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REGULATORY MECHANISMS IN OLFACTORY SYSTEM ASSEMBLY AND FUNCTION

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Cover image illustrates the developing main olfactory system at embryonic day E14.5 labeled with GFRα1 (red), GAD67 (green) and DAPI (blue).

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For Ottis Wilson,
the catalyst of these
scientific endeavors...

ABSTRACT

The mammalian sense of smell relies on the detection of odorants by a large family of G protein coupled receptors, encoded by ~1300 different genes in mice (Buck and Axel, 1991; Zhang et al., 2007). These odorant receptors (ORs) are expressed in olfactory sensory neurons (OSNs) in the main olfactory epithelium (MOE). A key feature of the mammalian olfactory system is that each OSN expresses only one of 2600 potential OR alleles (Chess et al., 1994; Malnic et al., 1999) such that the expression of a single specific OR protein establishes the odorant selectivity and identity of an OSN. Several layers of regulation control odorant receptor choice (Nguyen et al., 2007). Understanding this regulatory hierarchy of OR gene expression is critical in elucidating how odorant receptor choice is orchestrated and how ORs pattern the system.

Precise control of OR expression is fundamental for the assembly and operation of the MOE and the main olfactory bulb (MOB). ORs play an instructive role in OSN axon guidance and the generation of a topographic glomerular map in the MOB (Malnic et al., 1999; Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998). This first order glomerular map resides on the surface of both olfactory bulbs and is mirror symmetric, i.e. each bulb is comprised of identical lateral and medial glomeruli (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Belluscio and Katz, 2001). These isofunctional glomeruli are precisely and reciprocally connected by a second order intrabulbar map directly beneath the surface glomerular map (Schoenfeld et al., 1985; Belluscio et al., 2002; Lodovichi et al., 2003). Neural activity plays an important role in establishing and shaping the connections within these olfactory maps and defines the functional organization of the system (Cummings and Belluscio, 2008). Olfactory maps likely result from a combination of neuronal activity and chemical guidance cues.

While many families of guidance molecules have been found to be expressed in the olfactory system, their role in organization and function are not determined. GDNF and its receptor GFRα1 have been detected in the main olfactory system (Cao et al., 2006; Maroldt et al., 2005; Nosrat et al., 1997; Paratcha et al., 2006; Trupp et al., 1997), but their specific cellular localization and importance in the functional organization of the olfactory system is still unknown. Understanding the functional roles of GDNF signaling may provide a molecular basis for factors that pattern the olfactory system.

This thesis investigates the mechanisms that regulate five fundamental components of olfactory system assembly and function: 1) odorant receptor expression, 2) odorant receptor choice, 3) OSN axon guidance, 4) olfactory maps, and 5) guidance molecules. Thematically specified, these mechanisms include the OR coding sequence in OR gene regulation, timing of OR expression, activity in the intrabulbar map, GDNF signaling in assembly and function, and GFRα1 in olfactory interneuron development.

LIST OF PUBLICATIONS

I. Nguyen MQ, Zhou Z, Marks CA, Ryba NJ, Belluscio L. Prominent roles for odorant receptor coding sequences in allelic exclusion. Cell 2007 Nov 30; 131 (5):1009-17.

II. Nguyen MQ, Marks CA, Belluscio L, Ryba NJ. Early expression of odorant receptors distorts the olfactory circuitry. J Neurosci. 2010 Jul 7; 30 (27):9271-9.

III. Marks CA, Cheng K, Cummings DM, Belluscio L. Activity-dependent plasticity in the olfactory intrabulbar map. J Neurosci. 2006 Nov 1; 26 (44):11257-66.

IV. Marks CA, Belluscio L, Ibanez CF. Critical role of GFRα1 in the development and function of the mouse olfactory system. J Neurosci. 2012, In press.

V. Marks CA, Belluscio L, Ibanez CF. A cell-autonomous role of GFRα1 in the development of olfactory bulb GABAergic interneurons. Manuscript.

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LIST OF ABBREVIATIONS

AON	Anterior olfactory nucleus
ARTN	Artemin
CB	Calbindin
CCK	Cholecystokinin
CNS	Central nervous system
CR	Calretinin
E	Embryonic day
EC	Entorhinal cortex
EPL	External plexiform layer
ETC	External tufted cell
GABAergic	Gamma-Aminobutyric acid
GAD65	The 65-kDa isoform of glutamic acid decarboxylase
GAD67	The 67-kDa isoform of glutamic acid decarboxylase
GC	Granule cells
GDNF	Glial cell-line derived neurotrophic factor
GFP	Green fluorescent protein
GFRα	GDNF family ligand receptor alpha
GL	Glomerular layer
GPI	Glycosylphosphatidylinositol
IBM	Intrabulbar map
IBP	Intrabulbar projection
IRES	Internal ribosomal entry site
ISI	Intrinsic signal imaging
LGE	Lateral ganglionic eminence
MA	Medial amygdala
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
NCAM	Neural cell adhesion molecule
NRTN	Neurturin
OB	Olfactory bulb
OE	Olfactory epithelium
OEC	Olfactory ensheathing cell
OMP	Olfactory marker protein
ONL	Olfactory nerve layer
OR	Odorant receptor
OSN	Olfactory sensory neuron
PC	Piriform cortex
PG	Periglomerular
PNS	Peripheral nervous system
PSPN	Persephin
RMS	Rostral migratory stream
TH	Tyrosine hydroxylase
Tmx	Tamoxifen
TTA	Tetracycline transactivator

1 INTRODUCTION

1.1 Organization of the Olfactory System

The olfactory system is the sensory system used for olfaction, the sense of smell. While in humans olfaction is frequently considered an aesthetic sense, in many species it is crucial for survival, playing a central role in reproductive and rearing functions, neuroendocrine and emotional responses, as well as in the recognition of predators, prey and potential mates (Shipley and Ennis, 1996). The olfactory system is also essential for identifying food and plays a major role in taste perception (Shipley and Ennis, 1996). From a neuroanatomical perspective, the olfactory system is an excellent model system to study how the brain processes sensory information. The olfactory system is highly organized and precisely controlled. Even in humans, the olfactory system is the fastest modality to process sensory information to elicit a memory response. How does this dynamic system rapidly transduce chemical signals into perception? The olfactory system achieves this through the olfactory sensory pathway which is responsible for detecting, discriminating and distinguishing the meaning of odors.

Mammals have two distinct parts to their olfactory system: the main olfactory system and the accessory olfactory system. The main olfactory system detects volatile, airborne substances, while the accessory olfactory system senses fluid-phase stimuli. Behavioral evidence indicates that, most often, the stimuli detected by the accessory olfactory system are pheromones. The overall mechanism of the olfactory system can be divided into a peripheral one, sensing an odor stimulus and encoding it into an electrical signal, and a central one, where the odor-coded signals are integrated and processed in the central nervous system.

In mammals, the main olfactory system detects odorants that are inhaled through the nose, where they contact the main olfactory epithelium (MOE), which is comprised of various olfactory receptors. These odorant receptors (ORs) are membrane proteins of bipolar olfactory sensory neurons (OSNs) in the olfactory epithelium (OE) **(Figure 1)**. Rather than binding specific ligands like most receptors, odorant receptors display specialized affinity for a diverse range of odorant molecules. OSNs transduce receptor activation into electrical signals in neurons. The signals travel along the olfactory nerve, the axons of the OSN, which belongs to the peripheral nervous system (PNS). This nerve terminates in the main olfactory bulb (MOB), which belongs to the central nervous system (CNS). The multifaceted arrays of ORs that are individually expressed in different OSNs allow the system to distinguish a new odor from the background environmental odors and determine the odor concentration. In the CNS, odors are represented as patterns of neural activity. These odor

representations are encoded spatially, in a pattern of activated neurons across a specific region corresponding to the odor, temporally, in a pattern of action potentials from multiple neurons corresponding to the odor, or a combination of the two. These patterns of activity are integrated and further processed within the olfactory bulb (OB).

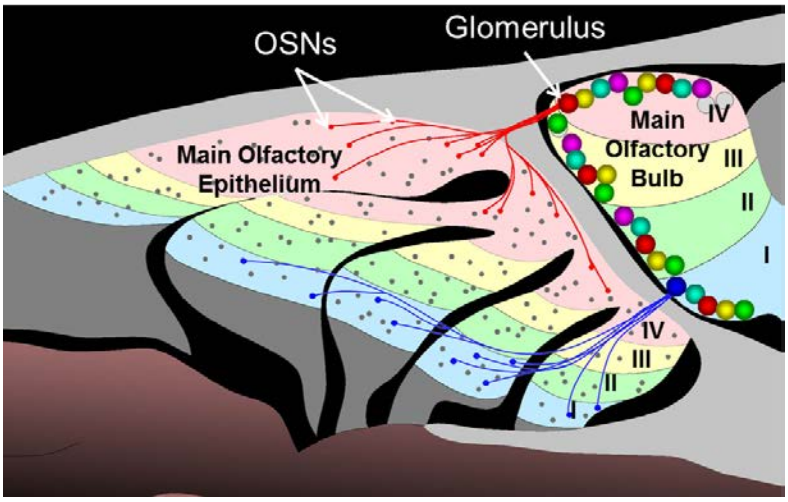

Figure 1: Schematic representation of the main olfactory system comprised of the MOE and MOB. Odors activate ORs which are broadly scattered in OSNs throughout the four zones (I-IV) of the MOE. OSNs expressing the same OR within each zone send axons that converge in corresponding regions (I-IV) of the MOB called glomeruli.

Axons from the OSNs precisely converge in the OB to form discrete spherical structures called glomeruli which cover the entire OB. The OB is architecturally structured such a way that most of the circuitry associated with an individual glomerulus is located in either the region immediately surrounding it or in the cells lying directly beneath it. The OB contains additional neuronal types: mitral/tufted cells which comprise the principal projection neurons of the bulb and two classes of interneurons, one called periglomerular (PG) cells, which are located in the glomerular layer (GL) at the surface of the bulb and the other called granule cells (GCs) which are located in the deepest layer of the bulb called the granule cell layer (GCL) **(Figure 2).**

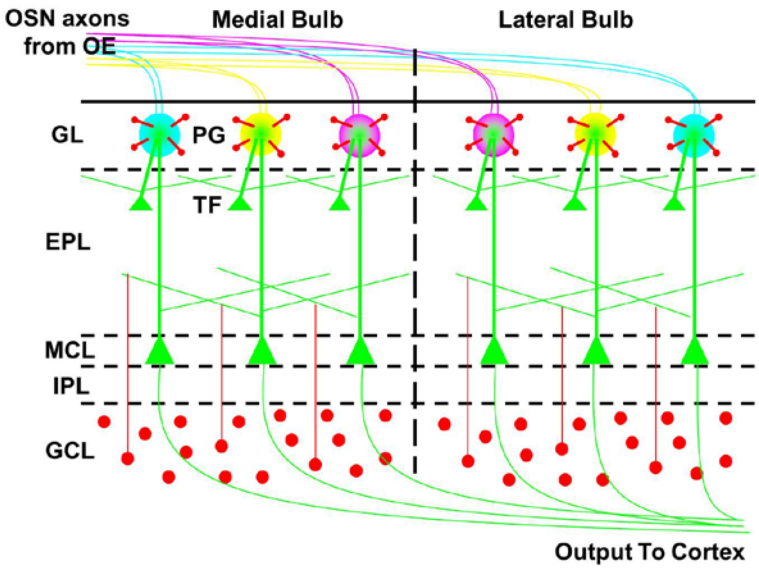

Figure 2: Schematic identifying OB cell types. OSN axons are shown entering the glomerular layer (GL). PG cells (red) are shown in the GL. Tufted(TF)/mitral cells are displayed (green). The granule cells (red) of the granule cell layer are located in the deepest layer.

Within glomeruli, OSNs form synapses directly with the principle output neurons – the mitral and tufted cells (Shipley and Ennis, 1996). Interestingly, each mitral/tufted neuron sends a single primary dendrite to receive input from only one glomerulus (Shipley and Ennis, 1996). Each mitral/tufted cell is therefore activated by a restricted range of odors. As both primary input and output neurons organize themselves around glomeruli, together they function as a local hub (Bozza et al., 2002). The PG cells are a large and diverse class of inhibitory cells that are located in and around the glomerulus. They are significant contributors of information processing and they form reciprocal synapses with both the axons of primary neurons and the dendrites of the secondary neurons (Pinching and Powell, 1971). Furthermore, PG neurons not only modulate both the input and output of the glomerulus that they immediately surround but also influence adjacent glomeruli. In addition to the processing that occurs within the GL, a second level of processing occurs deeper in the bulb mediated by the large population of granule cells. While GCs reside in the deepest layer of the bulb, they project dendrites toward the surface of the bulb to the external plexiform layer (EPL). There they form dendrodendritic synapses onto the lateral dendrites of mitral and tufted cells to modulate olfactory bulb output.

The final output of the OB is mediated by mitral cells. Mitral cells send their axons to a number of brain areas, including the anterior olfactory nucleus (AON), the piriform cortex (PC), the medial amygdala (MA), and the entorhinal cortex (EC). The PC is the area of the brain that is most closely associated with the identification of an odor. The MA is involved in social functions such as mating and the recognition of animals of the same species. The EC is associated with memory and serves to pair odors with memories. While the functions of these brain regions are not completely known and are certainly debatable, it is well known that, together, they are responsible for the final phase in the olfactory sensory pathway, the perception of smell.

Together, the olfactory sensory pathway consists of odor sensing, encoding, integration, and finally perception. How does the olfactory system process sensory information so rapidly? The olfactory system accomplishes this because it is highly organized, precisely connected, and tightly regulated. Several levels of control are in place to ensure its precision. This thesis explores such intricate levels of control in the mouse main olfactory system. In order to understand this precision, it is essential to appreciate how the system is exactly organized to accurately function. Thus, this thesis investigates and identifies mechanisms that regulate five fundamental components of olfactory system assembly and function. These five fundamental features include: odorant receptor expression, odorant receptor choice, axon guidance, the glomerular map, the intrabulbar map, and guidance molecules.

1.2 Odorant Receptor Expression and Odorant Receptor Choice

The mammalian olfactory system has evolved the ability to detect and discriminate thousands of different odorants. Odor detection begins with the expression of odorant receptors. ORs are a very large family of G protein coupled receptors (GPCR), which in mice are encoded by approximately 1300 genes (Buck and Axel, 1991; Zhang et al., 2007). As each gene consists of two alleles, a key feature of the main olfactory system is that each OSN expresses only one of the 2600 possible OR alleles (Chess et al., 1994; Malnic et al., 1999). How is a single OR allele chosen for expression out of thousands of possibilities?

In addressing this question, three important aspects of OR gene control have emerged. First, short DNA sequences upstream of the OR transcriptional start site are capable of driving OR expression and reporter genes in OSNs in similar patterns as endogenous ORs (Qasba and Reed, 1998; Vassalli et al., 2002). These results suggest that the choice of the OR to be expressed may be controlled by gene selection. Second, OR gene expression prevents activation of other endogenous OR genes by utilizing an OR feedback mechanism (Serizawa et al., 2003; Lewcock and Reed, 2004; Shykind et al., 2004), suggesting that once an OR is expressed; there seem to be additional levels of control that subsequently inhibit other ORs from being expressed. Third, a putative transactivating element (H) may be involved in initiating the expression of a single OR (Lomvardas et al., 2006). The H element serves as a target for feedback mechanisms to recognize the expression of a functional OR protein, and consequently prevents the activation of additional OR alleles (Serizawa et al., 2003; Lewcock and Reed, 2004; Shykind et al., 2004). Further evidence has demonstrated that while the H region is important in controlling OR expression of a local group of OR genes, it is not actually essential for the majority of ORs (Serizawa et al., 2000, 2003; Fuss et al., 2007).

While these studies provide useful information to help explain how a single OR might be chosen, many aspects of OR expression and odorant receptor choice still remain unclear. For example, a DNA transcription unit encoding for a protein contains both the olfactory coding sequence and regulatory sequences. It would be important to address which components are actually responsible for OR expression. Additionally, the feedback mechanism of OR expression and suppression is important, but it does not address how the first OR is actually expressed or chosen. What factors control the first OR to be expressed? Finally, perhaps elements of gene selection in addition to a feedback mechanism contribute to OR expression and OR choice. There may be several levels of control to insure the precision of when an OR is expressed and how it is selected. These aspects of OR expression and odorant receptor choice are investigated in Paper I.

1.3 OSN Axon Guidance and the Glomerular Map

ORs provide the molecular basis for the function of not only the OE, but also the OB, by establishing that each OSN expresses only a single odorant receptor gene and subsequently can respond to a small range of odorants, usually sharing a similar chemical structure. ORs provide an organizational framework for OB circuitry by instructing the guidance of axons from OSNs that express the same OR, leading them to converge into two small sets of isofunctional glomeruli at reproducible locations on each bulb: one on the medial and one on the lateral surface. Thus, the OR plays an important role in establishing the presence of two mirror symmetric glomerular maps of odorant receptor identity on the surface of the bulb. The expression of a particular OR in an OSN provides much of the molecular and functional organization of the olfactory circuitry, as their precise projections innervate the bulb and not only contribute to the formation of glomeruli but also give them their identity.

What factors control the accuracy of axon targeting and the formation of the glomerular map? The use of genetic techniques to swap OR genes, thereby altering OR expression, have defined the role of ORs in fixing OSN projections to specific glomeruli (Mombaerts et al., 1996; Wang et al., 1998). The complex mechanisms that control OR gene expression have prevented the use of simple transgenic methods to manipulate OR expression. In spite of this, studies have developed a tetracycline transactivator (TTA)-based approach to broadly express a single TetO-OR transgene in many OSNs throughout the MOE (Nguyen et al., 2007; Fleischmann et al., 2008). These studies revealed that when a single OR was expressed in 90% of mature OSNs, there were surprisingly few consequences in the OB. This suggests that either the OR is less important than is generally conceived or perhaps OR expression occurs too late in OSN development to influence OB circuitry.

While the initial expression of ORs occurs very early in embryonic development, much earlier than the first OSN synaptic connections are established (Sullivan et al., 1995), much of what is known about glomeruli and the formation of the glomerular map occurs after birth in the first three postnatal weeks (Potter et al., 2001). Precise information about exactly when OR expression begins in an OSN is necessary to examine the exact consequential influence on OB circuitry. Therefore, it would be important to identify if the timing of when an OR is expressed (early versus late) in OSN development can influence the precise targeting of OSN axons and the establishment of the glomerular map. This would provide important new insights as to how the bulb is wired to function. The influence of early timing of OR expression in OSNs on OB circuitry is explored in Paper II.

1.4 The Intrabulbar Map

OSNs that express the same odorant receptor send axons that converge to form two identical glomeruli on the surface of each olfactory bulb. These identical glomeruli are mirror symmetric, isofunctional and constitute the glomerular map. Directly beneath the surface of the glomerular map lies the intrabulbar map. The intrabulbar map specifically links isofunctional glomeruli of the glomerular map through a set of reciprocal intrabulbar projections (IBPs) .The intrabulbar map consists of external tufted cells (ETCs) directly associated with a glomerulus on one side of the bulb specifically projecting to the opposite side of the same bulb and terminating directly beneath the isofunctional glomerulus. The resulting map essentially mirrors the glomerular map at the surface (Belluscio et al., 2002; Lodovichi et al., 2003).

How does the precision of olfactory maps arise? Neural maps result from a combination of neural activity and chemical guidance cues. However, it remains unknown how these components regulate the map. In other sensory systems, many families of guidance molecules have been associated with the formation of maps (McLaughlin and O'Leary, 2005). While many of these molecules are expressed in the olfactory bulb, their role in patterning the circuitry of the bulb remains undetermined.

Odorant-induced activity has also been shown to play an important role both in establishing and shaping the connections within neural maps. Sensory input, in the visual system, dramatically alters specific cortical connections associated with right and left eye dominance as well as with orientation selectivity (Wiesel and Hubel, 1965; Katz and Shatz, 1996; White et al., 2001). However, the function of odorant-induced activity in organizing and maintaining the olfactory map is unclear. Surprisingly, previous studies using anosmic knock-out mice, in which key components of the odorant signaling cascade are disrupted, indicate that the glomerular map remains remarkably intact with very few alterations (Brunet et al., 1996; Belluscio et al., 1998; Wong et al., 2000; Zheng et al., 2000).

While the existence of the intrabulbar map in adulthood has been demonstrated, virtually nothing is known about the development of the intrabulbar map, nor how the projections precisely target such specific loci on the opposite side of the bulb. As a result, it would be important to determine how the intrabulbar map forms and the factors involved in achieving its specificity. The development of the intrabulbar map and the role of OR activity in its organization and maintenance are examined in Paper III.

1.5 Guidance Molecules

Many families of guidance molecules have found to be expressed in the olfactory system, but their role in development and function remains unclear and is only recently being investigated (Cummings and Belluscio, 2008). One family of particular interest is that of the glial cell line-derived neurotrophic factor (GDNF). GDNF and its receptor GFRα1 have been detected in the main olfactory system (Cao et al., 2006; Maroldt et al., 2005; Nosrat et al., 1997; Paratcha et al., 2006; Trupp et al., 1997), but the specific cellular localization of these guidance molecules and their importance in the functional organization of the olfactory system has not been determined.

GDNF has been found to promote differentiation and tangential migration of cortical inhibitory GABAergic neurons by stimulating axonal growth, enhancing cortical cell morphology and motility, and acting as a chemoattractant of GABAergic neurons (Pozas et al., 2005). Currently, there is only one study that has investigated the function of GDNF and its corresponding receptors within the main olfactory bulb. This study demonstrates that GDNF is produced in the olfactory bulb and acts as a chemoattractant factor for rostral migratory stream (RMS) derived neuronal precursors (Paratcha et al., 2006). It has been shown that GDNF is also distributed along the RMS, the path by which new olfactory interneurons travel to the olfactory bulb, in a pattern analogous to the expression of its GPI-anchored receptor GFRα1 (Paratcha et al., 2006). Although this work has provided valuable insights into the possible role that GDNF and GFRα1 plays in nervous system neurogenesis and olfactory bulb plasticity, it did not address the in vivo functions of this signaling system.

Thus, it would be important to establish the spatio-temporal expression patterns of both GDNF and GFRα1 throughout the main olfactory system and investigate the physiological roles of GDNF- GFRα1 signaling during development by examining their influence in overall organization. The expression patterns of GDNF and GFRα1 are characterized in Paper IV and the role of GFRα1 is identified in system assembly and function. Further analyses of the mechanisms that are mediated by GFRα1 are addressed in Paper V, which evaluates how GFRα1 influences the development of olfactory bulb interneurons.

2 AIMS

The principal objective of this thesis was to identify and examine mechanisms that control fundamental components of main olfactory system assembly and function.

Specifically:

1. Identify factors that regulate OR expression and how they play a role in odorant receptor choice.
2. Evaluate if the timing of when an odorant receptor is expressed influences olfactory bulb circuitry.
3. Determine how the intrabulbar map forms, is maintained and the factors involved in achieving its specificity.
4. Characterize the expression patterns of GDNF and GFRα1 within the olfactory system and investigate if they are important for normal system development and function.
5. Elucidate the role of GFRα1 in the development of olfactory bulb GABAergic interneurons.

3 METHODS

Comprehensive descriptions of all materials and methods used in this thesis are described in the Papers I-V. Please refer to the corresponding methods section of each paper for a detailed account. The following comments characterize the methods that were the most fundamental to this thesis and my doctoral education.

3.1 Tetracycline-Controlled Transcriptional Activation

Throughout this thesis, many mouse lines were employed, the majority of which were olfactory promoter driven tetracycline transactivator (TTA) lines crossed into TetO-OR transgenic mice. Tetracycline controlled transcriptional activation is a method of inducible gene expression where transcription is reversibly turned on or off in the presence of the antibiotic tetracycline or doxycycline. In nature, the P_{tet} promoter expresses TetR, the repressor, and TetA, the protein that pumps tetracycline antibiotic out of the cell. The difference between Tet-On and Tet-Off is not whether the transactivator turns a gene on or off, both proteins activate expression. The difference relates to their respective response to doxycycline. Tet-Off activates expression in the absence of Dox, whereas Tet-On activates in the presence of Dox (Allen N. et al., 2000).

The two most commonly used inducible expression systems for research of eukaryote cell biology are named Tet-Off and Tet-On. The Tet-Off system is for controlling expression of genes of interest in mammalian cells (Bujard et al., 1992). The Tet-Off system makes use of the TTA protein, which is created by fusing one protein, tetracycline repressor (TetR) found in Escherichia coli bacteria. The resulting TTA protein is able to bind to DNA at specific TetO operator sequences. In most Tet-Off systems, several repeats of such TetO sequences are placed upstream of a CMV promoter (Urlinger S et al., 2000). While in a Tet-Off system, TTA is capable of binding the operator only if not bound to tetracycline or one of its derivatives, for example doxycycline. In a Tet-On system, the TTA protein is capable of binding the operator only if bound by a tetracycline. Thus, doxycycline initiates the transcription once introduced. The Tet-On system is sometimes preferred over Tet-Off for its quick responsiveness. Tet-Off systems are used in generating transgenic mice, which conditionally express gene of interest (Zhou X et al., 2006).

The Tet system has advantages over Cre, FRT, and ER (estrogen receptor) conditional gene expression systems. In the Cre and FRT systems, activation or knockout of the gene is irreversible once recombination is accomplished, whereas, in Tet and ER systems, it is reversible (Zhou X et al., 2006). The Tet system has very tight control on expression, whereas ER system exhibits less control. However, the Tet system depends on transcription and subsequent translation of a target

gene and is therefore slower than the ER system, which stabilizes the already-expressed target protein upon hormone administration.

3.2 Iontophoretic Fluorescent Tracer Injections

This technique requires an iontophoresis unit. Current is applied through the electrode that contains the tracer solution at concentrations of about 1 - 5%. Tracer molecules carry an electrical charge and are thus driven by force of the electrical field. With appropriate electrode tip diameters, this technique is suited for both intracellular and extracellular applications. The major advantage of iontophoretic injections is the application of the tracer alone, without additional solvent. In addition, the application site may be precisely located prior to iontophoresis by conventional electrophysiological techniques with the same electrode.

We utilized the rI7→M71 line of mice in which a single odorant receptor and the corresponding glomeruli are labeled with green fluorescent protein (GFP) for targeted tracer injections. These mice enabled us to target our tracer injections to a defined glomerulus and measure the extent to which the projection site focused on the isofunctional glomerulus on the opposite side (Bozza et al., 2002) **(Figure 3)**.

For *in vivo* injections, mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride and maintained with 100% oxygen supplemented with a 1.0–3.0% isofluorane gas inhalant. Animals were then placed in a stereotaxic apparatus and all surgical procedures were performed under a Leica MZFL3 microscope. The scalp was retracted over the dorsal surface of the olfactory bulbs, and the bone was removed to allow access for tracer injections (Figure 1). A single microinjection of 10% dextran-tetramethylrhodamine (TMR) of 3000 molecular weight (Invitrogen, Carlsbad, CA) was iontophoresed (+10 μA, duration of 200 ms, interval of 2500 ms, 120 pulses, for 5 min) into each olfactory bulb through a quartz micropipette (5–10 μm tip diameter) **(Figure 3)**. Eight to 12 hours after injection, mice were killed with an overdose injection of 200 mg/kg ketamine hydrochloride and 20 mg/kg xylazine hydrochloride intraperitoneally and transcardially perfused with 1× PBS, followed by 4% paraformaldehyde (PFA). Olfactory bulbs were removed and postfixed in 4% PFA for 3 d, cryoprotected in 30% sucrose for 1 d, horizontally sectioned on a freezing microtome at 60 μm, placed onto Superfrost slides (Fisher Scientific, Houston, TX), and mounted with Vectashield containing 4′,6′-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

For *in vitro* injections, mice were anesthetized with isofluorane, decapitated, and rapidly dissected in low Ca^{+2} artificial CSF (ACSF) (McQuiston and Katz, 2001) bubbled with 95% O_2/5% CO_2. Medial and lateral sides were then exposed and injected using the same parameters

described above. After injection, the tissue was bubbled in oxygenated ACSF for 4–6 h, then fixed, and processed as described above.

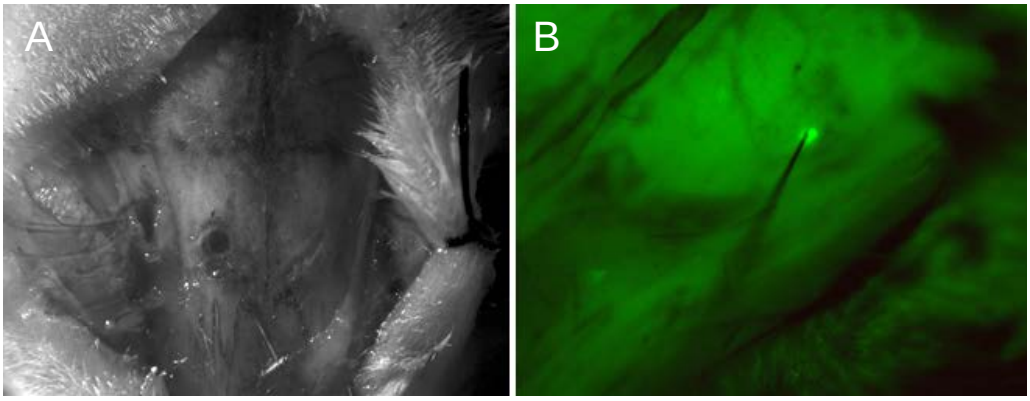

Figure 3: Targeted fluorescent tracer injections. Panel A depicts the area where the scalp was retracted over the dorsal surface of OBs, and bone was removed . Panel B illustrates in vivo tracer injection in to a defined glomerulus labeled with green fluorescent protein (GFP).

3.3 Naris Occlusion

Odor-induced activity has been shown to be important for normal olfactory system development. This has been demonstrated through naris occlusion in which odor-induced activity is blocked on one side of the olfactory system. This odor deprivation or loss of activity results in a cascade of changes in the physiology, biochemistry, and survival of neurons in the ipsilateral OE and OB without affecting the contralateral side (Brunjes, 1994). Recent work has verified that this type of unilateral naris closure disrupts the glomerular map formation that normally emerges during early rodent postnatal development (Zou et al., 2004). In Paper III, we adapted this technique to create discrete periods of anosmia. We could therefore evaluate olfactory deprivation or the loss of activity within the intrabulbar map. Naris occlusion experiments allow the capability to examine axonal targeting, development, and plasticity of the intrabulbar circuitry using permanent olfactory deprivation techniques in transgenic mice.

Wild-type mice at 4 and 10 weeks of age were anesthetized via intraperitoneal injection of a mixture of 80 mg/kg ketamine hydrochloride and 8 mg/kg xylazine hydrochloride and maintained with 100% oxygen supplemented with 1.0–3.0% isofluorane gas inhalant. Mice were then positioned in a small stereotaxic apparatus on their back without ear bars and placed under a low-magnification 10× Leica (Nussloch, Germany) microscope. Visualizing the naris region, a small 3% agar filled polyethylene tube (Becton Dickinson, Mountain View, CA) ~3–5 mm in length, coated with sterile ocular lubricant (Puralube Ointment; Fougera, Melville, NY), was inserted into the right nostril and sealed in place with quick dry adhesive (Instant Adhesive Loctite 404; Small Parts, Logansport, IN) to block odorants from entering the right naris **(Figure 4).** Animals were maintained in this unilateral anosmic state for either 3 weeks, when

occluded at 4 weeks of age, or for 4 weeks, when occluded at 10 weeks of age. Each block was checked biweekly and new adhesive was reapplied if necessary. Immediately before performing tracer injections, blocks were checked one last time by placing a drop of water over the occluded naris. The block was determined to have persisted when there was an absence of bubbles. Only mice with complete blocks were used for tracer injections.

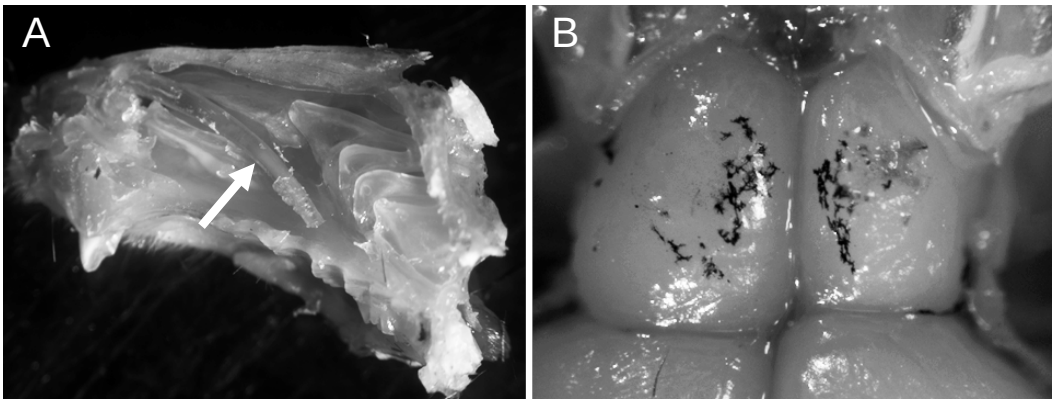

Figure 4: Representative image of permanent naris occlusion in the OE and OB from 4 to 7 weeks of age. Panel A depicts the permanent block within the olfactory sensory epithelium. Arrow highlights that the tubing does not affect the OE but serves as a method to block odorant-induced activity. Panel B illustrates the effect of this 3 week period of odor deprivation with the OB as only the right OB is dramatically reduced.

3.4 Intrinsic Signal Imaging

Intrinsic signal imaging (ISI) is an *in vivo* technique, adapted from studies in visual system, to examine functional responses of activity-dependent changes in blood flow and oxygenation state in the OB as a result of localized neural activity (Rubin and Katz, 1999; Belluscio and Katz, 2001). This imaging technique is designed to detect functional changes in the olfactory system. Intrinsic signal imaging detects changes in UV light reflection associated with neural activity. Mice were prepared and imaged using an Imager 3001 system (Optical Imaging). Approximately 5-week-old mice (at least six of each genotype) were used for measuring responses to ligands that activated transgenic ORs. To denote regions of highest activity, images were imported into IPLab (Scanalytics). Pixels >2 SDs above the mean pixel value for that image were color coded and superimposed onto the blood vessel image. A mask was drawn to omit pixels located in peripheral bone and medial vessel regions not located over the dorsal bulbs. Animals that were at least 20 weeks old (7 or more mice for each odorant) were used to determine the distorted olfactory map to aliphatic ligands. All odorants used were of the highest purity available from Sigma and were dissolved at 1% in mineral oil.

3.5 Behavioral Tests of Olfactory Function

Buried Food Test: The test was performed as previously described (Yang and Crawley, 2009). Adult male wild type and Gfra1 heterozygous littermate mice (7 to 10 weeks of age) were tested (n=20 for each genotype). Three consecutive days before the test, mice were given a uniform size palatable food stimulus (Teddy Grahams, Nabisco, Hanover, New Jersey) as odor familiarization. Mice were food deprived 16 hours prior to testing. Each mouse was then placed into the test cage (46 x 23.5 x 20cm) containing 3cm deep clean bedding, for a 5min acclimation period. Mice were then transferred to an empty clean cage. The food stimulus was placed in a random corner of the test cage 1cm deep under the bedding and the bedding surface was smoothed out. Mice were placed back in the test cage and the latency was recorded from 2m away (maximum 15mins) until the mouse found the buried food stimulus. Latency to find the buried food is determined as the time in seconds when the mouse has uncovered the buried food and begins to eat it holding the food with its forepaws. Values are shown as mean latency ± SEM.

Innate Olfactory Preference and Sensitivity Tests: Tests were performed as previously published (Kobayakawa et al., 2007; Witt et al., 2009). Adult male wild type and Gfra1 heterozygous littermate mice (7 to 10 weeks of age) were tested only once (n=14 for each genotype and odorant). Mice were individually habituated to the experimental environment for 30mins in a clean empty cage, identical to the test cage, and then transferred to a new cage. Habituation was repeated 3 additional times. Mice were then transferred to the test cage (20 x 15 x 13cm) and a filter paper (2cm x 2cm) scented with either water or a test odorant was introduced. Mouse behavior was recorded with a digital video camera for analysis. Investigation times for the filter paper were measured in seconds over a 3min test period. Investigation was defined by nasal contact with the filter paper within a 1 mm distance. Odorant concentrations used were: distilled water (20μL), peanut butter (10%w/v, 40μL), urine of female mice (20μL), vanillin (64μM, 20μL), eugenol (128μM, 20μL), 2-MB (8.7M, 20μL), and TMT (7.65M, 20μL). All odorants (Sigma) were dissolved in distilled water except peanut butter, which was dissolved in mineral oil. Values are shown as mean investigation time ± SEM.

4 RESULTS AND DISCUSSION

4.1 Paper I: Multiple layers of odorant receptor gene regulation

Mammalian odorant receptors (ORs) are crucial for establishing the functional organization of the olfactory system, but the mechanisms controlling their expression remain poorly understood. In Paper I, we examined the instructive role of the OR-coding sequence in OR gene expression and odorant receptor choice.

To achieve this, we utilized several transgenic strategies to broadly express single ORs in many OSNs and evaluated OR expression. First, we placed the OR-coding sequence, from single defined ORs, directly under the control of two general olfactory promoters, olfactory marker protein (OMP) which is a marker for mature OSNs (Rogers et al., 1987) and the heterotrimeric G protein subunit Gγ8 which is expressed in immature OSNs (Ryba and Tirindelli, 1995). We found that neither promoter was sufficient to drive the expression of ORs, as they never resulted in the expression of transgenic ORs in OSNs.

As a result, we designed an alternative strategy and placed the OR-coding sequence, from single defined ORs, downstream of a synthetic TetO promoter to generate TetO-OR-transgenic mice. These mice were labeled by using an internal ribosomal entry site (IRES) to drive the coexpression of GFP or lacZ. We then crossed these labeled TetO-OR- transgenes into animals expressing an OMP-driven tetracycline transactivator (TTA) (Yu et al., 2004) to induce and track OR expression in mature OSNs of the olfactory epithelium. We found that this approach was sufficient to induce and label transgene expression in mature OSNs, indicating that separation of the olfactory promoter from the OR-coding sequence can moderately overcome the suppression of OR transgene expression. However, transgene expression in the OSNs of these mice was very low and extremely variable. Thus, TetO-OR-transgenes were selectively silenced in the majority of OSNs.

To address the issue of transgenic OR silencing, we performed immunohistochemistry for MOR28, an endogenous OR expressed in many OSNs (Tsuboi et al., 1999; Serizawa et al., 2000; Barnea et al., 2004) to evaluate if endogenous ORs and transgenic ORs are coexpressed in the same OSN. We found that these transgenic ORs and the endogenous OR were never coexpressed. Either the transgenic OR was expressed and the endogenous OR was suppressed or the endogenous OR was expressed and the transgenic OR was suppressed, indicating two modes of OR suppression. Given that these mice contain only the OR coding sequence linked to a synthetic expression cassette, the two modes of OR suppression must be a result of elements in the OR-coding sequences that

are both capable of being silenced and have the ability to silence endogenous OR expression.

To determine why the majority of OSNs exhibited endogenous OR expression and transgenic OR suppression, we investigated if it was due to the timing of when the endogenous OR was expressed. Could transgenic ORs overrule endogenous OR expression if they were expressed before endogenous ORs in immature OSNs? To assess this, we generated mice in which the Gγ8 promoter drives expression of TTA (Gγ8-TTA). These G8-TTA mice were crossed to TetO-OR transgenic mice which resulted in the expression of TetO-OR transgenes in immature OSNs. Unlike the late-expressing driver (OMP-TTA), this early-expressing driver revealed broad transgene OR expression. Our results clearly demonstrated that transgenic ORs can predominate over endogenous OR expression and escape OR suppression. Furthermore, electrophysiological recordings from OSNs expressing these transgenic ORs revealed normal responses to their respective odorant ligands, thus demonstrating that these ORs are functional.

Given that our results indicate that the first OR to be expressed establishes cell identity and inhibits the expression of other ORs, we next evaluated what would happen if mice contained both early-expressing (Gγ8-TTA) and late-expressing (OMP-TTA) OR transgenes. These mice revealed broad and continuous transgenic OR expression in almost all OSNs (approximately 90%) and complete endogenous OR suppression. These results indicate that the silencing of other ORs is not dependent on whether the OR is transgenic or endogenous. The silencing of other ORs is dependent on the first OR that is expressed in an OSN.

Finally, to shed light on the complexity of OR selection, OR expression, and OR silencing which, when combined, instructs odorant receptor choice, we evaluated if an OSN could functionally express two ORs at the same time. To accomplish this, we generated mice which carry a single construct expressing two ORs from the same regulatory sequence to create TetO-x2OR transgenic mice. These mice were crossed to either the mature OSN driver OMP-TTA or the immature OSN driver Gγ8-TTA and resulted in transgene expression in mature OSNs or immature OSNs respectively, which contained these two ORs within the same OSN cell. Single cell electrophysiological recordings were employed using odorant ligands that activate these two ORs and showed that these single OSNs with two ORs respond to both odors. Thus, OSNs are capable of functionally expressing two different ORs at the same time.

Overall, we determined that OSNs are capable of supporting the expression of more than one functioning OR. However, several levels of control ensure that each OSN normally expresses only a single odorant receptor, which is the defining feature of odorant receptor choice. These levels of control are OR expression and OR silencing. Together, our results

have revealed that there multiple layers of regulation in the hierarchy that controls OR expression and odorant receptor choice, which is fundamental to olfactory system assembly and function. While many aspects of this hierarchy remain unclear, we have identified two important regulatory mechanisms: 1) the OR-coding sequence is paramount in regulating OR gene expression and 2) the timing of when an odorant receptor is expressed in an OSN controls the silencing of other ORs.

4.2 Paper II: Timing of OR expression shapes the olfactory circuitry

Odorant receptors have been shown to play instructive roles in guiding OSN axons to the bulb (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998) and in establishing the glomerular map (Malnic et al., 1999; Rubin and Katz, 1999; Belluscio and Katz, 2001), thereby shaping the internal circuitry of the olfactory bulb (OB). Mechanistically, how the precise regulation of OR expression influences these functions, the foundation of olfactory system assembly and operation, is unclear. Thus, in Paper II, we addressed if the timing of when an OR is expressed (early versus late) influences the overall architecture and function of the OB.

To accomplish this, we employed mice (described in Paper I) in which a single defined OR was expressed in OSNs using an OSN-directed tetracycline-dependent transactivator (TTA) to drive TetO-controlled OR transgenes (Nguyen et al., 2007). We were able to track the OSNs expressing transgenic ORs in these mice as they were labeled with an internal ribosomal entry site (IRES) to drive the coexpression of GFP. Given that the promoter that drives TTA defines the timing of OR expression, we used the OMP promoter to drive TTA in mature OSNs during the late stages of OSN development and the Gγ8 promoter (Ryba and Tirindelli, 1995) to drive TTA in immature OSNs early in development (Tirindelli and Ryba, 1996). We then crossed the late-expressing (OMP-TTA) and the early-expressing (Gγ8-TTA) mice into TetO-OR transgenic mice and evaluated the functional consequences in the bulb.

We examined the late-expressing OR mouse lines first. OMP-TTA mice crossed to TetO-GFP mice were used as a control to visualize and compare normal GFP expression, without transgenic ORs, to those with OR transgenes. We found that, when OMP drives the expression of transgenic ORs, OSN axons target the bulb normally and glomerular morphology was indistinguishable from control mice. This was apparent in 36 different transgenic OR mouse lines and was further supported by an independent study using a similar transgenic approach (Fleischmann et al., 2008). Thus, we concluded that OR expression late in OSN development has very little or no effect on the organization of the bulb. These results suggested to us that either the expression of ORs early in OSN development are responsible for the precise targeting of OSN axons to the OB and the formation of the glomerular map, thereby wiring the bulb, or the

expression of ORs and perhaps the odorant receptor itself is not as important as generally thought.

Accordingly, we evaluated the early-expressing (Gγ8-TTA) OR transgenic mice. These mice exhibited disorganized and dramatically widespread OSN axonal projections throughout the OB. Additionally, axons from these mice innervated abnormally large glomeruli. This was not the case in control mice, which consisted of Gγ8 driving the expression of TetO-GFP alone without the transgenic OR. Control mice displayed normal targeting of axons to the OB which were restricted to the olfactory nerve layer (ONL) and only very few axons entered the glomeruli layer, as expected of immature OSN axons projecting to the OB. Interestingly, expression of Gγ8 driven transgenic ORs continued even after Gγ8 was downregulated. Together, these results showed that the early expression of ORs grossly affects the projection and targeting of OSN axons to the OB in addition to the shape and size of glomeruli. Thus, early expression of ORs is pivotal in OSN axon guidance and glomerular map formation.

Next, we investigated the consequences in the OB of OR transgene expression in both early and late stages of OSN development. We performed this experiment to determine if the innervation of glomeruli that we observed in the Gγ8-OR transgenic mice was a temporary effect. These Gγ8+OMP-transgenic OR mice displayed axons that projected to stable but abnormally large glomeruli, comparable to those in the Gγ8-transgenic OR mice. Moreover, these axons innervated the enlarged glomeruli in both young and old mice. The size of GFP labeled glomeruli was quantified and revealed a two-fold increase in the average diameter in both Gγ8-transgenic OR mice and Gγ8+OMP-transgenic OR mice and a three-fold increase in the average glomerular volume of OSNs expressing only Gγ8-transgenic ORs compared to controls. The size of non-GFP labeled glomeruli, meaning glomeruli comprised of OSN axons from endogenous ORs, were also quantified and resulted in smaller glomeruli.

To assess the functional response properties of these distorted glomeruli, we employed intrinsic signal imaging (ISI) (see the Methods section) in Gγ8-OR transgenic mice. ISI in control animals revealed small discrete regions of glomerular activity elicited by specific odorant ligands. In contrast, Gγ8-OR transgenic mice exhibited robust and widespread ISI responses to the same odorant ligands. These responses corresponded with GFP labeled transgene expression. Most importantly, although the response signals were broad in Gγ8-OR transgenic mice, they were strongly selective for their appropriate odorant ligand.

Given that the non-GFP labeled glomeruli showed a reduction in glomerular size, we evaluated if the targeting of their axons was also affected in these mice. To achieve this, we used two lines of mice in which non-transgenic ORs were labeled by coexpression of LacZ (Mombaerts et al., 1996; Bozza et al., 2002). We found that, indeed, there was dramatic

mistargeting of axons from OSNs expressing endogenous ORs. Notably, the same degree of mistargeting was observed in older animals, after Gγ8 is downregulated, indicating that this mistargeting is constant and not subject to reorganization. We further evaluated the functional consequences of these glomeruli with ISI and analyzed their response to aliphatic odorants, as these odors have been shown to activate multiple glomeruli in the dorsal OB (Belluscio and Katz, 2001), coinciding with the location of most non-transgenic expressing ORs of older mice. We found the activation patterns of Gγ8-OR transgenic mice were remarkably broad and grossly distorted compared to controls, such that individual glomeruli were no longer distinguishable and the ISI signal was shifted in the rostral plane for all tested odors. These results demonstrated that the early expression of Gγ8-transgenic ORs disrupts normal targeting of OSN axons, leading to permanent changes in the glomerular map and the functional responses it generates.

Together, our results reveal that the timing of OR expression, specifically early timing of OR expression, has profound effects on the wiring and function of the olfactory system. While odorant receptors, themselves, are instructive in OSN axon guidance and the establishment of the glomerular map, thereby shaping the internal circuitry of the bulb, we have further identified the initiation of OR expression early in OSN development as a prominent regulatory mechanism that wires the olfactory circuitry and allows the system to function.

4.3 Paper III: Activity modulates intrabulbar map plasticity

Olfactory maps are fundamental to the assembly and operation of the main olfactory system, but the mechanisms governing their organization and maintenance remain unclear. In Paper III, we sought to determine how the intrabulbar map forms and what factors are involved in achieving its specificity. To accomplish this, we administered fluorescent tracer injections (see the Methods section) into the glomerular layer on one side of the bulb and examined the resulting projection on the opposite side. In adult mice, it has been previously established that the size of the projection tuft is directly proportional to the size of the injected region (Schoenfeld et al., 1985; Belluscio et al., 2002; Lodovichi et al., 2003). We utilized this 1:1 ratio as a measure of intrabulbar projection (IBP) and intrabulbar map (IBM) maturity.

To determine when IBPs are first established, we performed a series of fluorescent tracer injections from birth through adulthood. While we were unable to detect IBPs at birth, we were able to clearly label them by 1 week of age. However, these projections appeared very diffuse and targeted a much broader area on the opposite side of the bulb than in adults, indicating a less specific immature map. We quantified this specificity by calculating the ratio between the diameter of the projection site and the diameter of the injection site. This revealed an intrabulbar projection

developmental profile of 5:1 at 1 week of age, 3:1 at 2 weeks of age, 2:1 at 5 weeks of age, and 1:1 at 7, 10 and 14 weeks of age. We demonstrated that IBPs undergo dramatic developmental refinement from 1 week to 7 weeks of age, which is maintained in adulthood. This remarkable refinement clearly demonstrates that there is a developmental evolution to the intrabulbar projections that make up the intrabulbar map, from an immature to a mature state.

We investigated if there was any accuracy in the targeting of IBPs. We utilized the rI7→M71 mouse line to target our tracer injections to a defined glomerulus and measured the extent to which the projection site focused under the isofunctional glomerulus on the opposite side of the same OB (Bozza et al., 2002) (see the Methods section). We found the center of the projection and calculated the angle at which the isofunctional glomerulus was offset from the perpendicular of this center. This data revealed consistently precise IBP accuracy at all stages of development, with an angle offset of $\leq 6^{o}$ at 2 weeks and older. Even though the immature and maturing IBPs broadly target the opposite bulbar hemisphere, they are still accurately centered beneath the target glomerulus. We conclude that the intrabulbar map exhibits tremendous accuracy in linking isofunctional glomeruli regardless of map maturity. This suggests that both glomeruli and IBPs are accurately drawn to common regions of the bulb to establish specific communications between glomerular pairs.

Next, we evaluated if odorant-induced activity was involved in the formation of the intrabulbar map. We used an anosmic line of mice in which the α2-subunit of the olfactory cyclic-nucleotide-gated channel (OCNC-A2) had been disrupted, which allowed us to determine if IBPs are present and if the IBM forms in the absence of activity. Our results show that, in the absence of activity, IBPs are present and target the opposite side of the bulb**.** However, these projections are diffuse and broad, much like a 1 week old mouse, and possess a 4:1 ratio at 7 weeks of age, indicating a lack of refinement and immaturity in the intrabulbar map in the absence of activity. Since the IBPs are present and target the opposite side of the OB in the absence of odorant-induced activity, we concluded that the formation of the intrabulbar map is activity-independent. However, these IBPs never refine, suggesting that odorant-induced activity is necessary for IBM maturation.

Thus, we assessed if odorant-induced activity affects the maturation of the intrabulbar map. To achieve this, we performed naris occlusion experiments (see the Methods section) to create discrete periods of anosmia. We performed naris occlusion on 4 week old mice, at which time the intrabulbar map is only partially refined. Animals were then raised for 3 weeks with the block in place and injections were performed at 7 weeks of age, the time when a control map would have reached maturity and exhibited a 1:1 ratio. Our data revealed that the IBM did not continue to refine following occlusion. It actually regressed to an immature state,

displaying a broad 5:1 ratio. This suggests that when activity is lost, re-growth of axons occurs with increased branching. Overall, we concluded that activity is required for IBPs to refine. Thus, the maturation of the intrabulbar map, from immature to mature, is activity-dependent.

To determine whether activity is required to maintain map plasticity, we performed naris occlusion at 10 weeks of age and injections at 14 weeks of age. Interestingly, these projections also broadened to produce a 5:1 ratio, which was indistinguishable from the outcome of naris occlusion from 4 to 7 weeks. Our results revealed that activity is required for not only for maturation but also the maintenance of the IBM. Most notably, the activity-dependent plasticity of IBPs is not confined to a critical period but rather continues into maturity. Unlike other sensory systems which are defined by a critical period, the IBM retains the capacity to reorganize even after it has matured, demonstrating the importance of plasticity in a system that must maintain its ability to adapt to any odorant environment.

Given that the intrabulbar map regresses to an immature state when activity is lost, we then assessed if increased activity could accelerate map refinement. To achieve this, we exposed rI7→M71 mice to octanal, an odor known to activate only GFP labeled glomerular pairs. Mice were presented with octanal from birth until targeted tracer injections were performed at 2, 4, and 7 weeks of age. We found significantly more refined projections at both 2 and 4 weeks of age compared to controls, indicating that the intrabulbar map matured faster than normal. Interestingly, when the injection site was shifted in these octanal exposed mice, no longer targeting the activated rI7→M71 glomerulus, the resulting projection to injection ratios matched control mice. Overall, we concluded that not only can increased activity accelerate map refinement, but it does so very specifically at the level of glomerular pairs. This indicates that the intrabulbar map has a plastic capacity to shift maturation states, from immature to mature and mature to immature, which is regulated by activity. Moreover, the activity-dependent plasticity of the intrabulbar map is incredibly precise and glomerular-specific.

Finally, we evaluated if a change in the number of external tufted cells (ETCs) could account for either the refinement or the expansion of IBPs. We employed immunohistochemical and in situ hybridization assays to label cholecystokinin (CCK), a marker of external tufted cells, and quantified the number of ETCs throughout development. We found that the average density of ETCs increased from 1 week to 7 weeks of age, coinciding with the development of the intrabulbar map. We then assayed the bulbs of both naris-occluded and OCNC1-KO mice. Interestingly, while the OCNC1-KO mice showed a significant increase in ETC density at 7 weeks compared to age-matched wild type mice, the 10 to 14 week naris occluded mice showed only minor differences. This suggests that odorant-induced activity does not alter the number of ETCs once the intrabulbar map has matured and that the broadening of IBPs in the absence of

odorant-induced activity is not due to an increase in ETC number. Indeed, it is more probable that the activity-dependent plasticity we observe in the IBM is the direct result of local subcellular reorganization that occurs at the axonal level of the remaining neurons.

Based upon the columnar structure of the bulb, it is expected that IBPs modulate the output of the glomeruli they target. Given that IBPs are both specific and reciprocal, this circuitry provides a means by which isofunctional glomeruli can influence one another's output. Furthermore, the IBM is like the glomerular map in its ability to form in the absence of odorant-induced activity, but differs in its dependence on activity for maturation and maintenance. Our findings demonstrate a clear role for odorant-induced activity in shaping the internal circuitry of the bulb. While all factors that govern intrabulbar map plasticity have yet to be defined, we have distinguished odorant-induced activity as a key regulatory mechanism in intrabulbar map plasticity.

4.4 Paper IV: GDNF signaling guides olfactory assembly

Guidance molecules and their receptors are critical for olfactory system development and function. However, many have not been well characterized and their roles in the overall organization of the olfactory system have not been established. GDNF and its receptor GFRα1 have been detected in the main olfactory system (Cao et al., 2006; Maroldt et al., 2005; Nosrat et al., 1997; Paratcha et al., 2006; Trupp et al., 1997), but the specific cellular localization of these guidance molecules and their importance in the functional organization of the olfactory system is unknown. In Paper IV, we mapped the expression patterns of GDNF and GFRα1 in the developing mouse olfactory system and investigated the physiological role of GDNF- GFRα1 signaling in the development and function of the system.

To identify the cellular sources of GDNF in the developing mouse olfactory system, we performed in situ hybridization. We detected GDNF mRNA throughout the mature and immature OSN layers of the OE and in almost every cell type of the OB. As GDNF is a known diffusible ligand, our results suggested that GDNF is made in massive abundance throughout the olfactory system to be readily available for the cells and axons expressing GFRα1, its respective signaling receptor. Thus, we focused our subsequent analysis on GFRα1.

To determine if GFRα1 is important for olfactory system development, we mapped the expression of GFRα1to specific cell types of the OE and OB and evaluated mutant mice in which GFRα1 expression was either absent or diminished. The results from this expression and phenotypic analysis, summarized below, revealed a critical role for GFRα1 in the development of several of the major cell types of the olfactory epithelium and olfactory

bulb, including OSNs, OECs, projection neurons, and inhibitory interneurons.

In the OE, GFRα1 was localized to basal precursors, immature OSNs and OECs, but was not expressed in mature OSNs. The OE of mice lacking GFRα1 was thinner, contained fewer immature and mature OSNs but more dividing precursors, and demonstrated increased cell death, suggesting deficient neurogenesis. Thus, GFRα1 may function cell-autonomously in immature OSNs to regulate their differentiation and survival. Additionally, it is likely that fewer OSNs were generated in the mutants as a result of defects in stem cell differentiation and OSN neurogenesis. This claim is supported by the dramatically increased levels of proliferation and apoptosis we observed in the mutants, which suggests an attempt to compensate for the loss of OSNs. Furthermore, GFRα1 deficiency resulted in enlarged immature OSN axon bundles and increased OECs within the OE, indicating impairment in the migration of OECs and OSN axons.

In the OB, GFRα1 was distributed in immature OSN axons and OECs of the nerve layer, in addition to mitral and tufted projection neurons, but was excluded from the majority of GABAergic interneurons. In GFRα1 knockouts, the ONL was dramatically thinner, displayed fewer axons and OECs and demonstrated increased activation of caspase-3. These results demonstrate that GDNF-GFRα1 signaling is required for the maintenance of the ONL. Furthermore, mutant OBs were smaller, presented fewer and disorganized glomeruli and a demonstrated significant reduction in mitral cells. The glomerular abnormalities in *Gfra1* mutants were likely a result of deficits in the two major components of OB glomeruli, the OSN axons and mitral cell dendrites. Since GFRα1 has previously been shown to exhibit synaptogenic activity in developing neurons of the hippocampus and cerebral cortex (Ledda et al., 2007), then GFRα1-mediated GDNF signaling may also be essential for initial glomerular synapse formation and the stabilization process that occurs as immature OSNs transition into mature OSNs (Carson et al, 2005).

Moreover, tyrosine hydroxylase, calbindin, and calretinin expressing olfactory bulb interneurons were also dramatically reduced in newborn mice lacking *Gfra1* and in adult heterozygotes. Interestingly, the losses of interneurons were remarkably proportional in newborn knockouts and in adult heterozygotes, suggesting that OB interneurons are critically dependent on GFRα1, not only during development but also during postnatal stages. This was surprising, considering that GFRα1 has not been shown to display dose-dependent effects in vivo and no developmental abnormalities have been described in heterozygous GFRα1 mutants (Cacalano et al., 1998; Enomoto et al., 1998).

As a result, we performed behavioral tests in adult *Gfra1* heterozygotes to assess olfactory performance (see the Methods section). These mice

displayed diminished responses in behavioral tests of olfactory function, revealing decreased sensitivity in both the buried food assay and in the innate olfactory preference test. Taken together, these results highlight the exquisite sensitivity of the olfactory system to GFRα1 dosage and the critical role of this receptor in not only its development, but also in its functional output.

Finally, *Gdnf* knock-out mice displayed equivalent phenotypes in both the OE and the OB, including the loss of immature and mature OSNs, enlarged axon bundles, significantly reduced ONL, irregularly shaped glomeruli, and remarkably similar reductions in the number of TH-, CB-, and CR-expressing cells. These results indicated that GDNF is the most probable or principle ligand driving the GFRα1-mediated effects presented in this study.

We conclude that the guidance molecule GDNF and its receptor GFRα1 are essential for the proper development and function of the main olfactory system. GFRα1 contributes in a dose-dependent manner to the development and allocation of all major classes of neurons and glial cells. While the mechanisms by which GFRα1 orchestrates olfactory system development have yet to be determined, we can classify GFRα1 as a vital regulatory element in such mechanisms that instruct olfactory system assembly and function.

4.5 Paper V: GFRα1 directs olfactory interneuron development

GFRα1 has recently been reported as being critical to the development and function of the main olfactory system, by contributing to the allocation of all major classes of neurons and glial cells, including all classes of olfactory bulb OB GABAergic interneurons (Marks et al., n.d.). The means by which GFRα1 contributes to so many diverse classes of neurons and glial cells is not known. Thus, in Paper V, we investigated the specific role of GFRα1 in the development and allocation of one of these classes of neurons, the olfactory bulb interneurons.

GFRα1has been shown to be largely excluded from both GAD65- and GAD67-expressing GABAergic interneurons within the OB (Marks et al., n.d.). However, mice lacking GFRα1at birth exhibited remarkable reductions in all major interneuron subpopulations, including TH-, CB-, and CR-expressing cells (Marks et al., n.d.), indicating a role for GFRα1 in embryonic development for the generation and allocation of these cells. It is possible that these interneuron losses may be a downstream consequence of defects in OSNs and projection neurons, as other studies have demonstrated (de Carlos et al., 1995; Gong and Shipley, 1995; Bulfone et al., 1998; Menini et al., 2010). Thus, the loss of GFRα1 could affect olfactory interneuron development non-cell autonomously. Alternatively, and more likely, GFRα1 may be important for the development of OB interneuron precursors. Given that GDNF has been

shown to function as a chemoattractant factor for GFRα1-expressing cells migrating into the OB (Paratcha et al., 2003; Paratcha et al., 2006), GFRα1 could thus function cell-autonomously in OB interneuron precursors to regulate their differentiation, migration or survival prior to their final allocation in the OB.

To address this, we first identified if GFRα1 is expressed at the sites of origin of olfactory bulb interneurons. We found that GFRα1 is highly expressed in the GABAergic cells of one of the main sites of origin of OB interneurons, the embryonic septum. We observed strong GFRα1 coexpression with GAD67 throughout embryonic development in the septum. We further characterized these GFRα1 cells with different subpopulations of GABAergic interneurons in the septum and found they also colocalized with TH-, CB-, and CR-expressing cells. Our results show that while GFRα1is not localized in mature GABAergic neurons within the OB, it is expressed in GABAergic cells of a known prominent contributor of OB interneurons, the embryonic septum (Waclaw et al., 2006; Waclaw et al., 2009; Stenman et al., 2003). Thus, GFRα1 may be transiently expressed in OB interneuron precursors of the embryonic septum to regulate their development and final allocation within the OB.

To determine if GFRα1-expressing precursors of the embryonic septum give rise to GABAergic interneurons, we employed genetic fate-mapping studies. For these studies, we generated a knock-in line of mice expressing tamoxifen (Tmx)-inducible Cre-ERT2 fusion protein under the control of the *Gfra1* locus. These *Gfra1*$^{Cre\text{-}ERT2}$ mice were then crossed to a reporter line R26R^{tm} which expressed the dTomato reporter. These mice allowed us to assess the fate of cells that expressed GFRα1at the time of Tmx injection as revealed by persistent expression of the reporter gene following CRE-mediated recombination. We then performed Tmx injections at embryonic day 9.5 and 10.5 (E9.5-E10.5) and detected many dTomato (dTom) labeled cells in the embryonic septum. These cells overlapped with GFRα1 immunostaining at E14.5. These cells were further characterized with the GABAergic interneuron markers TH, CB, and CR, all of which overlapped in the E14.5 septum. Thus, we developed a method that labels GABAergic cells that are derived from GFRα1 precursors.

To assess if GFRα1 precursors migrate to the OB, we injected these mice at the same time embryonic time points and examined the OB at birth. Indeed, we found a significant number of dTom-expressing cells within the OB. Most importantly, these cells overlapped with the GABAergic interneuron marker GAD67, as visualized by immunohistochemical detection of GFP in GAD67-GFP mice. Moreover, several dTom-positive cells in the OB at birth overlapped with TH, CB, and CR. Overall, these results demonstrated that GFRα1-expressing precursors of the embryo can give rise to all major classes of GABAergic interneurons in the newborn OB. Since mature GABAergic interneurons of the OB do not express GFRα1 but are generated from GFRα1-expressing cells, then,

much like Gγ8, GFRα1 must be downregulated at some point in their developmental history. We presume that transient expression of GFRα1 contributes to some aspect of the development of these cells, such as proliferation, differentiation, or migration. GFRα1 may serve as a biological switch in the developmental programming of these cells that is switched off once they have reached their final destination in the OB.

Next, we examined the effects of *Gfra1* ablation in GABAergic cells on the TH-, CB-, and CR-expressing interneuron subpopulations within the OB. To achieve this, we employed mice expressing CRE recombinase from the GAD67 locus (Tolu et al., 2010) crossed into conditional *Gfra1* mice (Uesaka et al., 2007). Conditional deletion of *Gfra1* in these GABAergic cells resulted in dramatic losses of TH-, CB- and CR-expressing OB interneurons. Interestingly, the losses we observed in these mice phenocopied the deficits observed in OB GABAergic interneurons of the global *Gfra1* knockout (Marks et al., n.d.). Together with the results from cell-fate mapping studies, we concluded that GFRα1 contributes to the development of OB interneurons by regulating the development of their precursors in a cell-autonomous fashion.

Finally, we employed BrdU birthdating analysis in *Gfra1* knockouts to begin to discern how GFRα1 directs the development of OB GABAergic interneurons. We performed pulse injections of BrdU at E14.5 and quantified BrdU cell density in both the embryonic septum and in the OB. *Gfra1* knockouts displayed fewer BrdU labeled cells in both the embryonic septum and OB compared to controls, suggesting a reduced rate of proliferation in the absence of GFRα1. BrdU pulse injections at E14.5 which were then chased until birth revealed a much greater reduction in BrdU labeled cells in the OB of mutant mice. These results indicated that the migration of precursor cells to the OB is altered in the absence of GFRα1. Thus, GFRα1 influences cell proliferation in the embryonic septum and cell migration to the newborn OB.

Overall, our results demonstrate a cell autonomous role of GFRα1 in the development of olfactory bulb GABAergic interneurons. GFRα1 functions transiently and cell-autonomously in subpopulations of OB interneuron precursors of the embryonic septum to regulate their generation and final allocation in the OB. While neither the precise developmental processes that are regulated by GFRα1 nor the reason for its seemingly timed downregulation have been determined, we have distinguished GFRα1 as vital instructor in directing olfactory GABAergic interneurons to the bulb, which is fundamental in overall olfactory system assembly and function.

5 CONCLUSIONS

The main olfactory system is highly organized, precisely connected, and tightly regulated. This thesis has contributed to our understanding of key mechanisms that regulate fundamental components of olfactory system assembly and function. Thus, from the early stages when an odorant receptor is first expressed, to the guidance of axons which precisely target and define the glomerular map, to the underlying second order intrabulbar map, and finally the guidance molecules that assemble all major classes of neurons and glial cells. This thesis has contributed to our understanding of many factors that pattern the olfactory system and the mechanisms that control its functional organization by offering the following new insights:

- Discovery of multiple layers of odorant receptor gene regulation
- Identification of OR-coding sequences as regulators of OR expression
- Explanation of the first OR expressed in an OSN controls silencing of other ORs
- Demonstration that early OR expression disrupts axon targeting and alters the glomerular map and thus has profound effects on the wiring and function of the OB
- Evaluation of the role of activity in intrabulbar map formation, maturation and maintenance
- Distinguished activity-dependent plasticity in the intrabulbar map
- Characterization of GDNF and its receptor GFRα1 as essential for proper development and function of the main olfactory system
- Contribution of GFRα1 in a dose-dependent manner to the development and allocation of all major classes of olfactory neurons and glial cells
- Explanation of a cell autonomous role of GFRα1 in the development of olfactory bulb GABAergic interneurons
- Verified GFRα1 as vital instructor in directing olfactory GABAergic interneurons to the bulb

Overall, this thesis contributes to the understanding of how the multifaceted olfactory sensory system is accurately assembled to precisely function. Further investigation is essential in elucidating its contribution to nervous system development and exploiting its therapeutic potential. This information would allow us to better understand olfactory dysfunction.
Olfactory dysfunction has been highly linked with traumatic brain injury, cancer, and several neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Therefore, by deciphering the factors that define its organization, development, and connectivity we may contribute to progress in treating patients with neurodegenerative disorders in other brain regions where connections have been lost and stem cell therapies are underway.

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