanism thereby prevents the desilencing of other OR alleles. LSD1, the histone demethylase with the enzymatic activity for demethylation of both H3K9me2 and H3K4me2, was recently reported to be involved in the feedback mechanism. LSD1 derepress one selected *OR* gene expression that induce expression of adenylyl cyclase (Adcy3) that subsequently advance OSN maturation and downregulates LSD1. Removal of Adcy3 increases *OR* gene switching (Lyons et al., 2013).

Constitutive heterochromatin: hallmarks; H3K9me3 and H4K20me3. Moslty found in pericentomeric and telomeric repeats and remains compact during cell cycle and differentiation (Fodor et al., 2010). *Facultative heterochromatin*: hallmarks; hypoacetylated, H3K9me2 and H3K27me2/3. Found on silenced genes it is dynamic and developmentally regulated (Trojer and Reinberg, 2007).

Another feature supporting that silencing is critical in the control of OR expression is the overrepresentation of the Polycomb complex and chromatin remodeling transcripts in the not fully specified OSN (Sammeta et al., 2007). Additionally, Clowney and colleagues showed recently that silent *OR* genes from all chromosomes aggregated and localize to the periphery of the nucleus (Clowney et al., 2012). The active OR has limited heterochromatin markers and localize in euchromatin regions. Expression of the lamin B receptor in OSNs results in chromatin decompaction as well as coexpression of a large number of ORs and disruption of axon targeting. Thus, demonstrating that nuclear organization is critical for OR singularity.

Deterministic OR choice in *Drosophila*

Here, I will describe some of the findings prior to our work. In *Drosophila*, a negative feedback mechanism is for the moment ruled out since OR ectopic expression does not lead to alteration of the endogenous OR. The specification of OSNs is rather a consequence of lineage diversification as mentioned above that result in highly stereotypic expression. Studies on the regulatory logic of *OR* genes have suggested that a combinatorial code ensures strict organ *OR* gene expression. The Oligo-1 and Dyad-1 elements which are found upstream of *OR* genes in the maxillary palp, can promote *OR* expression in the maxillary palp and at the same time repress the antennal *OR* expression (Ray et al., 2007). Specific TFs that bind these elements await identification.

Before our screen only a few TFs was known to be involved in OSN specific expression of *OR* genes in Drosophila; *acj6, pdm3, lozenge*, and *Scalloped* (Bai et al., 2009; Clyne et al., 1999; Ray et al., 2007; Tichy et al., 2008). Acj6 (Abnormal chemosensory jump 6) is a POU-domain TF and was the first gene identified to control *OR* gene expression in a subset of OSNs in maxillary palp (Clyne et al., 1999). The binding site was found upstream of *OR* genes that were dependent on it for expression and mutation of the sites result in loss of expression (Bai et al., 2009). *Acj6* was also shown to have repressing function on some OR gene expression, possibly due to different splicing variant (Bai and Carlson, 2010). Pdm3, also a POU-domain TF, is expressed in a subset of OSN classes and is needed for expression of *ORs* in maxillary palp and only one *OR* in the antenna (Tichy et al., 2008). The binding site of the AML-1/Runt-like TF Lozenge (Lz) was predicted to be present upstream and downstream of some maxillary palp OR genes. A reduction of *OR* expression but not a total loss was seen in *Lz* mutant adult flies (Ray et al., 2007). A functional role for *lz* in *OR* expression that suggests that levels of expression can be altered after eclosion, perhaps via epigenetic modulation of odour receptor expression in *Drosophila.* Scalloped (Sd), a TEA domain transcription factor, was identified to play a repressor role in the maxillary palp OR. *Sd* is needed for expression of *OR* in correct OSN class within the same sensilla (Ray et al., 2008). *Acj6* and *Pdm3* have been found to genetically interact in *OR* gene expression and are essential for precise axon targeting of a subset of OSN classes. Results that indicate an overlapping developmental mechanism and a combinatorial code of POU gene TFs in OSN class specification (Tichy et al., 2008).

A classical approach that is used to delineate cis-acting DNA sequences is to cut out segments of variable sizes from the genome and test their ability to drive correct expression of a marker in cultured cells or transgenic animal. In *Drosophila*, it seems that short, proximal promoters control *OR* gene expression. A study of trichoid sensilla ORs demonstrated that relatively small cis-regulatory sequences (around 500bp) upstream of the *OR* gene are sufficient for expression. However, require multiple repressive functions to limit the expression to the correct OSN class (Miller and Carlson, 2010). A mechanism we further examine in paper I and II. Small regulatory regions may be caused by the fast and very plastic growth of the gene family through duplication and divergence. Analysis of the 500 bp regions upstream *OR* genes revealed remarkably little conservation, much less than has been observed upstream of mammalian *OR* genes. The flies are more dependent on their olfactory system (Asahina et al., 2008), thus, it is constantly evolving in response to change in the environment.

Little is known about the epigenetic mechanisms working in concert with TFs to regulate *OR* expression, except of the Atro complex (paper II), the multiprotein MMB/dREAM complex was identified in the regulation of the carbon dioxide receptor, Gr63a in the antenna. One of TFs in the complex, *Myb*, is required post developmentally to maintain receptor expression despite the lack of a DNA bindning domain. Myb binds indirectly the chromatin via the chromatin modifiers of the MMB/dREAM complex. The promoters of *ORs* in the antenna was found to be enriched by the repressive H3K9me2, a mark that is catalyzed by the histone methyltransferase Su(var)3-9. Consistent with these observations a genetic interaction between *myb* and *Su(var)3-9* was identified, where Myb opposes the repressive effects of Su(var)3-9 as a result the Gr63a is expressed (Sim et al., 2012).

Consistent with these reported data are examples from other diverse sensory systems such as the visual system. The analysis of rhodopsin gene cis-regulatory region in the *Drosophila* eye revealed a proximal region acting as a core promoter and a distal region acting to specify the expression to the right cell-type (Fortini and Rubin, 1990). In addition, several genes have been identified to act positively or negatively in the regulation of *opsin* gene expression (Morante et al., 2007). An example of dual regulation by a factor is also in the eye where Ots, a homodomain transcription factor acts as an activator for the rhodopsin genes *rh3* and *rh5* but represses *rh6* in other photoreceptor cells. It exerts its effect through a common bidning site, likely depending on cofactors that are available (Tahayato et al., 2003). Furthermore, the expressed rhodopsin ensures it singularity in the photoreceptor cell by a negative feedback mechanism (Vasiliauskas et al., 2011).

Aim

Recent findings have provided us with a high resolution of the neuronal types and complete olfactory map of the olfactory system in *Drosophila*. The molecular mechanisms behind the unique neuron identity are still not well understood. With the use of the available genetic tools and platform, the aim of my PhD project has been to address the following key questions:

How are various neuron classes produced? Is there a combinatorial code of transcription factors required for *OR* expression? ***Paper I***

How does a cell keep its cell fate? What role does the epigenetic system play to reinforce the cell fate? ***Paper II***

How can single neurons within a class change, so called neuron plasticity? What are the molecular pathways involved? **Paper *III and IV***

Materials and methods

The *Drosophila melanogaster* olfactory system is an excellent model for investigating the logic behind olfactory perception and diversification in the nervous system. The anatomic architecture of the *Drosophila* olfactory system is surprisingly similar that of the mammals (Strausfeld and Hildebrand, 1999), despite the 500 million year of evolution that separate the two. However, the drosophila olfactory system is anatomically and genetically simpler than that of mammals. Furthermore, a major advantage of *Drosophila* as a model organism is that it amenable to a large toolbox for genetical manipulation. *Drosophila* has been used in genetical studies since the early 20th century and is a well established system. Due to its rapid life cycle, well known genetics, effective use of GAL4/UAS trans-activation system, a full sequenced genome and a number of stock-, antibody-, and DNA centers, it has become a very useful model system for studying early embryogenesis to adult behavior. On cell biology level all the transcriptional regulators and signaling pathways are well conserved between insects and vertebrates, as studies have been shown to play surprisingly similar roles. At a molecular level, out of 2300 human disease genes identified, 70% have clear orthologues (http://superfly.ucsd.edu/homophila/). This means that we can by studying biological processes in insects understand human biology.

TARGET system.

The *GAL4/UAS* system is a standard technique for studying biological questions in *Drosophila*. The transgene expression can then only be targeted in the either the desired time or space, but not both. The TARGET system offers a method to direct transgene expression both temporally and spatially in the same time. Here the conventional *GAL4*-upstream activator, UAS, is regulated via a temperature sensitive *GAL4* repressor, GAL80^{ts} (McGuire et al., 2004). In the permissive temperature (18°C) the GAL4 transcriptional activity is repressed while at the restrictive temperature (29°C) the *GAL80^{ts}* is inactivated and repression is relieved.

OR markers

One main determinant for how informative a genetic experiment is pertains to the specificity of the available markers. This is one great benefit of the *Drosophila* olfactory system. *Drosophila* contains two olfactory organs, the antenna and maxillary palps that together

harbor 1300 OSNs. Each OSN expresses only one of the 60 *ORs* present in the *Drosophila* genome. Previously, Couto et al coupled each *OR* predicted promoter regions with the open reading frame encoding a fusion protein consisting of the *mCD8* extracellular and transmembrane domains and four tandem copies of *GFP*. The constructs are then inserted randomly in the fly genome and despite that the predicted promoters vary in sequence length they faithfully express in the right neurons (Couto et al., 2005). Consequential, the neurons expressing one specific receptor can be visualized from the scattered expression in the olfactory tissue to how their afferents all converge upon the same target in the olfactory centre, forming so called glomeruli. By this means an extensive anatomical, molecular and functional map of the *Drosophila* olfactory system are formed, This methodology allows for direct visualization of changes in i) establishment of neural identity during neurogenesis (loss or gain of *OR* expression), ii) axon guidance and iii) target and synapse formation.

RNAi methodology and Reverse genetic screening

This project rests on a revolutionary whole genome *Drosophila* inducible RNA interference (RNAi) library, first established in the Dickson laboratory (Dietzl et al., 2007). We have taken use of the multiple RNAi libraries around the world; Vienna Drosophila RNAi Centre (VDRC), DGRC Kyoto Japan, Bloomington Drosophila Stock Center (BDSC).

Traditionally forward genetic screening have been the method which genetics used find genes involved in biological processes. The method involves random mutagenesis, scoring of the desired phenotype and the identification of mutated gene by sequencing. This approach was time-consuming and genes with function early in development and other tissue could be missed. With the availability of complete genome sequences and the ability to inactivate gene function a systemic exploring by reverse genetic have arisen last decade. A gene that is predicted to be involved in a certain process can be inhibited and the phenotype outcome investigated.

The inducible RNAi method is based on the principle of endogenous gene silencing mediated by the siRNA system upon expression of a double stranded RNA or by microRNA (Lee et al., 1993; Pillai, 2005). The approach was first developed in cultured cells and in vivo in plants and *C. elegans* (Fire et al., 1998). In these in vivo models the RNAi was systemic and could not be restricted to single cell types. In *Drosophila*, RNAi is cell autonomous (Roignant et

al., 2003), and combined with the *GAL4-UAS* system for controlled gene expression; it allows the silencing of the gene of interest in almost any tissue. There are major advantages of this technique compared with the classical random mutagenesis screen. First, it circumvents lethality and positional cloning, thus preventing two major obstacles in classical random mutagenesis screens and reduces the work after the screen substantially. Second, the RNAi library makes it possible to choose groups of genes to screen, addressing specific questions. This property also makes the system flexible with possibilities to include interesting genes at anytime point also during the phenotypic analysis, thus facilitating addressing any questions along the way. However, no techniques are perfect and the main draw backs of RNAi is the risk of off targeting effects and that the knock down are not always complete, thus making the function of some genes difficult to reveal. To circumvent these draw backs we use different RNAi lines and when possible assess the knock down with RNA in situ hybridization or antibody staining. Furthermore, to increase the RNAi knock down efficiency, we have been using a sensitized background i.e. over expressing *Dicer2* which is the effector molecule of the RNAi machinery (Bernstein et al., 2001).

Paper I

In this paper we set out to identify the transcription factors (TFs) that are required for each OSN class to express the correct *OR*, the final step in specification. Although, some factors, *Acj6* and *Pdm3*, have been identified, and studied foremost in the maxillary palp of *Drosophila* (Bai et al., 2009; Ray et al., 2008; Tichy et al., 2008). Having a clear picture of the *Drosophila* receptor-neuron-glomeruli map we argued that a systemic approach to the specification of the olfactory system was doable. The completion of the *Drosophila* genome sequence and the ability to predict DNA binding factors that might function as TFs have resulted in a large amount of transgenic UAS-driven inverted repeats (IRs) for the annotated putative TFs in Vienna Drosophila RNAi Center (VDRC). Using the *pebbled-GAL4* that expresses in post mitotic sensory neurons we were able to knock down the gene late in development, since the aim was to identify late regulators of OR gene choice. A systematic RNAi screen was performed with all available putative TFs in Drosophila, 611 in total (www.flytf.org). As markers for *OR* expression we utilized the *OR* promoter fusions with *CD8:GFP* for two basiconic OSNs and two trichoid OSNs.

Principal findings

We identified 7 TFs that lead to a strong and penetrant loss of *OR* expression. The factors belong to different TF super families. Acj6 (POU-homeobox; Hox), E93 (Psq likehelix-turn-helix; HTH), Xbp1 (bZIP), Onecut (cut-Hox), Sim (PAS-bHLH), Fer1 (basic helix loop helix; bHLH) and Zf30c (C2H2; zinc finger). The last two genes, *Fer 1* and *Zf30c*, were not previously characterized. In situ hybridization and immunhistochemistry showed that the seven TFs are expressed in overlapping patterns in the adult fly antenna. The patterns were not direct restricted to domain or sensilla groups and *acj6* and *xbp1* were ubiquitously expressed in the whole antenna. The broad expression suggest that the factors are part of a regulatory network of *OR* gene expression and not sensilla and OSN lineage specification. Analysis of both the pan neuronal marker Elav and Neuroglia in the knock down of the 7 factors demonstrated no change in neuron identity and number. Furthermore, TARGET system experiments revealed that the factors are continuously required in the adult fly for expression of the *ORs* that were analyzed. One OR, *Or92a*, required most of the TFs identified for expression suggesting a combinatorial regulation. Furthermore, the broad expression of the TFs indicated that all ORs in the antenna are regulated by the 7 TFs. An

expansion of the RNAi analyses to the majority of ORs expressed in the antenna revealed that the 32 ORs tested require at least one of the seven TFs for expression and for most of them, indeed, multiple TFs are required in different combinations for the expression of the 32 ORs tested. 17 of the 32 ORs required unique TF combinations. The TF requirement demonstrated no correlation to OSN class location or sensilla group.

Next we asked if the identified TFs directly regulate *OR* gene expression by binding to upstream DNA sequences. Acj6, Xbp1 and Onecut have known DNA binding motifs in vertebrates and a bioinformatics search for the motifs upstream of OR genes were performed. The search revealed that most *OR* promoters included at least one binding motif for the required TF. It became also obvious that the region most proximally upstream the OR gene contained highest motif density and high sequence conservation in most ORs. Transgenes with only the proximal region of the promoter expressed in the right OSN class however half of them were also ectopically expressed in other OSN classes, indicating that the distal region acts to repress expression in some OSN classes.

Having identified the binding motifs for the TFs we constructed transgenes with different regions of the *OR* regulatory region. Mutating one motif in one *OR* promoter resulted in loss of expression for that particular *OR* in vivo, demonstrating that the motifs are necessary for OR expression. Another finding in the bioinformatics analysis was binding motifs for Xpb1 downstream the TATA box in *ORs* that did not show any *Xpb1* requirement. Mutating that particular Xpb1 binding motif resulted in ectopic expression in the antenna, indicating that dual role of TFs in OR expression can be direct defined by the position of the binding motif.

Conclusion and Discussion

Within this paper we performed a systematic analysis to demonstrate how TFs specify OSN class identity. The identified TFs belong to separate transcription factor super families with diverse DNA binding properties, most likely to avoid unspecific binding to other TFs motifs upstream non regulated OR genes. Two of the identified factors, Acj6 and Onecut, have two DNA binding domains. Onecut binds DNA with both the homeodomain and the Cut domain doubling the regulatory complexity, and is involved in regulation of some aspects of neural differentiation or maintenance in the eye (Nguyen et al., 2000). Vertebrate onecut orthologues bind DNA with the homeodomain and cut domain (Lannoy et al., 2000). Acj6 bind a homeodomain and a helix-turn-helix POU domain with two separate binding motifs either an ATAATTAAT motif or ATGCAAAT motif, respectively (Gruber et al., 1997; Klemm and

Pabo, 1996). In mouse OR gene promoter mapping an over-representation of homodomain, POU and EBF1 TF binding site motifs (Plessy et al., 2012).

Further analysis suggests that the identified TFs to be terminal selector genes for OR expression. First, the seven TFs are expressed in the mature OSNs and continuous expression of all TFs are required for OR expression and maintenance in the OSNs. Second, the binding motifs for the TFs are found upstream of the *OR* genes and the motifs are necessary for OR gene expression. It remains to be shown if the TFs can autoregulate themselves. Last, all 32 tested ORs require different combinations of the TFs for expression. The findings this far indicate that the large number of OR expression are achieved by a combination of a few TFs that function as OR terminal selector genes.

Our *OR* gene promoter analysis revealed that the identified TFs motifs are enriched within a short region immediate upstream the *TSS.* The short cis regulatory regions or proximal promoter are relatively conserved and sufficient for OSN expression. Similar enrichment of motifs has been found upstream mouse ORs TSS and these short cis regulatory regions were able to produce expression in the OSN (Plessy et al., 2012; Vassalli et al., 2002). Hence, there are related regulatory principle between vertebrates and *Drosophila* which could reflect a similar evolution process of the two *OR* gene families. Interestingly, the short cis regulatory regions are sufficient for expression but exhibit ectopic expression in other OSN classes, which indicate that restricted expression is due to repression from distal parts of the cis regulatory regions. By mechanisms that not well known but most probably include repressor factors. One such repressor complex is the dREAM complex that was found to control expression of carbon dioxide receptor to the correct OSN (Sim et al., 2012). Another is the Atro complex discussed in paper II. Further analysis showed that the TFs are not only necessary for receptor expression but also play a role in transcriptional repression. The function depended on where the bindning motif is located and most likely by the available co factors and other chromatin modulators. We conclude that a dual role of a relatively small set of factors can specify the class of neuron that will be expressed, producing diversity and making the regulation more economical.

OSN axon targeting in *Drosophila* does not require *OR* expression, but could share the same regulatory network used for *OR* expression. *Acj6* acts both on OR expression and OSN targeting and provides an example of a link between the two processes (Ray et al., 2008). This duality is economical allowing for each factor to act in two processes and reduces the number

of required factors. A feature that may be critical in the control of such a large number of *OR* genes in a large number of OSN classes. Our screen was carried out in post mitotic neuron and could therefore not elucidate this particular issue.

The regulatory machinery of an animal consists of many networks such as the ones described above. It controls the spatial and temporal differential expression o huge number of genes that is crucial for development. The similarities but also the differences in the regulatory machinery of organisms carry noteworthy information. The similarities are often critical evolutionary conserved components of developmental networks that give us the general principles. The differences are the key to understand mechanisms underlying phenotypic variations between animals. Possibly, when the receptor gene repertoire expanded in vertebrate evolution a deterministic choice became too complex regulatory and not sufficiently reliable, thus the deterministic mechanisms were replaced by a stochastic mechanism and a negative feedback mechanism.

Paper II

In vertebrates, there is increasing evidence that chromatin-mediated silencing is involved in the olfactory gene expression (Magklara et al., 2011; McClintock, 2010). By other words, the correct expression of one *OR* might be a result of silencing of all other *ORs*. Interestingly, our previous findings revealed a dual role for the TFs identified as activators and repressors Further, we found several cases where a surprisingly short promoter fragment is able to drive the *OR* expression, not only in the expected cells but also in cells in areas where the *OR* normally is not expressed (paper I). These findings suggests that there are repressive information in the longer fragment of the promoter used to silence expression in off target cell and argue for an involvement of different cofactors in the different OSNs.

To identify molecules that act together with the TFs to specify OR choice, we performed an RNAi screen for genes encoding epigenetic factors, that positively or negatively influenced *OR* gene expression. By bioinformatics we have pooled the biochemical and genetic available data and generated a set of conserved epigenetic factors that are: i) histone modifying enzymes ii) cofactors, genes that defines the complexes like sant domains iii) genes that homes the enzyme complexes to the right position on the chromosome like transcriptional cofactors and histone binding proteins.

An *Or59b* construct consisting 680 bp faithfully drives the reporter gene expression in a pattern very similar to the endogenous expression of *Or59b*. Whereas, we previously found that short proximal cis regulatory region of *Or59b* result in ectopic expression in a second OSN classes. Thus, *Or59b* require repression by the distal region to restrict expression to the correct OSN class. We used the *Or59b* promotor fused to *CD8::GFP* as our marker. By this, we could knock down an epigenetic factor and directly look at the Or59b OSNs in the antenna and see if we could "lift" any of the repressive events and get ubiquitous non-specific expression of the OR

Principal findings

RNAi lines representing 150 genes were screened, 14 were lethal and 32 candidate genes with an effect on *OR* expression was identified. The candidate genes were divided into two phenotype groups; one group with *more GFP expressing cells* and second group with *fewer GFP expressing cells*. Molecularly, the candidate genes were found in all different categories of epigenetic factors as well as some genes with unknown function. Histone modifying

enzymes such as acetylases, methylases as well as co-factors were found in both groups. Atrophin (Atro), a transcriptional co-repressor, was one of the most prominent phenotype in the group *more GFP expressing cells* and could consequently be involved in the restriction of *OR* expression.

Staining of the antenna with an Atro antibody showed the Atro protein is expressed broadly and in variable levels in the OSNs, indicating a possible broad function in regulation of *OR* expression. In a knockdown of *Atro* also *Or67c* showed ectopic expression in antenna. The additional cells stained positively with the pan neuronal marker Elav, indicating an induced switch of *OR* expression within the OSN fate. Examining the projections, additional glomeruli was observed in the AL. According to the OR glomeruli map we suspected it could be the projection of Or59b OSN class. By RNA in situ hybridization we were able to verify the co expression of *Or67c* and the *Or59b*, demonstrating that Atro is required to separate the expression of these two *ORs* to each OSN class. When expressing a functional form of *Atro* a loss of *Or67c* expression was observed, pinpointing a repressive function of Atro. A deletion of the distal cis regulatory region of *Or67c* was constructed and resulted in similar ectopic expression as the *Atro* knock down. Additionally, the *short Or67c* construct was not sensitive to over expression of *Atro*, demonstrating that Atro repress *OR* expression via distal cis regulatory regions.

Next, we performed a systematic analysis with in situ hybridization of all basiconic ORs in the antenna. Surprisingly, several *ORs* were lost in *Atro* knockdown whereas some showed increased expression similar to *Or67c*, implying that Atro has two different functions. The phenotypic results were assembled in a regulatory matrix. Consequently, from the matrix a correlation of Atro phenotypic groups with Notch OSN fates, Nab and Nba, were found. Disturbing Notch signalling, thus asymmetric division, typically give rise to a paired OR expression. No paired OR expression was observed indicating that Atro is functioning downstream of Notch signalling. From the co-expression of *Or67c* and *Or59b* it seems that Atro segregate *OR* expression within the Nba OSNs. The two function of Atro is temporally distinct, cause TARGET experiment demonstrated that Atro is required before *OR* expression to produce or establish the final Nab OSN fate while Atro is continuously required for correct *OR* expression in the Nba OSNs.

Atro is associated with nuclear receptors and the N-terminal region can bind the class I HDACs and recruit the H3K9 histone methyltransferase, G9a, to the nucleus thereby silencing

the target gene (de Ruijter et al., 2003; Wang et al., 2008). Having identified both *HDAC3*, a class I HDAC, and *G9a* belonging to the same phenotypic group as Atro in the screen. We asked whether Atro is exerting its effect via the epigenetic machinery and took an extra look at the histone acetylation mark. *HDAC3* knock down rescued the repression of *Or67c* in Atro over expression, demonstrating that HDAC3 is part of the Atro complex in the OSNs. Immunohistochemistry of antenna with the H3ac antibody confirmed that Atro controls H3ac levels in the OSNs. Global H3ac levels in nucleus were increased in the *Atro* knock down indicative of diminished HDAC activity and failure to target chromatin. While a decreased H3ac level was observed in *Atro* over expression. If the fate of OSNs is controlled by changing the global acetylation levels we wanted to determine the overall level of histone acetylation profiles of wild type OSNs. A quantification analysis of endogenous global H3ac levels in several OSN classes revealed segregated levels, specifically in the Nba OSNs expressing *Or59b* and *Or67c*. Furthermore, we noticed that Nba OSNs displayed in general higher H3ac levels than the Nab OSNs.

Conclusion and Discussion

In paper II, we focused on the cofactor Atro, also known as Grunge. The Atrophin proteins are conserved nuclear corepressors and includes vertebrate Atrophin-1 (ATN1), vertebrate arginine glutamic acid repeats encoded protein (RERE) and *Drosophila* Atrophin (Atro). Human ATN1 is a causative factor for development of the neurodegenerative disease dentatorubal-pallidoluysian atrophy (DRPLA) associated with histone acetylation imbalance (Shen and Peterson, 2009; Taylor et al., 2003; Wang and Tsai, 2008) and with similar symptoms to Huntington´s disease.

Until now the Atrophin genes has mostly been studied in development because of its fundamental effect on cell polarity and cell migration. Its function as a transcriptional co-regulator has emerged from studies in the fly development where it is needed for the correct segmentation during *Drosophila* embryogenesis (Haecker et al., 2007; Wang et al., 2006). However, the C-terminal region of human Atro is thought to bind the coactivator P300 which lead to the assumption that the atrophins function depend on the transcription factor it interacts with (Shen et al., 2007). Our findings demonstrate that Atro have a repressing role, Atro mediate the repressive function for *Or67c* expression. In the other group of ORs, *Atro* knock down resulted in loss of several *ORs*, a requirement of Atro that indicate an activator

function, though in that particular group of ORs also *Atro* overexpression resulted in a loss of OR expression, which favour an idea where the exact levels of Atro is crucial.

We find that the conserved co-repressor Atro plays a pivotal role in ensuring correct expression of many but not all basiconic odorant receptors. In fact, we find two groups that correlate, not with the anatomical organization or sensilla sub-types, but with the notch induced Nab and Nba cell fates. Intriguingly, these groups also exhibit different levels of histone acetylation; a modification we show is dependent on Atro. The different effect, divided in 2 phenotypic groups, is in distinct developmental windows. We show that Atro is required postmitotically for the maintenance of the first phenotypic group, Nba OSNs, whereas for the other group, Nab OSNs, Atro is required for the establishment of the OSN class.

Several pieces of evidence indicate that histone acetylation characterize OSN class identity. First, the OSN classes affected have a specific amount of H3ac. Second, alteration in the H3ac levels result in the co expression of *ORs* in OSN classes with similar levels of H3ac. Atro proteins have a SANT domain in their N termini. The SANT domain binds and stimulates class I HDAC enzyme catalytic activity, thereby promoting local heterochromatin formation and repression (Guenther et al., 2001; Wang et al., 2006). Interestingly, we found that Atro regulate global histone acetylation rather than for a subset of genes. Therefore, Atro is not likely to function solely as a cofactor to only some promoter regions through associations with transcriptional activators. Instead, it is probable that Atro has a function to retain HDACs within the complex and to stimulate the catalytic activity. This result thereby suggests that Atro has functions in addition to transcription-coupled gene-specific acetylation. It has for example been shown that global low levels of histone acetylation characterize glial cells in vertebrates whereas the CNS displays high levels of histone acetylation (H3K9ac) (Hsieh et al., 2004; Qi et al., 2004). Moreover, global differences in histone acetylation do not have to imply global differences in transcription activity (Flici et al., 2011). Consequently, the chromatin acetylation status can set the context for *OR* expression but also maintains the OR choice by priming or silencing promoters. Atro exerts its effect on the distal module which will repress gene expression in a specific level of H3ac.

Atro is viewed as a local cofactor that recruit HDAC and HMT enzymes exerts its repressive function by binding transcription factors such as Even-skipped, Teashirt, and Brakeless, (Haecker et al., 2007). Unfortunately, we were not able to identify a TF working in concert

with Atro but will definitely be an interesting question. A small subset of candidate factors has been investigated for genetic interaction with Atro with no success. Perhaps, as mentioned, TF binding is not required for Atro function since the SANT domain on the N-terminus of Atro are known to bind unmodified histone tails and recruit HATs as well as HDACs (Boyer et al., 2002; You et al., 2001). The SANT domain show clear similarity to Myb DNA-binding domain, thus, could also directly interact with DNA (de la Cruz et al., 2005), although no direct DNA interaction been reported. Groucho another corepressor have been shown to mediate long range repression by a "spreading mechanism" that modifies the chromatin over long regions (Chen and Courey, 2000; Martinez and Arnosti, 2008). A similar Atro spreading mechanism could potentially determine both local and global acetylation states.

Recently Endo et al demonstrated that chromatin modification directly on Notch target promoters via Hamlet can repress Notch signaling and determine cell fate. Hamlet does so in the last cell division in the sensilla lineage, allowing for a second response of Notch and giving rise the Naa, Nab and Nba fate (Endo et al., 2012). For the *ORs* that are ectopically expressed in *Atro* knock down, our finding is separate from this mechanism and downstream of the Nba fate since the OSN classes affected have all Nba identity. Thus, Atro must mediate fate choices later in this selection by defining the H3ac levels thereby setting an OSN class within the Nba fate post developmentally. This finding extend the Notch signaling events in sensory organs further of what has been reported previously. A predefined and limited repertoire of *OR* genes reduces the complexity of the choice.

In summary, we have identified an intriguing histone acetylation dependent mechanism of cell fate diversification. We show, for the first time, that a co factor, Atro, is setting this chromatin state possibly as a down-stream effect of Notch signalling and thereby support correct expression of olfactory receptors. Our work show how epigenetic switches dictate neuronal cell type sub-specification, a process fundamental to establishing the complex structure and function that comprises the brain.

Paper III, IV

There is broad consensus that Hedgehog (Hh) signaling has an evolutionarily conserved key role in metazoan development and its dysregulation has been implicated in many human diseases including cancers. Sonic hedgehog (Shh), is expressed in various adult neurons (Petralia et al., 2011). In cultured hippocampal neurons, Hh signalling components localize to primary cilia, dendrites, axons. Thus, Hh is found in adult brain but not much is known about the role it plays in homeostasis of tissues in adult life.

In mammals, Hh signaling is transduced via the primary cilia. The primary cilia, a slim microtubule-based organelle that is required for the response to developmental signals. Hh transduction is initiated when Hh binds to its receptor Patch1, thus, alleviating its inhibition and mediating the translocation of the seven transmembrane protein Smoothened (Smo) to the primary cilia, where it is activated and promotes downstream targets (Goetz and Anderson, 2010). Basically all human cells have a single non-motile primary cilium, however, in Drosophila, most cells are non ciliated and Smo localizes direct to the plasma membrane, which has led to the general view that *Drosophila* and mammalians transduce the Hh signal differently. Non-motile cilia are, though, found in the OSNs where the ORs are relocated. Sensory cilia, are thought to receive chemical and mechanical stimuli and initiate specific cellular signal transduction (Jana et al., 2011; Keil, 2012)

Principal findings

In *paper III*, we initially found *Hh* and its signaling components expressed in fully differentiated OSNs, more surprisingly we observed that the Smo protein also relocated to the cilia of the OSNs. *Drosophila* olfactory cilia share ultra structural features with the primary cilia involved in Shh signal transduction in vertebrates (Davenport and Yoder, 2005; Keil, 2012). It made us wonder if cilia mediated Hh signaling also exists in *Drosophila*. Expression of a GFP tagged version of Smo in the OSNs resulted in Smo:GFP localized to cilia in levels that varied between OSNs and made it possible to identify how and if Hh signalling regulate the cilia localization of Smo. Next, we genetically manipulated several levels of the pathway starting with Hh and its receptor Patched (Ptc). OSN specific knock down of *Hh* showed reduced cilia levels of Smo:GFP, which suggest that Hh is functioning partly autocrine in the OSNs and is required for Smo cilia relocation. In vertebrates, Patch1 reside in the cilium in absence of Hh and negatively control cilia entry (Rohatgi et al., 2007). *Drosophila* Ptc located

primarily to the OSN cellbodies and dendrites but less to the cilia indicating a different function. Further, the knock down of *Ptc* produced a very moderate increase of Smo whereas, overexpression produced a substantial reduction in the cilia. Therefore, our data suggest that Ptc rather than control cilia entry generally control Smo stability in *Drosophila*. Perhaps, as in the wing disc where Ptc has been shown to destabilize Smo (Khaliullina et al., 2009). Cilia lack local protein synthesis and hence have to import proteins via the intraflagellar transport (IFT) system (Berbari et al., 2009). In mammals and *Drosophila* mechanoreceptors the IFT system plays a critical role for proper formation and functioning of the cilium (Keil, 2012). Knock down of two IFT genes resulted in a disturbed OSN cilia or a loss of Smo in cilia, suggesting a function of the IFT machinery also in OSN ciliary transport. The kinesin-like protein Costal 2 (Cos2) has two vertebrate orthologs, Kif7a and Kif27. In the *Drosophila* wing disc, Smo accumulation and plasma membrane localization require Cos2 and in vertebrates Kif7 localize to cilia upon Shh stimulation (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Varjosalo and Taipale, 2008). In *Drosophila* OSNs, Cos2 localized to cilia and required *Hh* and *Smo* for its relocation. Another player in the *Drosophila* canonical Hh pahtway is Fused, a theronine kinase, that phosphorylate and activate Cos2 (Liu et al., 2007). A reduced Smo ciliary membrane localization was found when *Fused* was knocked down or a kinase dead version was expressed in the OSNs. Indicating that Hh transduction via the plasma membrane or cilia is similar in *Drosophila*.

The cilium is a trait of terminal differentiated OSNs (Jana et al., 2011) and the Hh pathway components are expressed in the antenna of the adult fly. What could be the post developmental function of Hh cilia signaling in the OSNs? We performed a T maze odorant response assay and observed an impaired odorant response to apple cider vinegar when *Smo* is knocked down in the OSNs. Mice and zebrafish Smo have a basic and hydrophobic amino acid cilia localization motif within the N terminus of the cytoplasmic tail. WRR Alanine replacement of the two first amino acids of the motif disrupts cilia location in mouse cell cultures. The motif and its´ location is conserved (Corbit et al., 2005). Introduction of the AAR mutation in *Drosophila* Smo (Smo^{AAR}) abolished entry to the cilia compartment demonstrating that cilia localization of Smo is a conserved feature from invertebrates to vertebrates. Moreover, the Smo^{AAR} flies displayed a decreased attraction to apple cider vinegar in the T maze assay suggesting that relocation of Smo to cilia is required for a proper olfactory response.

In *paper IV*, we moved on further in investigating the molecular mechanism behind the change in olfactory behaviour. In order to test whether this decrease in behavioural responsiveness observed is due to change in OSNs physiological sensitivity, we performed odor evoked calcium (Ca^{2+}) signal measurements (Akerboom et al., 2012; Nakai et al., 2001) from OSNs expressing receptors that respond to the broad ethyl acetate and the Or22a specific odour methyl octanoate. In *Smo* knock down flies, both odours evoked decreased calcium response compared to control flies, suggesting a peripheral change in sensitivity. Notably, lowering the dosage of *Ptc*, thereby decreasing the inhibition on Smo, resulted in a significantly enhanced calcium response to the fly attractant apple cider vinegar.

The change of response on the peripheral neuron might be due to receptor alteration or change in neuron excitability (ion channel or ion pump alteration) in the sensory neurons (Dawson et al., 1993; Stortkuhl et al., 1999). Next, we investigate the molecular role of Hh cilia transduction in the OSNs. *Drosophila* ORs, such as Or22a, are concentrated in the cilia. *Or22a* promoter construct fused to *CD8:GFP* visualized the expression of *Or22a* and demonstrated that the number of OSNs expressing *Or22a* was not changed in knock down of *Hh* and *Smo*. However, the number of cilia that stained for Or22a was reduced, indicating that Hh regulate OR transport to cilia. Is Smo cilia localization required for OR transport regulation? Smo^{AAR} expression produced loss of Or22a staining in one third of the cilia and a relocation of Or22a the dendrite, demonstrating a role of cilia mediated Hh signalling in OR transport. *Drosophila* ORs form a complex with a co-receptor, Orco. Co expression greatly helps tuning the response to ligands compared to the expression of the specific ORs alone, suggesting that the Orco and the specific OR forms a functional unit (Sato et al., 2008; Touhara, 2009). Furthermore, Orco is required for cilia transport of the OR complex (Benton et al., 2006). Surprisingly, we did not notice any change in Orco cilia location in *Hh* and *Smo* knock down flies, excluding Orco cilia transport as the mechanism for Hh regulation of OR transport.

Expression of Or43a fused with GFP in all OSNs revealed an additional feature. In control flies, Or43a:GFP produced a weak even staining in the cellbodies that colocalized with Orco while in *Smo* knock down flies, Or43a:GFP formed small bright puncta in the cellbodies separately from Orco, proposing that Hh signalling is required for OR transport and that the protein turnover possibly is disrupted. Protein turnover plays important role in the regulation of numerous cellular processes and Hh signalling pathway have been shown to be involved in the autophagy process (Jimenez-Sanchez et al., 2012). How about the other major type of

receptors in OSNs, the IRs? Ammonia, one of the odours that IRs responds to, resulted in a robust response in our calcium signal measurements in control flies while knock down of *Smo* attenuated the response, indicative of that IRs as well require Hh signaling for transport.

Conclusion and Discussion

Our work identifies an unanticipated non-developmental role for Hh signaling in the regulation of OR transport to cilia, a likely missing piece in how the Hh pathway has been adopted to use cilia. Moreover these results define a unifying view of Hh signaling in flies and mammals.

Adaption and sensitization in the olfactory system are crucial features in almost all organisms for coping with changes in the environment such as food availability and reproduction. There are multiple ways through which a sensory system can be modified, over the course of a milliseconds, seconds, minutes or weeks. The changes can occur at the periphery in the receptor level itself or via central mechanisms in higher order neuronal circuits (Dalton, 2000). In the olfactory centre in *Drosophila* certain neuropeptides has been associated in the modulation of olfactory perception (Carlsson et al., 2010; Farhan et al., 2013). However little is known whether plastic alteration of the odorant receptors peripherally in olfactory sensory neurons (OSNs) influence change in behaviour. Long term exposure to the bitter camphor diet lead to down regulation of TRPL (transient receptor potential-like) cation channel in gustatory receptor neurons dendrites and increased acceptance of camphor (Zhang et al., 2013). An ortholog gene, *osm-9* in C elegans is required for olfactory adaptation and is expressed in the olfactory neurons (Colbert et al., 1997). Because natural olfactory environments are complex and constantly changing, flies are likely to use multiple mechanisms to modulate the ORs.

Our results describe an Hh function that is independent of its established role in tissue patterning and cell proliferation during development, instead we suggest a modulator role in post mitotic sensory neurons. Hh signaling regulates nociceptive sensitization in both *Drosophila* and vertebrates, indicating a conserved modulator function (Babcock et al., 2011). Reusing the developmental signaling pathways might be an effective way for higher eukaryotes to continue the diversification in adult processes such as plasticity and adaptation. The Hh mechanism that produce a large number of different fates as a response to the different levels of Hh in a gradient could give rise to a graded adaption response that precisely interprets the external signals. Such gradients can have an autocrine source as we suggest in

paper III but also a paracrine source from support cells in the antenna. Paracrine regulation of the odor response has been demonstrated upon starvation (Farhan et al., 2013). A second developmental molecule has been shown to have a function in the modulation of odor perception and that is Notch. In *Drosophila,* Notch is expressed in adult tissue, in terminally differentiated neurons and glia. Notch is activated upon long-term exposure to different odors (environmental stimuli) and depends on OSN synaptic transmission. Suggesting an involvement in activity dependent plasticity in terminally differentiated neurons (Lieber et al., 2011).

In summary, we show that the non-motile OSN cilia in *Drosophila* transduce the Hh signal similar to primary cilia in vertebrates. Both the cilia and plasma membrane Hh transduction pathway are similar and are used in one organism. We demonstrate that cilia mediated Hh transduction is required for OR cilia localization. The conservation of cilia mediated Hh transduction suggests that the ancestral primary cilium was a sensory cilium that co-opted the Hh pathway to control sensory receptor localization. These results highlight Hh cilia signalling as a general modulator of olfaction in *Drosophila* OSNs.

Future perspectives

The complexity of structure and function that compromises the brain depends on highly specific and diverse neural types and classes. In olfactory neurons this specificity dictates that only one of 61 olfactory receptors is expressed. Clearly this strict regulation of olfactory receptors is dependent on a mosaic combination of TFs and cis regulatory elements but also chromatin-mediated silencing and activation. Despite the progress made in recent year's important question is still unresolved. One is the further elucidation of the cis regulatory code regulating *OR* genes, whereby different motifs critical for expression can be identified, as well as their location in relation to each other. Potentially, we will be able to build a synthetic enhancer driving *OR* expression. Indeed, computational methods are increasingly being used to identify novel enhancers in the genome.

A prominent question is how a terminal selector gene network is composed and regulated to support a large number of classes. What regulate the terminal selector genes in the adult state? It will be interesting to investigate whether the TF genes have terminal selector motifs in their own enhancers and upstream cis regulatory regions, a feature for auto regulation. Which additional milieu is required for the terminal selector genes to be expressed? Is there any general selector genes? For example are the sensilla-specific regulators early in the OSN lineage involved in regulation of the terminal selector genes as well as axon guidance genes. What is the role of Notch, are any of the TFs target genes downstream the last Notch mediated division?

Another challenge is the question what other aspects of the OSN identity the seven TFs regulate, how does the network downstream the selector genes and Atro look like? To have a chance to explore this issue we need to define what a class is. What are the general genes that are expressed in all OSNs and which are class specifically expressed besides the ORs? Generating a map of what is transcribed in the antenna is now days feasible by sequencing the mRNA from the antenna. To define what is expressed in one class require sequencing of the mRNA of one cell or at least a very limited number of cells. Since individual *OR* genes are expressed in a very small fraction of antennal OSNs and because neurons from a particular OSN class is a small fraction of the total cell population in the antenna (20-30 cells/antenna). In vertebrates that have larger neurons sequencing of single cells have been done but not in drosophila but the development is fast in this field and it is likely possible within the next

years. One could use this methodology to link what each factor regulate by sequencing mRNA from antenna with knock down of a particular TF and compare with the control mRNA.

ChIP-seq is another possible method that is increasingly becoming useful. With ability to sort out nuclei (Okada et al., 2011), the identification of the nuclei from one class, is potentially more efficient than sorting whole neurons. In addition, here the availability of high-quality antibodies is crucial. For two of the TFs, Acj6 and Fer1, reliable antibodies exist. Sequencing only the chromatin from one OSN class will provide us with information about other potentially regulated genes in specific OSN classes. Antibodies highly specific for histone modification, specially marks such as methylation and acetylation are also available which made it possible to analyze global prevalence H3 acetylation in the nuclei. It would though be interesting to investigate the histone acetylation marks on individual *OR* genes to make out if the levels correlated to our global findings.

Insects and vertebrates have evolved olfactory systems with many anatomical and functional similarities. The fact that cilia mediated Hh signaling exist in *Drosophila* OSN means that we have a novel, powerful in vivo model for studying Hh signaling and the regulation of transport within the cilia. Given the vital role of the Hh pathway in human ciliopaties and cancer it is critical to understand primary cilia Hh signaling. Studies on the mechanisms of cilia transport have revealed a complex system. Thus, the specific functions of the proteins involved need to be additionally investigated.

Having identified that Hh is involved in cilia mediated sensory activity the question is now how is Hh regulated in the adult OSN? Does neuronal activity lead to Hh induction and what in that case the molecular inducer, perhaps Ca^{+2}? Sensory experience and resultant neuronal activation lead to hyperpolarization and calcium influx into the neuron, which in turn triggers signals orchestrating short- and long-term changes in *Drosophila* and mice and by which extracellular stimuli are transformed into changes in activity-dependent gene expression. Trp $Ca2^{+}$ channel were shown to be required in olfactory adaptation, but also K^{+} and Na^{+} channel (Dubin et al., 1998; Stortkuhl et al., 1999).

Major advances have been made recent years within the field of neuroscience but new challenges are rising and the need for new knowledge seems endless. The rapid development of tools will definitely speed up the understanding.

Populärvetenskaplig sammanfattning

Hur styrs våra nervceller?

Det finns tusentals olika nervcellstyper och alla dessa har en specifik funktion och plats i en krets som tillsammans bildar vår hjärna. Hur varje cell får sin specifika instruktion om vad den skall göra är fortfarande oklart. För att studera och förstå denna process behövs en uppsättning nervceller som har väl definierade egenskaper. Därför har vi vänt oss till luktsystemet, där cellens egenskap bestäms av dess luktreceptor. Människan har tusentals olika luktreceptorgener och varje luktnervcell i näsan blir instruerad att använda endast en receptor. Med luktsystemet som modell kan vi nu börja besvara hur en nervcell får sina egenskaper; hur bestäms vilken av luktreceptorerna som skall användas och hur är det kopplat till luktkretsens bildande?

Eftersom alla organismers luktsystem i grunden ser lika ut har vi valt att studera ett mycket litet luktsinne; bananflugans (*Drosophila Melanogaster*). Den har bara 60 luktreceptorer och vi vet hur flugans lukt är uppbyggd i detalj, något som för närvarande är omöjligt i mus och människa med sina tusen grupper av luktnervceller. Vad vi nu vill göra är att förstå hur gener samverkar för att tillsammans bilda luktsystemet. Vår grupp har hittills hittat mer än hundra potentiella gener inblandade i denna process. Den stora utmaningen nu är att förstå hur de samverkar i cellen. Vi har funnit att luktreceptorvalet verkar bestämmas av olika kombinationer av gener i en möjlig kod och att även så kallade epigenetiska faktorer, mekanismer som bestämmer hur och när gener uttrycks, är involverade. Förändringar i sådana uppträder alltid tidigt i cancerutveckling, vilket får oss att tro att de är viktiga för både uppkomsten och tillväxten av cancer. Vi kan nu dessutom jämföra celler som använder olika receptorer och se vilka gener som styr de andra cellspecifika processerna, t ex förmågan att behålla sin funktion och/eller adaption till förändrad miljö. Rubbningar i dylika processer kan leda till förlorad cellidentitet och/eller ohämmad celltillväxt som i cancer men även till degenerering av celler såsom i Parkinson.

Vad kommer vi att lära oss av detta? Denna forskning kan ge en ökad förståelse för hur man i framtiden kan stödja tillväxt och nybildande av nervceller både i kroppen och i provrör. Den kan även bidra till utvecklingen av nya läkemedel för behandling av cancer.

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