THE ROLE OF THEODORANT RECEPTORCODINGSEQUENCE INTHEREGULATION OFODORANT RECEPTORTRANSGENE EXPRESSION IN ZEBRAFISH

by

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ABSTRACT

THE ROLE OF THE ODORANT RECEPTOR CODING SEQUENCE IN THE REGULATION OF ODORANT RECEPTOR TRANSGENE EXPRESSION IN ZEBRAFISH

Odorant receptors constitute the largest gene family in rodents and probably in all vertebrates. In addition to detecting the odorant molecules in the outside world, odorant receptorrs are responsible for some critical aspects of olfactory sensory neuron development including odorant receptor gene choice and axonal guidance. Although it is widely accepted that any given olfactory sensory neuron expresses only one odorant receptor out of a repertoire of almost a 1000 genes, it is still unknown how the singularity in odorant receptor gene expression is established. On the other hand, it has been suggested that a negative feedback signal is emitted from the OR proteins to suppress the expression of other ORs. Zebrafish, with only ~140 odorant receptors and very similar organization of olfactory system compared to mammals, is an ideal model organism to investigate the unknown features of odorant receptor gene choice. In the first part of this thesis, temporal dynamics of regulation of odorant receptor gene choice was analyzed by using a conditional zebrafish OR111-7 ablation transgene. In the second part, the importance of OR111-7 coding sequence in the negative feedback signal dependent inhibition of OR111-7 reporter transgene expression was investigated.

ÖZET

ZEBRA BALIĞINDA KOKU RESEPTÖRÜ KODLAYAN DİZİLERİN KOKU RESEPTÖRÜ TRANSGENİ ANLATIMININ DÜZENLENMESİNDEKİ RÖLÜ

Koku reseptörleri kemirgenlerde ve muhtemelen bütün omurgalılarda en büyük gen ailesini oluşturmaktadır. Dış dünyadaki koku moleküllerini algılamanın yanı sıra, koku reseptörleri kendi gen seçimlerinde ve anlatıldıkları koku duyu nöronlarının aksonal bağlantılarının oluşmasında önemli rol oynamaktadırlar. Genel olarak her bir koku duyu nöronunun sadece bir çeşit koku reseptörünü anlattığı kabul edilse de, tek bir nöronun nasıl sadece bir çeşit koku reseptör genini anlatmayı başarabildiği kesin olarak açıklanamamıştır. Öte yandan, anlatılan koku reseptörü proteinlerinin diğer koku reseptörlerinin anlatımını engellemek üzere bir negatif geri besleme sinyali gönderdiği önerilmektedir. Sadece yaklaşık 140 OR geni ve memelilere çok benzeyen koku alma sistemiyle zebra balığı koku reseptörü gen seçiminin bilinmeyen yönlerini araştırmak için ideal bir model organizmadır. Bu tezin ilk bölümünde, koku reseptör gen seçimi regülâsyonunun zamansal dinamikleri şartlı zebra balığı OR111-7 geni silinmesi transgenini kullanarak incelenmiştir. İkinci bölümde, OR111-7 kodlayan dizinin negatif geri besleme sinyaline bağlı OR111-7 raportör transgeni anlatımının engellenmesindeki rolü incelenmiştir.

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LIST OF ACRONYMS / ABBREVIATIONS

BAC	Bacterial Artificial Chromosome
bp	Base Pair
cAMP	Cyclic Adenosinemonophosphate
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
EYFP	Enhanced Yellow Fluorescent Protein
Gap43	Growth Associated Protein-43
GFP	Green Fluorescent Protein
GPCR	G-protein-coupled Receptor
IRES	Internal Ribosome Entry Site
kb	Kilobase Pair
mRNA	Messenger Ribonucleic Acid
OB	Olfactory Bulb
OE	Olfactory Epithelium
OMP	Olfactory Marker Protein
OR	Olfactory Receptor
OSN	Olfactory Sensory Neuron
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pOMP	Olfactory Marker Protein Promote
RNA	Ribonucleic Acid
RT-PCR	Reverse-transcription Polymerase Chain Reaction
UTR	Untranslated Region

1. INTRODUCTION

1.1. Anatomy of the olfactory system

In both insects and vertebrates, olfactory system is composed of peripheral structures for odor detection and more central structures for signal integration and discrimination. Although they evolved independently, the functional and anatomical organization of the olfactory systems in *Drosophila*, zebrafish and rodents is remarkably similar: Olfactory Sensory Neurons (OSNs) expressing a particular odorant receptor (OR) extend their axons in order to synapse with second order neurons. Here, they form distinct, spherical structures called glomeruli, which are composed of synapses between OSN axons, dendrites of second order neurons and innervations from local inhibitory neurons.

In insects, OSNs are found in special structures called sensillla. There are four different morphological types of sensilla in the fruit fly *Drosophila melanogaster*: basiconic, trichoid, coeloconic and intermediate sensilla. While the maxillary palp includes only the basiconic type, the antenna is equipped with all four types of sensilla (Shanbag *et al*., 1999). OSNs from both, the antenna and the maxillary palp extend their axons to the antennal lobe (AL) where they interact with projection neurons and local interneurons to form individual glomeruli. Projection neurons are responsible for transmitting signals from the antennal lobe to higher brain centerswhile local interneurons are responsible for lateral interactions and integration of the signals between different glomeruli.

In rodents, there are two distinct olfactory organs which are specialized in detecting different odorants. It is commonly thought that the main olfactory epithelium (MOE), which lines the nasal cavity (Buck, 2000) contains OSNs which are specialized to detect volatile odorants while the vomeronasal organ (VNO) is believed to detect pheromones, kairomones and non-volatile molecules (Firestein, 2001; Stowers *et al*., 2002; Papes *et al*., 2010). The MOE is mainly occupied by OSNs, sustentacular cells and horizontal and globose basal cells (stems cells in the MOE; Schwob, 2005). Ciliated OSNs in the MOE express odorant receptors (only one odorant receptor out of a repertoire of ~1000 odorant receptor genes), which mediate the first step of odorant signaling by binding to their

corresponding odorant ligands (Krautwurst *et al*., 1998, Bozza *et al*., 2002). These neurons are bipolar sensory neurons which protrude a ciliated dendrite into the nasal cavity and send an unbranched axon to the first relay station in the brain, the main olfactory bulb (MOB; Buck, 2000). Moreover, OSNs expressing the same receptor project their axons to a defined set of glomeruli in MOB and the positions of these glomeruli are highlyconserved between individuals (Mombaerts *et al*., 1996; Wang *et al*., 1998). In the VNO, vomeronasal receptor families, V1Rs and V2Rs are expressed in apical and basal cell populations, respectively (Belluscio *et al*., 1999, Rodriguez *et al*., 1999). While V1R-expressing VNs project their axons to the anterior AOB, V2R-expressing VNs send their axons to the posterior AOB (Munger *et al*., 2009). As in the MOB, each vomeronasal sensory neuron projects an unbranched axon to the accessory olfactory bulb. However, in contrast to OSNs, vomeronasal sensory neurons (VNs) expressing a particular receptor may project their axons into multiple glomeruli in the accessory olfactory bulb (Mombaerts *et al*., 1996; Wang *et al*., 1998; Belluscio *et al*., 1999; Rodriguez *et al*., 1999). Moreover, individual glomeruli in the AOB can be innervated by VNs expressing different receptors (Belluscio *et al*., 1999). It has been proposed that the AOB is a signal integration center (which is usually restricted to higher brain centers) and that it has a glomerular map in which neurons expressing receptors from the same subfamily are intermingled in shared domains as opposed to the individual spatial coding seen in the MOB. That is, information from highly related receptors is brought together in the glomerular layer and the mitral cells interacting with different domains have a role in integrating the signals received from multiple domains (Wagner *et al*., 2006).

Zebrafish has only one peripheral olfactory organ, unlike *Drosophila* and rodents. The zebrafish olfactory epithelium (OE) is a rosette like structure with a center (midline raphe) and lamella projecting radially from this center (Hansen and Zeiske, 1998). It has been shown that the zebrafish OE has at least three types of sensory neurons: ciliated, microvillous, and crypt cells (Hansen and Zeiske, 1998; Hamdani and Doving, 2007). These cells can be clearly differentiated from each other according to their cellular morphologiesand their relative basal-apical positions in the OE (Hansen and Zeiske, 1998; Sato *et al*., 2005, Oka *et al*., 2011). Ciliated OSNs are located basally and have long dendrites projecting apically, whereas microvillous cells located more apically and have shorter dendrites (Hansen *et al*., 2003). On the other hand, crypt cells are superficially

located globose cells in the OE with engulfed microvilli (Hansen and Zeiske, 1998). In addition, molecular markers allow to selectivelydistinguish between ciliated and microvillous OSNs. It has been shown that mature ciliated OSNs express zebrafish olfactory marker protein (zOMP) together with an OR or a (trace amine-associated receptor (TAAR), while microvillous OSNs express zebrafish transient receptor potential channel C2 (zTRPC2) along with a V2R-like OlfC gene (Sato *et al*., 2005, Korsching 2009). Although detailed molecular profiling has not been done for crypt cells, it has been shown that they exhibit S100 calcium binding protein-like immunoreactivity (Sato *et al*., 2005). Morever, Oka and colleagues have recently identified a novel family of highly conserved G-protein coupled receptors with only six members, namely V1R- like genes (2011). They have shown that one member of this family, ora4, is expressed in almost all crypt cells while the other five are not detected in any crypt cell.

The glomerular organization of the zebrafish olfactory bulb is strikingly similar to its rodent counterpart in terms of the positional stereotypy of glomerular units. By injecting the lipophilic tracer DiI into the nasal cavity, Baier and Korsching were previously able to identify ~80 glomeruli in adult zebrafish olfactory bulb (1994). While 22 of these glomeruli were clearly distinguishable, the rest were either found in glomerular plexus or form clusters. However, positions of these glomeruli and clusters were highly stereotypic among different individuals. Recently, a more detailed analysis of adult zebrafish glomerular array has been reported (Braubach *et al*., 2012). Theyused specific antibodies against neural markers, G proteins, and Calcium-binding proteins in order to label different OSN populations and to observe their convergence in the adult olfactory bulb. Briefly, they have identified ~140 glomeruli, 27 of which could be individually distinguished and further classified according to their immunoreactivities against different epitopes. However, the rest could not be identified as distinct structures; they were comparably small and clustered in broad regions. On the other hand, activity imaging in one of the unidentifiable regions, the lateral chain, has shown that discrete activity foci are formed upon the administration of certain amino acids (Friedrich and Korsching, 1997; Fuss *et al*., 2001), suggesting a functional/glomerular organization. Therefore, it might be possible to identify novel glomeruli in these regions by either using chemoreceptor reporter transgenes or additional cell type-specific markers.

1.2. Odorant Receptors

First discovered through the Nobel winning work in 1991, odorant receptors constitute the largest gene family in probably any given genome (Buck and Axel, 1991). Genome wide analysis has shown that the mammalian odorant receptor gene superfamily encodes for approximately 1300 ORs (Zhang and Firestein, 2002). OR genes are seven trans-membrane G protein-coupled receptors (GPCRs), distributed in clusters of different numbers of OR genes. Typically, OR coding regions do not have introns. In the mouse genome, OR genes can be found on all chromosomes, except chromosome 12 and the Y chromosome and they are distributed across 46 clusters (Zhang *et al*., 2004). Interestingly, viral coat proteins can be found in more than half of these clusters, which suggests a viral based gene duplication and relocation mechanism (Young *et al*., 2002; Zhang and Firestein, 2002).

Phylogenetic analysis based on OR protein sequence similarities allows for the separation of ORs into two main classes, the Class I and Class II ORs (Zhang and Firestein, 2002; Hirota *et al*., 2007). Class I OR genes were first identified in fish (Ngai *et al*., 1993) and are considered to be the more ancient class and which constitutes almost the entire fish OR gene repertoire (Alioto an Ngai, 2005). In mammals, Class I OR genes constitute only 10% of the OR gene repertoire and they are found exclusively on chromosome 7 in mouse. Therefore, the majority of mammalian OR genes are Class II ORs. Mammalian Class I and Class II OR genes do not only different in their sequences but also in their expression patterns and axonal projections of OSNs expressing them (see below).

The zebrafish OR repertoire is approximately 10-fold smaller than that of mammals with only ~140 OR genes. Most of the zebrafish OR genes are found in dense clusters and ORs from the same subfamily tend to be localized in the same cluster suggesting a tandem duplication mechanism during OR gene expansion. Unlike mammals, phylogenetic analysis of zebrafish OR superfamily has shown that there is only one Class II OR in zebrafish genome (Alioto and Ngai, 2005; Niimura and Nei, 2005). However, it is still unknown whether regulation of this gene is different from zebrafish Class I ORs.

In addition to ORs, mammals have around 35 V1R genes and ~150 V2R genes (Buck, 2000). V1Rs are seven trans-membrane GPCRs of family C with a large globular extracellular domain while ORs and V2Rs belong to family A (Zhang *et al*., 2004; Young et al 2005). Although V1Rs and V2Rs are thought to be involved in pheromone sensation, there is evidence that some are responsible for detection of amino acids in fish. In zebrafish, there are 62 V2R like genes (OlfC receptors) and six V1R genes (Pfister and Rodriguez, 2005; Alioto and Ngai, 2006; Saraiva and Korsching 2007). Yet another superfamily of GPCRs, the traceamine-associated receptors (TAARs) was identified as chemoreceptors in mouse (Liberles and Buck 2006). Bioinformatic analysis revealed ~100 TAAR coding sequences in zebrafish, while only 15 TAAR genes were identified in mouse (Gloriam *et al*., 2005; Hashiguchi and Nishida, 2007). Although it has been shown that mouse TAARs are expressed in the MOE in a similar pattern to ORs and activated by aminergic ligands, it has still not been elucidated whether TAAR genes are functional chemoreceptors in zebrafish (Liberles and Buck, 2006; Zucchi *et al*., 2006). However, the high number of TAAR genes in zebrafish genome suggests an important role for TAARs in zebrafish chemosensation.

The canonical olfactory signaling cascade is initiated with ligand binding to the extracellular surface of the OR. Several studies with different ORs have suggested essential amino acid residues for ligand binding residing in TM3, TM5 and TM6 (Abaffy *et al*., 2007). After ligand binding, the OR protein undergoes a conformational change and binds to olfactory specific G protein $G_{\alpha olf}$ through its cytoplasmic part. It has been shown that Asn-Arg-Tyr (DRY) motif in the cytoplasmic loop of TM3 is essential for this interaction (Belluscio *et al*., 1998; Imai *et al*., 2006). Next, $G_{\alpha olf}$ release GDP and goes into GTP-bound activated state (Von Dannecker *et al*., 2005). GTP-bound $G_{\alpha olf}$then stimulates Adenylyl cyclase type III (ACIII) which in turn produces cyclic AMP (cAMP). cAMP binds and opens nucleotide gated Calcium channels (CNG) and this results in the influx of calcium ions. Moreover calcium influx results in the opening of chloride channels, leading to chloride efflux. Combination of calcium influx with chloride efflux results in depolarization of the OSN membrane potentials (Imai and Sakano; 2008). Although the signaling mechanisms for V1Rs and V2Rs are not fully elucidated, it has been shown that V1Rs signal to TRPC2 through G_i and V2Rs through $G_{\alpha o}$ (Touhara, 2007).

1.3. Odorant receptor gene choice

The olfactory system has evolved a large number of OR genes in order to cope with the seemingly infinite number of odorants it might encounter. As it is seen in other sensory systems, a functional and topographical organization is also essential for olfactory system to receive and integrate the signals. Since the odorant receptor gene family outnumbers any other sensory receptor family, gene regulation in the olfactory system might employ very complex mechanisms.

The most interesting and maybe the least understood question about the olfactory system is how each sensory neuron is functionally specified. In vertebrates, a particular OSN expresses only one OR out of a repertoire of ~1300 genes in mouse and ~140 genes in zebrafish (Ressler *et al*., 1993; Chess *et al*., 1994; Barth *et al*., 1997). In rodents, expression of each OR is restricted to one of the four discrete OR expression zones lying through dorsomedial- ventrolateral axis of the MOE (Ressler *et al*., 1993; Vassar *et al*., 1993, Strotmann *et al*., 1994). In addition, OSNs expressing a particular OR send their axons to a defined number of glomeruli, typically two, in the olfactory bulb (Mombaerts *et al*., 1996).

Indirect evidence that one OSN expresses only one OR comes from relative cell counts of OSNs expressig specific ORs. Considering the fact that there are about 15 million OSNs in the adult rat MOE (Youngentob *et al*., 1997) and that there are ~1000 OR genes available for expression. Consequently there must be a few thousand OSNs expressing each OR. In situ hybridization studies with specific probes for different ORs revealed that each OR is expressed in approximately 0.1% of OSNs (Nef *et al*., 1992; Ressler *et al*., 1993; Vassar *et al*., 1993). However, it could not be ruled out that certain ORs could be systematically co-expressed in a single OSN.

In situ hybridization with dual probes has shown that three mouse ORs with high sequence similarity, MOR28, MOR10 and MOR83, are not coexpressed in the same OSN, even though they are expressed in the same zone (Tsuboi *et al*., 1999). In another study, serial sections of the olfactory epithelium were labeled by either specific probes against

each of the three members of the OR37 family or a common probe which labels all three members. Number of the cells labeled by the common probe highly correlated with the sum of the number of the cells labeled by probes against each member, suggesting that they were never or rarely co-expressed (Kubick *et al*., 1997).

By genetically labeling two different ORs from the OR37 subfamily, Strotmann and colleagues have shown that they are never coexpressed (2000). Although, these studies strongly suggest monogenic OR expression, they cannot rule out the possibility that specific combinations of ORs might be co-expressed, because the high number of OR genes makes it impossible to analyze each combination. Indeed, rare occasions of systematic coexpression have been reported in rat, mouse and zebrafish (Rawson *et al*., 2000; Sato *et al*., 2007; Tian and Ma, 2008).

Single cell reverse transcription polymerase chain reaction (Single cell RT-PCR) together with calcium imaging have also been used in order to detect how many ORs a single OSN can express (Malnic et al 1999; Touhara *et al*., 2001). Although only one OR could be detected per OSN in these studies, the complexity of the OR gene repertoire makes it impossible to analyze each individual OR. Moreover, a primer pair for a particular OR can be favored over another in RT-PCR reaction which can result in the failure of the amplification of the latter.

In addition to monogenic expression, ORs are also expressed monoallelically. First, Chess and his colleagues used RT-PCR on polymorphic alleles to show that only one allele is expressed per OSN (1994). They have crossed two different strains of mice which are polymorphic in OR I7 loci and performed RT-PCR reactions for the I7 gene on isolated OSN pools with different densities ranging from 200-10.000 cells. Thus, they have found the limiting dilution of cells in which probability of getting only one I7 OSN is the highest. Eventually only one polymorphic I7 allele could be detected for each OSN. Independent evidence based on relative cell counts in genetically manipulated mice, where a particular OR was tagged with a reporter protein. In these experiments, the number of OSNs labeled in homozygous mice is twice the numbers observed in heterozygous ones (Mombaerts *et al*., 1996; Khan *et al*., 2011). In other studies, each allele of a particular OR was tagged with a different marker. In independent works, it has been shown that both alleles are never

co-expressed in the same OSN (Conzelmann., 2000, Shykind, 2005). Lastly, combination of RNA and DNA fluorescent in situ hybridization (FISH) has shown that only one allele of the OR gene is actively transcribe per OSN (Ishii *et al*., 2001).

Although there is still not a comprehensive understanding of OR gene regulation, different models have been proposed as to how monogenic and monoallelic are established throughout the lifetime of an OSN. First, DNA rearrangements could enable expression of only a single OR in one neuron as it does for immunoglobulin and T-cell receptor genes in lymphocytes (Tonegawa, 1983). This possibility has been ruled out by Li and his colleagues (2004). They permanently marked OSNs expressing the odorant receptor M71 through bicistronic expression of Cre-recombinase and a reporter gene under the control of a ubiquitous promoter which is activated upon Cre-mediated recombination. They reconstituted oocytes by transferring nuclei of marked OSNs and it has been previously shown that DNA rearrangements are conserved during nuclear transfer (Hochedlinger and Jaenisch, 2002). Therefore if DNA rearrangements were involved in OR gene choice, a monoclonal nose which expresses only M71 gene should have been obtained. Proving that M71 is irreversibly expressed in donor OSNs, it has been shown in this study that OR gene choice is reset upon nuclear transfer and DNA rearrangements are not involved in OR gene regulation (Li *et al*., 2004). In a deterministic model, different transcription factors may define different cell types which are destined to express only one receptor. However, this model just raises the question as to how these transcription factors are differentially expressed in the first place. Alternatively, a combinatorial code of different transcription factors may activate different ORs in different OSNs which require transcription factor gradients along the olfactory epithelium or overlapping domains of transcription factors. In a stochastic model, ORs might compete for a limiting factor (ie. a transcription module) and the winner OR inhibits the expression of other ORs with so called ‘negative feedback’ mechanism or a single locus control region (LCR) stochastically activates a particular OR and forms a permanent interaction throughout the life of OSN. Indeed, the actual OR gene regulation mechanism seems to be a combination of deterministic and stochastic events.

1.4. Zonal expression of odorant receptors

Expression of a particular OR gene is restricted to a defined expression zone along the dorso-ventral axis of the olfactory epithelium. It has been suggested that main olfactory epithelium of mice can be divided into four expression zones with limited or no overlap and any given OR is expressed only in one of these zones (Ressler *et al*., 1993; Vassar *et al*., 1993). However, OSNs expressing a given OR are randomly distributed within the expression zone (punctate expression, see Mombaerts *et al*., 2004). An exception is found with the OR37 family, which is expressed in a small patch along ectoturbinate II and endoturbinate III (Strotmann *et al*., 1994). In recent studies it has been shown that zonal expression is more complex than the four zone model suggests; each OR appears to have its own expression zone along the dorsomedial-ventrolateral axis and these zones do not always fit into one of the four zones described before (Norlin *et al*., 2001, Iwema *et al*., 2004; Miyamichi *et al*., 2005; Tsuboi *et al*., 2006). Similar to the zonal organization in rodents, zebrafish ORs have been shown to be expressed in overlapping concentric rings/expression domains (Weth *et al*., 1996).

Different from the overlapping expression zones shown for Class II ORs, Class I ORs are exclusively expressed in the most dorsal zone of the olfactory epithelium (Miyamichi *et al*., 2005; Tsuboi *et al*., 2006). It has been shown that two genes, olfactory specific medium chain acyl coA synthetase (O-MACS) and olfactory cell adhesion molecule (OCAM) are differentially expressed in the mouse olfactory epithelium. Moreover, the most dorsal zone (zone 1) turns out to be O-MACS positive and OCAM negative (Yoshihara *et al*., 1997; Oka *et al*., 2003). Interestingly, Class I ORs are expressed only in this region of the olfactory epithelium (Tsuboi *et al*., 2006). Bioinformatic analysis has shown that O/E like (Olfactory/ B-cell factor) transcription factor sites are enriched in upstream regions of Class II ORs compared to Class I ORs (Hoppe *et al*., 2006). It is quite interesting that upstream regions of two exceptions to dorsal expression of Class I ORs, the MOR35-1 and MOR41-1 genes, show higher similarity to Class II OR promoter regions (Tsuboi *et al*., 2006; Hoppe *et al*., 2006). In a recent study it has been proposed that two different OSN types in the dorsal olfactory epithelium, OSN-DI and OSN-DII, are committed to express Class I and Class II ORs respectively. While cell bodies of these

OSN types are stochastically distributed in the dorsal zone, their axons sort out in the dorsal olfactory bulb according to the OR identity (Bozza et al, 2009; Imai *et al*., 2009).

In a previous study it has been shown that ORs found in the same cluster (with high homology in DNA sequence) are expressed in the same zone and project their axons to close but distinct glomeruli in the olfactory bulb (Tsuboi *et al*., 1999). However, further studies have shown that there is no correlation between genomic proximity, sequence homology and zonal restriction (Fuss *et al*., 2007, Nishizumi *et al*., 2007).

The identity of the OR is not essential for restriction of zonal expression. When the OR coding sequence was replaced with a fluorescent protein by gene targeting, labeled OSNs could only be found in the zone where the replaced OR is normally expressed (Serizawa *et al*., 2003; Lewcock and Reed, 2004). However, swapping coding sequences of two ORs expressed in different zones resulted in the ectopic expression of both ORs in the zone that is appropriate for the host locus (Mombaerts *et al*., 1996; Wang *et al*., 1998; Feinstein and Mombaerts, 2004).

Recently, specific locus control regions have been proposed to regulate both OR gene choice and zonal expression. A candidate sequence, the 2,1kb long H-region in mouse (H stands for homology, since this sequence displays high sequence similarity between human and mouse), has been shown to regulate the expression of seven OR genes in MOR28 cluster on mouse chromosome 14 in a range of 70-90kb (Serizawa *et al*., 2003; Nishizumi *et al*., 2007, Fuss *et al*., 2007). By using Chromosome Conformation Capture (3C) technique, Lomvardas *et al*. have shown that H-region interacts with different chromosomes and proposed that H-region is a trans-acting regulator of OR gene expression (2006). Although it seems to solve the OR gene regulation problem, two independent studies have proved that H- region is a cis-acting element. Moreover, they have shown that three genes in MOR28 cluster are expressed more ventrally while the rest are expressed in dorsal regions of MOE (Fuss *et al*., 2007; Nishizumi *et al*., 2007). Therefore, H-region may only act like a cis-acting regulator of OR gene expression and has no effect on zonal expression. Recently, another cis-acting locus control region, the P-region, has been shown to regulate the probability of OR gene choice from genes of the P2 cluster, but nottheir zonal expression (Khan *et al*., 2011). Similarly, candidate locus control regions have also

been defined in zebrafish but how they regulate OR gene expression or whether they regulate zonal expression still remains elusive (Nishizumi *et al*., 2007). Interestingly, it has been shown that fusion of H region with proximal OR upstream sequences increases the numbers of OSNs expressing the transgene with respect to the transgenes with only proximal upstream sequences, suggesting an evolutionary conserved mechanism (Nishizumi *et al*., 2007; this study). Thus, it would be reasonable to argue that similar locus control regions may also control the probability of OR gene choice but not zonal / spatial expression in zebrafish.

Several studies have shown that proximal cis-acting regions may be important for OR gene and zonal expression. Indeed, promoter transgenes which are composed of short genomic sequences upstream of OR coding sequences (as short as ~160bp) can recapitulate the zonal expression patterns of endogenous OR for a number of ORs in rat and mouse (Qasba and Reed 1998, Vassalli *et al*., 2002; Rothman *et al*., 2005). It's been demonstrated that proximal OR upstream regions harbor O/E and homeodomain like transcription factor binding sites (Vassalli *et al*., 2002; Michaloski *et al*., 2006). Consistent with the bioinformatic data, two homeodomain transcription factors, LHX2 and EMX2, were shown to be important for OR gene expression. Lhx2 is uniformly expressed throughout MOE. Interestingly, while expression of most Class I OR genes are not affected in LHX2 deficient mouse, Class II OR expression is severely impaired (Hirota *et al*., 2007). On the other hand, EMX2 mutant mice displaya severe reduction in the number of mature OSNs, in contrast to LHX2, which impairs both immature and mature OSNs (McIntyre *et al*., 2008). However, none of these studies were able to find a correlation between these transcription factors and zonal expression. Recently, a more comprehensive bioinformatic analysis of OR promoter motifs has also failed to detect differences in transcription factor binding sites among any of the conventional zones (Clowney *et al*., 2011).

1.5. Axonal targeting of the olfactory sensory neurons

Two fundamental principles are crucial for the organization of the peripheral olfactory system. One is the singularity of the odorant receptor gene choice and the other is the precise axonal projections of OSNs. Radioactive in situ hybridization studies demonstrated that OSNs expressing a particular OR project their axons to a pair of

glomeruli per olfactory bulb (Ressler *et al*., 1994; Vasssar *et al*., 1994). Later, genetic labeling of OSNs by introducing IRES (internal ribosome entry site) reporter genes into the 3'-UTR has reproduced stereotypic axonal projection patterns in various studies (Mombaerts *et al*., 1996, Wang *et al*., 1998; Bozza *et al*., 2002; Bozza *et al*., 2009).

It seems that epithelial positions of the OSNs, in part, affect the positioning of axonal convergence. It has been demonstrated that there is a zone to zone correlation between epithelial position and the position of glomeruli in the olfactory bulb. While OSN in the O-MACS-and NQO1- (NADPH: quinone oxidoreducatase) positive dorsal epithelial zone (all Class I ORs and some Class II ORs) project their axons to the dorsal part of the olfactory bulb (D Zone), OCAM-positive OSNs in the ventral MOE send their axons to the ventral olfactory bulb (V Zone, Yoshihara *et al*., 1997; Gussing and Bohm, 2004; Bozza *et al*., 2009). Tsuboi *et al*., have shown by radioactive in situ hybridization that OSNs expressing Class I ORs project their axons specifically to the most antero-dorsal part of the olfactory bulb (2006). In another study, retrograde DiI staining revealed that there is strong correlation between dorsal-ventral OSN positioning in the MOE and dorsomedial-ventrolateral glomeruli positioning in the olfactory bulb, respectively (Miyamichi *et al*. 2005). In the same study, expression of a zone 4 OR from a BAC transgene together with H-region has shifted the expression area of the OR to a more ventral position in the OE. Consistently, the corresponding glomerulus was also shifted more ventrally. Furthermore, it has been demonstrated that minigenes with broadened expression zones in the MOE also show glomerular shifts or formation for ectopic glomeruli in the ventral-dorsal axis of the olfactory bulb with no change in anterior posterior positioning (Vassalli *et al*., 2002). Lastly, OR swapping demonstrated similar effects on the glomerular positioning (Mombaerts *et al*., 1996).

Previous studies suggested that Neuropilin-2 (Nrp2) has an important role in dorsal-ventral patterning of the olfactory bulb (Norlin *et al*., 2001; Walz *et al*., 2002). Neuropilin is expressed in a low to high gradient along the dorsal-ventral axis of the olfactory bulb and axons of Neuropilin deficient OSNs display abnormalities in their axonal projections. Another pair of axon guidance cues, Robo2 and Slit1, is required for differential axonal segregation of OSNs projecting to D and V zones in the olfactory bulb (Cho *et al*., 2007). Robo2 is expressed in a gradient with high dorsomedial and low ventrolateral expression

and its repulsive ligand Slit1 expressed only ventrally. It has been demonstrated that ablation of Slit1 or Robo2 gene results in the mistargeting of NQO1 positive axons to the ventral zone, indicating that they are important for D-V zone distinction (Cho *et al*., 2007).

In addition to D-V zone distinction defined by the expression of NQO and OCAM, respectively, Bozza *et al*. have demonstrated that Class I ORs are restricted to a specific subzone in the D zone of the OB, which is distinct from zone occupied by Class II ORs, even though Class I and Class II expressing OSN are intermingled throughout the dorsal MOE (2009). Moreover, they could show that this segregation is not dependent on the OR identity but that it is determined by the distinct cell types, which are predestined to express a Class I or Class II OR, respectively. However, exact glomerular positions within the appropriate zone seem to be defined by the expressed OR (Bozza *et al*., 2009).

A detailed analysis of the dorsal ventral patterning of the olfactory bulb has been recently reported. In this study it has been demonstrated that D zone OSNs mature earlier than the V zone OSNs and that they arrive at the olfactory bulb earlier. Since D zone OSNs express Robo2 and Slit1 has a low to high gradient in dorsoventral axis of the OB, Robo2 expressing D zone OSNs project to the dorsal OB. Semaphorin 3F and Neuropilin2 have complementary expression patterns in the OE such that Semaphorin 3F is concentrated in the anterodorsal part of the OE and Neuropilin2 in the ventral part. D zone OSNs reach the OB earlier and deposit Semaphorin 3F to the dorsal olfactory bulb. Since V zone OSNs mature later their arrival to the OB is delayed with respect to the D zone OSNs and their axons are guided to the ventral OB by the repulsive interaction between Semaphorin 3F and Neuropilin 2 (Takeuchi *et al*., 2010).

In contrast to dorsoventral patterning, anterior-posterior positioning of glomeruli is affected by the OR identity rather than the epithelial positions of the OSNs. OR swapping has demonstrated that ORs expressed in ectopic positions results in anterior/posterior shift of the target glomerulus (Mombaerts *et al*., 1996). OR derived cAMP signals seem to be important for the anterior-posterior patterning. Axons of the OSNs expressing ORs mutated in their highly conserved G protein binding Asp-Arg-Tyr (DRY) stop short at the anterior edge of the OB and cannot innervate the glomerular layer (Imai *et al*., 2006). On the other hand, expressing constitutively active G_s protein results in a posterior shift of the

glomerular position (Imai *et al*., 2006) even though OR gene choice is not affected in the mutants. It should be noted that G_{olf} mutants and CNGA2 mutants do not show any defects in the initial formation of the glomerular map (Belluscio *et al*., 1998, Lin *et al*., 2000; Zheng *et al*., 2000). Therefore, it is suggested that axonal targeting and canonical receptor signaling are independent processes (Imai and Sakano, 2007). In other studies, deletion of the ACIII gene results in severe impairment of the glomerular map in anterior-posterior axis of the olfactory bulb (Chesler *et al*., 2007; Col *et al*., 2007) suggesting that cAMP signaling is important for anterior-posterior patterning. Moreover, dominant negative PKA or mutations in the CREB gene can cause an anterior shift (Imai *et al*., 2006). Therefore it has been proposed that Gs/cAMP/PKA/CREB signaling can regulate the axonal convergence along the anterior-posterior axis. Imai *et al*.(2006) have also identified target genes for this signaling pathway by using single cell microarray technique. Neuropilin 1 expression was shown to be correlated with high cAMP signals: Neuropilin1 is expressed in an anterior low-posterior high expression gradient in the olfactory bulb. Moreover, deletion of the repulsive ligand of Neuropilin 1, Semaphorin 3A, resulted in defects of anterior posterior patterning of the glomerular map (Imai *et al*., 2006; Schwarting *et al*., 2000). These results suggest that cAMP signal-dependent Neuropilin 1 levelsare a crucial regulator of the glomerular map along the anterior-posterior axis.

It has been shown that further fine-tuning of the glomerular position is determined in an activity-dpendent manner. CNGA null mice display abnormal glomerulus formation for some of the ORs examined (Zheng *et al*., 2000). In addition, inhibition of neuronal activity by overexpression of the inward rectifying potassium channel Kir2.1 severely impairs glomerulus formation (Yu *et al*., 2004). Expression of homophilic adhesion molecules Kirrel2 /3 and axon guidanc molecules Ephrin-A5/Eph-A5 is regulated in an activity dependent manner and their ablation results in duplicated glomeruli in the OB.

1.6. Negative feedback regulation of odorant receptor gene choice

It has been proposed that at least three steps of OR gene regulation contribute to the one neuron-one receptor phenomenon (Fuss and Ray, 2009; McClintock, 2010). First, one and only one OR allele is selected to be expressed, regardless whether this choice is

stochastic or deterministic. Second, expression of the selected OR must be maintained and third the expression of other OR genes must be inhibited throughout the life of the OSN. Serizawa *et al*., (2003) have proposed that a transcriptional activation complex including a locus control region is stably established on an OR promoter in a stochastic manner. Once the OR protein is expressed, it sends a negative feedback signal which ensures that the expression of other ORs is suppressed (Serizawa *et al*., 2003).

In last decade, several studies reinforced the idea of negative feedback regulation (Serizawa *et al*., 2003, Feinstein *et al*., 2004, Shykind *et al*., 2004; Lewcock and Reed, 2004; Bozza *et al*., 2009). Serizawa *et al*., (2003)deleted the entire coding sequence of the MOR28 gene in a yeast artificial chromosome, which contains a large part of the MOR28 gene cluster and demonstrated that in the absence of the OR coding sequence, OSNs which initially selected this allele expressed another OR.Typically these ORs overlap in their expression domain with that of MOR28. In addition, OSNS expressing naturally occurring frameshift mutants of OR genes with premature stop codons also expressed another OR from the same expression zone together with the frameshift mutation. A similar result is seen when the β-galactosidase gene was expressed under the control of proximal upstream genomic region of M4 gene. In this case, 1% of the labeled cells coexpressed the endogenous M4 gene (Qasba and Reed, 1998).

An outcome of this 'second choice' is a change in the number and positions of the labeled glomeruli when the OR coding sequence is replaced with a fluorescent protein (Serizawa *et al*., 2003, Feinstein *et al*., 2004; Bozza *et al*., 2009). When the M71 or M72 coding sequences were replaced with green fluorescent protein (GFP), OSNs expressing these alleles project their axons to multiple glomeruli in the OB. Interestingly, axons of mutant OSNs, which are normally expressed exclusively in the dorsal MOE, innervate only the OCAM negative dorsal OB, implying that second choice is not random (Feinstein *et al*., 2004). Moreover, calcium imaging in mutant cells has shown that they response to a variety of different odorant. OSNs expressing a functional M72 gene exclusively respond to acetophenone, thus suggesting that mutant OSNs express ORs other than M72 (Feinstein *et al*., 2004). Therefore, it would be reasonable to assume that the number of glomeruli targeted by mutant OSNs reflects the ORs that are selected for second choice.

Bozza *et al*.(2009) have shown that second choice is also class restricted. They have replaced the coding sequences of S50 (Class I) and M72 (Class II) with different fluorescent proteins and demonstrated that they converge to mutually exclusive regions in the olfactory bulb, corresponding to territories innervated by class I or class II expressing OSNs. They proposed that this class restriction for second choice is due to the presence of different OSN types which are destined to express Class I or Class II ORs.

There is strong evidence that it is not the DNA or RNA sequence that generates the negative feedback signal but the OR protein. In one study, the M4 OR gene without a start codon was expressed under the control of a short promoter. In this experiment, no protein is translated while RNA is transcribed. OSNs expressing this transgene undergo second choice, suggesting that the source of the negative feedback signal is the OR protein (Lewcock and Reed, 2004). Likewise, OSNs expressing naturally occurring frameshift mutants with a premature stop codon also make a second choice (Serizawa *et al*., 2003). Although there is little known about the nature of the negative feedback signal, it seems that it is not mediated by G protein signaling. Mutations in the highly conserved G protein binding motif of OR proteins, or OSNs expressing a constitutively active form of G_s did not induce second choice (Imai et al, 2006; Nguyen *et al*., 2007). However, it has been demonstrated that replacing the OR coding sequence with theβ_2-Adrenergic Receptor can prevent second choice; OSNs expressing β2 Adrenergic receptor from an OR locus are able to from distinct glomeruli in the olfactory bulb, suggesting that they do not express different receptors and that the adrenergic receptor can substitute for an OR. On the other hand OSNs expressing a V1R from an OR locus cannot form defined glomeruli (Feinstein *et al*., 2004). It has yet to be shown whether a common signaling pathway downstream of ORs and β2 Adrenergic Receptor but not of V1Rs may account for this phenotype.

How does the negative feedback signal generated by the expressed OR protein act on the other OR loci to prevent their expression? How is the negative feedback signal received by other OR loci? When is the specific time period in which singularity of OR gene expression is established? These questions are only partially answered so far. It has been demonstrated that the presence of an OR promoter is not a necessity for OR repression. When OR coding sequence is fused with other olfactory tissue-specific promoters, such as

OMP and Gγ8 no transgenic OSNs were observed. It is possible that proximity of OR coding sequence to an olfactory specific promoter results in transgene silencing. In order to test this hypothesis, Nguyen *et al*., (2009) have expressed OR proteins under the control of synthetic TetO promoter which is active only when genetically encoded tetracyclin transactivators (TTA) are present (Gossen et al, 1995). They have generated different knock-in mice, which express TTA under control of different olfactory specific promoters, namely OMP, Gγ8 and odorant receptor P2, and crossed them with TetO-OR lines. Interestingly, in situ hybridization together with antibody staining has shown that OSNs expressing the OR under TetO promoter control never coexpressed endogenous ORs and OSNs expressing endogenous ORs never coexpressed the transgene. Since the transgene did not have an endogenous promoter but only the OR coding sequence, it has been suggested that the negative feedback signal acts on the OR coding sequence rather than the OR promoter or sequences contained within the native OR locus. The signal can act on either the DNA or RNA level but not the OR protein because transgene OR mRNA could not be detected in the OSNs expressing endogenous ORs (Nguyen *et al*., 2007).

On the other hand, the timing of TTA expression was important for the heterologous expression of the OR transgene. In the mouse, OSNs are continuously generated by stem cells (globose basal cells) residing in the basal epithelium (Calof and Chikarashi, 1989). During maturation, OSNs migrate apically while they turn on and off different genes. Globose basal cells express cytokeratin 5 and cytokeratin 14. Immature OSNs express GAP43 while mature OSNs express OR proteins together with OMP. When Nguyen *et al*., used promoters of late markers (such as OMP and OR) to express TTA, only a small fraction cells express the TetO driven OR transgene. Indeed, when TTA is expressed in P2-IRES-TTA bicistronic transgene, less than 2% of odorant receptor P2 positive cells express the TetO-OR transgene, while 10-30% of cells express the OR transgene when TTA is expressed by the OMP promoter. Conversely, when TTA is expressed under the control of the Gγ8 promoter which is expressed mostly in immature OSNs, OR transgene is broadly expressed in the MOE (Nguyen *et al*., 2007). However, Gγ8 is predominantly expressed in the developing OSNs and silenced in mature neurons; under the control of TTA expressed by the Gγ8 promoter, OR transgene expression is only transient and the number of OSNs expressing the transgene is very low in the adult MOE. On the other hand, by

complementary expression of TTA under the control of markers expressed in different stages of OSN development (namely, Gγ8 and OMP), this problem is overcome, now majority of OSNs express the TetO driven OR transgene (Nguyen *et al*., 2007).

In the light of these results, several conclusions can be drawn. First there should be a critical time period at the end of which the singularity of OR gene is established. Presumably, this period corresponds to the early life of the OSNs when they are undergoing maturation, since driving the TetO-OR expression with an early promoter (Gγ8) gives significantly higher number of OR transgene-positive cells than expression under control of a late promoter (OMP). In addition, expressing TTA under an OR promoter (P2 promoter) results in very low OR transgene expression. Interestingly, the cells positive for both P2 and TetO driven OR transgene are mostly found in the basal epithelium where immature OSNs are found. Thus, it is reasonable to assume that there is an early stage in OSN development during which two ORs can be expressed simultaneously. Conceivably, appropriate amounts of the OR protein necessary to establish the negative feedback signal have to be accumulated during this period. Heterologous expression of the transgenic OR very early in the OSN development by using Gγ8 promoter makes it possible to accumulate sufficient amounts of transgenic OR protein to suppress the endogenous ORs in many OSNS resulting in high number of transgenic OSNs. However, using late markers such as OMP results in a much smaller number of transgenic OSNs. Most likely, OMP expression precedes OR expression for a small number of ORs while vast majority of ORs are expressed before OMP. Thus, transgenic OR expression could precede and suppress endogenous OR expression only for this small subset of ORs.

Singularity of OR gene expression is ensured in multiple regulatory steps, including OR gene choice, cis-regulatory sequence-dependent restriction/inhibition, and OR coding sequence-dependent silencing. In order to establish a neuronal identity in the adulthood, these steps of regulation might take place early in the development of an OSNbefore it establishes functional connections with the olfactory bulb. Indeed, Nguyen *et al*., have shown that an early developmental stage exists in which multiple ORs can be coexpressed. Eventually, at the end of this period, one of the ORs can win the race and accumulate

enough OR protein to silence the others. Then, one can imagine that if two different ORs are expressed simultaneously with the same expression levels throughout the life of the OSN, neither can silence the other. Indeed, OSNs are capable of expressing two different ORs from the same regulatory sequence in a bicistronic construct (M72-IRES-rI7-IRES-GFP) without any morphological and functional defect. Those cells responded to both octanal and acetophenone which are ligands for I7 and M72, respectively (Nguyen *et al*., 2007).

An OSN is competent to express one of the many OR genes expressed in a given zone (Ressler *et al*., 1993, Vassar *et al*., 1993). Moreover, it is also inherently capable of expressing multiple OR genes (Nguyen *et al*., 2007). How is then, one neuron-one receptor phenomena established? Previously, the H-region has been proposed to be a trans-acting locus control region which interacts with only one OR promoter at a time, thereby ensuring singular OR expression (Lomvardas *et al*., 2006). Later, deletion of the H-region has shown that it is a cis-acting element and is not capable of inter-chromosomal interactions (Nishizumi *et al*., 2007; Fuss *et al*., 2007). Therefore H region itself cannot ensure that a single odorant receptor is stably expressed per neuron.

Indeed, OR gene choice is not always stable, at least for two ORs switching of OR expression was demonstrated (Shykind *et al*., 2004). When IRES-Cre recombinase is targeted to the 3'-UTR of the MOR28 gene and the transgenic line is crossed with a Cre reporter line, 10% of MOR28-expressing OSNs switched to expression of another OR, which is expressed in the same zone as MOR28. Moreover, when MOR28 coding sequence was replaced with IRES-Cre, 97% of the cells expressing the deletion allele in 2 weeks old embryo extinguished its expression and switched to a functional OR by 12 weeks (Shykind *et al*., 2004). However it is contradictory toprevious data where the deletion allele was suppressed in the absence of a coding sequence (Nguyen *et al*., 2007). Perhaps, presence of the IRES sequence results in suppression of the allele by an unknown mechanism. In addition, OSNs may have an alternative regulatory mechanism which can act later than the previously defined coding sequence dependent mechanism (Nguyen *et al*., 2007) and ensure that cellular resources are not wasted on the expression of non-functional receptors. Actually this would be an appropriate regulatory checkpoint to inhibit the coexpression of naturally occurring pseudogenes. No matter how, it is obvious that

OSNs are inherently capable of switching OR gene expression (Shykind *et al*., 2004). Interestingly, expression of the mutant MOR28 allele is mostly observed in immature OSNs which do not yet express OMP (Iwema and Schwob, 2003) and diminished in mature OSNs. Consistent with the previous data (Nguyen *et al*., 2007), switching most likely occurs early in the OSN development when OSN has not established stable axonal connections with the olfactory bulb and gained a functional identity yet. Therefore, it is conceivable to suggest that OR switching is another layer of regulation that ensures adult OSNs express only one and a functional odorant receptor.

2. PURPOSE

The first part of this thesis aims to identify whether a critical period exists during the ontogenetic development of OSNs, at the end of which the singularity of OR gene expression is established, using zebrafish as a model system. It is assumed that early in sensory neuron development multiple olfactory receptors can be expressed simultaneously by a single OSN because negative feedback signaling is not completely established at this time. Although previous studies have proposed that this period might correspond to the early life of OSNs, there is no detailed information about its precise timing. Moreover, it is equally unknown whether OSNs have the plasticity to switch OR gene expression after they have established functional connections with the olfactory bulb. Until now, second OR gene choice has been reported in several studies only when the OR coding sequence has been deleted before the OR allele has been chosen for expression. Transgenic constructs were engineered for the conditional OR ablation of the zebrafish OR111-7 gene. This construct allows the introduction of an OR111-7 gene deletion in OSNs, which had previously expressed the OR111-7 but then undergo Cre/LoxP-mediated excision of the OR coding sequence. Thus, by temporally controlling the OR111-7 deletion in transgenesthe existence and the extent of a critical period during which OSNsare able to undergo second choice could be identified.

In the second part of this thesis, the concept of negative feedback signaling in OR gene choice is addressed. It has been suggested that a negative feedback signal acts on the OR coding sequence to suppress expression of more than one OR per sensory neuron. Consistent with this notion, transgenic constructs which include the OR111-7 coding sequence are expressed in a low number of OSNs when compared to transgenic constructs in which an identical promoter drives the expression of a fluorescent reporter gene but does not contain the OR coding sequence. By making serial deletions of the OR coding sequence in transgenic constructs and scoring their expression efficiencies and characteristics, the target sequences within the OR coding sequence, which are critical for receiving the suppressive negative feedback signal, could be identified.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Fish

AB/AB, AB/Tü and Tü/Tü zebrafish inbred strains (both embryos and adults) were purchased from the Zebrafish International Resource Center (ZIRC), at the University of Oregon, Eugene, USA and raised in our zebrafish facility.

3.1.2. Equipment and Supplies

The list of equipments, chemicals and consumables can be found in Appendix A and Appendix B.

3.1.3. Buffers and Solutions

The buffers and solutions were prepared according to Sambrook and Russell (1989) unless they are supplied directly by the manufacturers. Zebrafish specific solutions were prepared according to Westerfied (1997).

3.2. Methods

3.2.1. Fish Maintainance

Individuals from zebrafish strains Ab/Ab, Ab/Tü and Tü/Tü were kept in 14 hour light/10 hour dark cycle at 28.5 °C. Five to ten adult zebrafish were put in 3 liter (lt) tanks connected to a specific water system with aeration and 5 stage filtration (Stand Alone System, Aquatic Habitats, FL). Adult zebrafish were fed three times a day, twice with flake food (Sera Vipan, Germany) and once with live food, brine shrimp (*Artemia sp*.). Artifical fish water was prepared as follows: 2 g ocean salt, 7.5 g sodium bicarbonate and 0.84 g calcium sulfate were mixed thoroughly in 100 lt of dH_2O.

After fertilization zebrafish embryos were kept in a petri dish with E3 medium prepared according to the instructions by Westerfield (1993). Then they were kept in small, regularly cleaned, meshed and aerated tanks for between 6th and 30th days of development in a 28 °C aquarium. Zebrafish embryos were fed three times only with paramecia starting from 6dpf to 12 dpf (day post fertilization). Between 12 dpf and 30 dpf they were fed with paramecia, artemia and finely ground flake food once a day for each. After 30 dpf they were moved into the water system and fed according to regular adult feeding schedule.

For mating, two males and one female were put in 1 lt breeding tanks in the evening. Males and female were separated with a separator in the tank. Next day at the beginning of the light cycle the separators are removed and fishes were left for breeding. Fertilized eggs were collected into a petri dish, cleaned with fresh system water and transferred into petri dishes with E3 medium.

3.2.2. Microinjection into Zebrafish Oocytes

Fertilized zebrafish oocytes were collected after breeding in the morning and cleaned as it was explained above. They were placed into 2% agarose plate with specific grids in which the eggs can be aligned.

Fine capillary needles were filled with injection solution consisting of 80 ng/ul plasmid DNA (previously ethanol precipitated and dissolved in water), 0.1% KCl, and 0.01 % phenol red in distilled water. Approximately 2 nanoliters of plasmid DNA was injected into fertilized zebrafish at single-cell stage by using FemtoJet microinjection device (Eppendorf, Germany). Then injected embryos were transferred into E3 medium.

3.2.3. Polymerase Chain Reaction

Polymerase Chain Reactions (PCRs) were performed by using Titanium Taq or Advantage 2 Polymerase mix (Clontech, USA) according to manufacturer's instructions. Taq DNA Polymerase (Fermentas, USA) was used for colony PCRs according to manufacturer's protocols. Standard PCR protocol was used to amplify 5-50 nanogram (ng)

template (Final concentration: 0,5 micro-molar (μM) from forward and reverse primers, 1X reaction buffer, 1,5 milli-molar (mM) $MgCl_2$ if not supplied in the buffer, 0,2mM dNTP mix and 1-3 Units of Taq polymerase). Briefly, 4 min initial denaturation at 95 °C is followed by 16-24 cycles of 30 seconds at 95 °C°C, 30 seconds at the annealing temperature of the primer with the lowest T_m and 1minute (min) /1 kilobase (kb) target sequence at 72 °C. Then, 10 min of final elongation at 72 °C was followed by 30 min at 4 °C.

3.2.4. Restriction Endonuclease Digests of DNA

Two Units of different restriction endonucleases (NEB, USA) were used to digest 1ug DNA in reaction mixture with 1X reaction buffer at 37 °C for 1 to 8 hours. 1μg/ml bovine serum albumin (BSA, NEB, USA) was added if it is suggested by the manufacturer.

3.2.5. Agarose Gel Electrophoresis and DNA Extraction from the Gel

DNA samples were run in 1% agarose gel with ethidium bromide until the bands are clearly separated. 1kb DNA ladder (NEB, USA) was used as a molecular weight marker.

High Pure PCR Purification Kit (Roche,USA) was used to extract DNA from agarose gels according to instruction manual. Briefly, DNA samples were run in 1% agarose gel and DNA band of interest was cut under UV light by using a scalpel. 100μl of PCR binding buffer was added per 0.1g ofexcised gel, vortexed, incubated at 56 °C for 10 min, passed through a spin column, washed twice and eluted with 30-50μl of elution buffer.

3.2.6. PCR Purification

High Pure PCR Purification Kit (Roche,USA) was used to purify PCR products and plasmids. 100μl of PCR product was mixed with 500μl PCR binding buffer and passed through spin columns at 13.000 revolutions per minute (rpm). After two washes with wash buffer, DNA was eluted with elution buffer to the final volume of 30-50μl.

3.2.7. Ligation of DNA Fragments to Vectors

Vectors and DNA fragments were digested with appropriate restriction enzymes (NEB, USA) and run on 1% agarose gel. Desired bands were cut from gel and extracted by using High Pure PCR Purification Kit (Roche,USA). Extacted DNA samples were run on 1% agarose gel in equal volume to estimate the DNA concentration by relative intensity. For ligation, 1:3 vector:insert ratio was used. Total 100ng DNA was mixed with 1X ligation buffer (final concentration) and 1 unit of T4 DNA ligase (NEB, USA) and incubated at room temperature for 30 min. The ligation mixture was directly transformed into 50µl of chemically competent E. *Coli* strain TOP10.

PCR products were directly ligated to p-GEMT Easy vector (Promega, USA) by using TA Cloning. 3µl of PCR product was mixed with 1µl p-GEMT Easy vector, 5µl of 2X Ligase Buffer and 3 units of T4 DNA ligase (Promega, USA). The mixture was incubated 15 min and transformed into 50µl chemically competent TOP10 cells.

3.2.8. Preparation of Chemically Competent Cells

Five ml liquid culture of E. *Coli* strain TOP10 was grown overnight at 37 °C. Next morning, 500 ml of sterile LB was inoculated with 1ml of overnight culture and grown until the OD_{633nm} reaches 0.6-0.8. Then the culture was chilled on ice for 15 min and centrifuged at 4500rpm for 10 min. The supernatant was discarded and the bacteria pellet was resuspended in 30ml TfbI (100mM RbCl, 50mM $MnCl_2$-$4H_2O$, 30mM potassium acetate, 10mM $CaCl_2$-2H2O and 15% glycerol, chilled and filter sterilized) on ice by pipetting up and down. After complete resuspension the bacteria was chilled on ice for 15 min and centrifuged at 4000rpm for 5min. Supernatant was discarded and pellet was resuspended with 6ml Tfb II (10mM MOPS pH6.5, 10mM RbCl, 15% glycerol and 100mM $CaCl_2$, sterile filtered and kept on ice) on ice. 50µl aliquots of the chemically competent cells were prepared and stored at -80 °C.

3.2.9. Transformation of Plasmid DNA into Chemically Competent Cells

10µl of ligation mixture or 10ng of plasmid DNA was mixed with 50µl of competent cells. The mixture was kept on ice for 30 min, incubated at 42 °C for 90second (sec) and immediately transferred on ice. Whole transformation mixture was spread on an LB agar plate with appropriate antibiotics and grown overnight at 37 °C.

3.2.10. Analytical DNA digests with Restriction Endonucleases

Successful cloning was verified by digesting the plasmid with appropriate restriction endonucleases (NEB, USA) which gives unique DNA fragments when it is run on 1% agarose gel.

3.2.11. Bacterial Artifical Chromosome (BAC) Recombineering

BAC100G14 was transformed into SW102 E. *Coli* strain as follows: 5ml overnight culture of SW102 cells was grown at 32 °C. 50 µl of the overnight culture was inoculated into 25 ml LB in 250ml flask and grown at 32 °C. When OD_{633nm} reaches 0.6, the culture was chilled on ice for 15 min and centrifuged at 0 °C for 5 min at 4500rpm in 50 ml falcon tube. The supernatant was discarded carefully and the pellet was resuspended in ice without pipetting or vortexing using 1ml ice cold ddH_2O. Centrifugation and resuspension steps were repeated once more and finally the pellet was resuspended with ddH_2O in a final volume of 50µl. 25µl of electrocompetent SW102 cells were transformed with 1µg BAC100G14 DNA by using 0.1 cm cuvette with 1.75 kilovolts(kV) impulse. Transformed bacteria were spread on LB agar plate with 12.5µg /ml chloramphenicol and grown overnight at 32 °C. Homology arms flanking the target site on the BAC clone was amplified using appropriate primers and galK gene or the desired mutation was cloned in between these homology arms by conventional cloning. In the first step of recombineering the homology arm-galK-homology arm (HA-galK-HA) was cut from the plasmid with appropriate restriction enzymes and run on 1% agarose gel overnight. HA-galK-HA was carefully cut from the gel, purified by using High Pure PCR purification Kit (Roche) and eluted in 30µl ddH_2O. SW102 cells with BAC100G14 (see above) were prepared for

electrotransformation as it was done for BAC100G14 electrotransformation except that the bacteria were heat-shocked at 42 °C before the first centrifugation in order to activate the recombineering system. 200ng of purified HA-galK-HA was transformed into 25µl electrocompetent BAC100G14+ SW102 cells by using 0.1cm cuvettes and 1.75kV. Transformed bacteria were recovered in LB at 32 °C for 1 hour, pelleted, washed twice with M9 medium (1X M9: 6g N_2HPO_4, 3g KH_2PO_4, 1g NaH_4Cl and 0.5g NaCl in dH_2O total volume 1liter) and respsuspended in 1ml M9 medium. Then it was spread on 1X M63 minimal plates (5X M63: 10g $(NH_4)_2SO_4$, 68g KH_2PO_4, 2.5g $FeSO_4.7H_20$ and KOH to pH:7) with 0.001 M $MgSO_4.7H_2O$, 0.2% galactose, 45mg/lt leucine, 1mg/lt biotin and 12.5µg/ml chloramphenicol. The bacteria were grown at 32 °C overnight. Few colonies were picked and streaked on McConkey agar plates with 0.2% galactose and 12.5µg/ml chloramphenicol. Next day, bright red/pink colonies were selected (break down of galactose by galactokinase results in a pH change which results in this color) and grown in 5ml LB broth. Successful galK recombineering was screened by restriction enzyme digest and sequencing of BAC100G14. In the next step, these steps were repeated for the homology arm-desired mutation-homology arm fragment (HA-M-HA), however the colonies were selected against the presence of galK gene by using 0.2% 2-deoxygalactose (2-DOG) and glycerol instead of galactose. HA-M-HA was recombineered into galK+ BAC100G14 resulting in the replacement of HA-galK-HA with HA-M-HA. Break down of 2-DOG with galactokinase results in a toxic metabolite and thereby elimination of galK positive colonies. Therefore, the colonies survived on M63 minimal plates with 2-DOG and glycerol were positive for the desired mutation. Few colonies picked from the M63 minimal plates with 2-DOG and glycerol and grown in 5ml LB overnight at 32 °C. Successful recombineering was screened by restriction enzyme digest and sequencing of BAC100G14.

3.2.12. Generation of 111-7-T2A-eYFP BAC100G14

563 base pairs (bp) and 530 bp homology arms were cloned immediately upstream and downstream of the site of mutation using the primer pairs GB-111-7 EcoRV F with 111-7-XhoI R and GB_111-7-AscIMut F with GB_111-7-StuR, respectively. Then, the T2A-YFP sequence was cloned between these homology arms to be used in the

recombineering protocol (Figure 4.1.2.C, D down). It should be noted that the stop codon of the OR111-7 gene is deleted during the recombineering process in order to prevent the ribosome from stopping translation before reaching the T2A-eYFP sequence. The recombineering protocol explained above was performed to generate the 111-7-T2A-eYFP BAC100G14.

3.2.13. Generation of Venus > OR111-7 BAC100G14

The same homology arms that Sato *et al*. (2007) have used to replace the OR111-7 sequence in their experiments were used (Sato *et al*., 2007). Briefly, ~600 bp homology arms directly upstream and downstream of OR111-7 coding region were cloned using appropriate primers. Then, the galK cassette and Venus coding sequence were cloned between these homology arms to be used for the first and the second round of recombination, respectively. The recombineering was performed as it was explained above.

3.2.14. Generation of Conditional OR111-7 Ablation Transgene (Introduction of 5'loxp Sequence

LoxP sequence was recombineered into the 5' region of the OR111-7 gene in BAC100G14 by using the recombineering protocol described by Warming *et al*. (2005). LoxP sequence was cloned between two homology arms corresponding to the 5' region of OR111-7 genes using appropriate primers and restriction sites. Upstream homology arm was amplified by using the GB_111-7 EcoRI F and GB_111-7-SacI R primers. Dowstream homology arm was cloned by using the GB_111-7-SacI F and GB_111-7-EcoRV R primers. LoxP sequence flanked by two SacI sites was generated by annealing LoxP-SacI-Fwd and LoxP SacI-Rev oligos by incubating equal molar amounts of oligos in a temperature gradient starting from 95 C and ending at 55 °C. LoxP sequence was then cloned between two homology arms by using the SacI sites. Orientation was checked by sequencing.

3.2.15. Whole-Mount Antibody Staining of Zebrafish Embryos

Zebrafish embryos at different developmental stages were fixed in 4% PFA at room temperature for an hour. PFA was discarded and the embryos were dehydrated with 100% MeOH at room temperature for 15 min. Embryos were transferred into fresh 100% MeOH and left at -20 °C for 1 day to 2 months. Embryos were rehydrated with serial incubation in 75% MeOH, 50% MeOH and 25% MeOH at room temperature for 5 min each (without agitation). Then, they were incubated in 1X phosphate buffered saline (PBS) with 0.5% Triton-X (PBX5) for 4 times 5min. Then they were blocked in blocking buffer (PBX5 with 10% normal goat serum and 0.8% bovine serum albumin) for 2 hours. Primary antibodies were diluted in desired concentrations in blocking buffer and embryos were incubated with primary antibodies at 4 °C overnight. Next day, the embryos were washed with PBX5 3 times 20 min and 5 times 5 min. They were incubated in blocking buffer for an hour. Secondary antibodies were diluted in blocking buffer and the embryos were incubated with secondary antibodies for 2 hours then the embryos were washed with PBX5 3 times 20 min and 5 times 5 min. Lastly they were transferred into 1:1 PBS:glycerol and kept in 4 °C.

3.2.16. Whole Mount In situ Hybridization of Zebrafish Embryos

Chromogenic whole mount in situ hybridization with NBT/BCIP reaction was performed according to Thisse and Thisse (2007).

3.2.17. Imaging of Zebrafish Embryos

Embryos were mounted on 2% agarose gel and covered with a coverslip. Live embryos were anesthetized with 0.04% MS222 (Sigma, USA) before and during the imaging. Serial confocal images were captured by using SP5-AOBS laser scanning confocal microscope (Leica, Germany).

3.2.18. Generation of Partial Coding Sequence-eYFP Fusion Constructs

Initially, EcoRV restriction site that is present in the OR111-7 coding region was used to fuse the first 418 bp of the OR111-7 coding region to enhanced yellow fluorescent

protein in frame. Then appropriate degenerate primers with a 5' XhoI restriction endonuclease site were designed to increase the length of OR111-7 coding sequence step by step in the enhanced yellow fluorescent protein fusion. In this way, eYFP fusions of 517 bp and 721 bp of OR111-7 coding sequence in the pACSF vector were made using the XhoI restriction site upstream of eYFP sequence. Again, H-p571was used to drive the expression of the fusion proteins and named these constructs as H-p571-418bp-eYFP, H-p571-517bp-eYFP and H-p571-721bp-eYFP (Figure 4.2.3. and 4.2.5).

Table 3.1.: List of Antibodies Used in This Study.

Antibody	Company	Catalog No	Working Dilution
Anti-GFP,IgG Rabbit	Invitrogen	A-11122	1:500
Anti-GFP from mouse IgG	Roche	11814460001	1:500
DsRed Monoclonal Antibody, Rabbit	Clonetech	632496	1:500
Cre Monoclonal Antibody, Mouse	Covance	MMS-106R	1:500
Goat Anti Mouse Alexa 488	Invitrogen	A-11001	1:800
Goat Anti Rabbit Alexa 488	Invitrogen	A-11008	1:800
Goat Anti Mouse Alexa 555	Invitrogen	A-21422	1:800
Goat Anti Rabbit Alexa 555	Invitrogen	A-21428	1:800
Goat Anti Mouse Alexa 633	Invitrogen	A-21052	1:800

Table 3.2.: List of the Primers Used in This Study.

Primer	Sequence 5' to 3'
111-7-F	GCCTGTGCAAAACACTAAATG
111-7-R	CGTCTCAATGGGCAGAGATC
111-7-3CDS-F	GGCTTATGCTATTCCACCAATGC
111-7-3UTR-F	CTGGGGAGGCATGATATGTC
111-7-3UTR-R	GACATATCATGCCTCCCCAG
111-7-5Hind-F	GCTTTGAGAGCTAAAGCTCTGC
111-7-3Hind-R	GCTGTAAGGTATACCTGAAAGTCC
M13-F	CGCCAGGGTTTTCCCAGTCACGAC
M13-R	CAGGAAACAGCTATGACC
T7	TAATACGACTCACTATAGGG

Table 3.2.: List of Primers Used in This Study (cont.).

Primer	Sequence 5' to 3'
111_7_UTR_YFP_F	GTAAAGTTGCTATGGTGAGCAAGGGCGAGGAG
111_7_UTR_YFP_R	CTTGCTCACCATAGCAACTTTACATTTAGTGTTTTGC
GB_111_7_Poly_AscMut_F	GTCGACGATATCTGGCGCGCCAAGGCCTGTCGACGTAC
GB_111_7_Poly_AscMut_R	GTCGACAGGCCTTGGCGCGCCAGATATCGTCGACAGCT
LD_Poly_Pac_Sac_Asc_F	TAAGAGCTCTAGG
LD_Poly_Pac_Sac_Asc_R	CGCGCCTAGAGCTCTTAAT
GB_111_7_Poly 1_F	GTCGACGAATTCATGAGCTCTAGATATCGTCGACGTAC
GB_111_7_Poly 1_R	GTCGACGATATCATGAGCTCTAGAATTCGTCGACAGCT
GB111_7_AscIMut_F	GGCGCGCCAAACGTTTATTTTTTATTGTTTGATATCAC
GB111_7_AscIMut_R	TTGGCGCGCCTATTCACTTTGAAACCGTCTCAATGGG
GB_111_7_EcoRV_F	GCTTGCCATTAAGATATCATGTCATTGTG
CreFW1	ATGGCACCCAAGAAGAAGAGGAAG
CreRev1	GCCGCATAACCAGTGAAACAGCAT
GFP_F_819	GAGAAGCGCGATCACATGGTCCTG
GFP_F_up	GACAAGCAGAAGAACGGCATCAAGG
GFP_R_5	AACTTGTGGCCGTTTACGTCGC
mCherry_NcoI_F	ATCCATGGCCATCATCAAGGAGTTC
mCherry_NotI_R	ATGCGGCCGCTTACTTGTACAGCTCGTCCATGCCG
M13_R_highTM	GGAAACAGCTATGACCATGATTA
GFP_F	GCGACGTAAACGGCCACAAGTT
GFP_R	TACTTGTACAGCTCGTCCATGCCG
111_7_XhoI_R	TCTCGAGCTTTGAAACCGTCTCAATGGGC
111_7_myc2_AscI_R	GGCGCGCCTATTCACAAGTCCTCTTCAGAAATGAGCTT
111_7_myc1_R	CAGAAATGAGCTTTTGCTCCTTTGAAACCGTCTCAATG
GAP43_promoterF	CAAACAAGCTTGTGTGCACAAGCA
GAP43_promoterR	ATCCATGGTGGTATCTTCCCCTG
GFP_R_SalI	ATAGTCGACTTGTACAGCTCGTCCATG
TagRFP_T_NotI_R	AGCGGCCGCTTACTTGTACAGCTCG
TagRFP_T_NcoI_F	ATCCATGGTGTCTAAGGGCGAAG
GalK_F	TGCGTTGGCAAACAGAGATTGTGTT
GalK_R	TGAAACGTATGGGCGAGTTGATGG
creERT2_F	AACCTTTGGCCAAGCCCGCT
creERT2_R	CAGATTCATCATGCGGAACCGA
111_7_upstrm_Nco_R	GTCCATGGCAACTTTACATTTAGTG
111-7 3 prime Rec Control R	TTAGTTTGAAAAGCAATCTTTAAGCCTTAG
111_7_NCO_F	CACCATGGGTTCTTTAAATGCAAGC
GAP43_F	AAACCGGAGGAAAACGCTCA
GAP43_R	TTAAACACTCTCCTCTGTGCCGG
111-7 600bp R	ATCTCGAGTGTTGGTCACCAAAG
111-7 700bp R	ATCTCGAGATCCCATAATCTTATTT
111-7 800bp R	ATCTCGAGGTCTTTCCCAAGTTGCAA

RESULTS

3.3. Conditional OR gene Ablation Approach

3.3.1. Endogenous OR111-7 Expression

It was critical to pick up the right model odorant receptor before generating transgenic constructs to be used throughout this study. Three important criteria were considered to select the OR for further investigation. First, clear visualization and imaging of the peripheral olfactory system of the zebrafish is easier during the first seven days after fertilization since the progressive development of the olfactory placode, which undergoes complex folding to form the future rosette like olfactory epithelium, makes it difficult to perform whole mount imaging at later stages (Hansen and Zeiske, 1993). Therefore, an OR which is expressed during the first week of development was selected. Second, the OR should be expressed in a sufficiently high number of OSNs so that a statistically significant number of cells, including transgenic cells, could be obtained. Third, because the question of whether OSNs can undergo second choice at late developmental stages was aimed to be answered, it would be beneficial to know the target ORs, which can be selected for second OR gene choice. For this reason, the OR111-7 gene was selected to be used in the transgenic studies. OR111-7 expression begins around 30 hours post fertilization (30 hpf), and persists during the first week of development (Barth *et al*., 1997). Moreover, studies by Sato *et al*. (2007) used a BAC transgene to drive fluorescent reporter gene expression from the OR111-7 locus and identified a set of OR genes that are target for second OR gene choice. In addition, a study by Mori *et al*. (2000) identified a minimal promoter for this gene. For these advantageous reasons, the OR 111-7 gene was used for the transgenic studies.

3.3.2. OR111-7 is expressed in zebrafish embryos

First, in situ hybridization on whole-mount zebrafish embryos was performed to verify OR111-7 expression in OSNs. DIG-labeled antisense riboprobes were used to detect OR111-7 expression in the zebrafish olfactory epithelium. At 3 dpf, approximately 3-4

cells were labeled per olfactory placode (Figure 4.1.1.). Thus, it was decided to work with and analyze mostly 3dpf embryos since a sufficient number of OSNs expressing OR111-7 and axonal projections of these cells to the olfactory bulb are clearly visible at this stage (see below, Sato *et al*., 2007).

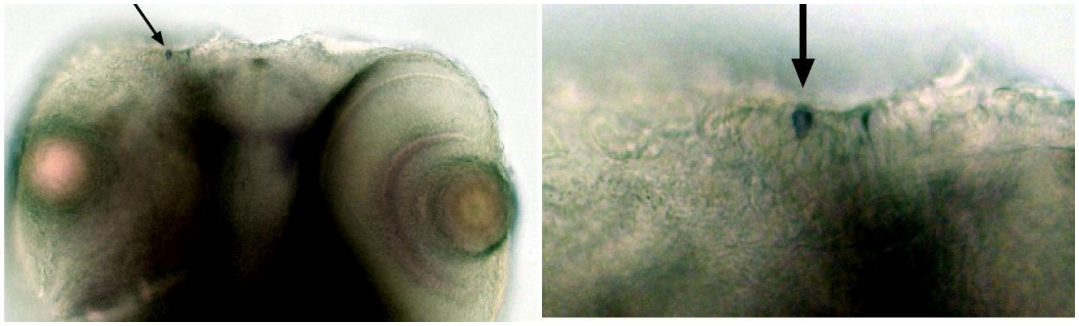


Figure 4.1.1: OR111-7 in situ hybridization on 3 dpf zebrafish embryos. Arrows indicate OR111-7 expressing OSNs.

3.3.3. OR111-7 Transgenic Constructs

In the conditional ablation approach, the OSNs expressing the OR111-7 transgene will undergo a deletion of the OR111-7 coding sequence upon Cre/LoxP-mediated recombination. Therefore, transgene-expressing OSNs will experience two scenarios. At an early stage, they would express the functional OR111-7 and follow their normal developmental program and eventually connect to appropriate glomeruli in the olfactory bulb. Next, upon recombination-based excision of the OR111-7 coding sequence, they will no longer be able to translate the OR111-7 protein from the transgenic construct. Thus, in principle these cells should no longer be able to emit a negative feedback signal and eventually switch OR gene expression. For this reason, it was necessary to confirm that OSNs expressing the transgene obey the general rules of odorant receptor gene choice, namely, the one neuron-one receptor rule, the formation of / connection to distinct glomeruli, and the maintenance of the negative feedback signal. However, it is still unknown for the zebrafish olfactory system whether OSNs expressing a particular odorant receptor project their axons to distinct glomeruli in the olfactory bulb. Therefore, it was decided to create an OR reporter construct in which the fluorescent reporter gene enhanced

yellow fluorescent protein (eYFP) is coexpressed with OR111-7. Second, it has been previously shown that when the OR111-7 coding sequence is replaced with a fluorescent reporter gene in a BAC transgenic context, OSNs selecting this allele coexpress another gene from the OR111 subfamily (Sato *et al*., 2007). Thus, it was also decided to generate an OR deletion construct in which the OR111-7 coding sequence has been replaced with the yellow fluorescent reporter gene Venus. Therefore, it was assumed that the OR reporter and OR deletion constructs represent the two extremes that OSN undergoing a conditional OR deletion would experience.

A two-way strategy was adopted to create OR reporter transgenes, a BAC transgenic and a short promoter transgenic approach. By doing so, it was aimed to guarantee that there is at least one platform available, which shows reasonable transgene expression and is sufficient for the intended experiments. In both strategies, the OR111-7 coding sequence was tagged with a T2A-EYFP reporter sequence. The rational was that conventionally used internal ribosome entry sites (IRESs) shows very low activity in zebrafish (Sato *et al*., 2007). Yet, it has been demonstrated that it is possible to translate two independent proteins from a single messenger RNA in a one to one ratio by using 2A peptides in zebrafish (Provost *et al*., 2007). T2A is a short viral peptide which promotes cleavage by exon skipping during translation (Holst *et al*., 2006). In this case, the T2A-eYFP sequence was cloned downstream of the OR111-7 coding sequence. After translation, OR protein and T2A-EYFP protein will be uncoupled eliminating the possibility that a bulky fusion of OR protein and the fluorescent reporter may interfere with the function of the OR protein.

3.3.4. OR111-7 Reporter BAC Transgenesis

In a previous study, a BAC clone bearing 16 functional zebrafish OR genes from different subfamilies including most of the OR111 subfamily (BAC100G14, Sato *et al*., 2007, Figure 4.1.2.A) was used. First, a BAC transgenic constructwas created using recombineering in bacteria, where a T2A-YFP sequence was engineered in frame with and immediately downstream of OR111-7 coding sequence in BAC100G14 (Warming *et al*., 2005).

In a first attempt to recombine the mutation downstream of the OR111-7 gene in BAC100G14, the recombineering protocol developed by Lalioti and Heath (2001), which utilizes a two-step recombineering strategy with a shuttle vector and a vector that drives the expression of the required recombination proteins under control of a heat shock promoter, was used. However, this system did not work efficiently in our hands. Therefore, an alternative recombineering system developed by Warming *et al*. was utilized (2005, see methods for details).

In the resulting BAC transgene, the T2A-eYFP sequence was successfully integrated immediately downstream and in frame with the OR111-7 coding sequence. The resulting BAC100G14 was designated OR111-7-T2A-eYFP BAC100G14 (Figure4.1.2. E, right).

3.3.5. Generation of a BAC transgene carrying a deletion of the OR111-7 Coding Sequence

For the BAC deletion construct, it was decided to replace the OR111-7 coding sequence with the sequence coding for the fluorescent reporter gene Venus in BAC100G14. For this purpose, exactly the same homology arms that Sato *et al*. (2007) have used to replace the OR111-7 sequence in their experiments were selected (Sato *et al*., 2007). After two rounds of recombineering, the recombinant BAC100G14, which contains the Venus sequence in place of OR111-7 coding region was obtained (Venus > OR111-7 BAC100G14) (Figure 4.1.2. E, left).

3.3.6. Low Efficiency of BAC Transgenesis

Considering the importance of positional effects on OR gene expression (Vassalli *et al*., 2002), BAC transgenesis seems to be the most appropriate approach in zebrafish to mimic the genomic context in which OR111-7 gene resides. Studies in zebrafish are limited because of the lack of tools to modify genes within their endogenous genomic locus. However, several problems with BAC transgenesis due to the high molecular weight of BAC100G14 (>100kb) were experienced. First, high concentrations of BAC DNA had to be injected in order to reach the optimal copy number of OR111-7 for efficient transgenesis. High DNA concentrations turned out to be lethal (Survival rate <10%,

n>1000 injected embryos). Second, finding an appropriate restriction enzyme to linearize the BAC is a limiting factor since only linearized DNA can be integrated into zebrafish genome. Third, high molecular weight can hinder nuclear transport of the BAC DNA.

Table 4.1: Constructs used throughout Section 4.1.

Construct:	**In:**	**Purpose**
OR111-7-T2A-eYFP BAC100G14	4.1.4., 4.1.6.	Visualization of OR111-7 expressing cells
Venus>OR111-7 BAC100G14	4.1.5., 4.1.6.	Visualization of OR111-7 deletion cells
p571-eYFP	4.1.7.	Visualization of OR111-7 deletion cells
p711-eYFP	4.1.7.	Visualization of OR111-7 deletion cells
H-p571-eYFP	4.1.7., 4.1.8.	More efficient visualization of OR111-7 deletion cells
H-p571-OR111-7-T2A-eYFP	4.1.9., 4.1.10.	Visualization of OR111-7 expressing cells
H-p571-eYFP-T2A-mCherry	4.1.9.	Analysis of T2A Cleavage Efficiency
pGAP43-eYFP-T2A-mCherry	4.1.9.	Analysis of T2A Cleavage Efficiency
hsp70-mCherry-T2A-CreERT2	4.1.9.	Analysis of T2A Cleavage Efficiency
H-p571-OR111-7-T2A-eYFP-3'UTR	4.1.9., 4.1.10.	Visualization of OR111-7 expressing cells
Conditional OR111-7 Ablation Construct	4.1.11., 4.1.14.	Analysis of late OR deletion
pOMP-mCherry	4.1.	Olfactory map visualization, Normalization of Injection Efficiency
pOMP-Cre	4.1.12.	Induction of conditional deletion after OR expression
pOMP-CreERT2	4.1.12.	Increased temporal control in conditional OR111-7 deletion
pGAP43-eGFP	4.1.13.	Testing promoters for earlier timing of conditional OR111-7 deletion

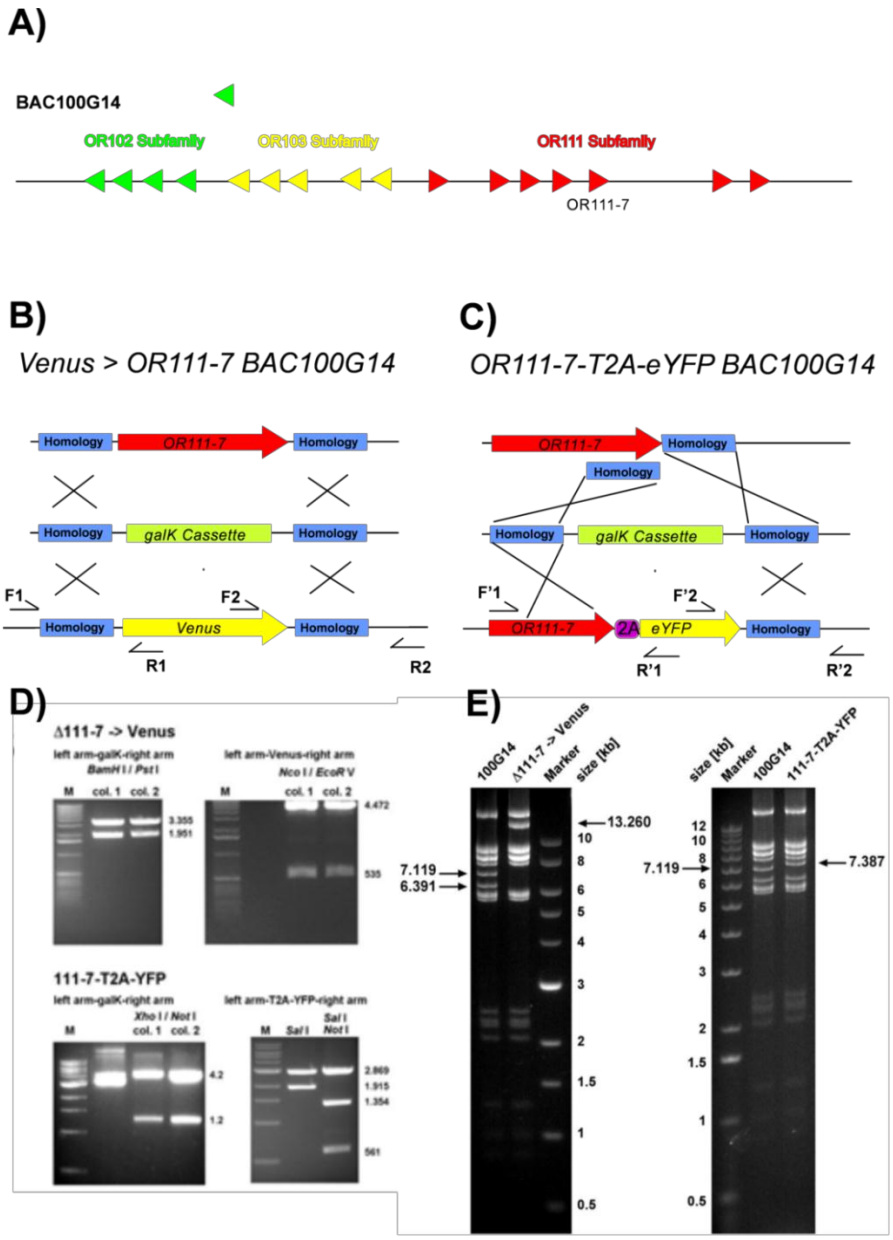
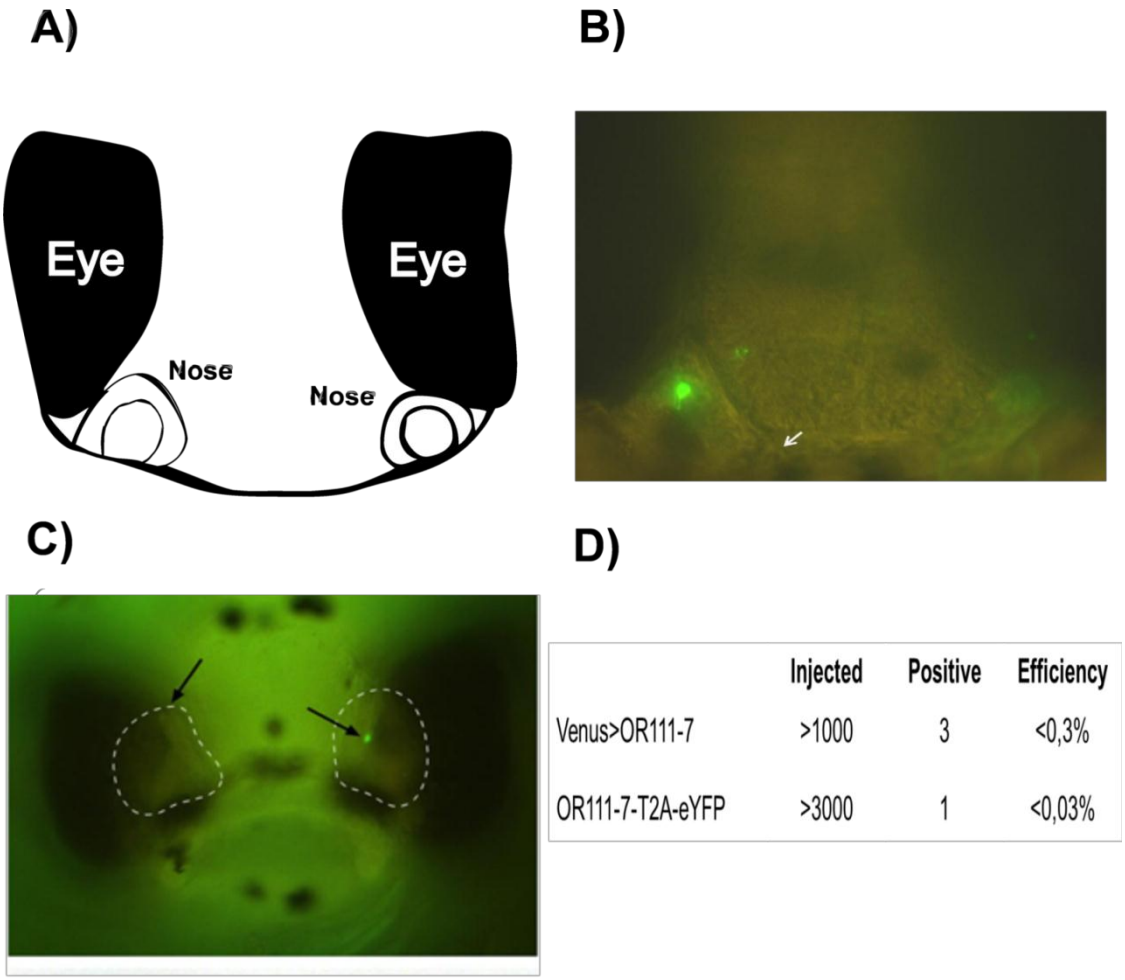


Figure 4.1.2.: Generation of Transgenic BAC Constructs.

A) Schematic representation of BAC Clone BAC100G14. B) Two-step recombineering for Venus >OR111-7 Mutation. C) Two-step recombineering for OR111-7-T2A-eYFP construct D) Verification of two-step targeting construct for Venus >OR111-7 (above) and OR111-7-T2A-eYFP (below) by restriction analysis. E) Left, verification of successful recombination for Venus > OR111-7 and OR111-7-T2A-eYFP recombinants BAC clones.

Indeed, when either Venus > OR111-7 BAC100G14 or OR111-7-T2A-eYFP BAC100G14 was injected, very low transgene efficiency was observed (Figure4.1.3.D). For Venus > OR111-7 BAC100G14, transgene expression could be detected only in 3

embryos (Figure4.1.3.B, D; n>1000 injected embryos). Similarly OR111-7-T2A-eYFP BAC microinjection resulted in only one positive embryo (Figure 4.1.3.C, D; n>3000 injected embryos). Although transgene expression was specific to the olfactory system, following experiments were not performed with BAC transgenesis because of its extremely low efficiency.



	Injected	Positive	Efficiency
Venus>OR111-7	>1000	3	<0,3%
OR111-7-T2A-eYFP	>3000	1	<0,03%

Figure 4.1.3.: Efficiency of BAC Transgenesis.

A) Schematic representation the anterodorsal view of 3dpf zebrafish embryo. B) Venus > OR111-7 injected 3dpf embryo. C) OR111-7-T2A-eYFP injected 3dpf embryo. Dashed lines indicate the left and right noses. Arrows indicate eYFP positive OSNs. D) A summary of BAC transgenesis efficiency.

3.3.7. OR111-7 Short Promoter Construct

In mouse, it has been shown that short genomic regions directly upstream of the OR coding region are sufficient to recapitulate endogenous OR expression (Qasba and Reed, 1998; Vassalli *et al*., 2002, Lewcock and Reed, 2004). Therefore, it was decided to search for a short promoter construct for OR111-7 which can drive robust expression with better efficiency. Previously, Mori *et al*. (2000) have identified a 571bp genomic region directly upstream of the OR111-7 coding region that can drive sufficient reporter expression. In an initial trial, the same sequence was used to drive the expression of enhanced yellow fluorescent protein (p571-eYFP construct, Figure 4.1.4.A, B). The p571-eYFP construct was microinjected into 230 fertilized zebrafish oocytes and 30 embryos that survived to 1dpf were obtained. Out of these 30 embryos only one showed eYFP expression in the olfactory placode (3% of survived embryos). To test whether longer upstream genomic regions drive more robust reporter expression, A 711 bp spaning genomic region directly upstream of the OR111-7 translational start site was cloned (Figure 4.1.4.A, B). After microinjection into single-cell oocytes, reporter expression could not be detected in the olfactory placode of 1 dpf embryos (132 injected oocytes, 60 survived embryos at 1 dpf, 0 positive for reporter gene expression). Thus, it was decided to utilize the shorter 571bp sequence to drive transgene expression.

However, only ~3% of embryos showed reporter gene expression when the p571-eYFP construct was used, which is still a too low efficiency for quantitative experiments. Moreover, the number of cells expressing the reporter gene in the positive embryos did not exceed 3 cells per fish. In search for a more robust driver, the 2,1 kb mouse H-region was fused with p571 and eYFP was cloned directly downstream of this fusion (H-p571-eYFP, Figure 4.1.4.B). It has been previously shown that the H-region increases the number of cells and transgene expressing fish for the zebrafish OR111-1 gene (Nishizumi *et al*., 2007). Similarly, H-p571-eYFP resulted in robust reporter expression in the olfactory placode of 1dpf zebrafish embryo. Out of 1340 embryos that survived after H-p571-eYFP injection, 650 embryos showed transgene expression (48, 5% of the survived embryos at 1dpf). In addition, the number of transgene-positive cells per nose significantly increased when compared to the p571-eYFP transgene. On average, about 7 cells per nose were obtained using the H-p571-eYFP construct. Using the red fluorescent reporter gene

mCherry as an alternative reporter, a similar efficiency of transgene expression was observed. Considering its much better activity, the H-p571 driver was utilized to promote expression of the transgenic OR constructs (Figure 4.1.5.).

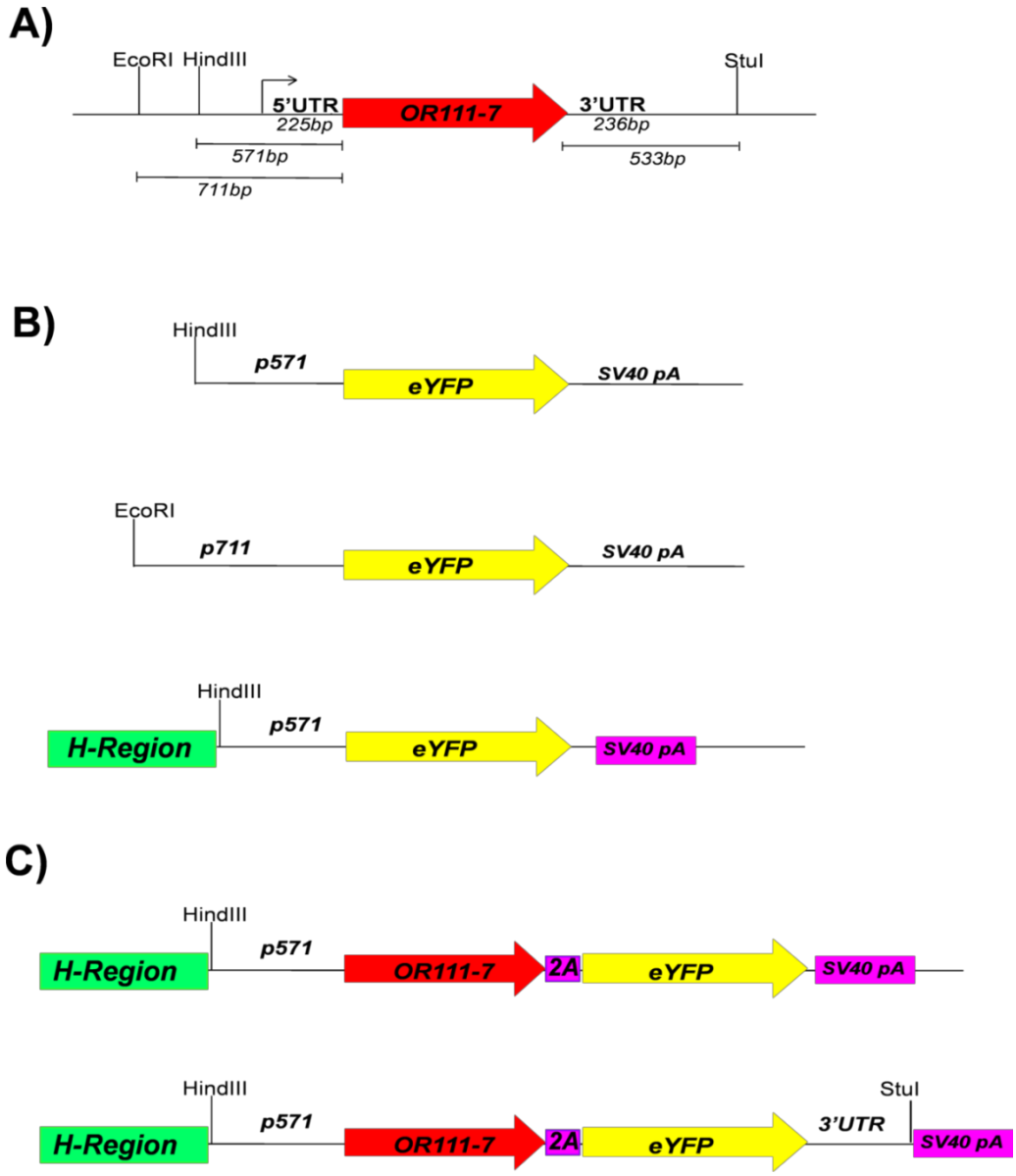


Figure 4.1.4.: Some of the short promoter transgenic construct used in this study. A) OR111-7 and genomic surround in zebrafish genome. B) OR111-7 short promoter constructs in which OR111-7 coding sequence has been replaced with eYFP. Upstream regions with different lengths were tested. The mouse H-region was used in order to increase transgene efficiency. C) OR-111-7 reporter constructs (See text for detailed information).

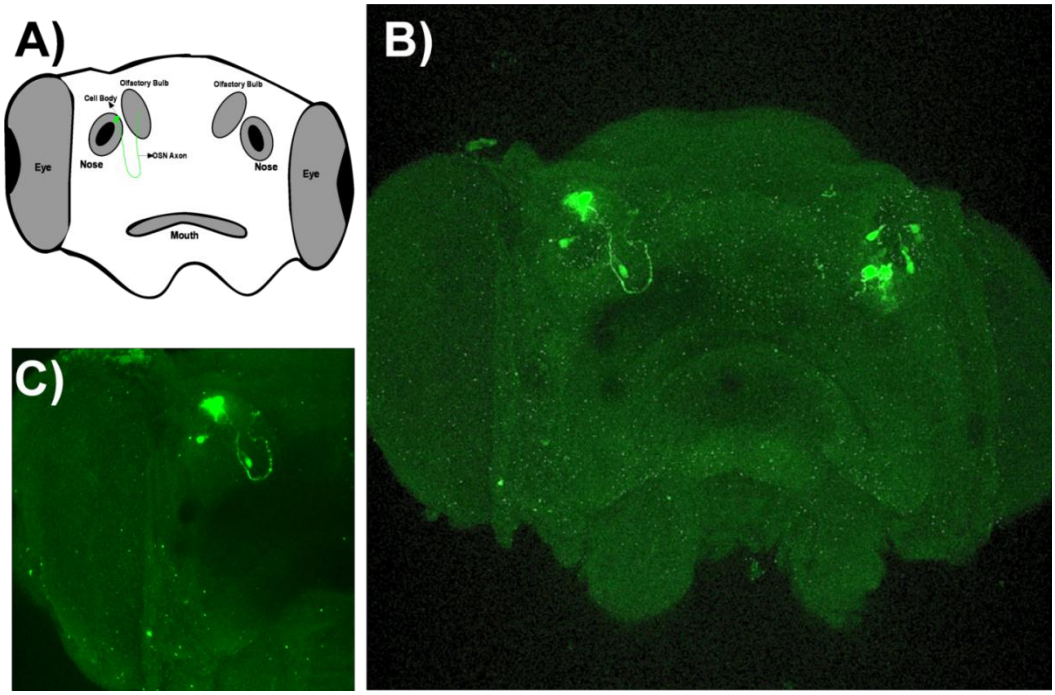


Figure 4.1.5.: H-p571-eYFP injected embryo.

A) Schematic representation of a zebrafish head, anterior view. B) H-p571-eYFP is robustly expressed in the olfactory epithelium (Compare to A). C) Separate view of the right nose.

3.3.8. Analysis of the H-p571-eYFP Transgene

Robust reporter gene expression was observed in H-p571-eYFP injected embryos. eYFP expression is first detectable as early as 24 hours post fertilization (24hpf). The number of cells expressing the transgene increased between 24 hpf and 72 hpf. At 3 dpf, the number of transgenic OSNs reaches a plateau and remains stable until the 5th day of embryonic development (~7 cells per nose, n=7). After 5dpf, the number of transgenic cells drops again to about 3 cells per nose at 7dpf (n= 7).

Previously, it has been demonstrated that OR111-7 is expressed in ciliated OSNs. The olfactory marker protein (OMP) is also expressed exclusively in all OR expressing ciliated OSNs while the transient receptor potential channel C2 (TRPC2) is a marker for microvillous OSNs which express vomeronasal receptors. In order to test which OSN subset expresses my transgene, H-p571-eYFP was coinjected with a construct in which mCherry reporter gene expression is driven by the zebrafish OMP promoter (pOMP-

mCherry; Celik *et al*., 2002; Atasoy, 2011). In 3 dpf embryos that were co-injected with H-p571-eYFP and pOMP-mCherry, 59% of H-p571-eYFP expressing cells also expressed pOMP-mCherry (Figure 4.1.6.B, D and E; n=71). Half of the remaining 41% appear to be immature OSNs. Those cells did neither have a clear dendrite or a visible axon (Figure 4.1.6.E, 48%, n=29). However OMP is a marker for mature OSNs and immature OSNs are not OMP positive. Altogether, these data suggest that H-p571-eYFP is mainly expressed in OR expressing ciliated OSNs. Moreover, high co-expression rate of the H-p571-eYFP and pOMP-Cherry constructs is particularly important since it was aimed to induce OR111-7 excision from a conditional allele by expressing Cre-recombinase under pOMP promoter control.

Next, it was analyzed, whether axons of H-p571-eYFP-expressing cells converge in the olfactory bulb. Previously Sato *et al*. (2007) have identified that OSNs expressing a transgenic locus in which the OR111-7 sequence has been deleted converge their axons to a specific cluster of glomeruli in the dorsomedial region of the olfactory bulb. When the H-p571-eYFP construct was co-injected with pomp-mCherry the majority of eYFP positive axons could be detected in the OMP-positive medial region of the olfactory bulb. However, some of the H-p571-eYFP-transgenic OSNs occasionally innervated the lateral olfactory bulb, which is devoid of OMP-positive axons (Figure 4.1.6.E). It could be hypothesized that the H- enhancer might induce expression of the transgene in cell types that would normally not express the OR111-7 gene, thereby resulting in unusual axonal targeting to the lateral OB.

3.3.9. Generation of a short promoter OR111-7 Reporter Construct

Having established the H-p571-eYFP construct as a first experimental control for the conditional ablation experiment, it was aimed to generate a OR111-7 reporter construct, which would co-express a fluorescent reporter with the intact OR111-7 gene. Similar to the construct explained above, the H-p571 sequence was utilized to drive the expression of OR111-7-T2A-YFP from a single open reading frame (H-p571-OR-T2A-eYFP). However, an extremely low transgenic efficiency was observed for this construct. Out of more than 3000 embryos that survived after microinjection in independent trials, only nine embryos positive for eYFP expression in the olfactory placode could be identified at 1dpf.

Moreover, transgene expression was transient and disappeared by 3dpf. By using reporter proteins different from eYFP, similarly low efficiencies were observed, indicating that the inhibition is not reporter gene-specific (mCherry: 0 positive embryos, n>100 injected embryos; Cre-recombinase: 0 positive embryos n=30 injected embryos).

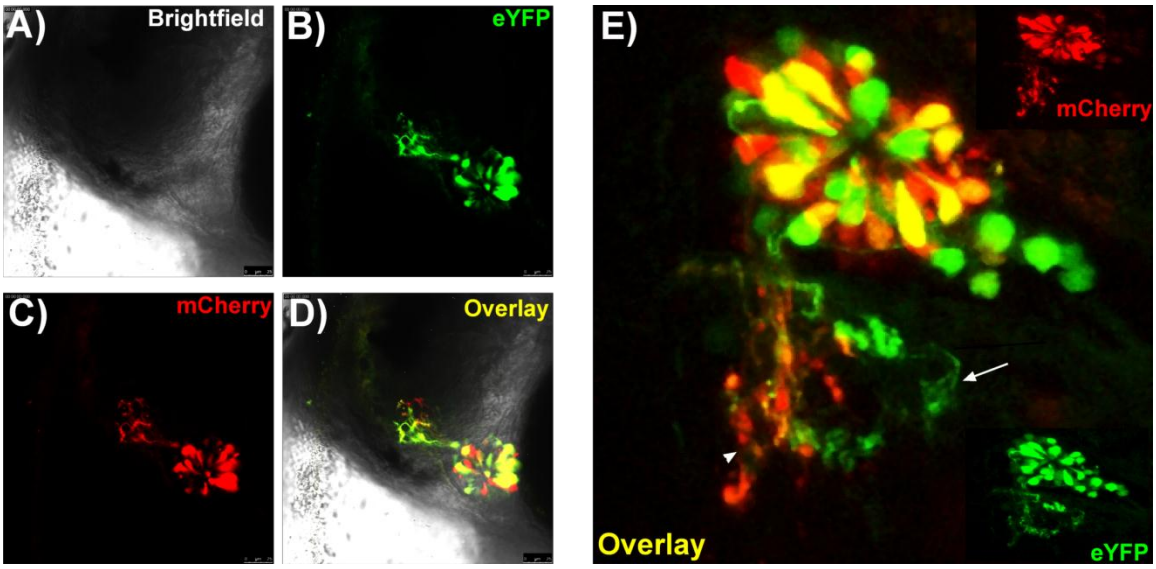


Figure 4.1.6.: pOMP-mCherry, H-p571-eYFP co-injection.
A-D) Majority of H-p571-eYFP expression OSNs also express pOMP-mCherry E)Axonal projections of H-p571-eYFP OSNs. Arrowhead OMP positive medial glomeruli and arrows lateral glomeruli devoid of OMP positive axons. eYFP positive and mCherry positive OSNs are separately shown in the insets.

At first glance, it was surprising that the H-p571-OR111-7-T2A-eYFP construct is expressed in such an extremely low number of embryos while the H-p571-eYFP construct was robustly expressed in 48.5% of injected embryos. There are several possibilities which could explain this phenomenon. First, inefficient cleavage of T2A peptide might result in inappropriate folding of the reporter proteins which in turn results in the lack of fluorescent signal. Alternatively, the absence of 3'-UTR sequence in my transgene constructs might cause rapid mRNA degradation due to RNA instability. Finally, specific sequence/sequences in the OR111-7 coding region might be selectively silence the transgene and transcription does not take place.

In order to test these possibilities, first the OR111-7 coding sequence in H-p571-OR-T2A-mcherry was replaced with a sequence coding for eYFP. If inefficient T2A peptide

cleavage inhibits functional reporter protein folding, it would not be expected to see reporter expression in H-p571-eYFP-T2A-mCherry injected embryos. Interestingly, co-expression of both reporters was observed in a high number of injected embryos, which was comparable to the H-p571-eYFP microinjection experiments (14 positive embryos, n=30 injected embryos, 46.6%). In addition, co-expression of the eYFP and mCherry reporter genes was observed in all analyzed cells (n=13 cells). Similarly, when a mCherry-T2A-CreERT2 construct was used to drive expression under control of the heat shock promoter 70 (hsp70:: mCherry-T2A-CreERT2; Hans *et al*., 2009; Figure 4.1.7.E-H) or a eYFP-T2A-mCherry construct driven by the ratGAP43 promoter sequence (Figure 4.1.7.A-D; Udvadia *et al*., 2001), 100% co-expression of both reporter proteins was observed (n=13 cells for pGAP43-YFP-T2A-mcherry; n=22 cells for hsp70-mcherry-T2A-CreERT2). Additionally, if the lack of expression were due to abnormal protein folding, reporter gene mRNA should be detected in H-p571-OR-T2A-eYFP injected embryos. Therefore, in situ hybridization on H-p571-OR-T2A-eYFP injected embryos was performed using antisense RNA probes against the eYFP sequence but no hybridization signal could be detected (n=32 embryos). These data suggest that the low expression of transgenes including the OR111-7 coding sequence might not be due to the inefficient cleavage of the T2A peptide because it worked properly in 3 positive control experiments.

In a previous study, OR111-1 transgene expression has been successfully driven by a short upstream region of OR111-1 gene fused with H-region (Nishizumi et al, 2007). However, 7,3kb of sequence immediately downstream of OR111-1 coding region were included in that transgenic construct. Therefore, it was hypothesized that there might be crucial sequences in the 3'-UTR region that are necessary to promote sufficient OR gene expression. The OR111-7 gene has single exon with 225 bp of 5'-UTR and 236bp of 3'-UTR. To test whether adding 3'-UTR sequences immediately downstream of the OR111-7 coding region in the transgenic constructs affects transgene expression, the first 530bp of genomic sequence was cloned directly downstream of the OR111-7 gene into the 3'-region of the construct (H-p571-OR111-7-T2A-YFP-3'UTR).

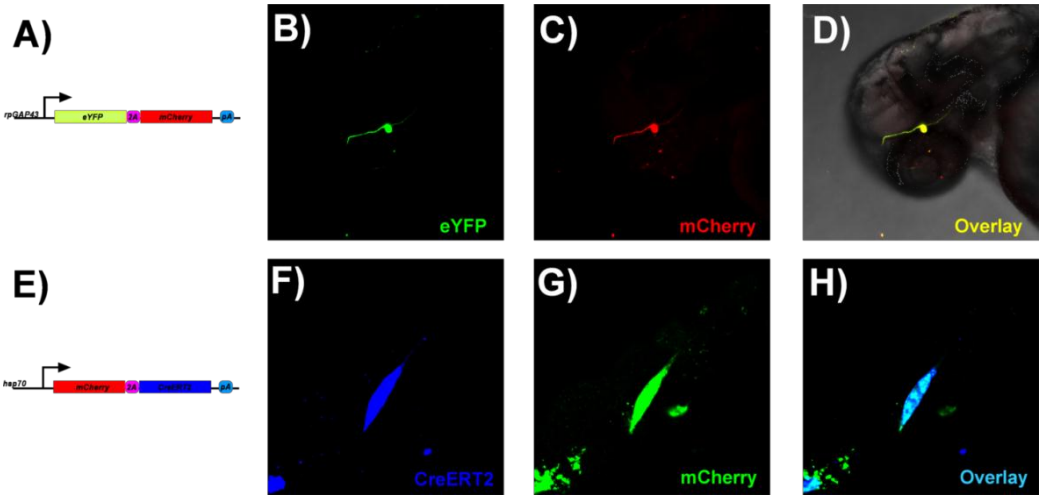


Figure 4.1.7.: Test for T2A Cleavage Efficiency.

A) YFP-T2A-mCherry is driven by rat GAP43 minimal promoter. B-D) A neuron expression both markers in the same cell E) mCherry-T2A-CreERT2 is driven by heat shock promoter F-H) A muscle cell express both CreERT2 and mCherry after a brief heat shock revealed by antibody staining using Anti-Cre antibody for CreERT2 and anti DsRed antibody for mCherry

When injected into fertilized zebrafish embryos at single-cell stage, 11,6% of the embryos displayed transgene expression at 1dpf (5 positive embryos, n=43 injected embryos). Although, adding 3'-UTR sequences increased the efficiency of transgenes containing the OR111-7 coding region to ~10%, it is still not comparable to efficiency of the coding sequence less construct H-p571-eYFP, for which expression was observed in 48,5% of injected embryos.

3.3.10. Analysis of OR111-7 Reporter Constructs

Due to the extreme inefficiency of the H-p571-OR111-7-T2A-eYFP construct, it was only possible to analyze the expression pattern of H-p571-OR111-7-T2A-eYFP-3'UTR. Similar to Hp571-eYFP, the H-p571-OR111-7-T2A-eYFP-3'UTR construct is first expressed at around 24hpf. On the other hand, the number of cells expressing the OR reporter transgene does not increase between 1dpf and 3dpf in contrast to H-p571-eYFP. Indeed, one of the olfactory epithelia analyzed completely extinguished OR reporter transgene expression at 3dpf (Figure 4.1.8., n=5), while the cell numbers remained

constant in other embryos. In addition, it was impossible to observe axonal projection in any of the transgenic OSNs in H-p571-OR111-7-T2A-eYFP-3'UTR injected embryos, while it was possible to see dendrites for some of the transgenic cells.

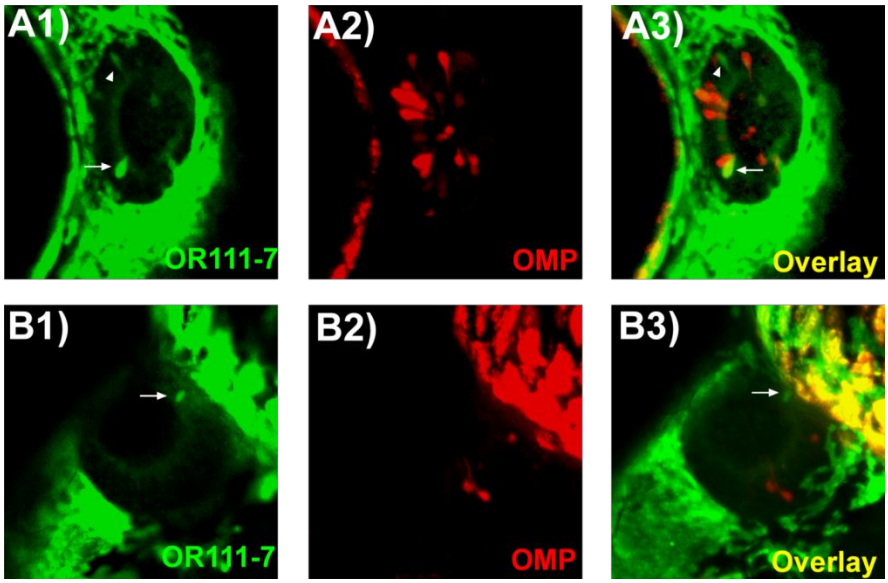


Figure 4.1.8.: OR111-7 Reporter Transgene.

A1-3) H-p571-OR111-7-T2A-eYFP-3'UTR injected embryo at 3dpf. B1-3) Another 3dpf embryo injected with H-p571-OR111-7-T2A-eYFP-3'UTR. Arrows indicate OR111-7 transgene expressing OSNs without dendrites and arrowhead indicates the transgenic OSN with a dendrite. Note that cell numbers are lower compared to H-p571-eYFP (Compare A1-3 to Figure 4.1.6.). Arrow in A3) points to a double positive OSN.

3.3.11. Generation of Conditional OR111-7 Ablation Transgene

After the crucial control constructs for the conditional gene ablation approach were generated, the conditional OR111-7 deletion transgene was analyzed. In a first attempt, it was tried to create a BAC transgene carrying the conditional deletion mutation explained above. In the first round of recombineering, a loxP sequence was introduced into the 5'-UTR region of OR111-7 in BAC100G14 using the SW102/galK recombination system. For the second round, the same homology arms used for the OR-T2A-eYFP recombination were used to introduce a complex cassette containing the OR111-7 fused in frame with T2A-mCherry, a transcription stop signal (3xpA), another loxP-site, a repeat of the 5'-UTR of the OR111-7 gene, and a sequence coding for eYFP followed by a polyadenylation

signal. Although BAC100G14 clones with a 5' loxP mutation were obtained, the 3'-cassette could not be introduced in several trials, probably due to the presence of internal homology in the OR111-7 5'-UTR that was included in the in the 3'-cassette. Indeed, several clones in which recombination took place between the 5'-UTR sequences were obtained, resulting in a OR deletion with an upstream loxP site.

Considering the success of short promoter transgenes and the failure in recombineering the conditional deletion transgene into BAC100G14, it was decided to generate a short promoter conditional ablation construct driven by H-p571 as a promoter. In this minigene, the OR111-7 sequence was tagged with a T2A-mCherry sequence and flanked with two loxP sites. A transcription stop signal, 3xpA (Soriano *et al*., 1999), was cloned between mCherry and the downstream loxP site, which was followed by a fusion of the OR111-7 5'-UTR and eYFP sequence, 530 bp genomic region downstream of OR111-7 (3'UTR sequence, see above) and a polyA signal.

3.3.12. Cre-recombinase expression and timing of Cre-dependent OR111-7 coding sequence excision

In the conditional deletion approach, it was aimed to target the OSNs which have already chosen the OR111-7 transgene for expression in order to see whether the OSNs which have undergone OR deletion late in their development are able to make second choices of OR gene expression. For this reason, precise timing of the excision of OR111-7 coding sequence is crucial. Since, it was aimed to utilize the Cre/loxP recombination system; it had to be ensured that Cre-recombinase was expressed in the same cells, which expressed OR111-7 but at a later timepoint than the OR111-7 transgene.

Previously, it has been shown that OR expression precedes OMP expression in the olfactory system of rodents (Iwema and Schwob, 2003). Moreover, it was shown in this study that ~60% of H-571-eYFP expressing OSNs were also positive for an OMP promoter transgene. For this reason, the zebrafish OMP promoter was used to drive late Cre-recombinase expression in OSNs. To do so, the sequence coding for Cre-recombinase with a nuclear localization signal was cloned downstream of the zebrafish OMP promoter (pOMP-nlsCre). By using antibodies against the Cre-recombinase protein, Cre-

recombinase expression in 3dpf pOMP-nlsCre-injected embryos was visualized. It was observed that pOMP-nlsCre was widely expressed in the olfactory placode, similar to pOMP-mCherry and that the Cre-recombinase protein was localized to the nuclei of OSNs due to the presence of a nuclear localization signal. A tamoxifen inducible version of Cre-recombinase, CreERT2, was also engineered under the control of zebrafish OMP promoter to be able to induce Cre-recombinase-mediated expression at a later developmental timepoint. This way, it was possible to temporally control the expression of Cre-recombinase even if expression under control of the OMP promoter would precede OR111-7 expression.

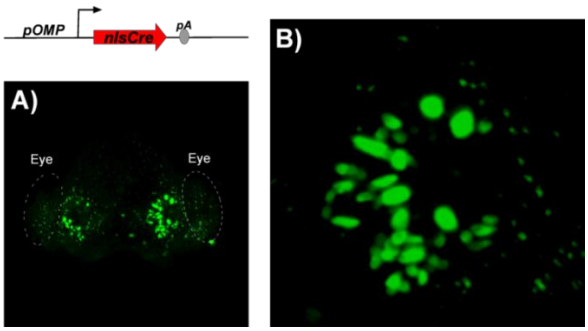


Figure 4.1.9.: pOMP-Cre Expression in the olfactory placode of 3dpf zebrafish embryo. A) Anterior view of 3dpf zebrafish embryo. Cre expression is visualized by fluorescent antibody staining against Cre protein using anti-Cre as the primary antibody and Alexa488 as secondary antibody. Dashed lines indicate the eyes. B) Closer view of the left nose. Nuclear localization of Cre protein is seen (compare to Figure 4.1.6.C, cytoplasmic localization of mCherry).

3.3.13. GAP43 Promoter Constructs

GAP43 is predominantly expressed in developing neurons, including immature OSNs. In a previous report, it has been shown that GAP43 expression starts in advance of OR gene expression and that it is down-regulated after the onset of OR and OMP gene expression (Iwema and Schwob, 2003).

As explained above, the period during which OR choice takes place was aimed to be studied by investigating at which ontogenetic stages second choice and/or OR gene

switching are possible. Since no information about the timing of OR111-7 was available with respect to other immature and mature OSN markers (such as GAP43 and OMP), it was decided to utilize the promoter of an earlier neuronal marker, GAP43, to drive Cre-recombinase expression. In this way, It was aimed to divide the experimental period by inducing OR111-7 excision with an early marker, GAP43 and a mature OSN marker, OMP to see whether second OR choice can take place at either time point.

For the purpose of early Cre-recombinase expression, a 1kb genomic region directly upstream of translation start site of the rat GAP43 gene was used. Previously it has been shown that reporter expression driven by this genomic fragment faithfully mimics the endogenous zebrafish GAP43 expression in a spatial and temporal fashion when it is injected into fertilized zebrafish oocytes (Reinhard *et al*., 1994; Udvadia *et al*., 2001). Therefore, the enhanced green fluorescent protein eGFP was engineered downstream of the 1kb rat GAP43 gene promoter (pGAP43-eGFP). When pGAP43-eGFP was injected into single cell zebrafish embryos, eGFP expression was obtained in immature OSNs characterized by the absence of clearly visible axons (Figure 4.1.10.C-D). Consistent with a study performed in rat (Iwema and Schwob, 2003), GAP43 expression overlapped with OMP expression only occasionally when pOMP-mCherry was co-injected with pGAP43-eGFP, suggesting that GAP43 is mostly expressed in immature OSNs in the zebrafish as well (Figure 4.1.10.D).

3.3.14. Analysis of Conditional Ablation Transgene

It has been previously shown in the mouse that a replacement of the OR coding sequences with fluorecent reporter genes or naturally occuring frameshift mutations in OR coding regions results in the coexpression of a functional OR by the same OSNs initially expressing the deletion/frameshift locus (Serizawa *et al*., 2003). This is a unique situation, because under this condition OSNs seem to break the ‘one neuron – one recptor’ rule. It has been proposed, and become a current concept in the field, that expression of a functional OR protein in OSNs elicits a negative feedback signal which is required for the silencing of other ORs in the same OSN. Interestingly, even transgenes in which expression of an OR gene is controlled by a heterologous promoter, such as the ‘artificial’ TetO promoter are also controlled by the same negative feedback regulation. This has led

to the idea that the OR coding sequence at the DNA level could be the receiver of the signal (Nguyen *et al*., 2007). These data suggest that expression of an OR protein has a crucial role in the regulation of OR gene expression through a negative feedback mechanism which normally inhibits the expression of other OR genes by acting on the OR coding region. To some extent, this possibility could explain the low or absent expression frequencies of my transgenes that contained OR coding sequences as opposed to the constructs in which the H-p571 driver was directly fused to a reporter gene. However, the exact time point at which the feedback inhibition-mediated singularity of OR gene expression is irreversibly established has not been elucidated. Interestingly, Shykind *et al*. (2004) have shown that OSNs are inherently capable of switching expression of different ORs during early OSN development.

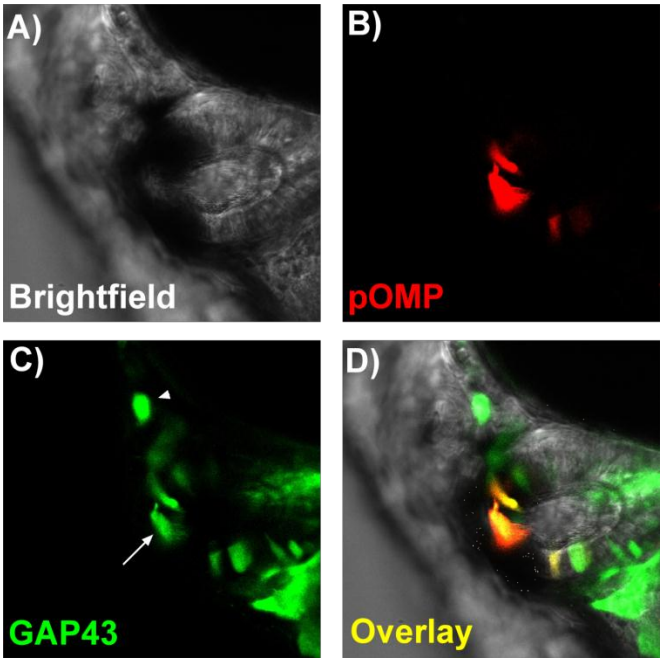


Figure 4.1.10.: 1dpf zebrafish embyo coinjected with pGAP43-eGFP and pOMP-mCherry. A) Brightfield image of the olfactory placode at 1dpf. B) pOMP-mCherry expression in the olfactory placode C) GAP43 expression in the olfactory placode of 1dpf embryo. Arrow indicates the OSN coexpressing OMP. Arrowhead indicates an immature OSN with only GAP43 expression. Note the remote positioning of the immature OSN with respect to the nose.

Similarly in zebrafish, OR coding replacement with fluorescent reporters for two different ORs in a BAC transgenic context resulted in the coexpression of the fluorescent reporter and a functional OR (Sato *et al*., 2007). However, throughout the literature, OR coding sequence deletions were always made at the embryonic stem cell (ESC) level or in transgenic constructs, thus long before the deleted OR gene locus is expressed. This approach, however, gives only limited information about the temporal dynamics of OR gene choice. On the other hand, a late deletion of the OR111-7 coding sequence in my conditional ablation approach would help us to determine the critical time point at which OR gene choice is irreversibly established. If, after a certain time point following the onset of OR gene expression stable OR gene choice would be established, no second OR gene choice should be possible.

As explained in detail above, the H-p571 sequence was used to drive expression of the conditional OR111-7 ablation transgene due to the failure in creating a BAC transgene and the expected low efficiency of BAC transgenic OSNs. The pOMP-Cre construct was used to express Cre-recombinase which will excise the OR111-7-T2A-mCherry-3xpA cassette that is flanked by two loxP sites. Since OR expression precedes OMP expression, OR111-7 will be expressed for a transient period before Cre-mediated recombination. In case that OMP expression takes place before OR111-7 expression in some OSNs due to the cell intrinsic expression dynamics or because the transgene expression from the OMP gene promoter does not faithfully mimic the temporal profile of endogenous gene expression a transgenic construct in which the zebrafish OMP promoter drives the expression of a modified Cre-recombinase, CreERT2, was also created. CreERT2 is a fusion of the human estrogen ligand binding domain and Cre-recombinase. In this fusion, Cre-recombinase protein does not show any enzymatic activity unless the estrogen antagonist tamoxifen is applied. This way, It was aimed to control Cre-mediated recombination temporally and shift the time of Cre-mediated excision to a later developmental time point at least for some of the OSNs expressing the OR111-7 transgene.

When the conditional deletion construct was co-injected with the pOMP-Cre transgene into single-cell embryos, enhanced yellow fluorescent protein expression was observed in the olfactory placode, indicating that Cre-mediated recombination took place. Importantly, no eYFP positive cells could be detected when the conditional deletion

construct but not the pOMP-Cre construct was injected alone, strongly supporting that eYFP is expressed only after Cre-recombinase–mediated excision of OR111-7-T2A-mCherry-3xpA. Unfortunately, it was impossible to detect any mCherry expression, even when not pOMP-Cre was co-injected with the conditional ablation construct. This might be due to the fact that transient expression of OR111-7-T2A-mCherry before Cre-mediated excision might not be sufficient to accumulate mCherry protein above detection threshold. This would suggest that maturation of OSNs is a relatively fast process. However, it was also impossible to detect any mCherry fluorescence when the conditional ablation construct was injected without any Cre-containing construct. Thus, the first half of the transgenic construct might not have worked as expected, but Cre-mediated excision and activation of the second reporter did. Surprisingly, a mutation in the mCherry coding sequence has disrupted the stop codon of mCherry in the construct, which could explain the absence of the mCherry signal. On the other hand, eYFP expression was first detectable at 24hpf, comparable to that of H-p571-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR transgenes. In addition, the number of cells per nose was approximately 3, ranging from 1 to 7. High variance in cell numbers for different noses was probably due to the random nature of having both conditional deletion transgene and pOMP-Cre simultaneously in a single cell after plasmid injection. As seen for pOMP-mCherry and H-p571-eGFP co-injections, not all transgenic OSNs expressed both transgenes and only about 50 % of cells were double positive for both reporters.

Surprisingly, OSNs expressing enhanced yellow fluorescent protein after Cre-mediated excision of the OR111-7 coding sequence could be classified according to different phenotypes in axonal projections. While, the majority of cells did not project any axon to the olfactory bulb during the analyzed period the remaining cells showed branched axonal projections while normal OSNs have unbranched axons.(between 1dpf and 7dpf; n=17). Therefore, the conditional deletion construct was co-injected with pOMP-Cre and pOMP-mCherry in order to see if the atypical axonal phenotype is exclusive to OSNs that have undergone the conditional deletion or whether it is a more widespread abnormality affecting the majority of OSNs. At 3dpf after injection, axonal projections of pOMP-mCherry-positive OSNs were clearly visible while YFP positive conditional deletion OSNs lack axonal outgrowth. The higher number of mCherry positive cells was due to the higher penetrance of pOMP promoter when compared to the H-p571 driver in combination with

the OR111-7 coding sequence. Thus, absence of axonal projections or branched axonal extensions was not a general phenotype and it is exclusively observed for the conditional deletion OSNs which have undergone Cre-mediated excision of the OR111-7 coding sequence. Therefore, it was hypothesized that OSNs expressing the conditional deletion allele show defects in axonal outgrowth and establishing proper connections in the olfactory bulb after Cre-mediated deletion of OR111-7 coding sequence takes place. Considering the crucial role of OR genes in axon guidance of OSNs (Wang *et al*., 1998; Feinstein *et al*., 2007; Imai and Sakano, 2007), it was thought that atypical axonal phenotypes observed is due to a defect in OR gene choice. It was assumed that OSNs cannot undergo second choice if the deletion of OR coding sequence at a later stage (after it has been expressed) occurs. Probably, transient expression of the OR111-7 gene is enough to irreversibly establish the singular OR gene choice but not enough to promote axon outgrowth or to establish appropriate axonal connections in the olfactory bulb. Therefore, this data suggest that the OR gene choice mechanism takes place over a short period during the early ontogenetic development of OSNs, before OR protein driven signals can elicit or guide appropriate axonal outgrowth. Using tamoxifen inducible CreERT2 would be a perfect way to delay the OR111-7 deletion beyond this developmental period and give OSNs sufficient time to project axons to the olfactory bulb. However, this experiment could not be performed since 4-hydroxytamoxifen did not become available in the laboratory.

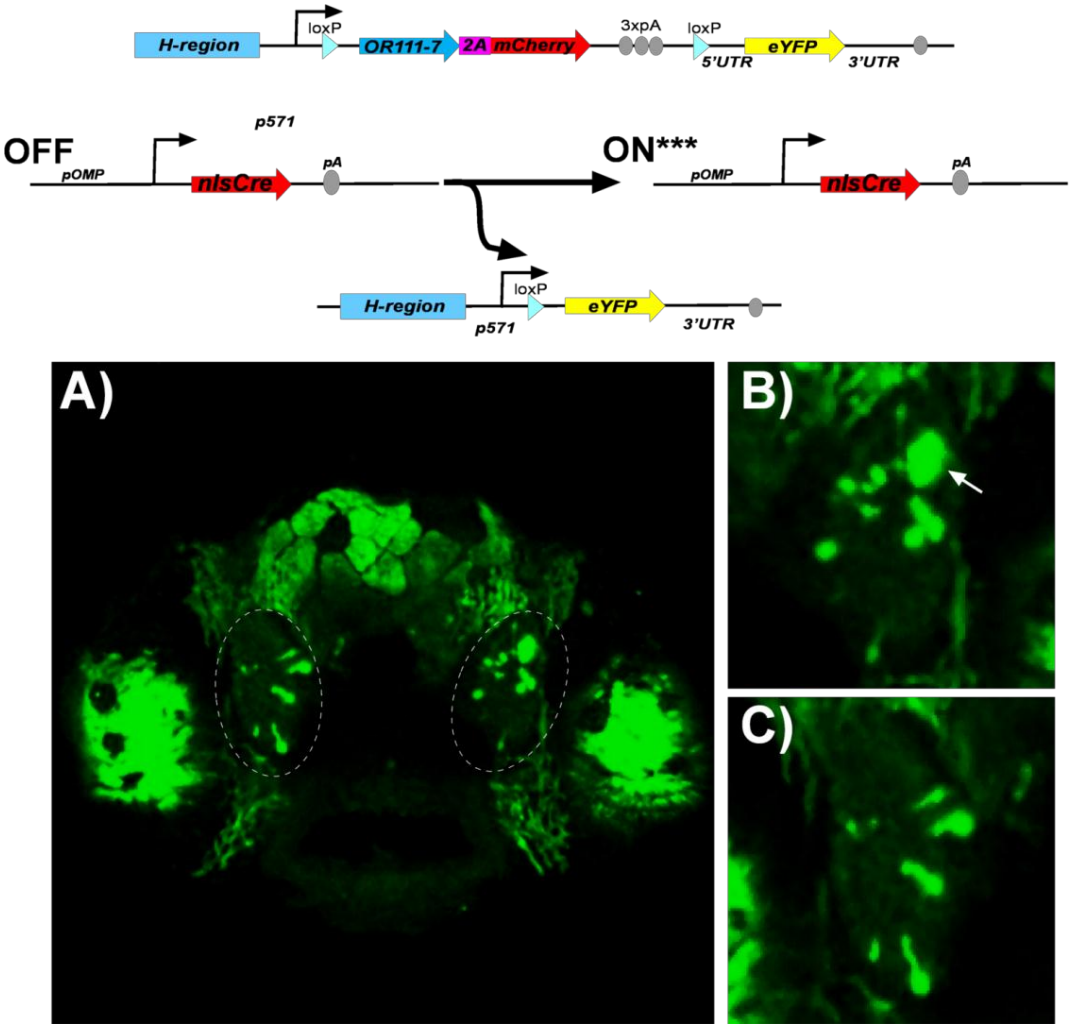


Figure 4.1.11.: Conditional Deletion Construct is coinjected with pOMP-Cre Construct. A) 3dpf embryo injected with conditional deletion construct and pOMP-Cre. Dashed lines indicate the noses. B) and C) Magnified image of the noses. Arrow indicate the OSN with abnormal morphology.

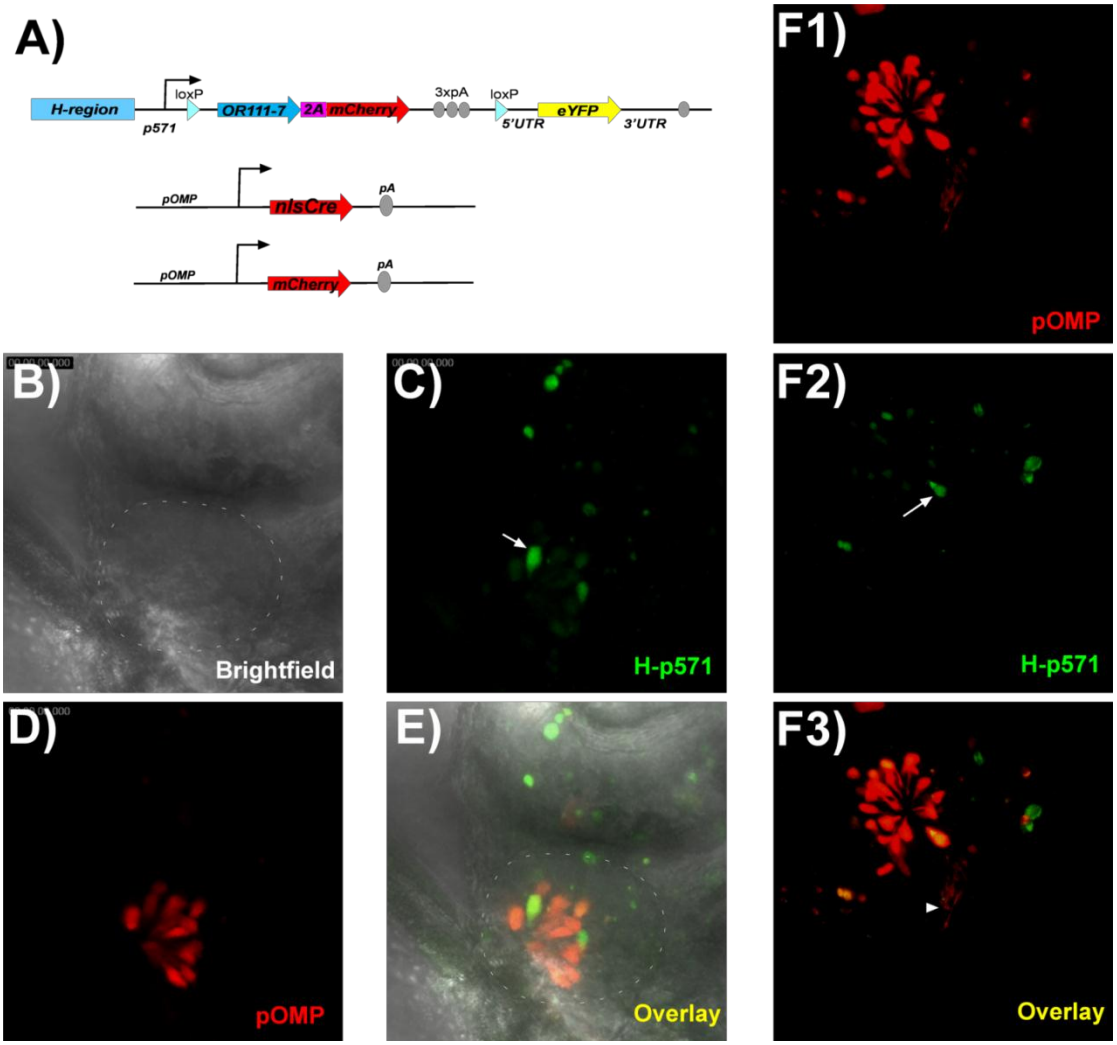


Figure 4.1.12.: Coinjection of Conditional deletion construct with pOMP-mCherry and pOMP-Cre.

B)-E) 3dpf embryo showing conditional deletion OSN coexpressing pOMP:mCherry F1)-F3) Another 3dpf embryo injected with the same constructs. Arrowhead indicates the typical axonal projections of OMP positive OSNs. No YFP positive axons are visible. Arrows indicate YFP positive conditional deletion OSNs.

3.4. OR111-7 Coding Sequence Dependent Inhibition of Transgene Expression

In the second part of this thesis, the inhibition of transgene expression by the presence of the OR coding sequence was investigated. In the first part, it was demonstrated that H-p571 is a powerful driver when fused upstream of a fluorescent reporter protein (Figure 4.1.5. and 4.2.6). On the other hand, an extremely low efficiency of transgene expression was observed, when the OR111-7 coding sequence was included in the transgenic construct. Initially, it was suspected that inefficient T2A cleavage might be the reason for low transgene efficiency. However, it was later demonstrated that H-p571 can drive the expression of eYFP-T2A-mCherry very efficiently at rates that were comparable to H-p571-eYFP. Moreover, both fluorescent proteins were efficiently co-expressed in all cells analyzed, suggesting that T2A is cleaved efficiently during translation (Figure 4.1.7.). In addition, when a myc epitope tag was fused to the OR111-7 sequence, no transgene expression could be observed after antibody staining against the myc tag. Similarly, a direct OR111-7-eYFP fusion also failed to show eYFP fluorescence. Another evidence for OR111-7 coding sequence-dependent transgene inhibition was that in situ hybridization against the eYFP sequence failed to show any signal in H-p571-OR111-7-T2A-eYFP injected embryos. Although the expression efficiency of the H-p571 driver was partially rescued by adding 530bp of 3'-UTR sequence downstream of the OR111-7 gene in a H-p571-OR111-7-T2A-eYFP construct (Figure 4.1.8.), the efficiency of H-p571-OR111-7-T2A-eYFP-3'UTR was only 10 % as compared to 48.5% for the H-p571-eYFP construct (Figure 4.2.2.).

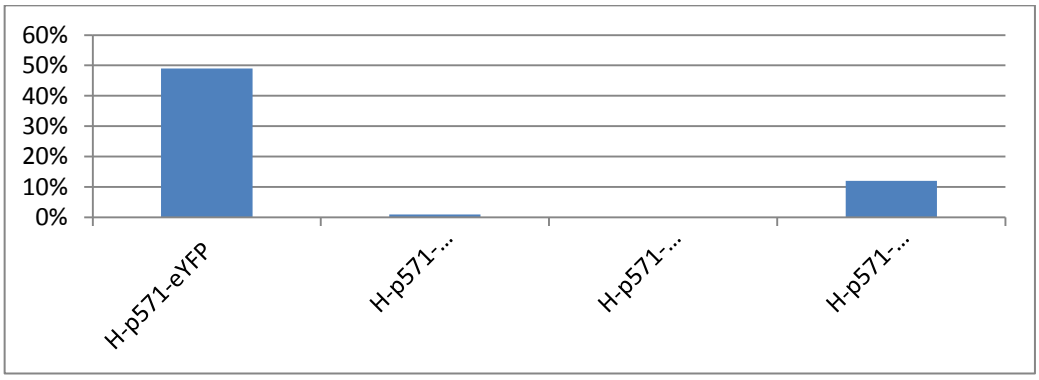


Figure 4.2.1: Transgene Efficiencies: Percentage of Reporter Positive embryos.

To further examine the coding sequence-dependent decrease in transgene efficiency, it was decided to analyze the number of transgene-positive OSN per nose for constructs that did or did not contain the OR111-7 coding sequence. In this approach, it was crucial to find a way to control the technical variability of the success of injection for independent experiments. In the previous experiments in this thesis work, whenever an H-p571 driven transgene was co-injected with pOMP-mCherry construct, it was observed that the embryos were always mCherry positive when they were H-p571 driven transgene positive but some mCherry positive embryos lacked the H-p571 derived signal. Therefore, it was decided to co-inject the H-p571-driven transgenes with pOMP-mCherry and perform the cell counts only for p-OMP-mCherry positive embryos since the absence of the mCherry signal indicates a failure of injection for this embryo. In this way, it would be possible to exclude those embryos from the analysis that did not get properly injected with the transgenic DNA and ensure that the cells only in embryos for which injection has successful were analyzed. By counting the cells at 3dpf for H-p571-eYFP and H-p571-OR-T2A-eYFP-3'UTR (Figure 4.2.3.), it became clear that constructs that include the OR111-7 coding sequence also express the transgenes in a lower number of cells per OMP-mCherry positive nose when compared to noses expressing the H-p571-eYFP construct (Mann-Whitney U Test, $p < 0.05$). Interestingly, the number of transgene-expressing cells per epithelium for the H-p571- OR-T2A-eYFP-3'UTR construct was similar to the frequency of expression of the endogenous alleles of OR111-7, while the number of OSNs expressing the H-p571-eYFP construct was 4-fold higher.

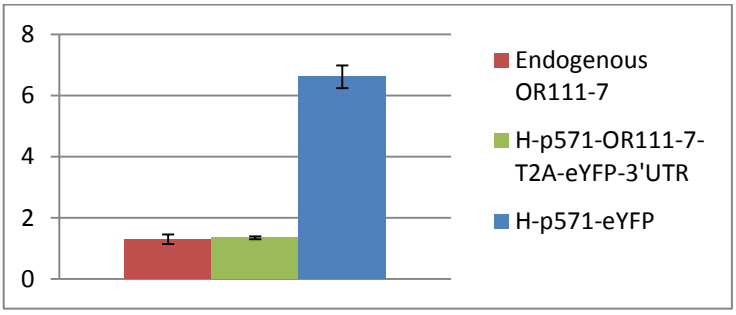


Figure 4.2.2: Cell Numbers per Nose for H-p571-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR.

Cell numbers for H-p571-eYFP and H-p571-OR111-7-T2A-eYFP are given per pOMP-mCherry positive nose (See text for details.)

These significant differences in transgene expression between theH-p571-eYFP and OR111-7 coding sequence constructs led to the idea that the OR111-7 coding region might contain specific DNA sequences that play a role in transgene expression. Indeed, it has been previously shown in the mouse that expression of an OR coding sequence driven by the heterologous TetO promoter system can be suppressed in OSNs expressing an endogenous OR (Nguyen *et al*., 2007). These findings suggest that the negative feedback signal can ensure expression of one receptor per neuron by acting on the OR coding sequence. Taken altogether, it was hypothesized that presence of OR111-7 coding sequence in H-p571-OR111-7-T2A-eYFP-3'UTR construct might have a similar effect and render the transgene suceptible to the negative feedback mechanism while the H-p571-eYFP construct would easily escape the negative feedback signal as it does not contain any OR coding sequence. In such a scenario, OSNs expressing an endogenous OR would be able to suppress the OR111-7 transgene and vice versa. Therefore, transgenes containing the OR111-7 coding sequence would be expressed at frequencies similar to the endogenous alleles. On the other hand, H-p571-eYFP could be expressed in higher numbers compared to H-p571-OR111-7-T2A-eYFP-3'UTR since it is not affected by the negative feedback signal. Thus, this transgene would be co-expressed with a functional OR from another OR gene locus in the same OSN. Indeed, at 3dpf the number of transgene-positive cells per nose for H-p571-eYFP is significantly higher than the number of cells per nose for both, the endogenous OR111-7 and the H-p571-OR111-7-T2A-eYFP-3'UTR transgenic construct. More importantly, there was no significant difference between the cell numbers for the endogenous OR111-7 and the H-p571-OR111-7-T2A-eYFP-3'UTR transgene (H-p571-eYFP vs. endogenous OR111-7 $p<0.01$, H-p571-eYFP vs. H-p571-OR111-7-T2A-eYFP-3'UTR $p<0.01$ and Endogenous OR111-7 vs. H-p571-OR111-7-T2A-eYFP-3'UTR $p=0.4$, Mann-Whitney U Test, Figure 4.2.2.). Considering these data and the published report (Nguyen *et al*., 2007), it was reasoned that the OR111-7 coding region possesses critical sequences related to OR gene choice and decided to make serial deletions of the OR111-7 coding sequence to pinpoint critical sequence motifs that might be involved in this process.

The OR111-7 gene comprises a single exon with a 978 bp long open reading frame. As other ORs, it has DNA sequences for seven transmembrane domains and a highly conserved sequence motif corresponding to the G-protein binding site (DRL). As it was

explained in methods, partial OR111-7 coding sequence eYFP fusions with the first 418bp, 517bp and 723bp of OR111-7 coding sequence were generated and named as H-p571-418bp-eYFP, H-p571-517bp-eYFP and H-p571-721bp-eYFP, respectively (Figure 4.2.3. and 4.2.5.).

```
  1 atgagttctt taaatgcaag cttctccctg aatatttctg ttgttcgtcc tgaatacttt
    Met
                                                   TM1
 61 tttatccttg gactttcagg tataccttac agcaatttgt actatatttt catattcatt

121 atttatttca tcactgtaat tggcaacttt ctagtcattc ttcttatagt tcttgaccgg
                                   TM2
181 agtctgcaca gtccaaagta cattggtgtg tttaacttgg ccttggctga tattggtgaa

241 actaatgctc tgattcctaa catgatgaag acttttctgt ttgactcgca gtacatctcc
                                   TM3
301 tacaatgctt gtttggtgaa catgtttttt gtgtttcttt ttagctctat gcagagtctc

361 acccttgttg ttatggcgta tgatcgttta attgcaattt gcttgccatt aagatatcat
                DRL                                        EcoRV
                                   TM4                      400
421 gtcattgtga acaacaccag tatgatttca attttctcag ctgttttcat gttcaattcg

481 attattgttg cctccatggt gtctttggtg accaacatat cattctgtaa atccaatgtg
                                500 R

541 atacagagtt atttttgtga tcacggacca atgtttagga tggcatgcaa tgacaataac
                                   TM5
601 ataaataaga ttatggatt tctctacaca actttatacc ttatagcacc catgcttgtc

661 attttcttgt catatctggg cattttcttg gttgtaagca agattgcaac ttgggaaaga
                                                          700 R
721 cgtttgaaag cactgaagac ctgtgtttca catctgttat tagtggggat ttattttctc
            TM6
781 cccatattct tcacatacct tacttcatta ttgcttttta gcacctccaa ttctagagtc
                                   TM7
841 atcagcacgt ctttggctta tgctattcca ccaatgctta atcccattat ttatgtatta

901 aacacagctg aaatcaaaaa cacaattcga aaaggtttta aaaacagatc tctgcccatt

961 gagacggttt caaagtga
                   Stop
```

Figure 4.2.3.: OR111-7 Coding Sequence.

Transmembrane regions, Endogenous EcoRV site, primers for 517 and 712 bp pieces and G protein binding region DRL are shown.

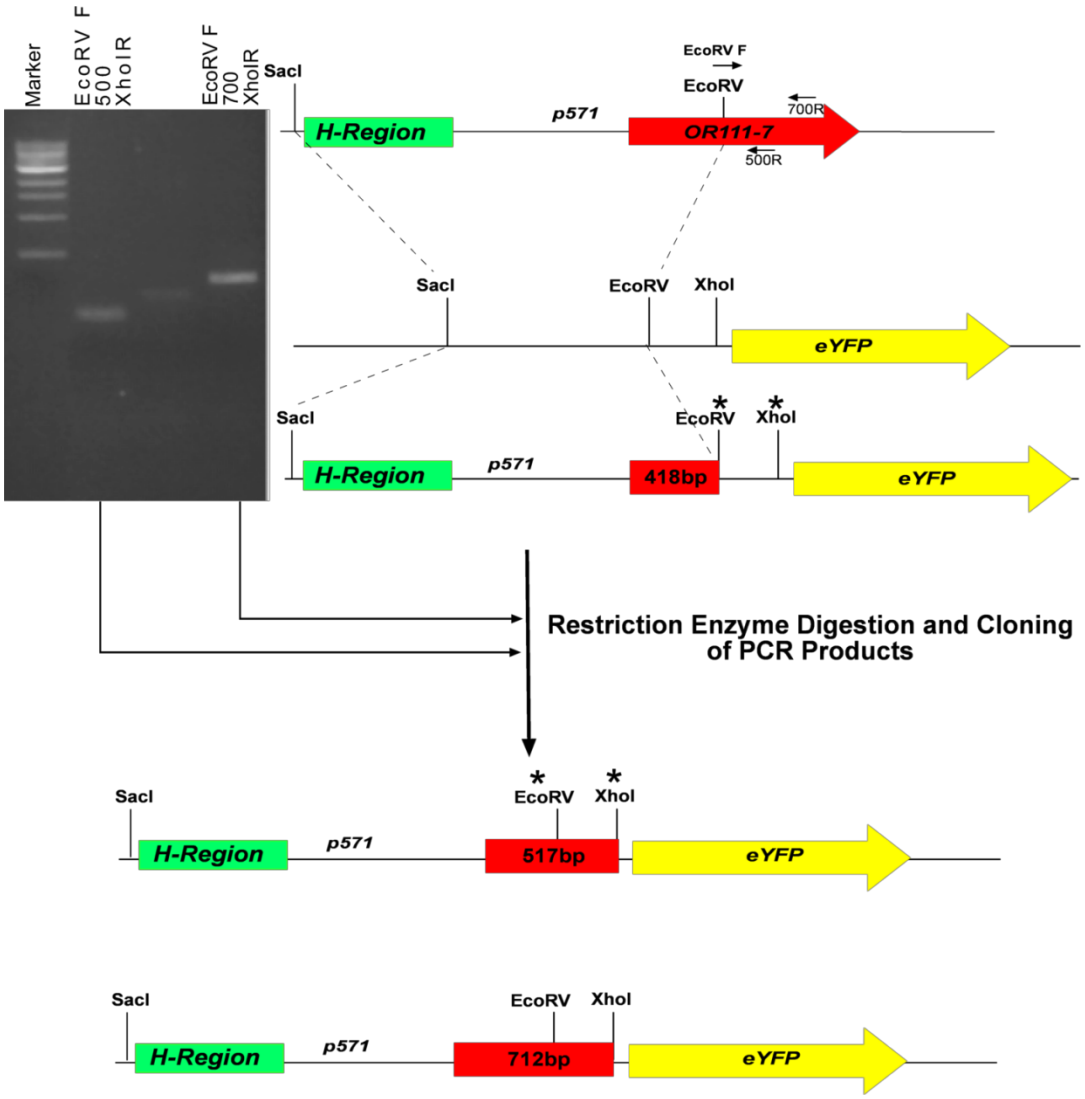


Figure 4.2.4.: Cloning Strategy for different lengths of coding sequence fused with eYFP.

3.4.1. Analysis of H-p571-418bp-eYFP, H-p571-517bp-eYFP and H-p571-712bp-eYFP Constructs

Table 4.2.1.:Constructs used in Section 4.2.

Construct:	In:	Purpose:
H-p571-eYFP	4.2., 4.2.1.	OR coding sequence dependent Transgene Inhibition
H-p571-OR111-7-T2A-eYFP	4.2., 4.2.1.	OR coding sequence dependent Transgene Inhibition
H-p571-OR111-7-T2A-eYFP-3'UTR	4.2., 4.2.1.	OR coding sequence dependent Transgene Inhibition
pOMP-mCherry	4.2., 4.2.1.	Olfactory map visualization, Normalization of Injection Efficiency
H-p571-418bp-eYFP	4.2., 4.2.1.	OR coding sequence dependent Transgene Inhibition
H-p571-571bp-eYFP	4.2., 4.2.1.	OR coding sequence dependent Transgene Inhibition
H-p571-712bp-eYFP	4.2., 4.2.1.	OR coding sequence dependent Transgene Inhibition

As explained above, it was aimed to investigate whether there are specific sequences which could be targets of the negative feedback signal and thereby affect transgene efficiency when they are present in a transgenic construct. For this purpose, a strategy to analyze a variety of parameters for each of the transgenic constructs in this series of experiments was designed. First of all, it was crucial to compare the transgenic embryos at a fixed developmental stage so that the comparison would not be affected by any temporal fluctuation in OR gene expression (Barth *et al*., 1996; Argo *et al*., 2003). Therefore, it was decided to analyze the embryos at 3dpf because previously it was observed that OR111-7 expression level reached a peak in cell number at this time and axonal projections to the developing olfactory bulb were clearly visible, which at this time contains about 12 protoglomeruli allowing unequivocal visualization of axonal projections (Li *et al*., 2005). Second, cell counts should be performed in such a way that technical variability in injection efficiency should not affect the results. For this reason, the partial coding sequence reporter constructs were again co-injected with pOMP-mCherry. In addition, analysis of pOMP-mCherrry positive axons enables the visualization of olfactory epithelium development and the formation of axonal projections. Lastly, the presence of the dendrites and axon in reporter-positive OSNs, which are morphological markers of OSN maturity, were analyzed. This experimental design will allow the quantitative

expression in which OSN population (immature/mature), the partial coding sequence reporter transgenes are predominantly expressed.

First H-p571-418bp-eYFP injected embryos were analyzed at 3dpf according to the parameters outlined above. The H-p571-418bp-eYFP transgene includes transmembrane domains 1, 2 and 3 together with the highly conserved G-protein binding motif DLR (Figure 4.2.4.). When the construct was injected into fertilized zebrafish oocytes, reporter expression could first be detected around 24hpf similar to H-p571-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR. After quantifying the results, it was observed that there was no significant difference in the number of transgene-positive cells per nose for H-p571-418bp-eYFP and H-p571-eYFP (Table 3; 5.08 vs. 6.06 cells per nose, n=26, p=0.2, Mann Whitney U Test). As for the ratio of dendrite-positive/dendrite-negative transgenic OSNs at 3dpf for H-p571-418bp-eYFP , I observed that 87.7% of eYFP-positive cells also had a clear dendrite(116 OSNs, n=132). On the other hand, almost all of the pOMP-mCherry positive embryos possessed dendrites for H-p571-eYFP (n>150, only 6 cells did not have a dendrite).

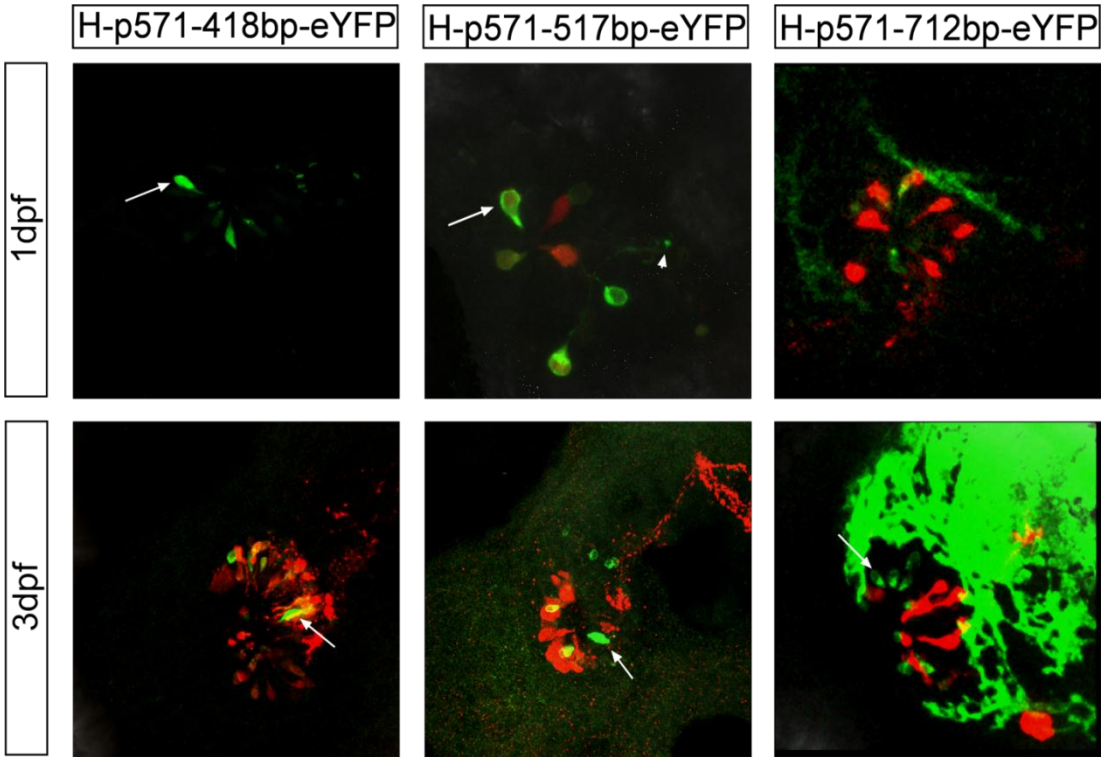


Figure 4.2.5.: Confocal z-stacks for different lengths of coding sequence reporter transgenes at 1dpf and 3dpf.

Red cells are pOMP-mCherry positive cells. Arrows indicate the transgene expressing OSN cell bodies and arrowhead indicate the defined projection of H-p571-517bp-eYFP expressing OSNs at 1dpf. However, it was impossible to visualize axons at 3dpf.

The 99 bp longer partial coding sequence reporter transgene H-p571-517bp-eYFP additionally includes the 4th transmembrane domain and thus encodes for the first 172 amino acids of OR111-7 protein (Figure 4.2.4.). Similar to the other constructs, H-p571-517bp-eYFP expression was first seen around 24hpf and its injection resulted in 3.76 eYFP positive cells per nose at 3dpf (Figure 4.2.5., n=21 olfactory epithelia).Interestingly, only 35.6% of eYFP positive OSNs possessed a clearly visible dendrite in contrast to 87.9% that were observed for H-p571-418bp-eYFP transgene.

The longest partial coding sequence reporter transgene H-p571-712bp-eYFP includes the first 237 amino acids of OR111-7 protein located between the N terminal and 6th transmembrane domain. H-p571-712bp-eYFP transgene was expressed in 4.6 cells per nose at 3dpf with an onset of expression as early as 24hpf (n=14 noses, Figure 4.2.5.).

Only 22.2 % of transgene positive OSNs extended dendrites into the nasal cavity for H-p571-712bp-eYFP.

In order to understand whether partial coding sequence transgenes with different lengths have different expression profiles, they were compared for the cell numbers per nose, percentage of OSNs having a dendrite and positions in the embryonic olfactory epithelium at 3dpf. However the presence of axons could not be used as a comparison parameter since the axons of transgene-expressing cells could not be clearly visualized. Perhaps, eYFP protein levels in the axons were below detection limits due to the fusion of the partial OR protein to eYFP.

When the cell numbers for the partial coding sequence reporter constructs were statistically compared to H-p571-eYFP or to each other, no significant difference for any pairing was observed. On the other hand, each of the H-p571-418bp-eYFP, H-p571-517bp-eYFP and H-p571-712bp-eYFP constructs had a significantly higher number of transgene-positive cells per nose when compared to H-p571-OR111-7-T2A-eYFP-3'UTR ($p<0,01$, Mann Whitney U Test with Bonferroni Correction). Thus, it was impossible to find any sequence in the first 712bp of OR111-7 coding sequence which could be responsible for the significant reduction in transgene expression, as all partial coding sequence constructs were more similar and not statistically different from H-p571-eYFP but statistically different from H-p571-OR111-7-T2A-eYFP-3'UTR.

Table 4.2.2.: Cell counts for different transgenes.

H-p571	eYFP	OR111-7-T2A-eYFP	418 bp	517 bp	712 bp
Cell Number/OE	6.61(13)	1.35(23)	5.08(26)	3.76(21)	4.57(14)
Dendrite +/Dendrite -	75/11	12/19	116/16	28/50	12/52

Interestingly, the proportion of OSNs having a dendrite differs among the different transgenes. While, 87.2% of H-p571-eYFP expressing OSNs had a dendrite, only 38.7% of H-p571-OR111-7-T2A-eYFP-3'UTR expressing cells possessed a clearly visible dendrite ($p<0.01$, Mann-Whitney U Test with Bonferronni Correction). Since these two constructs represent the two extremes of OR111-7 transgenes, one with the full coding region and the

one without it, it was reasoned that the difference in dendrite formation might be an (unexpected) outcome of the OR111-7 coding sequence dependent suppression of transgene expression. Thus, it was hypothesized that OR111-7 transgene expression might be free of coding sequence dependent suppression in the early development when OSNs did not grow out a dendrite into the nasal cavity yet. However, OR coding sequence-specific suppression might be activated later in development which result in the down-regulation of OR111-7 coding sequence including transgenes in mature OSNs. Likewise, it would be expected that there are specific sequences in the OR111-7 coding region that have a role in late suppression of H-p571-OR111-7-T2A-eYFP-3'UTR transgene.

Therefore, the same partial coding sequence-eYFP fusion approach was utilized to test this hypothesis. Cells that have a dendrite for each of the H-p571-418bp-eYFP, H-p571-517bp-eYFP and H-p571-712bp-eYFP transgenes were quantified and it was observed that 87.8%, 35.6% and 22.1% of transgene expressing cells possessed a dendrite, respectively (Figure 4.2.6.). Interestingly, the addition of the DNA sequence between the 418th and 517th base pairs of OR111-7 coding region resulted in a significant decrease in the proportion of dendrite positive cells ($p<0.01$) while no difference was seen between H-p571-517bp-eYFP and H-p571-712bp-eYFP ($p>0.01$, Kruskal-Wallis Variance Analysis followed by Mann-Whitney U Test with Bonferronni Correction).

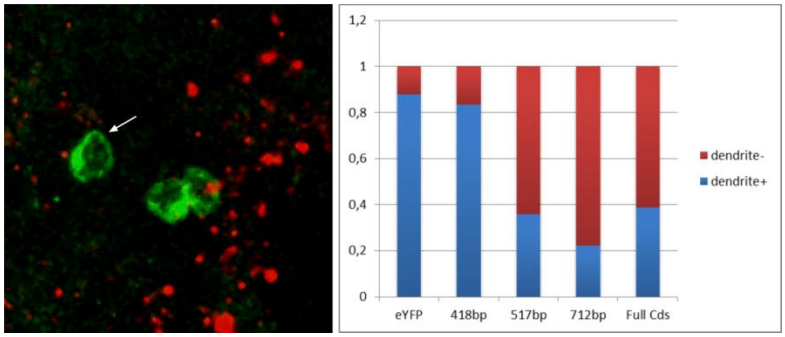


Figure 4.2.6.: Proportions of dendrite positive and dendrite negative transgenic OSNs for different partial coding sequence reporter constructs.

On the left, transgenic OSNs without a dendrite are shown. Arrow indicate an H-p571-517bp-eYFP positive, dendrite negative OSN. On the right, stacked bar graph showing the proportions of dendrite+ /dendrite- transgenic OSNs are shown for different constructs (Full cds: entire OR111-7 coding sequence, eYFP stands for H-p571-eYFP transgene).

Moreover, there was no significant difference between H-p571-eYFP and H-p571-418bp-eYFP in terms of dendrite formation ($p>0.01$) while H-p571-OR111-7-T2A-eYFP-3'UTR has significantly low number of dendrite positive cells than H-p571-eYFP and H-p571-418bp-eYFP ($p<0.01$). On the other hand, H-p571-517bp-EYFP, H-p571-712bp-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR were significantly indifferent from each other (Figure 4.2.6., $p>0.01$, Kruskal-Wallis Variance Analysis followed by Mann-Whitney U Test with Bonferronni Correction).

4. DISCUSSION

Various studies have shown that a mammalian olfactory sensory neuron expresses only one odorant receptor from ~1000 OR genes that it could theoretically choose from (for a review see Mombaerts et al., 2004). Moreover, an OSN expresses only paternal or maternal allele of the selected OR gene (Chess *et al*., 1994, Ishii *et al*., 2001). Although many studies have been performed to unravel the regulatory mechanisms underlying the one neuron-one receptor rule, we still lack an in depth understanding of how and when the singularity of OR gene expression is established. However, it seems that OR protein itself has a critical role in the expression of only one functional receptor per OSN. When OR coding regions are replaced with a fluorescent protein or a frameshift mutation causing a premature stop codon is introduced into the OR coding sequences, it has been shown that the OSN which would normally express the modified OR allele choose another OR from a limited subset of ORs (Serizawa et al., 2003, Bozza et al., 2009). Therefore, it has been widely accepted that the functional OR protein emits a negative feedback signal which suppresses the expression of other ORs. Then another question is raised: How is a particular OR selected over many others? First, it has been demonstrated that the expression of a given OR is restricted to only a particular area (zone) of the olfactory epithelium (Vassar et al., 1993; Ressler et al., 1993). Short range cis-acting sequences directly upstream of OR coding regions seem to be important for zonal specificity. Minigenes with short genomic sequences directly upstream of OR coding sequences are able to recapitulate the zonal expression patterns of the endogenous OR (Qasba and Reed 1998, Vassalli *et al*., 2002; Rothman *et al*., 2005). Second, it has been shown that there are at least two types of OSNs each of which is destined to express either Class I or Class II ORs. Therefore, a multistep regulatory mechanism exists to confine the expression of an OR to a particular subset of OSNs in a particular expression zone. Despite of these regulatory steps, there are still many ORs to be selected for an OSN in a given zone. It has been proposed that a stochastic OR selection process precedes the stable expression of only one receptor one neuron. In this model, after a particular OR is stochastically chosen for expression, it sends a negative feedback signal to other OR loci which suppresses their expression and somehow maintains its own expression through an unknown mechanism. It has been proposed that, long range locus control regions might form stable interactions with OR

promoter regions and maintains the expression of the selected OR throughout the life of the OSN (Serizawa et al., 2003). Indeed, a highly conserved genomic sequence upstream of MOR28 locus (H-region) seems to active any OR gene in the MOR28 locus but it activates only one of them (Serizawa *et al*., 2003, Fuss *et al*., 2007). Although it has previously been proposed that H region can act in trans to regulate the expression of ORs from different chromosomes, two independent studies have shown that it can act only in cis to regulate MOR28 locus (Fuss et al., 2007; Nishizumi et al., 2007). On the other hand, it is reasonable that there exists a locus control region for every loci in the genome and they can compete among each other to establish the singularity of OR gene expression in an early bird catches the worm manner. Indeed, Khan *et al*. defined another locus control region (P-region) which regulates the expression of the genes in the P2 locus (Khan *et al*., 2011).

On the other hand, OR gene choice is not always stable. Lineage tracing experiments have shown that 10% of MOR28 expressing OSNs switch to another OR which is expressed in the same zone with MOR28 (Shykind *et al*., 2004) suggesting that the interaction between the H region and the MOR28 promoter is not always stable as it is proposed. Interestingly, the switching seems to occur mostly in the immature stage of the olfactory sensory neuron development when the OSNs did not establish their functional connections yet (Shykind *et al*., 2004). Consistently, it has been shown that immature OSNs are inherently capable of expressing multiple ORs simultaneously (oligogenic expression, Mombaerts, 2004; Nguyen *et al*., 2007). Therefore, we hypothesized that there exists a critical period during which OSNs are able to express multiple ORs at the same time. Presumably, this period corresponds to the early life of the OSNs (Shykind et al, 2004; Nguyen *et al*., 2007) and sufficient levels of negative feedback signal is could not be accumulated yet because of the low levels of OR expression. Later in the OSN development, a particular OR might win over the others and be able to suppress the expression of other ORs by emitting sufficient levels of negative feedback signal.

In previous studies, OR coding regions were replaced with a fluorescent protein long before they were selected for expression. However, it is impossible to study the temporal aspects of OR gene choice by adopting this approach. Therefore it was

necessary to find a strategy that enables us to test whether a period of oligogenic expression (which ends up with the singular OR expression) exists during the olfactory sensory neuron development. Presumably, OSNs would be flexible enough to switch to another OR when the OR they express is deleted. That is, OSNs would be able to gain new specificities during this period. Consistently, it would be impossible for an olfactory sensory neuron to pick another OR if the coding region of the odorant receptor which is already expressed is deleted after this period.

In order to test the possibilities explain above, a conditional OR gene ablation transgene allowing us to delete the OR111-7 coding sequence after it has already been expressed. In this construct, OR111-7 coding region was flanked by two loxP sequences and the excision of the OR111-7 coding sequence is controlled by Cre recombinase expression. In order the OR111-7 expression to take place before the Cre expression (thereby Cre-mediated excision), zebrafish OMP promoter was used to drive Cre recombinase expression since it has been previously shown that OMP expression takes place before OR expression at least for some Ors (Iwema and Schwob, 2003).

However, critical controls must have been performed before the analysis of conditional OR111-7 ablation transgene for several reasons. First OR111-7 was selected as the odorant receptor model for the reasons explained in 4.1.1. Second, it has to be determined what kind of transgenesis should be performed for optimal transgene expression. For this reason, BAC transgenesis and short promoter transgenesis approaches have been tried. In order to test the feasibility of BAC transgenesis, a BAC clone (BAC100G14) including OR111-7 coding region has been modified such that OR111-7 coding sequence was either replaced with a fluorescent protein or tagged with T2A-eYFP sequence (see 4.1.3. for details). However, low penetrance of either BAC transgenes makes it impossible to continue the conditional ablation experiment by using a BAC transgene. In mice, it has previously demonstrated that short genomic regions upstream of OR genes are able to recapitulate endogenous OR gene expression (Qasba and Reed 1998; Vassalli et al., 2002). Therefore, short genomic regions directly upstream of OR111-7 coding sequence were tested to drive fluorescent protein expression. In our experiments, 571bp directly upstream of OR111-7 translation start site was able to drive eYFP expression, albeit with low

frequency. Longer or shorter genomic regions were also tried but it was observed that 571bp has the highest efficiency possible. Nishizumi *et al*. have shown that transgene efficiency increases when proximal promoter region of zebrafish 111-1 gene is fused with mouse H-region (Nishizumi *et al*., 2007). Therefore, H-p571 fusion has been used in order to drive the expression of OR111-7 transgenes. Indeed, transgene efficiencies increased drastically when H-p571 was used. However, it has been observed that some of the OSNs expressing H-p571-eYFP transgene innervate the lateral glomeruli which are devoid of pOMP-mCherry positive fibers. Perhaps, H region increased the probability of transgene expression so much that some of the sensory neurons that normally would not express OR111-7 choose H-p571-eYFP for expression (Figure 4.1.6.).

Third, there is no information about whether one neuron- one receptor rule is valid for the zebrafish olfactory system and the olfactory sensory neurons expressing a particular OR converge their axons to distinct glomeruli in the olfactory bulb. Therefore, a short promoter OR111-7 reporter transgene was generated by tagging OR111-7 coding sequence with self-cleavable T2A-eYFP sequence (see 4.1.3. for details) since internal ribosome entry site (IRES) has very low efficiency in zebrafish (Sato et al., 2007). Much to our surprise, transgene efficiency was too low (only nine positive embryos out of 3000 survived after injection, each embryo expressed only one transgenic cell) when H-p571-OR111-7-T2A-eYFP was injected into fertilized zebrafish oocytes. Initially, it has been thought that inefficient T2A cleavage might account for low transgene penetrance. For this purpose, T2A peptide was used to link two different fluorescent proteins and expression of this fusion (eYFP-T2A-mCherry) was driven by different promoters. When pGAP43- eYFP-T2A-mCherry was injected into fertilized zebrafish eggs, expression levels comparable to pGAP43-eGFP was observed (data not shown) and all of the transgenic cells expressed both reporter genes simultaneously. Consistent results were obtained when mCherry-T2A-CreERT2 expression was driven by heat shock promoter hsp70.

Nishizumi et al successfully expressed the OR111-1-GFP fusion when they drive the expression of their transgene with H region and a short proximal genomic region upstream of OR111-1 gene (Nishizumi *et al*., 2007). The difference between their construct and ours was that they included a long genomic region directly downstream of OR111-1 gene

directly downstream of GFP. To test whether 3' sequences have a role in odorant receptor expression 530bp downstream of OR111-7 gene was cloned to the 3' region of H-p571-OR111-7-T2A-eYFP construct. This 530bp genomic region includes complete 3'UTR sequences of OR111-7 gene and ~200bp downstream of 3'UTR. Interestingly, transgene efficiency was increased to 10% when 3'UTR sequence was included into the transgene. Alternatively, a reverse eYFP sequence was cloned downstream of H-p571-OR111-7-T2A-eYFP construct to see whether increase in transgene expression specifically depends on he 3' OR111-7 sequences or elongating the 3' region of the transgene simply prevents/delays the degradation of OR111-7-T2A-eYFP mRNA. As a result, no expression was observed when H-p571-OR111-7-T2A-eYFP-reverse eYFP was injected into zebrafish embryos at the single-cell stage (data not shown). Therefore, it seems that OR111-7 3' sequences are specifically able to increase transgene expression and 530bp downstream sequence of OR111-7 gene was cloned directly downstream of conditional OR111-7 gene ablation transgene. However, the role of 3' sequences in the regulation of OR gene expression is still to be elucidated.

As it is explained above and in 4.1.14.,that it is still unknown whether there is really a period of oligogenic OR expression during the olfactory sensory neuron development and when the expression of only one odorant receptor per one olfactory sensory neuron is irreversibly established. In the first part of this study, conditional OR111-7 ablation transgene was used to answer these questions. The pOMP-Cre construct was utilized to express Cre-recombinase which will excise the OR111-7 coding region that is flanked by two loxP sites. Since OR expression precedes OMP expression, OR111-7 will be expressed for a transient period before Cre-mediated recombination. When the conditional OR111-7 ablation transgene was injected together with pOMP-Cre construct, the OSNs expressing the conditional deletion transgene showed eYFP expression upon Cre recombinase mediated excision. On the other hand, injecting the conditional deletion construct without pOMP-Cre did not show eYFP expression in any of the embryos analyzed suggesting that eYFP is expressed exclusively after Cre-mediated recombination.

Odorant receptors are responsible for regulating different but interconnected events during the OSN development such as maintaining singularity of OR gene expression and guiding the functional connections to the first relay center in the brain. The most pronounced phenotypic outcome of appropriate OR expression (singular OR expression) in a particular OSN is perhaps the extension of an unbranched axon to a distinct glomerulus in the olfactory bulb (Mombaerts et al., 1996). Therefore, analysis of axonal projections seemed to be a reasonable assay to analyze the outcomes of the late OR111-7 deletion.

Cre-mediated excision of OR111-7 coding sequence might occur in OSN in different stages of development depending on the timing of Cre recombinase expression determined by the cell intrinsic timing of pOMP-Cre transgene transcription. First, Cre-mediated recombination might occur in early development before the OR111-7 transgene is selected. In this case, it would be expected to see an axonal phenotype similar to H-p571-eYFP. Second, Cre-mediated excision might happen during the (hypothetical) critical period when the transgenic OSNs are capable of switching to another OR and later able to embrace the characteristics (axonal projection to distinct glomeruli) of the OR they have reselected. If OR111-7 expression precedes Cre mediated expression during this period, the OSNs expressing the conditional deletion transgene, would be flexible enough to express another OR and gain new specificities determined by the second OR choice they have made. Lastly, transgenic OSNs would be unable to make a second choice, if the deletion of OR111-7 coding sequence occurs after the neuronal identity is irreversibly established in terms of OR gene choice. Under these circumstances, defects in axonal projections and neuronal cell death seem to be possible outcomes.

When conditional OR111-7 ablation transgene was coinjected with pOMP-Cre and pOMP-mCherry, two different kind of axonal abnormality were observed. The embryos were analyzed until the 7dpf and it was observed that most cells did not send any axons to the olfactory bulb during this period while pOMP-mCherry axons were clearly visible. This data suggest that late deletion of OR111-7 coding sequence cause developmental defects which result in no-axon phenotype and there is not a general defect in the development of the olfactory system of the embryos injected with the conditional deletion transgene. On the other hand, olfactory sensory neurons with

branched axons which cannot really innervate the olfactory bulb were also observed. Considering the role of proper OR gene choice in the axonal projections of the olfactory sensory neurons (Mombaerts et al., 1996; Imai and Sakano, 2007), axonal defects in the conditional deletion OSNs can be interpreted as an outcome of defective OR gene choice. Therefore, absence of axonal projections and branching of OSN axons suggest that Cre-mediated excision occurs after the OR gene choice is already irreversibly established. It is conceivable that transient expression of OR111-7 from the conditional ablation transgene was sufficient enough to stably set up the OSN identity and put an end to OR gene choice event. Moreover, it can be suggested that OR gene choice mechanism takes place in a short period at the earlier stages of ontogenic development of olfactory sensory neurons and before the OR protein dependent axon guidance establishes neuronal connections.

It would be very interesting to see how the OSNs behave after they form stable connections with the olfactory bulb. In future experiments, the Cre-mediated excision of OR111-7 coding sequence can be delayed by using the tamoxifen inducible form of Cre recombinase (CreERT2, Hans et al., 2009). Administering the inducing chemical 4-hydroxytamoxifen later in the development (after the proper axonal connections are formed) would allow us to delay the excision of OR111-7 coding sequence after at least some of the transgenic OSNs establish their connections with the olfactory bulb. Alternatively, it is possible to use the promoter of an earlier marker such as GAP43 to induce the deletion of OR111-7 coding sequence before the transgene is already expressed. In this way, it is aimed to control the deletion of OR111-7 coding region in different points of temporal dimension of the olfactory sensory neuron development and to define the periods/time points when the oligogenic expression is possible and the OR gene choice is irreversibly established by analyzing the axonal phenotypes.

The second part of this thesis focused on the OR111-7 coding sequence dependent inhibition of transgene expression. While H-p571 sufficiently drives the expression of different fluorescent proteins, almost no expression was seen when H-571 is used to drive the expression of OR111-7-T2A-eYFP. Initially, inefficient T2A cleavage was thought to result in inappropriate folding of eYFP protein causing the absence of

fluorescent signal. However, series of experiments have shown that T2A cleavage occurs when different proteins werefused to each other with T2A peptide. Moreover, H-p571 is also unable to drive theexpression of either OR111-7-eYFP fusion or OR111-7-myc tag fusion. For the reasons explained above and depending on a previous study (Nishizumi *et al*., 2007), 3'UTR region of OR111-7 gene was cloned directly downstream of H-p571-OR111-7-T2A-eYFP sequence. Although 3'UTR sequences increased the efficiency to 10%, there was still significantdifference between H-p571-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR. Moreover, the inefficiency was also represented as cell numbers per nose.

The work of Nguyen and his colleagues in 2007 has shown that negative feedback signal is able to suppress the expression of other ORs by acting on the OR coding sequence in the absence of the regions downstream or upstream of OR coding region. Since OR coding sequences are intronless, it is suggested that the sequences coding for the OR proteins are the receivers of the negative feedback signal. Considering the fact that OR111-7 coding sequence including constructs were inefficiently expressed, it is hypothesized that the presence of OR111-7 coding region renders our transgenes prone to negative feedback signal. On the other hand, H-p571-eYFP sequence can easily escape the negative feedback regulation since it does not have the coding region and can be expressed in high levels. Indeed, this kind of regulation could explain the huge difference in expression levels between H-p571-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR.

It was reasoned that in the OR coding regions, there might be specific sequences which are responsible for receiving the negative feedback signal. For this reason, OR111-7 coding sequence was chopped into smaller pieces (partial coding sequences) and each piece was fused to eYFP sequence. By driving the expression of partial coding sequence-eYFP fusion with H-p571, the part of the OR111-7 coding sequence which would possibly receive the negative feedback signal. 3 different constructs were designed for this purpose: First 418 bp or 517 bp or 712 bp parts of OR111-7 coding sequence was fused to eYFP and cloned downstream of Hp571 driver.

Initially, cell numbers per nose were analyzed for each of the partial coding sequence construct and they were compared to each other and to H-p571-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR. Unfortunately, no significant difference was observed between any of them. Much to our surprise, however, there exists a difference in the percentage of dendrite positive OSNs expressing the partial coding sequence transgenes. Therefore, dendrite+ and dendrite- OSNs were counted for each partial coding sequence transgene and for H-p571-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR (Table 2). Interestingly, H-p571-eYFP had significantly higher number of dendrite positive OSNs compared to H-p571-OR111-7-T2A-eYFP-3'UTR. Interestingly, there was no significant difference between H-p571-eYFP and H-p571-418bp-eYFP while H-p571-OR111-7-T2A-eYFP-3'UTR had significantly lower number of dendrite positive cells compared to both of them. When the percentage of dendrite positive cell numbers for H-p571-517bp-eYFP was analyzed, it was seen that it has significantly lower number of dendrite positive cells compared to both H-p571-eYFP and H-p571-418bp-eYFP. Moreover, there was no significant difference between H-p571-517bp-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR. On the other hand, H-p571-712bp-eYFP was not different from either H-p571-517bp-eYFP or H-p571-OR111-7-T2A-eYFP-3'UTR while it has significantly lower number of dendrite positive cells compared to H-p571-eYFP and H-p571-418bp-eYFP. Taken altogether, it would be suggested that the sequences between 418th and 517th base pairs of OR111-7 coding sequence might account for the difference in presence or absence of a dendrite.

How can then the difference in the percentages of dendrite positive transgenic cells be explained? Perhaps, there is really a transient period in the ontogenic development of the olfactory sensory neurons during which the negative feedback signal is not fully established and the OSNs are capable of expressing multiple ORs simultaneously. As the results of the conditional OR111-7 ablation suggest, this period should correspond to the very early life of the OSNs when they could still not extend a functional dendrite or axon. Since the negative feedback signal is not established during this transient (and early) stage of oligogenic expression, the transgenes which have the sequences responsible for receiving the negative feedback signal are expressed indifferently compared to the transgenes devoid of these sequences. Therefore, H-p571-OR111-7-T2A-eYFP-3'UTR could be expressed in the olfactory sensory

neurons undergoing the transient period of oligogenic expression while the establishment of the negative feedback signal decreases the number of H-p571-OR111-7-T2A-eYFP-3'UTR positive cells in a stochastic manner. On the other hand, H-p571-eYFP could be freely expressed before or after this period since it does not have any sequence which can receive the negative feedback signal. Because this transient period presumably corresponds to the immature stage of OSN development (when OSNs are devoid of clearly visible dendrites or axons), the difference in the percentages of dendrite+ and dendrite – cells between H-p571-OR111-7-T2A-eYFP-3'UTR and H-p571-eYFP could be a phenotypic outcome of the OR coding sequence dependent suppression of OR111-7 reporter transgenes. Likewise, it was expected that the partial coding sequence transgenes including the receiver of negative feedback signal would behave like full coding sequence OR111-7 reporter construct while the ones without the receiver sequence would behave like the H-p571-YFP transgene. Here it was demonstrated that H-571-418bp-eYFP is indistinguishable from H-p571-eYFP in terms of expression in dendrite positive cells while H-p571-517bp-eYFP was expressed in significantly lower number of dendrite positive cells compared to H-p571-418bp-eYFP and H-p571-eYFP. Moreover, there was no statistical difference between H-p571-517bp-eYFP and H-p571-OR111-7-T2A-eYFP-3'UTR transgenes. Therefore, it was assumed that the sequence responsible for receiving the negative feedback signal stays between the 418th and 517th base pairs of OR 111-7 coding region. To test whether this sequence is confined between 418th and 517th base pairs or there are additional sequences downstream of 517th base pair, H-p571-712bp-eYFP construct was also analyzed in terms of expression in dendrite positive cells. As a result, it was shown that there is no difference between H-p571-517bp-eYFP and H-p571-5712bp-eYFP further reinforcing the idea that the receiver of negative feedback signal is between 418th and 517th base pairs. Interestingly, nine H-p571-OR111-7-T2A-eYFP OSNs out of 3000 survived embryos after injection were only positive at 1dpf and the fluorescent signal disappears in 12 hours. Perhaps, H-p571-OR111-7-T2A-eYFP could also be expressed only in the transient period when there is no sufficient negative feedback signal and it is suppressed later at the end of this stage. Moreover, all of the nine cells were devoid of dendrites further suggesting the presence of a transient, negative feedback signal free period during olfactory sensory neuron development. However, it is still to be elucidated how the addition of 3' UTR sequence specifically increase the

probability that the OR111-7 coding sequence including transgenes are expressed in the OSNs at later stages of OSN development. Perhaps, 3'UTR regions possess sequences which has a role in zebrafish OR gene choice in contrast to previous data in mice (Vassalli *et al*., 2001).

In conclusion, several critical and interconnected aspects of zebrafish OR gene choice are covered in this thesis work. First, conditional OR111-7 ablation experiments suggested that there OR gene choice is irreversibly established early in the ontogenic development of the OSNs. Second, it was demonstrated that there exist an OR coding sequence dependent inhibition of OR reporter transgenes. Further analysis of this suppression mechanism interestingly revealed that coding sequence dependent suppression is not active earlier in OSN development (monitored by the absence of a dendrite) and proportion of dendrite positive transgenic cells (older olfactory sensory neurons) significantly decreased when OR coding sequence was included in the constructs. It is reasonable to assume that OR reporter transgene positive and dendrite negative OSNs are in the transient period of oligogenic expression when the negative feedback signal acting on the OR coding sequences is not still established. Following the irreversible establishment of the negative feedback signal, OR reporter transgenes would be silenced in a stochastic manner. Interestingly, at 3 dpf the number of cells expressing the OR reporter transgene is not significantly different from the number of cells expressing the endogenous OR111-7 revealed by cell counts after in situ hybridization further suggesting that the presence of the OR111-7 coding sequence in H-p571-OR111-7-T2A-eYFP-3'UTR construct makes the transgene prone to negative feedback signal and the transgenic cell numbers are maintained in the levels of endogenous OR111-7 expression even though a very strong driver, H-p571 was used to promote the expression. On the other hand, H-p571-eYFP cannot be suppressed by the negative feedback signal and expressed in high number of cells thanks to the strong driver H-p571. In addition, it was aimed to identify the specific sequences which receive the negative feedback signal by generating reporter transgenes with partial OR111-7 coding sequences. As a result, it is strongly suggested that, this sequence resides between the 418th and 517th base pairs of OR111-7 coding region. Recently it has been shown that OR loci are epigenetically silenced in the mouse olfactory system (Magklara *et al*., 2011). It would be very interesting to further dissect this sequence in smaller pieces to

narrow down the specific sequences responsible for negative feedback inhibition and to find whether specific DNA binding proteins responsible for epigenetic silencing can bind to these sequences. Taken altogether, we believe that this study helps us understand both the temporal aspects of OR gene choice and the role of specific sequences in OR coding regions in the negative feedback regulation.

APPENDIX A:EQUIPMENTS

Table 5.1.: List of Equipments.

4 °C Room	Birikim Elektrik, Turkey
Autoclaves	Astell Scientific, UK
Centrifuge	Eppendorf, Germany (5417R)
Confocal Microscope	Leica SP5-AOBS, USA
Electronic Balance	Sartorius, Germany (TE412)
Electrophoresis Supplies	Bio-Rad Labs, USA (ReadySub-Cell GT Cells)
Fluorescence Microscope	Leica Microsystems, USA (MZ16FA)
Freezer 1	-20 °C Arçelik, Turkey
Freezer 2	-80 °C Thermo Electron Corp., USA (Thermo Farma 723)
Gel Documentation	Bio-Rad Labs, USA (GelDoc XR)
Glass Bottles	Isolab, Germany
Incubator 1	Weiss Gallenkamp, UK
Incubator 2	Nuve, Turkey
Incubating Shaker	Thermo Electron Corp., USA
Micropipetters	Gilson, USA (Pipetman)
Microwave Oven	Vestel, Turkey
Microinjector	Eppendorf, Germany (FemtoJet)
Refrigrator	Arçelik, Turkey
Softwares	Invitrogen, USA (Vector NTI)
Thermal Cyclers	Bio-Rad Labs, USA (C1000)
Vortex	Scientific Industries, USA

APPENDIX B: SUPPLIES

Table 6.1.: Disposables.

14 ml Culture Tubes	Greiner Bio-One,Belgium (187261)
CELLSTAR Centrifuge Tubes, 15 ml	Greiner Bio-One,Belgium (186161)
CELLSTAR Centrifuge Tubes, 50 ml	Greiner Bio-One,Belgium (227261)
Filtered Pipette Tips	Greiner Bio-One,Belgium (771288, 772288, 740288)
Micro-centrifuge tubes	Citotest, China (34730015)
PCR Tubes	Bio-Rad, U.S.A. (TBS0201)

Table 6.2.: Chemical Supplies.

1 kb DNA Ladder	New England Biolabs, U.S.A. (N3232)
100 bp DNA Ladder	New England Biolabs, U.S.A. (N3231)
5X GoTaq Flexi Buffer	Clontech, U.S.A. (639201)
Advantage 2 Polymerase Mix	Promega, U.S.A. (M890A)
AscI	New England Biolabs, U.S.A. (R0558 L)
AseI	New England Biolabs, U.S.A. (R0526 M)
BamHI	New England Biolabs, U.S.A. (R0136 L)
Bovine Serum Albumin	New England Biolabs, U.S.A. (B9001)
EcoRI	New England Biolabs, U.S.A. (R0101 M)
EcoRV	New England Biolabs, U.S.A. (R0195 L)
Ethanol Absolute	Sigma-Aldrich, U.S.A. (34870)
Ethidium Bromide	Sigma Life Sciences, U.S.A. (E1510-1 ml)
Ethlyenediaminetetraaceticacid (EDTA) Disodium Salt	Sigma-Aldrich., U.S.A. (E5134 - 1 kg).
Glycerol	Sigma-Aldrich, U.S.A. (G5516-500 ml)
GoTaq Flexi DNA Polymerase	Promega, U.S.A. (M830B)
LB Agar	Sigma Life Sciences, U.S.A. (SL08394)
LB Broth	Sigma-Aldrich, U.S.A. (L7658- 1 kg)

Table 6.2.: Chemical Supplies (cont.).

Magnesium Chloride, 25 mM	Promega, U.S.A. (A3511)
Magnesium Sulfate	Sigma-Aldrich, U.S.A. (M7506)
NcoI	New England Biolabs, U.S.A. (R0193 L)
NotI	New England Biolabs, U.S.A. (R0189 L)
pGEM®-T Easy Vector System	Promega, U.S.A. (A1360)
Phenol : Chloroform : Isoamyl alcohol	Sigma-Aldrich, U.S.A. (P2069)
Potassium Chloride	Sigma-Aldrich, U.S.A. (P9541)
PstI	New England Biolabs, U.S.A. (R0140 L)
SalI	New England Biolabs, U.S.A. (R0138 L)
SeaKem® Agarose	Cambrex, U.S.A. (50004)
Sodium Acetate	Sigma-Aldrich, U.S.A. (S8625)
Sodium Chloride	Sigma-Aldrich, U.S.A. (S7653 - 1 kg)
Sodium Hydroxide	Sigma-Aldrich, U.S.A. (S8045 - 1 kg)
SpeI	New England Biolabs, U.S.A (R0133 L)
SphI	New England Biolabs, U.S.A (R0182 L)
T4 DNA Ligase	New England Biolabs, U.S.A (M0202L)
Trizma® Base	Sigma-Aldrich, U.S.A. (T6066)
XhoI	New England Biolabs, U.S.A. (R0146 L)

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