The Role Of Class 3 Semaphorins In Guiding Olfactory Sensory Axons To Their Protaglomerular Targets In The Zebrafish Olfactory Bulb

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Abstract
The convergence of olfactory sensory axons onto topographically fixed, odorant receptor specific glomeruli in the olfactory bulb (OB) is a complex biological problem. Early in development, axons from olfactory sensory neurons (OSNs) extend into the OB where they innervate distinct neuropilar structures called protoglomeruli. Protoglomeruli subsequently segregate into mature glomeruli. The zebrafish is an excellent system in which to study early protoglomerular targeting. At 72 hours post-fertilization, the zebrafish OB contains 12 easily identifiable protoglomeruli. The objective of this thesis work was to identify axon guidance cues and receptors that direct olfactory sensory axons to their protoglomerular targets.

Here, I describe a novel role for Sema3D/Nrp1a signaling in protoglomerular targeting. An OR111-7:IRES:Gal4 transgene labels a subset of olfactory axons that target the ventromedially located central zone (CZ) protoglomerulus. Sema3D is expressed in the anterior OB. OR111-7 transgene expressing axons terminate posterior to sema3D expression. In sema3D mutants, a subset of OR111-7 transgene expressing axons misproject to the dorsal zone (DZ) protoglomerulus which is dorsal and anterior to the CZ. Four zebrafish neuropilins (nrps), essential receptor components for class 3 semaphorins, are differentially expressed in OSNs. OR111-7 transgene expressing neurons express nrp1a, nrp1b, and/or nrp2b. Loss of either nrp1a or nrp2b phenocopies sema3D mutants. OR111-7 transgene expressing axons are misguided in nrp1a-/+;sema3D-/+ embryos, but not in nrp2b-/+;sema3D-/+ embryos. The phenotype is not accentuated in sema3D;nrp1a double mutants as compared to sema3D or nrp1a mutants. Re-expression of nrp1a in OR111-7 transgene expressing OSNs corrects the DZ targeting error observed in nrp1a mutants. These data support a model wherein sema3D expressed in the anterior OB repels nrp1a and OR111-7 transgene expressing axons towards the CZ.

Additional class 3 semaphorins, not previously implicated in olfactory development, are expressed in the olfactory system during development. Mutations in nrp1a, nrp1b and nrp2b result in sema3D independent targeting errors. These findings suggest that many class 3 semaphorins cooperate in protoglomerular map formation. These studies highlight the utility of the zebrafish as a model for the study of early olfactory axon guidance, and provide the foundation for the future study of Semaphorin/Neuropilin signaling in the developing olfactory system.

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THE ROLE OF CLASS 3 SEMAPHORINS IN GUIDING Olfactory Sensory Axons to Their Protoglomerular Targets in the Zebrafish Olfactory Bulb

Alemji A. Taku

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ABSTRACT

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Alemji A. Taku
Dr. Jonathan A. Raper

The convergence of olfactory sensory axons onto topographically fixed, odorant receptor specific glomeruli in the olfactory bulb (OB) is a complex biological problem. Early in development, axons from olfactory sensory neurons (OSNs) extend into the OB where they innervate distinct neuropilar structures called protoglomeruli. Protoglomeruli subsequently segregate into mature glomeruli. The zebrafish is an excellent system in which to study early protoglomerular targeting. At 72 hours post-fertilization, the zebrafish OB contains 12 easily identifiable protoglomeruli. The objective of this thesis work was to identify axon guidance cues and receptors that direct olfactory sensory axons to their protoglomerular targets.

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Additional class 3 semaphorins, not previously implicated in olfactory development, are expressed in the olfactory system during development. Mutations in nrp1a, nrp1b and nrp2b result in sema3D independent targeting errors. These findings suggest that many class 3 semaphorins cooperate in protoglomerular map formation. These studies highlight the utility of the zebrafish as a model for the study of early olfactory axon guidance, and provide the foundation for the future study of Semaphorin/Neuropilin signaling in the developing olfactory system.
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CHAPTER 1. GENERAL INTRODUCTION

During nervous system development, axons must extend through a chemically and physically complex environment to contact specific synaptic partners. The motile tip of the growing axon or growth cone responds to repulsive and attractive cues in the extracellular environment. Accurate axon guidance is critical for the establishment of functional neural networks. In this thesis, I use the olfactory system as a model to study axon guidance.

The olfactory system, one of first sensory systems to evolve, is specialized to detect and discriminate between volatile airborne or water soluble odorants (Hoover, 2010). Olfaction mediates learned and innate behaviors including food detection, mate selection, and predator avoidance (Mori and Sakano, 2011). Like all sensory systems, the olfactory system has the task of mapping an accurate representation of the external environment onto the brain. The physical senses of vision and proprioception map physical space using spatial coordinates to generate a topographic map that defines sensory stimuli based on location within a receptive field. In contrast, olfactory topography must be generated without reference to spatial coordinates. The olfactory system has the challenge of mapping odorant space which contains numerous odorant stimuli. The olfactory system is uniquely organized to accomplish this task and its anatomy has been conserved over 500 million years of vertebrate evolution and across phyla (Hildebrand and Shepherd, 1997; Hoover, 2010; Imai et al., 2010).
The organization of the mouse olfactory system

The mouse is the preeminent vertebrate model used thus far for studying olfactory circuitry. The first direct observations of the convergence of axons from neurons expressing a particular odorant receptor (OR) onto the brain were made using this system (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998). The ability to label specific subsets of sensory neurons has proven instrumental in the characterization of olfactory system organization.

Olfactory sensory neurons (OSNs) are embedded in an olfactory epithelium (OE) lining the nasal cavity. In mice, there are two anatomically distinct olfactory systems; the main olfactory system and the vomeronasal or accessory olfactory system. Sensory neurons in the main olfactory system are located in the roof of the nasal cavity in the main olfactory epithelium (MOE) and in the septal organ (Ma et al., 2003; Imai et al., 2010). OSNs extend cilia into the nasal mucosa and monoallelically express 1 or 2 ORs (Chess et al., 1994; Serizawa et al., 2000; Ishii et al., 2001; Shykind, 2005). Although, the cell bodies expressing a particular receptor are sparsely scattered within epithelial zones, the axons of neurons expressing the same OR coalesce and converge on one or two (one medial and one lateral) topographically fixed glomeruli in the ipsilateral main olfactory bulb (MOB) (Ressler et al., 1993; Vassar et al., 1993, 1994; Mombaerts et al., 1996). There they form synapses with mitral and tufted projection neurons as well as juxtaglomerular interneurons (Mori, 1999). Thus, in the mouse main olfactory system, OR gene choice establishes a neuronal identity that is linked to axon targeting.

Sensory neurons that respond to pheromones are located at the base of the nasal septum in the vomeronasal organ (VNO). Microvillus vomeronasal receptor neurons (VRNs)
express vomeronasal-type receptors V1R or V2R (Dulac and Axel, 1995; Ryba and Tirindelli, 1997). Each VRN expresses 1 or 2 receptor species out of ~137 V1R genes or ~120 V2R genes. Unlike axons in the main olfactory system however, axons expressing the same vomeronasal receptor target 6-30 glomeruli in the accessory olfactory bulb (AOB) which is dorsocaudal to the MOB (Belluscio et al., 1999; Rodriguez et al., 1999, 2002; Serizawa et al., 2000; Yang et al., 2005; Wagner et al., 2006; Ishii and Mombaerts, 2011).

The role of classical guidance cues in olfactory map formation

Several guidance molecules play a role in olfactory map formation. It has been proposed that dorsal-ventral patterning in the OB is guided by the actions of Slit/Robo and Nrp2/Sema3F signaling, while Sema3A/Nrp1a and Eph/Ephrin signaling direct patterning along the anterior-posterior axis (Mori and Sakano, 2011).

The first wave of OSNs to reach the mouse MOB express the guidance receptor Robo2 as early as embryonic day 13 (E13) and project to the dorsal MOB (Cho et al., 2007). The repulsive ligands Slit1 and Slit3 are expressed in the ventral OB at this time-point. Slit2 is expressed by OSNs (Cho et al., 2007; Nguyen-Ba-Charvet et al., 2008). Loss of Robo2 or Slit1 causes OSN axons to shift ventrally in the OB (Cho et al., 2007). Although Robo1 is expressed by ensheathing cells surrounding the olfactory nerve and not by OSNs, errors are exaggerated in Robo1−/−;Robo2−/− animals, where many axons fail to enter the OB (Nguyen-Ba-Charvet et al., 2008). This suggests that Robo1 may have a non-cell autonomous function.
Sema3F, a secreted member of the semaphorin class of repulsive guidance cues also contributes to OSN positioning along the dorsal-ventral axis of the OB. As early as E14, Sema3F is expressed by OSNs that target the dorsal OB. Sema3F deposited by these neurons is thought to restrict late-arriving axons expressing the receptor Neuropilin 2 (Nrp2) to the ventral OB (Norlin et al., 2001; Takeuchi et al., 2010). In sema3F or Nrp2 mutants, axons overshoot the OB (Walz et al., 2002; Cloutier et al., 2004). Loss of Nrp2 shifts axons more dorsally, while overexpression of Nrp2 shifts axons more ventrally in the OB (Takeuchi et al., 2010).

It has been proposed that Sema3A guides olfactory patterning along the anterior-posterior (AP) axis of the mouse OB (Scheving et al., 2000; Taniguchi et al., 2003; Imai et al., 2009). Sema3A is expressed in anteromedial and ventral regions of the olfactory nerve layer as early as E13 (Schwerting et al., 2000). It has been reported that Sema3A is detected in the anterior OB at E16 but is not in older animals (Imai et al., 2009). Cross sections through the olfactory nerve at P0 reveal that Sema3A is expressed by a subset of axons in the central part of the axon bundle, while axons expressing its receptor, Nrp1, are positioned laterally (Imai et al., 2009). Loss of Sema3A/Nrp1 signaling disrupts axon sorting in the olfactory nerve and glomerular positioning in the bulb where Nrp1 expressing axons are shifted anteriorly (Taniguchi et al., 2003; Schwarting et al., 2004; Imai et al., 2009). Nrp1 expressing axons are shifted anteriorly in the OBs of npr1, sema3A, or OSN specific sema3A mutants (Taniguchi et al., 2003; Schwarting et al., 2004; Imai et al., 2009). Conversely, overexpression of Nrp1 in a subset of OSNs that project to a specific glomerulus shifts the position of that glomerulus posteriorly (Imai et al., 2009). While OSN specific knockdown of sema3A may shift a subset of Nrp1 expressing axons anteriorly, the disruption of axon sorting in OSN specific knockout is less severe than that seen in
total knockouts indicating that Sema3A expressed outside of OSNs also plays a role in orienting axons along the correct axis (Imai et al., 2009).

The large family of Eph receptor tyrosine kinases and their ligands, the ephrins, have also been implicated in olfactory sensory map formation. EphrinA3 and EphrinA5 are expressed by OSNs and their EphA receptors are expressed in the OB. The glomeruli innervated by axons expressing either the SR1 or P2 ORs are shifted posteriorly in ephrinA3/ephrinA5 knockouts (Cutforth et al., 2003).

Many of the same guidance cues found to direct glomerular map formation in the main olfactory bulb also contribute to vomeronasal axon targeting. The expression of Slits in the anterior AOB repels Robo expressing axons, pushing them into the posterior AOB (Knöll et al., 2003; Cloutier et al., 2004). Sema3F expressed in the posterior AOB helps restrict Nrp2 expressing axons to the anterior AOB (Cloutier et al., 2002, 2004; Walz et al., 2002). Sema3F/Nrp2 signaling is required for the segregation of vomeronasal axons from the main olfactory system. In sema3F knockouts, vomeronasal axons are defasciculated and a subset of vomeronasal axons invades the MOB (Cloutier et al., 2002, 2004). EphA6 in the AOB, attract axons expressing high levels of EphrinA5 (Knöll et al., 2001). Thus, the same families of guidance molecules direct axons from two different classes of olfactory sensory neuron.

**Odorant receptors instruct olfactory map formation**

Odorant receptors themselves are thought to play an instructive role in olfactory map formation. When the genomic coding sequence of a particular OR (recipient) is replaced by homologous recombination with that of another OR (donor), OSN axons from neurons
expressing from the donor locus converge on a new glomerulus that is distinct from the predicted locations of either the recipient or donor glomeruli (Mombaerts et al., 1996; Wang et al., 1998; Feinstein et al., 2004). Although these results demonstrate a role for ORs in glomerular map formation, the formation of a glomerulus at an intermediate location suggests that ORs are not the sole determinants of targeting. It was originally speculated that ORs expressed on the axonal growth cone might act as guidance receptors or homophilic adhesion molecules (Feinstein and Mombaerts, 2004; Strotmann et al., 2004; Schwarzenbacher et al., 2006). This hypothesis remains unsupported. However more recent experiments demonstrate that ORs can affect olfactory map formation through the transcriptional regulation of guidance molecules (Imai et al., 2006; Nakashima et al., 2013).

ORs are G-protein coupled receptors that can signal through either Golf or Gs. Both couple to adenylyl cyclase III (ACIII) which activates the production of cAMP. This opens a cyclic nucleotide-gated cation channel (CNG), ultimately resulting in the firing of action potentials (DeMaria and Ngai, 2010). OR-derived cAMP levels differ between ORs and it is thought that these differences result in differential expression of guidance and adhesive molecules providing a link between OR choice and map formation (Serizawa et al., 2006; Imai et al., 2009; Nakashima et al., 2013). OR-derived cAMP positively regulates the transcription of Nrp1 and negatively regulates expression of sema3A (Imai et al., 2006). It also regulates the expression of the homophilic adhesion molecules Kirrel2 and Kirrel3, which are required for glomerular segregation but not for initial targeting (Serizawa et al., 2006; Nakashima et al., 2013).
There may be a mechanistic distinction between the actions of OR-derived cAMP on initial targeting compared to glomerular segregation. Gs is expressed in immature neurons before Golf or CNG expression (Nakashima et al., 2013). In Gs knockouts, Nrp1 expression is abolished in a subset of neurons whereas the expression level of Kirrel2 is unaffected (Nakashima et al., 2013). Conversely, Golf is necessary for odor-evoked neuronal activity but is not required for early OSN axon targeting (Belluscio et al., 1998). Kirrel2 expression is eliminated but Nrp1 expression is unaffected in Golf knockouts (Belluscio et al., 1998; Nakashima et al., 2013). Nakashima et al., 2013 propose that OR induced, agonist-independent, spontaneous Gs activity regulates transcription of genes that control early targeting along the anterior-posterior axis, while neuronal activity mediated by Golf regulates genes that control glomerular segregation. Consistent with this hypothesis, naris closure or disruption of neuronal activity by either knocking out CNG2 or by overexpression of Kir2.1, affects levels of Kirrel2/3 but not Nrp1a (Serizawa et al., 2006; Nakashima et al., 2013).

OR-derived cAMP may not regulate the transcription of guidance cues that guide dorsal-ventral patterning. Although sema3F and Nrp2 expression is correlated with OR expression, the expression of these cues is reportedly cAMP-independent (Takeuchi et al., 2010). Instead, the expression pattern of Nrp2 is correlated with OSN position within the OE (Norlin et al., 2001; Takahashi et al., 2010; Takeuchi et al., 2010). Like ORs and guidance cues, the expression of lozenge-like transcription factors is also correlated with epithelial topography as are the transcription factors Msx1 and Foxg1 (Norlin et al., 2001; Miyamichi et al., 2005; Tietjen et al., 2005; Duggan et al., 2008). It has been proposed that cell lineage dictates the cohort of transcription factors expressed by a particular OSN, which in turn, determines the identity of both the OR and guidance molecules that are
expressed (Takeuchi et al., 2010). Consistent with this idea, when the coding sequence of a transgenic OR is replaced with a fluorescent reporter, the choice of a second OR is not random but is restricted to ORs that were expressed in the same zone as the initial OR and with comparable levels of Nrp2 (Serizawa et al., 2003).

**The olfactory system develops in at least two stages**

The precise convergence of OSN axons onto glomeruli is preceded by the formation of a crude protoglomerular map. Mouse OSN axons leave the OE by E12 and reach the telencephalon as early as E15 (Conzelmann et al., 2001; Miller et al., 2010). During the first stage of development OSN axons display a diffuse rather than restricted targeting pattern (Royal and Key, 1999; Potter et al., 2001). Axons from neurons expressing a particular OR first innervate glomerulus-like structures that contain axons from OSNs expressing other OR genes, called protoglomeruli (Treloar et al., 1996, 1999; Conzelmann et al., 2001). Protoglomeruli have also been described in insects (Oland et al., 1990). In the second stage of development, protoglomeruli segregate into mature glomeruli that contain axons from neurons expressing the same OR. In new born mice (postnatal day 0, P0), axons from neurons expressing the related ORs MOR37A and MOR37C are intermingled in the same protoglomerulus. These protoglomeruli then segregate into OR-specific glomeruli by P3 (Conzelmann et al., 2001). Experiments conducted by Zou et al., 2004 indicate that the latter process of segregation is at least in part, activity-dependent. Mice in which naris closure is performed at P0 display heterogeneous glomeruli containing axons from neurons that express different ORs into adulthood in the ipsilateral bulb but not in the contralateral control (Zou et al., 2004). An attractive hypothesis is that the initial targeting of axons to protoglomeruli is primarily driven by the actions of classical axon
guidance cues, while the segregation of protoglomeruli into mature glomeruli is driven by OR-dependent and/or activity dependent mechanisms.

While the organization of the olfactory system is well characterized, the developmental mechanisms driving the precise convergence of OSN axons onto specific protoglomeruli remain unclear. This is in part because the majority of studies on olfactory system development have been carried out in the mouse where in utero observations are difficult and for which there is no defined protoglomerular map. Although expression analysis of guidance cues is often carried out in the embryo, axon targeting is often assayed postnatally once glomeruli have matured. Thus, while many guidance cues have been shown to impact olfactory map formation, distinguishing their role in the initial targeting axons to protoglomeruli versus glomerular segregation is impossible.

The ability to label neurons expressing a particular odorant receptor is a major advantage of using the mouse model. The OR coding sequence can be replaced with an OR:IRES:reporter gene (Mombaerts et al., 1996; Wang et al., 1998). This technique allows direct visualization of axon convergence onto one or two specific glomeruli. OSNs can also be selectively labeled by expressing a minigene construct consisting of a short upstream sequence, the OR coding region followed by an IRES:reporter cassette and a short 3’ non-coding sequence (Vassalli et al., 2002). The inclusion of the OR coding sequence in this strategy suppresses expression of other OR’s. The ability to visualize axons from neurons expressing a particular OR is critical for understanding olfactory system development. Yet, access to the developing olfactory system is equally critical.
Conservation of olfactory organization across phyla

Despite many distinctions, several basic organizational principles are shared by invertebrate and vertebrate olfactory systems, suggesting convergent evolution (Hildebrand and Shepherd, 1997). First, there are a large number of odorant receptor genes. There are over 1000 in mouse and ~62 OR genes in Drosophila (Hallem and Carlson, 2004). Second, each OSN expresses 1 or 2 ORs out of this large repertoire. Third, axons from neurons expressing the same OR converge on the same neuropilar glomerulus where they synapse with second order neurons (Hildebrand and Shepherd, 1997; Imai et al., 2010). Fourth, odors are encoded in a combinatorial manor. Each OR has a specific ligand binding profile and can bind multiple odorants, while each odorant can bind multiple ORs (Malnic et al., 1999). This generates a chemosensory map in which odors are represented in the olfactory bulb as maps of glomerular activity (Uchida et al., 2000). Finally, similar guidance cues are involved in establishing the olfactory projection in invertebrates and vertebrates. In both the mouse and Drosophila Slit/Robo and Semaphorin/Neuropilin signaling are required for proper olfactory map formation (Schwarting et al., 2000; Jhaveri et al., 2004; Sweeney et al., 2007; Nguyen-Ba-Charvet et al., 2008; Takeuchi et al., 2010; Joo et al., 2013)

In Drosophila, ORs are expressed after axons reach their target and are not required for axon targeting (Dobritsa et al., 2003). Several regulatory elements that promote the expression of specific ORs have been identified in Drosophila. These elements are also found upstream of guidance molecules, including robo2, and sema1a (Ray et al., 2008). This suggests that, at least in Drosophila, OR choice is linked to axon targeting via a common regulatory mechanism. While this kind of mechanism has yet to be described in vertebrates, it remains a possibility (Serizawa et al., 2003).
The zebrafish as a model to study early olfactory map formation

Unlike the mouse, the zebrafish offers a simple vertebrate model in which the olfactory system is accessible throughout development. There are only 143 OR genes and 88 identifiable glomeruli compared to ~1000 OR genes and 1800 glomeruli in the mouse (Royet et al., 1988; Buck and Axel, 1991; Alioto and Ngai, 2005). Embryos develop externally and are transparent throughout development making observation of early developmental events straightforward. Zebrafish are also amenable to transgenesis and targeted genome editing (Rembold et al., 2006; Auer and Del Bene, 2014). Importantly, a defined protoglomerular map forms within the first 3 days post fertilization, making it possible to assay the accuracy of OSN axon targeting in mutant larvae (Dynes and Ngai, 1998; Sato et al., 2005; Lakhina et al., 2012).

As early as 18 hours post fertilization (hpf) olfactory placodes arise from ectodermal thickenings (Hansen and Zeiske, 1993). By 24hpf, 10 unipolar pioneer axons exit the OE, extend dorsally and then anteriorly towards the OB (Whitlock and Westerfield, 1998). These neurons do not express ORs and succumb to apoptosis around 48hpf. Pioneers are required for proper guidance of subsequently born sensory neurons (Whitlock and Westerfield, 1998). By 48hpf protoglomerular structures begin to emerge in the bulb and by 72hpf there are 12 identifiable and stereotyped protoglomeruli (Wilson et al., 1990; Dynes and Ngai, 1998; Li et al., 2005; Miyasaka et al., 2005; Lakhina et al., 2012). As in mouse, these initial protoglomeruli segregate over time. By 6 days post fertilization (dpf) there are 25 protoglomeruli and there are 80 glomeruli by adulthood (Baier and Korsching, 1994; Friedrich and Korsching, 1997; Li et al., 2005).
Both main olfactory-type sensory neurons and vomeronasal-type neurons occupy the same olfactory epithelium in zebrafish. There are ~ 143 OR genes that are expressed by ciliated neurons located in the deep layers of the OE (Alioto and Ngai, 2005; Sato et al., 2005). These neurons are olfactory marker protein positive (OMP) and project to ~80 medial glomeruli in zebrafish (Baier and Korsching, 1994; Friedrich and Korsching, 1997; Sato et al., 2005). Microvillious neurons located in the superficial layers of the OE express one of ~70 V2R-type receptors (Hashiguchi and Nishida, 2005; Sato et al., 2005). These neurons also express the transient receptor potential channel 2 (TRPC2) and project predominantly to lateral glomeruli in the olfactory bulb (Sato et al., 2005). The axons from OMP and TRPC2 positive neurons project to mutually exclusive glomeruli in the same olfactory bulb. As in mouse, main-olfactory type OSNs express only one OR and axons expressing a particular OR presumably converge on the same glomerulus. In zebrafish, main-olfactory type OSNs are thought to detect bile acids, steroids and prostaglandins and mediate social behaviors. TRPC2 positive neurons detect amino acids and mediate feeding behaviors (Friedrich and Korsching, 1997, 1998; Koide et al., 2009).

At 72hpf OMP positive and TRPC2 positive axons innervate mutually exclusive protoglomeruli. Axons from OSNs expressing OMP target the central zone, dorsal zone, lateral protoglomerulus 3 (LG3) and the medial glomeruli, while TRPC expressing OSNs target LG1, LG2, LG4, the olfactory plexus (OP), and the ventro-posterior protoglomerulus (VPG) (Dynes and Ngai, 1998; Sato et al., 2005; Lakhina et al., 2012). These protoglomeruli contain functional synapses. At 72hpf, as in the adult, amino acids evoke activity in the lateral OB, while bile acids evoke activity in the medial olfactory bulb (Li et al., 2005).
As in the mouse, subsets of OSNs can be readily visualized in the zebrafish. Recent work from our lab used a minigene strategy similar to that pioneered by Vassalli et al., 2002 to label a specific subset of zebrafish olfactory neurons expressing a transgenic OR111-7 receptor. This transgene contains the E15-1 enhancer element near the OR111 gene family cluster 4kb genomic sequence upstream to the OR111-7 coding region; the OR111-7 coding region; an IRES:Gal4VP16 cassette; and 1kb genomic downstream of the coding region (Nishizumi et al., 2007; Lakhina et al., 2012). The developmental accessibility of the zebrafish and the existence of identifiable protoglomeruli will allow us to separate early targeting events from glomerular maturation.

The identification of guidance cues required for protoglomerular targeting

Early work in zebrafish demonstrated that, as in mouse, Slit/Robo signaling directs the pathfinding of early and later born neurons and is required to guide a subset of axons into the telencephalon (Miyasaka et al., 2005). Robo2 is detected as early as 20hpf in the zebrafish OE and is strongly expressed between 24 and 36hpf. After exiting the OE pioneer axons misroute ventromedially or posteriorly without entering the OB in Robo2 mutants. At 72hfp, OSN axons in Robo2 mutants are defasciculated as compared to controls and, like the pioneers, fail to penetrate the OB. They instead project posteriorly (Miyasaka et al., 2005). All four zebrafish slits (slit1a, slit1b, slit2, and slit3) are expressed posteriorly or ventromedially to the OSN axon pathway (Miyasaka et al., 2005). Loss of Robo2 in zebrafish also alters the segregation of the lateral protoglomeruli that, based on their location, likely express vomeronasal-type receptors (Miyasaka et al., 2005; Sato et al., 2005). Errors observed in Robo2 mutants persist into adulthood in both zebrafish and mouse (Miyasaka et al., 2005; Nguyen-Ba-Charvet et al., 2008). Although this work
provides evidence that the same guidance cues are required for olfactory map formation in mouse and zebrafish, it does not address the question of whether Slit/Robo signaling directs axons to specific protoglomerular targets.

Using an OR111-7:ires:Gal4 transgenic zebrafish line, which labels axons that project to the central zone (CZ) protoglomerulus, Lakhina et al., 2012 described a novel role for attractive Netrin/DCC signaling in zebrafish protoglomerular targeting. This is first time that an attractive molecule has been implicated in olfactory map formation and the first time a guidance cue has been shown to specifically instruct protoglomerular targeting. The DCC receptor is expressed by sensory neurons as early as 24hpf, a time-point when axons are just exiting the OE. Netrin1a is expressed at the medial border of the OB and netrin1b is expressed in the ventral OB. Perturbations of Netrin/DCC signaling cause this specific subset of axons to misproject inappropriately to the dorsal zone protoglomerulus (Lakhina et al., 2012). These results are consistent with Netrin acting as an attractant to guide OR111-7 transgene expressing axons to the CZ.

**Multiple class 3 semaphorins may play a role in protoglomerular targeting**

The objective of this thesis was to identify other guidance cues that are required for protoglomerular targeting. Specifically, I sought to determine whether class 3 semaphorins are required for protoglomerular targeting. Class 3 semaphorins are secreted members of the semaphorin family of guidance molecules which is divided into 8 classes. Class 1 and 2 semaphorins are encoded by invertebrates, classes 3-7 are encoded by vertebrates and class V Semaphorins are encoded by viruses (Yazdani and Terman, 2006). Class 3 semaphorins are the best described class of semaphorins. In mammals there are 7 class 3 semaphorins, Sema3A-Sema3G (Yazdani and Terman,
The first vertebrate semaphorin, Sema3A, was identified as a strong axonal repellent (Luo et al., 1993). Later work demonstrates that, in addition to acting as axon guidance molecules, class 3 semaphorins mediate many cellular functions including neuronal migration, synapse formation, apoptosis, vasculature development, and immune response (Roth et al., 2009). Class 3 semaphorins are required for proper axon targeting throughout the nervous system. In several cases, multiple semaphorins act on the same population of axons. For example, both Sema3A and Sema3F are required for the proper innervation of dorsal limb muscles by the axons of the Lateral motor column in mice (Huber et al., 2005). Similarly, in the visual system, in the absence of either Sema3D or Sema3E, retinal ganglion cell axons misproject ipsilaterally at the zebrafish optic chiasm (Sakai and Halloran, 2006; Dell et al., 2013).

In the mouse olfactory system, Sema3A and Sema3F are required for olfactory map formation (Mori and Sakano, 2011). However, the expression of additional class 3 semaphorins has been reported in the developing olfactory system of the mouse (sema3C and sema3B) and in the chick (Sema3C, Sema3D, and sema3E) (Giger et al., 2000; Renzi et al., 2000; Cloutier et al., 2002). Due to a genome duplication there are 12 zebrafish class 3 semaphorins. The studies reported here reveal that at least 9 of them are expressed in the developing olfactory bulb, making them attractive candidate guidance cues that could contribute to protoglomerular targeting (Chapter 3). Like sema3A and sema3F in mouse, several zebrafish semaphorins are expressed by sensory neurons, indicating potential roles in pre-target axon sorting (Chapter 3). Four neuropilins are differentially expressed in the OE, raising the possibility that axons expressing different Neuropilins respond differently to various Semaphorins. I focused on studying the role of Sema3D in protoglomerular targeting. I show that sema3D is
expressed in the anterior OB and is required for OR11-7 transgene expressing OSN axons to correctly target the central zone protoglomerulus (Chapter 2). This is the first time that a role for Sema3D has been demonstrated in olfactory map formation and the first time any semaphorin has been shown to instruct protoglomerular targeting. My findings also suggest that many other class 3 semaphorins are positioned to cooperate in guiding axons to their initial protoglomerular targets.
CHAPTER 2. SEMAPHORIN3D HELPS GUIDE A SPECIFIC SUBSET OF
OLFACTORY SENSORY AXONS TO THE CENTRAL ZONE PROTOGLOMERULUS

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Abstract

Olfactory sensory neurons project to topographically fixed glomeruli in the olfactory bulb. The mature olfactory sensory map is preceded in development by a cruder protoglomerular map. The axonal guidance cues that direct the formation of this rudimentary map are not well characterized. In this study we ask whether the guidance cue semaphorin3D (sema3D) is required for protoglomerular map formation in the zebrafish. Sema3D is expressed in the anterior olfactory bulb at a time-point after olfactory axons first enter the bulb but before protoglomerulus formation. A subset of olfactory sensory neurons whose axons target the central zone protoglomerulus are labeled by the expression of an OR111-7:IRES:Gal4 transgene. These axons terminate posterior to a region of sema3D expression. In sema3D mutants, a subset of OR111-7 transgene labeled axons misprojects to the dorsal zone protoglomerulus which is positioned dorsal and anterior to the central zone. OR111-7 transgene expressing neurons express multiple neuropilin (nrp) receptors, but only the nrp1a mutant both phenocopies and interacts genetically with the sema3D mutant. Selective expression of nrp1a in OR111-7 transgene expressing neurons corrects the dorsal zone protoglomerular targeting error seen in nrp1a mutants. Together these data support a model in which Sema3D in the anterior olfactory bulb acts as a repellent that guides OR111-7 and nrp1a expressing axons to the central zone protoglomerulus.
Introduction

The targeting of axons to their post-synaptic partners is a critical step in the formation of functional neural circuits. The olfactory system is an attractive model for the study of axon of guidance because odorant receptor (OR) selection defines a clear neuronal identity that is linked to axon targeting. Each olfactory sensory neuron (OSN) stochastically expresses one or a few ORs from a large repertoire of OR genes (Rodriguez, 2013). Although OSNs expressing a particular OR are stochastically distributed within a broad zone within the olfactory epithelium, their axons ultimately converge upon the same topographically fixed glomerulus in the olfactory bulb where they synapse with second order neurons (Ramon y Cajal, 1892; Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). Since each glomerulus contains axons from cells that express a single OR species (Treloar et al., 2002), and each OR responds to specific odorants, odors are represented as distinct patterns of glomerular activity within the bulb (Friedrich and Korsching, 1997; Malnic et al., 1999).

Early in development, a coarse sensory map forms in the olfactory bulb consisting of protoglomeruli which contain axons from OSNs that expresses different but potentially related ORs (Royal and Key, 1999; Strotmann et al., 2000; Conzelmann et al., 2001). Later, receptor-specific and activity-dependent local sorting drives the segregation of protoglomeruli into individual OR-specific glomeruli (Zou et al., 2004; Serizawa et al., 2006). Several canonical guidance molecules are known to play a role in early OSN axon pathfinding (Renzi et al., 2000; Schwarting et al., 2000; Cutforth et al., 2003; Cho et al., 2007; Takeuchi et al., 2010; Lakhina et al., 2012). However, given the complexity of the
initial olfactory projection, it is likely that many additional guidance cues cooperate to establish the protoglomerular map.

In this study we show for the first time that Semaphorin3D (Sema3D) is required for OSN axons to target a specific protoglomerulus in zebrafish. OSN axons that express an OR111-7 transgene target the central zone protoglomerulus (CZ) in wild type larvae. A subset of OR111-7 transgene expressing axons project inappropriately to the dorsal zone protoglomerulus (DZ) in sema3D mutants. OR111-7 transgenic OSNs express nrp1a, nrp1b, and nrp2b. Although loss of either nrp1a or nrp2b phenocopies sema3D mutants, we find that sema3D only interacts genetically with nrp1a. Further, nrp1a expressed exclusively within OR111-7 transgene expressing OSNs is sufficient for correct axonal targeting in nrp1a mutants. Our results support a model in which Sema3D acts through Nrp1a to repel axons away from the anterior bulb, directing them to the more posteriorly positioned central zone protoglomerulus.

Materials and methods

Zebrafish maintenance and transgenic lines. Adult zebrafish were raised and maintained according to standard practices (Mullins et al., 1994). Larvae were staged based on hours post fertilization (hpf) and were raised at 28°C. For some of the in situ experiments described in Figure 4 the 36 hpf time point was obtained by incubating for 1 day at 28°C and 1 day at 25°C (Kimmel et al., 1995). Tg(omp:lyn-RFP) and Tg(trpc2:gap-VENUS) lines were a gift from the Yoshihara laboratory (Miyasaka et al., 2005; Sato et al., 2005). The Tg(or111-7:or111-7-IRES:GAL4), Tg(omp:GAL4) and Tg(UAS:gap43-Citrine) lines were previously described by Lakhina et al., (2012). A double transgenic line, Tg(or111-7:GAL4;UAS:Citrine) was generated by crossing Tg(or111-7:or111-7-
IRES:GAL4) to Tg(UAS:gap43-Citrine). A UAS:nrp1a rescue line was generated by injecting a tol2 UAS:nrp1a;UAS:Citrine construct (Dell et al., 2013) into embryos produced from a nrp1a\(^{sa1485}\) +/- incross using standard procedures (Fisher et al., 2006). These fish were raised to adulthood, screened for germline transmission of the transgene, and genotyped. Np1a\(^{sa1485}\) +/- founders carrying the UAS:nrp1a;UAS:Citrine were mated with the Tg(or111-7:or111-7-IRES:GAL4) line and the resultant larvae were analyzed. Transgenic lines were used alone or crossed into various mutant strains.

**Zebrafish mutants.** Sema3D\(^{sa1661}\), and nrp1a\(^{sa1485}\) mutants were generated by the Sanger Center’s Zebrafish Mutation Project and obtained from The Zebrafish International Resource Center (ZIRC) (Kettleborough et al., 2013). These mutants were genotyped using a KASP genotyping assay (LGC Genomics; sema3D SNP ID 554-1608.1, nrp1a SNP ID 554-1410.1). The nrp1b\(^{th278}\) mutant was identified by the Zebrafish Tilling Project and ordered from ZIRC. Nrp2b\(^{nn0126GT}\) mutants were a gift from the Ekker laboratory (Clark et al., 2011). Standard PCR based methods were used to genotype nrp1b\(^{th278}\) (forward primer: TCTCTCTTGGGAGGTTCTGC, reverse primer: TGTCTTTGTGTGTCATT, MseI cuts the mutant sequence into 161bp and 34bp fragments); and Nrp2b\(^{nn0126GT}\) (nrp2b forward primer: GCTGAAGATCGGTATCAGACGAAAAACA, nrp2b reverse primer: AGACCTGCCATATTGAGTGACTACCGA, RFP reverse primer: CCTTGAAGGCATGAACCTCTTGAT ) lines.

**Whole-mount Immunohistochemistry.** Immunohistochemistry was performed as previously described (Lakhina et al., 2012). Larvae were fixed in 4% paraformaldehyde in PBS and dehydrated in methanol. To visualize Citrine positive axons, larvae were
permeabilized in acetone for 20 minutes at -20°C and stained with goat anti-GFP (1:300; Rockland Immunochemicals, 600-101-215) and donkey anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen). To visualize RFP positive axons, fish were permeabilized for 30 minutes in 0.1% collagenase at room temperature and stained with rabbit anti-dsRed (1:300; Clonetech, 632496) and donkey anti-rabbit IgG Alexa Fluor 647 (1:500; Invitrogen). Propidium iodide staining was performed following secondary antibody treatment as described by Brend and Holley (2009) with the exception that larvae were not treated with RNase. Confocal microscopy was performed on an inverted Leica SP5 using a 40X oil-immersion lens. Stacks were acquired through the entire olfactory bulb with optical sections taken 1 micron apart.

**Whole-mount fluorescent in situ hybridization.** Single label *in situ* hybridization was performed using antisense Digoxigenin (DIG) labeled RNA probes as previously described (Chalasani, 2007). *In situ* signals were amplified using a cyanine 5-coupled tyramide kit (TSA Plus cyanine 5, PerkinElmer, NEL745001KT). Double label *in situ* hybridization was performed using DIG and fluorescein labeled probes as previously described (Brend and Holley, 2009), with the exception that larvae were not dehydrated in between detection of the first and second probes. The DIG label was amplified using a cyanine 5-coupled tyramide kit (PerkinElmer, NEL745001KT) and the fluorescein label was amplified using a cyanine 3-coupled tyramide kit (PerkinElmer, NEL744001KT). Prior to tyramide amplification embryos were incubated in either anti-DIG-POD (1:500; Roche, 11207733910) or anti-Fluorescein-POD (1:500:Roche, 11426346910).

Immunohistochemistry, propidium iodide labeling, and imaging were performed following tyramide amplification as described above. The plasmids used to make probes targeting *sema3D, sema3E, and nrp1a* were as described in Dell et al., (2013). The plasmids used
to make probes for *sema3Aa*, *sema3Ab*, *sema3Fa*, *sema3Fb*, *sema3Ga*, *sema3Gb*, and *nrp2b* were gifts from the Moens laboratory (Yu et al., 2004; Yu and Moens, 2005). The plasmid used to make *sema3H* probe was a gift from the Halloran laboratory (Stevens and Halloran, 2005). For *sema3C* (refseq accession number XM_687755.5, nucleotides 1539-2400); *nrp1b* (refseq accession number NM_205674.1, nucleotides 2-972); and *nrp2a* (refseq accession number NM_212965.1, nucleotides 138-1108) sequences were amplified from cDNA and cloned into pcRII (Invitrogen, K460001) for probe synthesis. Full-length probes were used in all hybridization experiments.

**Quantification of targeting errors.** After larvae were processed and imaged, the number of olfactory bulbs containing axons terminating in either individual protoglomeruli or non-protoglomerular regions were counted. The percentage of olfactory bulbs with axons in particular locations was recorded and Fisher’s exact (two-tailed) test was used to determine statistical significance.

**Results**

Olfactory sensory neuron (OSN) axons exiting the olfactory epithelium project dorsally and anteriorly into the telencephalon, entering the nascent olfactory bulb (OB) by 24 hours post fertilization (hpf) (Wilson et al., 1990; Hansen and Zeiske, 1993). Although distinct protoglomeruli cannot be easily distinguished at 48 hpf, three distinct projections thought to correspond to the DZ, CZ and MG can be observed (Dynes and Ngai, 1998).

By 72 hpf the zebrafish olfactory bulb contains 12 distinct, individually identifiable protoglomeruli, making it an ideal system in which to study protoglomerular targeting (Dynes and Ngai, 1998; Lakhina et al., 2012). Unlike rodents, which have two anatomically
separate olfactory systems (main and vomeronasal), the zebrafish olfactory epithelium (OE) contains at least two classes of sensory neurons which project to mutually exclusive protoglomerulari in a single olfactory bulb (Sato et al., 2005). Ciliated sensory neurons which express olfactory marker protein (OMP) and classical main olfactory bulb-type odorant receptors (ORs) innervate the central zone (CZ), dorsal zone (DZ), lateral protoglomeruls 3 (LG3), and to a lesser extent, the medial protoglomeruli (MG). Microvillous sensory neurons expressing V2R-type vomeronasal receptors along with the transient receptor potential channel C2 (TRPC2) innervate lateral protoglomeruli 1, 2 and 4 (LG1, LG2, LG4), the ventral posterior glomerulus (VPG), and the olfactory plexus (OP) (Celik et al., 2002; Sato et al., 2005; Lakhina et al., 2012). The segregation of OMP and TRPC2 positive neurons is apparent by 48hpf (Sato et al., 2005).

**Sema3D is expressed in the anterior telencephalon at 36hpf.**

Class 3 Semaphorins are secreted members of the larger semaphorin family of axon guidance molecules. In mammals there are 7 class 3 semaphorins, Sema3A-Sema3G. The first discovered were characterized as repulsive guidance cues, but some family members have since been shown to act as attractants (He et al., 2002). Sema3A and Sema3F are required for correct OSN axon guidance in the mouse (Kobayashi et al., 1997; Schwarting et al., 2000; Cloutier et al., 2004; Imai et al., 2009; Takeuchi et al., 2010). The expression of additional class 3 semaphorins has been reported in mouse (sema3C and sema3B) and in chick (Sema3C, Sema3D, and sema3E) during olfactory system development (Renzi et al., 2000; Cloutier et al., 2002).

Sema3D is one of the least studied class 3 semaphorins. Disruption of Sema3D signaling perturbs retinal axon guidance and neural crest migration in zebrafish (Sakai and Halloran,
In zebrafish, it is expressed as early as 16hpf in the diencephalon and by 27hpf it is detectable in the boundary between the olfactory epithelium and the brain (Halloran et al., 1999; data not shown). By 36hpf sema3D mRNA is also detected in the anterior-most region of the primordial olfactory bulb (Fig. 2.1). At this age, OMP positive olfactory sensory axons project close to, and partially overlap with, sema3D expressing cells (Fig. 2.1A-B, D, and E).

The OR111-7:IRES:Gal4 transgene is expressed in a subset of OMP positive neurons that project predominantly to the CZ at 72hpf (Lakhina et al., 2012). Axons expressing this transgene terminate posterior to the sema3D expression domain at 36hfp (Fig. 2.1C, and G-I). A small subset OR111-7 transgene expressing axons can occasionally be detected projecting anteriorly but not quite overlapping with sema3D expressing cells (Fig. 2.1C). Imaging along the dorsoventral axis reveals that, in the anterior bulb, sema3D is strongly expressed ventrally and medially (Fig. 2.1D-I). Sema3D expressing cells are also detected just dorsal to the most anterior of the OR111-7 transgene expressing axons (Fig. 2.1H, white arrowhead). As axons exit the olfactory epithelium, they project through a sema3D expressing region at the boundary between the olfactory epithelium and bulb (Fig. 2.1A-C). By 48hpf sema3D expression is undetectable in the anterior part of the bulb, but remains strong between the olfactory epithelium and bulb (data not shown). Thus, sem3D is temporally and spatially positioned to affect early OSN axon pathfinding.

**OR111-7 transgene expressing axons project ectopically into the dorsal zone protoglomerulus in sema3D mutants.**

To test the requirement for Sema3D in olfactory axon guidance, we analyzed the olfactory projections in larvae harboring a presumptive null allele, sema3Dsa1661. This mutant allele
was identified by the Sanger Center’s Zebrafish Mutation Project and contains a nonsense codon that generates a premature stop at amino acid 257. This stop is within the Sema domain (amino acids 74-521, Ensembl genome browser 75, ENSDARG00000017369) which is required for the interaction of class 3 semaphorins with their receptors and is necessary for their biological activity (Feiner et al., 1997; Koppel et al., 1997). *Sema3D* homozygotes were obtained at a frequency of ~23% and are viable. Because protoglomeruli are composed exclusively of neuropil, propidium iodide staining allows their visualization as pronounced acellular regions in the OB (Lakhina et al., 2012). There were no gross differences in OB morphology or protoglomerular arrangement in *sema3D* mutants as compared to wild-type larvae at 72 hpf (Fig. 2.2A-B). To determine whether Sema3D plays a role in targeting of axons to the CZ, we interbred *sema3D<sup>+/−</sup>;OR111-7:ires:Gal4;UAS:Citrine* zebrafish. Their fluorescent progeny were collected at 72hpf and genomic DNA was extracted from their tails for genotyping. Matched heads were processed for immunohistochemistry and imaged using confocal microscopy. The percentage of OBs with a labeled axonal projection to each protoglomerulus was calculated. Axons were scored as projecting to a particular protoglomerulus only if they terminated in that protoglomerulus and not if they passed through it en route to another location.

*OR111-7* transgene expressing axons projected to the CZ in 100% of the bulbs examined in wild-type, heterozygous, and *sema3D* mutant larvae (Fig.2.3A-E). However, in *sema3D* mutants bulbs, *OR111-7* transgene expressing axons inappropriately targeted the DZ in 37% (N= 53) of larvae as compared to 14% in wild-type (N= 44) and 16% in heterozygous (N=45) siblings. Using Fisher's exact test we estimate that the chance that axons in mutant larvae project to the CZ at the same frequency as axons in wild-type larvae as p=.02; or
comparing mutant to heterozygotes larvae as $p=.04$ (Fig. 2.3). No additional significant misprojections to protoglomerular targets were observed. Ectopic projections that did not terminate in a protoglomerulus were scored as “other” and characterized based on their region of termination in the bulb. OR111-7 transgenic axons do not display an increase in non-protoglomerular errors in *sema3D* mutants (Fig. 2.2E-F).

To determine whether the loss of *sema3D* has any broad effects on OSN pathfinding, we intercrossed either *Sema3D*<sup>+/−</sup>;OMP:RFP or *Sema3D*<sup>+/−</sup>;TRPC2:Venus lines. OMP expressing OSN axons project to the correct protoglomeruli (CZ, DZ, LG3, and MG) with the same frequency as controls in *Sema3D* mutants (Fig. 3A-B, E). There were no apparent differences in innervation density between mutants and controls. Nor do OMP expressing sensory axons display an increase in non-protoglomerular targeting errors in *sema3D* mutants (Fig. 2.3A-B, E). Similarly, there were also no significant differences in the protoglomerular or non-protoglomerular targeting of TRPC2 expressing sensory axons detected in *sem3D* mutants as compared to controls (Fig. 2.3C-D, F).

Thus, Sema3D is required to specifically direct protoglomerular targeting of a subset of OMP positive axons that project to the CZ. Because, the OMP:RFP line does not permit analysis of specific protoglomerular targeting errors it remains possible that Sema3D influences the targeting of other subsets of axons within this class, for example those that normally target the DZ or LG3 protoglomeruli.

**All four zebrafish neuropilins are expressed in the developing olfactory system**

Class 3 semaphorins signal though neuropilin/plexinA receptor complexes (Sharma et al., 2012). There are two mammalian neuropilins, Nrp1 and Nrp2, which differ in their binding
affinities for various class 3 semaphorins (Chen et al., 1997). Chick Sema3D can bind Nrp1 \textit{in vitro} (Feiner et al., 1997). Although it is unknown whether Sema3D can bind Nrp2, experiments in zebrafish suggest that it can signal through either Nrp1 or Nrp1/Nrp2 with differing functional consequences (Wolman et al., 2004).

To identify potential candidate receptor(s) for sema3D, we examined neuropilin expression in the 36hpf olfactory system. There are four zebrafish neuropilins: \textit{nrp1a}, \textit{nrp1b}, \textit{nrp2a}, and \textit{nrp2b} (Bovenkamp et al., 2004; Yu et al., 2004). Any of the four can be detected in subsets of OMP expressing sensory neurons at 36hpf (Fig. 2.4A-D, M). The most frequently expressed are \textit{nrp1a} in 40% of OMP expressing sensory cells and \textit{nrp1b} in 34% of OMP expressing sensory cells (Fig. 2.4A-B, and M). \textit{Nrp2a} and \textit{nrp2b} are expressed in 19% and 23% of OMP expressing sensory neurons respectively (Fig. 2.4C-D, M). \textit{OR111-7} transgene expressing neurons express \textit{nrp1a} (59%), \textit{nrp1b} (68%) and \textit{nrp2b} (34%) (Fig. 2.4E-H, and M). \textit{Nrp2a} is only detected in 3% of \textit{OR111-7} transgenic neurons at this age (Fig. 2.4G, and M). Double \textit{in situ} hybridization experiments revealed that \textit{nrp1a} and \textit{nrp1b} can be co-expressed in \textit{OR111-7} transgenic neurons (data not shown). Similarly, \textit{nrp1a} is co-expressed with \textit{nrp2b} in in a subset of \textit{OR111-7} transgenic neurons (data not shown). \textit{Nrp1a} is only detectable in 5% of TRPC2 positive neurons but they do express \textit{nrp1b} (67%), \textit{nrp2a} (69%), and \textit{nrp2b} (54%) (Fig. 2.4I-L, and M). The differential expression of neuropilins in OSNs raises the possibility that axons expressing different combinations of neuropilins may be directed by class 3 semaphorins to distinct targets in the bulb. The presence of \textit{nrp1a}, \textit{nrp1b}, and \textit{nrp2b} in \textit{OR111-7} transgene expressing neurons make any of them receptor potential candidates for mediating the effects of sema3D in \textit{OR111-7} transgene expressing OSNs.
Loss of nrp1a or nrp2b phenocopies sema3D mutants

To further identify candidate receptor(s) for sema3D, we examined OR111-7 transgene expressing OSN axon targeting in nrp1a, nrp1b, or nrp2b presumptive null mutants. The nrp1a*sa1485 allele contains a nonsense mutation that results in a premature stop codon at amino acid 206 within the second CUB domain (Ensembl genome browser 75, ENSDARG00000071865). The nrp1b*fh278 allele contains a nonsense mutation that results in a premature stop codon at amino acid 116 within the first CUB domain (Ensembl genome browser 75, ENSDARG00000027290). The nrp2b*mno126GT allele obtained from the Ekker laboratory contains an RFP and polyadenylation site inserted at amino acid 427 just before the second Coagulation-factor homology domain (Clark et al., 2011). The CUB domains and the amino-terminal Coagulation-factor homology domain are necessary for Neuropilin to bind class 3 semaphorins (Gu et al., 2002).

Olfactory bulb morphology and protoglomerular positioning appear normal in all three mutants at 72hpf (Fig. 2.6B, 6F, and data not shown). Similar to sema3D mutants, OR111-7 transgene expressing axons terminate incorrectly within the DZ in nrp1a mutants. Mistargeting to the DZ was observed in 28% (N=47) of nrp1a mutant bulbs as compared to 8% in wild-type (N=50, p=.02) and 6% in heterozygotes (N=54, p=.003) (Fig. 2.5A-D and Fig. 2.6A). In contrast to sema3D mutants, OR111-7 transgene expressing axons also misproject to non-protoglomerular locations dorsal to the CZ in nrp1a mutants (Fig. 2.6B and data not shown). OR111-7 transgene expressing axons inappropriately targeted the DZ in 48% (N=42) of nrp2b mutant bulbs as compared to only 7% (N=30, p=.0002) in wild-type and 14% (N=42, p=.002) in heterozygote embryos (Fig. 2.5E-H and Fig. 2.6E). No non-protoglomerular targeting errors were detected in nrp2b mutant embryos (Fig. 2.6E-
OR111-7 transgene expressing axons do not have a significant increase in projections to the DZ in *nrp1b* mutants. They do, however, have increased projections to the LG1 and LG2 protoglomeruli (Fig. 2.6C and data not shown).

**Sema3D interacts with *nrp1a* to promote targeting of OR111-7 transgene expressing axons to the CZ**

We next asked whether loss of *nrp1a* or *nrp2b* affects the *sema3D* phenotype. For example, if a one of these neuropilins acts as a receptor component for *sema3D*, embryos that are heterozygous for both *nrp* and *sema3D* mutations might have a sufficient reduction in signaling activity to phenocopy *sema3D* mutants. We generated *sema3D*/*nrp* trans-heterozygotes by mating *sema3D*/*-;OR111-7:IRES:Gal4;UAS:Citrine fish with *nrp1a*/*-;UAS:Citrine or *nrp2b*/*-;OR111-7:IRES:Gal4;UAS:Citrine fish. Larvae carrying heterozygous mutations for either *sema3D*, *nrp1a*, or *nrp2b* alone do not have any detectable targeting errors (Fig. 2.6A, E). However, OR111-7 transgene expressing axons terminate inappropriately in the DZ in 29% of *sema3D*/*-;*nrp1a*/*- trans-heterozygotes (N=68, p=.02) (Fig. 2.7A-G). In contrast, *sema3D*/*-;*nrp2b*/*- trans-heterozygotes have no detectable increase in DZ targeting errors (Fig. 7H). OR111-7 transgene expressing axons are misguided to the DZ in 28% of *Sema3D*/*-;*nrp1a*/*- double mutant larvae (N=50, p=.02) (Fig. 2.8). These larvae also display the non-protoglomerular dorsal error observed in *nrp1a* mutants (Fig. 2.8J). There were no additional errors observed in the double mutants as compared to the single mutant siblings. Importantly, the DZ targeting error seen in double mutants is similar in frequency and severity to the errors in either *sema3D* or *nrp1a* single mutants. These data support the hypothesis that sema3D signals via Nrp1a to promote targeting of OR111-7 transgene expressing axons to the CZ.
Selective expression of *nrp1a* in *OR111-7* transgene expressing OSNs corrects errors seen in *nrp1a* mutants

Because *nrp1a* is prominently expressed in OR111-7 transgene expressing sensory neurons at 36hpf, we tested whether *nrp1a* is required cell-autonomously for normal *OR111-7* transgene expressing axon guidance. We generated a *nrp1a+/-;UAS:nrp1a;UAS:Citrine* transgenic line. Three different *nrp1a+/-;UAS:nrp1a;UAS:Citrine* founders were mated to *nrp1a+/-;OR111-7:Gal4* fish. *Nrp1a* mutants expressing the UAS:*nrp1a* transgene do not project ectopically to the DZ (Fig. 2.9). No protoglomerular mistargeting was detected in *OR111-7* transgene expressing axons when Nrp1a was overexpressed in wild-type embryos (Fig. 2.9). Slightly fewer sensory neurons were labeled in wild-type and mutant larvae expressing the UAS:*nrp1a* rescue construct (16 cells/pit, *N*=24) as compared to larvae expressing the UAS:Citrine control construct (20 cells/pit, *N*=20). A smaller number of labeled axons reduces the chance of detecting targeting errors. With this caveat in mind, the ability of Nrp1a expressed in *OR111-7* transgene expressing neurons to correct *nrp1a* mutant-induced targeting errors is consistent with its acting cell autonomously to guide these axons to their correct target.

**Discussion**

Each olfactory sensory neuron expresses only one olfactory receptor (OR) out of a large repertoire of ~1296 genes in mouse and ~143 genes in zebrafish (Zhang and Firestein, 2002; Alioto and Ngai, 2005). The precise convergence of sensory neuron axons that express a particular OR onto specific glomeruli in the olfactory bulb (OB) generates a sensory map relating odorant experience to map position. Sensory map formation can be divided into at least two stages. In the first stage, axons exit the epithelium and project
into the primordial OB where they converge into specific reproducible locations to form protoglomeruli. Each protoglomerulus likely contains axons from neurons expressing related ORs. Protoglomeruli have been detected in the developing rodent, insect, and fish olfactory systems (Oland et al., 1990; Dynes and Ngai, 1998; Treloar et al., 1999; Conzelmann et al., 2001). In the second stage, this crude map is refined by the segregation of distinct OR-specific glomeruli from each larger protoglomerulus.

Recent work highlights the importance of early axon targeting events in the establishment and maintenance of the olfactory sensory map. Studies in mouse have identified several axonal guidance cues that contribute to olfactory map formation including the guidance cues slit1, slit2, sema3A, and sema3F (Schwarting et al., 2000; Cloutier et al., 2004; Cho et al., 2007; Nguyen-Ba-Charvet et al., 2008; Takeuchi et al., 2010). Work from our laboratory has taken advantage of the relative simplicity of the zebrafish olfactory system to identify a role for Netrin/DCC signaling in protoglomerular targeting (Lakhina et al., 2012). This study identifies Sema3D as an important guidance cue required for normal protoglomerular map formation.

Sema3D is required to guide a subset of sensory neurons to the central zone protoglomerulus. A subpopulation of axons identified by their expression of the OR111-7 transgene project ectopically to the dorsal zone (DZ) protoglomerulus in sema3D mutants. The DZ is both dorsal and anterior to their normal target, the central zone (CZ) protoglomerulus. Sema3D expression is detected in the anterior OB at 36hpf, decreases markedly by 48hpf, and is undetectable at 72hpf (data not shown). Although the protein product may perdure longer, peak expression is early, before and during the formation of protoglomeruli. We asked whether errors in axon positioning could be observed at earlier
time-points. We examined the OR111-7 transgene expressing projection at 36hpf or 48hpf and found no obvious differences in axon position between mutants and controls (data not shown). At these time-points, a small subset of OR111-7 transgene expressing axons normally projects toward the anterior OB (Fig. 2.1C). It is possible that in the absence of Sema3D, these projections fail to be redirected posteriorward and persist abnormally in anterior territories until 72hpf. Protoglomeruli are undetectable at these ages and without a transgene that labels the dorsal zone projection, it is unclear whether these axons are intermingled with those that will ultimately target the dorsal zone. Our findings support a model where the early repellent actions of Sema3D in anterior olfactory OB direct OR111-7 transgenic axons away from the dorsal zone. A very early requirement for some guidance cues in establishing the protoglomerular map may be a common theme in olfactory development. The guidance receptor DCC has been shown to instruct protoglomerular targeting in zebrafish and the proportion of OSNs expressing DCC decreases markedly from between 27hpf and 53hpf (Lakhina et al., 2012).

Neuropilin is the ligand-binding subunit of the class 3 semaphorin receptor complex. Sema3D has been reported to bind Nrp1 but not Nrp2 in vitro (Feiner et al., 1997; Degenhardt et al., 2013). However, in vitro binding affinity is not always predictive of in vivo binding profiles (Feiner et al., 1997). There are four neuropilins in zebrafish: nrp1a, nrp1b, nrp2a, and nrp2b (Bovenkamp et al., 2004; Yu et al., 2004). It has been suggested that Nrp1a mediates a Sema3D evoked repellent response from axons originating in the nucleus of the medial longitudinal fasciculus, but that Nrp1a-Nrp2b heterodimers mediate an Sema3D evoked attractive response from axons in the anterior commissure (Wolman et al., 2004). OR111-7 transgene expressing neurons express nrp1a, nrp1b, and nrp2b (Fig. 2.4E-H). Loss of either nrp1a or nrp2b phenocopies sema3D mutants, but a genetic
interaction between \textit{nrp1a} and \textit{sema3D} can be detected in double heterozygotic mutants (Fig. 2.5, 2.6 and 2.7). \textit{Sema3D};\textit{nrp1a} double mutants are similar to \textit{nrp1a} or \textit{sema3D} single mutants in both penetrance and severity (Fig. 2.8). The penetrance of the \textit{nrp2b} mutant phenotype (48\%) is markedly higher than that of \textit{nrp1a} or \textit{sema3D} mutants (28\%) (Fig. 2.6). It is therefore likely that an additional semaphorin acts through Nrp2b to promote targeting to the central zone. Our results support the hypothesis that Nrp1a is the most important neuropilin mediating sema3D repulsion in \textit{OR111-7} transgene expressing neurons. The repulsive nature of sema3D/Nrp1a signaling we infer from our results is consistent with previous reports; however, \textit{OR111-7} transgene expressing neurons can co-express \textit{nrp1a} and \textit{nrp2b} together (data not shown), raising the possibility that axons from this population respond differently to Sema3D as compared to axons from neurons that express Nrp1a alone (Wolman et al., 2004).

\textit{Nrp1a, nrp1b, and nrp2b} mutants all display \textit{sema3D} independent phenotypes. In \textit{Nrp1a} mutants, in addition to misprojecting to the DZ, \textit{OR111-7} transgenic axons also project to non-glomerular regions that are dorsal to their intended target (Fig. 2.6A-B, data not shown). These projections are not observed in \textit{sema3D} mutants (Fig. 2.2). In \textit{nrp1b} mutants, \textit{OR111-7} transgenic axons misproject to LG1 and LG2, an error that is not observed in \textit{sema3D} mutants. Finally, the greater severity of \textit{OR111-7} transgenic axon mistargeting in \textit{nrp2b} as compared to \textit{sema3D} mutants and the lack of a detectable genetic interaction between \textit{nrp2b} and \textit{sema3D} transheterozygotes suggest that the \textit{nrp2b} phenotype cannot be fully explained by Sema3D acting as its sole ligand (Fig. 2.7H).

We propose that additional class 3 semaphorins direct protoglomerular map formation. Owing to a genome duplication, there are 12 identified class 3 semaphorins in zebrafish: 3Aa, 3Ab, 3B, 3bl, 3C, 3D, 3E, 3Fa, 3Fb, 3Ga, 3Gb, and 3H (Amores, 1998; Halloran et
al., 1999; Roos et al., 1999; Yee et al., 1999; Stevens and Halloran, 2005; Yu and Moens, 2005). We generated RNA probes for 10 class 3 semaphorin and assayed their expression in the developing olfactory system at 36hpf. At least 9 class 3 semaphorins are expressed in overlapping but distinct patterns at this time point (data not shown). Thus, multiple class 3 semaphorins are spatially and temporally positioned to cooperate in promoting protoglomerular targeting.

Sema3A/Nrp1 signaling has been proposed to affect OSN axon targeting along the anterior-posterior (AP) axis in the mouse OB (Schwarting et al., 2000, 2004; Taniguchi et al., 2003; Imai et al., 2009). Sema3A is expressed in anteromedial and ventral regions of the olfactory nerve layer (Schwarting et al., 2000). It is also expressed in a subset of sensory neurons (Imai et al., 2009). Glomeruli formed by Nrp1 expressing axons are shifted anteriorly in the OBs of nrp1, sema3A, or OSN specific sema3A mutants (Taniguchi et al., 2003; Imai et al., 2009). Conversely, overexpression of Nrp1 in a subset of OSNs shifts the position of their glomerulus posteriorly (Imai et al., 2009). Our results show that sema3D/Nrp1a signaling affects OSN targeting along the anterior-posterior axis in the zebrafish OB. Zebrafish sema3Aa is expressed in the dorsal part of the anterior OB, in the lateral OB, and in a subset of OMP positive sensory neurons at 36hpf (Data not shown). Sema3Ab is expressed in the dorsal part of the posterior OB, in the lateral OB, and also in OMP positive sensory (Data not shown). Based on these expression patterns, sema3Aa and sema3Ab could contribute to guidance along the anterior-posterior axis in zebrafish. While Sema3D affects anterior-posterior positioning of OR111-7 transgene expressing axons via nrp1a; Sema3Aa or Sema3Ab could potentially affect positioning via Nrp1b alone or in combination with Nrp1a. OR111-7 transgene expressing axons
misproject to LG1 and LG2 in \textit{nrp1b} mutants (Fig. 2. 6). This phenotype could be explained if Nrp1b mediates a repulsive activity to Sema3a expressed in the lateral bulb.

In this study we show for the first time that \textit{sema3D} is required for normal olfactory sensory map formation. It further suggests that additional class 3 semaphorins could play a role in olfactory axon guidance. Our results support a model in which Sema3D/Nrp1a mediated repulsion directs a specific subset of sensory axons towards the CZ protoglomerulus. Previously, we showed that the same subset of olfactory sensory axons is drawn to the CZ protoglomerulus by netrins expressed at the ventral and medial margins of the bulb (Lakhina et al., 2012). Together, these findings demonstrate semaphorin/neuropilin mediated repulsion working cooperatively with netrin/DCC mediated attraction to guide axons to their initial target location in the olfactory bulb (Fig. 2.10). They are consistent with the idea that many guidance cues are required to work in concert to establish complex neuronal circuitry in the developing nervous system. This may explain why the loss any single axonal guidance cue generally produces only a partially penetrant axonal guidance phenotype (Hedgecock et al., 1987).
Figure 2.1

**sema3D mRNA**

- **OMP:GAL4;UAS:Citrine**
- **OR111-7;IRES:GAL4;UAS:Citrine**

![Diagram of neuronal structure with images of sema3D mRNA expression in different regions](image-url)
**Figure 2.1. Sema3D is expressed in the anterior telencephalon at 36hpf.**

**A-C,** Maximum intensity projections through 36hpf larvae from a ventral view. Anterior is up and medial is to the right. Axons are shown in green and *sema3D* mRNA in red. **A-C,** *Sema3D* is expressed between the olfactory epithelium and nascent olfactory bulb (white arrowhead in **B**). **A-B,** A subset of OMP:Gal4;UAS:Citrine expressing axons overlaps with the *sema3D* expression domain in the anterior bulb (white arrowhead in **A**). **C,** *OR111-7* transgene expressing axons are positioned posterior to *sema3D* expression. **D-I,** Single optical sections through 36hpf larvae from a frontal view. Dorsal is up and medial it to the right. Sections are arranged from anterior (left) to posterior (right). The distance from each section to the anterior most part of the telencephalon is denoted in the bottom right corners. **D-F,** In the most anterior parts of the telencephalon, *sema3D* (red) is detected in the same plane as OMP positive axons (green, white arrowhead in **E**). **G-I,** *OR111-7* transgenic axons are not present in the most anterior portion of the telencephalon. *Sema3D* is detected in the region just dorsal to the most anterior portion of the *OR111-7* transgenic projection (white arrowhead in **H**). Scale bar (in **C**): **A-I,** 50μm. OE, olfactory epithelium; OB, olfactory bulb.
Figure 2.2
**Figure 2.2. OR111-7 transgenic axons misproject to the DZ in sema3D mutants.**  
**A-B,** Single optical sections through 72hpf OR111-7:IRES:GAL4;UAS:Citrine larvae (frontal view). Axons shown in green. Dorsal is up and medial is to the right of the image. Propidium iodide (blue) labels cell bodies revealing protoglomeruli as cell-free regions.  
**C-D,** Maximum intensity projections of serial optical sections from the larvae shown directly above. Scale bar (in **D**): **A–D,** 50μm.  
**E-F,** The percentage of olfactory bulbs that have a labeled projection to a particular protoglomerulus or non-protoglomerular region. Sema3D mutants (black bars) are compared to heterozygous (grey bars) and wild-type (white bars) siblings. Statistical significance was estimated using two-tailed Fisher’s exact tests (p < 0.05*, p < 0.01**, p < 0.001***). Error bars represent SEM.  
**A, C,** Wild-type OR111-7 transgenic axons project to the CZ and LG1 (white arrowheads).  
**B, D,** In sem3D mutants, a subset of OR111-7 transgenic axons inappropriately projects to the DZ (yellow arrowheads).  
CZ, central zone; DZ, dorsal zone; MG, medial protoglomeruli; LG1, lateral protoglomerulus 1; LG2, lateral protoglomerulus 2, LG3, lateral protoglomerulus 3; LG4, lateral protoglomerulus 4, VPG, ventral posterior protoglomerulus; OP, olfactory plexus.
Figure 2.3
Figure 2.3. Sensory neurons expressing OMP or TRPC2 have no detectable targeting errors in sema3D mutants.

**A-B**, Single optical sections through 72hpf OMP:RFP larvae (frontal view). Axons shown in red. Dorsal is up and medial is to the right of the image. **C-D**, Single optical sections through 72hpf TRPC2:Venus larvae (frontal view). Axons shown in green. Propidium iodide (blue) labels cell bodies revealing protoglomeruli as cell-free regions. Scale bar (in **D**): **A–D**, 50µm. **E-F**, The percentage of olfactory bulbs displaying a projection to a particular protoglomerulus or non-protoglomerular regions (other) are shown. Homozygous *sema3D* mutants (black bars) are compared to heterozygous (grey bars) and wild-type (white bars) siblings. Statistical significance was estimated using two-tailed Fisher’s exact test (*p* < 0.05*, *p* < 0.01**, *p* < 0.001***). Error bars represent SEM. **A, B, E**, OMP positive axons project to the same protoglomeruli in *sema3D* mutants as in controls. **C, D, F**, The TRPC2:Venus projection is unaltered in *sema3D* mutants.
Figure 2.4

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Figure 2.4. Zebrafish neuropilins are differentially expressed in the developing olfactory epithelium.

A-L, Single optical sections through olfactory epithelia of 36hpf larvae (frontal view). Propidium iodide (blue) labels cell bodies and mRNA is shown in red. A-D, Subsets of OMP neurons (green) express \( \text{nrp1a} \) (arrowheads in A), \( \text{nrp1b} \) (arrowhead in B), \( \text{nrp2a} \) (arrowhead in C) and \( \text{nrp2b} \) (arrowhead in D). E-H, \( \text{OR111-7} \) transgenic neurons (green) express \( \text{nrp1a} \) (arrowheads in E), \( \text{nrp1b} \) (arrowhead in F), and \( \text{nrp2b} \) (arrowhead in H). They do not express \( \text{nrp2a} \) at this age (arrow in G). I-L, TRPC2 positive neurons (green) express \( \text{nrp1b} \) (arrowhead in J), \( \text{nrp2a} \) (arrowhead in K), and \( \text{nrp2b} \) (arrowhead in L), but not \( \text{nrp1a} \) (arrow in I). Scale bar (in L): A–L, 10 µm. M, Summary showing the percent of cells expressing a given neuropilin in either OMP, \( \text{OR111-7} \) or TRPC2 expressing neurons.
Figure 2.5. Loss of either nrp1a or nrp2b phenocopies sema3D mutants.

A-B, E-F, Single optical sections through 72hpf OR111-7 transgenic larvae (frontal view). Axons shown in green. Dorsal is up and medial is to the right of the image. Propidium iodide (blue) labels cell bodies revealing protoglomeruli as cell-free regions. C-D, G-H, Maximum intensity projections of serial optical sections from the larvae shown directly above. A, C, E, G OR111-7 transgenic axons project to the CZ in wild-type larvae. B, D, A subset of OR111-7 transgene labeled axons misprojects to the DZ in nrp1a mutants (yellow arrowheads). F, H A subset of OR111-7 transgene labeled axons also inappropriately targets the DZ in nrp2b mutants (yellow arrowheads). Scale bar (in H): A–H, 50µm.
Figure 2.6. Quantification of OR111-7 transgene labeled axon targeting observed in nrp1a, nrp1b and nrp2b mutants.

A-F, The percentage of olfactory bulbs displaying a projection to a particular protoglomerulus or non-protoglomerular regions (other) are shown. Statistical significance was estimated using two-tailed Fisher’s exact tests ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$). Error bars represent SEM. Mutants (black bars) are compared with wild-type (white bars) and heterozygous (gray bars) siblings. A-B, OR111-7 transgene labeled axons have increased frequency of projections to the DZ and to non-protoglomerular regions dorsal to the CZ in nrp1a mutants. C-D, OR111-7 transgene labeled axons have increased frequency of projections to LG1 and LG2 in nrp1b mutants. Nrp1b mutants do not display an increase in non-protoglomerular errors. E, F, OR111-7 transgene labeled axons misproject to the DZ in nrp2b mutants. Nrp2b mutants do not display an increase in non-protoglomerular errors.
Figure 2.7

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OR111-7; IRES: Gal4, UAS: Citrine

G

% olfactory bulbs with axons targeting a particular population/dience

H

% olfactory bulbs with axons targeting a particular population/dience
Figure 2.7. Sema3D interacts genetically with nrp1a to promote axon targeting of OR111-7 transgene labeled axons to the CZ.

A–C, Single optical sections through 72hpf OR111-7 transgenic larvae (frontal view). Axons shown in green. Dorsal is up and medial is to the right of the image. Propidium iodide (blue) labels cell bodies revealing protoglomeruli as cell-free regions. D–F, Maximum intensity projections of serial optical sections from the larvae shown directly above. G–H, The percentage of olfactory bulbs with projections to a particular protoglomerulus or non-protoglomerular region are shown. Statistical significance was estimated using two-tailed Fisher’s exact tests ($p < 0.05^*, p < 0.01^{**}, p < 0.001^{***}$). Error bars represent SEM. A–G, sema3D<sup>+/−</sup>;nrp1a<sup>+/−</sup> transheterozygotic larvae (C, F, and black bars in G) have an increase in projections to the DZ compared to sema3D<sup>+/−</sup> heterozygotic (A, D, and white bars in G) and nrp1a<sup>+/−</sup> heterozygotic (B, E, and gray bars in G) siblings. 
H, sema3D<sup>+/−</sup>;nrp2b<sup>+/−</sup> transheterozygotes (black bars) do not display an increase in projections to the DZ when compared to sema3D<sup>+/−</sup> heterozygotic (white bars) and nrp2b<sup>+/−</sup> heterozygotic (gray bars in H) siblings. Scale bar (in F): A–F, 50μm
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**Figure 2.8**
Figure 2.8. In sema3D−/ −;nrp1a−/− double mutants, OR111-7 transgenic axons project to the DZ protoglomerulus with a similar penetrance and severity as single mutants.

A-D, Single optical sections through 72hpf OR111-7 transgenic larvae (frontal view). Axons shown in green. Dorsal is up and medial is to the right of the image. Propidium iodide (blue) labels cell bodies revealing protoglomeruli as cell-free regions. E-H, Maximum intensity projections of serial optical sections from the larvae shown directly above. I-J, The percentage of olfactory bulbs with a projection to a particular protoglomerulus or non-protoglomerular region are shown. Statistical significance was estimated using two-tailed Fisher’s exact tests (*p < 0.05, **p < 0.01, ***p < 0.001). Error bars represent SEM. A-J, Sema3D−/− mutants (B, F and gray bars in I and J), nrp1a mutants (C, G and black bars in I and J) and sema3D−/−;nrp1a−/− double mutants (D, H and purple bars in I and J) display an increase in projections to the DZ compared to wild-type (A, E and white bars in I and J). Sema3D−/−;nrp1a−/− double mutants and nrp1a−/− mutants also display an increase in misprojections to dorsal non-protoglomerular regions of the bulb not seen in sema3D mutants (I and J). Scale bar (in H): A–H, 50μm
Figure 2.9

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E:

- Uas:nrp1a,nrp1a<sup>+/+</sup> (n=45)
- Uas:nrp1a,nrp1a<sup>++/−</sup> (n=58)
- Uas:nrp1a,nrp1a<sup>−/−</sup> (n=52)

% of olfactory bulbs with axons targeting a particular protocerebral nucleus:

- CZ
- DZ
- MG
- LG1
- LG2
- LG3
- LG4
- VPG
- OP
- Other

Legend:

- Open bars: Uas:nrp1a,nrp1a<sup>+/+</sup> (n=45)
- Gray bars: Uas:nrp1a,nrp1a<sup>++/−</sup> (n=58)
- Black bars: Uas:nrp1a,nrp1a<sup>−/−</sup> (n=52)

ns: not significant
Figure 2.9. Selective expression of nrp1a in OR111-7 transgene expressing neurons corrects DZ errors seen in nrp1a mutants.

**A-B,** Single optical sections through 72hpf OR111-7:Gal4;UAS:nrp1a:UAS:Citrine transgenic larvae (frontal view). Axons shown in green. Dorsal is up and medial is to the right of the image. Propidium iodide (blue) labels cell bodies revealing protoglomeruli as cell-free regions. **C-D,** Maximum intensity projections of serial optical sections from the larvae shown directly above. **E,** The percentage of olfactory bulbs displaying a projection to a particular protoglomerulus or non-protoglomerular region are shown. Statistical significance was estimated using two-tailed Fisher’s exact tests ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$). Error bars represent SEM. **A-E,** Nrp1a mutants expressing the UAS:nrp1a:UAS:Citrine transgene (**B, D** and black bars in **E**), do not display an increase in misprojections to the DZ or non-protoglomerular errors compared to wild-type (**A, C** and white bars in **E**), and heterozygous (gray bars in **E**), siblings. Scale bar (in **D**): **A–D,** 50µm.
Figure 2.10. Semaphorin/Neuropilin mediated repulsion may work cooperatively with Netrin/DCC mediated attraction to guide OR111-7 transgene expressing axons to their initial target in the olfactory bulb.

Ventral view, anterior is up and medial is to the left. OR111-7 transgene expressing neurons (green) project to the central zone protoglomerulus (CZ). Sema3D is expressed in the anterior olfactory bulb and in the boundary between the olfactory pit and olfactory bulb (shown in red). netrin1a (light blue) and netrin1b (dark blue) are expressed in medially, ventrally, and posteriorly in the olfactory bulb. Loss of either Sema3D mediated repulsion or Netrin mediated attraction induces a subset of OR111-7 transgene expressing axons to target the more dorsally and anteriorly located dorsal zone protoglomerulus (DZ).
CHAPTER 3. MULTIPLE CLASS 3 SEMAPHORINS ARE EXPRESSED DURING PROTOGLOMERULAR TARGETING

Introduction

Each olfactory sensory neuron expresses 1 to 2 odorant receptors (OR) out of a large repertoire of OR genes. There are approximately ~1200 mouse, 300 human, 143 zebrafish, and 60 Drosophila functional OR genes (Alioto and Ngai, 2005; DeMaria and Ngai, 2010; Brochtrup and Hummel, 2011). Olfactory sensory neurons expressing a particular OR converge on the same topographically fixed glomerulus in the olfactory bulb (OB) (Ramon y Cajal, 1892; Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). Each glomerulus contains axons from cells that express the same OR (Treloar et al., 2002), and each OR responds to specific odorants. For this reason odors are represented as distinct patterns of glomerular activity within the bulb (Friedrich and Korsching, 1997; Malnic et al., 1999). Perturbations in glomerular targeting by manipulation of guidance cues or olfactory receptor expression can perturb olfactory induced behaviors (Fleischmann et al., 2008; Cho et al., 2011).

The olfactory map develops in at least two stages. In the first stage axons from neurons expressing different ORs project to glomerulus-like structures called protoglomeruli. In the second stage these axons segregate into mature glomeruli which contain axons from neurons expressing the same OR gene (Conzelmann et al., 2001). Although, several guidance cues have been identified that play a role in olfactory map formation, the complexity of this system suggests that coordinated actions of many cues may guide axons to their glomerular targets. However, because most studies examine the mature olfactory projection, it is unclear which cues are required for initial protoglomerular targeting or for glomerular segregation.
Class 3 semaphorins, the secreted members of the semaphorin family of guidance molecules, are excellent candidates to guide axons to their protoglomerular targets. Semaphorins were first described as strong repellents, however, in some systems, they can act as attractants (Sharma et al., 2012). Two class 3 semaphorins, Sema3A and Sema3F, have previously been studied in the mouse. It has been reported that Sema3A contributes to OSN targeting along the anterior-posterior axis, while Sema3F is required for targeting along the dorsal-ventral axis (Imai et al., 2009; Takeuchi et al., 2010). Additional class 3 semaphorins have been detected in the mouse (Sema3C and Sema3B) and chick (Sema3C, Sema3D, and Sema3E) olfactory systems (Giger et al., 2000; Renzi et al., 2000; Cloutier et al., 2002). My work in the zebrafish demonstrates that sema3D is required for normal protoglomerular targeting (Chapter 2).

Here I show that at least 8 of the 12 identified zebrafish class 3 semaphorins are expressed in the olfactory system during initial protoglomerular targeting, and that at least 5 of these are expressed in olfactory sensory neurons. Class 3 semaphorins signal through neuropilin/plexin complexes (Sharma et al., 2012). All four zebrafish neuropilins are differentially expressed in subsets of OSNs in the olfactory epithelium (OE) (Chapter 2). Here I show that some OSNs co-express more than one neuropilin and that neuropilin mutants display sema3D independent phenotypes. Together these data suggest that multiple class 3 semaphorins cooperate to guide olfactory axons to their protoglomerular targets.

Materials and Methods

Zebrafish maintenance and transgenic lines. Adult zebrafish were raised and maintained according to standard practices (Mullins, 1994). Larvae were staged based on
hours post fertilization (hpf) and were raised at 28°C. For the in situ experiments the 36 hpf time point was obtained by incubating for 1 day at 28°C and 1 day at 25°C (Kimmel, 1995). Tg(omp:lyn-RFP) and Tg(trpc2:gap-VENUS) lines were gifts from the Yoshihara laboratory (Sato et al., 2005). The Tg(or111-7:or111-7-IRES:GAL4), Tg(omp:GAL4) and Tg(UAS:gap43-Citrine) lines were previously described by Lakhina et al., (2012). A double transgenic line, Tg(or111-7:GAL4;UAS:Citrine) was generated by crossing Tg(or111-7:or111-7-IRES:GAL4) to Tg(UAS:gap43-Citrine). Transgenic lines were used alone or crossed into various mutant strains.

**Zebrafish mutants.** Nrp1a<sup>ha1485</sup> mutants were generated by the Sanger Center's Zebrafish Mutation Project and obtained from The Zebrafish International Resource Center (ZIRC). Larvae were genotyped using a KASP genotyping assay (LGC Genomics; nrp1a SNP ID 554-1410.1). The nrp1b<sup>fh278</sup> mutant was identified by the Zebrafish Tilling Project and ordered from ZIRC. Nrp2b<sup>mn0126GT</sup> mutants were a gift from the Ekker laboratory (Clark et al., 2011). Standard PCR based methods were used to genotype nrp1b<sup>fh278</sup> (forward primer: TCTCTTTTGGAGTCTCTGC, reverse primer: TGTCTTTGTGTGTGTGTCTAG, MseI cuts the mutant sequence into 161bp and 34bp fragments); and Nrp2b<sup>mn0126GT</sup> (nrp2b forward primer: GCTGAAGATCGGTATCAGACGAAAAACA, nrp2b reverse primer: AGACCTGCCATATTGGATGAGTACCAGA, RFP reverse primer: CCTTGAAAGCGAATCCTGGTAC) lines.

**Whole-mount Immunohistochemistry.** Immunohistochemistry was performed as described by Lakhina et al., (2012). Larvae were fixed in 4% paraformaldehyde in PBS and dehydrated in methanol. To stain Citrine positive axons, larvae were permeabilized in
acetone for 20 minutes at -20°C and stained with goat anti-GFP (1:300; Rockland Immunochemicals, 600-101-215) and donkey anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen). To stain RFP positive axons, fish were treated for 30 minutes in 0.1% collagenase at room temperature and stained with rabbit anti-dsRed (1:300; Clontech, 632496) and donkey anti-rabbit IgG Alexa Fluor 647 (1:500; Invitrogen). Propidium iodide staining was performed following secondary antibody treatment as described by Brend and Holley (2009) with the exception that larvae were not treated with RNase. Confocal microscopy was performed on an inverted Leica SP5 using a 40X oil-immersion lens. Stacks were acquired through the entire olfactory bulb with optical sections taken 1 micron apart.

**Whole-mount fluorescent in situ hybridization.** Single label *in situ* hybridization was performed using antisense Digoxigenin (DIG) labeled RNA probes as described by Chalasani (2007). *In situ* signals were amplified using a cyanine 5-coupled tyramide kit (TSA Plus cyanine 5, PerkinElmer, NEL745001KT). Double label *in situ* hybridization was performed using DIG and fluorescein labeled probes as previously described (Brend and Holley, 2009) with the exception that larvae were not dehydrated in between detection of the first and second probes. 36hpf Larvae were permeabilized with proteinase K for 18 minutes and 72hpf larvae were permeabilized with 0.2% collagenase for 2 hours at room temperature. The DIG label was amplified using a cyanine 5-coupled tyramide kit (PerkinElmer, NEL745001KT) and the fluorescein label was amplified using a cyanine 3-coupled tyramide kit (PerkinElmer, NEL744001KT). Prior to tyramide amplification embryos were incubated in either anti-DIG-POD (1:500; Roche, 11207733910) or anti-Fluorescein-POD (1:500;Roche, 11426346910). Immunohistochemistry, propidium iodide labeling, and imaging were performed following tyramide amplification as described
above. The plasmids used to make probes targeting \textit{sema3E}, and \textit{nrp1a} were as described in Dell et al., (2013). The plasmids used to make probes for \textit{sema3Aa}, \textit{sema3Ab}, \textit{sema3Fa}, \textit{sema3Fb}, \textit{sema3Ga}, \textit{sema3Gb}, and \textit{nrp2b} were gifts from the Moens laboratory (Yu et al., 2004; Yu et al., 2005). The plasmid used to make \textit{sema3H} probe was a gift from the Halloran laboratory (Stevens and Halloran, 2005). For \textit{sema3C} (refseq accession number XM_687755.5, nucleotides 1539-2400); \textit{nrp1b} (refseq accession number NM_205674.1, nucleotides 2-972); and \textit{nrp2a} (refseq accession number NM_212965.1, nucleotides 138-1108) sequences were amplified from cDNA and cloned into pcRII (Invitrogen, K460001) for probe synthesis. Full-length probes were used in all hybridization experiments.

\textbf{Quantification of targeting errors.} After larvae were processed and imaged, the number of olfactory bulbs containing axons terminating in either individual protoglomeruli or non-protoglomerular regions were counted. The percentage of olfactory bulbs with axons in particular locations was recorded and Fisher’s exact (two-tailed) test was used to determine statistical significance.

\textbf{Results}

During development, OSN axons exit the OE and project dorsally and anteriorly into the telencephalon, reaching the nascent olfactory bulb (OB) by 24 hours post fertilization (hpf) (Wilson et al., 1990; Hansen and Zeiske, 1993). By 72hpf, the OB contains 12 identifiable protoglomeruli (Dynes and Ngai, 1998; Lakhina et al., 2012). Protoglomeruli are indistinguishable prior to this stage. The zebrafish olfactory epithelium contains at least two classes of sensory neurons which innervate mutually exclusive protoglomeruli (Sato et al., 2005). OMP positive neurons are ciliated, express classical main-olfactory type ORs
and innervate the central zone (CZ), dorsal zone (DZ), lateral protoglomerulus 3 (LG3) and medial protoglomeruli (MG) (Celik et al., 2002; Sato et al., 2005; Lakhina et al., 2012). Microvillous neurons express the transient receptor potential channel 2 (TRPC2) and vomeronasal V2R-type receptors (Sato et al., 2005). TRPC2 positive axons innervate lateral protoglomeruli 1, 2, and 4 (LG1, LG2 and LG4), the ventral posterior protoglomerulus (VPG) and the olfactory plexus (OP) (Dynes and Ngai, 1998; Lakhina et al., 2012).

**Multiple class 3 semaphorins are expressed in the developing zebrafish olfactory system.**

The semaphorin family of guidance molecules is divided into 8 classes. Classes 1 and 2 are found in invertebrates and classes 3 through 7 are found in vertebrates. The 8th class is encoded by viruses (Yazdani and Terman, 2006). Class 3 semaphorins are the only vertebrate semaphorins that are secreted.

There are at least 12 identified zebrafish class 3 semaphorins: *sema3Aa*, *sema3Ab*, *sema3B*, *Sema3Bl*, *sema3C*, *sema3D*, *sema3E*, *sema3Fa*, *sema3Fb*, *sema3Ga*, *sema3Gb and sema3H* (Amores, 1998; Halloran et al., 1999; Roos et al., 1999; Yee et al., 1999; Stevens and Halloran, 2005; Yu and Moens, 2005). To determine which class 3 semaphorins are expressed during zebrafish protoglomerular targeting, RNA probes targeting 10 (*sema3Aa, sema3Ab, sema3C, sema3D, sema3E, sema3Fa, sema3Fb, sema3Ga, sema3Gb and sema3H*) semaphorins were generated and hybridized with 36hfp larvae.
Sema3A is one of the best studied class 3 semaphorins. In mouse, Sema3A is expressed in anteromedial and ventral regions of the olfactory nerve layer (Schwarting et al., 2000). It is also expressed in subset of sensory neurons (Imai et al., 2009). Glomeruli formed by axons expressing the receptor Nrp1 are shifted anteriorly in the OBs of nrp1, sema3A, or OSN specific sema3A mutants (Taniguchi et al., 2003; Imai et al., 2009). In the zebrafish olfactory system, Sema3Aa is expressed in the lateral and dorsal olfactory bulb (Fig. 3.1A-D). Sema3Ab is expressed in the OB laterally and dorsally to OMP positive axons (Fig. 3.1E-H).

The role of Sema3F in olfactory map formation has also been studied in mouse. It is expressed by OSNs that target the dorsal OB. These axons secrete sema3F into the dorsal OB where it is thought to restrict late-arriving, Nrp2 expressing axons to the ventral OB (Norlin et al., 2001; Takeuchi et al., 2010). Sema3Fa RNA is detected in the anterior zebrafish OB overlapping sensory neuron axons (Fig. 3.1I-J). Sema3Fb is detected dorsomedial to the OB (Fig. 3.1M-P).

Many semaphorins yet to be implicated in olfactory map formation are expressed in the 36hpf zebrafish olfactory system. Sema3C RNA is detected in the medial olfactory bulb (data not shown). Sema3D RNA is detected in the anterior OB (Chapter 2). Sema3E RNA is detected between the OE and the OB. It is expressed in the same plane as OMP positive axons in the anterior OB. It is also expressed just medial and ventral to the olfactory projection (Fig. 3.2 A-D). Sema3Ga is expressed in OE and sparsely in the OB (Fig. 3.2E-H). Sema3Gb was not detected near the OB or in the OE at 36hpf (data not shown). Sema3H, a novel zebrafish semaphorin, shares no more than 42% amino acid identity.
with any mouse class 3 semaphorin (Stevens and Halloran, 2005). Sema3H is strongly expressed in the OB and overlaps extensively with sensory axons (Fig. 3.3A-D).

Sema3A and Sema3F are expressed in subsets of mouse OSNs (Imai et al., 2009; Takeuchi et al., 2010). We examined semaphorin expression in the zebrafish OE and found 5 semaphorins expressed in OMP positive sensory neurons. Sema3Aa, sema3Ab, sema3Fa and Sema3Ga RNA can be detected in small subsets of OMP positive neurons at 36hpf (Fig. 3.4A-D). Sema3H RNA is strongly expressed in a subset of OSNs at 36hpf (Fig. 4E). Sema3D, sema3E, sema3Fb, and sema3Gb were not detected in the OE at this time-point (data not shown). Although expression in the OB was greatly reduced sema3Ab, sema3D, sema3Fa and sema3Ga RNA could be detected at 72hpf (data not shown). Overall, 8 class semaphorins are expressed in overlapping but distinct patterns in the developing zebrafish olfactory system.

**OMP positive sensory neurons can co-express more than one neuropilin**

Class 3 Semaphorins signal through Neuropilin/Plexin complexes with the exception of Sema3E which can signal via PlexinD1 in the absence of a neuropilin (Gu et al., 2005). There are two neuropilins in mammals, Nrp1 and Nrp2, which differ in their binding affinities for various class 3 semaphorins (Chen et al., 1997). There is evidence that Sema3A, Sema3B, Sema3C, Sema3E can signal through Nrp1, while Sema3B, Sema3C, Sema3F and Sema3G signal via Nrp2 (Sharma et al., 2012).

There are four zebrafish neuropilins: nrp1a, nrp1b, nrp2a, and nrp2b (Bovenkamp et al., 2004; Yu et al., 2004). Nrp1a and Nrp1b share 54% amino acid identity, while Nrp2a and Nrp2b share 75% amino acid identity (Bovenkamp et al., 2004). These neuropilins are
differentially expressed in the OE. Each neuropilin can be detected in OMP positive neurons at 36hpf. At this age, 40% of OMP positive cells express nrp1a, 34% express nrp1b, 19% express nrp2a and 23% express nrp2b. Many TRPC2 positive neurons express nrp1b (67%), nrp2a (69%) or nrp2b (54%). Only 5% of TRPC2 positive neurons express nrp1a (Chapter 2). OR111-7 labeled neurons are a subset of OMP positive neurons that project predominantly to the central zone protoglomerulus and express nrp1a (59%), nrp1b (68%) and nrp2b (34%). Nrp2a is detected in only 3% of OR111-7 labeled neurons at this age (Chapter 2). We asked whether OSNs might co-express neuropilins and found that subsets of OMP positive neurons express nrp1a only, nrp1b only or co-express nrp1a and nrp1b (Fig 3.5A). Similarly, OMP subsets of positive neurons express nrp2a only, nrp2b only or co-express nrp2a and nrp2b (Fig. 3.5B). There is evidence suggesting that Sema3B can signal through Nrp1 or Nrp2 and that Sema3C can signal through Nrp1/Nrp2 complexes (Takahashi et al., 1998; Sharma et al., 2012). We next asked whether nrp1a and nrp2b are co-expressed. Indeed, subsets of OMP expressing neurons co-express nrp1a and nrp2b (Fig. 3.5C).

**Neuropilin mutants display sema3D independent phenotypes**

To further explore the possibility that multiple class 3 semaphorins might influence protoglomerular targeting, we examined the OMP, TRPC2 and OR111-7 transgene expressing axons in nrp1a, nrp1b or nrp2b neuropilin mutants. The \textit{nrp1a}^{sa1485} allele contains a nonsense mutation that results in a premature stop codon at amino acid 206 within the second CUB domain (Ensembl genome browser 75, ENSDARG00000071865). The \textit{nrp1b}^{fh278} allele contains a nonsense mutation that results in a premature stop codon at amino acid 116 within the first CUB domain (Ensembl genome browser 75,
The nrp2b mno126GT allele obtained from the Ekker laboratory contains an RFP and polyadenylation site inserted at amino acid 427 just before the second Coagulation-factor homology domain (Clark et al., 2011). Olfactory bulb morphology and protoglomerular positioning appear normal in all three mutants at 72hpf (Fig. 6B, 6F, and data not shown). In sema3D mutants, a subset of OR111-7 expressing OSN axons inappropriately target the DZ instead of the CZ protoglomerulus. When the entire OMP positive projection or the TRPC2 projection is observed, no errors were detected in sema3D mutants (Chapter 2). There is convincing evidence that Sema3D works through Nrp1a to prevent OR111-7 transgene expressing axons from entering the DZ (Chapter 2). However, each of the three neuropilin mutants display phenotypes that are not observed in sema3D mutants, indicating that these receptors are working, at least in part, through different semaphorins.

In nrp1a mutants, OMP:RFP expressing axons project to the CZ, DZ, LG3 and MG with the same frequency as axons in wild-type larvae (Fig. 3.6A). However, there was a slight increase in ectopic projections to LG2 in nrp1a mutants (16%, N=58) compared to wild-type (2%, N=75) larvae (Fisher’s exact, p=.006) (Fig. 3.6A). LG2 is normally innervated by TRPC2 positive axons (Lakhina et al., 2012). Projections that do not terminate in a protoglomerulus are scored as “other” and characterized based on their region of termination in the bulb. OMP positive axons do not display an increase in non-protoglomerular errors in nrp1a mutants (Fig 3.6A).

Although TRPC2 expressing axons normally project to LG2, in nrp1a mutants there is an increase in the frequency of projections to this protoglomerulus (Fig. 3.6B). Wild-type TRPC2 axons project to LG2 in 73% (N=22) of the OBs examined, while mutant axons
projected to LG2 in 95% (N=39) of bulbs (Fisher’s exact, p=.002). There is also an
increase in non-protoglomerular targeting errors in nrp1a mutant TRPC2 axons; 4% in
wild-type (N=22) compared to 41% in mutants (N=39) (Fisher’s exact, p=.009). This may
be attributed to an increase in dorsal misprojections in mutants (33%, N=39) compared to
wild-type larvae (4%, N=22) (Fisher’s exact, p=.01) (data not shown; Fig. 3.6B).

In addition to projecting ectopically to the DZ, OR111-7 transgene expressing axons also
make dorsal non-protoglomerular target errors (Fig. 3.7; Chapter 2). These errors were
present in 51% (N=47) of the mutant bulbs that were examined and 26% (N=50) of the
wild-type bulbs (Fisher’s exact, p=.01).

Nrp1b mutants were also examined for protoglomerular targeting errors. OMP positive
axons did not display targeting errors in nrp1b mutants, however, OR111-7 transgene
expressing axons project inappropriately to LG1 in 57% (N=49) of mutant bulbs as
compared to 33% (N=43) of wild-type bulbs (Fisher’s exact, p=.02). In nrp1b mutants,
OR111-7 transgene expressing axons also display a significant increase in projections to
LG2 (Chapter 3 and data not shown). OR111-7 transgene expressing axons project LG2
in 20% of mutant bulbs (N=43) compared to only 2% of wild-type bulbs (N= 49) (Fisher’s
exact, p=.03) (Chapter 2, data not shown).

OR111-7 transgenic axons misproject to the DZ in nrp2b mutants. Although nrp2b mutants
do phenocopy sema3D mutants, the severity and penetrance are markedly greater in
nrp2b mutants as compared to sema3D mutants. Further, we detected no genetic
interaction between sema3D and nrp2b (Chapter 2). This suggests that Sema3D is not
the sole ligand for Nrp2b. Preliminary studies indicate that there are no observable defects
in OMP positive axon targeting in nrp2b mutants, but TRPC2 positive axons misproject to
the LG3 protoglomerulus in nrp2b mutants (Fig. 3.8). TRPC2 positive axons innervate LG3 in 30% of OBs (N=44) in nrp2b mutants as compared to 5% of wild-type bulbs (N=57) (Fisher’s exact, p=.002) (Fig. 3.8E). This is particularly interesting because TRPC2 positive axons do not make targeting errors in sema3D mutants, further evidence that a different semaphorin might be the ligand for Nrp2b.

Discussion

Sema3A is reported to control olfactory map formation along the anterior-posterior axis, while Sema3F is thought to control targeting along the dorsal-ventral axis in the mouse (Imai et al., 2009; Takeuchi et al., 2010). Our previous finding that sema3D is required for protoglomerular targeting in zebrafish and the finding that there are 9 class 3 semaphorins expressed during the developing olfactory system raise the possibility that many class 3 semaphorins contribute to this complex task. Understanding which Semaphorins and Neuropilins contribute to olfactory axons guidance and how they interact with each other will not only expand our understanding of olfactory development but may also provide insight into other systems where multiple semaphorins are expressed. For example, subsets of spinal motor neurons can be defined by differential expression of 6 different class 3 semaphorins (3A, 3B, 3C, 3D, 3E, 3F) and their receptors and at least 8 semaphorins (3Aa, 3Fa, 3Fb, 3C, 3E, 3D, 3Ga, 3Gb) are expressed near the developing retina-tectal projection (Cohen et al., 2005; Sakai and Halloran, 2006; Callander et al., 2007; Dell et al., 2013).

As in mouse, sema3Aa/b and sema3Fa/b are expressed in the zebrafish olfactory system. Interestingly, sema3Aa and sema3Ab display distinct expression patterns. Both are expressed in the dorsal OB, but sema3Aa is detected strongly in the lateral OB. This raises
the question of whether these cues have redundant or specific functions. Similarly, 
sema3Fa is detected mainly in the anterior OB, while sema3Fb is strongest in the 
dorsomedial bulb. To determine their functions, it will be necessary to examine loss-of-
function mutants for each cue. These mutants can either be obtained from the Sanger's 
Zebrafish Mutation Project or generated using targeted genome editing technology such 
as transcription activator-like effector nucleases (TALENs) or the clustered regularly 
interspaced short palindromic repeats (CRISPR)/Cas9 system (Bedell et al., 2012; 
Kettleborough et al., 2013; Auer and Del Bene, 2014). It will be important to determine 
whether the functions of these cues are analogous to what has been described in mouse. 
This can only be done after further characterization of neuropilin expression and binding 
and a careful examination of targeting of specific subsets of OSN axons in mutants.

Sema3E, sema3Ga and sema3H have not previously been implicated in olfactory axon 
guidance, but are expressed by cells in close proximity to extending olfactory axons. 
Sema3H is of particular interest because there is no mammalian homolog and it is 
expressed strongly in the OB (Fig. 3.3). If it does play a role in olfactory axon guidance its 
expression pattern suggests that it may be an attractant.

It is unclear whether Nrp1a and Nrp1b or Nrp2a and Nrp2b are functionally redundant or 
specialized. However, the phenotypic differences between nrp1a and nrp1b mutants 
suggest that each can function independently (Chapter 2). For example, OR111-7 
expressing axons misproject to the DZ and to dorsal non-protoglomerular areas in the bulb 
in nrp1a mutants, whereas they display an increase in ectopic projections to LG1 and LG2 
in nrp1b mutants (Fig. 3.7; Chapter 2). Importantly, Nrp1a and Nrp1b may share a 
redundant function that cannot be detected in single mutants. Nrp1a−/−;nrp1b−/− double
mutants must be generated to address this possibility. Once \( nrp2a \) mutants are available, \( nrp2a^{-/-};nrp2b^{-/-} \) double mutants can also be examined.

The observation that subsets of OMP positive neurons co-express \( nrp1a \) and \( nrp1b \) or \( nrp2a \) and \( nrp2b \) raises the possibility that the response properties of these neurons differ from those of neurons expressing either \( nrp1a \) or \( nrp1b \) alone. \( Nrp1a \) and \( nrp2b \) are co-expressed in a subset of OMP neurons (Fig. 3.5). In the mouse, Sema3C binds and signals through Nrp1/Nrp2 complexes (Takahashi et al., 1998). Further, it has been suggested that Sema3D acts as a repellent through Nrp1a but as an attractant through Nrp1/Nrp2 complexes (Wolman et al., 2004). It will be important to determine whether neurons expressing different combinations of neuropilins project to different protoglomeruli and whether they recognize different semaphorins.

\( \text{Sema3Aa, sema3Ab, sema3Fa, sema3Ga and sema3H are each expressed in subsets of OMP positive neurons at 36hpf (Fig. 3.4). Is it not known whether the semaphorins expressed in zebrafish OSNs control pre-target axon sorting as Sema3A does in mouse, or whether particular semaphorins are secreted by axons in the OB comparable to what has been described for Sema3F in the mouse (Imai et al., 2009; Takeuchi et al., 2010).} \)

Although we have previously shown that Sema3D/Nrp1a signaling guides \( OR111-7 \) transgene expressing axons to the CZ, \( nrp1a, nrp1b \) and \( nrp2b \) mutants display sema3D independent phenotypes. \( OR111-7 \) transgene expressing neurons project to non-protoglomerular regions dorsal to the CZ in \( nrp1a \) mutants (Fig. 3.7 and Chapter 2). \( \text{Sema3Aa, sema3Ab, and sema3Fb are each expressed in the dorsal olfactory bulb (Fig. 3.1). It is possible that one or more of these cues acts through Nrp1a to repel axons away from the dorsal OB. It is also possible that Sema3H/Nrp1a mediated attraction keeps} \)
axons from projecting dorsally. OR111-7 transgene expressing axons misproject to LG1 and LG2 in nrp1b mutants (Chapter 2). Sema3Aa, sema3Ab, sema3E, and to a lesser extent sema3Fa are expressed in the lateral OB at 36hfp where they may signal through Nrp1b to repel axons from the lateral bulb (Fig. 3.1 and 3.2). In nrp2b mutants, both OR111-7 transgene expressing and TRPC2 positive axons make guidance errors (Fig. 3.8 and Chapter 2). TRPC2 axons misproject to LG3 in the dorsal lateral OB which is normally innervated by axons from neurons expressing OMP and classical ORs in nrp2b mutants (Fig. 3.8). Potential Nrp2b ligands in the dorsal lateral OB include Sema3Aa, Sema3Ab, Sema3Fa, and sema3Ga (Fig. 3.1 and 3.2).

The impact of Sema/Nrp signaling on OSN targeting may be exceedingly complex. First, multiple Semaphorins may work through the same Neuropilin. While Sema3D signals through Nrp1a to promote targeting to the dorsal zone, nrp1a mutants also display a non-protoglomerular dorsal errors which are not seen in sema3D mutants (Fig. 3.7 and Chapter 2). Second, a single OSN can express multiple Neuropilins (Fig. 3.5). Third, Semaphorins can signal through Neuropilin heterodimers and axonal response to a particular Semaphorin may vary based on the cohort of Neuropilins that are expressed (Wolman et al., 2004; Sharma et al., 2012). The olfactory map is remarkably complex and it is fitting that an equally complex mechanism is required for its development. Our findings suggest that Semaphorin/Neuropillin signaling may be a key component of this mechanism.
Figure 3.1

<table>
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Anterior ➔ Posterior

2μm, 8μm, 18μm, 5μm, 11μm, 18μm, 4μm, 10μm, 16μm, 2μm, 8μm, 17μm
Figure 3.1. Sema3Aa/b and sema3Fa/b expression at 36hpf.

A-C, E-G, I-K, M-O, Single optical sections through 36hpf larvae from a frontal view. Dorsal is up and medial it to the right. Sections are arranged from anterior (left) to posterior (right). Axons from OMP:Gal4;UAS:Citrine expressing neurons are shown in green and mRNA in red. The distance from each section to the anterior most part of the telencephalon is denoted in the bottom right corners. D, H, L, P, Maximum intensity projections through the larvae in adjacent sections. A-D, Sema3Aa is expressed in the lateral and dorsal olfactory bulb. E-H, Sema3Ab is expressed in the lateral and dorsal to OMP positive axons. I-J, Sema3Fa is expressed in the anterior olfactory bulb. Expression overlaps olfactory sensory axons. M-P, Sema3Fb is expressed in the dorsomedially to olfactory bulb. Scale bar (in P): A–P, 50μm. OE, olfactory epithelium; OB, olfactory bulb.
Figure 3.2

Figure 3.2. Sema3E and sema3Ga expression at 36hpf.

A-C, E-G, Single optical sections through 36hpf larvae from a frontal view. Dorsal is up and medial it to the right. Sections are arranged from anterior (left) to posterior (right). Axons from OMP:Gal4;UAS:Citrine expressing neurons are shown in green and mRNA in red. The distance from each section to the anterior most part of the telencephalon is denoted in the bottom right corners. D, H, Maximum intensity projections through the larvae in adjacent sections. A-D, Sema3E is expressed between the OE and the OB. It is expressed in the same plane as OMP positive axons in the anterior OB. It is also expressed just medial and ventral to the olfactory projection (white arrowheads). E-H, Sema3Ga is expressed in the OE, and sparsely in the OB. Scale bar (in H): A–H, 50μm.

OE, olfactory epithelium; OB, olfactory bulb.
Figure 3.3

Figure 3.3. Figure Sema3H expression at 36hpf.

A-C, Single optical sections through 36hpf larvae from a frontal view. Dorsal is up and medial is to the right. Sections are arranged from anterior (left) to posterior (right). Axons from OMP:Gal4;UAS:Citrine expressing neurons are shown in green and mRNA in red. The distance from each section to the anterior most part of the telencephalon is denoted in the bottom right corners. D, Maximum intensity projection through the larvae in adjacent sections. A-D, Sema3H is expressed strongly throughout the OB and in the OE. Scale bar (in D): A–D, 50µm. OE, olfactory epithelium; OB, olfactory bulb.
Figure 3.4
**Figure 3.4. Five class 3 semaphorins are expressed in olfactory sensory neurons.**

A-E, Single optical sections through 36hpf larval olfactory epithelia from a frontal view. Dorsal is up. OMP:Gal4;UAS:Citrine expressing cell bodies are shown in green and mRNA in red. A-D, Sema3Aa (A), sema3Ab (B), sema3Fa (C) and Sema3Ga (B) RNA is detected in small subsets of OMP positive neurons at 36hpf (white arrowheads). E, Sema3H RNA is strongly expressed in OMP positive neurons at 36hpf (white arrowheads). Scale bar (in E): A–E, 25µm.
Figure 3.5. Olfactory sensory neurons can express multiple neuropilins.

A-C. Single optical sections through olfactory epithelia of 36hpf OMP:Gal4;UAS:Citrine larvae (frontal view). Insets are enlarged below. 

A, Subsets of OMP neurons (green) express nrp1a (inset 1, red), nrp1b (inset 2, blue) or both (inset 3).

B, Subsets of OMP neurons (green) express nrp2a (inset 1, red), nrp2b (inset 2, blue) or both (inset 3).

C, Subsets of OMP neurons (green) express nrp1a (inset 1, red), nrp2b (inset 2, blue) or both (inset 3). Scale bar (in C): A–L, 12μm.
Figure 3.6. OMP and TRPC positive axons display minor errors in nrp1a mutants.

A-B, The percentage of olfactory bulbs displaying a projection to a particular protoglomerulus or non-protoglomerular regions (other) are shown. Statistical significance was estimated using two-tailed Fisher’s exact tests ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$). Error bars represent SEM. Mutants (black bars) are compared with wild-type (white bars) and heterozygous (gray bars) siblings. A, OMP positive neurons have a slight increase in projections to LG2 in nrp1a mutants. B, TRPC2 positive axons show an increase in the frequency of projections to LG2 and an increase in non-protoglomerular projections (other) in nrp1a mutants.
<table>
<thead>
<tr>
<th>OR111-7: IRES: Gal4; UAS: Citrine</th>
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Figure 3.7
Figure 3.7. OR111-7 expressing axons make non-protoglomerular dorsal errors in nrp1a mutants.

A-B, Single optical sections through 72hpf OR111-7:IRES:GAL4;UAS:Citrine larvae (frontal view). Axons shown in green. Dorsal is up and medial is to the right of the image. Propidium iodide (blue) labels cell bodies revealing protoglomeruli as cell-free regions. C-D, Maximum intensity projections of serial optical sections from the larvae shown directly above. Scale bar (in D): A–D, 50μm. A, C, Wild-type OR111-7 transgenic axons project to the CZ and LG1. B, D, In nrp1a mutants, a subset of OR111-7 transgenic axons inappropriately projects dorsally (yellow arrowheads).
Figure 3.8. TRPC positive axons ectopically project to LG3 in nrp2b mutants.

A-B, Single optical sections through 72hpf TRPC2:Venus larvae (frontal view). Axons shown in green. Dorsal is up and medial is to the right of the image. Propidium iodide (blue) labels cell bodies revealing protoglomeruli as cell-free regions. C-D, Maximum intensity projections of serial optical sections from the larvae shown directly above. Scale bar (in D): A–D, 50μm. A, C, Wild-type axons project to LG1, LG2, VPG, and OP (white arrowheads) and do not project to LG3 (white arrowhead). B, D, In nrp2b mutants, a subset of TRPC2 positive axons projects inappropriately to LG3 (yellow arrowheads). LG1, lateral protoglomerulus 1; LG2, lateral protoglomerulus 2, LG3, lateral protoglomerulus 3; VPG, ventral posterior protoglomerulus; OP, olfactory plexus. E, The percentage of olfactory bulbs that have a labeled projection to a particular protoglomerulus or non-protoglomerular region. Nrp2b mutants (black bars) are compared to heterozygous (grey bars) and wild-type (white bars) siblings. Statistical significance was estimated using two-tailed Fisher’s exact tests (p < 0.05*, p < 0.01**, p < 0.001***). Error bars represent SEM.
CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

The convergence of sensory axons onto topographically fixed positions in the olfactory bulb is a complex biological problem. It is clear that multiple cues and in particular, multiple class 3 semaphorins participate in this process. Several questions deserve further attention.

**Which guidance cues are required for protoglomerular targeting?**

In insects, mice and fish, olfactory sensory axons initially target protoglomerular structures in the olfactory bulb (Oland et al., 1990; Royal and Key, 1999; Conzelmann et al., 2001; Potter et al., 2001; Li et al., 2005; Lakhina et al., 2012). Here axons from neurons expressing different odorant receptors (OR) are intermingled (Treloar et al., 1996; Conzelmann et al., 2001). Over time, these axons segregate into mature, OR specific glomeruli, conforming to the one receptor-one glomerulus rule (Conzelmann et al., 2001; Zou et al., 2004; Li et al., 2005). To fully understand the mechanisms that guide olfactory map formation, the cues that direct protoglomerular targeting must be identified and characterized.

While several guidance cues required for olfactory map formation have been identified in the mouse, the lack of a well-defined protoglomerular map in this model complicates the examination of protoglomerular targeting. In contrast, by 72hpf the larval zebrafish has 12 identifiable protoglomeruli that are invariant and reproducible between larvae (Dynes and Ngai, 1998; Lakhina et al., 2012). Our lab has previously used a transgenic zebrafish in which axons projecting to the central zone (CZ) protoglomerulus are selectively labeled to identify the requirement for Netrin/DCC signaling in protoglomerular targeting. During the course of my thesis work I used this model to identify a novel role for Sema3D in
protoglomerular targeting (Chapter 2). Further, I show that many additional class 3 semaphorins that have not previously been implicated in olfactory development are expressed during protoglomerular targeting (Chapter 3).

Class 3 semaphorins bind neuropilins (nrps), of which there are four in zebrafish (Bovenkamp et al., 2004; Yu et al., 2004). Examination of nrp mutants revealed several phenotypes that appear to be sema3D-independent indicating that other class 3 semaphorins may contribute to early targeting (Chapter 3). Future experiments will focus on determining the extent to which this particular class of guidance molecule affects protoglomerular targeting.

In the past, Zebrafish loss-of-functions experiments were challenging and relied heavily on the use of morpholinos, which are known to have off-target affects (Bill et al., 2009). Currently, however, several groups, including the Sanger Institute’s Zebrafish Mutation Project are working to generate mutations in every zebrafish protein coding gene (Kettleborough et al., 2013). When I started this project the only class 3 semaphorin mutant available was sema3D. Today there are mutants for 9 additional class 3 semaphorin mutants available from the Zebrafish Mutation Project. A nrp2a mutant is also available. The advancement of targeted genome editing techniques will facilitate the generation of additional mutants. Both transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system have been used to successfully introduce mutations into the zebrafish genome (Bedell et al., 2012; Auer and Del Bene, 2014).

Both OR111-7 transgene expressing and TRPC positive neurons make protoglomerular targeting errors in nrp2b mutants (Chapter 2; Chapter 3). Future efforts will be aimed at
identifying the Semaphorin(s) that work through Nrp2b. There is evidence that Sema3B, Sema3C, Sema3F and Sema3G can interact with Nrp2 (Sharma et al., 2012). Using available or generated mutants, it should be possible to determine which semaphorin mutants phenocopy and/or interact genetically with nrp2b mutants. It will be interesting to determine whether the same ligand guides OR111-7 labeled and TRPC positive axons.

The OR111-7:IRES:Gal4 transgene only allows us to investigate targeting to the central zone protoglomerulus. To investigate targeting to other protoglomeruli, it is necessary to generate transgenic zebrafish in which axons projecting to other protoglomeruli are labeled, particularly the dorsal zone and lateral glomerulus 3 which are thought to be innervated by neurons expressing main-olfactory type ORs (Celik et al., 2002; Sato et al., 2005; Lakhina et al., 2012). These lines are currently being developed in our lab using bacterial artificial chromosomes (BACs) containing large clusters of OR genes. An IRES:Gal4 cassette is inserted behind the coding sequence of a particular OR. When these lines are mated with a UAS:Citrine line, the axons of neurons expressing the OR directly upstream of the IRES:Gal4 cassette are selectively labeled. For example, axons from neurons expressing the OR130-1 BAC transgene target the dorsal zone protoglomerulus (Shao et al., unpublished observations). These fish can be used to identify the cues necessary for targeting axons specifically to the dorsal zone protoglomerulus. It will also be possible to explore the roles of other guidance molecule families such as Slits or Ephrins in protoglomerular targeting. As the mechanisms that guide zebrafish protoglomerular targeting become clear, it will be possible to make comparisons between this system and what is known in mouse or Drosophila.
How do multiple class 3 Semaphorins cooperate in the establishment of the protoglomerular map?

One of the objectives of this thesis project was to explore the possibility that class 3 semaphorins other than Sema3A and Sema3F might impact olfactory map formation. Future experiments will explore how the coordinated actions of many class 3 semaphorins establish the protoglomerular map. Moving forward, it will be important to determine the extent to which different Semaphorins and Neuropilins function independently or redundantly. This is of particular interest in the zebrafish where several Semaphorins and both Neuropilins have duplicate family members. For example, Sema3Fa and Sema3Fb may be functionally separable or redundant. It is also possible that different family members, such as Sema3Aa and Sema3Ga which are both expressed in the lateral OB, have overlapping functions (Chapter 3). Once the mutants for each class 3 Semaphorin have been examined, double mutants can be generated to investigate this possibility. Similarly, although OR111-7 transgene expressing axons in nrp1a or nrp1b mutants display different phenotypes, it is possible that they share a function that is masked in single mutants. Axons from OR111-7 transgene expressing and OMP positive neurons will be examined in nrp1a⁻⁻;nrp1b⁻⁻ double mutants. After obtaining or generating a nrp2a mutant it will be possible to conduct a similar analysis using nrp2a⁻⁻;nrp2b⁻⁻ larvae.

In vitro binding assays using AP-tagged Semaphorins could be used to match each Semaphorin with potential Neuropilins, providing additional information regarding specificity and redundancy in this system. It will be interesting to compare the binding profiles of Nrp1a against Nrp1b and Nrp2a against Nrp2b. It may be possible to use these tagged Semaphorins to stain wholemount zebrafish larvae. This could provide valuable
information as to whether different subsets of neurons bind different Semaphorins \textit{in vivo}. When used in conjunction with BAC transgenic lines, we could also determine whether cells expressing a particular OR recognize specific Semaphorins.

\textbf{Are multiple class 3 semaphorins required for pre-target axon sorting?}

Consistent with the expression of Sema3A and Sema3F in mouse olfactory sensory neurons (OSNs), \textit{sema3Aa, sema3Ab} and \textit{sema3Fa} were detected in zebrafish OSNs (Chapter 3). \textit{Sema3Ga} and \textit{sema3H} are also expressed in OSNs (Chapter 3). Mouse Sema3A is required for sorting of axons in the olfactory nerve before they arrive at their target, while Sema3F does not appear to sort axons in the nerve. Instead Sema3F is secreted into the dorsal OB by axons were it repels Nrp2 expressing axons (Takeuchi et al., 2010). It will be interesting to establish which, if any, zebrafish Semaphorins contribute to pre-target axon sorting. An OMP:RFP;TRPC2:Venus double transgenic line can be crossed into various mutant backgrounds to determine whether Sema/Nrp signaling is required to maintain segregation between these broad populations in the olfactory nerve.

Axons expressing the same OR are segregated in the mature mouse olfactory bulb (Treloar et al., 2002; Miller et al., 2010). In the BAC lines described previously, axons expressing a particular OR are selectively labeled. This will allow us to assess the extent to which axons expressing the same OR are fasciculated in the nerve prior to entering the OB. When crossed into semaphorin or neuropilin mutant backgrounds, these lines can be used to determine which cues are required for axon sorting within the olfactory nerve.

Nrp1 expressing axons are segregated within the mouse olfactory nerve (Imai et al., 2009). It is possible that axons expressing different cohorts of Neuropilins are sorted
together in the zebrafish olfactory nerve. To explore this possibility, axons expressing different Neuropilins must be labeled. This can be achieved either by generating antibodies that recognize each of the four neuropilins, or by inserting different reporter cassettes into the coding regions of each neuropilin gene. The latter strategy can be achieved using TALENs or CRISPR/Cas9 system. These lines can be designed to function as reporters of neuropilin expression as well as mutants. It should be possible to visualize the axons that express semaphorins in the olfactory nerve using this technique. If selective sorting is detected between axons expressing different Neuropilins, it will be possible to identify the candidate Semaphorins required for this segregation using the available expression data and mutants. Semaphorins that are not required for pre-target sorting could be deposited in the bulb.

The semaphorins expressed by OSNs are also expressed in the OB (Chapter 3). To separate the effects of OSN versus OB derived Semaphorins, it may be necessary to generate OSN specific mutants. It is possible to insert a mloxp site into the zebrafish genome using either TALENs or the CRISPR/Cas9 system (Auer and Del Bene, 2014). Cell-type specific knockdown can be achieved by selectively expressing Cre recombinase in subsets of OSNs using standard transgenesis or targeted genome editing.

**How are neuropilin and semaphorin expression related to OR choice?**

Four zebrafish neuropilins are differentially expressed in the OE and several class 3 semaphorins are expressed in small subsets of OSNs. Future experiments will aim to determine the relationship between OR expression and the expression of these guidance molecules in zebrafish. Several approaches can be used to demine whether there is a correlation between OR and guidance molecule expression. First, RNA probes targeting
either individual ORs or entire OR families can be combined with probes targeting individual guidance molecules in double in situ hybridization studies. Second, BAC transgenic lines in which specific subsets of OSNs are labeled can be hybridized with RNA probe targeting different neuropilins and semaphorins. Third, if TALENs or the CRISPR/Cas9 system is successfully used to generate neuropilin reporter lines, as described previously, these lines could be mated with OR BAC transgenic lines to look for co-expression.

Odorant receptor choice establishes a neuronal identity that is linked to axon targeting in the OB. There are at least two non-mutually exclusive hypotheses to explain the relationship between OR choice and guidance molecule expression. One possibility is that OR-derived cAMP, the level of which may vary between ORs, regulates the transcription of guidance molecules. This is the case for Sema3A and Nrp1 in mouse (Imai et al., 2006; Nakashima et al., 2013). A second possibility is that ORs and guidance molecules share common regulatory elements. This mechanism has been proposed for the control of Nrp2 expression in mouse OSNs and has been demonstrated in Drosophila (Dobritsa et al., 2003; Serizawa et al., 2003).

OR swap experiments can be attempted to determine whether the OR dictates guidance molecule expression. For example, a particular guidance receptor (GR) may be expressed by OR111-7 positive OSNs and not by OR130-1 expressing OSNs. It may be possible to use the CRISPR/Cas9 system to replace the OR111-7 coding sequence with that of OR130-1. If ORs regulate expression of the GR that is normally expressed by OR111-7 neurons, then in this experiment, that GR would not be expressed by cells expressing the donor OR130-1. However, if OR111-7 and the GR are under the control of the same
regulatory elements, these would remain intact and the cell expressing the donor OR130-1 would also express the GR.

**Do guidance molecules play distinct roles in protoglomerular targeting versus glomerular segregation?**

The mouse model has been instrumental in identifying cues that participate in olfactory map formation, yet it remains unclear whether these cues contribute to initial targeting, glomerular segregation or both. Although expression studies have been carried out at embryonic stages in mouse, analyses of olfactory map formation are typically conducted after glomeruli begin to mature. The simplicity, developmental accessibility, and identifiable protoglomeruli of the zebrafish allow us to isolate protoglomerular targeting. Over longer developmental time courses this system could also be used to explore glomerular segregation. Axons targeting a specific protoglomerulus can be monitored temporally in fixed larvae or in real-time using live imaging. *OR111-7* transgene expression decreases dramatically by 7 days post fertilization (dpf), however protoglomeruli begin to segregate as early as 96hpf (Li et al., 2005). This line can be used to examine segregation taking place between 72hpf and 6dpf. Additional lines made using BACs, TALENs or the CRISPR/Cas9 system may allow visualization of specific subsets of axons into adulthood. Expression analyses at latter ages are needed to determine which cues are present in OSNs during glomerular segregation.

In conclusion, the studies described in this thesis are the first to examine the role of class 3 semaphorins in protoglomerular targeting. Here, I identify Sema3D as a novel guidance cue required for olfactory map formation and reveal the expression of many class 3
semaphorins during protoglomerular development. These findings provide an impetus for the future study of Semaphorin/Neuropilin signaling in the developing olfactory system.
REFERENCES


