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The Guidance of Olfactory Sensory Axons to Identifiable Protoglomeruli in the Larval Zebrafish Olfactory Bulb

Abstract

During development, sensory neurons in the olfactory epithelium extend axons into the olfactory bulb. The earliest axons to enter the bulb terminate onto distinct neuropilar condensations called protoglomeruli. Protoglomeruli are thought to segregate into individual glomeruli later in development. The three day old larval zebrafish olfactory bulb contains 12 stereotyped, identifiable protoglomeruli, rendering it a good system to investigate mechanisms of initial axonal targeting in the bulb. In this thesis, I describe the generation of transgenic zebrafish lines in which neurons expressing odorant receptors along with the olfactory marker protein (OMP), V2R vomeronasal receptors along with the transient receptor potential channel2 (TRPC2) or the odorant receptor OR111-7 are selectively labeled. OMP and TRPC2 expressing neurons innervate multiple, non-overlapping protoglomeruli. Transgenic neurons expressing OR111-7:IRES:Gal4; UAS:Citrine transgenes primarily target a single protoglomerulus, the central zone, allowing the investigation of mechanisms directing axonal navigation to an individual protoglomerulus. Using this transgenic line, I show for the first time in any system that netrin/DCC signaling is required to guide olfactory sensory axons to a specific location within the olfactory bulb. Interestingly, I find that the central zone protoglomerulus is innervated by neurons expressing related odorant receptors of the OR111 subfamily. Upon replacing the coding sequence of OR111-7 in the OR111-7:IRES:Gal4 transgenic construct with RFP, axons continue to target the central zone, suggesting that the OR111-7 is not required for the protoglomerular targeting of transgene expressing axons. Rather, it is likely that the transgenic construct is selectively expressed in neurons destined to target the central zone. Based on these observations, I propose a model hypothesizing that the zebrafish olfactory epithelium consists of distinct neuronal subsets. Each subset innervates a specific protoglomerulus and is restricted to express a predetermined set of odorant receptors and axon guidance receptors, which mediate the navigation of axons to particular protoglomeruli. These studies have laid the groundwork for future investigations into the mechanisms of axonal targeting to protoglomeruli in the larval zebrafish olfactory bulb.

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THE GUIDANCE OF OLFACTORY SENSORY AXONS TO IDENTIFIABLE PROTOGLOMERULI IN THE LARVAL ZEBRAFISH OLFACTORY BULB

Vanisha Lakhina

A DISSERTATION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2011

Supervisor of Dissertation

____

Dr. Jonathan Raper

Professor, Department of Neuroscience

Graduate Group Chairperson

____

Dr. Rita Balice-Gordon

Professor, Department of Neuroscience

Dissertation Committee

Dr. Minghong Ma, Associate Professor, Department of Neuroscience

Dr. Michael Granato, Professor, Department of Cell and Developmental Biology

Dr. Steven S. Scherer, Professor, Department of Neurology

Dr. Greg Bashaw, Associate Professor, Department of Neuroscience

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ABSTRACT

THE GUIDANCE OF OLFACTORY SENSORY AXONS TO IDENTIFIABLE PROTOGLOMERULI IN THE LARVAL ZEBRAFISH OLFACTORY BULB

Vanisha Lakhina

Supervisor: Dr. Jonathan A. Raper

During development, sensory neurons in the olfactory epithelium extend axons into the olfactory bulb. The earliest axons to enter the bulb terminate onto distinct neuropilar condensations called protoglomeruli. Protoglomeruli are thought to segregate into individual glomeruli later in development. The three day old larval zebrafish olfactory bulb contains 12 stereotyped, identifiable protoglomeruli, rendering it a good system to investigate mechanisms of initial axonal targeting in the bulb. In this thesis, I describe the generation of transgenic zebrafish lines in which neurons expressing odorant receptors along with the olfactory marker protein (OMP), V2R vomeronasal receptors along with the transient receptor potential channel2 (TRPC2) or the odorant receptor OR111-7 are selectively labeled. OMP and TRPC2 expressing neurons innervate multiple, non-overlapping protoglomeruli. Transgenic neurons expressing OR111-7:IRES:Gal4; UAS:Citrine transgenes primarily target a single protoglomerulus, the central zone, allowing the investigation of mechanisms directing axonal navigation to an individual protoglomerulus. Using this transgenic line, I show for the first time in any system that netrin/DCC signaling is required to guide olfactory sensory axons to a specific location within the olfactory bulb. Interestingly, I find that the central zone protoglomerulus is innervated by neurons expressing related odorant receptors of the OR111 subfamily. Upon replacing the coding sequence of OR111-7 in the OR111-

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CHAPTER 1. GENERAL INTRODUCTION

The organization of olfactory systems in vertebrates

The chemical senses of olfaction (smell), gustation (taste), and chemesthesis (pain, touch, and thermal somatosensation) are phylogenetically the oldest senses and quite likely the first means of communication between organisms (Finger et al., 2000). The basic anatomy underlying olfaction has been conserved across 500 million years of vertebrate evolution (Hoover, 2010). Vertebrate sensory neurons embedded in nasal epithelia detect odorants via chemoreceptors called odorant receptors or vomeronasal receptors. In mice, olfactory sensory neurons expressing odorant receptors (ORs) are located in the main olfactory epithelium (MOE) lining the nasal cavity and the septal organ, present bilaterally at the ventral base of the nasal septum (Ma et al., 2003; Storan and Key, 2006). Vomeronasal sensory neurons expressing V1R or V2R vomeronasal receptors reside in a separate olfactory structure, the vomeronasal organ (VNO) (Dulac and Axel, 1995; Ryba and Tirindelli, 1997). Neurons in the MOE and the septal organ project axons to the main olfactory bulb (MOB) while VNO neurons target their axons to the accessory olfactory bulb (AOB). In the MOB and AOB, sensory axons terminate in discrete synaptic condensations called glomeruli. Within a glomerulus, axons form synapses with the dendrites of tufted and mitral cells whose cell bodies reside in the olfactory bulb or accessory olfactory bulb. Tufted and mitral cells in turn project their axons to higher processing centers.

Each olfactory sensory neuron in the main olfactory epithelium is thought to express only one odorant receptor (OR) from a single allele (Chess et. al. 1994; Shykind, 2005). Mouse olfactory sensory neurons choose to express one receptor from a genomic repertoire of more than 1000 receptors, while zebrafish choose from about 150 (Alioto

and Ngai, 2005). The mechanism by which a single olfactory neuron chooses the expression of a unique receptor gene remains a mystery. Various hypotheses have been formulated to explain this phenomenon. These include the expression of complex combinations of transcription factors to specifically activate an odorant receptor, chromatin remodeling to position cis- or trans-acting regulatory elements upstream of the chosen receptor gene or heterochromatization mediated silencing of odorant receptor genes followed by stochastic activation of an allele (Serizawa et al., 2004; Tietjen et al., 2005; Magklara et al., 2011).

Neurons expressing a particular odorant receptor are interspersed in the main olfactory epithelium with neurons expressing different odorant receptors, yet their axons converge onto bilaterally symmetric, almost spatially invariant OR-specific glomeruli within the main olfactory bulb. Since glomeruli are homogenously innervated by neurons expressing a given odorant receptor, the binding of an odorant to a receptor elicits activity in the corresponding glomeruli (Mombaerts et al., 1996; Wang et al., 1998; Malnic et al., 1999; Tan et al., 2010). The pattern of activated glomeruli is thought to encode the primary neural representation of an odorant (Rubin and Katz, 1999). Thus, the generation of distinct, stereotyped glomeruli in the olfactory bulb is a prerequisite for olfactory perception.

Tremendous progress has been made in describing the anatomy and organizational principles underlying glomerular map formation in the mouse olfactory bulb. Yet, the mechanisms underlying the precise targeting of olfactory sensory axons to stereotyped glomeruli remain unclear. Several properties of the mouse primary olfactory pathway significantly limit its use as a model system. Mice contain more than 1000 odorant receptors and since most neurons expressing a given odorant receptor project axons to

one medial and one lateral glomerulus, about 1800 glomeruli are present in the main olfactory bulb (Mombaerts et al., 1996; Wang et al., 1998; Royal and Key, 1999; Levai et al., 2003). Development is slow and navigating axons are relatively inaccessible to observation in utero. In contrast, the zebrafish genome encodes ~150 receptors and the adult zebrafish olfactory bulb contains about 80 distinct glomeruli and perhaps as many as 120 'glomerular modules', glomerulus-like regions compressed together within the lateral bulb (Baier and Korsching, 1994; Friedrich and Korsching, 1997). Zebrafish larvae are relatively more accessible to embryonic manipulations. Their transparency allows for real time observation of developmental events providing fresh insight into these processes (Korsching et. al., 1997; Niell and Smith, 2004; Beis and Stanier, 2006). For these reasons, I wished to establish the zebrafish as an alternate vertebrate model system to study the targeting of sensory axons to the olfactory bulb.

The zebrafish olfactory epithelium is relatively simple and contains two major classes of olfactory sensory neurons. One class expresses classical odorant receptors resembling those in higher vertebrates that innervate the main olfactory bulb. The other class of neurons expresses ORs more closely related to the V2R family of receptors in the vomeronasal organ of rodents. There are currently estimated to be 143 ORs and 70 V2Rs in the zebrafish (Alioto and Ngai., 2005; Niimura and Nei, 2005; Hashiguchi and Nishida, 2005). Odorant receptors and the olfactory marker protein (OMP) are expressed in ciliated sensory neurons residing deep in the olfactory epithelium while V2R receptors and the transient receptor channel (TRPC2) are expressed in microvillus sensory neurons located more superficially. Transgenic zebrafish in which fluorophores are specifically expressed under the OMP or TRPC2 promoters revealed that sensory axons containing ORs project to medial glomeruli while those expressing V2Rs project to lateral glomeruli (Sato et. al, 2005). Medial glomeruli are activated in response to bile

acids, steroids, and prostaglandins. They are thought to control social behaviors. Lateral glomeruli respond to amino acids and nucleotides and influence feeding behaviors (Sato et. al., 2005; Koide et al., 2009).

Protoglomeruli are precursors to mature glomeruli

Zebrafish olfactory placodes are recognizable as thickenings in the ectoderm as early as 18 hours post fertilization (hpf) and nasal pits form by 32 hpf (Hansen and Zeiske, 1993). Around 24hpf, about 10 pioneer axons emerge from the olfactory epithelium and grow towards the bulb (Whitlock and Westerfield, 1998). Pioneer neurons subsequently die via apoptosis at around 2 days post fertilization. As opposed to sensory neurons, pioneer neurons do not express odorant receptors. They serve a true pioneer role as their ablation induces the misrouting of olfactory sensory axons extending later in development.

Protoglomerular condensations of olfactory sensory axons begin to emerge at 2 days post fertilization (dpf) within the bulb (Wilson et. al., 1990; Dynes and Ngai, 1998; Miyasaka et. al., 2005; Li et. al, 2005). Protoglomeruli are thought to be immature glomerular precursors that ultimately give rise to mature glomeruli. Similar to adult activation patterns, amino acids and bile acids predominantly evoked activity in the lateral and medial olfactory bulb in three day old larvae (Li et al., 2005). This chemotopic activation is thought to arise because OMP and TRPC expressing axons converge onto 12 identifiable, non-overlapping protoglomeruli in the three day old larval zebrafish olfactory bulb (Sato et al., 2005; Dynes and Ngai. 1998). My experiments indicate that OMP expressing axons target the central zone, dorsal zone, lateral glomerulus 3 and the medial glomeruli in 3 day old larval olfactory bulbs, while TRPC expressing neurons project to the olfactory plexus, ventro-posterior glomerulus, and lateral glomeruli 1, 2,

and 4 (Chapter 2, 3). Since there are only 12 stereotyped protoglomeruli in the three day old larval zebrafish olfactory bulb, this is a good system to study how subsets of olfactory sensory axons are specifically targeted to distinct protoglomeruli in the larval olfactory bulb.

Protoglomeruli are stereotyped in their number and position, but they evolve over time. Twelve large protoglomeruli observed at 3dpf appear to break up into about 25 distinct glomerulus-like structures at 6dpf (Li et al., 2005). The increasing number of glomerulus-like condensations may be related to the entry of newly generated sensory axons expressing late-onset odorant receptors. Alternatively, the increasing number of condensations could represent the refinement of projections that are at first diffuse. In rodents, axons expressing different ORs co-mingle within protoglomeruli before segregating into homogenously innervated glomeruli (Conzelmann et al. 2001; Zou et. al., 2004). Glomeruli innervated by axons expressing related receptors are located adjacent to each other in the mouse olfactory bulb (Tsuboi et al., 1999). One attractive hypothesis is that initial condensations contain sensory axons that express many different odorant receptors which subdivide over time into distinct glomeruli, each composed of axons expressing a single odorant receptor. Supporting this hypothesis, I show in chapter 4 of this thesis that a particular protoglomerulus, the central zone, is heterogeneously innervated by related odorant receptors of the OR111 subfamily in 3 day old larvae.

Visualizing neurons expressing a particular odorant receptor and their axonal projections

To examine protoglomerular targeting in detail, it is essential to selectively visualize neurons expressing a particular odorant receptor whose axons likely converge into a

specific protoglomerulus. In mice, but not zebrafish, odorant receptor mRNAs can be detected in axonal terminals. mRNAs encoding a particular odorant receptor localize in distinct glomeruli in the olfactory bulb, suggesting that the axons of neurons expressing a particular odorant receptor target a specific glomerulus (Vassar et al., 1994). This principle was subsequently observed directly in genetically engineered mice in which an odorant receptor coding sequence was replaced with an OR:IRES:reporter gene (Mombaerts et al., 1996; Wang et al., 1998). These landmark studies allowed the selective visualization of subsets of neurons that express a particular OR and demonstrate that their axons converge upon one or two glomeruli. Subsequently, Serizawa and colleagues (2000) generated 460-kb and 200-kb yeast artificial chromosome (YAC) constructs to transgenically express the odorant receptor MOR28. Neurons expressing the transgenic MOR28 innervate glomeruli adjacent to, but distinct from the endogenous MOR28 glomeruli. In 2002, Anne Vassalli and colleagues created 'OR minigenes' to selectively label OR expressing neurons. The minigene constructs consist of short genomic sequences driving OR expression followed by an IRES:reporter cassette and short non coding sequences 3' to the odorant receptor added to ensure mRNA stability. Transgenic minigene expressing axons projected to the correct glomerulus. Like endogenous odorant receptors, transgenic odorant receptors expressed using either the YAC or minigene strategies suppressed the expression of other odorant receptors (Serizawa et al., 2000; Vassalli et al., 2002).

The organization of odorant receptors in the zebrafish genome is ideal for minigene construction. Zebrafish odorant receptors that share greater than 40% amino acid identity are classified into families and further divided into subfamilies if receptors share greater than 60% amino acid identity (Lancet and Ben-Arie, 1993; Alioto and Ngai, 2005). Highly related odorant receptors belonging to the same subfamily are densely

packed in gene clusters with only 4-10 kb of non-coding sequence between each receptor (Dugas and Ngai, 2001). Thus, the regulatory sequences upstream of individual odorant receptors are small and can potentially be used to drive odorant receptor expression. In chapter 2 of this thesis, I describe the generation of a zebrafish transgenic line that selectively labels neurons expressing a transgenic OR111-7 receptor and whose design was based on the 'minigene' strategy pioneered by Vassalli et al., 2002. The OR111-7:IRES:Gal4 transgenic was valuable since it marked axons primarily targeting the central zone protoglomerulus. I used this transgenic line to study the role of the classical axonal guidance cues netrin1a and netrin1b in targeting axons to the central zone (Chapter 3).

Guiding olfactory sensory axons within the bulb – Guidance Cues

Several guidance cues have been implicated in targeting subsets of olfactory sensory axons to stereotyped glomeruli in the olfactory bulb. The repulsive cues slit1 and slit3 present in the ventral mouse olfactory bulb restrict sensory neurons expressing the slit receptor, Robo2, to the dorsal region of the bulb (Cho et al., 2007). In the Robo2 mutant zebrafish olfactory bulb, some glomeruli are either absent or ectopically located. This phenotype has been ascribed to a more global disruption of protoglomeruli in Robo2 mutant larvae (Miyasaka et al., 2005). The repulsive ligand Sema3F is present in the mouse dorsal olfactory bulb and confines axons expressing its receptor component, neuropilin2, to the ventral bulb. (Takeuchi et al., 2010). Sema3a is expressed by ensheathing cells in the nerve layer of the ventral mouse olfactory bulb. Olfactory sensory neurons that express its receptor, Neuropilin1a, are repelled by Sema3a and extend axons to the lateral region of the anterior olfactory bulb and medial region of the posterior bulb. In Sema3A deficient mice, many neuropilin1a positive glomeruli are ectopically positioned in the anteromedial and ventral bulb (Schwarting et al., 2000,

2004; Taniguchi et al., 2003). Chick olfactory sensory neurons expressing a dominant negative neuropilin1 enter the brain prematurely and overshoot semaphorin expressing regions in the bulb to project into the forebrain (Renzi et al., 2000). Knocking out both ephrinA3 and ephrinA5 in mice shifts glomeruli formed by SR1- or P2- expressing olfactory sensory neurons posteriorly, while overexpression of ephrinA5 specifically in P2 neurons shifts its glomerulus anteriorly (Cutforth et al., 2003).

Ephrins, slits, and semaphorins also contribute to the anterior vs. posterior mapping of VNO axons in the AOB of mice. The basal cells of the VNO express Robo receptors causing their axons to avoid high levels of slits in the anterior AOB, thereby restricting them to the posterior AOB (Knöll et. al., 2003; Cloutier et. al., 2004). Apical VNO cells containing higher concentrations of the ephrins A3 and A5 are attracted by higher levels of ephA3 and A6 present in the anterior AOB (Knöll et. al., 2001; Knöll et. al., 2003). Apical VNO neurons also express neuropilin-2 which prevents their axons from terminating in sema3F expressing posterior regions of the AOB (Cloutier et. al., 2002, 2004; Walz et. al., 2002).

In all the experiments mentioned above, the levels of axonal guidance cues or their receptors were perturbed and the targeting of subsets of olfactory sensory axons to mature glomeruli was examined. In contrast, very little is known about the initial targeting of axons to protoglomeruli in the mouse olfactory bulb, presumably because of the relative experimental intractability of mouse embryos. In chapter 3, I have examined the requirement for netrin/DCC signaling in targeting OR111-7 transgene expressing axons to the central zone protoglomerulus. I find, for the first time in any system, that netrin1a and netrin1b signal via the DCC receptor to attract sensory axons to the ventrally positioned central zone protoglomerulus.

Guiding olfactory sensory axons within the bulb - Odorant receptors

In the mouse, odorant receptors expressed in olfactory sensory neurons themselves participate in determining glomerular position. Neurons expressing an odorant receptor which has been inserted into the genomic position of another reroute their axons to ectopic glomeruli that are often positioned between the predicted locations of endogenous glomeruli corresponding to the individual host and donor odorant receptors (Mombaerts et al., 1996; Wang et. al., 1998). These results suggest that while odorant receptors contribute to axonal targeting, they are not the sole determinant of glomerular positioning. The mechanism through which odorant receptors influence axonal guidance is unclear. One hypothesis is that odorant receptors are homophilic adhesion molecules that promote the selective adherence of sensory axons expressing a particular receptor (Strotmann et al., 2004; Schwarzenbacher et al., 2006). An alternative hypothesis is that the expression of a particular odorant receptor is correlated with the expression of a specific set of adhesive or repulsive molecules which mediate the selective fasciculation of axons expressing a given receptor (Serizawa et al., 2006). This provides a reasonable explanation for how axons expressing a particular receptor converge together, but is an unsatisfactory explanation for how glomerular position is determined.

A third hypothesis to explain how ORs influence axonal targeting is that the expression of a particular odorant receptor is correlated with the expression of specific guidance receptors. For example, odorant receptor expression in sensory axons is correlated with the relative expression levels of ephrin-As and EphAs (Cutforth et. al., 2003). It has also been reported that OR related activity levels control the expression levels of guidance molecules that in turn contribute to glomerular positioning (Serizawa et. al., 2006).Odorant receptors are G-protein coupled seven transmembrane receptors. They signal via Gs/olf, which in turn activates adenylate cyclase type-3 and thereby stimulates

the production of cAMP. Increased cAMP levels induce the opening of cyclic nucleotide gated (CNG) ion channels that permit the entry of extracellular calcium. The levels of cAMP generated downstream to odorant receptor signaling regulate the mRNA levels of the guidance receptor, Neuropilin1a (Imai et al., 2006).

A fourth hypothesis for the mechanism of OR dependent axonal guidance is that signaling via G-proteins downstream to the odorant receptor alters how sensory axons respond to guidance cues. This is based on the observation that the response of axonal growth cones to guidance cues can be modulated by altering their intracellular concentrations of cAMP and $Ca2+$ (Song et al., 1997; Ming et al., 1997). Similarly, the activation of G protein coupled receptors (GPCRs) can either directly attract or repel axons, reduce the effectiveness of repellants or convert an attractive response to a repulsive one (Xiang et al., 2002; Bonnin et al., 2007; Chalasani et al., 2003, 2007; Kreibich et al., 2004). Our lab has shown previously that in chick dorsal root ganglion cells and retinal ganglion cells, signaling via GPCRs such as CXCR4 or mGluR1 activates $G\alpha i$, $G\alpha q$ and $G\beta\gamma$ mediated signaling. In embryonic neurons, this elevates cAMP levels and inactivates Rho GTPase, thereby reducing the responsiveness of growth cones to axonal repellents. In chapter 5 of this thesis, I explore the contribution of G-protein signaling to olfactory axonal guidance. I find that reducing $G\alpha s$/olf causes OMP expressing axons to ectopically project into lateral protoglomeruli. In contrast, I find that reducing G-protein signaling in TRPC or OR111-7 expressing neurons simply reduces their number. I speculate that this is due to improper specification or differentiation of olfactory sensory neurons.

Guiding olfactory sensory axons within the bulb – Epithelial zone specific targeting

The mouse olfactory epithelium is divided into four zones from dorso-medial (zone I) to ventro-lateral (zone IV) based on the restricted expression of odorant receptors within these zones. The fish-like Class I odorant receptors are expressed in zone I (Tsuboi et. al., 2006). The more predominant Class II receptors are expressed in discrete but overlapping regions within all four zones (Iwema et. al., 2004; Miyamichi et. al., 2005). All neurons expressing the same receptor are randomly dispersed within one of these zones (Buck and Axel, 1991, Malnic et al., 1999, Ressler et al., 1993, Vassar et al., 1993). The restriction of receptor expression into zones of the olfactory epithelium implies that the epithelium is spatially patterned. Like odorant receptors, transcription factors of the lozenge-like family of proteins display restricted expression in zones of the olfactory epithelium (Tietjen et al., 2005). The homeobox gene Msx1, the axon guidance receptor Neuropilin 2, BMP type I receptor Alk6 and a retinioic acid synthesizing enzyme RALDH2 are expressed in gradients in the olfactory epithelium that correlate with zonal topography (Norlin et al., 2001).

Neurons located in zone I of the epithelium project to the dorso-medial part of the bulb and neurons located in zone IV target axons to the ventro-lateral part of the bulb (Yoshihara and Mori, 1997). In the odorant receptor swap experiments described previously, replacing an odorant receptor with another odorant receptor expressed in a different zone reroutes axons to a new glomerulus. The new glomerulus remains in the zone corresponding to the original receptor and not the newly inserted receptor (Mombaerts et al., 1996; Yoshihara and Mori, 1997). Thus, axonal targeting to glomeruli is determined, in part, by the zone that the neuron is expressed in and presumably its transcription factor profile. In chapter 2, I show that replacing the OR111-7 in the OR111-7:IRES:Gal4 construct with an RFP does not alter the targeting of axons to the central zone protoglomerulus. This suggests that OR111-7 is not specifically required for

axonal targeting to the central zone. Rather, it is likely that the transgenic construct is selectively switched ON in neurons that are destined to target the central zone. In chapter 4 of this thesis, I show that related ORs of the OR111 subfamily target their axons to the central zone. I propose a model hypothesizing that subsets of neurons are restricted to target their axons to protoglomeruli, similar to zonal targeting of mouse olfactory axons to limited areas in the bulb.

In summary, I have generated new tools for examining axonal targeting to protoglomeruli in the zebrafish olfactory bulb. These tools include transgenic zebrafish lines in which the Gal4 transcription factor is specifically expressed in neurons expressing OMP and all odorant receptors, TRPC and all V2R vomeronasal receptors, and neurons that contain the odorant receptor OR111-7. OR111-7 transgene expressing axons require signaling via netrin/DCC for proper targeting to the central zone protoglomerulus. Inhibiting signaling via Gαs/olf in axons containing OMP induces ectopic projections into lateral protoglomeruli. Reducing signaling via Gαs/olf, Gαq, Gβγ or PKA in OR111-7 or TRPC expressing axons reduces their number, which could reflect defects in specification or differentiation. I further show that the central zone protoglomerulus is heterogeneously innervated by neurons expressing related receptors of the OR111 subfamily. These studies have opened up new and exciting avenues for investigating the initial innervation of olfactory protoglomeruli in the zebrafish olfactory bulb.

CHAPTER 2. CHARACTERIZATION OF THE OR111-7:IRES:GAL4 TRANSGENIC ZEBRAFISH LINE

INTRODUCTION

Neural circuits mediating olfaction perform the daunting task of identifying and discriminating between a plethora of chemical odorants. Vertebrate olfactory sensory neurons stochastically express just one odorant receptor (OR) from a large genomic repertoire (Chess et al., 1994; Yoshihara, 2009). The newly generated OR protein suppresses the transcription of other OR genes (Serizawa et al., 2003; Lewcock and Reed, 2004). Neurons expressing a given odorant receptor project their axons to non-overlapping stereotyped locations called glomeruli in the olfactory bulb (Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998).

The direct visualization of subsets of olfactory sensory axons targeting specific glomeruli was a powerful breakthrough that led to insights into the mechanisms of axonal targeting within the olfactory bulb. Neurons expressing a particular odorant receptor and their axons were directly observed in mice in which a particular odorant receptor was replaced with an OR:IRES:reporter gene via homologous recombination (Mombaerts et al., 1996; Wang et al., 1998). Neurons expressing a specific odorant receptor were also visualized in YAC transgenic mice or transgenic mice harboring odorant receptor minigene constructs in which OR expression was controlled by its upstream non-coding regulatory sequences (Serizawa et al., 2000; Vassalli et al., 2002). Thus far, several axonal guidance cues and cell adhesion molecules have been shown to guide olfactory sensory axons into and within the olfactory bulb (Renzi et al., 2000; Schwarting et al., 2000, 2004; Taniguchi et al., 2003; Cutforth et al., 2003; Miyasaka et al., 2005; Cho et al., 2007; Takeuchi et al., 2010; Lipscomb et al., 2003). Mice in which neurons

expressing a particular odorant receptor were selectively labeled were used to demonstrate that odorant receptors themselves contribute in axonal targeting to glomeruli in the mouse olfactory bulb (Mombaerts et al., 1996; Wang et al., 1998; Imai et al., 2006). Neurons expressing an odorant receptor that replaced a different receptor in the genome reroute their axons to ectopic glomeruli often positioned between endogenous glomeruli corresponding to the individual donor and host odorant receptors (Mombaerts et al., 1996; Wang et. al., 1998). Perturbing odorant receptor mediated signaling reduces the level of cAMP and the neuropilin1 axon guidance receptor, and shifts glomeruli anteriorly in the olfactory bulb (Imai et al., 2006).

Despite tremendous progress, much remains to be learned about how diverse axonal guidance mechanisms work together to target axons to distinct glomerular positions in the olfactory bulb. It is likely that there are as yet undiscovered guidance cues that guide axons to domains within the olfactory bulb and to particular glomeruli. These questions are difficult to address in the mouse olfactory system, which consists of more than 1000 odorant receptors and about 1800 glomeruli. I wished to employ the zebrafish as a simpler vertebrate model for studying the targeting of olfactory sensory axons to stereotyped locations in the olfactory bulb. The zebrafish genome encodes only about 150 odorant receptors and about 150 glomeruli are present in the adult olfactory bulb. I restricted my studies to an even simpler olfactory circuit present in three day old zebrafish olfactory bulbs which consists of 12 protoglomeruli, or immature glomeruli (Dynes and Ngai, 1998; Lakhina et al., submitted). The simplicity of this circuit makes it an attractive system to study the mechanisms of axonal targeting to protoglomeruli. However, the lack of transgenic lines that selectively label a subset of neurons targeting specific protoglomeruli was a major hindrance to these studies.

To directly visualize neurons whose axons converged primarily onto a particular protoglomerulus, I generated a transgenic zebrafish line in which neurons expressing odorant receptor OR111-7 and their axonal projections were selectively labeled. I chose to study OR111-7 since its mRNA is detected around 30 hours post fertilization, making it one of the earliest odorant receptors expressed in the olfactory epithelium (Barth et al., 1997). In this chapter, I characterize the properties of the OR111-7:IRES:Gal4 transgenic line. Transgenic OR111-7 expressing neurons are selectively detected in the olfactory epithelium and they recapitulate the onset of endogenous OR111-7 expression. They target their axons primarily to the central zone protoglomerulus while a few axons project to the lateral glomerulus 1 (LG1). Since axons primarily targeting a single protoglomerulus are labeled in this transgenic line, our lab can now begin to investigate mechanisms of axonal targeting to the central zone protoglomerulus. Indeed, the OR111-7:IRES:Gal4 transgenic line served as a valuable tool to study the contribution of netrin/DCC signaling in guiding axons to the central zone protoglomerulus (Chapter 3, this thesis; Lakhina et al., submitted). Unfortunately, transgenic OR111-7 expressing neurons exhibit three undesirable and unforeseen properties despite their usefulness as markers for a subset of axons with stereotyped projections in the olfactory bulb. First, the number of OR111-7 expressing neurons steadily increases until 3 days post fertilization and then decreases with age in stable transgenic lines. This hinders their examination at older ages. Second, OR111-7 mRNA is abnormally localized as compared to the distribution pattern observed in wild type neurons. Third, OR111-7 transgene expressing neurons express ORs of the OR106 subfamily. Replacing the OR111-7 with mRFP in the transgenic construct does not alter axonal targeting to the central zone, suggesting that the OR111-7 encoded in the construct is not strictly required for central zone targeting. It is likely that OR111-7 transgene expression is selectively switched ON in a subset of neurons destined to target the central zone protoglomerulus.

MATERIALS AND METHODS

Microinjection constructs. All constructs were cloned into the mini-Tol2 vector kindly provided by the Ekker laboratory (Balciunas et al., 2006).

(1) OR111-7:IRES:Gal4: The design of this construct is based on previous studies in the mouse (Vassalli et. al. 2002; Bozza et. al., 2009). This construct is schematized in Figure 2.5 and is described in detail in the Results section. (2) OR111-7:mRFP:IRES:Gal4: This construct was generated by replacing the OR111-7 coding sequence in the OR111-7:IRES:Gal4 construct with the mRFP protein coding sequence (schematized in Figure 2.5). (3) UAS:gap43-Citrine: 14X UAS sequences and the E1b carp β-actin minimal promoter were used to control the expression of a Citrine fluorophore fused with the membrane targeting region of the GAP43 protein.

Microinjection into zebrafish embryos. Microinjection of DNA constructs along with Tol2 Transposase RNA into one-celled zebrafish embryos was performed as described in Fisher et al., 2006. For transient transgenesis experiments, injected embryos were raised until 3 days post fertilization, fluorescent larvae were collected and processed for immunohistochemistry.

Transgenic zebrafish lines. The OMP:RFP and TRPC2:Venus transgenic lines were obtained from the Yoshihara laboratory (Sato et al., 2005). The OR111-7:IRES:Gal4 construct or the UAS:gap43-Citrine construct was injected into single celled zebrafish embryos which were raised to adulthood (Fisher et al., 2006). Two independent transgenic lines were generated from individual founders. These transgenic lines and wild type zebrafish were raised at 28.5^{o} C and maintained as described in Mullins et al. (1994).

Immunohistochemistry. Three day old zebrafish larvae were fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer. Larvae were treated with acetone for 20 minutes at -20° C to facilitate tissue permeabilization. Goat anti-GFP (1:100, Rockland Immunochemicals, catalog #600-101-215) and anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen, catalog#A11055) were used to visualize GFP-positive neurons. Rabbit anti-DsRed (1:50, Clontech, catalog #632496) and anti-rabbit Cy3 (1:500, Jackson ImmunoResearch, catalog # 711-165-152) were used for visualizing RFP expressing axons. Mouse anti-zns2 (1:50, Zebrafish International Resource Center) and anti-mouse Alexa Fluor 546 (1:500, Invitrogen, catalog #A10036) label the pioneer axons (Whitlock and Westerfield, 1998). Propidium iodide staining was performed using the protocol of Brend and Holley (2009) with the omission of the RNase treatment step. Larvae were then mounted face down (frontal orientation) to visualize projections along the dorso-ventral axis. Larvae were imaged using a 40X oil immersion lens on a Leica TSP2 confocal microscope. Confocal sections at 1 micron intervals were taken through the entire olfactory bulb.

Quantification of neuronal number. Stacks of single confocal sections through the entire larval olfactory epithelium were used for analysis. The outline of each neuron in individual sections was traced out on a transparent sheet and counted to accurately determine neuronal number. One way ANOVA was used to determine whether the number of OR111-7 transgene expressing neurons is significantly different in three day old, seven day old or nine day old larvae.

Analysis of axonal distribution within the central zone. Three day old or nine day old larvae were processed for immunohistochemistry, propidium iodide staining and imaging via confocal microsopy as described above. The number of sections in which

the central zone was present were counted. On average, the central zone spanned about 21 sections for 3 day old olfactory bulbs and 27 sections for 9 day old olfactory bulbs. OR111-7 transgene expressing axons that were thin and fibrous were judged to be passing through the section while those that appeared thickened, bulbous, or which ended abruptly were judged to be terminating. Individual sections were scored as empty, containing transient axons, or containing terminating axons. Their positions along the antero-posterier axis of the bulb were normalized by expressing them as a percentage of the length of the central zone protoglomerulus.

Whole mount fluorescent in situ hybridization. Full length coding sequences of OR111-7, 106-1, -2, -3, -4, -6, -7, -10, -11 and OR128-1, -5, -10 were cloned into the pCRII-TOPO vector. Antisense digoxigenin labeled RNA probes were generated for each odorant receptor. *In situ* hybridization was performed as described previously (Chalasani et al., 2007). Briefly, wild type embryos were hybridized with probes for individual odorant receptors and *in situ* signals were amplified using a Cyanine 3-coupled tyramide system (TSA™ Plus Cyanine 3 System, Perkin Elmer, Product number: NEL744001KT). For microinjected or transgenic larvae, *in situ* signals were amplified using a Cyanine 5-coupled tyramide system (TSA™ Plus Cyanine 5 System, Perkin Elmer, Product number NEL745001KT). *In situ* hybridization was followed by immunohistochemistry using goat anti-GFP (1:100, Rockland Immunochemicals, catalog #600-101-215) and anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen, catalog#A11055) to visualize OR111-7 expressing transgenic neurons in the context of RNA expression. This was followed by propidium iodide staining (as above). Processed larvae were mounted in the frontal orientation and imaged using a 40X oil immersion lens on an

inverted Leica TSP2 confocal microscope. Images were captured every micrometer through the entire extent of the olfactory bulb.

Quantification of protoglomerular targeting. After immunohistochemistry and propidium iodide staining, entire olfactory bulbs of three day old larvae were optically sectioned via confocal microscopy. Individual protoglomeruli were identified based on the characterization and nomenclature described in Dynes and Ngai (1998). The number of larval olfactory bulbs with axonal projections to individual protoglomeruli were counted. The Fisher exact test was used to determine whether the control group was statistically different from the experimental group. The graphs represent the percentage of larvae with axonal projections to the specified protoglomerulus in the olfactory bulb.

RESULTS

The zebrafish olfactory epithelium consists of two major neuronal types; neurons that simultaneously express classical odorant receptors and olfactory marker protein (OMP), and neurons that express V2R vomeronasal receptors and the transient receptor potential channel TRPC2. By three days post fertilization, OMP and TRPC2 expressing axons converge onto protoglomeruli, acellular regions consisting of olfactory sensory axons and mitral cell dendrites (Dynes and Ngai, 1998; Li et al., 2005). Protoglomeruli are observed to segregate into smaller units which presumably mature into glomeruli (Li et al., 2005). Dynes and Ngai (1998) identified protoglomeruli as unstained regions observed upon treating larvae with the cellular stain, BODIPY. I have used the nuclear stain propidium iodide to identify protoglomeruli (Figure 2.1D-I; Lakhina et al., submitted; Chapter 3, this thesis). OMP expressing axons (red) target the central zone, dorsal zone, medial glomeruli and lateral glomerulus 3, while TRPC2 expressing axons (green) innervate the olfactory plexus, lateral glomeruli 1, 2 and 4, and the ventral posterior

glomerulus (Figure 2.1A; Lakhina et al., submitted; Chapter 3, this thesis). The OMP and TRPC2 transgenics are useful in selectively visualizing the two major classes of zebrafish olfactory sensory neurons, but since they innervate multiple protoglomeruli, they are not good tools for studying axonal targeting to a specific protoglomerulus.

The OR111-7 transgenic construct was designed based on the minigene strategy employed in the mouse

Since homologous recombination methods are not available for zebrafish, I designed a transgenic strategy to selectively visualize a subset of olfactory sensory neurons that target a specific protoglomerulus. This strategy was based on an approach established by Vassalli and colleagues (2002) who generated "minigene" constructs to transgenically label neurons expressing the mouse M71 or MOR23 odorant receptors. In the minigene constructs, varying lengths of 5' non-coding sequences proximal to the odorant receptor were used to drive odorant receptor expression. The odorant receptor coding sequence was followed by an IRES:tauLaCZ or IRES:tauGFP and varying lengths of non coding sequences 3' to the odorant receptor coding sequence. Extensive characterization of transgenic mice harboring M71 or MOR23 minigene constructs revealed that the transgene expressing neurons and their axonal projections are comparable to neurons and their projections labeled via homologous recombination. Thus, the minigene construct retains important biological properties of the odorant receptors themselves and their regulatory elements.

In my transgenic construct (see Figure 2.5), OR111-7 expression is regulated by its presumptive endogenous promoter (OR111-7 5') and the E15-1 enhancer element which promotes odorant receptor expression (Nishizumi et al., 2007). The presumptive OR111-7 promoter is comprised of the entire 4kb of genomic sequence between the coding

sequences of OR111-7 and its closest 5' upstream gene, OR111-8. The cis-acting E15-1 and E15-2 enhancer elements are present in the midst of odorant receptor clusters belonging to the 111, 116, 117, 118 and 119 subfamilies. They are required to switch ON odorant receptor expression and are interchangeable in their ability to activate the transcription of odorant receptors from these clusters (Nishizumi et al., 2007). An IRES:Gal4 cassette was inserted after the OR111-7 coding sequence. Thus, neurons that would transcribe the odorant receptor OR111-7 from the transgenic construct would also transcribe the Gal4 transactivator. 1 kb of untranslated sequence downstream from the OR111-7 coding sequence was added immediately after the IRES:Gal4 sequence in an effort to replicate endogenous mRNA stability and localization.

OR111-7 transgene expressing neurons are detected selectively in the olfactory epithelium in transiently transgenic larvae

The OR111-7:IRES:Gal4 construct was injected in combination with UAS:Citrine and mRNA encoding the Tol2 transposase in single celled OMP:RFP transgenic embryos to visualize OR111-7 transgene expressing neurons along with the rest of the odorant receptor expressing neurons. OR111-7 transgene expressing neurons are expected to represent a subset of OMP expressing neurons (Figure 2.1C). I also injected the OR111-7:IRES:Gal4 and UAS:Citrine transgenic constructs into wild type embryos (Figure 2.1G-I). Using this method of transient expression, an average of five OR111-7:IRES:Gal4; UAS:Citrine neurons were labeled. Transgene expressing neurons were observed in the olfactory epithelium, suggesting that the regulatory sequences contained in our minigene construct are sufficient to retain endogenous tissue specificity of expression. My previous attempts to generate a functional minigene construct without the E15-1 enhancer element were unsuccessful. This observation is consistent with a previous report showing that either the E15-1 or E15-2 enhancer is essential for the

expression of OR111-1 and OR116-1 and presumably other odorant receptors of the OR111 and OR116 subfamilies (Nishizumi et al., 2007).

Axons of OR111-7 transgene expressing olfactory sensory neurons primarily target the central zone protoglomerulus

OR111-7 expressing axons primarily target the central zone protoglomerulus. This protoglomerulus is also innervated by OMP expressing axons (Figure 2.1C). The central zone protoglomerulus is a large ventrally positioned protoglomerulus with its longest axis oriented antero-posteriorly. It is thought to be composed of approximately five smaller “sub”-protoglomeruli (Dynes and Ngai, 1998). Our experiments indicate that the central zone is endogenously innervated by neurons expressing receptors of the OR111 subfamily, suggesting that OR111-7 transgene expressing neurons recapitulate the endogenous targeting of neurons expressing OR111-7 (Chapter 4, this thesis). Consistent with this observation, in a bacterial artificial chromosome (BAC) transgenic in which the OR111-7 coding region was replaced with YFP, YFP expressing axons were detected in the central zone (Sato et al., 2007). Surprisingly, a small minority of OR111-7:IRES:Gal4; UAS:Citrine axons also project to the LG1 protoglomerulus, which is normally innervated by axons that express V2R receptors and the TRPC2 channel (Figure 2.1A, B, H). The significance of this LG1 projection is unclear.

OR111-7 expressing transgenic neurons primarily innervate the central zone protoglomerulus in stable transgenic lines

After confirming the efficacy of our construct, I generated two independent stable lines of transgenic zebrafish carrying the OR111-7:IRES:Gal4 transgene. Both lines appear equivalent in their properties. Crossing adult OR111-7:IRES:Gal4; UAS:Citrine transgenics with OMP:RFP transgenic animals generated progeny that harbored all

three transgenes. In our transgenic lines, about 27 neurons are labeled at 3 days post fertilization and as expected, their axons primarily target the central zone while a few axons project to LG1 (Figure 2.1D-F). These results are consistent with the idea that the OR111-7 transgene is largely expressed in OMP type neurons that project exclusively to the central zone, and perhaps less frequently in TRPC2 and V2R type neurons that extend to LG1. In ongoing experiments, I am trying test this idea by visualizing OR111-7 transgene expressing neurons in the context of TRPC expressing neurons. Despite the minor LG1 projection, the OR111-7:IRES:Gal4 transgenic line is a useful tool for studying axon guidance to the central zone protoglomerulus.

The axons of olfactory sensory neurons expressing the OR111-7 transgene enter the olfactory bulb early in development before protoglomeruli are apparent

I wished to characterize the temporal properties of OR111-7 transgene expression and the targeting of transgene expressing axons in the olfactory bulb. OR111-7 mRNA is detected around 30 hours post fertilization (Barth et al., 1997). OR111-7 transgene expressing neurons are first detected around 27hpf. Thus, the regulatory sequences present in the minigene construct recapitulate the onset of endogenous OR111-7 expression. By 28hpf, Zns2+ pioneer axons (red, Figure 2.2) have entered the primordial olfactory bulb (OB) in which protoglomeruli are not yet discernable (Figure 2.2A-C). The zns2+ pioneer axons establish the first axonal pathway between the olfactory epithelium and the bulb before the outgrowth of the sensory neurons. They are a transient class of neurons that undergo apoptosis soon after the sensory neurons reach the bulb (Whitlock and Westerfield, 1998). In single confocal sections, OR111-7 transgene expressing axons (green) extend along the pioneer axonal scaffold (Figure 2.2A-C). Thus, OR111-7 transgene expressing axons are one of the first axonal populations to enter the olfactory bulb during development.

The number of OR111-7 transgene expressing neurons decreases with developmental age after three days post fertilization

Surprisingly, I observe that the number of labeled transgene expressing neurons increases until three days post fertilization and then decreases as development proceeds. At 3 dpf, on average about 27 neurons in each olfactory pit express the OR111-7 transgene, but only 13 neurons in 7 dpf larvae, and 6 neurons in 9 dpf larvae express the transgene (Figure 2.3A-C). The decline in transgene expressing neurons suggests that it may be switched off over time. It is possible that regulatory elements required for continued stable expression of the transgenic odorant receptor are missing in our minigene construct. At 3 dpf, OR111-7 transgene expressing axons are not localized to any specific sub-compartment within the central zone (Figure 2.3D). Rather, axons are distributed and appear to terminate throughout nearly the whole anterior to posterior extent of the protoglomerulus. The projection of OR111-7 transgene expressing neurons is largely restricted to the posterior half of the central zone protoglomerulus by 9 days post fertilization (Figure 2.3E). This may reflect the arrival of new olfactory sensory axons that terminate specifically in the anterior portion of the central zone, the selective loss of anteriorly projecting axons as the transgene is switched OFF with time or the coalescence of OR111-7 expressing axons into a more posterior territory as the central zone protoglomerulus matures (Li et al., 2005).

OR111-7 mRNA expressed in OR111-7 transgene expressing olfactory sensory neurons is aberrantly localized in puncta

I wished to confirm that OR111-7 expressing transgenic neurons express OR111-7 mRNA. Using a digoxigenin labeled antisense RNA probe against OR111-7, I performed in situ hybridization in three day old OR111-7:IRES:Gal4; UAS:Citrine larvae.

Surprisingly, I observed two types of in situ hybridization signals. One of these resembled the wild type pattern. In wild type neurons, OR111-7 mRNA is detected as large diameter spheres or ovoids (N = 108 neurons from 20 olfactory pits, arrow, Figure 2.4A). In OR111-7:IRES:Gal4, UAS:Citrine neurons, OR111-7 mRNA appears in this pattern in only about 4 out of 29 neurons on average, i.e. in 15% of transgene expressing neurons (N= 272 neurons from 10 olfactory pits, arrow in Figure 2.4B, grey bars in Figure 2.4E, F). OR111-7 mRNA in OR111-7 transgene expressing neurons appears as puncta in 22 out of 29 neurons on average, i.e. 79% total OR111-7:IRES:Gal4; UAS:Citrine transgene expressing neurons (N= 272 neurons from 10 olfactory pits, white arrowhead in Figure 2.4B, red bars in Figure 2.4E, F). These puncta are observed in OR111-7 expressing neurons from both OR111-7:IRES:Gal4 transgenic lines (data not shown). OR111-7 mRNA puncta are also observed upon in situ hybridization for Gal4 mRNA (data not shown). The significance of this abnormal punctate mRNA distribution remains unclear. On average, OR111-7 mRNA expression is not detected in 2 out of 29 OR111-7 transgene expressing neurons, i.e. 6% of OR111-7:IRES:Gal4; UAS:Citrine neurons (N= 272 neurons from 10 olfactory pits, white bars in Figure 2.4E, F).

Olfactory sensory neurons expressing the OR111-7 transgene also express receptors of the OR106 subfamily but not the OR128 subfamily

I expected that OR111-7:IRES:Gal4; UAS:Citrine expressing neurons would exclusively express OR111-7 mRNA based on previous observations that zebrafish olfactory neurons, like mouse olfactory neurons, express only one odorant receptor (Barth et al., 1997; Sato et al., 2007; Yoshihara, 2009). Surprisingly, I find that neurons expressing the OR111-7 transgene also sometimes express mRNA encoding receptors of the OR106 subfamily including OR106-1, -2, -3, -4, -6, -7, -10, -11 (Figure 2.4C, E, F).

OR106 subfamily mRNA is primarily detected as puncta, in about 12 out of 22 neurons on average, i.e. 54% of OR111-7 transgene expressing neurons (N= 194 neurons from 10 olfactory pits, white arrowhead in Figure 2.4C, red bars in Figure 2.4 E, F). OR106 subfamily mRNA is also detected as spheres or ovoids, similar to the wild type expression pattern in about 1 out of 22 transgene expressing neurons on average, i.e. 3% of all OR111-7:IRES:Gal4; UAS:Citrine neurons (N= 194 neurons from 10 olfactory pits). About 43% of OR111-7 transgene expressing neurons do not express mRNA belonging to the OR106 subfamily (N= 194 neurons from 10 olfactory pits, blue arrowhead in Figure 2.4C, white bar in Figure 2.4E, F). The presence of OR106 subfamily members in OR111-7 transgene expressing neurons appears to be specific, since 91% of OR111-7 transgene expressing neurons do not express odorant receptors belonging to the OR128 subfamily (N= 206 neurons from 8 olfactory pits, blue arrowhead in Figure 2.4D, white bars in Figure 2.4E, F). These data suggest that OR111-7:IRES:Gal4; UAS:Citrine expressing neurons exhibit inappropriate intracellular distribution of OR111-7 mRNA and may express odorant receptors of the OR106 subfamily.

Replacing OR111-7 with mRFP in the OR111-7 transgenic construct does not alter axonal targeting to the central zone protoglomerulus

Despite the observation that OR111-7:IRES:Gal4; UAS:Citrine expressing neurons display altered subcellular distribution of OR111-7 mRNA and can express more than one odorant receptor, OR111-7 transgene expressing axons still primarily target the central zone protoglomerulus. I wondered whether removing the OR111-7 coding sequence within this transgenic construct would alter the axons' ability to target the central zone. I replaced the OR111-7 coding sequence with the mRFP protein coding sequence to generate OR111-7:mRFP:IRES:Gal4 (schematized in Figure 2.5). I injected

either the OR111-7:mRFP:IRES:Gal4 experimental construct or the control OR111-7:IRES:Gal4 construct (referred to as OR111-7:111-7:IRES:Gal4 in this experiment for clarity) into UAS:Citrine transgenic embryos at the one cell stage and observed the labeled axonal projections at 3 days post fertilization. Both OR111-7:111-7:IRES:Gal4/UAS:Citrine expressing neurons and OR111-7:mRFP:IRES:Gal4/UAS:Citrine neurons project their axons to the central zone, indicating that the replacement of the OR111-7 coding region with an mRFP did not affect the axons' ability to navigate properly to the central zone protoglomerulus (Figure 2.5A-C). Neurons that express the RFP replacement construct can potentially express odorant receptors other than OR111-7, yet their axons target to the central zone. This suggests that the minigene construct is selected to be expressed in a subset of neurons destined to project to the central zone.

DISCUSSION

In order to selectively label neurons expressing OR111-7 and their axonal projections, I generated a transgenic zebrafish line harboring a minigene construct which was designed based on previous studies in the mouse (Vassalli et al., 2002). The OR111-7 minigene construct encodes the OR111-7 receptor driven by its endogenous 5' non-coding regulatory region followed by an IRES:Gal4 cassette and its 3' non-coding sequences. Neurons expressing OR111-7:IRES:Gal4; UAS:Citrine are found in the olfactory epithelium and recapitulate the endogenous onset of OR111-7 expression, indicating that regulatory sequences required to switch ON OR111-7 expression are present in the minigene construct. Axons of neurons expressing OR111-7:IRES:Gal4; UAS:Citrine primarily target the central zone protoglomerulus. Thus, like higher vertebrates, the axons of zebrafish olfactory sensory neurons expressing a particular OR converge onto a specific location in the olfactory bulb. Neurons expressing the OR111-

7:IRES:Gal4; UAS:Citrine transgenes also target the LG1 protoglomerulus. This is presumably an aberrant projection since LG1 is normally innervated by TRPC and V2R expressing axons. Since most OR111-7 transgene expressing axons target the central zone, this transgenic line was valuable in testing the role of netrin/DCC signaling in axon targeting to the central zone (Chapter 3, this thesis; Lakhina et al., submitted).

Aberrant mRNA expression and transgene instability in OR111-transgene expressing olfactory sensory neurons

OR111-7 mRNA in OR111-7 transgene expressing neurons is primarily detected as puncta. This punctate staining is never observed in wild type neurons. The punctate localization of OR111-7 mRNA and Gal4 mRNA in the OR111-7:IRES:Gal4; UAS:Citrine expressing neurons could represent OR111-7:IRES:Gal4 mRNA mislocalized in RNA granules, large ribonucleoprotein particles that transport or locally store mRNAs to control their stability and onset of translation (Knowles et al., 1996; Anderson and Kedersha, 2006; Kiebler and Bassell, 2006). The puncta could also represent mRNA localization at active translation sites (Rodriguez et al., 2006). Altered OR111-7 mRNA distribution is detected by 28hpf (data not shown), around the time the expression of the OR111-7 transgene is first observed. Thus, the puncta are not gradually generated, but seem to be a property of OR111-7:IRES:Gal4 mRNA transcribed from the transgenic construct. The anomalous OR111-7 mRNA expression in OR111-7 expressing neurons could occur due to missing regulatory sequences in the minigene construct. About 60% of OR111-7 expressing neurons also express odorant receptors of the OR106 subfamily. mRNA encoding receptors of the OR106 subfamily is also detected as puncta in OR111-7 transgene expressing neurons, suggesting that the OR111-7 transgene is able to influence odorant receptor expression in trans. These abnormal mRNA expression properties were observed in both OR111-7 transgenic lines that I generated. It is

possible that these properties emerged as a result of transgene insertion into a regulatory locus important for odorant receptor expression. The rate of transgenesis for the OR111-7:IRES:Gal4 line was about 5%, significantly lower than the success rate for other transgenes (~30%), suggesting that perhaps the OR111-7:IRES:Gal4 transgene is functional only when inserted into specific genomic loci.

The presence of odorant receptors other than OR111-7 in OR111-7 expressing neurons suggests that transgenically expressed OR111-7 protein is unable to fully suppress the transcription of other odorant receptor genes. When immature mouse olfactory sensory neurons are forced to express a mutant odorant receptor containing a premature stop codon, they begin to transcribe other odorant receptors with high probability (Shykind et al., 2004). Once a functional odorant receptor is expressed, it elicits a feedback signal that stabilizes the expression of that receptor and terminates the transcription of other odorant receptor genes. If the expression of the transgenically expressed OR111-7 is unstable, then neurons that chose to transcribe the transgene may subsequently switch to a different odorant receptor, which in turn will suppress transcription from all other genomic loci including the OR111-7 transgene. This would cause a decrease in the number of OR111-7:IRES:Gal4; UAS:Citrine neurons with time.

The involvement of odorant receptors in targeting of zebrafish olfactory sensory axons to protoglomeruli in the bulb

Replacing the coding sequence of the OR111-7 with an RFP in our minigene construct does not alter the central zone projection of the transgene expressing axons. RFP expressing neurons may transcribe odorant receptors other than OR111-7, yet axons do not change their protoglomerular targeting. This suggests that OR111-7 in the transgenic construct is not required to direct axons to the central zone. Our data are consistent with

a previous observation that neurons in which the OR111-7 coding region is replaced with YFP in a BAC transgenic project axons to the central zone (Sato et al., 2007). This experimental result has two distinct implications for the involvement of odorant receptors in guiding zebrafish olfactory sensory axons.

First, it is possible that zebrafish odorant receptors, like those in *Drosophila,* do not contribute to the targeting of olfactory sensory axons to glomeruli. The *Drosophila* odorant receptor Or22a is present in the ab3A neuron and deleting Or22a does not alter the targeting of olfactory sensory axons expressing Or22a to glomeruli in the antennal lobe (Dobrista et al., 2003). In fact, when Or22a is deleted, the ab3a neuron loses sensitivity to all odorants tested, suggesting that unlike mouse olfactory sensory neurons, fly neurons do not transcribe a different odorant receptor in the absence of a functional receptor. Rather, the identity of a neuron correlates with the expression of odorant receptors as well as axonal guidance receptors which regulate axonal pathfinding to specific glomeruli (Ray et al., 2008). Using a bioinformatics approach followed by genetic analysis, Ray et al. (2008) identified novel regulatory elements that dictate proper expression of odorant receptor genes in subsets of sensory neurons. Intriguingly, these regulatory sequences were also found upstream of axon guidance receptors such as robo2, N-cadherin and Semaphorin-1a. Thus, the identity of a neuron and not the odorant receptor it expresses dictates which glomerulus its axon will innervate.

A second possibility is that while odorant receptors do not contribute to the initial targeting of olfactory sensory axons to protoglomeruli, they are required at later stages for axonal segregation into glomeruli. Protoglomeruli in mice are heterogeneously innervated by axons expressing related odorant receptors, suggesting that individual

odorant receptors are not required for axonal targeting to protoglomeruli (Conzelmann et al., 2001). So far, my experiments indicate that this is true for zebrafish as well. The central zone protoglomerulus is heterogeneously innervated by neurons expressing receptors of the OR111 subfamily (Chapter 4, this thesis). This would appear to be inconsistent with the well established idea that odorant receptor mediated signaling is required for axonal guidance along the antero-posterior axis in the mouse olfactory bulb (Mombaerts et. al., 1996; Wang et. al., 1998; Imai et al., 2006). However, it is important to note that all the studies investigating the requirement for odorant receptors in axonal guidance were performed in postnatal or adult mice and assessed the formation of mature glomeruli. It is possible that odorant receptors do not contribute in the initial targeting of mouse olfactory axons to protoglomeruli, but are required later during glomerular maturation and segregation.

If odorant receptors are not required for the initial targeting of mouse olfactory sensory axons, then how do axons target specific locations in the bulb? It is likely that different subsets of olfactory sensory neurons are specified to target their axons to particular protoglomeruli. Mouse olfactory sensory neurons are located in distinct zones in the epithelium and they generally express zone-specific subsets of odorant receptors, axon guidance receptors and other zonally restricted markers, suggesting that neurons belonging to different zones represent distinct neuronal subsets (Yoshihara et al., 1997; Gussing and Bohm, 2004). Neurons positioned in the dorsomedial epithelium project to the dorsal region of the bulb, whereas neurons located in the ventrolateral region of the epithelium innervate the most ventral region of the bulb (Strotmann et al., 1992; Ressler et al., 1993; Vassar et al., 1993; Cho et al., 2007). The guidance of axons along the dorso-ventral axis depends on the zonal location of the neurons in the olfactory epithelium (Wang et al., 1998; Cho et al., 2007). Whether zebrafish olfactory sensory

neurons are localized in zones remains unclear. Weth et al. (1996) suggest that neurons expressing a particular receptor are arranged in concentric, but overlapping expression domains around the adult olfactory epithelium, while Vogt et al. (1997) report that neurons expressing a given odorant receptor are randomly distributed in the embryonic epithelium. Irrespective of the physical distribution of neurons expressing a particular receptor into zones, it is still possible that distinct subsets of sensory neurons exist in the zebrafish olfactory epithelium. Support for this idea stems from a study showing that zebrafish olfactory sensory neurons are restricted to stochastically express an odorant receptor from a predetermined set of receptor genes (Argo et al., 2003).

I propose that distinct subsets of olfactory sensory axons are specified to target particular protoglomeruli in the zebrafish olfactory bulb (Figure 4.3). These neuronal subsets are restricted in the odorant receptor that they can transcribe. Further, I hypothesize that neurons belonging to a given subset express a set of axonal guidance receptors that target their axons to a particular protoglomerulus. Whether zebrafish odorant receptors contribute to axonal segregation into mature glomeruli can only be addressed when neurons expressing a particular odorant receptor can be labeled in more mature animals. Our results show that while generating minigenes to visualize a particular subset of neurons is valuable in marking protoglomerular projections, a different approach is required to generate stable transgenic lines that maintain transgene expression until maturity. We are currently exploring the possibility of generating transgenic zebrafish harboring bacterial artificial chromosome (BAC) constructs in which an IRES:Gal4 sequence is inserted right after the odorant receptor coding sequence. Since more regulatory elements are present in the BAC, these transgenics could potentially regulate the odorant receptor mRNA appropriately and maintain sustained

temporal expression. The potential for understanding the simple yet stereotyped larval olfactory circuit can be fully exploited once such transgenic lines become available.

Acknowledgements

I thank Mark Lush for help with cloning the OR111-7:IRES:Gal4 construct. I also thank the Yoshihara laboratory for kindly providing us with the OMP:RFP and TRPC2:Venus transgenic zebrafish lines.

Figure 2.1

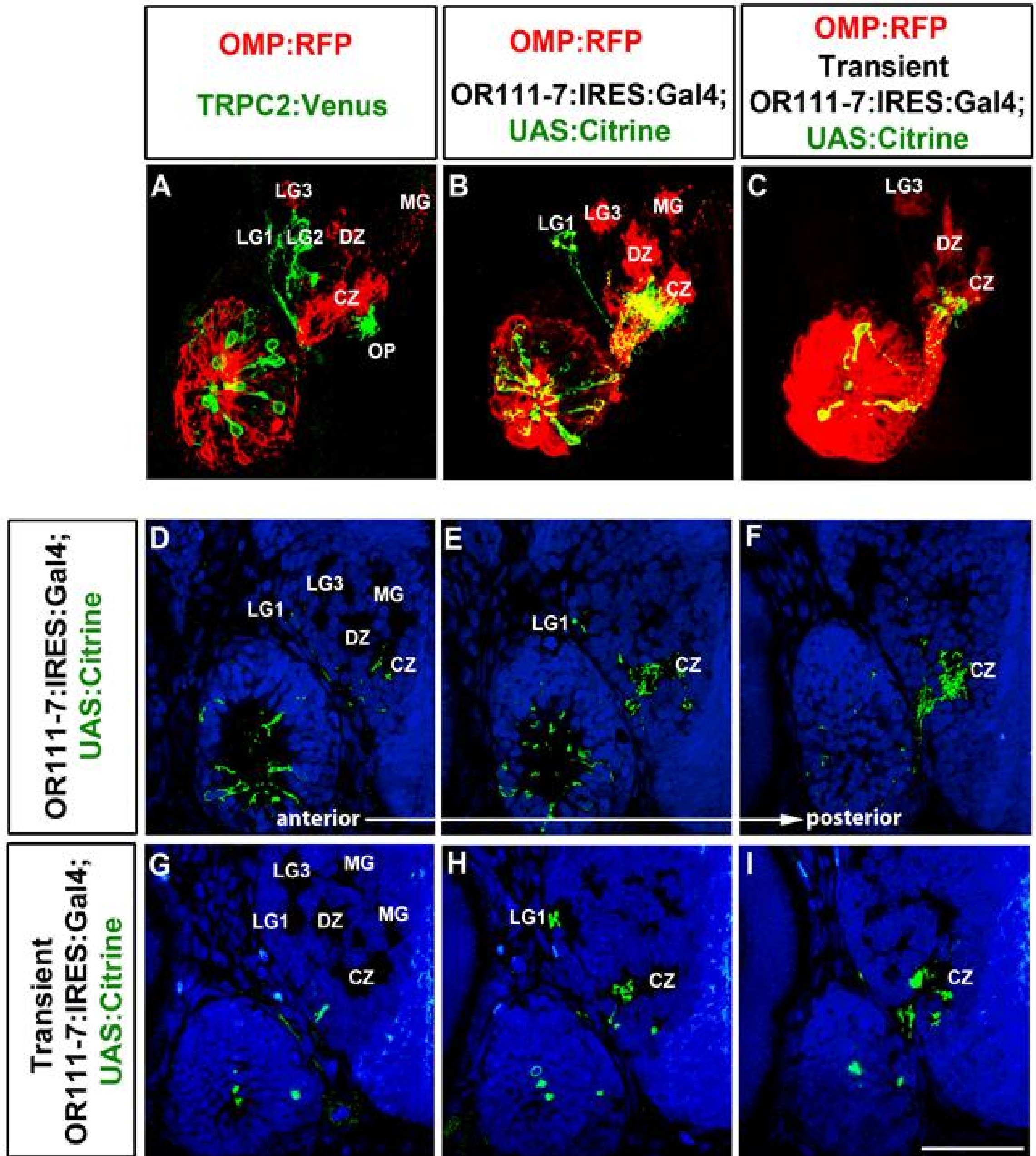

Figure 2.1. OR111-7 transgene expressing neurons primarily project axons to the central zone protoglomerulus in the 3 day old zebrafish olfactory bulb

A-C, Maximum projections of single confocal sections through the entire 3 day old olfactory epithelium and bulb (frontal view). Dorsal is to the top and midline to the right of the image. OMP expressing axons (red) target the CZ, DZ, MG and LG3. **A,** TRPC axons (green) project to the LG1, LG2 and OP. **D-I,** Single confocal optical sections through 3 day old OR111-7:IRES:Gal4; UAS:Citrine larvae (frontal view). Propidium iodide (blue) delineates distinct protoglomeruli in the olfactory bulb. **B, D-F,** OR111-7:IRES:Gal4; UAS:Citrine expressing neurons primarily target the CZ in stable transgenic zebrafish lines. **C, G-I,** OR111-7:IRES:Gal4; UAS:Citrine expressing neurons primarily target the CZ in transiently transgenic larvae. **B, E, H,** A few OR111-7 transgene expressing neurons project to the LG1.

CZ: *central zone,* DZ: *dorsal zone,* MG: *medial glomeruli 1-4,* LG1-4: *lateral glomeruli 1-4,* OP*: olfactory plexus*. Scale bar in I: 50 μm.

Figure 2.2

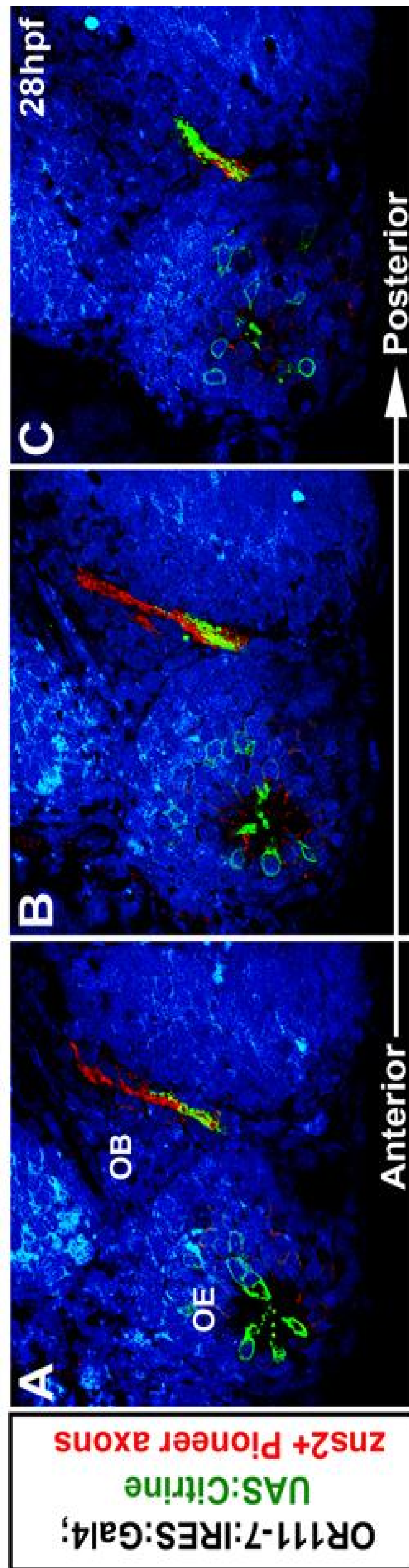

Figure 2.2. OR111-7 transgene expressing neurons associate closely with pioneer axons and enter the bulb by 28hpf

A-C, Single confocal optical sections through 28 hour old OR111-7:IRES:Gal4; UAS:Citrine expressing larvae (frontal view). Dorsal is to the top and midline to the right of the image. Propidium iodide (blue) staining reveals that protoglomeruli are not yet evident in the olfactory bulb. Zns2+ pioneer axons (red) are detected along the antero-posterior extent of the bulb. OR111-7 transgene expressing axons (green) are closely associated with pioneer axons.

Figure 2.3

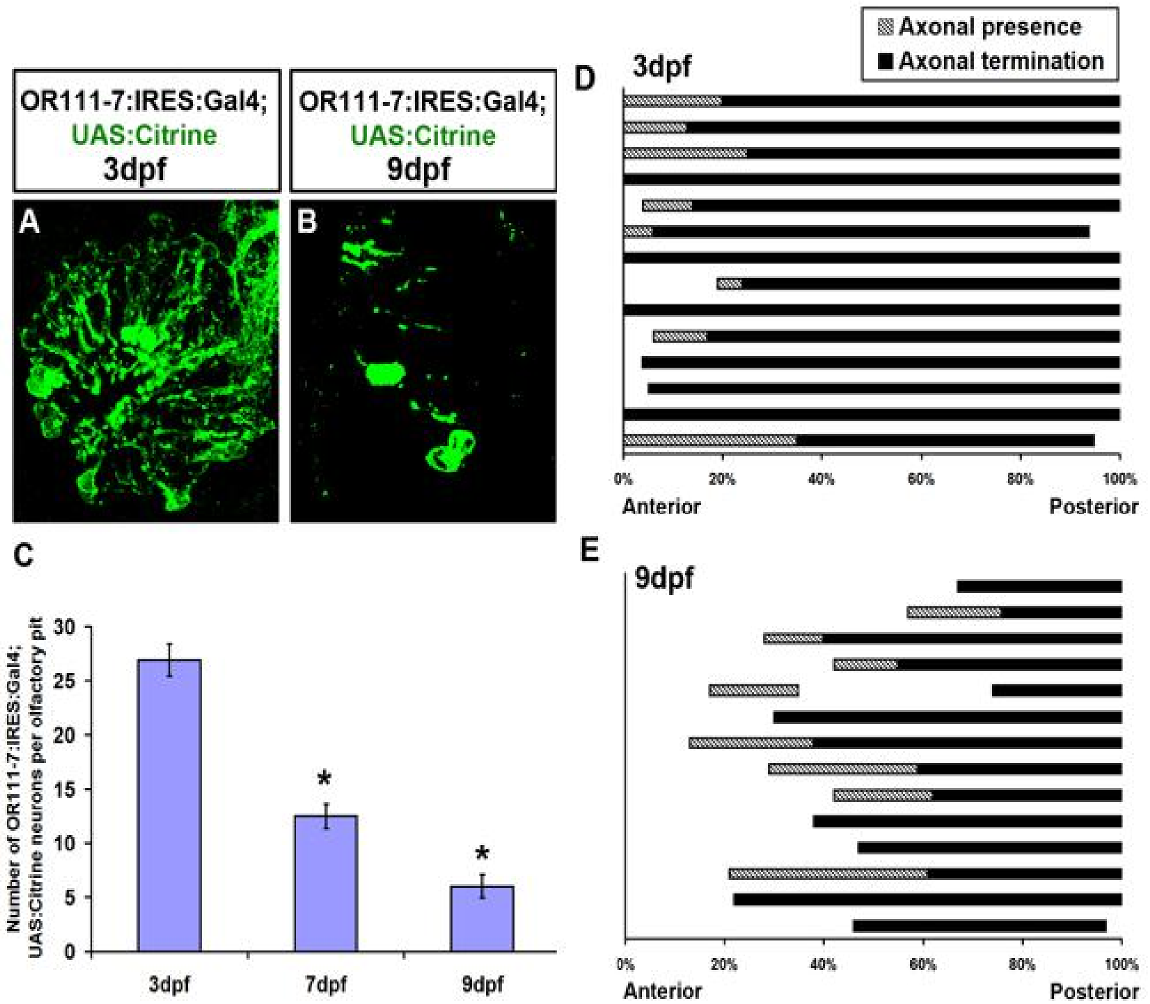

Figure 2.3. The number of OR111-7 transgene expressing neurons reduces with time and their axons are detected posteriorly in the central zone

A-C, The number of OR111-7:IRES:Gal4; UAS:Citrine expressing neurons (green) decreases from 27 neurons in 3dpf larve to 13 neurons in 7dpf larvae and 6 neurons in 9dpf larvae. **D,** OR111-7 transgene expressing neurons are distributed all along the antero-posterior extent of the central zone in 3 day old larvae. **E,** OR111-7 transgene expressing neurons are localized in more posterior regions of the central zone protoglomerulus. One way ANOVA was used to test for statistical significance, $P<0.05$.

Figure 2.4

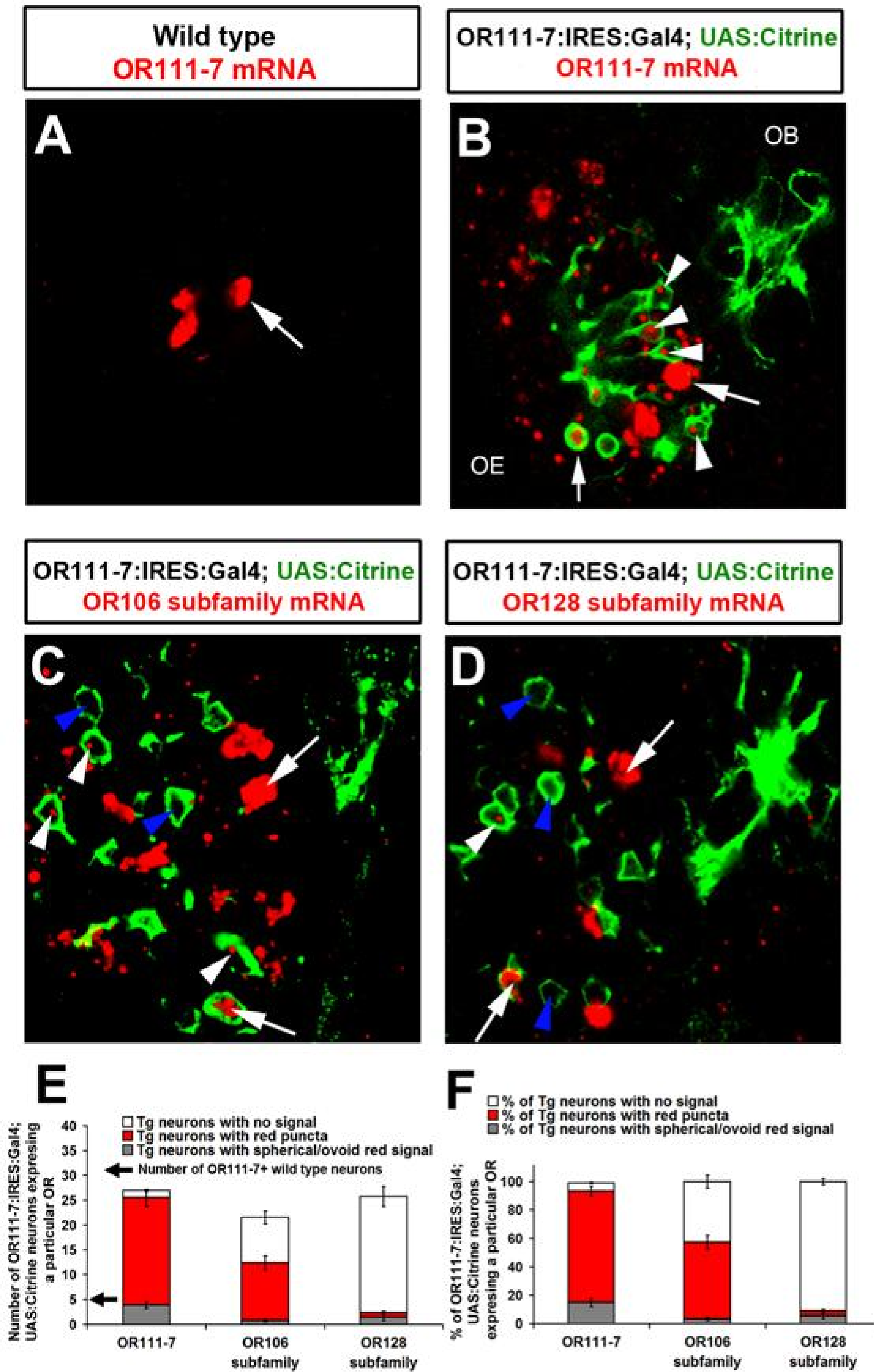

Figure 2.4. OR111-7 transgene expressing neurons display altered OR111-7 mRNA expression and ectopic expression of receptors belonging to the OR106 subfamily
A, OR111-7 mRNA (red) is expressed in a spherical/ovoid pattern in wild type 3 day old olfactory sensory neurons (arrow). **B, E, F,** OR111-7 mRNA (red) is detected in the spherical/ovoid pattern (arrow) in a few neurons but is mostly present in puncta (arrowhead) in OR111-7 transgene expressing neurons (green). **C, E, F,** Multiple receptors of the OR106 subfamily mRNA (red) are expressed as puncta (arrowhead), rarely detected in the spherical/ovoid pattern (arrow) or not expressed at all (blue arrowhead) in OR111-7 transgene expressing neurons (green).

Figure 2.5

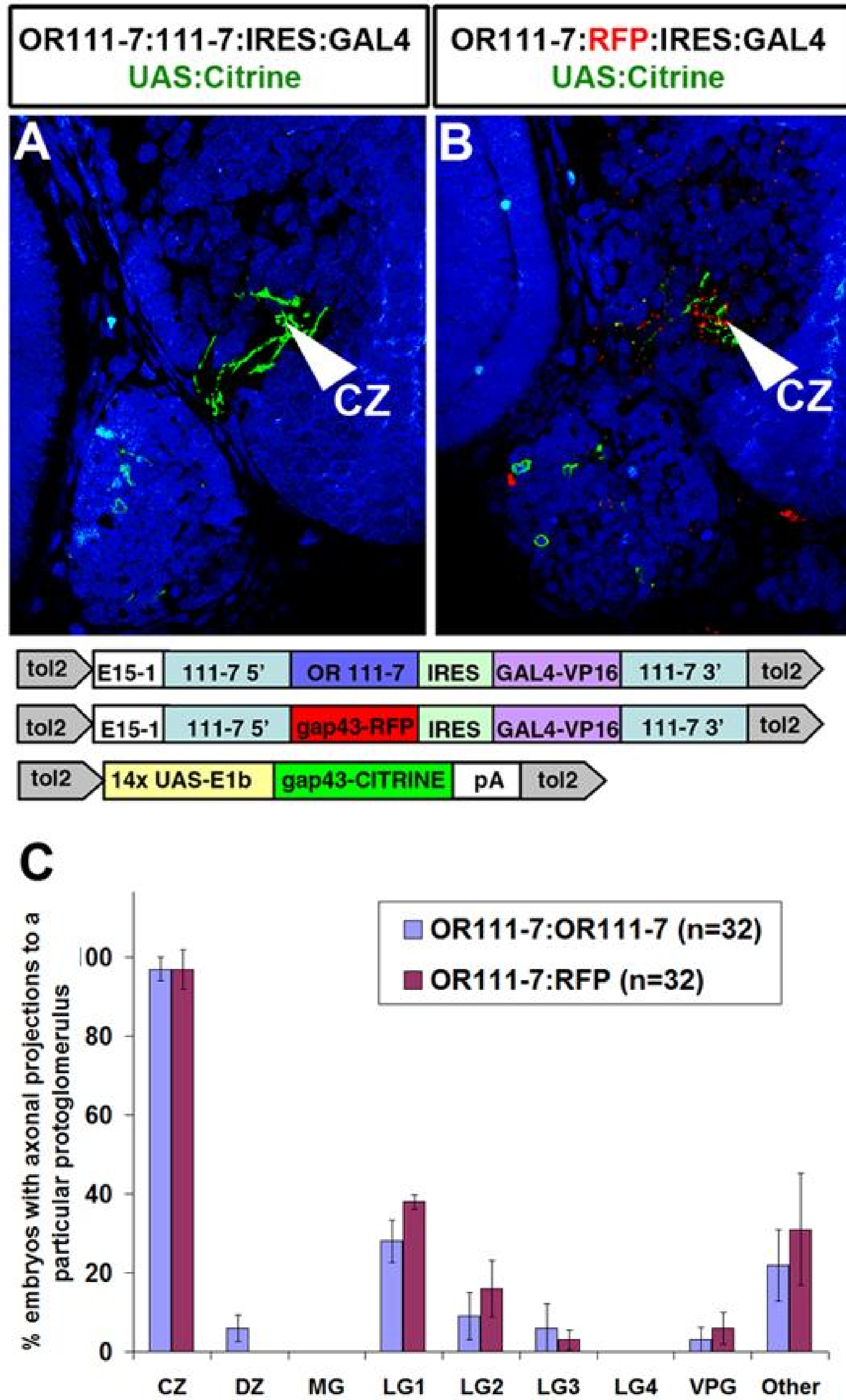

Figure 2.5. Replacing the OR111-7 coding region with mRFP in the OR111-7 transgenic construct does not alter axonal targeting to the central zone protoglomerulus

A, B, Single confocal optical sections through 3 day old OR111-7:111-7:IRES:Gal4; UAS:Citrine expressing larvae or OR111-7:mRFP:IRES:Gal4; UAS:Citrine expressing larvae (frontal view). Dorsal is to the top and midline to the right of the image. Propidium iodide (blue) delineates distinct protoglomeruli in the olfactory bulb. **A, B, C,** Both OR111-7:111-7:IRES:Gal4; UAS:Citrine expressing axons (green) and OR111-7:mRFP:IRES:Gal4; UAS:Citrine expressing axons (red) primarily target their axons to the central zone protoglomerulus. **C,** Axonal distribution to protoglomeruli does not differ between OR111-7:111-7:IRES:Gal4; UAS:Citrine expressing or OR111-7:mRFP:IRES:Gal4; UAS:Citrine expressing axons. Fisher's exact test was used to test for statistical significance, $P<0.05$.

CZ: *central zone,* DZ: *dorsal zone,* MG: *medial glomeruli 1-4,* LG1-4: *lateral glomeruli 1-4,* VPG: *ventral posterior glomerulus,* Other: *axonal projections to non-protoglomerular regions of the olfactory bulb.*

Chapter 3. NETRIN/DCC SIGNALING GUIDES OLFACTORY SENSORY AXONS TO THEIR CORRECT LOCATION IN THE OLFACTORY BULB

Vanisha Lakhina[1], Christina L. Marcaccio[1], Mark E. Lush[1,2], Roshan A. Jain[3], Esther Fujimoto[4,5,6], Joshua L. Bonkowsky[4,5], Michael Granato[3] and Jonathan A. Raper[1]

[1] Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

[2] Stowers Institute for Medical Research, 1000 50th Street, Kansas City, MO 64110, USA.

[3] Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

[4] Department of Neurobiology and Anatomy, University of Utah School of Medicine, Salt Lake City, Utah 84132

[5] Division of Pediatric Neurology, Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, Utah 84132

[6] Deceased

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ABSTRACT

Olfactory sensory neurons expressing particular olfactory receptors project to specific reproducible locations within the bulb. The axonal guidance cues that organize this precise projection pattern are only beginning to be identified. To aid in their identification and characterization, we generated a transgenic zebrafish line, OR111-7:IRES:Gal4, in which a small subset of olfactory sensory neurons is labeled. Most sensory neurons expressing the OR111-7 transgene project to a specific location within the bulb, the central zone protoglomerulus, while a smaller number project to the LG1 protoglomerulus. Inhibiting netrin/DCC signaling perturbs the ability of OR111-7 expressing axons to enter the olfactory bulb and alters their patterns of termination within the bulb. The netrin receptor DCC is expressed in olfactory sensory neurons around the time that they elaborate their axons, netrin1a is expressed near the medial-most margin of the olfactory bulb, and netrin1b is expressed within the ventral region of the bulb. Loss of netrin/DCC signaling components causes some OR111-7 expressing sensory axons to wander posteriorly after exiting the olfactory pit, away from netrin expressing areas in the bulb. OR111-7 expressing axons that enter the bulb target the central zone less precisely than normal, spreading away from netrin expressing regions. These pathfinding errors can be corrected by the re-expression of DCC within OR111-7 transgene expressing neurons in DCC morphant embryos. These findings suggest for the first time that netrins act as attractants for olfactory sensory neurons, first drawing OR111-7 expressing axons into the bulb and then into the ventromedially positioned central zone protoglomerulus.

INTRODUCTION

Olfactory sensory neurons in the olfactory epithelia of vertebrates stochastically choose to express a single specific receptor from a large repertoire of odorant receptors (ORs), thereby conferring a distinct molecular and functional identity upon each neuron. They project axons to the olfactory bulb where they synapse onto second order neurons. The axons of sensory neurons expressing the same OR converge into glomeruli that are located in reproducible locations within the olfactory bulb. This pattern of convergence into OR specific glomeruli is essential for normal olfactory perception (Sakano, 2010). Understanding how sensory axons find their way to their appropriate glomeruli is a key step in understanding how the olfactory system is assembled during development and how it functions when mature.

A large body of evidence suggests that the olfactory receptors themselves contribute to glomerulus formation and placement in the mouse olfactory bulb (Mombaerts et al., 1996; Wang et al., 1998; Imai et al., 2006). In addition, several canonical axonal guidance cues and their receptors have also been shown to contribute to sensory axon targeting in the bulb (Renzi et al., 2000; Schwarting et al., 2000, 2004; Taniguchi et al., 2003; Cutforth et al., 2003; Miyasaka et al., 2005; Cho et al., 2007; Takeuchi et al., 2010). However, the precise sequence of cues that direct sensory axons to their targets has not yet been defined. The potential guidance roles of many candidate cues have not even been examined. In this study, we explored the role of netrin/DCC signaling in olfactory sensory axon guidance. Netrin1 was one of the first axonal guidance cues to be identified (Hedgecock et al., 1990; Ishii et al., 1992), was the founding member of a small family of guidance cues (Serafini et al., 1994), and depending upon the receptors expressed by a responding axon, can act as either an attractant or a repellent (Hong et al., 1999).

In this study we define a role for netrins in the guidance of a small group of olfactory sensory neurons that project to two specific, identified locations in the olfactory bulb. We first generated lines of transgenic zebrafish in which the odorant receptor OR111-7 is expressed along with the transactivator GAL4. GAL4 expression in turn induced the expression of a fluorescent axonal tracer. The highly specific projection pattern of these labeled axons allowed us to analyze the effects of knocking down or misexpressing candidate guidance receptors and ligands.

Here we show that OR111-7 transgene expressing neurons express the netrin receptor DCC. Netrin1a is expressed medially while netrin1b is detected ventrally in the bulb. Loss of netrin/DCC signaling induces ectopic projections of transgene expressing axons both between the olfactory epithelium and the bulb and within the bulb itself. We show that netrins act directly on olfactory sensory neurons. Our results are consistent with netrins acting as attractants for a subset of olfactory sensory axons, drawing them both medially and ventrally. Thus, for the first time, netrin/DCC signaling is shown to contribute to the initial targeting of olfactory sensory axons and to be required for the generation of a correct protoglomerular map in the olfactory bulb.

MATERIALS AND METHODS

Transgenic zebrafish lines. OMP:lyn-RFP and TRPC2:gap-Venus transgenic lines (hereby referred to as OMP:RFP and TRPC2:Venus respectively) were obtained from the Yoshihara laboratory (Sato et al., 2005). The UAS:DCC transgenic line was generated using the full length DCC coding sequence with 7 of the first 27 base pairs mutated (ATGGGaTGtGTaACaGGcGAcATcCGC) so that it was resistant to the translation blocking morpholino we used in our experiments. This sequence was cloned

into a Gateway middle clone, pME-dcc$_m$, and confirmed by full-length sequencing (Lim et al., 2011). The UAS:DCC transgenesis clone was built using the Tol2 kit (Kwan et. al., 2007) and multisite Gateway (Invitrogen) recombination reactions. p5E-10xUAS, pME-dcc$_m$, and p3E-pA were recombined into pDestTol2CG2, which provided Tol2 transposon ends and a *cmlc2:*EGFP transgenesis marker. Injection of this DNA construct and generation of the stable transgenic line Tg(*UAS:dcc$_m$; myl7:EGFP*)^{zc79} was performed as described in Bonkowsky et al (2008).

The following constructs were cloned into the mini-Tol2 vector (Balciunas et al., 2006) and injected into single celled zebrafish embryos which were raised to adulthood (Fisher et al., 2006). Two or more independent founders were obtained for all transgenic lines made with the following constructs: (1) OMP:Gal4: The 2.1 kb OMP promoter, obtained from the Yoshihara laboratory (Sato et al., 2005), was cloned upstream from a Gal4 coding sequence. (2) OR111-7:IRES:Gal4: We designed our transgenic approach based on studies that were pioneered in the mouse (Vassalli et. al. 2002; Bozza et. al., 2009). In our construct (depicted in Figure 3.1), OR111-7 expression is regulated by its endogenous promoter and the E15-1 enhancer element which promotes odorant receptor expression (Nishizumi et al., 2007). The entire 4 kb of genomic sequence between the coding sequences of OR111-7 and its 5' upstream gene, OR111-8, were included in the construct (111-5 5'). An IRES:Gal4 cassette was inserted after the OR111-7 coding sequence. This was followed by 1 kb of untranslated genomic sequence downstream from the OR111-7 coding sequence (OR111-7 3'). (3) UAS:gap43-Citrine: 14 tandom UAS sequences were used to drive the expression of a citrine fluorophore that was fused with the first 20 amino acids of the GAP43 protein to promote axon labeling.

DCC mutant. A DCC mutant was isolated by an ENU induced screen (Granato et al., 1996), and mutant embryos were identified using standard PCR genotyping (Jain & Granato, in preparation).

Immunohistochemistry. Three day old zebrafish larvae were fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer. Larvae were treated with acetone for 20 minutes at -20° C to facilitate tissue permeabilization. Goat anti-GFP (1:100, Rockland Immunochemicals, catalog #600-101-215) and anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen) were used to visualize GFP-positive neurons. Propidium iodide staining was performed using the protocol of Brend and Holley (2009) with the omission of the RNase treatment step. Larvae were mounted face down or ventral side down to visualize projections along the dorso-ventral axis or antero-posterior axes respectively. Larvae were imaged using a 40X oil immersion lens on an inverted Leica TSP2 confocal microscope. Sections of 1 micron thickness were taken through the entire olfactory bulb.

Whole mount fluorescent in situ hybridization. Antisense digoxigenin labeled RNA probes for DCC, netrin1a, or netrin1b were generated using plasmids obtained from the Chien laboratory (Fricke and Chien 2005; Suli et al., 2006). *In situ* hybridization was performed as described previously (Chalasani et al., 2007). In situ signals were amplified using a cyanine 5-coupled tyramide system (TSA^{TM} Plus cyanine 5 System, Perkin Elmer, Product number NEL745001KT). *In situ* hybridization was followed by immunohistochemistry using goat anti-GFP (1:100, Rockland Immunochemicals, catalog #600-101-215) and anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen) to visualize axons in the context of RNA expression. This was followed by propidium iodide staining (as above). Processed larvae were mounted in the frontal or ventral orientation and imaged

using a 40X oil immersion lens on an inverted confocal microscope. Images were captured every micrometer through the entire extent of the olfactory bulb

Morpholino injections into zebrafish embryos. Morpholinos (MOs) were obtained from Gene Tools (Philomath, OR). They were suspended in autoclaved water at a final stock concentration of 5mM (~41.5 ng/nl) and were injected into the embryo yolk immediately after fertilization at the one cell stage. Morpholino sequences were identical to those used by Suli et al. (2006). 9 ng/embryo of either DCC MO1, DCC MO2, netrin1a MO, or netrin1b MO was injected along with 5 ng of p53 morpholino (Robu et al., 2007). The p53 MO was routinely added to reduce cell death occurring as a side effect of morpholino treatment.

Quantification of targeting errors. Larvae were processed for immunohistochemistry and imaged using confocal microscopy as described previously. The number of larval olfactory bulbs with axonal projections to either individual protoglomeruli or non-protoglomerular regions (posterior, ventral, midline, lateral or anterior) were counted. The Fisher exact test was used to determine whether the control group was statistically different from the experimental group. The graphs represent the percentage of larval olfactory bulbs with axonal projections to the specified locations.

RESULTS

Two general classes of olfactory sensory neurons project to specific defined neuropilar regions in the olfactory bulb

The zebrafish olfactory epithelium contains two well characterized sensory neuron populations that can be defined based on their position in the epithelium, their morphologies, and their molecular characteristics (Hamdani and Doving. 2007; Sato et

al., 2005). Ciliated olfactory sensory neurons have long dendrites and are located deep within the olfactory epithelium. They express classical main olfactory bulb type odorant receptors along with the olfactory marker protein OMP (Celik et al., 2002; Sato et al., 2005). Microvillous olfactory sensory neurons have shorter dendrites and are located more superficially within the epithelium. These neurons express V2R type vomeronasal receptors and also the transient receptor potential channel 2 (TRPC2). The axons of these two classes of OMP and TRPC2 expressing neurons project to non-overlapping regions of the olfactory bulb (Sato et al., 2005).

By 3 days post fertilization (3 dpf), OMP and TRPC2 projections have coalesced into distinct protoglomeruli (Figure 3.1; Sato et al., 2005). Protoglomeruli are specialized neuropilar regions comprised of olfactory sensory axons and the dendrites of olfactory bulb neurons (Li et al., 2005). Since they are the only acellular regions in the olfactory bulb, they can be demarcated using cellular dyes such as BODIPY that label cells within the bulb while leaving protoglomeruli unlabeled (Dynes and Ngai, 1998). Individual protoglomeruli identified in this manner are stereotyped and consistently recognizable between larvae. In this study, we have used the protoglomerular nomenclature proposed by Dynes and Ngai (1998).

We used propidium iodide instead of BODIPY to label cells (presented in the blue color channel) and were able to identify 12 protoglomeruli as unstained regions in the olfactory bulb surrounded by olfactory bulb cells (Figure 3.1). Using OMP:RFP and TRPC2:Venus double transgenic animals in combination with propidium iodide staining, we have identified the specific protoglomeruli that are targeted by either OMP or TRPC2 expressing axons in 3 day old olfactory bulbs (Figure 3.1A-C, schematized in Figure 3.1D). OMP expressing neurons (green) project to the central zone, dorsal zone, and

lateral glomerulus 3 (LG3). A few OMP expressing axons also innervate the medial glomeruli 1-4 (MG1-4). TRPC2 expressing neurons (red) target the olfactory plexus (OP), lateral glomeruli 1, 2 and 4 (LG1, LG2, LG4), and the ventero-posterior glomerulus (VPG). This pattern of targeting is repeatable from fish to fish, demonstrating that the axons of specific subsets of olfactory neurons target particular protoglomeruli. Aside from the small OMP projection, the MG protoglomeruli are unfilled by axons labeled in the OMP and TRPC2 transgenics. It is possible that the MG protoglomeruli receive axonal input from other less well characterized classes of olfactory sensory neurons, perhaps the recently identified TAAR and/or V1R expressing neurons (Hussain et al., 2009; Saraiva and Korsching, 2007).

Construction of a transgenic line that expresses OR111-7 under the control of its flanking control sequences

The study of axonal targeting in the zebrafish system has been impeded by an inability to visualize the axonal projections of sensory neurons that express a single odorant receptor. Inspired by the transgenic approach pioneered in the mouse by Vassalli et. al. (2002), we generated two independent transgenic zebrafish lines in which a specific odorant receptor, OR111-7, is expressed along with a reporter under the control of its flanking non-coding sequences. We expected that transgene expressing sensory neurons would project axons to a single specific protoglomerulus. We chose to study OR111-7 because its expression is detected around 30 hours post fertilization (hpf) when some of the earliest axons are extending into the olfactory bulb (Barth et al., 1997). Our construct is schematized in Figure 3.1 (bottom left) and is composed of the following elements: (1) the E15-1 enhancer element identified by Nishizumi et al. (2007) that is near the OR111 gene family cluster and acts in cis to promote odorant receptor expression, (2) 4 kb of genomic sequence upstream to the OR111-7 coding sequence

(OR 5'), (3) The OR111-7 coding sequence, (4) an IRES sequence followed by a sequence encoding the transactivating factor Gal4/VP16, and (5) 1kb of genomic sequence located just 3' to the OR111-7 coding region. This construct links olfactory receptor expression to the simultaneous expression of the transactivating factor Gal4. Two independent transgenic lines were made with this construct and appear equivalent in their properties.

OR111-7:IRES:Gal4 transgenic fish were mated with fish containing a UAS driven Gap43-Citrine transgene. The fluorescent fusion protein is transported into axons and brightly labels axonal projections. Those progeny that contain both transgenes have a small number of labeled cells that are restricted exclusively to the olfactory epithelium. As would be expected, fluorescently labeled cells are also labeled *in situ* with a probe for OR111-7 (data not shown). They elaborate axons that extend into the olfactory bulb. OR111-7 transgene expressing neurons are first detected around 26-27 hpf. Surprisingly, we observe that the number of labeled transgene expressing neurons decreases with developmental age. At 3 dpf, an average of about 27 neurons in each olfactory pit express the OR111-7 transgene, but only 13 neurons in 7 dpf larvae, and 6 neurons in 9 dpf larvae express the transgene. The decline in transgene expressing neurons suggests that it may be inactivated over time. It is possible that regulatory elements required for continued stable expression of the transgenic odorant receptor are missing in our transgene construct. Alternatively, the expression of endogenous odorant receptors may suppress the expression of the transgenic odorant receptor through processes analogous to those that limit odorant receptor expression to a single allele in each sensory neuron (Shykind, 2005).

The axons of OR111-7 transgene expressing olfactory sensory neurons project to the Central zone protoglomerulus and sometimes to the LG1 protoglomerulus

At 3 dpf the majority of axons extending from transgene expressing neurons converge within the bulb upon the Central zone protoglomerulus (Figure 3.1E-H). This is reminiscent of a previous study in which the OR111-7 coding sequence was replaced with a YFP reporter in a BAC transgenic animal and the resulting YFP labeled axons were shown to target a ventromedial area likely to be the central zone protoglomerulus (Sato et al., 2007). The central zone protoglomerulus is large and ventrally positioned with its longest axis oriented anterior-posteriorly. It is thought to be composed of approximately five smaller “sub”-protoglomeruli (Dynes and Ngai, 1998). At 3 dpf OR111-7 transgene expressing axons are not localized to any specific sub-compartment within the central zone. Rather, axons are distributed and appear to terminate throughout the whole anterior to posterior extent of the protoglomerulus. In about 60% of larvae, a small number of OR111-7 transgene expressing axons project to LG1. This was surprising since LG1 is normally innervated exclusively by TRPC2 and V2R type receptor expressing axons (Figure 3.1A-D). Crossing the OR111-7 transgene into an OMP:RFP background, we found that the axons of all OR111-7 and OMP transgene expressing neurons project to the central zone, suggesting that these OMP and OR111-7 expressing cells are ciliated olfactory sensory neurons. A small number of sensory neurons expressing only the OR111-7 transgene project to LG1 (see figures 3.1E, F; 3.3A, D, G, J; 3.4A, E, M; 3.6A; 3.7A, E and 3.9A). These results are consistent with the idea that the OR111-7 transgene is largely expressed in OMP type neurons that project exclusively to the central zone, and less frequently in TRPC2 and V2R type neurons that extend to LG1. Since the predominant projection of OR111-7 transgene expressing neurons is to the central zone, these transgenic fish are a valuable tool for testing the roles of candidate guidance cues in targeting axons to a consistent and identifiable

location in the developing olfactory bulb. In this study we assessed the contribution of netrin/DCC signaling in properly targeting axons to the central zone.

DCC is expressed in OMP and OR111-7 transgene expressing olfactory sensory neurons

OMP:Gal4 expressing olfactory sensory axons (green, Figure 3.2A) have entered the primordial olfactory bulb in 24 hpf embryos. At this time, there are on average of 24 labeled olfactory sensory neurons in each olfactory pit and approximately 75% of them express DCC mRNA (red, arrowheads in Figure 3.2A. N=241 neurons from 8 olfactory pits). The axons of OR111-7 transgene expressing neurons enter the bulb by 27 hpf. At this age, there are on average 8 labeled olfactory sensory neurons in each olfactory pit and approximately 87% of them express DCC mRNA (red, arrowheads in Figure 3.2B, N=87 neurons from 11 olfactory pits). At 53 hpf, when axons have already reached the presumptive central zone, there are on average 23 Citrine labeled OR111-7 transgene expressing neurons and approximately 35% of them express DCC mRNA (data not shown, N=232 neurons from 10 olfactory pits). These results indicate that the netrin receptor DCC is expressed in OMP expressing sensory neurons and in most OR111-7 transgene expressing neurons early as their axons are growing into the olfactory bulb.

Netrin1a is expressed at the telencephalic midline and netrin1b is expressed ventrally in the olfactory bulb

Netrin1a and netrin1b expression patterns were examined along the dorso-ventral axis (in frontal views) and along the antero-posterior axis (in ventral views). At 53 hpf, netrin1a mRNA (red) is expressed in a dense strip of cells near the margin of the bulb at the telencephalic midline (arrow, Figure 3.2C) all along the antero-posterior axis (arrow, Figure 3.2E). At the same age, netrin1b mRNA (red) is present more ventrally in close

proximity to where olfactory sensory axons enter the bulb (green, arrows in Figure 3.2D, F). These findings suggest that these two secreted netrins are expressed at a time and in a place where they could influence the trajectories of olfactory sensory axons.

OR111-7 transgene expressing sensory neurons misproject into dorsal protoglomeruli in DCC morphants and in a DCC mutant

We next examined whether reducing DCC expression affects the protoglomerular targeting of OR111-7 transgene expressing axons. Two separate approaches were employed to decrease DCC levels *in vivo*. First, OR111-7:IRES:Gal4 and UAS:Citrine transgenic fish were crossed together. The resulting embryos were injected with one of two different translation blocking morpholinos, DCC MO1 or DCC MO2, both of which were previously characterized by Suli et al. (2006). OR111-7:IRES:Gal4; UAS:Citrine double transgenic larvae with fluorescent olfactory sensory neurons were identified and processed for immunohistochemistry and propidium iodide staining. Axonal projections of uninjected controls were compared with larvae that were injected with morpholinos (morphants). Second, we examined the projections of OR111-7 transgenic axons in larvae with a hypomorphic DCC allele that has a missense mutation in the DCC coding sequence (Granato et al., 1996; Jain and Granato, in preparation). Adult OR111-7:Gal4; UAS:Citrine double transgenics that carried a single DCC mutant allele were generated. Mating these transgenics with their siblings generated fluorescent progeny of which 25% were also homozygous mutant for DCC. Genomic DNA was extracted from the tails of 3 day old fluorescent larvae to test for the presence of the DCC mutant allele while their heads were processed for immunohistochemistry. Axonal projections in individual mutant larvae were compared to siblings that carried wild type DCC alleles. In general, the axonal guidance phenotypes observed in morphant and mutant fish are similar but stronger in morphants. In both DCC morphants and DCC mutant larvae, axons of

OR111-7 transgene expressing axons misproject into the dorsal zone (DZ) and Medial (MG) protoglomeruli (Figure 3.3A-F, G-L, M, N, quantified in Figure 3.5A-C). These errors were incompletely penetrant, but consistent in all three experimental conditions. Projections to dorsal protoglomeruli are rare in control larvae. Additionally, DCC MO1 or MO2 morphants also show increased axonal misprojections into the lateral glomeruli LG2 and LG3 (Figure 3.5A, B). Since we observed similar misprojections using two different morpholinos to DCC and confirmed dorsal misprojections in the DCC mutant, we are confident that these errors are not caused by off target effects of the morpholinos or undefined genetic variation in the mutant fish.

OR111-7 transgene expressing sensory neurons misproject into dorsal protoglomeruli in netrin1b morphants

We next tested whether decreasing levels of the likely ligands of the DCC receptor, netrin1a or netrin1b, phenocopy the targeting errors induced in DCC knockdown fish. Netrin1a or netrin1b were targeted with morpholinos previously characterized by Suli et al. (2006). Knockdown of netrin1b alone induces axonal misprojections of OR111-7 transgene expressing neurons into both the DZ protoglomerulus (Figure 3.4A, C, E, G, Q, S, quantified in Figure 3.5E), and the MG protoglomerulus (Figure 3.4I, K, M, O, Q, S, quantified in Figure 3.5E). This observation recapitulates the finding that mistargeting to these more dorsal protoglomeruli is greatly enhanced when DCC expression is reduced. In contrast, knockdown of netrin1a did not induce any significant axonal mistargeting of OR111-7 transgene expressing neurons to any protoglomeruli (Figure 3.4A, B, E, F, I, J, M, N, Q, R, quantified in Figure 3.5D). Knockdown of both netrin1a and netrin1b induced the same protoglomerular mistargeting as netrin1b knockdown alone (Figure 3.4A, D, E, H, I, L, M, P, Q, T, quantified in Figure 3.5F). We presume that the netrin1a morpholino is at least partially effective since its knockdown causes other guidance errors (Figure

3.7A, B, E, F, I, J, M, N, quantified in Figure 3.8D). These data suggest that netrin1b works together with DCC to help target the axons of OR111-7 transgene expressing axons ventrally.

Netrin/DCC signaling contributes to the entry of olfactory sensory axons into the olfactory bulb

In addition to mistargeting of protoglomeruli by the axons of OR111-7 transgene expressing neurons in DCC and netrin1b knockdown larvae, we also observed errors in transgene labeled axon trajectories between the olfactory epithelium and the bulb. Normally, olfactory axons exit the epithelium together in a tight bundle and traverse the short distance between the epithelium and the bulb by the most direct route. In contrast, in a majority of DCC MO1 or DCC MO2 treated larvae, the axons of at least some OR111-7 transgene expressing neurons project posteriorly and away from the olfactory bulb (Figure 3.6B; 'posterior' category in Figure 3.8A, B). Even in these animals, however, the majority of labeled sensory axons enter the bulb normally where they make the targeting errors described above. The same posterior misprojections between the olfactory epithelium and the bulb are observed at a much lower but still statistically significant rate in DCC mutant fish (Figure 3.6C, 'posterior' category in Figure 3.8C). Additional non-protoglomerular misprojections are detected in DCC knockdown fish including small numbers of axons extending along the lateral-most margin of the bulb (Figure 3.6E, F, H, Figure 3.3N, 'lateral' category in Figure 3.8A-C). Overall, the axons of a larger number OR111-7 transgene expressing neurons end in a variety of locations outside protoglomeruli in DCC knockdown as compared to normal larvae.

Very similar results were obtained in larvae injected with netrin1a or netrin1b morpholinos, or both morpholinos together. Frequently the axons of OR111-7 transgene expressing neurons were observed to turn posteriorly away from the bulb after exiting

the olfactory epithelium (Figure 3.7A-D, M-P, 'posterior' category in Figure 3.8D-F). Netrin1a or netrin1b knockdown was also observed to induce ectopic anterior trajectories of transgene labeled axons (Figure 3.7E-G, M-O, 'anterior' category in Figure 3.8D,E). Treatment of embryos with netrin1a or with both netrin 1a and netrin1b morpholinos together induced ventral misprojections of transgene labeled axons (Figure 3.7I, J, L, Figure 3.4Q, R, T, 'ventral' category Figure 3.8D, F). The netrin1a and netrin1b morpholinos together induced an even higher rate of ectopic ventral, lateral, and anterior misprojections than either morpholino alone (Figure 3.7D, H, L, P, Figure 3.8D-F). The parallel findings that both DCC and netrin knockdown induce similar misguidance effects supports a role for netrin/DCC signaling in the axonal pathfinding of OR 111-7 transgene expressing sensory neurons.

Expression of DCC in OR111-7 expressing neurons corrects mistargeting induced by DCC knockdown.

DCC is expressed in the olfactory epithelium, including most of the OR111-7 transgene expressing subpopulation, and also in the olfactory bulb. In principle, netrin or DCC knockdown could affect sensory axon pathfinding either through a direct action on sensory axons themselves, or indirectly by influencing the development of netrin sensitive cells within the bulb. To determine whether the axonal pathfinding defects we observed upon DCC knockdown were cell-autonomous to the OR111-7 transgene expressing sensory neurons, we sought to increase DCC levels within this subpopulation in DCC morphants using a UAS:DCC transgenic line (Lim et al., 2011). The UAS driven DCC gene was modified so that it cannot be knocked down by the translation blocking DCC morpholino that we used in this study (See Methods). The UAS:DCC transgenic construct contains a *cardiac myosin light chain2 (cmlc2)* promoter driving expression of mCherry. Thus, the presence of the UAS:DCC transgene was detected by selective red

fluorescence in cardiac cells. OR111-7:IRES:Gal4;UAS:Citrine double transgenics were crossed to the UAS:DCC transgenic and injected with DCC MO1.

This generated two sets of larvae with fluorescently labeled olfactory sensory neurons: a set that overexpressed DCC in OR111-7 transgene and Citrine expressing neurons and a set that did not. Within each set, larvae were further subdivided into those injected with DCC morpholino at the single cell stage or those that were not. Altogether there were a total of four sets of larvae: uninjected or morpholino injected larvae that either overexpressed DCC and Citrine or just Citrine alone exclusively in OR111-7 transgene expressing neurons. We compared the axonal trajectories of uninjected fish that did not express the DCC transgene (Figure 3.9A, E, I, M, red bars in Q and R), fish injected with a DCC morpholino that did not express the DCC transgene (Figure 3.9B, F, J, N, blue bars in Q and R), uninjected fish that expressed the DCC transgene (Figure 3.9C, G, K, O, green bars in Q and R), and fish injected with a DCC morpholino that expressed the DCC transgene (Figure 3.9D, H, L, P, white bars in Q and R). As expected, knocking down DCC levels induced posterior misprojections (Figure 3.9A, B, blue ‘Post’ bar in Q), and also dorsal misprojections to the DZ and MG protoglomeruli (Figure 3.9I, J, M, N, R, blue ‘DZ’ and ‘MG’ bars in R). These morpholino induced errors were partially corrected to a significant degree in larvae that over-expressed DCC only in the OR111-7 transgene expressing neurons (Figure 3.9D, H, L, P, white ‘Post’ bar in Q, white ‘DZ’ and ‘MG’ bars in R). Overexpression of DCC in the OR111-7 expressing neurons of non-morpholino treated fish had no significant effect on these particular errors. But interestingly, overexpression of DCC in OR111-7 expressing neurons did increase the chance that their axons would misproject ventrally and to the midline (Figure 3.9G, O, green ‘Vent’ and ‘Mid’ bars in Q). These results are consistent with a requirement for DCC in the subset of olfactory sensory neurons that express the OR111-7 transgene for

correct targeting of the central zone protoglomerulus. They also suggest that the overexpression of DCC in the same cells can drive their axons closer to the midline and ventral margin of the olfactory bulb where netrins are expressed, further supporting the idea that netrins act as attractants for these axons.

DISCUSSION

Vertebrate olfactory sensory neurons choose to express a single odorant receptor from a large genomic repertoire. This choice of odorant receptor determines the ligand specificity of the neuron. The axons of neurons that express a particular odorant receptor reproducibly target selected glomeruli in the olfactory bulb. Odorants evoke a characteristic activation pattern of one or more specific glomeruli, revealing an underlying coding mechanism for odorants in the primary olfactory circuit (Rubin and Katz., 1999; Belluscio and Katz., 2001; Friedrich and Korsching, 1997; 1998). The formation of an accurate map of olfactory sensory axons into reproducibly located glomeruli in the bulb requires that sensory neurons expressing particular receptors be guided to specific locations in the bulb. However, they do not converge into glomeruli immediately, but first form protoglomerular condensations from which individual glomeruli segregate over time.

Protoglomeruli have been observed not only in zebrafish (Li et al., 2005), but also in insects and rodents (Oland et al., 1990; Treloar et al., 1999; Conzelmann et al., 2001). There are 12 distinct, identifiable protoglomeruli in 3 day old zebrafish olfactory bulbs (Dynes and Ngai, 1998). They are innervated by at least two classes of olfactory sensory neurons in the olfactory epithelium: ciliated neurons expressing classical ORs along with the olfactory marker protein (OMP), and microvillous neurons expressing V2R type vomeronasal receptors along with the transient receptor potential channel 2 (TRPC2).

Selective expression of fluorophores directed by OMP or TRPC2 promoters in transgenic animals showed that OMP and TRPC2 axons target distinct protoglomeruli in the bulb (Sato et. al., 2005). Our work extends these findings by defining the particular protoglomeruli innervated by these two general classes of sensory axons. The OMP expressing class of sensory axons terminate in the central zone, dorsal zone, lateral protoglomerulus 3, and a very few axons in MG 1-4 protoglomeruli. TRPC2 expressing axons terminate in the olfactory plexus, lateral protoglomeruli 1, 2 and 4; and the ventro-posterior protoglomerulus. Our work further suggests that there is at least one additional class of sensory neurons that express neither OMP nor TRPC2. Since the medial protoglomeruli are only very sparsely innervated by either of these two classes, it is attractive to hypothesize that they are innervated by olfactory sensory neurons expressing the recently discovered TAAR and/or V1R class of receptors (Hussain et al., 2009; Saraiva and Korsching, 2007).

Our results reveal that the netrin receptor DCC is required cell autonomously within a small subpopulation of sensory neurons for their axons to correctly target the central zone protoglomerulus. Reducing the expression or activity of DCC, netrin1a, or netrin1b interferes with the ability of axons from the OR111-7 transgene expressing subpopulation of neurons to enter the olfactory bulb. These findings are consistent with an attractive function for netrin1a expressed at the midline and netrin1b expressed ventrally where sensory axons enter the bulb. Reducing DCC or netrin1b levels induces the axons of transgene expressing neurons that enter the bulb to mistarget dorsal protoglomeruli. These data suggest that netrin1b expressed in the ventral portion of the bulb helps draw these axons into the central zone protoglomerulus and prevents them from wandering into more dorsal protoglomeruli. Altogether, we propose that a combination of midline netrin1a and ventral netrin1b signaling helps to guide this specific

population of axons into the bulb and to terminate within the central zone protoglomerulus.

Netrin/DCC signaling has been shown to mediate attractive effects in a wide variety of axons in invertebrates (Hedgecok et al., 1990; Mitchell et al., 1996; Kolodziej et al., 1996), in vertebrate central commissures (Kennedy et al., 1994, Fazeli et al., 1997; Serafini et al., 1996), and in non-commissural projections in vertebrates including corticofugal and thalamocortical axons (Metin et al., 1997; Braisted et al., 2000). DCC has been detected on rat olfactory axons that are extending towards the telencephalon and netrin1 is present along their trajectory, suggesting that netrin/DCC signaling could potentially play a role in early olfactory map formation in rodents (Astic et al., 2002). Netrins can bind and act through other alternative receptors. Unc-5, in combination with DCC or on its own, mediates a repulsive response to netrins (Hong et al., 1999). In principle, a repellent reaction to netrin expressed at the midline in zebrafish could help guide axons that terminate in more lateral protoglomeruli. There are three known Unc5 genes in zebrafish: Unc5a, Unc5b, and Unc5c. We did not detect any of their mRNAs by *in situ* hybridization in the developing olfactory epithelium. Unc5a and Unc5c mRNA were, however, detected in the developing olfactory bulb along with DCC mRNA. It is therefore unlikely that Unc5 mediated repellent responses to netrins guide olfactory sensory axons within the bulb, but it remains a possibility that repellent netrin signaling contributes to the protoglomerular targeting of mitral cell dendrites.

Several other traditional guidance cues have been implicated in organizing the mouse glomerular map. The repulsive cues slit1 and slit3 are present in the ventral olfactory bulb in mice while OSNs in the epithelium express the slit receptor, Robo2. In both Robo2 mutant mice and slit1 mutant mice, a subset of OSNs that normally targets the

dorsal region of the bulb misprojects ventrally and forms ectopic glomeruli (Cho et al., 2007). Some olfactory glomeruli in Robo2 mutant zebrafish are either absent or ectopically located. This phenotype is thought to be a consequence of the gross protoglomerular disruption observed in Robo2 mutant larvae (Miyasaka et al., 2005). We have examined the trajectories of OR111-7 transgene expressing axons in zebrafish embryos with a null mutation in robo-2 and find no consistent errors (data not shown). However, our results do not exclude the possibility that the targeting of other olfactory sensory axons is affected by the loss of robo-2 mediated signaling.

The repulsive ligand Sema3F is expressed in the dorsal bulb of mice and repels axons that express its receptor, neuropilin2, thus confining these axons to the ventral bulb. Knocking down either Sema3F or neuropilin2 in olfactory sensory neurons mistargets ventrally projecting axons to the dorsal olfactory bulb (Takeuchi et al., 2010). Sema3a is expressed in mice by ensheathing cells in the nerve layer of the ventral olfactory bulb while some olfactory sensory neurons express its receptor, neuropilin1a. Neuropilin1a positive axons extend to the lateral region of the anterior olfactory bulb and medial region of the posterior bulb, avoiding Sema3A expressing regions. In Sema3A deficient mice, many neuropilin1a positive glomeruli are ectopically positioned in the anteromedial and ventral bulb (Schwarting et al., 2000, 2004; Taniguchi et al., 2003). The repulsive guidance mediated by neuropilin-1 receptor signaling in olfactory sensory axons seems to be conserved to some degree across vertebrate species. Chick olfactory sensory neurons expressing a dominant negative neuropilin1 enter the brain prematurely and overshoot semaphorin expressing regions in the bulb to project into the forebrain (Renzi et al., 2000). Finally, deleting ephrinA3 and ephrinA5 together in mice shifts glomeruli formed by SR1- or P2- expressing olfactory sensory neurons posteriorly, while

overexpression of ephrinA5 specifically in P2 neurons shifts its glomerulus anteriorly (Cutforth et al., 2003).

Although studies in the mouse have identified several cues that contribute to olfactory axon guidance, the complexity and inaccessibility of its developing olfactory system has limited most observations to postnatal animals in which the glomeruli are almost fully differentiated. Thus the developmental contribution of these signaling molecules to initial targeting is not always clear, and the processes that drive the segregation of axons into glomeruli may confound the analysis of some axonal guidance mutants. By comparison, the zebrafish is an excellent vertebrate system in which to study the targeting of olfactory sensory axons to the olfactory bulb. The system is relatively simple, with approximately 150 ORs as compared to more than 1000 in mice (Alioto and Ngai, 2005). Development is rapid, with initial axon targeting occurring within the first three days of fertilization (Whitlock and Westerfield, 1998; Wilson et al., 1990; Sato et al., 2005). The embryo is small and transparent, making it possible to observe sensory axon pathfinding even in a living animal (Dynes and Ngai, 1998). Most importantly, sensory axons entering the bulb first terminate in distinct individually identifiable neuropilar protoglomeruli.

Our results highlight the advantages of a relatively simple vertebrate olfactory system for studying the initial targeting of olfactory sensory axons to defined protoglomeruli in the olfactory bulb. In this study, we identified for the first time in any model system, an important role for netrin/DCC signaling in the correct initial targeting of axons from the olfactory epithelium to the bulb and within the bulb itself. To deduce the overall molecular logic underlying protoglomerular targeting, it will be necessary to generate additional transgenic lines that label axons that target protoglomeruli other than the central zone. Using the OR111-7 transgenic line and additional lines as they become

available, it will be possible to assess the specific contributions of almost any candidate guidance cue to the mapping of specific olfactory sensory neurons to their targets in the olfactory bulb.

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Figure 3.1.

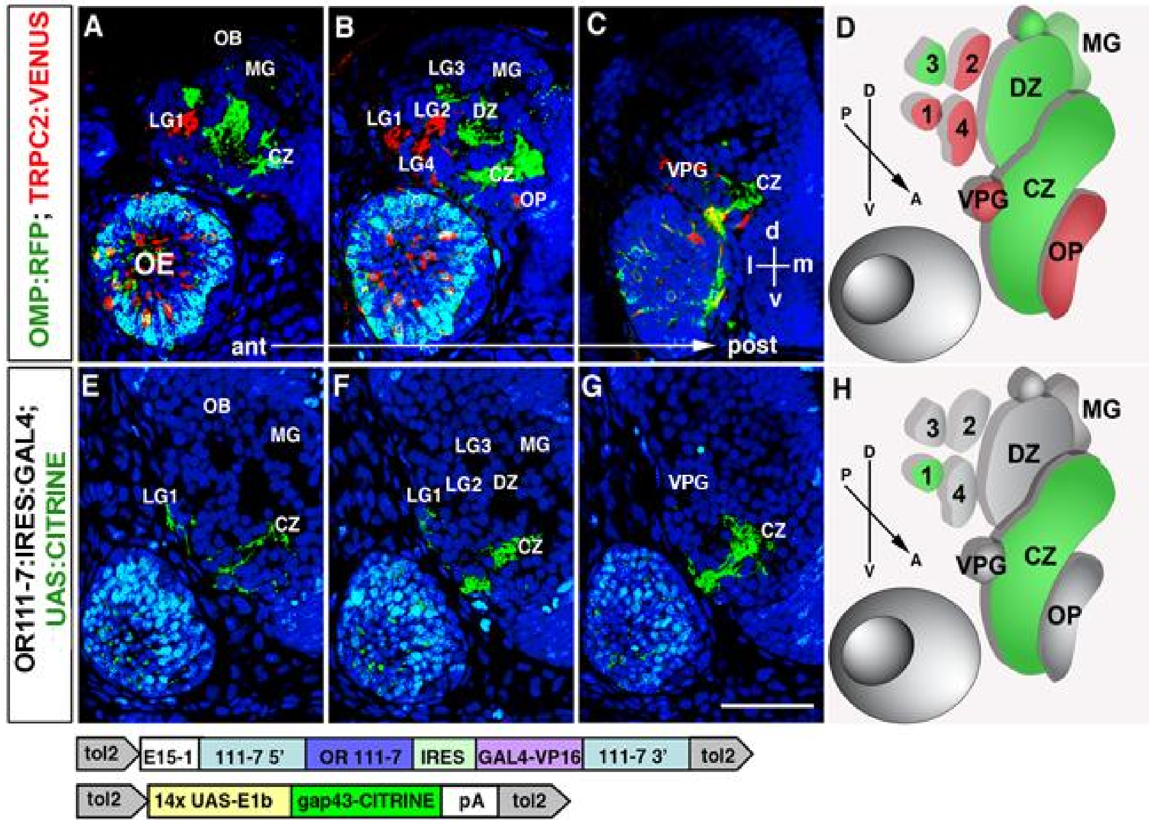


Figure 3.1. Olfactory sensory axons converge into distinct identifiable protoglomeruli in the 3 day old zebrafish olfactory bulb.

A-C, E-G, Single confocal sections through a 3 day old olfactory bulb (frontal view). The absence of propidium iodide staining (blue) delineates distinct protoglomeruli in single sections along the antero-posterior axis. **A-D,** In OMP:RFP; TRPC2:Venus double transgenics, OMP:RFP (green) labeled sensory axons project to the CZ, DZ, MG and LG3 protoglomeruli while TRPC2:Venus (red) labeled axons project to the VPG, OP, LG1, LG2 and LG4 protoglomeruli. **E-H,** OR111-7:IRES:Gal4; UAS:Citrine expressing axons primarily target the CZ protoglomerulus. Some OR111-7 transgene expressing axons project to the LG1 protoglomerulus. CZ: *central zone,* DZ: *dorsal zone,* MG: *medial glomeruli 1-4,* LG1-4: *lateral glomeruli 1-4,* VPG: *ventral posterior glomerulus,* OP*: olfactory plexus*. Scale bar in G: 50 μm.

Figure 3.2.

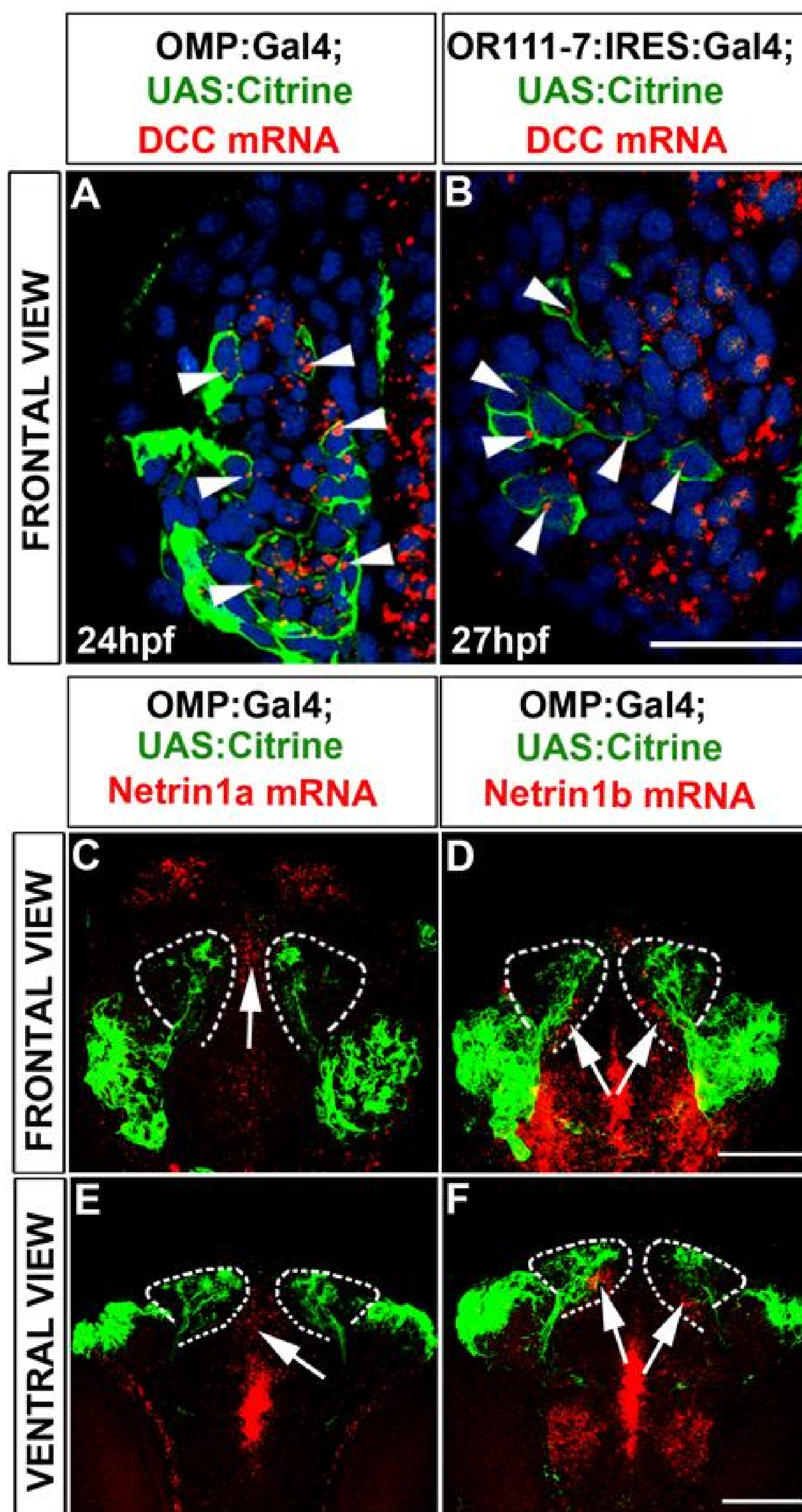


Figure 3.2. DCC is detected in OR111-7 transgene expressing neurons while Netrin1a is expressed at the telencephalic midline and netrin1b is present ventrally within the olfactory bulb

A, B Propidium iodide (blue) demarcates cells in the olfactory epithelium in frontal view of zebrafish embryos. **A,** DCC mRNA (red, arrowheads) is detected in OMP:Gal4; UAS:Citrine (green) neurons at 24 hours post fertilization (hpf) as axons first extend into the olfactory bulb. **B,** At 27 hpf, most OR111-7 transgene expressing neurons (green) express DCC mRNA (red, arrowheads). **C-F,** Maximum intensity projections of serial confocal optical sections through OMP:Gal4; UAS:Citrine transgenic embryos. **C, D,** In frontal views, dorsal is to the top and ventral towards the bottom of the image. **E, F,** In ventral views, anterior is to the top and posterior towards the bottom. **C, E,** Netrin1a mRNA (red) is detected at the telencephalic midline along the antero-posterior axis of the olfactory bulb (arrow). **D, F,** Netrin1b mRNA is expressed within the ventral region of the olfactory bulb (arrow), close to the presumptive central zone. The olfactory bulb is outlined with dashed lines. Scale bar in B: 25 μm; D, F: 50 μm.

Figure 3.3.

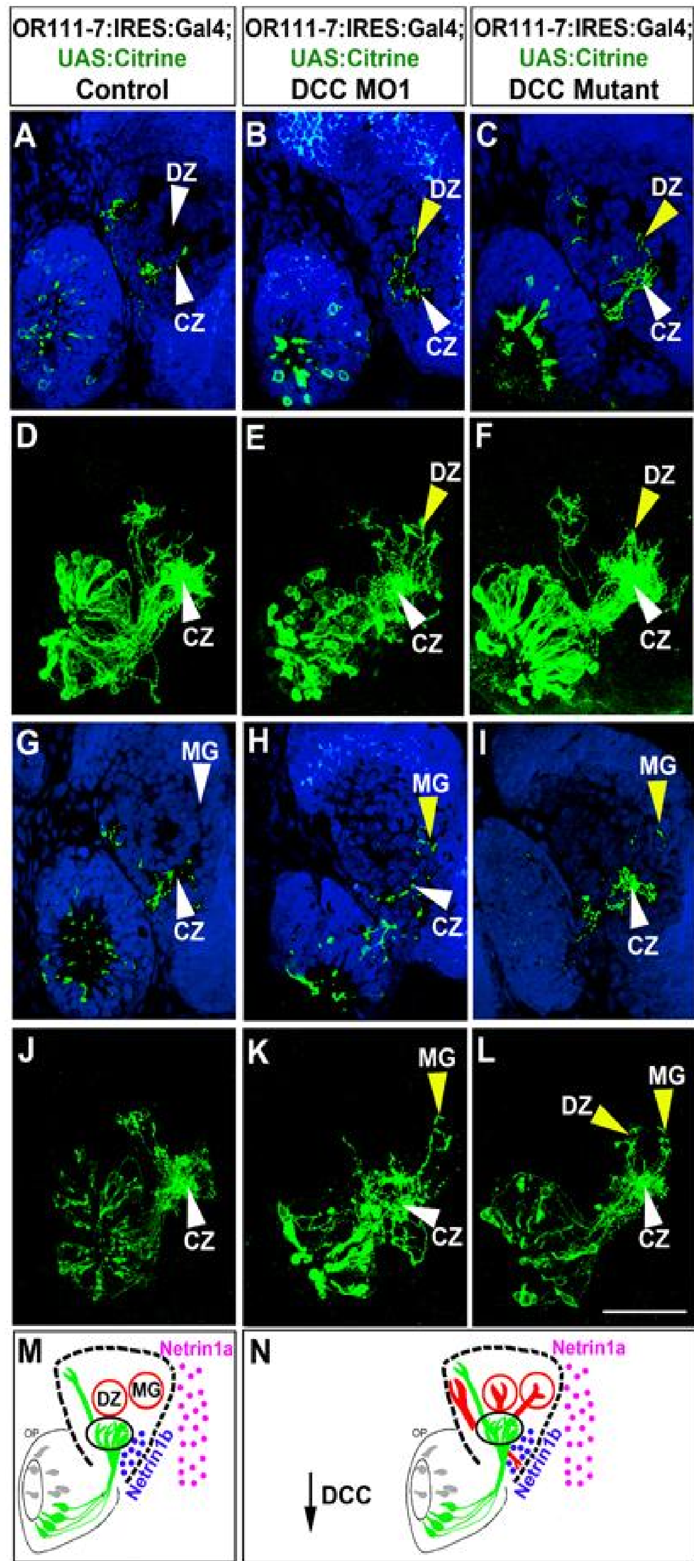


Figure 3.3. OR111-7 transgene expressing sensory neurons misproject into dorsal protoglomeruli in DCC morphants and in a DCC mutant

A-C, G-I, Single confocal optical sections through 3 day old OR111-7:IRES:Gal4; UAS:Citrine larvae (frontal view). Dorsal is to the top and midline towards the right of the image. Propidium iodide (blue) allows the identification of distinct protoglomeruli. **D-F, J-L,** Maximum intensity projections of serial confocal optical sections from the same larvae shown immediately above. **M, N,** Schematics showing control OR111-7:IRES:Gal4; UAS:Citrine projections (green) and mistargeted axons (red) observed upon DCC knockdown. The thickness of the red lines corresponds roughly to the penetrance of the indicated phenotypes. Netrin1a mRNA (pink dots) is expressed at the telencephalic midline and netrin1b (blue dots) is expressed in the ventral bulb. Black circle = central zone, Red circles = dorsal zone, medial glomeruli. **A, D, G, J, M,** In uninjected embryos, OR111-7 transgene expressing axons (green) target the central zone (CZ, white arrowhead) and LG1. **A, M,** Control OR111-7 transgenic axons do not enter the dorsal zone (DZ, white arrowhead) **B, E, C, F, N,** In embryos injected with DCC MO1 or in DCC mutants, OR111-7 transgenic axons target the CZ (white arrowheads), but also inappropriately enter the dorsal zone (DZ, yellow arrowheads). **G, M,** Uninjected control OR111-7 transgene expressing axons do not enter the Medial Glomerulus (MG, white arrowhead) **H, I, K, L, N** OR111-7 transgene expressing axons misproject into MG and DZ protoglomeruli in DCC morphants and DCC mutant larvae (yellow arrowheads). Scale bar in L: 50 μm.

Figure 3.4.

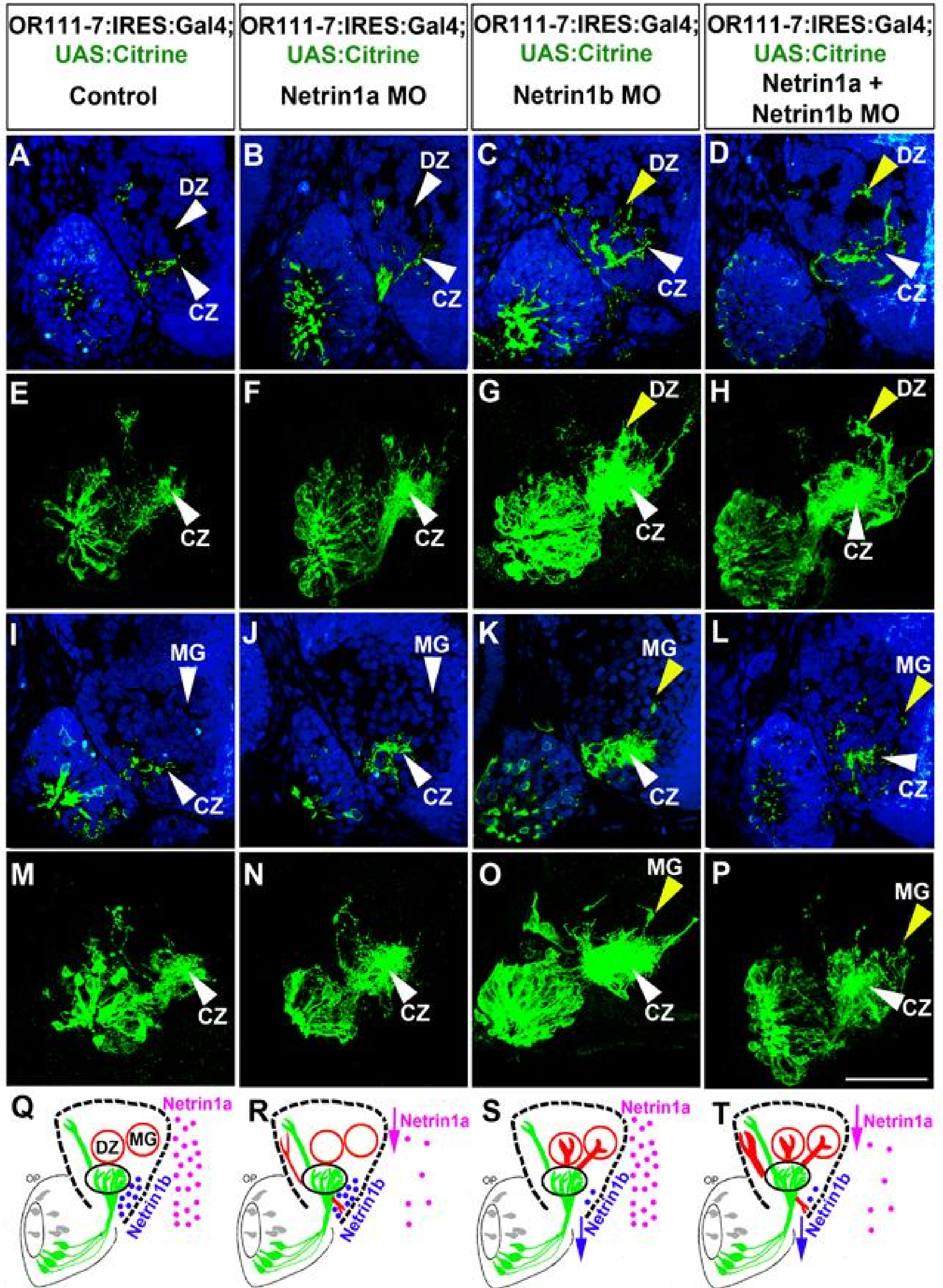


Figure 3.4. Inhibiting netrin1b, but not netrin1a, phenocopies the dorsal protoglomerular misprojections of OR111-7 transgenic axons observed in DCC morphants.

A-D, I-L, Single confocal optical sections through 3 day old OR111-7:IRES:Gal4; UAS:Citrine larvae (frontal view). Dorsal is to the top and midline to the right of the image. Propidium iodide (blue) delineates distinct protoglomeruli in the olfactory bulb. **E-H, M-P,** Maximum intensity projections of serial confocal optical sections of the same larvae shown immediately above. **Q-T,** Schematics showing control OR111-7:IRES:Gal4; UAS:Citrine projections (green) and mistargeted axons (red) observed upon reducing netrin1a or netrin1b levels. The thickness of the red lines corresponds roughly to the penetrance of the indicated phenotypes. Netrin1a mRNA (pink dots) is expressed at the telencephalic midline and netrin1b (blue dots) is expressed in the ventral bulb. Black circle = central zone, Red circles = dorsal zone, medial glomeruli. **A, E, I, M, Q,** Uninjected control OR111-7 transgene expressing axons (green) project to the central zone (CZ, white arrowhead) and LG1. **A, B, Q, R,** OR111-7 transgene expressing axons do not enter the dorsal zone (DZ, white arrowhead) in uninjected control embryos or in netrin1a morphants. **C, G, D, H, S, T,** Knocking down netrin1b alone or netrin1a and netrin1b together causes OR111-7 transgene expressing axons to inappropriately enter the dorsal zone (DZ, yellow arrowheads). **I, J, Q, R,** OR111-7:IRES:Gal4; UAS:Citrine axons do not enter the medial glomerulus (MG, white arrowheads) in controls or in netrin1a morphants. **K, O, L, P, S, T,** Reducing the level of netrin1b alone or netrin1a and netrin1b together causes axonal misprojections into MG (yellow arrowheads). Scale bar in P: 50 μm.

Figure 3.5.

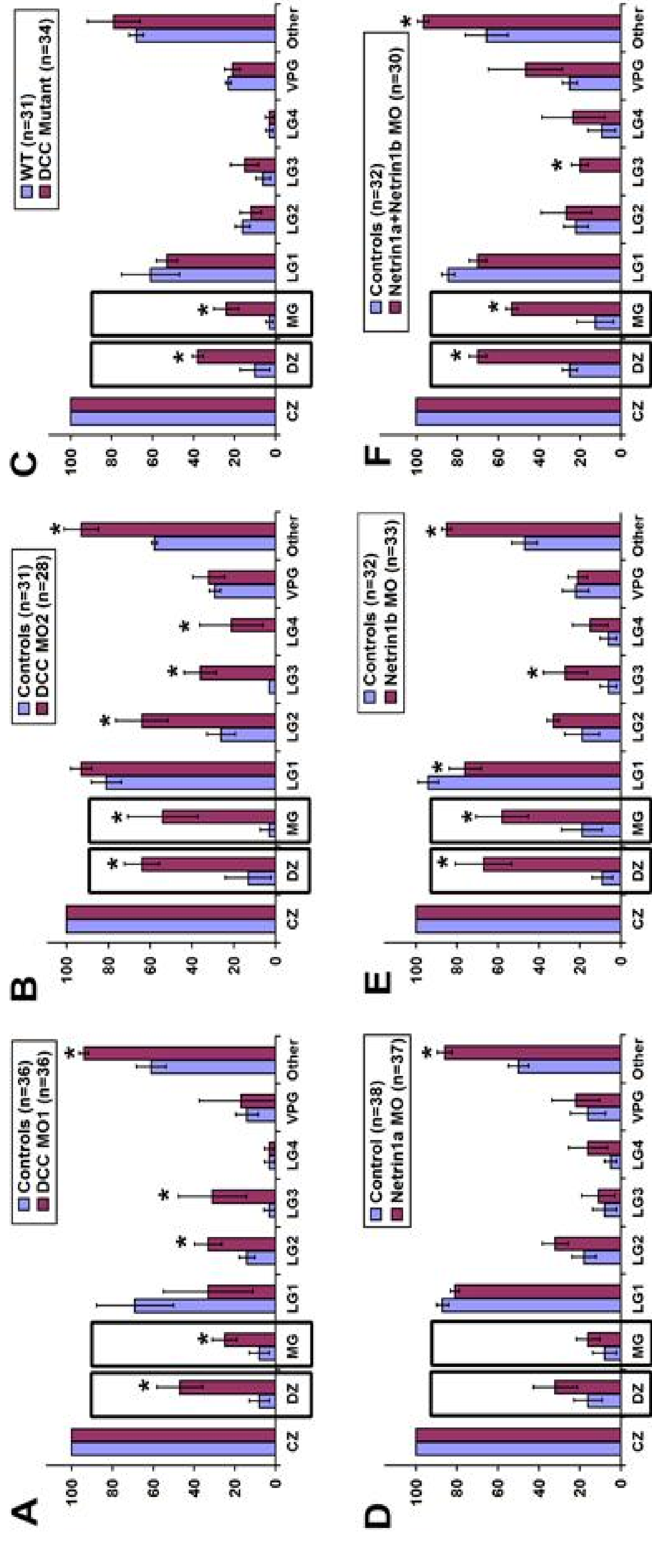


Figure 3.5. Quantification of protoglomerular targeting errors observed upon knocking down DCC, netrin1a, netrin1b, or both netrins together

A-F, The percentage of embryos with OR111-7:IRES:Gal4 axonal projections into the specified protoglomeruli is shown. Error bars indicate the SEM. **A-C,** Embryos injected with DCC MO1, DCC MO2, or DCC mutant larvae (purple bars) are compared with sibling control larvae (blue bars). **D-F,** Embryos injected with netrin1a MO, netrin1b MO, or netrin1a and netrin1b MOs together (purple bars) are compared with uninjected control siblings (blue bars). **A-C,** The percentages of larval olfactory bulbs with OR111-7 transgenic axonal projections to the DZ and MG (boxed) significantly increase upon DCC inhibition using DCC MO1, DCC MO2, or in the DCC mutant. **D-F,** OR111-7 transgenic axons inappropriately enter the DZ and MG at a statistically higher rate in netrin1b and in netrin1a+netrin1b morphants but not netrin1a morphants. **A-C,** DCC MO1 or MO2 morphants also show increased misprojections into LG2 and LG3, while DCC mutant larvae do not have misprojections into any lateral protoglomeruli. **E, F,** Inhibiting netrin1b alone or netrin1a and netrin1b together also causes increased axonal projections to the LG3. **A, B, D, E, F,** Inhibiting DCC, netrin1a, netrin1b, or both netrins together causes increased non-protoglomerular misprojections of OR111-7 transgenic axons (‘Other’, described in detail in Figures 6, 7, 8). Fisher’s exact test was used to test for statistical significance, $P<0.05$. CZ: *central zone,* DZ: *dorsal zone,* MG: *medial glomeruli,* LG1-4: *lateral glomeruli 1-4,* VPG: *ventral posterior glomerulus,* Other: *axonal projections to non-protoglomerular regions of the olfactory bulb.*

Figure 3.6.

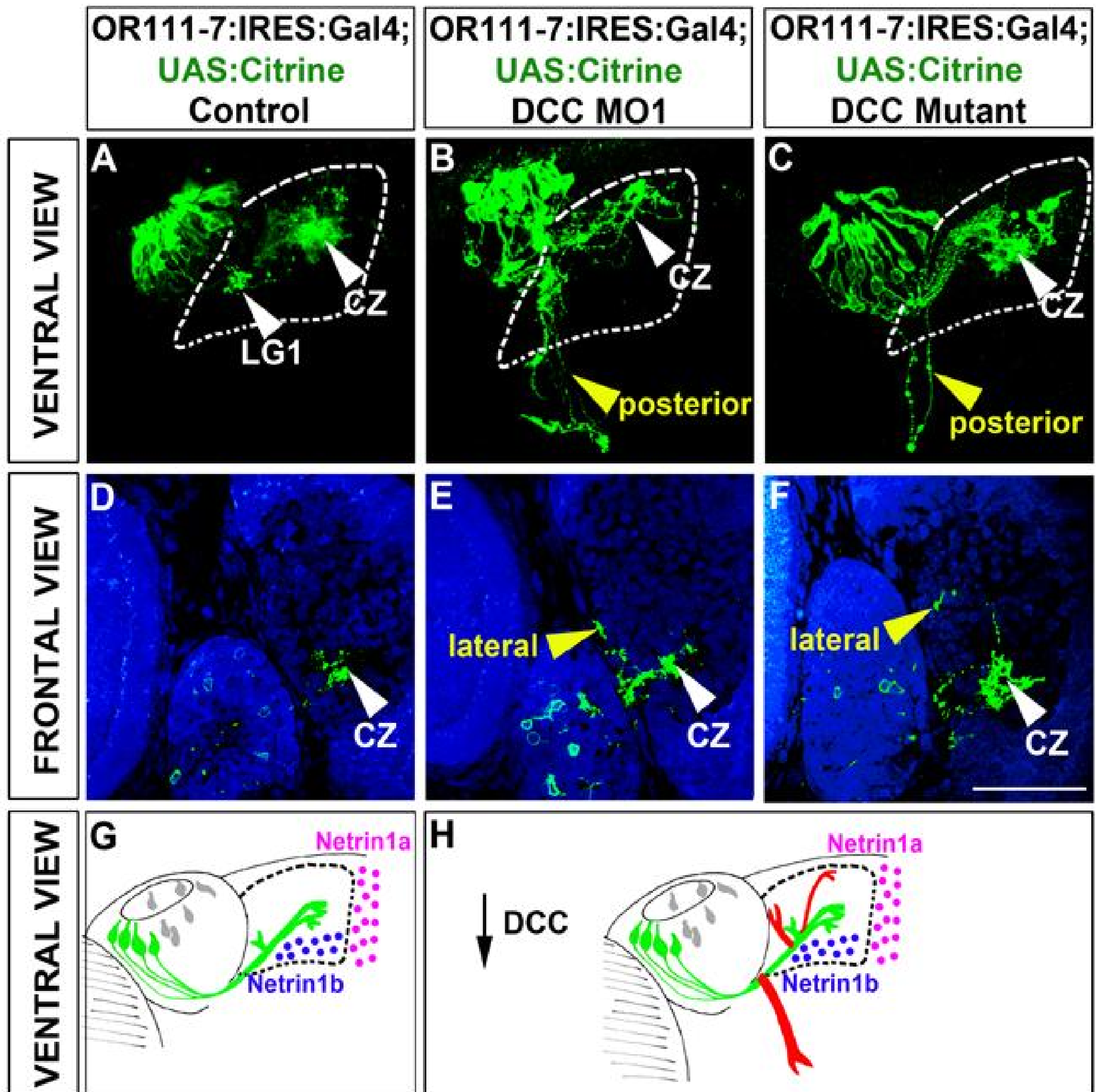


Figure 3.6. DCC signaling helps OR111-7 transgene expressing axons enter the olfactory bulb and prevents non-protoglomerular mistargeting within the bulb.

A-C, Maximum intensity projections of serial confocal optical sections of 3 day old larvae (ventral view). Anterior is towards the top and the midline to the right of the image. The olfactory bulb is outlined with dashed lines. **G, H,** Schematics showing control OR111-7:IRES:Gal4; UAS:Citrine projections (green) and mistargeted axons (red) observed upon inhibiting DCC. The thickness of the red lines corresponds roughly to the penetrance of the indicated phenotypes. Netrin1a mRNA (pink dots) is expressed at the telencephalic midline and netrin1b (blue dots) is expressed in the ventral bulb. **A, G,** OR111-7 transgene expressing axons (green) primarily target the central zone (CZ, white arrowhead) and LG1. **B, C, H,** In DCC MO1 morphants and in the DCC mutant, some OR111-7 transgene expressing axons extend posteriorly away from the olfactory bulb and do not enter it ('posterior', yellow arrowhead). **D-F,** Single confocal optical sections through frontally mounted 3 day old OR111-7:IRES:Gal4; UAS:Citrine larvae. Propidium iodide (blue) delineates distinct protoglomeruli in the olfactory bulb. **D,** OR111-7:IRES:Gal4; UAS:Citrine axons are detected in the central zone (CZ, white arrowhead). **E, F,** Reducing DCC levels via DCC MO1 morpholino or in the hypomorphic DCC mutant causes some OR111-7 transgenic axons to mistarget towards the lateral border of the olfactory bulb posterior to the LG1 protoglomerulus ('lateral', yellow arrowhead, schematized in Figure 3M, N). Scale bar in F: 50 μm.

Figure 3.7.

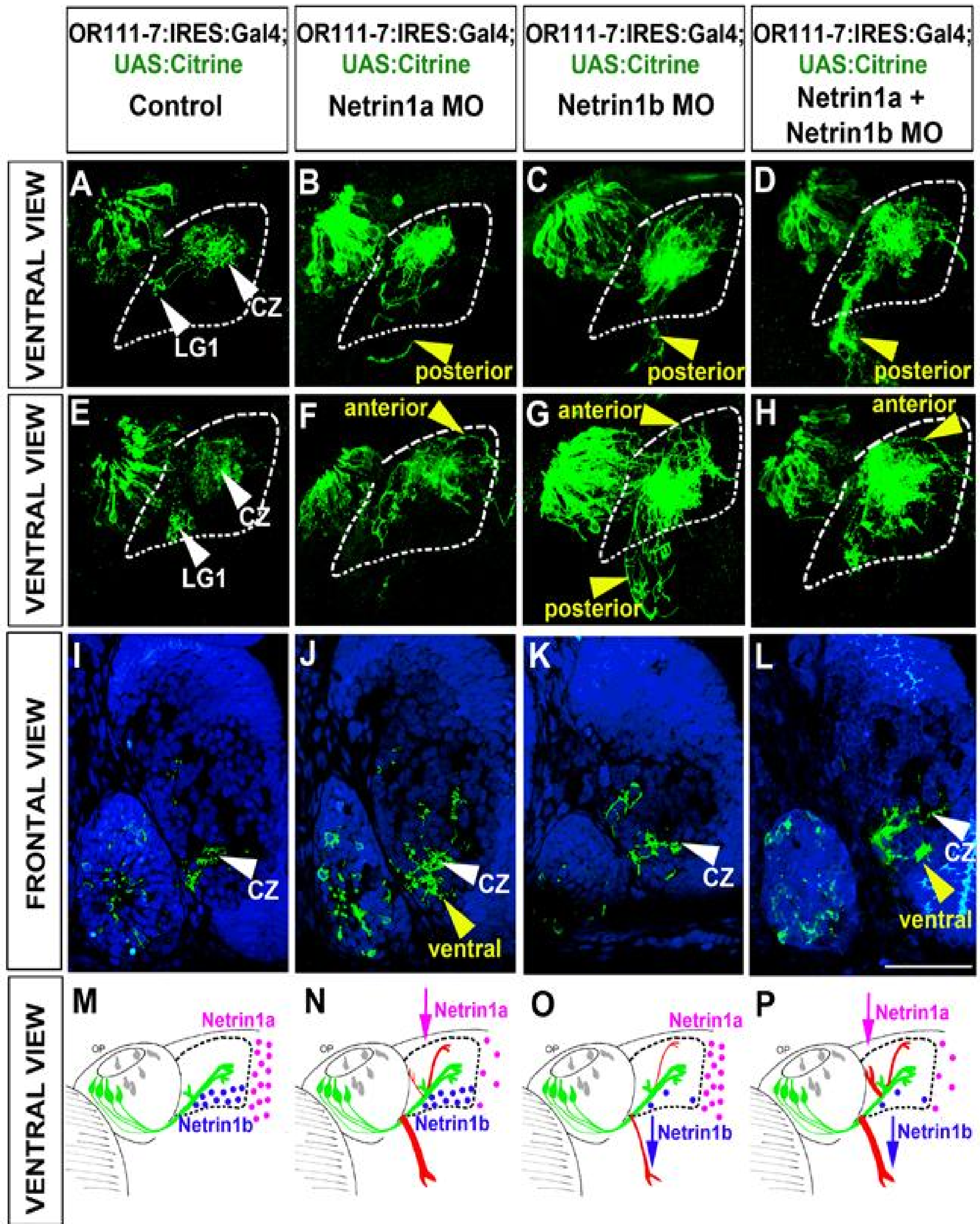


Figure 3.7. Netrin1a and netrin1b contribute to the proper pathfinding of OR111-7 transgenic axons into and within the olfactory bulb

A-H, Maximum intensity projections of serial confocal optical sections of 3 day old larvae (ventral view). Anterior is towards the top and the midline to the right. The olfactory bulb is outlined with dashed lines. **M-P,** Schematics showing control OR111-7:IRES:Gal4; UAS:Citrine projections (green) and mistargeted axons (red) observed upon inhibiting netrin1a, netrin1b or netrin1a and netrin1b together. The thickness of the red lines corresponds roughly to the penetrance of the indicated phenotypes. Netrin1a mRNA (pink dots) is expressed at the telencephalic midline and netrin1b (blue dots) is expressed in the ventral bulb. **A, E, M,** OR111-7:IRES:Gal4; UAS:Citrine axons (green) project to the central zone (CZ, white arrowhead) and LG1. **B, C, D, N, O, P,** Some OR111-7 transgenic axons misproject posteriorly ('posterior', yellow arrowheads) rather than entering the olfactory bulb when netrin1a, netrin1b, or both netrin1a and netrin1b levels are reduced. **F, G, H, N, O, P,** OR111-7:IRES:Gal4; UAS:Citrine axons also inappropriately stray anteriorly ('anterior', yellow arrowheads) in netrin1a, netrin1b, or netrin1 and netrin1b double morphants. **I-L,** Single confocal optical sections of 3 day old zebrafish larvae (frontal view). Dorsal is towards the top of the image. Propidium iodide (blue) labels olfactory bulb cells and allows identification of distinct protoglomeruli. **I,** OR111-7 transgene expressing axons are observed in the central zone (CZ, white arrowhead) in uninjected Control larvae. **J, L,** Reducing netrin1a levels or netrin1a and netrin1b levels together causes ventral mistargeting of OR111-7 transgene expressing axons ('ventral', yellow arrowhead, schematized in Figure 4R, T). Scale bar in L: 50 μm

Figure 3.8.

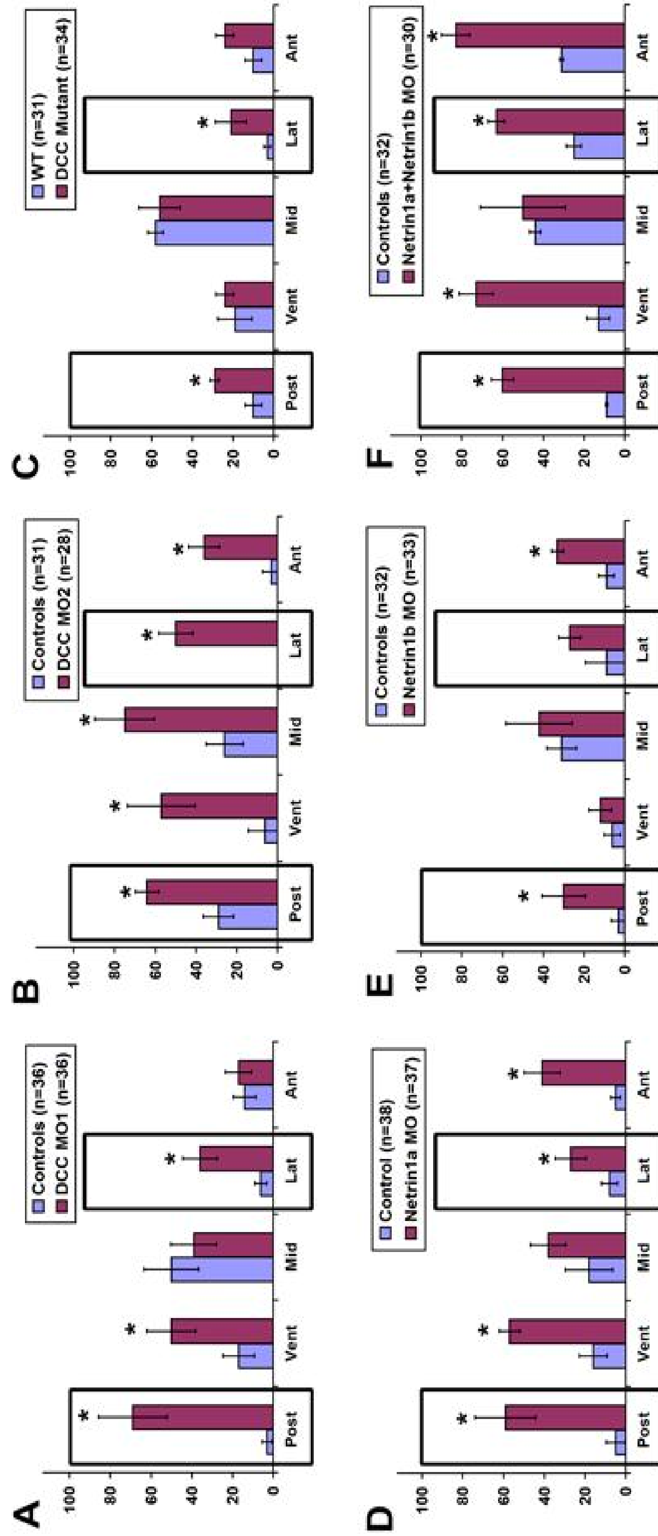


Figure 3.8. Quantification of non-protoglomerular targeting errors observed upon knocking down DCC, netrin1a, netrin1b, or both netrins together

A-F, The percentages of larval olfactory bulbs in which OR111-7 transgene expressing axons display the specified targeting errors are quantified. Error bars show the SEM. **A-C,** DCC MO1, DCC MO2 morphants, or DCC mutants (purple bars) are compared with control siblings (blue bars). **D-F,** Embryos injected with netrin1a MO, netrin1b MO, or netrin1a and netrin1b MOs together (purple bars) are compared with uninjected control siblings (blue bars). **A-F,** The proportion of larvae with posterior misprojections (boxed) increases in DCC MO1, DCC MO2, netrin1a, netrin1b and netrin1a + netrin1b morphants, and in DCC mutant larvae. **A-C, D, F,** Reducing the levels of DCC, netrin1a or netrin1a together with netrin1b increases the number of larvae in which OR111-7 transgene expressing axons make lateral pathfinding errors. **A, B, D, F,** DCC MO1, DCC MO2, netrin1a and netrin1a + netrin1b morphants display increased ventral misprojections. **B, D-F,** Larvae injected with DCC MO2, netrin1a, netrin1b or netrin1a and netrin1b morpholinos together display increased anterior misprojections. Fisher's exact test was used to test for statistical significance, $P<0.05$. Post: *posterior,* Vent: *ventral,* Mid: *midline,* Lat: *lateral,* Ant: *anterior. These individual categories were previously grouped together as 'Other' in Figure 5.*

Figure 3.9.

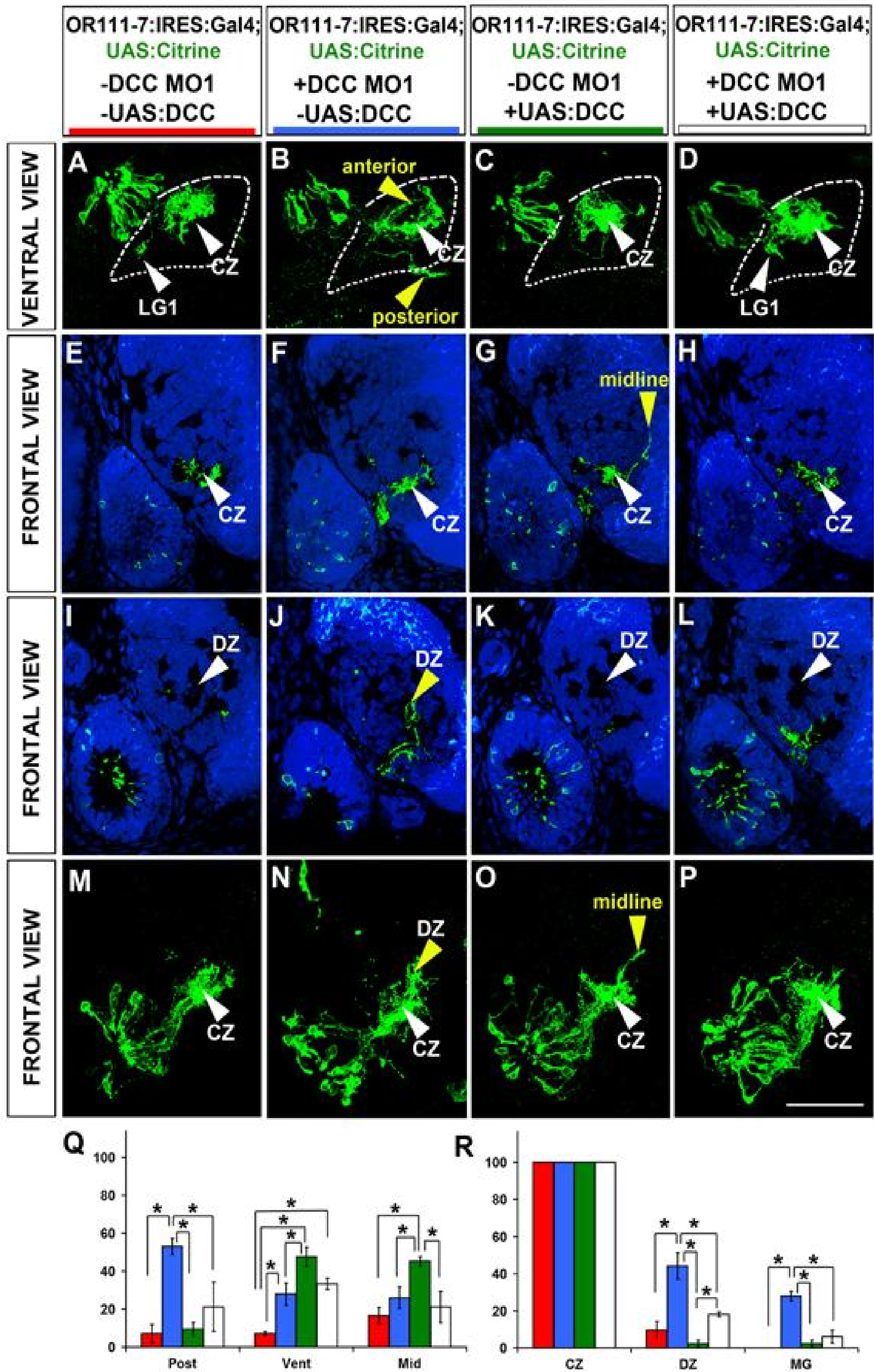


Figure 3.9. Expression of DCC in OR111-7 expressing neurons corrects mistargeting induced by DCC knockdown.

A-D, Maximum intensity projections of serial confocal optical sections of 3 day old larvae (ventral view). **E-L,** Single confocal optical sections through 3 day old OR111-7 transgene expressing larvae (frontal view). Propidium iodide (blue) labels olfactory bulb cells and allows identification of distinct protoglomeruli. **M-P,** Maximum intensity projections of serial confocal optical sections of the same larvae. **Q, R,** The percentages of larval olfactory bulbs with the specified axonal misprojections are shown. Bars are color coded based on the larval groups indicated at the top of the figure. **A, E, I, M, red bars in Q, R,** Three day old control uninjected OR111-7:IRES:Gal4; UAS:Citrine larvae without the UAS:DCC transgene. **B, F, J, N, blue bars in Q, R,** Three day old OR111-7:IRES:Gal4; UAS:Citrine larvae without UAS:DCC were injected with DCC MO1. **C, G, K, O, green bars in Q, R,** Three day old uninjected OR111-7:IRES:Gal4; UAS:Citrine larvae that also carry the UAS:DCC transgene. **D, H, L, P, white bars in Q, R,** Three day old OR111-7:IRES:Gal4; UAS:Citrine larvae carrying UAS:DCC were injected with DCC MO1 (Rescue condition). **B, blue 'Post' bar in Q,** Reducing DCC levels causes posterior misprojections. **A, C, red and green 'Post' bars in Q,** Posterior targeting errors are not observed in the absence of DCC MO. OR111-7 transgenic axons (green) are present in the central zone (CZ, white arrowhead) and in the lateral glomerulus (LG1, white arrowhead). **D, white 'Post' bar in Q,** Posterior targeting errors in DCC morphants are reduced in OR111-7 axons also expressing the UAS:DCC transgene. **J, N, blue 'DZ' bar in R,** Reducing DCC levels causes OR111-7 transgenic neurons to inappropriately enter the dorsal zone (DZ, yellow arrowhead). **I, M, K, O, red and green 'DZ' bars in R,** OR111-7 transgene expressing axons do not enter the DZ in the absence of DCC morpholino irrespective of whether the UAS:DCC transgene is present. **L, P, white 'DZ' bar in R,** Axonal mistargeting into the DZ is reduced in DCC morphants

that contain the morpholino resistant UAS:DCC transgene. **Green 'Vent' and 'Mid' bars in Q,** Increased ventral and midline misprojections are observed in OR111-7 transgenic larvae that express the UAS:DCC transgene. Fisher's exact test was used to test for statistical significance, $P<0.05$. Scale bar in P: 50μm. Post: *posterior,* Vent: *ventral,* Mid: *midline,* CZ: *central zone,* DZ: *dorsal zone,* MG: *medial glomeruli*

CHAPTER 4. RELATED RECEPTORS OF THE OR111 SUBFAMILY TARGET THE CENTRAL ZONE PROTOGLOMERULUS

INTRODUCTION

In vertebrates, odorant receptor expressing olfactory sensory neurons (OSNs) located in the olfactory epithelium extend their axons to sterotyped locations in the olfactory bulb (Baier and Korsching, 1994; Pinching and Powell, 1971). Each olfactory sensory neuron expresses only one odorant receptor (OR) from a large genomic repertoire (Chess et al., 1994; Sato et al., 2007). Sensory axons expressing a given odorant receptor converge onto specific synaptic condensations in the olfactory bulb called glomeruli. Within each glomerulus, the axons of olfactory sensory neurons synapse with the dendrites of olfactory bulb neurons (Pinching and Powell, 1971). Since glomeruli are homogenously innervated by axons expressing just one odorant receptor, the binding of an odorant to an odorant receptor elicits the activation of the corresponding glomeruli. The glomerular activation pattern elicited by an odorant is thought to be its primary neural representation and forms the basis of olfactory coding (Belluscio and Katz, 2001). How the characteristic one odorant receptor-one glomerulus organization is established during development is not well understood.

In both mouse and zebrafish, the onset of odorant receptor expression is asynchronous and detected early in development, around the time that olfactory axons first enter the bulb (Barth et al., 1996; Rodriguez-Gil et al., 2010). Several mouse odorant receptors are detected in the epithelium by E11.5, while zebrafish receptors are detected by 24 hours post fertilization. The 3 day old larval zebrafish olfactory bulb consists of 12 protoglomeruli, thought to be glomerular precursors that have not yet fully differentiated (Li et al., 2005). They are innervated by at least two major classes of sensory neurons,

those that express OMP (olfactory marker protein) along with odorant receptors and those that express TRPC2 (transient receptor potential channel 2) along with V2R vomeronasal receptors. We have previously characterized the protoglomerular projection patterns of OMP and TRPC2 expressing neurons in 3 day old zebrafish (Chapter 3, this thesis; Lakhina et al., submitted). Neurons that express OMP and odorant receptors innervate the central zone, dorsal zone, lateral glomerulus 3 (LG3), and the medial glomeruli1-4 (Lakhina et al., submitted). Thus, odorant receptor expressing OMP neurons project axons to not more than seven protoglomeruli in 3 day old zebrafish larvae. It is unknown whether protoglomeruli, like mature glomeruli, are homogenously innervated by neurons expressing the same odorant receptor. We wished to explore which odorant receptors are expressed by OMP expressing neurons in 3 day old larvae.

The zebrafish genome encodes 143 ORs that belong to 8 OR families and 40 subfamilies (Alioto and Ngai, 2005). Odorant receptors with greater than 40% amino acid identity are classified into a family ranging from family A to family H, while those with greater than 60% amino acid identity are categorized into the same subfamily (Lancet and Ben-Arie, 1993; Alioto and Ngai, 2005). Related odorant receptors belonging to the same subfamily are clustered together in the genome. These receptor clusters are distributed across at least six chromosomes, but most clusters are present in chromosome 10, 15 or 21 (Alioto and Ngai, 2005; Dugas and Ngai, 2001). To understand the relationship between odorant receptor expression and protoglomerular targeting of axons in 3 day old larvae, it was important to first identify which odorant receptors are expressed at that age. To this end, odorant receptors belonging to six distinct subfamilies - OR111, OR106, OR128, OR133, OR125 and OR103 - were cloned and their expression in 3 day old olfactory epithelia was characterized. These odorant

receptor subfamilies were chosen either based on previous reports showing that at least some subfamily members were expressed in 3 day old olfactory epithelia or if their expression was detected in high levels in adult epithelia (Barth et al., 1996; Byrd et al., 1996; Argo et al., 2003; Sato et al., 2007). Further, the receptor subfamilies that we chose to study are classified into different families and are present at genomically distinct positions. The OR106 subfamily belongs to OR family G and is located on chromosome 10, the OR111 (family D), OR128 (family E) and OR103 (family C) subfamilies are on chromosome 15 and the OR133 (family H) and OR125 (family E) subfamilies are positioned on chromosome 21.

These experiments reveal that mRNAs encoding 35 distinct odorant receptors are expressed in 3 day old olfactory epithelia. At the same age, a maximum of 7 protoglomeruli in the bulb are innervated by odorant receptor expressing neurons. These data suggest that a protoglomerulus is heterogenously innervated by neurons expressing a variety of odorant receptors. Consistent with this hypothesis, I find that axons of neurons expressing related ORs belonging to the OR111 subfamily all project to the central zone protoglomerulus. In chapter 2, I demonstrated that replacing the coding region of OR111-7 with RFP in the transgenic construct did not alter axonal targeting to the central zone. This suggests that the transgenically expressed OR111-7 is not required for axons to innervate the central zone. Rather, it is likely that the transgenic construct is selectively expressed in neurons destined to target the central zone. In chapter 3, I showed that OR111-7 expressing neurons also express the DCC receptor which is required for axons to properly navigate into the central zone. Based on all these data, I propose a model hypothesizing that the zebrafish olfactory epithelium consists of distinct neuronal subsets that are restricted to express a predetermined set of odorant receptors and axonal guidance receptors. Axons of neurons belonging to a

particular subset will innervate a specific protoglomerulus. For example, the neuronal subset in which the OR111-7:IRES:Gal4 transgene is expressed is likely to be the one in which other ORs of the OR111 subfamily can be transcribed. Axons of this neuronal subset specifically innervate the central zone protoglomerulus which is mediated, at least in part, by the guidance receptor DCC. It is possible that other neuronal subsets innervate individual protoglomeruli aside from the central zone in the three day old larval zebrafish olfactory bulb.

MATERIALS AND METHODS

Isolation of odorant receptor sequences for in situ hybridization probes. RNA was extracted from the heads of 3 day old wild type zebrafish larvae. This was used to perform RT-PCR with degenerate primers that amplified individual odorant receptors belonging to the OR111, OR106, OR128, OR133, OR125 and OR103 odorant receptor subfamilies. The degenerate primer sequences are as follows:

OR111 Forward primer = 5' GTGTTTAACTTRGCCTTRGCTGA 3'

OR111 Reverse primer = 5' ACACAGGTYTTCARWGCYTTY 3'

OR106 Forward primer = 5' ACTGGATTTGATCACCTGCARAACC 3'

OR106 Reverse primer = 5' TGACTYAAGCARGTRTTGATCATTTGC 3'

OR128 Forward primer = 5' CACMAGCCYATGTACATTCTG 3'

OR128 Reverse primer = 5' CACTATGTGKGGAAYACAVGTTTGA 3'

OR133 Forward primer = 5' TCCTYTATGTCAAYGSDGTYATG 3',

OR133 Reverse primer = 5' ACCCAGCTGTAKTAGATGCAGAAG 3'

OR125 Forward primer = 5' CTKCATSRACCRATGTWCATYTTC 3'

OR125 Reverse primer = 5' GTTTYACHAYHGACCAGTTGTCAC 3'

OR103 Forward primer = 5' AACAYCAGCTTTGYAAAGGA 3'

OR103 Reverse primer = 5' CAGGTGGAGAAAGTCTTCAK 3'

PCR products were cloned into the pCR II-TOPO vector and sequenced to determine their identity. Individual odorant receptor clones were subsequently used to generate digoxigenin labeled antisense RNA probes for in situ hybridization by in vitro transcription.

Whole mount fluorescent in situ hybridization. *In situ* hybridization was performed as described previously (Chalasani et al., 2007). Briefly, wild type embryos were hybridized with probes for individual odorant receptors and *in situ* signals were amplified using a Cyanine 3-coupled tyramide system (TSA™ Plus Cyanine 3 System, Perkin Elmer, Product number: NEL744001KT). For larvae microinjected with OMP:Gal4 and UAS:Citrine or UAS:CD8-GFP, *in situ* signals were amplified using Cyanine 5-coupled tyramide system (TSA™ Plus Cyanine 5 System, Perkin Elmer, Product number NEL745001KT). *In situ* hybridization was followed by immunohistochemistry and propidium iodide staining. Goat anti-GFP (1:100, Rockland Immunochemicals, catalog #600-101-215) and anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen) were used to visualize GFP-positive neurons. Rabbit anti-DsRed (1:50, Clontech, catalog #632496) and anti-rabbit Cy3 (1:500, Jackson ImmunoResearch, catalog # 711-165-152) were used for visualizing RFP expressing axons. Propidium iodide staining was performed using the protocol of Brend and Holley (2009) with the omission of the RNase treatment step. Processed larvae were mounted on coverslips in the frontal orientation and imaged using a 40X oil immersion lens on a Leica TSP2 confocal microscope. Images were captured every micrometer through the entire extent of the olfactory bulb.

Microinjection constructs. All constructs were cloned into the mini-Tol2 vector kindly provided by the Ekker laboratory (Balciunas et al., 2006). (1) OMP:Gal4: The 2.1 kb

OMP promoter, obtained from the Yoshihara laboratory (Sato et al., 2005) was cloned upstream to a Gal4-VP16 coding sequence. (2) UAS:gap43-Citrine: 14X UAS sequences and the E1b carp β-actin minimal promoter were used to control the expression of a Citrine fluorophore fused with the membrane targeting region of the GAP43 protein. (3) UAS:CD8-GFP: 14X UAS sequences and the E1b carp β-actin minimal promoter were used to control the expression of GFP fused with the transmembrane CD8 protein. CD8-GFP is therefore targeted to the membrane and allows for better visualization of axons and growth cones (Lee and Luo, 1999). No significant difference in axonal labeling was found between embryos injected with UAS:gap43-Citrine or UAS:CD8-GFP, and I therefore used them interchangeably for microinjection experiments.

Microinjection into zebrafish embryos. Microinjection of DNA constructs along with Tol2 Transposase RNA into one celled wild type zebrafish embryos was performed as described in Fisher et al., 2006. Larvae in which one to three olfactory sensory neurons were labeled were sorted, fixed and processed for in situ hybridization and immunohistochemistry.

RESULTS

Cloning of odorant receptors expressed in three day old zebrafish larvae

We wished to identify odorant receptors that are expressed in 3 day old OMP expressing olfactory sensory neurons. To this end, Jiwei He and Xin Shao in the Raper laboratory designed degenerate primers to amplify odorant receptors belonging to six distinct receptor subfamilies. They used these subfamily-specific degenerate primers to perform PCR on cDNA derived from 3 day old zebrafish larvae and cloned a total of 39 odorant receptors into pCRII TOPO vectors. Using this technique, they obtained all receptors of

the OR111, OR128 and OR103 subfamilies, but only 8 out of 12 receptors of the OR106 subfamily, 2 out of 10 receptors of the OR133 subfamily and 1 out of 8 receptors of the OR125 subfamily. It is possible that odorant receptors that are highly expressed in the epithelium are preferentially amplified. It is possible that other receptors of the OR106, OR133 and OR125 subfamilies are expressed in the 3 day old olfactory epithelium. Since we only examined the expression of odorant receptors belonging to six out of forty subfamilies encoded in the zebrafish genome, it is likely that the odorant receptors detected by these methods are an underestimation of the total number of odorant receptors expressed in 3 day old epithelia.

At least thirty-five odorant receptors are expressed in the three day old zebrafish olfactory epithelium

To confirm that the odorant receptors recovered by RT-PCR were indeed expressed in the 3 day old olfactory epithelium, we generated antisense RNA probes using in vitro transcription. Using individual odorant receptor probes, I performed in situ hybridization on 3 day old larvae and subsequently counted the number of neurons expressing a given odorant receptor. Owing to sequence similarity, the in situ hybridization probe for OR128-5 is likely to detect OR128-11. Thus, we cannot distinguish between these two odorant receptors. Instead, we can assume that the expression of at least OR128-5 or OR128-11 is detected by our method. Of the 39 cloned receptors, at least 35 were confirmed to be expressed in 3 day old olfactory epithelia. These 35 odorant receptors represent 24% of all odorant receptors encoded in the zebrafish genome (Figure 4.1). Individual receptors within each subfamily are expressed in a variable number of neurons. On average, 1 to 11 neurons express OR111 subfamily members (red bars, Figure 4.1), 6 to 14 neurons express OR106 subfamily members (blue bars, Figure 4.1), 0 to 6 neurons express the OR128 subfamily members (green bars, Figure 4.1), 2 to 3

neurons express the OR133 subfamily members (yellow bars, Figure 4.1), 1 neuron expresses OR125-8 (grey bar, Figure 4.1) and 1 to 8 neurons express receptors of the OR103 subfamily (purple bars, Figure 4.1). There is no obvious relationship between the number of neurons expressing a given receptor and its location within the genomic cluster. The expression of OR111-8 and OR128-6 was not detected despite repeating the experiment with two batches of freshly generated RNA probe (data not shown). OR128-9 was detected very infrequently, in only 2 neurons out of 10 olfactory pits. The sample size for these in situ hybridization experiments was between 10 to 20 olfactory pits.

Neurons expressing related receptors of the OR111 subfamily target their axons to the central zone protoglomerulus

Strikingly, the number of odorant receptors expressed in 3 day old olfactory epithelia (at least 35) is higher than the seven OMP+ protoglomeruli that are reliably detected at this age. These data suggest that a given protoglomerulus is likely innervated by axons expressing more than one odorant receptor. I hypothesized that genomically linked, related odorant receptors belonging to the same subfamily are likely to project to the same protoglomerulus. To test this idea, Christina Marcaccio and I microinjected 5ng of OMP:Gal4 and UAS:gap43-Citrine or UAS:CD8-GFP to stochastically label one to three olfactory sensory neurons in each olfactory pit. We then fixed these larvae at 3 days post fertilization and performed *in situ* hybridization with a mixture of odorant receptor probes consisting of OR111-1, -2, -3, -4, -5, -6, -7, -9, -10, and -11. Since OR111-8 mRNA was not detected by in situ hybridization despite repeated attempts, the pool of probes that we used represents all OR111 subfamily receptors expressed in three day old olfactory epithelia. Since protoglomeruli are acellular, we were able to identify them as regions in the olfactory bulb that remained unstained upon propidium iodide treatment (Figure

4.2A-D). One possible outcome of this experiment is that each neuron that co-expresses the OMP driven neuronal tracer and OR111 subfamily mRNA projects its axon to the same protoglomerulus every time. This would suggest that neurons expressing genomically linked odorant receptors of the OR111 subfamily are likely to project to the same protoglomerulus. Alternately, neurons expressing related receptors of the OR111 subfamily could target their axons to multiple protoglomeruli. Our results indicate that 100% of neurons co-labeled with GFP and expressing OR111 subfamily mRNA project axons to the central zone protoglomerulus (N = 10 neurons, white arrowhead, Figure 4.2A-D, purple bar in E). As expected, OMP expressing neurons that do not express OR111 subfamily receptors project their axons to many protoglomeruli. 32% of OMP:Gal4; UAS:Citrine expressing neurons (i.e. 24 out of 75 neurons) target the central zone, 17% (i.e. 13 out of 75) neurons target the dorsal zone, 25% (i.e. 19 out of 75) neurons innervate the medial glomeruli, while the remaining 25% of OMP:Gal4; UAS:Citrine expressing axons target the lateral glomeruli, ventral posterior glomerulus or do not innervate protoglomeruli (blue bars in Figure 4.2E). Although we cannot be sure which individual members of the OR111 subfamily co-localize with the stochastically labeled neurons targeting the central zone, it is likely that several members of the OR111 subfamily are sampled since the probability of expression of individual odorant receptors is about the same (5 to 8 neurons). These results suggest that the central zone protoglomerulus is innervated by neurons expressing related receptors of the OR111 subfamily. Thus, it is likely that endogenous OR111-7 expressing neurons project axons to the central zone, similar to our previous observations that transgenic OR111-7 expressing axons project to the central zone protoglomerulus (Lakhina et al., submitted; Chapter 3, this thesis). Some stochastically labeled OMP neurons did not co-localize with OR111 subfamily mRNA, yet they targeted the central zone (Figure 4.2E, blue bar labeled ‘CZ’). It is therefore likely that neurons expressing odorant receptors

that are not members of the OR111 subfamily also innervate the central zone protoglomerulus.

DISCUSSION

In the adult zebrafish olfactory bulb there are estimated to be about 80 clearly distinct glomeruli and perhaps as many as 120 'glomerular modules' (Baier and Korsching, 1994; Friedrich and Korsching, 1997). Glomerular modules are distinct glomerulus-like regions that are compressed together into neuropil within the lateral bulb. As in rodents, there is an approximate correspondence between the total number of odorant receptors (~150) and the combined total of glomeruli and glomerular modules in the zebrafish olfactory bulb (~120). Protoglomeruli are immature glomeruli consisting of OSN axons and dendrites of olfactory bulb neurons. They have been described in insects, zebrafish and rodents (Oland et al., 1990., Li et al., 2005; Treolar et al., 1999). Zebrafish olfactory protoglomeruli emerge at around 2 days post fertilization as condensations of olfactory sensory fibers within the bulb (Wilson et. al., 1990; Dynes and Ngai, 1998; Miyasaka et. al., 2005; Li et. al, 2005). By 3 days post fertilization, about 12 protoglomeruli are detected in the olfactory bulb. Of these 12 protoglomeruli, 5 receive input from V2R expressing neurons while 7 are innervated by odorant receptor (OR) expressing neurons (Lakhina et al., submitted).

Here I find that neurons in the 3 day old zebrafish olfactory epithelium express at least 35 odorant receptors and most likely many more, yet they project their axons to a maximum of seven protoglomeruli. I show that the central zone protoglomerulus is heterogeneously innervated by neurons expressing related odorant receptors of the OR111 subfamily. Similarly, some mouse olfactory axons expressing related odorant receptors are intermingled within the presumptive olfactory bulb at E15.5 (Conzelmann

et al., 2001; Zou et al., 2004). At postnatal ages, neurons expressing one odorant receptor coalesce into distinct glomeruli. Neurons expressing genomically linked, related odorant receptors target their axons to neighboring glomeruli in the mouse olfactory bulb (Tsuboi et al., 1999). Zebrafish olfactory sensory axons targeting a particular protoglomerulus at 3dpf have been observed to segregate into smaller units as development proceeds (Li et al., 2005). It is tempting to speculate that these smaller units will eventually mature into glomeruli consisting of axons expressing just one odorant receptor. Once transgenic lines labeling neurons expressing related odorant receptors become available, the intermingling and subsequent segregation of axons expressing related odorant receptors can be observed directly.

Our data indicate that the expression of odorant receptors of the OR111 subfamily is restricted to neurons that target the central zone protoglomerulus. Mouse olfactory sensory neurons targeting a specific region of the olfactory bulb are similarly limited to the type of odorant receptors that they can express based on their location in one of four zones of the olfactory epithelium. Their zonal location in the epithelium also restricts the repertoire of axonal guidance receptors they can stochastically express and the targeting of their axons along the dorso-ventral axis (Wang et al., 1998; Cho et al., 2007). Neurons located in different zones express other zonally restricted markers as well, suggesting that neurons located in different zones represent distinct neuronal subsets (Yoshihara et al., 1997; Gussing and Bohm, 2004). Weth et al. (1996) report that adult zebrafish olfactory sensory neurons expressing a particular receptor are arranged in concentric, but overlapping expression domains analogous to zones in the mouse olfactory epithelium. In embryonic zebrafish however, neurons expressing a given odorant receptor are randomly distributed in the epithelium (Vogt et al., 1997). Whether or not zebrafish olfactory sensory neurons expressing a particular receptor are

distributed into zones, it is still possible that distinct subsets of sensory neurons exist in the zebrafish olfactory epithelium. This hypothesis is supported by the observation that zebrafish olfactory sensory neurons are restricted to stochastically express an odorant receptor from a predetermined set of receptor genes (Argo et al., 2003).

I propose a model hypothesizing that the zebrafish olfactory epithelium consists of distinct neuronal subsets that are restricted to express a predetermined set of odorant receptors and axonal guidance receptors in a manner similar to zonally restricted subsets of mouse olfactory sensory neurons (Figure 4.3). Axons of neurons belonging to a particular subset will innervate a specific protoglomerulus. Our previous studies using the OR111-7:IRES:Gal4 transgenic line are consistent with this model (Chapter 2, this thesis). OR111-7:IRES:Gal4; UAS:Citrine neurons target the central zone protoglomerulus. Replacing OR111-7 with RFP in this transgenic construct did not alter the targeting of axons to the central zone, suggesting that the construct is selectively expressed in neurons that were destined to innervate the central zone. The neuronal subset in which the OR111-7:IRES:Gal4 transgene is expressed is likely to have the ability to transcribe odorant receptors of the OR111 subfamily. OR111-7:IRES:Gal4; UAS:Citrine neurons also express the DCC receptor which is required for proper axonal targeting to the central zone (Lakhina et al., submitted; Chapter 3, this thesis).

This model predicts that like the central zone, every other protoglomerulus innervated by a specific subset of OMP and OR expressing neurons that have a unique transcriptional profile and express related odorant receptors. To test this model, it is essential to first determine whether protoglomeruli other than the central zone in the 3 day old zebrafish olfactory bulb are also heterogeneously innervated by related odorant receptors of the same subfamily. Since the experiments described here are very tedious and slow, we

are currently exploring a different method to correlate odorant receptor expression with axonal targeting. After stochastically labeling a neuron, we propose to trace its axonal projection, extract RNA from the labeled neuron and perform single cell PCR with degenerate primers to determine the identity of the expressed odorant receptor. If every protoglomerulus is indeed innervated by neurons expressing related odorant receptors, it will be interesting in the future to examine whether neurons targeting different protoglomeruli express a distinct combination of transcription factors which might correlate with the expression of different axonal guidance receptors.

Using multiple transgenic lines each labeling neurons that express a different, related odorant receptor, it will be possible to directly visualize our inference that neurons expressing related odorant receptors innervate the same protoglomerulus. An important feature of the model in Figure 4.3 is that while it maintains that the initial targeting of sensory axons to protoglomeruli is likely a consequence of neuronal subset specific targeting mechanisms, it leaves open the possibility that odorant receptor mediated signaling is required later in development for glomerular maturation and segregation. These studies have opened the door for exciting discoveries about the organization and development of olfactory protoglomeruli.

ACKNOWLEDGEMENTS

I thank Jiwei He and Xin Shao for cloning the various odorant receptors used in this study. I also thank Christina Marcaccio for help with zebrafish embryo injections and in situ hybridization experiments with pooled probes of the OR111 subfamily.

Figure 4.1.

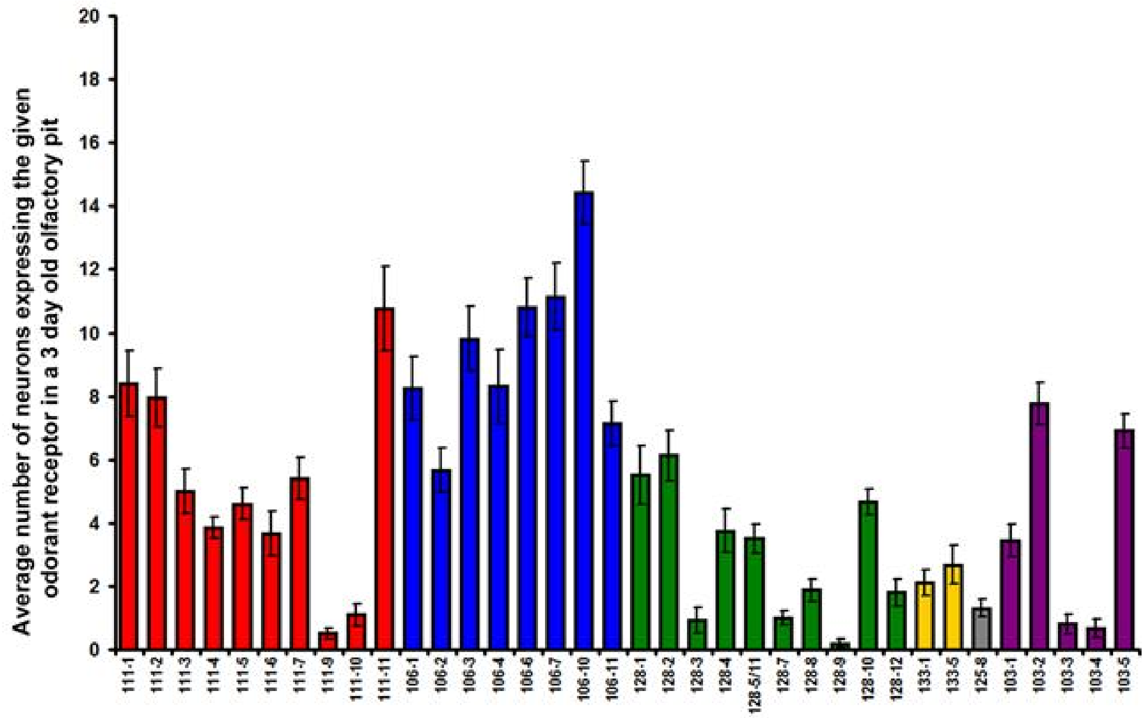


Figure 4.1. Thirty five distinct odorant receptors belonging to six subfamilies are expressed in the 3 day old zebrafish olfactory epithelium

On average, mRNA encoding the OR111 subfamily members is detected in 1 to 11 neurons (red bars), OR106 subfamily members are expressed in 6 to 14 neurons (blue bars), 0 to 6 neurons express the OR128 subfamily members (green bars), 2 to 3 neurons express the OR133 subfamily members (yellow bars), OR125-8 is detected in 1 neuron (grey bar) and 1 to 8 neurons express receptors of the OR103 subfamily (purple bars).

Figure 4.2.

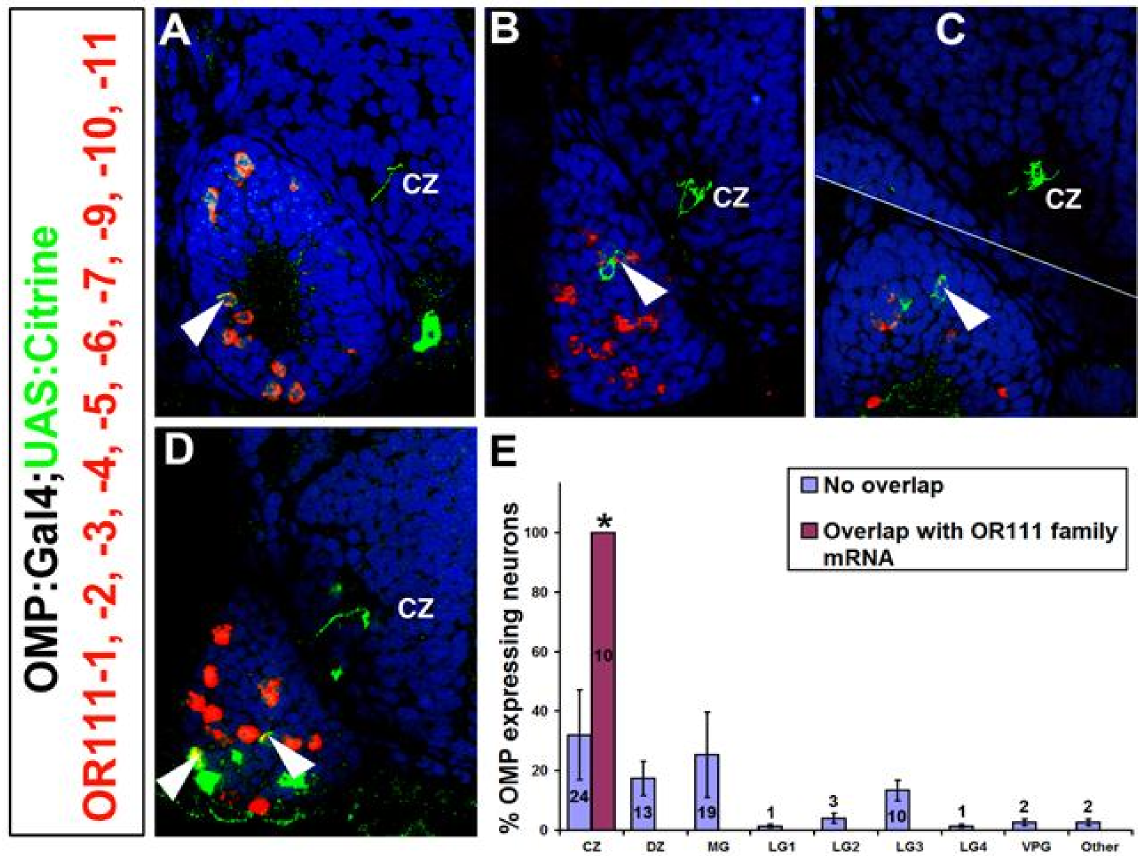


Figure 4.2. Neurons expressing receptors of the OR111 subfamily target their axons to the central zone protoglomerulus in 3 day old zebrafish larvae

A-D, Single confocal optical sections through 3 day old larvae that stochastically express OMP:Gal4; UAS:Citrine in one to three neurons (frontal view). Dorsal is to the top and midline to the right of the image. mRNA encoding OR111-1, -2, -3, -4, -5, -6, -7, -9, -10 and -11 (red) was detected by in situ hybridization. Propidium iodide (blue) delineates distinct protoglomeruli in the olfactory bulb. **A-D, purple bar in E,** Stochastically labeled OMP:Gal4; UAS:Citrine neurons (green) that express mRNA encoding members of the OR111-7 subfamily (red, white arrowhead) project their axons to the central zone protoglomerulus (CZ). **Blue bars in E,** Stochastically labeled OMP:Gal4; UAS:Citrine neurons (green) that do not express mRNA encoding members of the OR111-7 subfamily target axons to multiple protoglomeruli. Fisher's exact test was used to test for statistical significance, $P<0.05$. **E,** The number of stochastically labeled OMP neurons that project axons to a particular protoglomerulus are indicated either inside the bar or immediately above it.

CZ: *central zone,* DZ: *dorsal zone,* MG: *medial glomeruli 1-4,* LG1-4: *lateral glomeruli 1-4,* VPG: *ventral posterior glomerulus,* Other: *axonal projections to non-protoglomerular regions of the olfactory bulb.*

Figure 4.3.

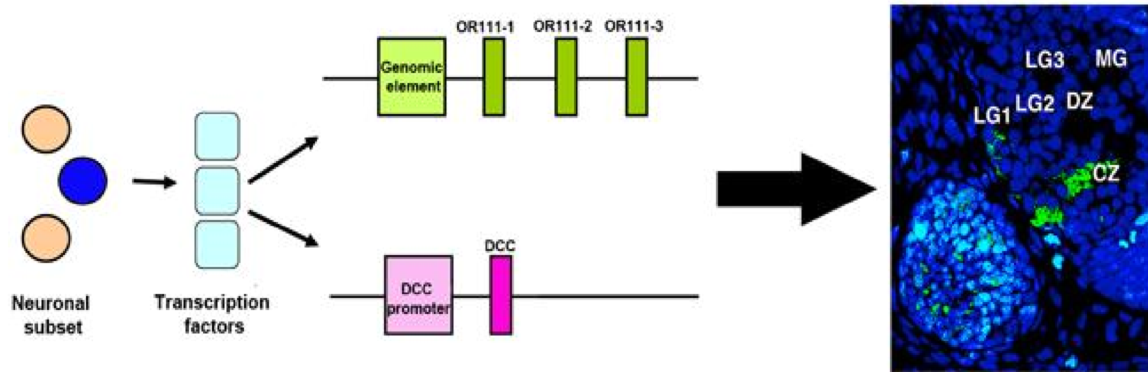


Figure 4.3. A model proposing that a protoglomerulus is innervated by axons expressing a restricted subset of odorant receptors and axon guidance receptors. Neurons expressing members of the OR111 subfamily are restricted to target the central zone. OR111-7 expressing neurons express the DCC receptor which contributes to axonal guidance to the central zone. It is possible that the central zone protoglomerulus is innervated by axons destined to target the central zone based on neuronal lineage, which in turn determines which odorant receptors and axon guidance receptors the neuron can express.

CHAPTER 5. THE ROLE OF G-PROTEIN SIGNALING IN OLFACTORY SENSORY AXON GUIDANCE

INTRODUCTION

Vertebrate olfactory sensory neurons located in the olfactory epithelium express a single odorant receptor and reproducibly project their axons to distinct synaptic condensations, glomeruli, in the olfactory bulb. The mouse olfactory bulb contains more than 1000 glomeruli, each of which is homogenously innervated by neurons expressing a particular odorant receptor. This topographic convergence of axons into receptor-specific glomeruli is essential for normal olfactory perception (Sakano, 2010). The mechanisms underlying the targeting of subsets of olfactory sensory axons to reproducibly positioned glomeruli in the bulb are not entirely understood.

During development, axonal growth cones navigate through complex environments by either moving towards attractants or turning away from repellents. The behavior of growth cones is further modulated depending on their intracellular levels of cyclic nucleotides and calcium ions (Hong and Nishiyama, 2010). Both in vitro and in vivo, activation of G protein coupled receptors (GPCRs) can either directly attract or repel axons, reduce the effectiveness of repellants or switch the valence of growth cone responses to guidance cues (Xiang et al., 2002; Bonnin et al., 2007; Chalasani et al., 2003; Kreibich et al., 2004; Sabol et al., 2007). For example, an increase in cAMP levels or PKA activation downstream to the 5HT1B/D receptor switches the response of thalamic axons to Netrin1 from attraction to repulsion (Bonnin et al., 2007). In Xenopus spinal neurons, activation of $GABA_B$ receptor switches on the PLC signaling pathway and repels axons. This repulsion can be switched to attraction by activating IP3 signaling and increasing intracellular cGMP (Xiang et al., 2002). Our lab has shown previously in

embryonic chick dorsal root ganglion cells and retinal ganglion cells that signaling via GPCRs such as CXCR4 or mGluR1 activates Gαi, Gαq and Gβγ mediated signaling. This elevates cAMP levels and inactivates Rho GTPase, thereby reducing the responsiveness of growth cones to axonal repellents (Chalasani et al., 2003; Kreibich et al., 2004; Sabol et al., 2007; Twery and Raper, 2011).

Odorant receptors expressed by olfactory sensory neurons are the largest family of G-protein coupled receptors in vertebrate genomes (Buck and Axel, 1991; Firestein, 2001). Odorant receptor activation turns on a signaling pathway mediated via Gαs/olf proteins which stimulates adenylate cyclase 3. This causes an increase in the levels of cAMP and Ca2+, and the activation of protein kinase A (PKA) in cilia and growth cones (Sklar et al., 1986; Sato et al., 1991; Maritan et al., 2009; Jones and Reed, 1989; Schild and Restropo, 1998). In adenylate cyclase type-3 knockout mice, sensory neurons expressing a given odorant receptor project axons to multiple ectopic glomeruli (Zou et. al., 2007). Replacing the odorant receptor coding region with another receptor reroutes mouse olfactory axons to ectopic glomeruli, suggesting that odorant receptors contribute to axonal targeting (Mombaerts et al., 1996; Wang et al., 1998). Altering cAMP levels in a subset of mouse olfactory neurons that express a particular OR changes glomerulus positioning and the mRNA levels of Neuropilin1, an axon guidance receptor which binds to the semaphorin family of guidance cues (Imai et. al., 2006; Chesler et. al; 2007). Thus, G protein mediated signaling participates in axonal pathfinding by either modulating the local responsiveness of axons to guidance cues or by altering the transcription or translation of axon guidance receptors (Jassen et al., 2006; Col et al., 2007).

Zebrafish olfactory epithelia contain two major sensory neuronal classes with distinct molecular characteristics, morphologies and axonal projections (Sato et al., 2005). Ciliated olfactory sensory neurons express the olfactory marker protein (OMP) and odorant receptors coupled with Gαs/olf proteins. Microvillous neurons express transient receptor potential channel 2 (TRPC) along with Gαo proteins coupled with V2R vomeronasal receptors. Olfactory sensory neurons also express Gαi and perhaps Gαq coupled with V1R vomeronasal receptors and Gαi/αo proteins that signal via the CXCR4 receptor (Hamdani and Doving, 2007; Miyasaka et al., 2007). By three days post fertilization, OMP and TRPC expressing axons converge onto distinct, non overlapping regions in the olfactory bulb called protoglomeruli (Sato et al., 2005). Protoglomerular condensations are thought to represent intermingled axons expressing different odorant receptors which eventually segregate into mature glomeruli that are homogenously innervated by neurons expressing a single odorant receptor. I have previously shown that OMP expressing axons target the central zone, dorsal zone, medial glomeruli 1-4 and lateral glomerulus 3 (Lakhina et al., submitted; Chapter 3, this thesis). TRPC neurons project to lateral glomeruli 1, 2 and 4, ventral posterior glomerulus and the olfactory plexus.

In this study, I tested whether G-protein mediated signaling in olfactory sensory neurons contributes to the proper targeting of axons to distinct protoglomeruli in the larval zebrafish olfactory bulb. To this end, I selectively inhibited Gαs/olf, Gαq, Gαi/o, Gβγ, or a downstream effector, protein kinase A (PKA), in subpopulations of olfactory sensory neurons. I then examined whether axonal targeting to protoglomeruli was altered. For selective inhibition of G-proteins, I used dominant negative (DN) G-proteins, short peptides that compete with the endogenous G-protein for GPCR binding (Gilchrist et al., 1999). Expression of Gα blocking peptides has been shown to abrogate targeted

signaling pathways in many systems (Ghahremani et al., 1999; Gilchrist et. al., 2002; Vanhauwe et. al., 2002; Lin et. al., 2005; Kim and Kim, 2005). I perturbed signaling via the Gβγ subunit by overexpressing a C-terminal peptide encoding the G-protein coupled receptor kinase 2 (GRK2) that scavenges Gβγ subunits (Ghahremani et al., 1999). Signaling via protein kinase A (PKA), an effector downstream to cAMP signaling was reducing by overexpressing a mutant in the regulatory subunit of the PKA enzyme (Clegg et al., 1987). Reducing Gαs/olf in OMP expressing neurons causes axonal mistargeting into lateral protoglomeruli. Inhibiting G-protein mediated signaling in OMP, TRPC or OR111-7 expressing neurons reduces the number of transgenic fluorescent neurons, possibly due to improper maintenance of sensory neuron identity. All these phenotypes are cell-autonomous, since G-protein inhibition was performed selectively within specific neuronal subsets. The reduction of the number of olfactory sensory neurons prevents the analysis of axonal guidance errors upon G-protein inhibition. Given that the axonal guidance phenotype that I did observe upon reducing signaling via Gαs/olf in OMP expressing neurons was relatively minor, I did not extend these studies to comprehensively characterize the obtained phenotypes.

MATERIALS AND METHODS

Transgenic zebrafish lines. The construction of the OMP:Gal4, OR111-7:IRES:Gal4 and UAS:Citrine lines is described in Lakhina et al. (submitted) and Chapter 3 of this thesis. The TRPC2:Gal4 construct contains the 4.5 kb TRPC2 promoter (obtained from the Yoshihara laboratory) cloned upstream to a Gal4-VP16 sequence. The dominant negative G proteins specifically prevent heterotrimeric G-protein complexes that contain a particular Gα subunit from binding to their receptors (Gilchrist et. al., 1999). Dominant negative Gαs/olf, Gαq and Gαi/o encode 11 amino acids at the C-terminus of human

Gαs/olf, Gαq or Gαi/o respectively. They function as dominant negative inhibitors by competing with the endogenous Gα subunit for binding to GPCRs. The dominant negative protein kinase A (PKA) subunit contains a mutation in the regulatory type I subunit of the mouse PKA rendering it resistant to cAMP binding thus preventing subsequent activation of the enzyme (Clegg et al., 1987). cDNA encoding dominant negative PKA was kindly provided by Dr. G.S. McKnight (Clegg et. al., 1987). This dominant negative version of PKA has been used previously to knock down PKA activity in zebrafish and mouse olfactory sensory neurons (Yoshida et al., 2002; Imai et al., 2006). The GRK-CT protein encodes the 1506-bp COOH-terminal domain of the G Protein-Coupled Receptor Kinase 2 (GRK2). This protein fragment binds to and scavenges Gβγ subunits and in doing so blocks signaling downstream to Gβγ (Ghahremani et. al., 1999). Alison Dell in the Raper laboratory cloned each dominant negative Gα peptide within the multiple cloning site (MCS) of the UAS:MCS:UAS:gap43-Citrine plasmid to generate a UAS:DN peptide:UAS:gap43-Citrine which is flanked by Tol2 sites (schematized in Figure 5.1). The 'UAS' elements in these constructs contain 14X UAS sequences and the E1b carp β-actin minimal promoter. Alison then confirmed that these constructs interfere with specific signaling steps in the anti-repellent pathway in *in vitro* collapse assays. We injected the TRPC2:Gal4 construct and each UAS:DN peptide:UAS:gap43-Citrine along with mRNA for the Tol2 transposase into wild type zebrafish embryos at the single cell stage. Injected embryos were raised to adulthood and screened to identify transgenic founders. At least two independent transgenic lines were established for each injected construct.

Immunohistochemistry. Three day old zebrafish larvae were fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer. Larvae were treated with acetone for 20

minutes at -20° C to facilitate tissue permeabilization. Goat anti-GFP (1:100, Rockland Immunochemicals, catalog #600-101-215) and anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen) were used to visualize GFP-positive neurons. Propidium iodide staining was performed using the protocol of Brend and Holley (2009) with the omission of the RNase treatment step. Larvae were mounted face down to visualize projections along the dorso-ventral axis (frontal view). Larvae were imaged using a 40X oil immersion lens on a Leica TSP2 confocal microscope. Sections of 1 micron thickness were taken through the entire olfactory bulb.

Quantification of targeting errors. Larvae were processed for immunohistochemistry and imaged using confocal microscopy as described above. The number of larval olfactory bulbs with axonal projections to individual protoglomeruli were counted. The Fisher exact test was used to determine whether the control group was statistically different from the experimental group. The graphs represent the percentage of larvae with axonal projections to the specified location in the olfactory bulb.

Quantification of neuronal number. Stacks of single confocal sections through the entire larval olfactory epithelium were used for analysis. The outline of each neuron in individual sections was traced out on a transparent sheet and counted to accurately determine neuronal number.

RESULTS

Construction of transgenic zebrafish lines used to specifically inhibit signaling via Gαs/olf, Gαq, Gαi/o, PKA and Gβγ in subpopulations of olfactory sensory neurons

I used the Gal4/UAS binary expression system to specifically express dominant negative proteins within subpopulations of labeled olfactory sensory neurons. I generated the

OMP:Gal4 and TRPC:Gal4 transgenic lines to label ciliated and microvillous neurons respectively (Figures 3.1, 5.1). I also made the OR111-7:IRES:Gal4 transgenic line which is expressed in a subset of sensory neurons that primarily target the central zone protoglomerulus (Figures 3.1, 5.1, Lakhina et al., submitted). Alison Dell in the Raper laboratory generated UAS driven dominant negative Gαs/olf, Gαq, Gαi/o, Gβγ transgenic zebrafish lines while I generated the UAS driven dominant negative protein kinase A (PKA) transgenic line (Figure 5.1). To test the role of G-protein signaling in guiding axons to specific protoglomeruli in the developing olfactory bulb, I expressed UAS driven dominant negative Gαs/olf, Gαq, Gαi/o, Gβγ or PKA in OMP, TRPC or OR111-7 transgenic neurons. This was performed by mating adult zebrafish carrying the Gal4 driver constructs with adults harboring the UAS:Citrine control construct or the UAS:DN peptide:UAS:Citrine experimental construct. Embryos generated from this cross were raised until 3 days post fertilization, sorted for the expression of the fluorescent reporter and processed for immunohistochemistry and propidium iodide staining. They were then imaged using confocal microscopy and their axonal projections were analyzed.

Inhibiting Gαs/olf in OMP neurons causes axonal misprojections into lateral protoglomeruli

In control larvae, OMP expressing axons are detected in the central zone, dorsal zone, medial glomeruli and LG3 (Figure 5.2A, B, blue bars in E). OMP:Gal4 expressing axons rarely target the LG1, LG2, VPG or non-protoglomerular regions of the bulb which are labeled as ‘other’ in Figure 5.2E (white arrows, Figure 5.2A, B, blue bars in E). When dominant negative Gαs/olf is expressed in OMP:Gal4 neurons, axons ectopically project to the LG1 and LG2 protoglomeruli at a frequency that is significantly higher than in control larvae (yellow arrows, Figure 5.2C, D, purple bars in E). Transgenic OMP

neurons expressing dominant negative Gαq or dominant negative PKA displayed no axonal guidance errors (Figure 5.2F, H, Table 1A). Upon reducing Gβγ signaling, OMP expressing axons did not target ectopic protoglomeruli (Figure 5.2G, Table 1A).

Inhibiting signaling via Gαs/olf, Gαq, Gαi/o, Gβγ or PKA in OMP neurons causes a reduction in the number of fluorescently labeled neurons

Fewer transgenic OMP expressing neurons that also expressed dominant negative Gαs/olf, Gαq, Gαi/o, Gβγ or PKA were detected (Table 1A). The OMP:Gal4 transgenic line labels the majority of olfactory sensory neurons in the epithelium. Thus, it is impossible to accurately count the number of OMP expressing neurons in 3 day old larvae. Assuming that ~400 neurons are present in each olfactory pit, upon reducing signaling via Gαs/olf, about 300 neurons are detected (~1 fold decrease). Reducing signaling via Gαq causes approximately a 2 fold decrease, while inhibiting signaling via Gβγ or PKA results in approximately a 10 fold decrease in labeled neurons (Table 1A). The decrease in labeled transgenic OMP expressing neurons upon reducing Gβγ signaling results in decreased axonal projections to several protoglomeruli (Figure 5.2G, Table 1A). Upon mating OMP:Gal4 transgenic zebrafish with UAS:DN Gαi/o:UAS:Citrine transgenics, very few fluorescent progeny were obtained (Table 1A). Of the about 400 progeny screened, 25% (i.e. 100 larvae, or 200 olfactory pits) are expected to harbor both Gal4 and UAS transgenes, but only 5 larvae with an average of three fluorescent neurons in their olfactory epithelia were detected. These residual neurons did not target their axons to inappropriate protoglomeruli (data not shown).

Inhibiting signaling via Gαs/olf, Gαq, Gαi/o, Gβγ and PKA specifically in OR111-7 expressing neurons reduces their number

I have previously characterized the axonal projections of neurons expressing OR111-7:IRES:Gal4; UAS:Citrine transgenes (Chapter 2, this thesis; Lakhina et al., submitted). OR111-7 transgene expressing neurons primarily target their axons to the central zone and a few axons to the LG1 protoglomerulus (Figure 5.3A). I wondered whether inhibiting Gαs/olf mediated signaling specifically in OR111-7 expressing neurons would affect their protoglomerular targeting. Reducing signaling via Golf/s in OR111-7 neurons decreased the number of OR111-7 expressing neurons in 3 day old larvae from an average of 26 neurons in controls to about 8 neurons in the OR111-7:IRES:Gal4; UAS:DN Gαs/olf:UAS:Citrine larvae (Figure 5.3A, B, F; Table 1B). The remaining axons still target the central zone protoglomerulus (Figure 5.3A, B). A decrease in the number of transgenic OR111-7 expressing neurons was also observed when Gαq, Gβγ or PKA mediated signaling was inhibited. On average, 4 neurons expressing the OR111-7 transgene and dominant negative Gαq were detected, while 2 OR111-7:IRES:Gal4; UAS:GRKCT:UAS:Citrine neurons and 3 OR111-7:IRES:Gal4; UAS:DN PKA:UAS:Citrine neurons were detected in three day old olfactory epithelia (Figure 5.3C, D, E, F; Table 1B). In all cases, neurons that were still present projected their axons to the central zone protoglomerulus (Figure 5.3C, D, E). Mating OR111-7:IRES:Gal4 transgenic animals with those harboring the UAS:DN Gαi/o:UAS:Citrine transgene did not generate progeny with fluorescently labeled neurons, despite screening about 400 larvae, 25% of which (i.e. 100 larvae, 200 olfactory pits) were expected to harbor both Gal4 and UAS transgenes (Table 1B).

Expression of dominant negative G-protein signaling components in TRPC neurons reduces their number

I next examined the effects of inhibiting G-protein mediated signaling in TRPC expressing neurons. Reducing signaling via Gαs/olf, Gαq, Gβγ or PKA reduces the

number of fluorescent TRPC neurons detected (Figure 5.4). On average about 17 neurons are labeled in control TRPC:Gal4; UAS:Citrine larvae, while only 6 neurons are detected upon Gαs/olf inhibition and only 2 neurons are detected upon PKA inhibition (Figure 5.4A-E). Surprisingly, mating transgenic TRPC:Gal4 zebrafish with UAS:DN Gαq:UAS:Citrine transgenics generated no fluorescent progeny in eight clutches of embryos (a total of 416 embryos). Since 25% of the progeny should have harbored both the Gal4 and UAS transgenes, I expected to observe around 104 larvae (i.e. 208 olfactory pits) with fluorescently labeled neurons (Table 1C). Similarly, mating the TRPC:Gal4 transgenics with UAS:GRKCT:UAS:Citrine transgenics reduced the number of progeny with fluorescently labeled neurons. Upon screening a total of 204 embryos obtained from 4 separate clutches, only 3 larvae (i.e. 6 olfactory pits) with an average of 9 fluorescent TRPC neurons were obtained. As before, 25% of the progeny were expected positives, i.e. 51 embryos or 102 olfactory pits; Table 1C). Despite the reduction in neuronal number, reducing signaling via Gαs/olf, Gβγ or PKA in TRPC neurons does not cause axons to innervate ectopic protoglomeruli (Figure 5.4A-D). Mating the TRPC:Gal4 transgenics with UAS:DN Gαi/o:UAS:Citrine transgenic animals, did not generate progeny with labeled neurons, despite screening around 400 larvae, of which 25% were expected to carry both Gal4 and UAS transgenes (Table 1C).

DISCUSSION

In this study, I investigated whether inhibiting G-protein signaling in different subsets of olfactory sensory neurons affected axonal targeting to protoglomeruli. In most cases, inhibiting G-protein mediated signaling reduced the numbers of fluorescently labeled olfactory sensory neurons. This either precludes the analysis of axonal targeting to protoglomeruli or complicates the interpretations of the phenotypes obtained. I therefore did not perform an extensive characterization of the phenotypes obtained.

Axonal guidance errors observed upon expressing dominant negative Gαs/olf in OMP neurons could occur due to decreased midline attraction

Inhibiting Gαs/olf signaling in OMP neurons caused aberrant axonal projections into lateral glomeruli 1 and 2. Preliminary data suggests that reducing signaling via the netrin receptor, DCC in OMP expressing neurons also causes inappropriate axonal projections into LG1 and LG2 (data not shown). Thus, aberrant lateral projections away from the midline could represent a decrease in midline attraction to netrin1a (Lakhina et al., submitted; Chapter 3, this thesis). If this hypothesis is correct, overexpressing DCC using the UAS:DCC transgene in OMP:Gal4; UAS:dominant negative Gαs/olf:UAS:Citrine larvae should decrease the number of misprojections into lateral protoglomeruli. Alternately, reducing signaling via Gαs/olf could alter the sensitivity of a subset of OMP expressing neurons to other guidance cues in the bulb.

Olfactory sensory neurons may be particularly sensitive to alterations in G-protein signaling

Expressing dominant negative G-proteins in TRPC neurons and OR111-7 expressing neurons caused a dramatic reduction in the number of fluorescent neurons detected. Expression of the same UAS:dominant negative G-protein:UAS:Citrine transgene in retinal ganglion cells does not cause a dramatic reduction in fluorescent neurons (Alison Dell, personal communication). Further, expressing a dominant negative Gαi/o coupled GABA-B receptor in OMP expressing olfactory sensory neurons and not retinal ganglion cells also reduces the number of fluorescent neurons (Alemji Taku, personal communication). These data suggest that olfactory sensory neurons are particularly sensitive to alterations in G protein signaling. Furthermore, it is unlikely that the decrease in fluorescent olfactory sensory neurons is a consequence of silencing of the

UAS transgene, a problem that frequently occurs in zebrafish embryos (Akitake et al., 2011). It is possible that G protein signaling is permissive for proper development of olfactory sensory neurons since the reduction of signaling pathways downstream of Gαs/olf, Gαq, Gαi/o, Gβγ and PKA had the common effect of reducing the number of transgenic neurons.

Altering G-protein mediated signaling could affect the survival, proliferation or maintenance of the fate of olfactory sensory neurons

G-protein mediated signaling promotes the survival of olfactory sensory neurons, hippocampal neurons, retinal ganglion cells, cerebellar Purkinje cells and cortical neurons (Tanaka et al., 1999; Gingerich et al., 2010; Chalasani et al., 2003; Catania et al., 2001; Nicolai et al., 2010). It is therefore possible that the decrease in fluorescent neurons observed upon inhibiting G-protein signaling occurs due to increased apoptosis. This could be explored by performing TUNEL staining using larvae in which dominant negative G-proteins are expressed in different neuronal subsets.

G-protein signaling is also required for neuronal differentiation and proliferation of neuronal precursors. Activation of the Gαq-coupled purinergic P2Y2 receptor enhances NGF-induced neuronal survival and differentiation (Arthur et al., 2005). Activation of the endocannabinoid receptor CB1 promotes the proliferation of neuronal precursor cells (Aguado et al., 2005; Mulder et al., 2008). It is possible that the observed reduction in olfactory sensory neurons upon inhibiting G-protein signaling arises due to defective proliferation which can be tested by performing BrDU incorporation experiments to compare the proliferation rate of olfactory sensory neurons between control groups and larvae in which G protein signaling has been reduced.

Neurons expressing either OMP or TRPC are specified to adopt two distinct fates. They are morphologically dissimilar and express a variety of cell specific markers (Sato et al., 2005). Since I have used OMP or TRPC driven Gal4 lines in my experiments, dominant negative G-proteins are expressed only after neurons are restricted to the OMP or TRPC fate. It is possible that interfering with G-protein signaling prevents the maintenance of these sensory neuron fates, thus causing a decrease in labeled, fate-restricted OMP or TRPC neurons. In a different context, cAMP signaling via the cAMP response element binding protein (CREB) promotes the maintenance of gene expression required for the maturation of olfactory bulb interneurons in the mouse subventricular zone (Herold et al., 2011).

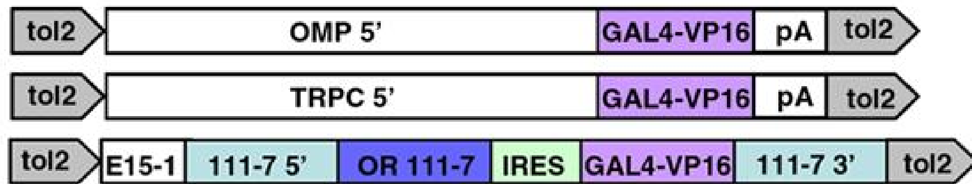
In conclusion, I find that inhibiting Gαs/olf signaling in OMP expressing olfactory sensory neurons causes aberrant axonal projections into LG1 and LG2 protoglomeruli. A dramatic decrease in fluorescent OMP neurons is observed upon inhibiting Gαi/o and Gβγ. Similarly, expressing dominant negative Gαs/olf, Gαq, Gαi/o, Gβγ and PKA in OR111-7 or TRPC expressing neurons reduces the number of fluorescent neurons detected. Detailed characterization of the neuronal reduction phenotypes will reveal whether these phenotypes arise due to problems with neuronal proliferation, survival or fate maintenance. Future experiments in which G-protein mediated signaling is inhibited with greater temporal control could be performed to fully explore the role of G-protein signaling in the guidance of olfactory sensory axons. Once Gal4 lines labeling other subsets of olfactory sensory neurons become available, it would be interesting to examine whether G-protein signaling is required for their pathfinding. Over-expressing constitutively active G-protein subunits in different subsets of olfactory sensory neurons is another approach to test whether altering G-protein signaling affects axonal targeting to protoglomeruli.

ACKNOWLEDGEMENTS

I thank Alison Dell for generating the UAS driven dominant negative Gαs/olf, Gαq, Gαi/o and Gβγ transgenic zebrafish lines and testing the efficacy of these transgenics using collapse assays.

Figure 5.1.

A) DRIVER CONSTRUCTS



B) REPORTER CONSTRUCTS

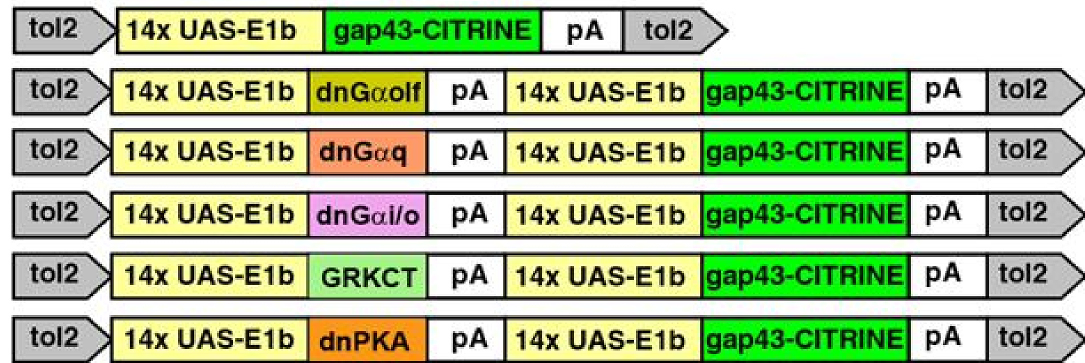


Figure 5.1. Schematics showing the Gal4 driver and UAS reporter constructs used to inhibit G-protein signaling in different subsets of zebrafish olfactory sensory neurons

A, OMP:Gal4 is selectively expressed in ciliated neurons that express olfactory marker protein (OMP) and classical odorant receptors. The TRPC:Gal4 driver selectively labels microvillous neurons expressing the transient receptor potential channel 2 (TRPC2) and V2R vomeronasal receptors. OR111-7:IRES:Gal4 is primarily expressed in a subset of OMP expressing neurons that target the central zone protoglomerulus. **B,** The control construct consists of a UAS-E1b minimal promoter driving the Citrine fluorophore. In the experimental constructs, a UAS-E1b minimal promoter drives the expression of dominant negative Gα peptides, GRKCT, or dominant negative PKA while a second UAS-E1b minimal promoter drives the Citrine fluorophore.

Figure 5.2.

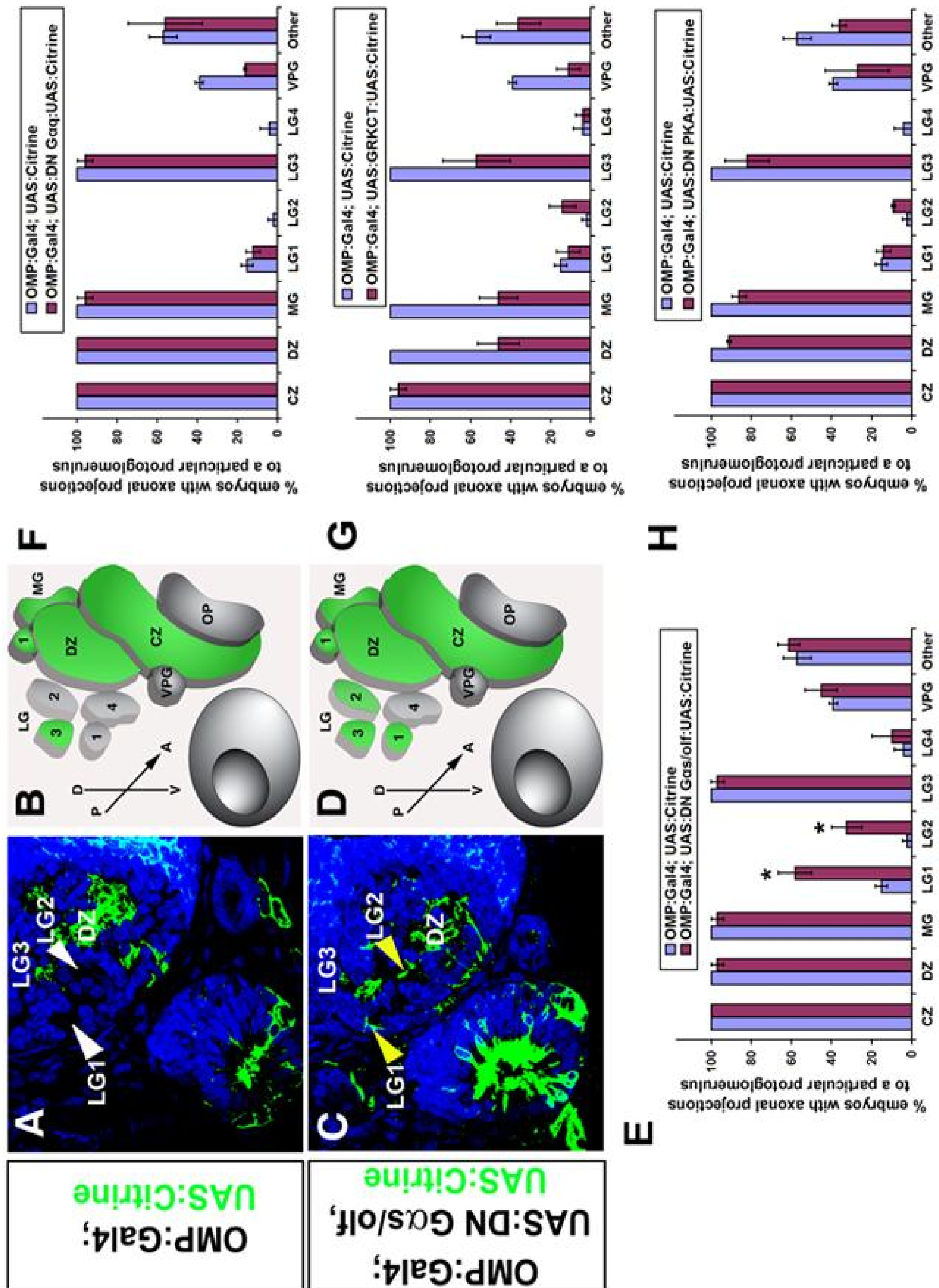


Figure 5.2. Reducing Gαs/olf signaling in OMP expressing neurons causes axonal mistargeting into lateral protoglomeruli

A, C, Single confocal optical sections through 3 day old OR111-7:IRES:Gal4; UAS:Citrine larvae or OR111-7:IRES:Gal4; UAS:DN Gαs/olf:UAS:Citrine larvae (frontal view).). Dorsal is to the top and midline to the right. Propidium iodide (blue) delineates distinct protoglomeruli in the olfactory bulb. **A, B, blue bars in E,** OMP expressing neurons project axons to the CZ, DZ, MG and LG3 and rarely innervate LG1 and LG2. **C, D, purple bars in E,** OMP neurons expressing dominant negative Gαs/olf misproject into the LG1 and LG2 protoglomeruli at a significantly greater rate than controls. **F, H,** No axonal guidance errors are observed in transgenic OMP neurons expressing dominant negative Gαq or PKA. **G,** Expression of GRK-CT in OMP expressing neurons reduces the percentage of olfactory epithelia in which OMP neurons target the DZ< MG, LG3 and VPG, but this is due to a reduction in the number of labeled neurons (see Figure 5). Fisher's exact test was used to test for statistical significance, $P<0.05$.

CZ: *central zone,* DZ: *dorsal zone,* MG: *medial glomeruli 1-4,* LG1-4: *lateral glomeruli 1-4,* VPG: *ventral posterior glomerulus,* Other: *axonal projections to non-protoglomerular regions of the olfactory bulb.*

Figure 5.3.

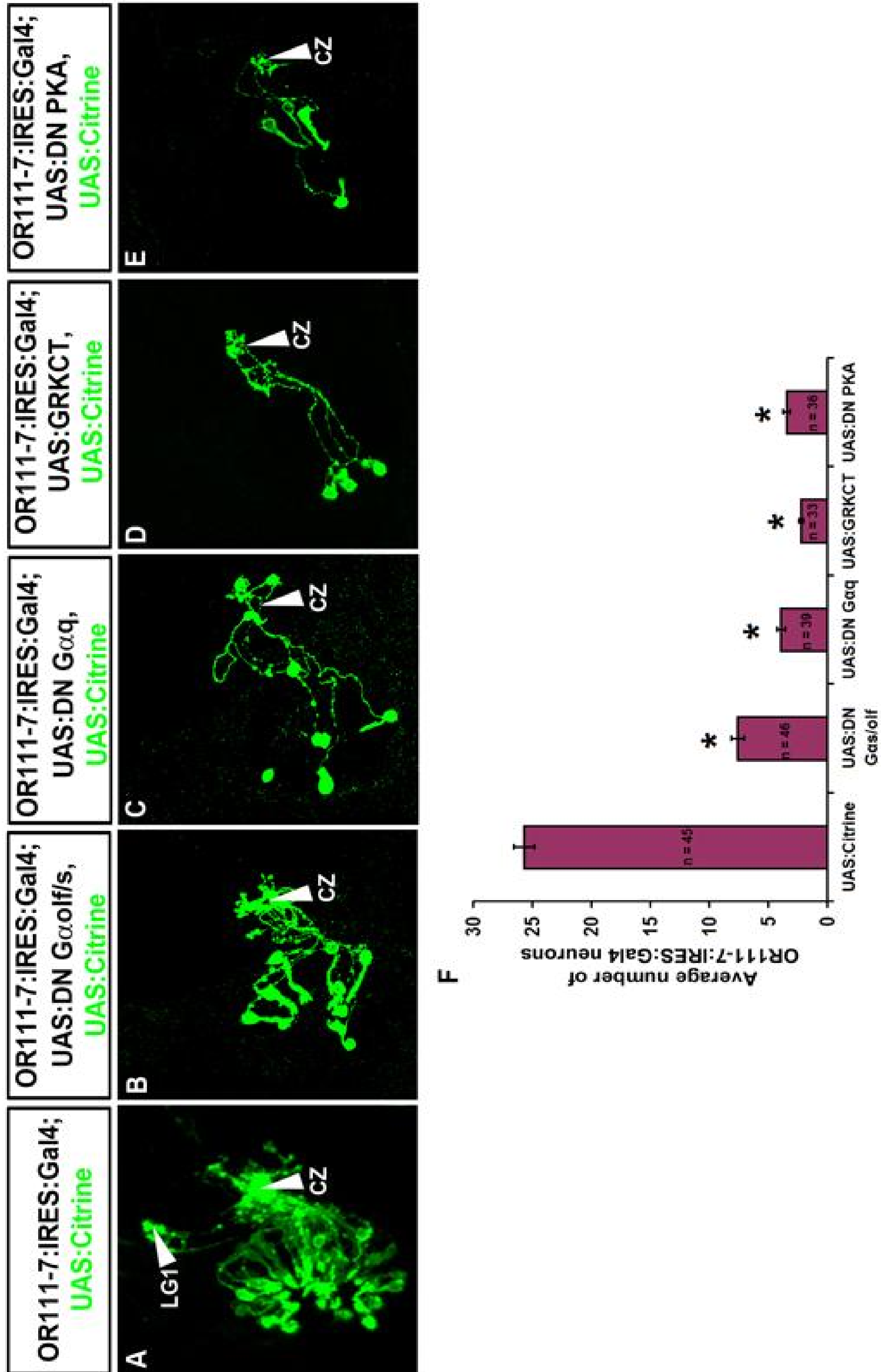


Figure 5.3. A decrease in signaling via Gαs/olf, Gαq, Gβγ and PKA reduces the number of OR111-7 transgenic neurons

A, F, On average, 26 neurons expressing the OR111-7:IRES:Gal4; UAS:Citrine transgenes are detected in 3 day old olfactory epithelia. **B, F,** Driving the expression of dominant negative Gαs/olf in OR111-7 expressing neurons reduces their number to an average of about 8 neurons. **C, F,** On average, 4 OR111-7 transgenic neurons expressing the dominant negative Gαq are detected. **D, F,** Expressing the Gβγ scavenger, GRKCT in OR111-7 transgenic neurons reduces their number to about 2 neurons. **E, F,** About 3 transgenic OR111-7 neurons expressing dominant negative PKA were seen in 3 day old olfactory epithelia. **A-F,** OR111-7 expressing axons are detected in the central zone (CZ) in control larvae and larvae with reduced G-protein signaling. One way ANOVA was used to test for statistical significance, $P<0.05$.

Figure 5.4.

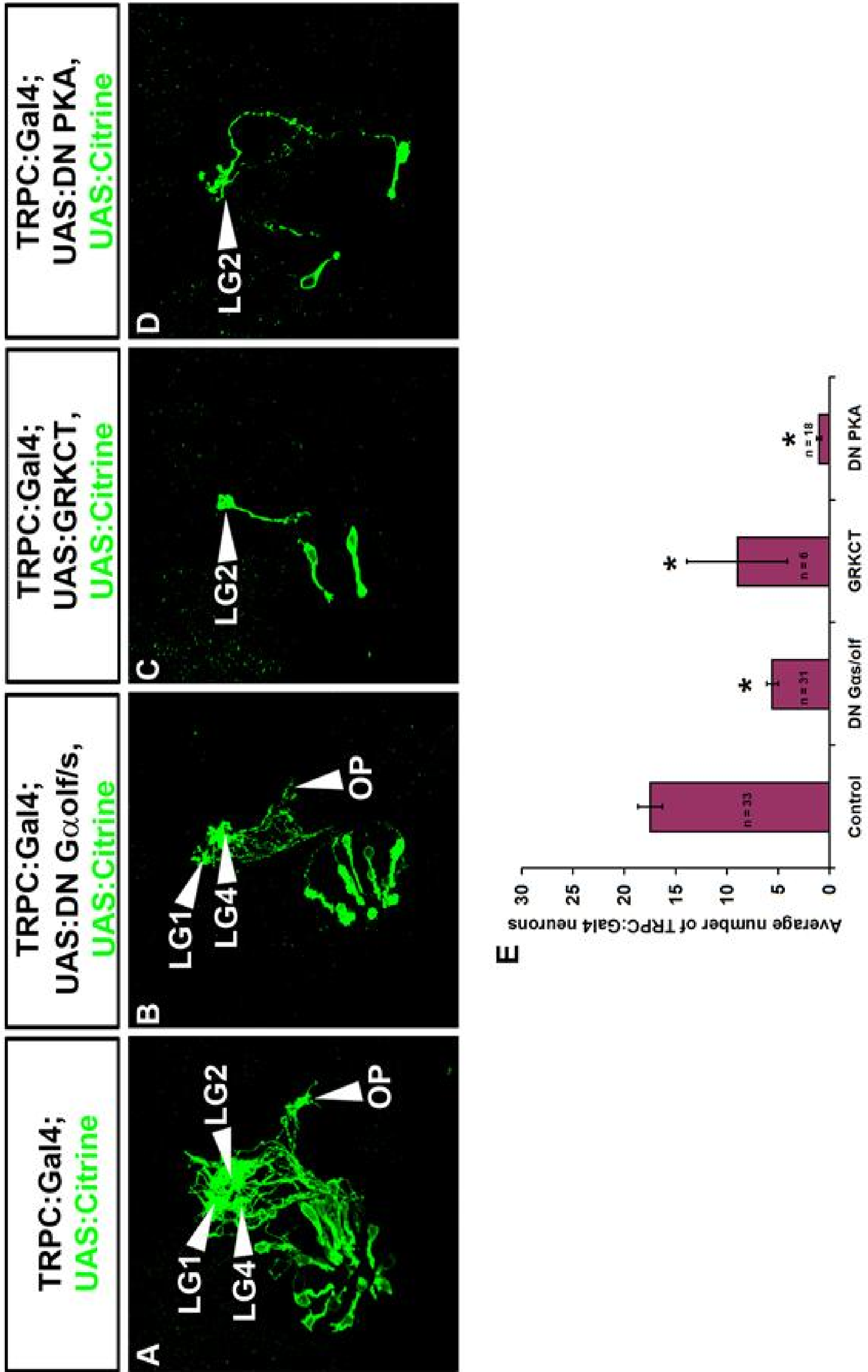


Figure 5.4. Inhibiting signaling via Gαs/olf, Gβγ and PKA reduces the number of TRPC expressing microvillous olfactory sensory neurons

A-D, TRPC:Gal4 labels microvillous olfactory sensory neurons that express the transient receptor potential channel 2 (TRPC2) and V2R vomeronasal receptors. **A, E,** On average about 17 neurons are labeled in control TRPC:Gal4; UAS:Citrine larvae. TRPC expressing axons target the LG1, LG2, LG4 and OP. **B, E,** Upon Gαs/olf inhibition, only 6 TRPC neurons are detected. These neurons project axons to protoglomeruli that are normally innervated by TRPC expressing neurons. **C, E,** Inhibiting Gβγ using GRKCT in TRPC expressing neurons reduced the number of fluorescent progeny obtained (see Table 1). Only 6 olfactory pits with fluorescent neurons were obtained from 4 separate clutches of embryos (expected 102 positive olfactory pits). Of the olfactory pits expressing fluorescent neurons, an average of 9 TRPC neurons were detected. **D, E,** Only 2 TRPC expressing neurons are detected upon PKA inhibition. One way ANOVA was used to test for statistical significance, $P<0.05$.

LG1-4: *lateral glomeruli 1-4,* OP: *olfactory plexus*

Table 1.

A

OMP:Gal4	Qualitative reduction in neuronal number	Axonal guidance errors
UAS:DN Gαolf/s:UAS:Citrine (n=31)	~1 fold decrease	Mistargeting to LG1 and LG2
UAS:DN Gαq:UAS:Citrine (n=24)	~2 fold decrease	None
UAS:DN Gαi/o:UAS:Citrine (n=10)	Severe (n=10 fluorescent olfactory pits obtained, expected = around 200 olfactory pits)	None
UAS:GRKCT:UAS:Citrine (n=28)	~10 fold decrease	None
UAS:DN PKA:UAS:Citrine (n=22)	~10 fold decrease	None

B

OR111-7:IRES:Gal4	Reduction in neuronal number	Axonal guidance errors
UAS:DN Gαolf/s:UAS:Citrine (n=46)	3 fold decrease	None
UAS:DN Gαq:UAS:Citrine (n=39)	6 fold decrease	None
UAS:DN Gαi/o:UAS:Citrine	Severe (no fluorescent progeny obtained, expected around 200 olfactory pits)	Unable to assay
UAS:GRKCT:UAS:Citrine (n=33)	12 fold decrease	None
UAS:DN PKA:UAS:Citrine (n=36)	6 fold decrease	None

C

TRPC:Gal4	Reduction in neuronal number	Axonal guidance errors
UAS:DN Gαolf/s:UAS:Citrine (n=33)	3 fold decrease	None
UAS:DN Gαq:UAS:Citrine	Severe (No fluorescent progeny obtained, expected = 208 olfactory pits)	Unable to assay
UAS:DN Gαi/o:UAS:Citrine	Severe (no fluorescent progeny obtained, expected around 200 olfactory pits)	Unable to assay
UAS:GRKCT:UAS:Citrine (n=6)	Severe (n=6 fluorescent olfactory pits obtained, expected = 102 larvae)	None
UAS:DN PKA:UAS:Citrine (n=18)	17 fold decrease	None

Table 1. Summary of the phenotypes obtained upon inhibiting signaling via Gαs/olf, Gαq, Gαi/o, Gβγ and PKA in OMP-, OR111-7- or TRPC-expressing olfactory sensory neurons

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

The three day old larval zebrafish olfactory bulb consisting of only 12 stereotyped protoglomeruli has the potential to be developed into an excellent vertebrate system to study the initial targeting of olfactory sensory axons to specific locations within the olfactory bulb. In 2005, when I was embarking upon my thesis projects, transgenic zebrafish lines were available in which the two major neuronal subsets in the olfactory epithelium (those expressing either OMP or TRPC) were selectively labeled. Since OMP- and TRPC-expressing neurons target multiple protoglomeruli, these transgenic lines are not useful for examining mechanisms mediating axonal targeting to individual protoglomeruli (Sato et al., 2005). For example, Miyasaka et al (2005) observed gross protoglomerular disorganization in larvae in which the Robo2 axonal guidance receptor was knocked out, but were unable to extend these studies to examine whether axonal subsets mistargeted into inappropriate OMP+ protoglomeruli (Miyasaka et al., 2005). Subsequently, to selectively visualize neuronal subsets, Sato et al. (2007) generated transgenic zebrafish carrying a BAC construct in which the OR111-7 and OR103-1 were replaced with YFP and CFP respectively. YFP and CFP expressing axons converged onto the central zone. Adult YFP/CFP expressing neurons also expressed a variety of other odorant receptors, thus it was not known whether the endogenous targeting of neurons expressing OR111-7 or OR103-1 was recapitulated. Consequently, it was essential to generate better tools to selectively label subsets of olfactory sensory neurons. The availability of such tools would allow researchers to investigate mechanisms mediating axonal targeting into and within the olfactory bulb. Furthermore, at the time that I began my studies, only receptors belonging to an odorant receptor cluster in chromosome 15 were known to be expressed in the larval olfactory epithelium (Barth and Ngai, 1996; 1997). Thus, it was necessary to determine which odorant

receptors are expressed in three day old larvae to gain a better understanding of the correlation between the odorant receptor expressed by a neuron and the protoglomerulus that its axon innervates.

Generating tools to develop the larval zebrafish as a viable model for studying protoglomerular axonal targeting

The first goal of my thesis project was to use the Gal4/UAS binary expression system to generate transgenic zebrafish lines in which subsets of olfactory sensory neurons and their axons would be selectively labeled. I generated OMP and TRPC promoter driven Gal4 transgenic zebrafish lines to make possible neuronal subset specific misexpression of UAS driven dominant negative reagents. Another important goal was to generate transgenic zebrafish lines in which a subset of OMP neurons expressing a particular odorant receptor could be selectively visualized. As described in chapter 2, the OR111-7:IRES:Gal4 trangenic line labels a subset of neurons that primarily target axons to the central zone protoglomerulus. This served as an excellent tool that allowed for the discovery that netrin/DCC signaling contributes to the guidance of OR111-7 expressing axons to the central zone protoglomerulus (Chapter 3). Unfortunately, the number of transgenic OR111-7:IRES:Gal4; UAS:Citrine decreases with age, thus obstructing the analysis of axonal targeting events occurring later in development. This is likely a consequence of missing regulatory elements in the OR111-7 minigene construct. It is possible that for an odorant receptor such as OR111-7 that is present in odorant receptor-rich clusters, regulatory elements that influence its expression are distributed in multiple locations within the cluster. A minigene construct only contains limited cis upstream and downstream genomic elements and may not recapitulate endogenous odorant receptor expression.

To resolve this problem, in future experiments, transgenic zebrafish lines can be generated using bacterial artificial chromosomes encompassing the entire OR111 subfamily cluster. The BAC construct would contain an IRES:Gal4 cassette inserted immediately after the OR111-7 coding sequence. Since repetitious sequences can interfere with the recombineering required to generate such constructs, perhaps it might be prudent to choose to study an odorant receptor that is not surrounded by many other odorant receptor genes. For example, OR135-1 is located on chromosome 8 and is not surrounded by any other odorant receptors for at least 200 kb upstream or downstream (Ensembl zebrafish genome browser; Alioto and Ngai, 2005). OR129-1 and OR130-1 are located on chromosome 8 about 40 kb apart and at least 250 kb of genomic sequence flanking them on either side does not contain other odorant receptors (Ensembl zebrafish genome browser).

The relationship between odorant receptor expression and axonal targeting to protoglomeruli

As a first step towards understanding the relationship between the odorant receptor expressed by a neuron and the protoglomerulus its axon innervates, Jiwei He, Xin Shao and I cloned and characterized the expression of 35 odorant receptors expressed in the three day old zebrafish olfactory epithelium. Christina Marcaccio and I subsequently showed that neurons expressing genomically linked, related receptors of the OR111 subfamily innervate the central zone protoglomerulus (Chapter 4). Replacing the OR111-7 coding sequence in the OR111-7:IRES:Gal4 transgenic construct with RFP did not alter axonal targeting to the central zone (Chapter 2). It will be interesting to examine whether neurons expressing the minigene construct in which the OR111-7 coding region was replaced with RFP switch ON the expression of other receptors of the OR111 subfamily.

Future experiments will determine whether, like the central zone protoglomerulus, other protoglomeruli in the zebrafish olfactory bulb are innervated by neurons expressing related, but different odorant receptors. Correlating the odorant receptor expressed by a neuron and its axonal projection can be achieved as described previously in Figure 4.2. Briefly, a single OMP expressing neuron and its axonal projection can be labeled in transiently transgenic larvae and in situ hybridization using a mixture of related odorant receptor probes can be performed to examine whether the labeled neuron expresses those odorant receptors. However, these experiments were very low throughput and technically challenging. Future experiments could employ two different strategies to probe the relationship between odorant receptor expression and protoglomerular innervation. First, the protoglomerular projections of neurons that are singly and randomly labeled using a low dose of the OMP:Gal4 transgene can be mapped. Next, using a patch electrode, the cytoplasm of that neuron can be recovered and used to perform RT-PCR with odorant receptor subfamily specific degenerate primers. Performing repeated single cell experiments will reveal which odorant receptors are expressed by neurons that target particular protoglomeruli. The information obtained from these experiments can subsequently be used to generate transgenic zebrafish lines in which neurons expressing a particular odorant receptor and their axonal projections to a specific protoglomerulus are selectively visualized. BAC transgenics with an IRES:Gal4 cassette inserted immediately after an odorant receptor coding sequence are likely to retain endogenous expression properties. By generating BAC transgenics in which neurons expressing related odorant receptors express differently colored fluorophores, heterogenous innervation of protoglomeruli can be directly observed. Importantly, BAC transgenic lines in which neurons targeting protoglomeruli other than the central zone can be generated. The availability of such transgenic lines is crucial to enable the study of axonal targeting to different protoglomeruli in the larval zebrafish

olfactory bulb. If every protoglomerulus is indeed innervated by neurons expressing related odorant receptors, it will be interesting to examine whether neurons targeting different protoglomeruli express a distinct combination of transcription factors which might correlate with the expression of axonal guidance receptors. Transgenic lines labeling neurons that innervate different protoglomeruli can be used to perform microarray experiments to identify unique transcription factors that distinguish between neuronal subtypes.

Axonal guidance to stereotyped protoglomeruli and fasciculation of like axons

My studies using the OR111-7:IRES:Gal4 transgenic line showed that the OR111-7 expressing neurons require netrin/DCC signaling to appropriately target the central zone. Since not all axons exhibit ectopic projections upon reducing netrin or DCC levels, it is likely that other guidance cues also contribute to the correct targeting of OR111-7 expressing axons. Possible candidates include ligands of the semaphorin family of axonal repellants, Sema3a, Sema3d and Sema3e and their receptors neuroplin1a and neuropilin1b, which are expressed in the developing olfactory epithelium (Yu et al., 2004; Thisse and Thisse, 2005; Callander et al., 2007; Kuan et al., 2007). Larvae in which the Robo2 receptor is knocked out have disrupted protoglomeruli, but it is unclear whether subsets of olfactory sensory neurons are mistargeted into ectopic protoglomeruli (Miyasaka et al., 2005). Xin Shao in the Raper laboratory did not observe axonal guidance errors of OR111-7:IRES:Gal4; UAS:Citrine axons in Robo2 mutant larvae. When BAC transgenic lines labeling different subsets of olfactory sensory neurons become available, the contribution of Robo2 signaling to their protoglomerular targeting can be examined.

It remains unclear how the axons of neurons expressing the same odorant receptor fasciculate together. The odorant receptor expressed by a neuron could determine the expression levels of homophilic adhesive molecules Kirrel2/Kirrel3 and repulsive molecules ephrin-A5/EphA5 (Serizawa et al., 2006). Olfactory cell adhesion molecule (OCAM), a novel homophilic adhesion molecule belonging to the immunoglobulin superfamily, and Rb-8 neural cell adhesion molecule (RNCAM) are expressed by sensory neurons in restricted chemoreceptor expression zones (Yoshihara et al., 1997; Alenius and Bohm, 1997). Cadherin-1 and cadherin-2 are expressed in zebrafish olfactory sensory neurons and neurons with different amounts of cadherin-1 protein expression were detected in the epithelium, which could reflect neuronal subset specific variation in expression (Liu et al., 2004). The expression patterns of cadherins and other homophilic adhesion molecules can be examined in OMP-, TRPC-, or OR111-7 expressing neurons. To test the hypothesis that homophilic adhesion between like axons is required for their proper convergence onto protoglomeruli, dominant negative cell adhesion molecules or their effectors can be expressed in different subsets of olfactory sensory neurons. For example, UAS driven dominant negative cadherin-1 (Zhu and Watt, 1996) or a dominant negative alpha catenin (Bajpai et al., 2009) which reduces the strength of intercellular cadherin-1 bonds can be selectively expressed using OMP:Gal4, TRPC:Gal4 or OR111-7:IRES:Gal4 driver lines, or in other neurons as more subset-specific Gal4 lines become available.

The requirement for G-protein signaling during axonal segregation

The model that I describe in chapter 4 predicts that axonal targeting to protoglomeruli is independent of odorant receptor mediated signaling. It is possible that subsequent steps involving glomerular segregation and maturation require G-protein mediated signaling via odorant receptors. Temporally restricted inhibition of G-proteins in OR111-

7:IRES:Gal4 expressing neurons at the precise time when protoglomeruli are segregating into glomeruli can be performed to test this hypothesis. One way to do this experiment is to generate transgenic zebrafish larvae that harbor the following transgenes: a heat shock driven Cre recombinase transgene line (hsp70-Cre), the OR111-7:IRES:Gal4 minigene and a UAS:LoxP-RFP-LoxP-DN G-protein:UAS:Citrine (modified from the design of Le et al., 2007). In the absence of Cre recombinase, RFP and Citrine will be expressed in a tissue specific manner, but due to the presence of a stop codon in the RFP gene, the DN G-protein will not be expressed. Expression of the Cre recombinase subsequent to heat shock will result in recombination at the LoxP sites and loss of RFP expression. At the same time, the DN G-protein and the Citrine will be expressed specifically in OR111-7 expressing sensory neurons.

Another approach is to generate transgenic zebrafish harboring minigene constructs encoding odorant receptors deficient in downstream G-protein mediated signaling. Imai et al. (2006) generated mutant odorant receptors that had a point mutation into the Gαs/olf interacting domain which abrogated downstream signaling. Neurons expressing this type of mutant receptor suppress the expression of other odorant receptors and do not express a different, functional odorant receptor (Imai et al., 2006). Assuming that this property is conserved in zebrafish, a mutant odorant receptor expressed from a BAC construct can be used to test whether G-protein mediated signaling is required for proper axonal segregation into glomeruli and glomerular maturation. If zebrafish larval olfactory sensory neurons require odorant receptor mediated signaling for glomerular segregation, neurons expressing the mutant receptor would target to the appropriate protoglomerulus, but would perhaps be unable to appropriately segregate into glomeruli.

In conclusion, the studies described in this thesis are among the first to address the role of axonal guidance cues, odorant receptors and G-protein signaling in the initial targeting of zebrafish olfactory sensory axons to stereotyped protoglomeruli. These studies, along with the experimental tools that I generated have laid the foundation for future work exploring basic mechanisms that mediate the pathfinding of olfactory sensory axons to correct locations in the olfactory bulb.

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