RESEARCH ARTICLE

Attractant and repellent cues cooperate in guiding a subset of olfactory sensory axons to a well-defined protoglomerular target

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ABSTRACT

Olfactory sensory axons target well-defined intermediate targets in the zebrafish olfactory bulb called protoglomeruli well before they form odorant receptor-specific glomeruli. A subset of olfactory sensory neurons are labeled by expression of the or111-7:ires:GAL4 transgene whose axons terminate in the central zone (CZ) protoglomerulus. Previous work has shown that some of these axons misproject to the more dorsal and anterior dorsal zone (DZ) protoglomerulus in the absence of Netrin 1/Dcc signaling. In search of additional cues that guide these axons to the CZ, we found that Semaphorin 3D (Sema3D) is expressed in the anterior bulb and acts as a repellent that pushes them towards the CZ. Further analysis indicates that Sema3D signaling is mediated through Nr p1a, while Nrp2b also promotes CZ targeting but in a Sema3D-independent manner. n p1a, n rp2b and d cc transcripts are detected in or111-7 transgene-expressing neurons early in development and both Nr p1a and Dcc act cell-autonomously in sensory neurons to promote accurate targeting to the CZ. d cc and n p1a double mutants have significantly more DZ misprojections than either single mutant, suggesting that the two signaling systems act independently and in parallel to direct a specific subset of sensory axons to their initial protoglomerular target.

KEY WORDS: Axon guidance, Olfactory development, Protoglomeruli, Sema3D, Semaphorin, Zebrafish

INTRODUCTION

The targeting of axons to their correct postsynaptic partners is a critical step in circuit formation. Studying this process is made challenging by the extraordinary degree of neuronal diversity within the nervous system and the specificity with which neuronal partners interact. The olfactory system is an attractive model for the study of axon targeting because each olfactory sensory neuron (OSN) assumes a specific identity as it chooses a single odorant receptor (OR) from a large gene repertoire and since OR choice is closely coordinated with axonal targeting (Alioto and Ngai, 2005; Zhang and Firestein, 2002). Although sensory neurons expressing a particular OR are broadly dispersed in the olfactory epithelium (OE), their axons converge upon one or a few reproducibly located OR-specific glomeruli in the olfactory bulb (OB); there, they synapse with the dendrites of second order neurons that communicate olfactory information deeper into the brain (Ramon y Cajal, 1892; Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1997; Sosulski et al., 2011; Miyasaka et al., 2014). The OR-specific glomerular map relates odorant experience to neuronal activity at specific locations within the OB, providing a basis for the identification and discrimination of odorants (Bozza et al., 2002). This mapping is, in a sense, an early stage of olfactory information processing.

Our working hypothesis is that the development of the olfactory projection can be divided into at least two broad stages. In the first stage, rather than projecting directly to OR-specific glomeruli, early axons diffusely target larger intermediate regions called protoglomeruli that contain axons from neurons that express a specific subset of different ORs. In a second stage, later in development, OR-specific glomeruli segregate from these larger regions (Royal and Key, 1999; Treloar et al., 1999; Conzelmann et al., 2001; Potter et al., 2001; Li et al., 2005). In the mouse, the first olfactory sensory axons enter the bulb at ~E15, while glomeruli are only detectable postnatally (Doucette, 1989; Royal and Key, 1999; Conzelmann et al., 2001; Potter et al., 2001). Targeting of sensory axons from the OE to the bulb has been studied extensively in mice (Wang et al., 1998; Schwarting et al., 2000; Vassalli et al., 2002; Cutforth et al., 2003; Imai et al., 2006, 2009; Serizawa et al., 2006; Cho et al., 2007; Col et al., 2007; Kaneko-Goto et al., 2008; Nguyen-Ba-Charvet et al., 2008; Takeuchi et al., 2010). With few exceptions, most of this work has concentrated on glomerular formation near the time of birth or thereafter. The relative accessibility of postpartum animals and the convenience of using glomerulus formation to define target position has facilitated significant advances. Much less attention has been devoted to the initial acquisition of targets before glomeruli form. One important question yet to be addressed is the identity of the molecular cues that define the initial intermediate protoglomerular targets in the bulb and how are they recognized by sensory axons.

The zebrafish is an ideal vertebrate model system for identifying the cues that guide sensory axons as they first reach the bulb. Embryos are accessible at all embryonic stages and have a relatively simple olfactory system. More importantly, sensory axons initially target 12 protoglomerular neuropilar regions, which are discrete, identifiable and spatially reproducible (Dynes and Ngai, 1998; Li et al., 2005; Lakshma et al., 2012). OSN axons exiting the OE project dorsally and anteriorly into the telencephalon, entering the nascent OB by 24 hpf (Wilson et al., 1990; Hansen and Zeiske, 1993; Whitlock and Westerfield, 1998). By 48 hpf, three distinct projection branches can be observed that are thought to correspond to what will develop into the dorsal, central, and medial protoglomeruli (Dynes and Ngai, 1998). Twelve individually identifiable protoglomeruli emerge by 72 hpf (Dynes and Ngai, 1998; Li et al., 2005; Lakshman et al., 2012). Two classes of sensory neurons project to mutually exclusive protoglomeruli in each OB (Sato et al., 2005). Ciliated sensory neurons express classical main OB-type ORs along with olfactory marker protein (OMP). They innervate the central zone (CZ), dorsal zone (DZ), lateral protoglomerulus 3 (LG3), and also sparsely innervate the medial protoglomeruli (MG). Microvillous sensory neurons express V2R-type vomeronasal receptors along
with the transient receptor potential channel C2 (Trpc2). They innervate lateral protoglomeruli 1, 2 and 4 (LG1, LG2 and LG4), the ventral posterior glomerulus (VPG) and the olfactory plexus (OP) (Celik et al., 2002; Sato et al., 2005; Lakhina et al., 2012). Individual glomeruli are seen to bud off from protoglomeruli starting at ~96 hpf (Li et al., 2005). Zebrafish OSNs are first responsive to odorants between 60 and 96 hpf (Li et al., 2005). A long-term aim of our studies is to relate developmental mechanisms to the functional architecture of the bulb.

Previous work has shown that Netrins expressed near the ventral border and the midline of the OB help to guide a subset of olfactory sensory axons into the bulb and towards the CZ protoglomerulus (Lakhina et al., 2012). This subset of sensory neurons is labeled by expression of a transgene in which the or111-7 coding sequence and an IRES:GAL4 reporter are embedded in a minigene construct containing the non-coding sequences surrounding or111-7 along with an enhancer element that is near the or111 gene cluster (Lakhina et al., 2012). In this study we show that sensory axons labeled by the same transgene are repelled by Sema3D expressed at the anterior margin of the OB, pushing them towards the more posteriorly positioned CZ protoglomerulus. We show that the Semaphorin receptor component Neuropilin 1a (Nrp1a) is required for Sema3D repulsion of or111-7 transgene-labeled sensory neurons, and that Nrp2b contributes to their targeting through an independent signaling pathway mediated by an as yet unidentified ligand. Our results identify multiple parallel signaling pathways that cooperate to guide a subset of sensory axons to a particular protoglomerulus.

RESULTS

**sema3d and netrin 1b have complementary expression patterns in the OB**

Semaphorins play key roles in the regional targeting of OSNs in both fly and mouse (reviewed by Mori and Sakano, 2011; Lattemann et al., 2007; Joo et al., 2013). Sema3A and Sema3F, secreted members of the larger semaphorin family of axon guidance molecules, are important determinants of olfactory sensory axon targeting in vertebrate systems (Schwartz 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 (Giger et al., 2000; Renzi et al., 2000; Cloutier et al., 2002). As a first step towards understanding their contribution to protoglomerular targeting in the zebrafish, we examined the patterns of class 3 semaphorin mRNA expression in the olfactory system at 36 hpf, when sensory axons have reached the OB but are not yet organized into protoglomeruli. There are 12 identified class 3 zebrafish semaphorins: 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). Although many are expressed in distinct patterns (Table S1) within the bulb, sema3d is expressed in a complementary pattern to netrin 1b, an attractant that helps guide sensory neurons expressing an or111-7 transgene to the CZ protoglomerulus (Lakhina et al., 2012). At 36 hpf, sema3d mRNA is not detected in OSNs but is strongly expressed in the anterior-most region of the primordial OB (Fig. 1). The axons of OSNs expressing the or111-7 transgene project just posteriorly to sema3d-expressing regions of the bulb. By contrast, netrin 1b mRNA expression overlaps sema3d expression in the anterior bulb, but extends further posteriorly where it is coincident with or111-7 transgene-expressing axons (Fig. 1). Thus, sema3d expression is temporally and spatially positioned in a way that could direct the early axon pathfinding of or111-7 transgene-expressing axons, perhaps by working in opposition to netrin 1b.

**or111-7 transgene-expressing axons project ectopically into the DZ protoglomerulus in sema3d mutants**

To test whether Sema3D is required in the guidance of or111-7 transgene-expressing sensory axons, we analyzed the olfactory projections in larvae harboring the presumptive null allele sema3d-1661. This allele was identified by the Sanger Centre Zebrafish Mutant 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 (Feiner et al., 1997; Koppel et al., 1997); it is in an exon that cannot be skipped without throwing the translated sequence out of frame. sema3d homozygous mutants 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. There were no gross differences in OB morphology or protoglomerular pattern in sema3d mutants as compared with wild-type larvae at 72 hpf (Fig. 2A,B).

or111-7 transgene-expressing axons projected to the CZ in wild-type, sema3d+/− and sema3d−/− larvae (Fig. 2C,D). However, in sema3d−/− bulbs, one or more or111-7 transgene-expressing axons inappropriately targeted the DZ in 37% of bulbs (n=53) as compared with 14% in wild-type siblings (n=44) and 16% in sema3d+/− siblings (n=45). Using Fisher’s exact test we estimate the chance of axons projecting to the DZ at the same frequency in sema3d−/− and wild-type larvae as P=0.02; similarly, when comparing sema3d+/− with sema3d−/− larvae as P=0.02 (Fig. 2). No additional significant misprojections to other protoglomerular targets were observed. Ectopic projections that did not terminate in a protoglomerulus were scored as ‘other’. or111-7 transgenic axons do not display an increase in non-protoglomerular errors in sema3d−/− mutants (Fig. S1G).

To determine whether the loss of sema3d has any broader effects on OSN pathfinding, we incrossed sema3d−/−;Omp:RFP or sema3d+/−;Trpc2:venus lines. Omp-expressing OSN axons project to the CZ, DZ, LG3, and MG protoglomeruli with the same frequency in sema3d−/− as compared with controls and there were no apparent differences in innervation density between mutants and controls (Fig. S1A,B,E). Nor do Omp-expressing sensory axons display any increase in non-protoglomerular targeting errors in sema3d−/− mutants (Fig. S1E). Similarly, there were no significant differences in the protoglomerular or non-protoglomerular targeting of Trpc2-expressing sensory axons in sema3d−/− as compared with controls (Fig. S1C,D,F). The or111-7 transgene also labels a small subset of Omp-negative sensory neurons that project to the Trpc2-specific protoglomerulus LG1 (Lakhina et al., 2012). The lack of a sema3d−/− phenotype in Trpc2-expressing axons indicates that the errors observed in the or111-7 transgenic background originate from Omp-positive neurons that normally target the CZ. Furthermore, there was no significant difference in the number of bulbs with projections to LG1 in sema3d−/− mutants (Fig. S1G).

Thus, Sema3D is required to specifically direct protoglomerular targeting of a subset of Omp-positive axons that project to the CZ. Since 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.
sema3d is strongly expressed in the anterior OB at 36 hpf. Although Sema3D protein may perdure longer, mRNA expression decreases markedly by 48 hpf, and is undetectable in the anterior OB by 72 hpf (data not shown). We examined whether errors in axon positioning could be observed at very early time points. We examined the or111-7 transgene-expressing projection at 36 hpf and 48 hpf. We found no obvious differences at these early stages in axon trajectories between mutants and controls (data not shown). At these time points, we occasionally saw a small subset of or111-7 transgene-expressing axons that project toward the anterior OB in wild-type larvae (Fig. 1; data not shown). It is possible that, in the absence of Sema3D, these projections fail to be redirected posteriorward and persist in abnormal anterior territories through 72 hpf. Protoglomeruli are not detectable before 72 hpf and, without a transgene that labels the DZ projection, it is unclear whether these axons are intermingled with those that will ultimately target the DZ. Our findings support a model whereby the early repellent actions of Sema3D in the anterior olfactory OB direct or111-7 transgene-labeled axons posteriorly away from the nascent DZ (see Fig. 7).

Loss of nrp1a, nrp1b or nrp2b induces protoglomerular mistargeting
To further identify candidate receptors for Sema3D, we examined neuropilin expression in the olfactory system at 36 hpf. There are four zebrafish neuropilins: nrp1a, nrp1b, nrp2a and nrp2b (Bovenkamp et al., 2004; Yu et al., 2004). or111-7 transgenic axons are not present in the anteriormost portion of the telencephalon. sema3d expression wraps around the edge of the olfactory pit and is also present between the OE and nascent OB. OE, olfactory epithelium; OB, olfactory bulb.

**All four zebrafish neuropilins are expressed in the developing olfactory system**
Class 3 semaphorins signal through neuropilin/plexin A 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). Although mouse Sema3D has been reported to bind Nrp1 and not Nrp2 in vitro, experiments in zebrafish suggest that Sema3d can signal through Nrp1a/Nrp2b complexes (Wolman et al., 2004; Degenhardt et al., 2013).

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**Fig. 1. sema3d and netrin 1b have complementary expression patterns in the zebrafish OB at 36 hpf.** (A) Maximum intensity projection spanning 30 µm through a 36 hpf or111-7:RES:GAL4:UAS:citrine embryo. Ventral view, anterior is up and medial is to the right. Axons are shown in green, sema3d mRNA in red and netrin 1b mRNA in blue. (B–B″) A single optical section through the inset in A. (C,C′) Single optical sections through a 36 hpf embryo. Frontal view, dorsal is up and medial is to the right. Sections are arranged from anterior (left) to posterior (right). The distance from each section to the anteriormost part of the telencephalon is denoted in bottom left. (D) Diagram of a 36 hpf embryo in lateral view. **sema3d** is expressed in the anterior OB. Some **netrin 1b** is detected in the anterior bulb but it extends further posteriorly. or111-7 transgene-expressing axons are positioned posterior to sema3d expression but within the netrin 1b expression domain. or111-7 transgenic axons are not present in the anteriormost portion of the telencephalon. sema3d expression wraps around the edge of the olfactory pit and is also present between the OE and nascent OB. OE, olfactory epithelium; OB, olfactory bulb.
amino acid 427 just before the second Coagulation factor homology domain (Clark et al., 2011).

OB morphology and protoglomerular positioning appear normal in all three mutants at 72 hpf (Fig. 4). Similar to sema3d−/− mutants, or111-7 transgene-expressing axons misproject to the DZ in nrp1a−/− mutants. Mistargeting to the DZ was observed in 28% (n=47) of nrp1a−/− bulbs as compared with 8% in wild-type siblings (n=50, P=0.002) and 6% in heterozygote siblings (n=54, P=0.003) (Fig. 4B,E). In contrast to sema3d−/− mutants, or111-7 transgene-expressing axons also misproject to non-protoglomerular locations in nrp1a−/− mutants (Fig. 4E). Axons misproject to regions of the bulb that are dorsal to the CZ in 51% of mutants as compared with 24% of heterozygous siblings (P=0.007). Additionally, we found that or111-7 transgene-expressing axons inappropriately targeted the DZ in 48% (n=42) of nrp2b−/− bulbs as compared with only 7% (n=30, P=0.0002) in wild-type siblings and 14% (n=42, P=0.002) in heterozygous sibling larvae (Fig. 4D,G).

Fig. 3. Four zebrafish neuropilins are expressed in or111-7 transgene-labeled neurons during development. (A-D) Single optical sections through the OE of 36 hpf embryos (frontal view). mRNA is in magenta. Subsets of or111-7 transgene-labeled neurons (green) express (A) nrp1a (N=56 pits/764 cells), (B) nrp1b (N=16 pits/191 cells), (C) nrp2a (N=29 pits/610 cells) and (D) nrp2b (N=26 pits/384 cells). Scale bar: 25 µm. (E) The percentage of or111-7 transgene-expressing cells expressing a given neuropilin. Error bars represent s.d. (A,B) Single optical sections through 72 hpf or111-7:ires;GAL4;UAS:citrine larvae (frontal view). Axons are in green. Dorsal is up and medial is to the right. 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 in A,B. (A,C) Wild-type or111-7 transgenic axons project to the CZ and LG1. (B,D) In sema3d mutants, a subset of or111-7 transgenic axons inappropriately projects to the CZ. Scale bar: 25 µm. (E) The percentage of OBs that have a labeled projection to the CZ protoglomerulus. sema3d homozygous mutants are compared with heterozygous and wild-type siblings. Statistical significance was estimated using two-tailed Fisher’s exact test (P<0.05*). Error bars represent s.e. of the sample proportion. CZ, central zone; DZ, dorsal zone; LG1, lateral protoglomerulus 1; OE, olfactory epithelium.

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sema3d interacts with nrp1a to promote targeting of or111-7 transgene-expressing axons to the CZ

We next tested whether nrp1a or nrp2b genetically interact with sema3d. If either of these neuropilins acts as a receptor component for Sema3D, larvae that are heterozygous for both nrp1a or nrp2b and sema3d might have a sufficient reduction in signaling activity to induce ectopic misprojections to the CZ, similar to those observed in sema3d−/− mutants. Larvae carrying heterozygous mutations for either sema3d, nrp1a or nrp2b alone had no detectable CZ targeting errors (Fig. 5A,B,D,E,G). or111-7 transgene-expressing axons terminated inappropriately in the CZ in 29% of sema3d−/−;nrp1a−/− transheterozygotes (n=68, P=0.003) as compared with 10% in
transgene-expressing sensory axons mistargeted the DZ in relative contributions of Nrp1a or Nrp2b to Sema3D signaling.

**Fig. 4. Loss of npr1a phenocopies sema3d mutants.** (A-D) Single optical sections through 72 hpf or111-7 transgenic larvae (frontal view). Axons are in green. Dorsal is up and medial is to the right. Propidium iodide (blue) labels cell bodies revealing protoglomeruli as cell-free regions. WT, wild type. Scale bar: 25 µm. (E-G) Percentage of OBs displaying a projection to a particular protoglomerulus or all non-protoglomerular regions (other) is shown. Statistical significance was estimated using two-tailed Fisher’s exact tests (P<0.05*, P<0.01**, P<0.001***; ns, not significant). Homozygous mutants are compared with wild-type and heterozygous siblings. Error bars represent s.e. of the sample proportion. (B,E) A subset of or111-7 transgene-labeled axons misproject to the DZ and to non-protoglomerular areas in npr1a mutants. (C,F) npr1b mutants do not have increased mistargeting errors to the DZ but do have increased misprojections to LG2. (D,G) A subset of or111-7 transgene-labeled axons also inappropriately targets the DZ in npr2b mutants. CZ, central zone; DZ, dorsal zone; LG1, lateral protoglomerulus 1; LG2, lateral protoglomerulus 2.

**sem3d+/− or 8% in npr1a+/− siblings (Fig. 5C,F,G). Similarly, we tested for interactions between sema3d and npr2b. In contrast to the sema3d+/−;npr1a+/− transheterozygotes, no excess DZ misprojections were observed in sema3d+/−;npr2b+/− fish (Fig. 5G). Thus, a genetic interaction is detected between sema3d and npr1a but not between sema3d and npr2b.**

We next examined double-mutant larvae to further probe the relative contributions of Nrp1a or Nrp2b to Sema3D signaling. or111-7 transgene-expressing sensory axons mistargeted the DZ in 29% of sema3d+/− mutants, in 31% of npr1a+/− siblings, and in 28% of sema3d+/−;npr1a+/− double-mutant siblings. Thus, the frequency of misprojections is not augmented in double mutants. This is consistent with sema3d and npr1a working in the same signaling pathway, assuming that both mutants are null, a reasonable assumption given the nature of these mutations. By contrast, or111-7 transgene-expressing axons mistargeted to the DZ in 29% of sema3d+/− mutants, in 43% of npr2b+/− siblings, and in 64% of sema3d+/−;npr2b+/− siblings. npr2b mutants thus have a higher frequency of DZ misprojections than sema3d mutants, and errors increase further in sema3d+/−;npr2b+/− double mutants (Fig. 5G). This finding is consistent with Sema3D and Nrp2b participating in parallel signaling pathways. Together, these data indicate that Nrp1a is acting as the primary receptor for Sema3D in this context, and that Nrp2b is likely to be mediating the activity of another ligand or ligands.

We examined npr1a or npr2b mutants in the Trpc2:venus background and did not observe an increase in projections to the DZ (data not shown). This indicates that, as with sema3d+/− mutants, the or111-7 transgene-labeled axons that misproject to the DZ in npr1a−/− and npr2b−/− mutants are likely to be Omp-positive neurons that normally project to the CZ. They are unlikely to be recruited from or111-7 transgene-labeled (and Trpc2-expressing) axons that sometimes terminate in LG1. To further verify that Nrp1a-expressing axons that respond to Sema3D are a subset of Omp-positive neurons rather than Trpc2-positive neurons, we examined sema3d+/−;npr1a+/− transheterozygotes in which both Omp and or111-7 transgene-expressing neurons are labeled. As expected, axons mistargeting to the DZ in sema3d+/−;npr1a−/− transheterozygotes are Omp positive (Fig. S1H).

**Selective expression of npr1a in or111-7 transgene-expressing OSNs corrects targeting errors in npr1a mutants**

These results support a model in which Nrp1a serves as an essential receptor component for Sema3D in or111-7 transgene-expressing sensory axons, and that in normal circumstances its presence helps prevent their entry into the DZ protoglomerulus. To test whether npr1a is required cell-autonomously for normal or111-7 transgene-expressing axon guidance, we generated an npr1a−/−;UAS:npr1a; UAS:citrine transgenic line. Three different npr1a−/−;UAS:npr1a; UAS:citrine founders were mated to npr1a−/−;or111-7:GAL4 fish. No protoglomerular mistargeting was detected in or111-7 transgene-expressing axons when Nrp1a was overexpressed in otherwise wild-type larvae (Fig. 5G). npr1a mutants expressing the UAS:npr1a transgene do not project ectopically to the DZ (Fig. 5G). The ability of Nrp1a expressed in or111-7 transgene-expressing axons to correct targeting errors seen in npr1a mutants is consistent with its acting cell-autonomously to guide these axons to their correct target.
nrp1a and dcc are transiently co-expressed in a subset of or111-7 transgene-expressing neurons

Previous work showed that or111-7 transgene-expressing sensory neurons express the netrin receptor Dcc, and that Netrin 1b acts as an attractant that draws this subgroup of axons to the CZ protoglomerulus (Lakhina et al., 2012). We hypothesized that Sema3D/Nrp1a and Netrin 1b/Dcc signaling cooperate to target protoglomerulus (Lakhina et al., 2012). We hypothesized that attractant that draws this subgroup of axons to the CZ neurons express the netrin receptor Dcc, and that Netrin 1b acts as an

OR111-7:RES:Ga4/UAS:Citrine

G

Fig. 5. sema3d interacts genetically with nrp1a to promote axon targeting of or111-7 transgene-labeled axons to the CZ. (A-C) Single optical sections through 72 hpf or111-7 transgenic larvae (frontal view). Axons are in green. Dorsal is up and medial is to the right. 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 in A-C. Scale bar: 25 µm. (G) The percentage of OBs with projections to the CZ protoglomerulus. Statistical significance was estimated using two-tailed (*P<0.05, **P<0.01**, ***P<0.001***') or one-tailed (**P<0.05, ***P<0.01***') Fisher’s exact tests. Error bars represent s.e. of the sample proportion. sema3d⁺/−;nrp1a⁺/− transheterozygotic larvae (C,F; 1 in G) have an increase in projections to the CZ compared with sema3d⁺/− heterozygotic (A,D) or nrp1a⁺/− heterozygotic (B,E) siblings. sema3d⁺/− mutants, nrp1a⁺/− mutants and sema3d⁺/−;nrp1a⁺/− double mutants have a similar increase in projections to the DZ as compared with wild-type siblings (2 in G). By contrast, sema3d⁺/−;nrp2b⁺/− transheterozygotes do not have an increase in projections to the CZ when compared with sema3d⁺/− heterozygotic or nrp2b⁺/− heterozygotic siblings (3 in G). sema3d⁺/−;nrp2b⁺/− double mutants have a greater frequency of projections to the DZ than sema3d⁺/− or nrp2b⁺/− siblings (4 in G). nrp1a⁺/− mutants expressing the UAS:nrp1a:UAS:Citrine transgene do not have an increase in misprojections to the DZ or non-protoglomerular regions as compared with wild-type or heterozygotic siblings (5 in G). CZ, central zone; DZ, dorsal zone.
DISCUSSION

This study identifies Sema3D as a guidance cue that is required for a subset of olfactory sensory axons to target a well-defined intermediate protoglomerular target. or111-7 transgene-expressing axons normally extend just posteriorly to sema3d-expressing cells at the anteriormost margin of the OB. In wild-type larvae these axons congregate in the CZ protoglomerulus, but in sema3d mutants they frequently misproject into the more anterior and dorsal DZ protoglomerulus. These observations can be most simply explained by Sema3D acting as a repellent for sema3d-expressing neurons. Additionally, our results strongly suggest that Sema3D plays a role in fish reminiscent of the role that Sema3A is proposed to play in mice. We also observed a Sema3D– mediated repulsion in or111-7 transgene-expressing axons to the CZ.

Nrp1-mediated signaling has been proposed to affect OSN axon targeting along the anterior-posterior (AP) axis in the main OB of the mouse (Schwarting et al., 2000, 2004; Taniguchi et al., 2003; Imai et al., 2009). Sema3a is thought to be its activating ligand and is expressed in anteromedial and ventral regions of the olfactory nerve layer (Schwarting et al., 2000); it is also expressed in a subset of OSNs (Imai et al., 2009). Selected glomeruli formed by Nrp1-expressing axons are shifted anteriorly in the OBs of Nrp1, Sema3a, or OSN-specific Sema3a mouse 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 Nrp1 signaling also affects OSN targeting along the AP axis in the zebrafish OB but, at least in the context of our studies, this effect is largely Sema3D dependent. This suggests that Sema3D plays a role in fish reminiscent of the role that Sema3A is proposed to play in mice. We also observed a Sema3D-independent increase in non–protoglomerular dorsally directed errors in nrla mutants (Fig. 4), implying that Nrp1 is likely to act as a receptor for additional ligands. Zebrafish sema3aa and sema3ab are expressed in overlapping regions in dorsal and lateral regions of the OB at 36 hpf (Table S1; data not shown). Based on these expression patterns, sema3aa and sema3ab might be candidate guidance cues affecting dorsal-ventral targeting in zebrafish.
different set of receptors might regulate the segregation of OR-a distinct set of axon guidance genes compared with mature shown). It has been suggested that immature mouse OSNs express (Miyasaka et al., 2005; Lakhina et al., 2012) (Fig. 6F; data not nrp2b expression of all of the guidance receptors thus far implicated in protoglomerular map seems to be emerging. In zebrafish, the expression of the guidance receptors that establish the discrete protoglomerular neuropils. A very early requirement for development, before the emergence of OR-specific glomeruli. During this period, OSNs target a relatively small number (12) of initial target in the OB, the CZ. or111-7 transgene-expressing neurons (green) project to the CZ protoglomerulus. Early in development (36 hpf), sema3d is expressed in the anterior OB and in the boundary between the olfactory pit and OB (red), netrin 1b (blue) is expressed more posteriorly in the OB. or111-7 transgene-expressing axons invade netrin 1b-expressing but not sema3d-expressing areas of the 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 CZ protoglomerulus at 72 hpf. Ventral views, anterior is up and medial is to the left. CZ, central zone; DZ, dorsal zone.

The guidance events that we are studying occur early in development, before the emergence of OR-specific glomeruli. During this period, OSNs target a relatively small number (12) of discrete protoglomerular neuropils. A very early requirement for the expression of the guidance receptors that establish the protoglomerular map seems to be emerging. In zebrafish, expression of all of the guidance receptors thus far implicated in early OSN guidance, namely robo2, dcc, and now nrp1a and nrp2b, are all downregulated between 27 hpf and 48 hpf (Miyasaka et al., 2005; Lakhina et al., 2012) (Fig. 6F; data not shown). It has been suggested that immature mouse OSNs express a distinct set of axon guidance genes compared with mature neurons (McIntyre et al., 2010). It is possible that accurate targeting relies upon the early expression of guidance receptors that either pre-sort axons in the olfactory nerve or direct them as they first enter the bulb (Imai et al., 2009; Miller et al., 2010). A different set of receptors might regulate the segregation of OR-specific glomeruli in more mature OSNs.

In this study we show for the first time that sema3d is required for normal OSN targeting in the bulb. Our results support a model in which Sema3D/Nrp1a-mediated repulsion directs a specific subset of sensory axons towards a well-defined intermediate target, the CZ protoglomerulus. One such subset of sensory axons was previously shown to be drawn towards the same intermediate target by Netrin 1b acting through the Dcc receptor (Fig. 7) (Lakhina et al., 2012). Our dcc and nrp1a double-mutant data support a model in which Sema3D/Nrp1a and Netrin 1b/Dcc signal independently and in parallel through competing attractive and repulsive mechanisms to direct a subset of axons to the same target. Additionally, Nrp1b, and especially Nrp2b, participate in the normal guidance of this subset of sensory axons though Sema3D-independent signaling pathways. These findings suggest that the first targeting events within the bulb are more complex than previously appreciated, with many different ligand-receptor pairs cooperating to guide sensory axons to particular protoglomeruli. Each cue that we have identified contributes to normal pathfinding, but none is decisive on its own. This should not be surprising. For example, no single mutant was found to be fully penetrant in a collection of mutants affecting axon and neuroblast migration in C. elegans (Hedgecock et al., 1985, 1990). The development of complex neural circuitry originates through an evolutionary process that is likely to add additional guidance cues and receptors on top of those already in use, thereby either increasing the robustness of targeting or altering connectivity. The resulting complexity of cooperating and competing guidance pathways is likely to be a common feature of how neural circuits are assembled.

**MATERIALS AND METHODS**

**Zebrafish maintenance and transgenic lines**

Adult zebrafish were raised and maintained according to standard procedures (Mullins, 1994). All experiments were conducted with the approval of the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Veterinary care was supervised by University Laboratory Animal Resources (ULAR). Larvae were staged based on hours post fertilization (hpf) and were raised at 28°C. For some experiments, 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). Before fixation, larvae were staged based on morphology (Westerfield, 1995). Tg(omp:lyn-RFP) and Tg(trpc2:gap-Venus) lines were gifts from the Yoshihara laboratory at the RIKEN Brain Science Institute, Saitama, Japan (Sato et al., 2005). The Tg(or111-7:or111-7-ires:Gal4), Tg(omp:Gal4) and Tg(UAS:gap43-citrine) lines were 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 an nrp1a+/--; UAS:nrp1a;UAS:citrine founders were mated with the nrp1a+/--; 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**

sema3dmutA1661 and nrp1a+1485 mutants were generated by the Sanger Centre Zebrafish Mutation Project and obtained from The Zebrafish International Resource Center (ZIRC). These were genotyped using KASPR assays (LG Genomics; sema3d SNP ID 554-1608.1, nrp1a SNP ID 554-1410.1). The nrp1aR1278 mutant was identified by the Zebrafish Tilling Project and ordered from ZIRC. The nrp2b+/- mutant was obtained from the Zebrafish International Resource Center (ZIRC). These were genotyped using KASPR assays (LG Genomics; nrp2b SNP ID 554-3590.2). The nrp1a+1485 mutant was identified by the Zebrafish Tilling Project and ordered from ZIRC. The nrp1a+1485 mutant was identified by the Zebrafish Tilling Project and ordered from ZIRC. The nrp2b+/- mutant was obtained from the Zebrafish International Resource Center (ZIRC). These were genotyped using KASPR assays (LG Genomics; nrp2b SNP ID 554-3590.2). The nrp1a+1485 mutant was identified by the Zebrafish Tilling Project and ordered from ZIRC. The nrp2b+/- mutant was obtained from the Zebrafish International Resource Center (ZIRC). These were genotyped using KASPR assays (LG Genomics; nrp2b SNP ID 554-3590.2). The nrp1a+1485 mutant was identified by the Zebrafish Tilling Project and ordered from ZIRC. The nrp2b+/- mutant was obtained from the Zebrafish International Resource Center (ZIRC). These were genotyped using KASPR assays (LG Genomics; nrp2b SNP ID 554-3590.2). The nrp1a+1485 mutant was identified by the Zebrafish Tilling Project and ordered from ZIRC. The nrp2b+/- mutant was obtained from the Zebrafish International Resource Center (ZIRC). These were genotyped using KASPR assays (LG Genomics; nrp2b SNP ID 554-3590.2).
heads were processed for immunohistochemistry and imaged using confocal microscopy.

**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 min at −20°C and stained with goat anti-GFP (1:300; Rockland Immunocore, 600-101-215) and donkey anti-goat Alexa Fluor 647 (1:500; Invitrogen). To visualize RFP-positive axons, fish were permeabilized for 30 min in 0.1% collagenase at room temperature and stained with rabbit anti-dsRed (1:300; Clontech, 632496) and donkey anti-rabbit 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 40× oil-immersion lens. Stacks were acquired through the entire OB with optical sections taken 1 μm apart.

**Whole-mount fluorescent in situ hybridization**

Single-label in situ hybridization was performed using antisense digoxigenin (DIG) RNA probes as previously described (Chalasani, 2007). In situ signals were amplified using a cyanine 3-coupled tyramide kit (TSA Plus Cyanine 3; PerkinElmer, NEL744001KT). Double-label in situ hybridization was performed using DIG and fluorescein-labeled probes as previously described (Brend and Holley, 2009), with the exception that embryos were not dehydrated in between detection of the first and second probes and RNase (RNase A, 10 μg/ml; RNase T1, 100 U/ml; Roche, 10109193001) treatment was included after probe removal. The DIG label was amplified using the cyanine 3-coupled tyramide kit and the fluorescein label was amplified using a fluorescein-coupled tyramide kit (PerkinElmer, NEL741001KT). 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 npr1a were as described by Dell et al. (2013). The plasmids used to make probes for sema3a, sema3b, sema3f, sema3g, sema3h and npr2b were gifts from the Moens laboratory at the Fred Hutchinson Cancer Research Center, Seattle, WA, USA (Yu et al., 2004; Yu and Moens, 2005). The plasmid used to make the sema3h probe was a gift from the Halloran laboratory at the University of Wisconsin, Madison, WI, USA (Stevens and Halloran, 2005). For sema3c (refseq accession number XM_687755.5, nucleotides 1539-2400), npr1b (refseq accession number NM_205674.1, nucleotides 2-972) and npr2a (refseq accession number NM_212965.1, nucleotides 138-1108) sequences were amplified from cDNA and cloned into pcRII (Invitrogen, K160001) for probe synthesis. Full-length probes were used in all hybridization experiments. The plasmids used to make probes targeting acc and netrin 1b were as described by Lakhina et al. (2012).

**Quantification of targeting errors**

The number of OBs containing axons terminating in either individual protoglomeruli or non-protoglomerular regions were counted. The percentage of OBs with axons in particular locations was recorded and Fisher’s exact test (two-tailed) was used to determine statistical significance. To determine whether the number of errors was statistically greater in double mutants as compared with single mutants, a one-tailed Fisher percentage of OBs with axons in particular locations was recorded and the number of OBs containing axons terminating in either individual protoglomeruli and not if they passed though it en route to another location.

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**Competing interests**

The authors declare no competing or financial interests.


