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# Neuropilin-2 Mediates Axonal Fasciculation, Zonal Segregation, but Not Axonal Convergence, of Primary Accessory Olfactory Neurons

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## Summary

**The mechanisms that underlie axonal pathfinding of vomeronasal neurons from the vomeronasal organ (VNO) in the periphery to select glomeruli in the accessory olfactory bulb (AOB) are not well understood. Neuropilin-2, a receptor for secreted semaphorins, is expressed in V1R- and V3R-expressing, but not V2R-expressing, postnatal vomeronasal neurons. Analysis of the vomeronasal nerve in *neuropilin-2* (*npn-2*) mutant mice reveals pathfinding defects at multiple choice points. Vomeronasal sensory axons are severely defasciculated and a subset innervates the main olfactory bulb (MOB). While most axons of V1R-expressing neurons reach the AOB and converge into distinct glomeruli in stereotypic locations, they are no longer restricted to their normal anterior AOB target zone. Thus, Npn-2 and candidate pheromone receptors play distinct and complementary roles in promoting the wiring and patterning of sensory neurons in the accessory olfactory system.**

## Introduction

The olfactory system of most terrestrial vertebrates is comprised of two anatomically and functionally distinct chemosensory systems that provide information about the chemical environment. Primary sensory neurons within the main olfactory epithelium located in the principal nasal cavity can relay odor information to second-order neurons in the MOB. In contrast, pheromone-responsive sensory neurons are found in the VNO, a bilateral sensory structure located at the base of the nasal septum, and relay their information to second-order neurons in the AOB (reviewed in Halpern, 1987; Dulac, 2000).

Axons of chemosensory neurons in both olfactory systems form specific and highly stereotyped projections to higher-order central nervous system (CNS) neurons. Neurons distributed within four distinct zones of the main olfactory epithelium project their axons to four specific zones in the MOB (Strotmann et al., 1992; Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1994). Neurons of the main olfactory epithelium each express one of approximately 1000 odorant receptors. Axons of neurons expressing the same odorant receptor converge on two bilaterally symmetric and topographically fixed glomeruli of the approximately 1800 glomeruli in the MOB (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). Zonal segregation is also observed in the accessory olfactory system; cell bodies of chemosensory neurons found within the apical and basal zones of the VNO extend axons that project to either the anterior or posterior region of the ipsilateral AOB, respectively (Halpern et al., 1995). However, in contrast to the remarkable degree of axonal convergence into single glomeruli at fixed locations in the MOB, axons of vomeronasal neurons that express the same pheromone receptor innervate multiple glomeruli clustered within large, but topographically conserved, domains within the AOB (Belluscio et al., 1999; Rodriguez et al., 1999).

Vomeronasal neurons express putative pheromone receptors (VRs), which are members of the large superfamily of G protein-coupled seven-transmembrane spanning receptors (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Pantages and Dulac, 2000). Neurons located in the apical (luminal) portion of the vomeronasal epithelium express either V1R or V3R receptors and, also, the G protein $\alpha$ subunit $G_{\alpha i2}$. In contrast, sensory neurons located in the basal region of the vomeronasal epithelium express members of the V2R receptor family and the G protein $\alpha$ subunit $G_{\alpha o}$. V1R-expressing neurons project their axons via the vomeronasal nerve to glomeruli restricted to the anterior half of the AOB, whereas V2R neurons in the basal vomeronasal epithelium project to glomeruli restricted to the posterior half of the AOB. Relatively little is known about the molecular and cellular mechanisms that govern the guidance of sensory neuron axons to the AOB and the segregation of these axons into either the anterior or posterior zones of the AOB.

It is well established that growth cones of extending axons respond to a wide range of environmental cues that guide them to their targets. The semaphorins are a large family of glycoproteins that have been implicated in the guidance of growing axons (Raper, 2000; Nakamura et al., 2000). The well-characterized class 3 secreted semaphorins act as chemorepellents for specific, yet partially overlapping populations of developing neurons. Neuropilin-1 (Npn-1) and neuropilin-2 (Npn-2) are members of a small family of type-I transmembrane proteins that are essential ligand-binding subunits of class

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3 semaphorin holoreceptors (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Giger et al., 1998). Recent work shows that certain members of the Plexin family of transmembrane proteins associate with neuropilins and propagate intracellular signals upon binding of secreted semaphorins to the receptor complex (Takahashi et al., 1999; Tamagnone et al., 1999; Rohm et al., 2000; Takahashi and Strittmatter, 2001).

Npn-1 and Npn-2 bind secreted semaphorins with high affinity and have been shown to impart functional specificity toward different secreted semaphorins by discrete populations of neurons in vitro and in vivo. For example, Npn-1 and Npn-2 are receptors for Sema3A and Sema3F, respectively, while Npn-1/Npn-2 heterodimers have been implicated as receptors for Sema3C (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Chen et al., 1998; Giger et al., 1998; Takahashi et al., 1998). In the developing nervous system, Npn-1 and Npn-2 are expressed in specific neuronal populations in complementary, yet partially overlapping patterns (Kawakami et al., 1996; Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Giger et al., 1998). Likewise, the neuropilin ligands show distinct developmental distribution patterns. Mice with targeted deletions in the *npn-1* (Kitsukawa et al., 1997) or *npn-2* (Chen et al., 2000; Giger et al., 2000) loci display severe but distinct defects in nervous system wiring and loss of responsiveness to Sema3A and Sema3F, directly demonstrating that Npn-1 and Npn-2 are selective receptors for class 3 semaphorins required for the development of overlapping but distinct sets of central and peripheral nervous system projections. In contrast to *npn-1* null mice, *npn-2* null mice are viable into adulthood, allowing for analysis of Npn-2 function throughout neural development.

Very little is known about the function of either Npn-2 or its secreted semaphorin ligands during development of the olfactory system. Analysis of *neuropilin* distribution revealed that *npn-2*, but not *npn-1*, is strongly expressed in a subset of vomeronasal sensory neurons (Kawakami et al., 1996; Chen et al., 1997; Giger et al., 1998). Here, we report on the role of Npn-2 and class 3 secreted semaphorins in the patterning of vomeronasal sensory neuron projections to their targets in the AOB. Our results demonstrate that Npn-2 is required for axonal fasciculation, zonal segregation, but not convergence of axons, of VNO neurons into AOB glomeruli. The phenotypic defects in *npn-2* mutant mice are in striking contrast to the wiring defects observed in VNO neurons lacking individual V1R receptors, where the vomeronasal nerve and zonal targeting of these neurons in the AOB are unaffected, but axonal convergence is lost (Belluscio et al., 1999; Rodriguez et al., 1999). Thus, Npn-2 and pheromone receptors function independently and play complementary roles during the establishment and patterning of primary accessory olfactory neuron projections.

## Results

### *npn-2* Is Expressed in the Vomeronasal Organ

The molecular cues involved in guiding vomeronasal projections to their target, the AOB, are unknown. Previous work showed that *npn-2* is expressed in both the VNO and AOB (Chen et al., 1997; Giger et al., 1998). To further assess the potential involvement of secreted semaphorins and their receptors, the neuropilins, in the development and guidance of VNO projections, we performed in situ hybridization experiments to determine the pattern of expression of neuropilins and secreted semaphorins in the developing and adult accessory olfactory system.

Vomeronasal neurons express putative pheromone receptors and either $G_{\alpha i2}$ or $G_{\alpha o}$ subunits of heterotrimeric G proteins. During late embryogenesis, segregation of these two populations of neurons begins to take place within the VNO (Halpern et al., 1995; Dulac and Axel, 1995; Berghard and Buck, 1996); $G_{\alpha i2}$-expressing neurons are eventually restricted to the apical region of the VNO, while $G_{\alpha o}$-expressing neurons are found in the basal region (Figures 1B, 1C, 1E, and 1F). While *npn-1* transcripts were not detected in the VNO (Kawakami et al., 1996; data not shown), *npn-2* is strongly expressed in developing neurons of the VNO as early as embryonic day (E)13, and somewhat weaker expression persists throughout adulthood (Chen et al., 1997; Giger et al., 1998; Figures 1A and 1D; data not shown). *Npn-2* is at first expressed widely in the vomeronasal epithelium, and its expression then becomes gradually more restricted to the apical region of the VNO during late embryogenesis and early postnatal development, ultimately being confined to the apical portion of the vomeronasal epithelium by postnatal day (P)15 (Figures 1A and 1D). Immunohistochemical analysis using antibodies specific for Npn-2 revealed that Npn-2 is found on axons of the vomeronasal nerve, both adjacent to and along the nasal septum (Figures 1G and 1H). Furthermore, analysis of the target area, the AOB, detected Npn-2-expressing fibers exclusively in the anterior half of the AOB (Figures 7A and 7B; see below). Taken together, these observations indicate that Npn-2 is a candidate for imparting guidance information to extending axons of $G_{\alpha i2}$-expressing vomeronasal neurons.

### Expression of Secreted Semaphorins along Vomeronasal Projections and in the Accessory Olfactory Bulb

Before reaching the AOB, axons of vomeronasal neurons run along the medial surface of the MOB. In situ hybridization experiments revealed expression of secreted semaphorins in regions near vomeronasal fibers and in their target field in the AOB. In caudal sections of the MOB at E16, *sema3A* and *sema3B* expression is found throughout the MOB. *sema3A* appears to be expressed in a more ventral-lateral pattern in caudal sections of the MOB at an earlier developmental time point (E13) (Schwarting et al., 2000). In contrast, *sema3C* and *sema3F* transcripts are found at higher levels in the medial MOB, compared to the lateral MOB during embryogenesis (Figures 2A–2D, Figure 3D). It is interesting to note that Npn-2 immunohistochemistry and AP-Sema3F binding analyses indicate that Npn-2-expressing primary olfactory neurons innervate the ventral-lateral side in the caudal region of the MOB, complementary to *sema3F* and *sema3C* expression (Figures 3A, 3B,

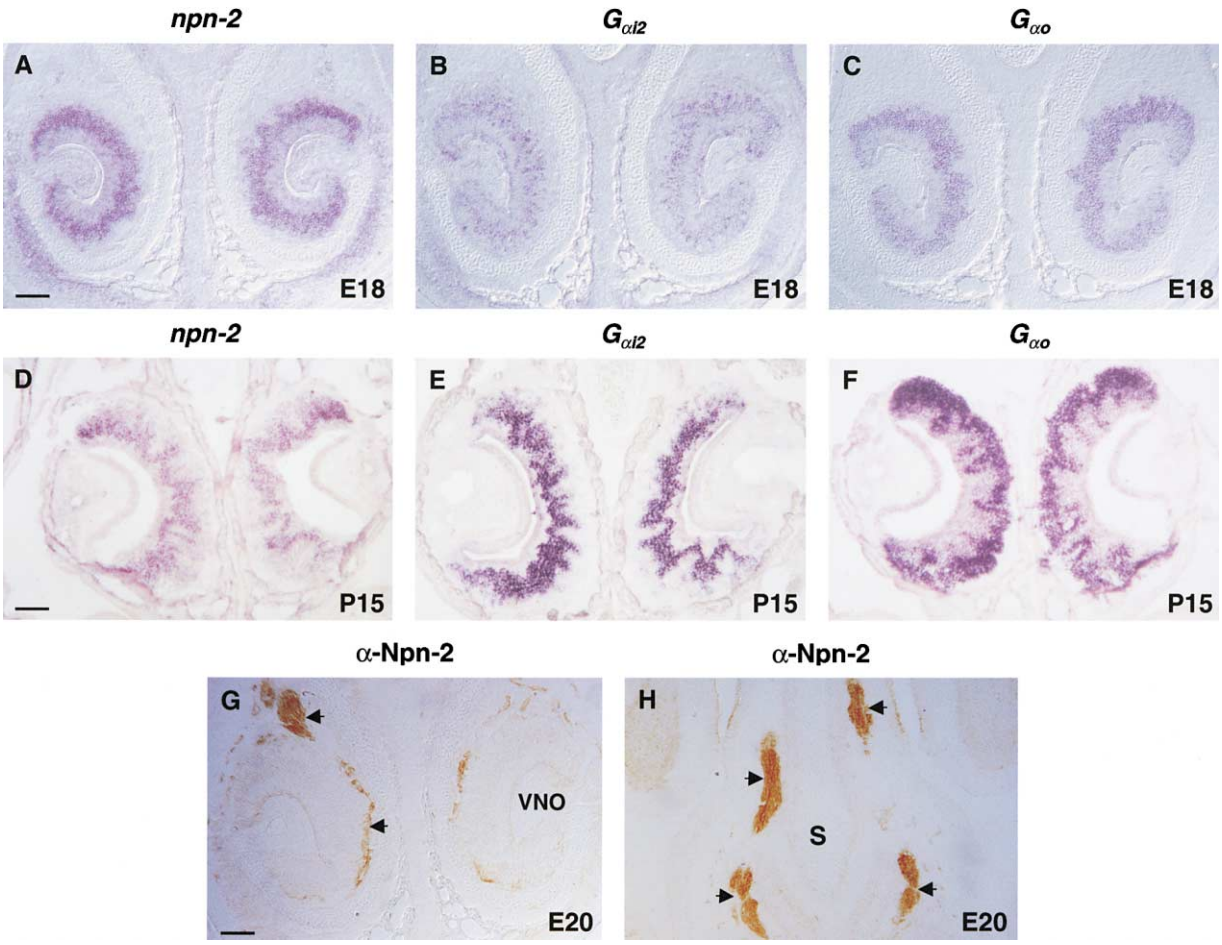


Figure 1. Expression of Npn-2 in the Vomeronasal Organ (VNO)

(A–F) In situ hybridization of coronal sections of VNO at embryonic day (E)18 (A, B, and C) and postnatal day (P)15 (D, E, and F) with cRNA probes specific for *npn-2* (A and D), $G_{\alpha i2}$ (B and E), $G_{\alpha o}$ (C and F). Expression of *npn-2* is restricted to the apical portion of the VNO at P15 (D), in contrast to E18 (A), when *npn-2* expression is detected in both apical and basal regions. These apical VNO neurons project their axons to the anterior region of the AOB.

(G and H) Npn-2 is expressed on axons of the projecting vomeronasal nerve. Coronal sections of the nasal septum at E20 immunostained with anti-Npn-2. (G) shows a cross-section of the VNO, while (H) shows a section caudal to the one in (G) with the Npn-2-positive vomeronasal fibers projecting along the nasal septum. Arrowheads, vomeronasal nerve; S, septum; VNO, vomeronasal organ.

Scale bar: 100 μm (A–C); 110 μm (D–H).

and 3D). In contrast, Npn-1-expressing main olfactory neurons project to the medial region of the caudal MOB (Nagao et al., 2000; Schwarting et al., 2000) (Figure 3C). Thus, like VNO neurons, Npn-2-expressing primary olfactory neurons do not innervate the medial MOB, a structure that expresses relatively high levels of Npn-2 ligands.

Secreted semaphorins are also expressed in the target region of the vomeronasal nerve. In contrast to the MOB, where expression of certain secreted semaphorins differs spatially, *sema3A, sema3B, sema3C, sema3F* (Figures 2E–2L), and *sema3E* (data not shown) appear evenly expressed in the mitral cell layer of the AOB during embryonic and postnatal periods. *sema3D* transcripts are undetectable in the AOB at these developmental stages (data not shown). Differential expression of this complete set of known class 3 secreted semaphorins in specific brain structures confirmed the specificity of all probes used in our in situ hybridizations (data not shown).

Interestingly, in contrast to the uniform patterns of expression of five class 3 semaphorins in the mitral cell layer of the AOB, Npn-2 protein is found at higher levels in the anterior half of the mitral cell layer at E16, E18, and P1(Figures 4D–4F). However, Npn-2 protein levels in the AOB mitral cells are significantly decreased by P5 and into adulthood (data not shown). In situ hybridization experiments reveal that *npn-2* shows somewhat higher expression in mitral cells of the anterior AOB at E16 but is expressed evenly throughout the AOB at P0 (data not shown).This late embryonic pattern of Npn-2 distribution is complementary to that observed for the cell adhesion molecule OCAM, which is restricted to the posterior half of the AOB mitral cell layer at these same developmental times (Figures 4A–4C; von Campenhausen et al., 1997). Thus, the expression patterns of certain class 3 sema-

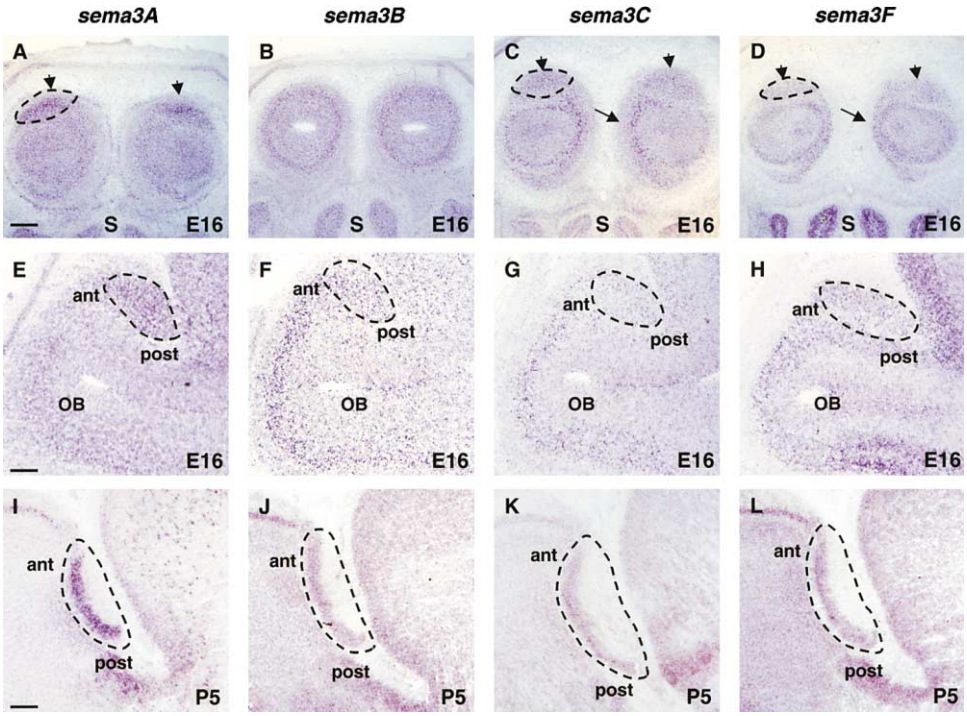


Figure 2. Expression of Secreted Semaphorins in the Main and Accessory Olfactory Bulbs

In situ hybridization of coronal (A–D) or parasagittal sections (E–L) of mouse E16 (A–H) or P5 olfactory bulbs (I–L) with cRNA probes specific for *sema3A* (A, E, and I), *sema3B* (B, F, and J), *sema3C* (C, G, and K), and *sema3F* (D, H, and L). The secreted semaphorins *sema3A*, *sema3B*, *sema3C*, and *sema3F* are expressed in the mitral cells of the AOB (I and J). Arrowhead, AOB; S, septum. Arrows in (C) and (D) point to higher levels of expression of *sema3C* (C) and *sema3F* (D) on the medial side of the olfactory bulb. The AOB is outlined by a dashed black line. Scale bar: 225 μm (A–D); 500 μm (E–H); 200 μm (I–L).

phorins and their receptor, Npn-2, are consistent with these molecules playing a role in establishing the trajectories and targeting of *npn-2*-expressing vomeronasal neurons.

### The Vomeronasal Nerve Is Defasciculated and Vomeronasal Neurons Ectopically Innervate the Main Olfactory Bulb in *npn-2*$^{-/-}$ Mice

To assess the involvement of Npn-2 in the development and guidance of vomeronasal neuron axons, we looked at the integrity of VNO projections in mice harboring a null mutation at the *npn-2* locus (Giger et al., 2000). In initial experiments, coronal sections of the MOBs from 3-month-old wild-type and *npn-2*$^{-/-}$ mice were stained with Erythrina Cristagalli (EC) lectin to visualize all axons in the vomeronasal nerve (Tanaka et al., 1999). While vomeronasal axons are bundled into two large, bilateral nerves in wild-type and *npn-2*$^{+/-}$ mice (Figure 5A; data not shown), many smaller bundles of fibers spanning a large portion of the dorsal-ventral axis between the MOBs were observed in *npn-2*$^{-/-}$ mice (Figure 5B). This strongly suggests that Npn-2 function is necessary for maintaining the integrity of the vomeronasal nerve as it projects along the medial MOB.

Genetically modified mouse lines in which neurons expressing specific V1R receptors harbor the axonal marker tau-β-galactosidase (tau-lacZ) have provided insight into the neuronal wiring of the accessory olfactory system (Belluscio et al., 1999; Rodriguez et al., 1999). To further evaluate the role played by Npn-2 in vomeronasal axon pathfinding, *npn-2*$^{-/-}$ mice expressing *tau-lacZ* in specific V1R-expressing populations of vomeronasal neurons were generated. The expression of *tau-lacZ* in neurons expressing either VN2 or VN12 pheromone receptors allowed us to visualize the projections of these two populations of $G_{\alpha i2}$-expressing sensory neurons in *npn-2*$^{-/-}$ mice. Whole-mount X-Gal staining of 3-month-old *npn-2*$^{+/-}$ mice illustrates VN12-expressing neurons projecting normally and in a tight fascicle as they grow along the medial surface of the MOB toward the AOB (Figure 5C). In contrast, we found in *npn-2*$^{-/-}$ mice marked defasciculation of these fibers at the level of the medial surface of the MOB (Figure 5D). A similar defect was observed for VN2-expressing neurons (data not shown). Much less, if any, defasciculation of *tau-lacZ*-positive fibers was observed in the peripheral portion of the vomeronasal nerve in *npn-2*$^{-/-}$ mice. Four to six tightly fasciculated fiber bundles projected from the VNO along the nasal septum to the cribriform plate in both *npn-2*$^{+/-}$ and *npn-2*$^{-/-}$ mice; however, occasionally in *npn-2*$^{-/-}$ mice, single VNO axons were observed adjacent to the main axon bundles (data not shown). The severe defasciculated state of the central projection of the vomeronasal nerve does not appear to impede the ability of most vomeronasal axons to reach the AOB (see Figures 7 and 8). However, a significant number of defasciculated VNO fibers were observed to terminate in the MOB. These VNO axons project below the outer

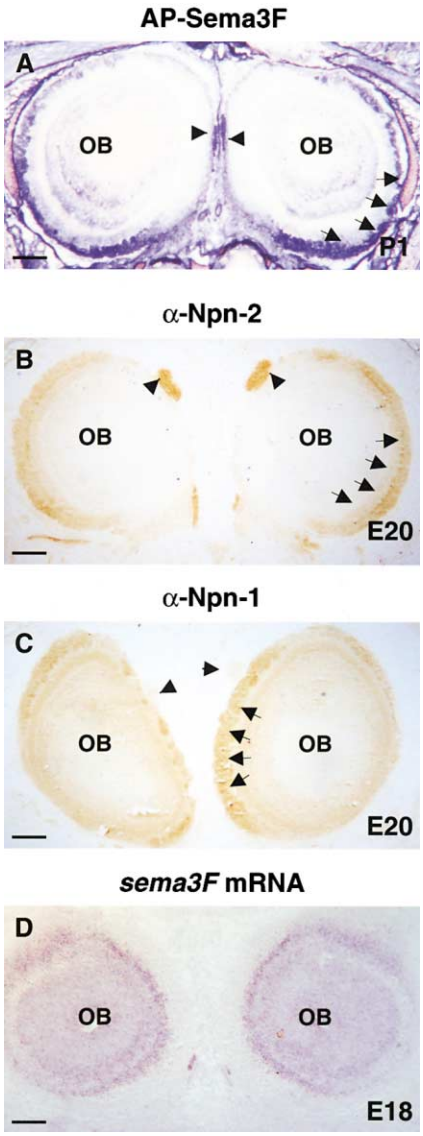


Figure 3. Axons of Primary Olfactory Neurons Expressing Npn-2 Project to the Lateral Side of the Olfactory Bulbs

(A) Coronal section of a P1 brain incubated with AP-Sema3F. AP-Sema3F binding is detected on the ventral-lateral portion of the olfactory bulbs (arrows) and on the projecting VN (arrowhead). Scale bar: 320 μm.

(B and C) Immunostaining of a coronal section of an E20 brain with anti-Npn-2 (B) or anti-Npn1 (C). Npn-2 staining is restricted to the ventral-lateral side of the olfactory bulb (arrows) and to the VN projecting to the AOB (arrowhead). Npn-1 is restricted to the dorsal-medial side of the olfactory bulb (arrows on right olfactory bulb) and absent from the VN (arrowhead in [C]). In (C), the number of medially located glomeruli stained by anti-Npn-1 is lower in the left olfactory bulb as a result of a different plane of sectioning through the bulb. Scale bar: 280 μm.

(D) In situ hybridization of a coronal section from an E18 brain with a cRNA probe for *sema3F*. *sema3F* expression is observed on the medial side of the olfactory bulbs. OB, olfactory bulb. Scale bar: 200 μm.

nerve layer and enter the glomerular layer, ectopically innervating the medial and, occasionally, the ventral and dorsal regions of the MOB (Figure 5E; data not shown). These errant vomeronasal fibers do not form new, dedicated glomeruli but, instead, appear to terminate within MOB glomeruli (Figure 5F). These observations indicate that Npn-2 is necessary for the segregation of projections between the main and accessory olfactory systems. The fasciculation of axons of vomeronasal neurons may prevent the ectopic innervation by vomeronasal neurons of the MOB glomerular neuropil.

### Vomeronasal Axons Are Responsive to a Npn-2 Repellent Secreted by the Medial Surface of the Olfactory Bulb

The distribution of secreted semaphorins in the main and accessory olfactory systems, combined with vomeronasal nerve defects observed in *npn-2*$^{-/-}$ mice, suggest that secreted semaphorins provide guidance information to VNO neurons. To determine whether secreted semaphorins that are ligands for Npn-2 can act as repellents for vomeronasal neurons, vomeronasal epithelia from E14 mouse embryos were isolated and cocultured in close proximity to 293T cell aggregates transfected with control or AP-Sema3F expression vectors. Unfortunately, our attempts to grow vomeronasal epithelia isolated from older embryos (E16–E18) in which Npn-2 expression may become restricted to $G_{\alpha i2}$-expressing neurons failed (data not shown). The growth of vomeronasal fibers from either wild-type or *npn-2*$^{-/-}$ epithelia was unaffected by control 293T cell aggregates (Figure 6A; data not shown). In contrast, AP-Sema3F-expressing aggregates strongly repelled murine vomeronasal axons (Figure 6B). The repulsive effect of AP-Sema3F is mediated by its receptor, Npn-2, since vomeronasal projections from epithelia isolated from *npn-2*$^{-/-}$ embryos are completely unresponsive to AP-Sema3F (Figure 6C). Intriguingly, all vomeronasal axons emanating from these explants were repelled by AP-Sema3F in vitro. Immunostaining of the explants with Npn-2 antibodies revealed that all projecting fibers express Npn-2 (data not shown). Likewise, all fibers were found to express the cell adhesion molecule OCAM (data not shown), which is normally restricted to $G_{\alpha i2}$-expressing neurons by late embryogenesis (Yoshihara et al., 1997). While it is possible that Npn-2 is expressed by all VNO neurons at E14 in vivo, this pattern of expression could also result from the in vitro growth conditions.

The defasciculated state of the vomeronasal nerve in *npn-2*$^{-/-}$ mice suggests that a Npn-2 ligand expressed on the medial surface of the MOB promotes fasciculation of the VN and prevents ectopic innervation of the MOB. To test this possibility, vomeronasal epithelia were cocultured in close proximity to coronal slices of MOBs, and the growth of axons from vomeronasal neurons was monitored. Because of the relatively poor growth observed with murine vomeronasal neurons in vitro, we used rat vomeronasal epithelia, which grow much better in our culture conditions. As observed with mouse epithelia, all fibers growing out of rat epithelia were found to express Npn-2 and OCAM. We first tested the responsiveness of rat vomeronasal neurons to Sema3F. All explants were scored according to the degree of observed repulsion, as shown schematically in Figure 6D (results are represented graphically in Figure 6H). Robust growth of axons from vomeronasal neurons was

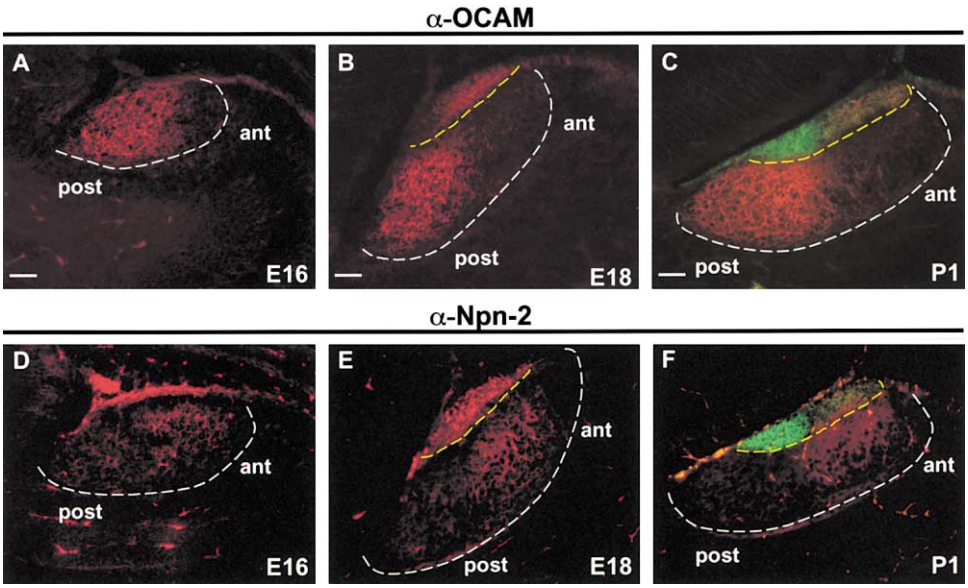


Figure 4. Complementary Expression of Npn-2 and OCAM in Mitral/Tufted Cells of the Accessory Olfactory Bulb

(A–F) Immunostaining of sagittal sections of E16 (A and D), E18 (B and E), and P1 (C and F) brains with anti-OCAM (A–C) or anti-Npn-2 (D–F) (both red). All sections were incubated with FITC-conjugated Erythrina Cristagalli (EC) lectin (green) to visualize axons of vomeronasal neurons. Binding of the lectin to the glomerular layer of the AOB was detected in P1, but not in E16 or E18 brains. In the external plexiform layer, OCAM staining is restricted to the posterior half of the AOB, while Npn-2 staining is restricted to the anterior half. The glomerular layer of the AOB is outlined by a yellow dashed line, while the external plexiform layer containing mitral and tufted cells is outlined by a white dashed line. Ant, anterior; post, posterior. Scale bars: 80 μm (A, B, D, and E); 85 μm (C and F).

observed when E15 rat epithelia were cultured with 293T cell aggregates transfected with a control vector (Figures 6E and 6H). As observed for murine vomeronasal neurons, AP-Sema3F strongly repelled axons emanating from rat vomeronasal epithelia (Figures 6F and 6H). Similar to our observations in mouse explants, the repulsive effect of Sema3F appeared to be mediated through Npn-2, since addition of function-blocking antibodies raised against Npn-2 (Giger et al., 1998) to the growth medium attenuated this response (Figures 6G and 6H). As previously described, these antibodies do not completely block Sema3F signaling through Npn-2 (Giger et al., 1998). In accordance with the observation that Npn-1 is not expressed in vomeronasal neurons, Sema3A and Sema3C do not repel vomeronasal axons (data not shown).

We next asked whether a Npn-2 ligand expressed in the main olfactory bulb serves as a repellent for VNO axons. For these experiments, rat vomeronasal epithelia were cultured in proximity to either the medial or lateral surface of coronal slices of main olfactory bulbs isolated from early postnatal mice. While axons projecting from epithelia grown on the lateral side of the main olfactory bulb were not obviously repelled (Figures 6I and 6L), axons projecting from the epithelia grown close to the medial side were strongly repelled (Figures 6J and 6L). To test whether the medial bulb chemorepellent acts on vomeronasal axons in a Npn-2-dependent fashion, Npn-2 function-blocking antibodies were added to the growth medium of explants cultured next to the medial side of the olfactory bulb. The Npn-2 antibodies attenuated the repulsive effect of the medial region of the MOB (Figures 6K and 6L). Taken together with the robust expression of *sema3F* in the medial region of the MOB, these in vitro repulsion experiments suggest that Sema3F and its receptor Npn-2 promote fasciculation of the vomeronasal nerve to prevent ectopic innervation of the MOB by vomeronasal neurons and, thereby, contribute to the segregation of fiber of the main and accessory olfactory systems.

### Ectopic Innervation of the Posterior AOB by $G_{\alpha i2}$-Expressing Vomeronasal Neurons in *npn-2*$^{-/-}$ Mice

The overlapping expression patterns of *npn-2* and $G_{\alpha i2}$ in the VNO suggested that *npn-2*-expressing VNO neurons are targeted to the anterior half of the AOB. To identify the region of the AOB that is innervated by Npn-2-positive VNO neurons, two types of analyses were performed. In the first series of experiments, immunohistochemical analysis using Npn-2 antibodies was performed on parasagittal sections of AOB from adult mouse (Figure 7). In a second series of experiments, whole-mount X-Gal staining of *tau-lacZ*-expressing mice was used to assess the innervation pattern of the entire AOB (Figure 8).

In parasagittal sections of the AOB, Npn-2 protein was found exclusively in the anterior half of the AOB glomerular layer, where $G_{\alpha i2}$-positive neurons normally project (Figure 7A). Accordingly, AP-Sema3F binding sites were also found in the anterior, but not the posterior, AOB glomerular layer (Figure 7B). Additionally, in situ hybridization analysis revealed that *npn-2* is expressed throughout the mitral cell layer in adult AOB (data not shown). This is in contrast to the graded expression of Npn-2 protein in E18 and P1 AOBs (Figure

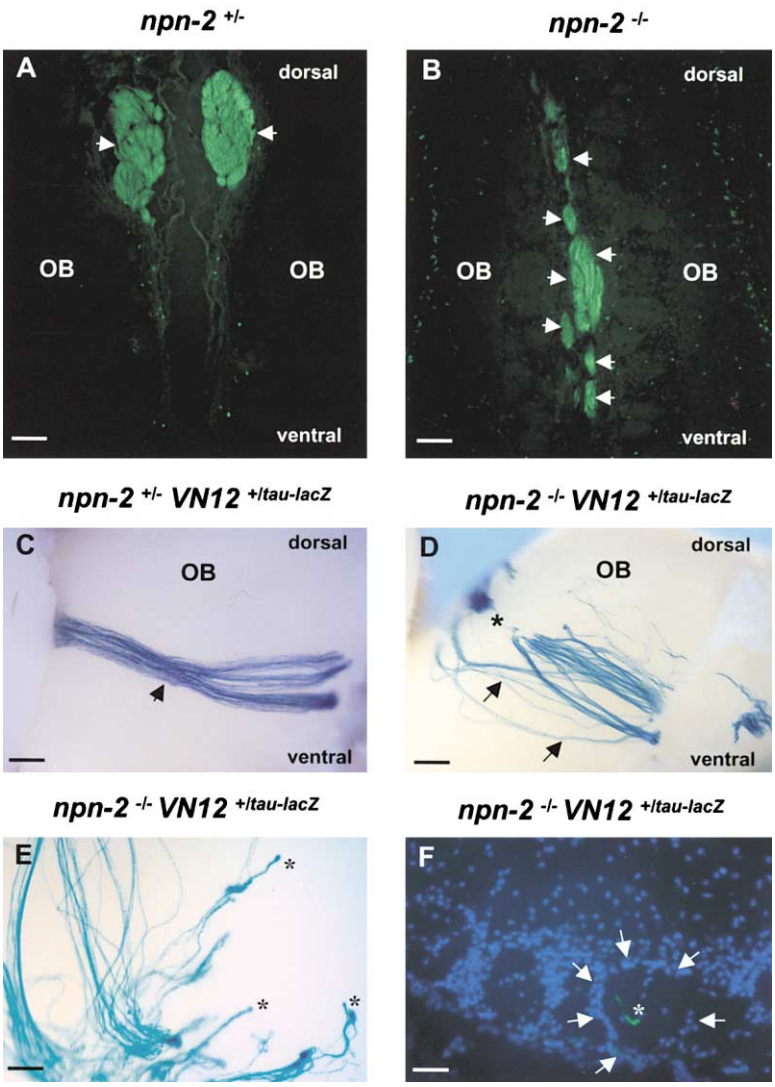


Figure 5. Defasciculation of the Vomeronasal Nerve in *npn-2*$^{-/-}$ Mice Leads to Ectopic Innervation of the Main Olfactory Bulb

(A and B) Coronal sections of olfactory bulbs at similar rostral-caudal axial levels from 3-month-old *npn-2*$^{+/-}$ (A) and *npn-2*$^{-/-}$ (B) stained with EC lectin. The large bundles of vomeronasal fibers observed in *npn-2*$^{+/-}$ mice are found to be defasciculated into several small bundles distributed over a large dorsal-ventral area in *npn-2*$^{-/-}$ mice. Arrowheads, vomeronasal nerve. n = 1 (+/+), 2 (+/−), and 3 (−/−). Scale bar: 30 μm.

(C and D) Whole-mount high-powered magnification view of the primary accessory olfactory projections stained with X-Gal in 3-month-old *npn-2*$^{+/-}$;*VN12*$^{+/tau\text{-}lacZ}$ (C) and *npn-2*$^{-/-}$;*VN12*$^{+/tau\text{-}lacZ}$ (D) mice. The arrowhead in (C) shows vomeronasal fibers projecting on the medial surface of the main olfactory bulb toward the AOB, while the arrows in (D) point to defasciculated fibers of the vomeronasal nerve. The asterisk in (D) denotes an area in which fibers were severed during the dissection procedure. OB, olfactory bulb. For *npn-2*;*VN12-tau-lacZ* mice, n = 2 (+/+), 2 (+/−), and 4 (−/−). For *npn-2*;*VN2-tau-lacZ* mice (data not shown), n = 1 (+/+), 2 (+/−), and 3 (−/−). Scale bar: 225 μm.

(E and F) Vomeronasal axons ectopically innervate the main olfactory bulb. Whole-mount high-magnification view of the vomeronasal nerve stained with X-Gal in a 3-month-old *npn-2*$^{-/-}$;*VN12*$^{+/tau\text{-}lacZ}$ mouse (E). The asterisk denotes vomeronasal fibers innervating the main olfactory bulb at a medial-dorsal level. The majority of ectopic innervations observed in *npn-2*$^{-/-}$ were found in the medial portion of the main olfactory bulb and occasionally in dorsal and ventral regions. (F) A high-magnification view of a section through the main olfactory bulb stained with anti-β-galactosidase to show glomerular innervation by vomeronasal axons and Hoechst stain to label nuclei of periglomerular cells (arrows) demarcates the boundaries of a single glomerulus. Ectopic innervation of the main olfactory bulb was observed in all *npn-2*$^{-/-}$ mice analyzed, while none of the *npn-2*$^{+/+}$ or *npn-2*$^{+/-}$ mice showed this phenotype. For *npn-2*;*VN12-tau-lacZ* mice, n = 2 (+/+), 3 (+/−), and 4 (−/−). For *npn-2*;*VN2-tau-lacZ* mice (data not shown), n = 1 (+/+), 3 (+/−), and 3 (−/−). Scale bars: 90 μm (F); 60 μm (G).

4). Thus, the restricted distribution of Npn-2 protein in the anterior half of the glomerular layer of the adult AOB is likely derived from the axon projections of Npn-2-positive/$G_{\alpha i2}$-positive VNO neurons, rather than from protein derived from AOB mitral cells.

To determine whether Npn-2 is necessary for the axonal projections of $G_{\alpha i2}$-expressing VNO neurons to the anterior region of the AOB, parasagittal sections of AOB from wild-type, *npn-2*$^{+/-}$, and *npn-2*$^{-/-}$ mice were immunostained with antibodies to OCAM, a cell adhesion molecule expressed exclusively on $G_{\alpha i2}$-positive VNO neurons (Yoshihara et al., 1997). The anterior-posterior border of the AOB was defined using BS lectin, which binds strongly to $G_{\alpha o}$-expressing axons that innervate the posterior AOB (Figures 7C and 7D) (Tanaka et al., 1999). In *npn-2*$^{+/-}$ mice, all OCAM-expressing fibers were found to innervate the anterior region of the AOB (Figure 7E). This observation is in accordance with previously reported results obtained in mouse and rat (von Campenhausen et al., 1997; Yoshihara et al., 1997). In contrast, a very large number of improperly targeted OCAM-positive fibers was detected in the posterior half of the AOB in *npn-2*$^{-/-}$ mice (Figure 7F). This effect is a result of aberrant axon targeting within the AOB, rather than disorganization of sensory neurons within the VNO itself, since the VNO of *npn-2*$^{-/-}$ mice had a normal structure and normal spatial patterns of expression of $G_{\alpha o}$, $G_{\alpha i2}$, and class 3 semaphorins (data not shown). Likewise, mitral cells in the AOB of *npn-2*$^{-/-}$ mice were clearly present and expressed normal levels of secreted semaphorins (data not shown).

Axonal projections originating from V1R-expressing VNO neurons have been shown to form complex glomerular maps in the anterior AOB. To assess the role of Npn-2 in the formation of these maps, we visualized the axonal projections of two populations of V1R-positive VNO neurons, respectively expressing VN2 and VN12 candidate pheromone receptors, in AOBs obtained from

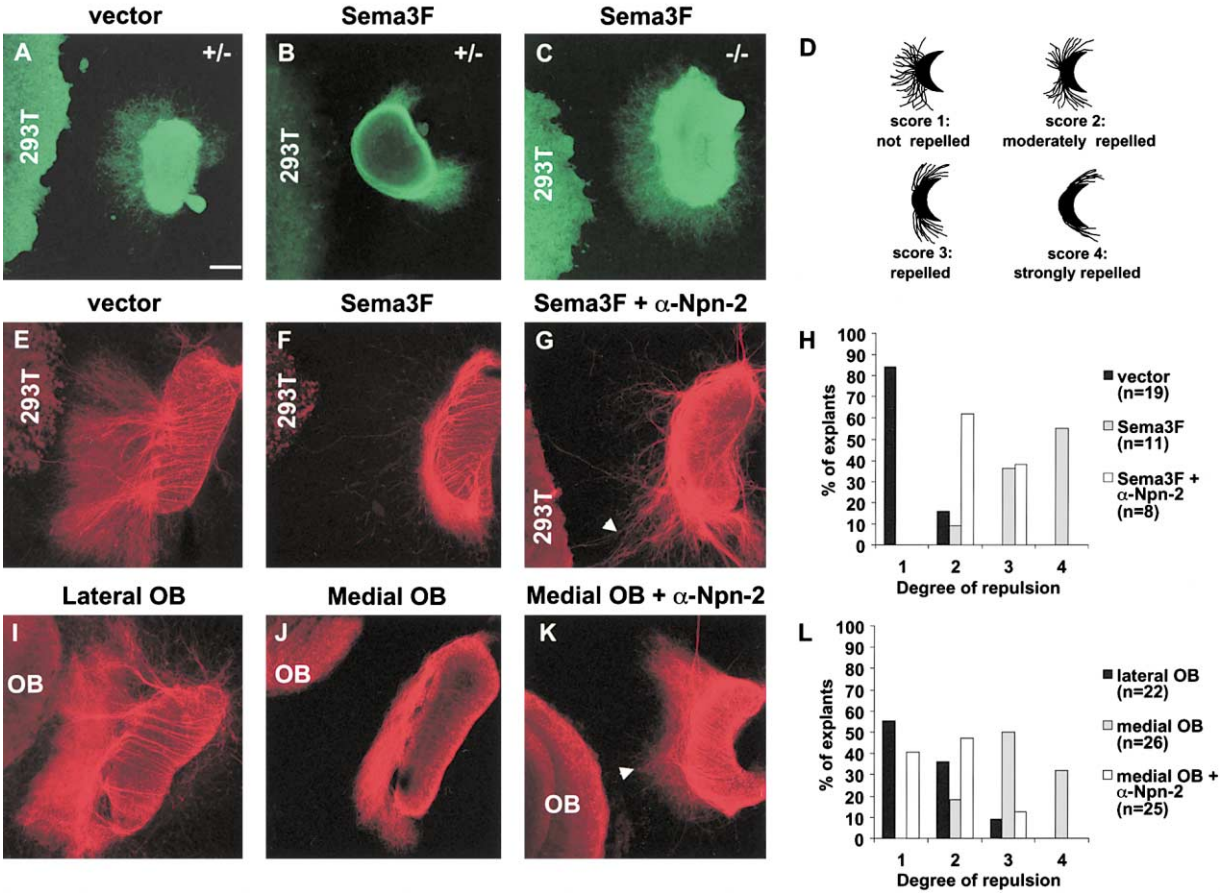


Figure 6. Axons of Vomeronasal Neurons Are Repelled by Sema3F and Are Selectively Responsive to a Npn-2 Ligand Secreted by the Medial Side of the Olfactory Bulb In Vitro

(A–C) Vomeronasal epithelia from *npn-2*$^{+/-}$ (A and B) or *npn-2*$^{-/-}$ (C) E14 mouse embryos were cocultured with 293T cell aggregates transfected with either a control (A) or a AP-Sema3F expression vector (B and C). Axons growing out of vomeronasal epithelia from *npn-2*$^{+/-}$ mice (A) are repelled by Sema3F, while axons from *npn-2*$^{-/-}$ mice (C) epithelia are unresponsive to Sema3F. n = 19 (+/+), 24 (+/−), and 3 (−/−). Scale bar: 80 μm.

(D–H) Vomeronasal epithelia from E15 rat embryos were cultured with 293T cell aggregates transfected with a control (E) or AP-sema3F expression vector (F and G). Like murine vomeronasal axons, rat vomeronasal axons are repelled by Sema3F (F). Addition of anti-Npn-2 to the growth medium partly blocked the Sema3F-induced repulsion (G). Arrowhead indicates nonrepelled fibers. Rat vomeronasal explants were scored blind by assigning each explant a score of one to four, representing the degree of repulsion observed as presented in (D). The percentage of explants assigned to each score is represented graphically in (H). Comparisons between vector and AP-Sema3F, $p < 0.0001$; between AP-Sema3F and anti-Npn-2, $p = 0.0001$. Scale bar: 40 μm.

(I–K) Vomeronasal epithelia from E15 rat embryos cultured in the proximity of the lateral (I) or medial (J and I) side of an olfactory bulb slice from P2 mice. Vomeronasal axons are repelled by factor(s) secreted by the medial side of the olfactory bulb (J), but are not repelled by the lateral side of the same bulb (I). Addition of anti-Npn-2 antibody to the growth medium partially inhibits the repulsion of vomeronasal axons by the medial portion of the olfactory bulb (K). Arrowhead indicates nonrepelled fibers. The explants were scored blind by two observers using criteria presented in (D). The percentage of explants assigned to each score by one of the observers is represented graphically in (L). Comparisons between responsiveness to the lateral and medial sides, $p < 0.0001$ for each observer; between medial and anti-Npn-2, $p < 0.0001$ for both observers. Scale bar: 40 μm.

*npn-2*$^{-/-}$*;VN2*$^{+/tau\text{-}lacZ}$ or *npn-2*$^{-/-}$*;VN12*$^{+/tau\text{-}lacZ}$ mice. Whole-mount X-Gal staining was performed on AOBs to compare the VN2-and VN12 projection maps in *npn-2*$^{+/-}$ and *npn-2*$^{-/-}$ mice. Detailed analysis of the number, location, and depth of blue glomeruli throughout the AOB allowed us to generate three-dimensional glomerular maps. These analyses revealed that β-gal-positive fibers appear almost evenly distributed between the anterior and the posterior halves of the AOB in *npn-2*$^{-/-}$ (Figures 8B–8C′ and 8E–8F′), whereas they are restricted to the anterior AOB in *npn-2*$^{+/-}$ and wild-type animals (Figures 8A, 8A′, 8D, and 8D′). This result demonstrates that Npn-2 plays a major role in restricting the projection of $G_{\alpha i2}$-positive VNO neurons to the anterior AOB. Furthermore, β-gal-positive fibers are seen converging into multiple glomeruli of the anterior AOB, suggesting that the absence of *npn-2* expression does not affect glomerular convergence of V1R-positive axons.

Taken together, these results indicate that *npn-2* is necessary for proper axonal targeting of $G_{\alpha i2}$-expressing neurons to the anterior region of the AOB. Are the axons originating from the $G_{\alpha o}$ population of VNO neurons similarly affected in the *npn-2* mutant? The accuracy of axonal projections from $G_{\alpha o}$-positive neurons to the poste-

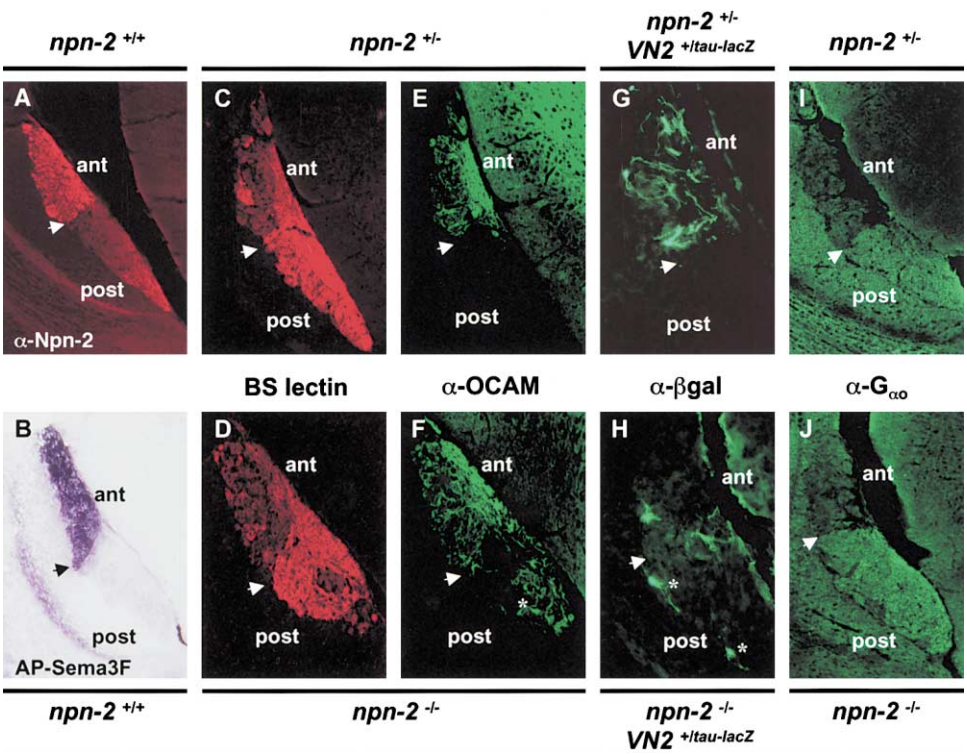


Figure 7. Loss of Zonal Targeting of Axons from $G_{\alpha i2}$-Expressing Vomeronasal Sensory Neurons within the Accessory Olfactory Bulb in *npn-2*$^{-/-}$ Mice

(A and B) Npn-2-expressing vomeronasal axons of wild-type mice innervate the anterior accessory olfactory bulb. Parasagittal sections of AOB from 2-month-old (A) or 2-week-old old *npn-2*$^{+/+}$ mice (B) stained with anti-Npn-2 (A) and AP-Sema3F (B). Scale bar: 120 μm.

(C–H) Parasagittal sections of AOB from 3-month-old *npn-2*$^{+/-}$ (C and E), *npn-2*$^{+/-}$ (D and F), *npn-2*$^{+/-}$;*VN2*$^{+/tau\text{-}lacZ}$ (G), and *npn-2*$^{-/-}$;*VN2*$^{+/tau\text{-}lacZ}$ (H) mice were stained with BS lectin (C and D), anti-OCAM (E and F), and anti-β-galactosidase (G and H). Projections of axons from $G_{\alpha i2}$-expressing neurons, detected using anti-OCAM (E and F) or anti-β-galactosidase (G and H) immunohistochemistry, are restricted to the anterior region of the AOB in *npn-2*$^{+/-}$ mice (E and I), while many of these fibers innervate the posterior AOB in *npn-2*$^{-/-}$ mice (F and H). An asterisk indicates ectopic OCAM or β-galactosidase-positive fibers in the posterior half of the AOB from *npn-2*$^{-/-}$ mice. Sections (C) and (E) and (D) and (F) represent pairs of the same section doubly stained. For anti-OCAM staining, n = 2 (+/+), 2 (+/−), and 4 (−/−). For anti-β-galactosidase staining, n = 2 (+/+), 3 (+/−), and 5 (−/−).

(I and J) Parasagittal sections of AOB from 3-month-old *npn-2*$^{+/-}$ (I) or *npn-2*$^{-/-}$ (J) mice were stained with an anti-$G_{\alpha o}$ antibody. In contrast to axons of $G_{\alpha i2}$-expressing neurons, axons of $G_{\alpha o}$-expressing neurons are properly restricted to the posterior region of the AOB in *npn-2*$^{-/-}$ mice. Arrowheads indicate the border between the anterior (ant) and posterior (post) regions of the AOB. n = 1 (+/+), 3 (+/−), and 4 (−/−).

rior AOB was assessed by anti-$G_{\alpha o}$ immunostaining and BS lectin binding in *npn-2*$^{+/-}$ and *npn-2*$^{-/-}$ mice. Our results show that $G_{\alpha o}$-expressing neurons are comparably restricted to the posterior half of the AOB in the *npn-2*$^{+/-}$ and *npn-2*$^{-/-}$ (Figures 7I and 7J). This observation is further supported by the similar binding we observe of BS lectin to AOBs from *npn-2*$^{+/-}$ and *npn-2*$^{-/-}$ mice (data not shown). Grossly disrupted targeting, therefore, appears restricted to $G_{\alpha i2}$-positive VNO neurons to which *npn-2* expression is normally confined.

### Vomeronasal Axons Are Responsive to a Npn-2 Ligand Secreted by the Posterior Half of the Accessory Olfactory Bulb

The ectopic innervation of the posterior half of the AOB by axons of $G_{\alpha i2}$-positive VNO neurons observed in *npn-2*$^{-/-}$ mice suggests that these fibers may normally be prevented from entering this region through Npn-2-dependent repulsion. To test this idea, we performed collagen gel coculture experiments in which E15 rat vomeronasal epithelia were grown in proximity to explants of either anterior or posterior AOBs isolated from early postnatal rat brains and then processed for immunostaining with anti-tau-1. Rat vomeronasal epithelia were used for these experiments because the poor outgrowth of axons from mouse vomeronasal epithelia precluded use of the mouse tissue for these experiments. While a large number of vomeronasal axons grew robustly into explants of anterior AOB (Figures 9A, 9B, and 9Q), they grew away from explants of posterior AOB (Figures 9E, 9F, and 9Q).

The axons observed projecting into the anterior AOB explants must emanate from the VNO epithelial explants for the following reasons. Fluorescent axons were detected in cocultures in which DiI crystals were placed in the VNO explant (Figure 9D). In contrast, fluorescent axons were never observed in cocultures in which DiI crystals were placed in the AOB explant (data not shown). Likewise, anti-tau staining of VNO/AOB cocultures at a time following axon outgrowth but prior to contacting the AOB revealed robust projections from the VNO, but never from the AOB (Figure 9C). Taken

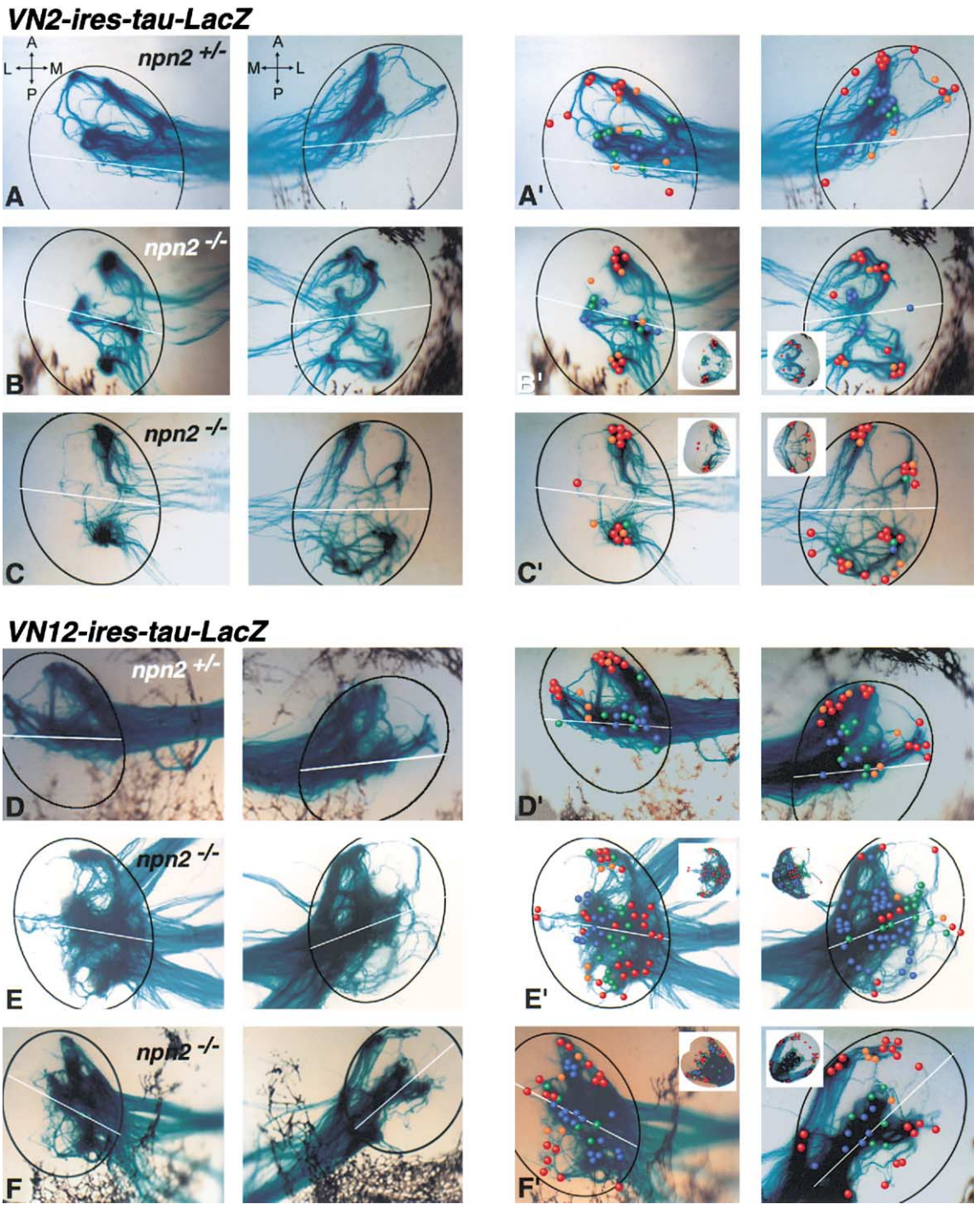


Figure 8. Spatial Distribution of Glomeruli Formed by VN2- and VN12-Expressing Vomeronasal Neurons in the AOB of *npn-2*$^{-/-}$ Mice

(A–F′) Whole-mount view of the AOB from 3-month-old *npn-2*$^{+/-}$;*VN2*$^{+/tau\text{-}lacZ}$ (A and A′), *npn-2*$^{-/-}$;*VN2*$^{+/tau\text{-}lacZ}$ (B–C′), *npn-2*$^{+/-}$;*VN12*$^{+/tau\text{-}lacZ}$ (D and D′), and *npn-2*$^{-/-}$;*VN12*$^{+/tau\text{-}lacZ}$ (E–F′). The spatial distribution and depth of glomeruli in the AOB glomerular layer is depicted using colored spheres where red is for a superficial, green is for a medium deep, and blue is for a deep glomerulus (Belluscio et al., 1999). Prospective AOB antero-posterior boundary is indicated by a white line. In *npn-2*$^{+/-}$ mice, VN2- and VN12-expressing vomeronasal fibers are segregated to the anterior half of the AOB and stereotypical glomerular mapping is observed (A, A′, D, and D′). In contrast, a large proportion of axons from VN2- and VN12-expressing vomeronasal neurons are misrouted to the posterior half of the AOB ([B–C′] and [E–F′]). Insets are computer generated maps of the pattern of posterior glomeruli expected if perfect mirror symmetry of anterior innervation is observed along the anterior-posterior boundary of the AOB (B′, C′, E′, and F′). A, anterior; P, posterior; L, lateral; M, medial. For *npn-2*;*VN2-tau-LacZ* mice, n = 1 (+/+), 5 (+/−), and 5 (−/−). For *npn-2*;*VN12-tau-LacZ* mice, n = 2 (+/+), 5 (+/−), and 6 (−/−).

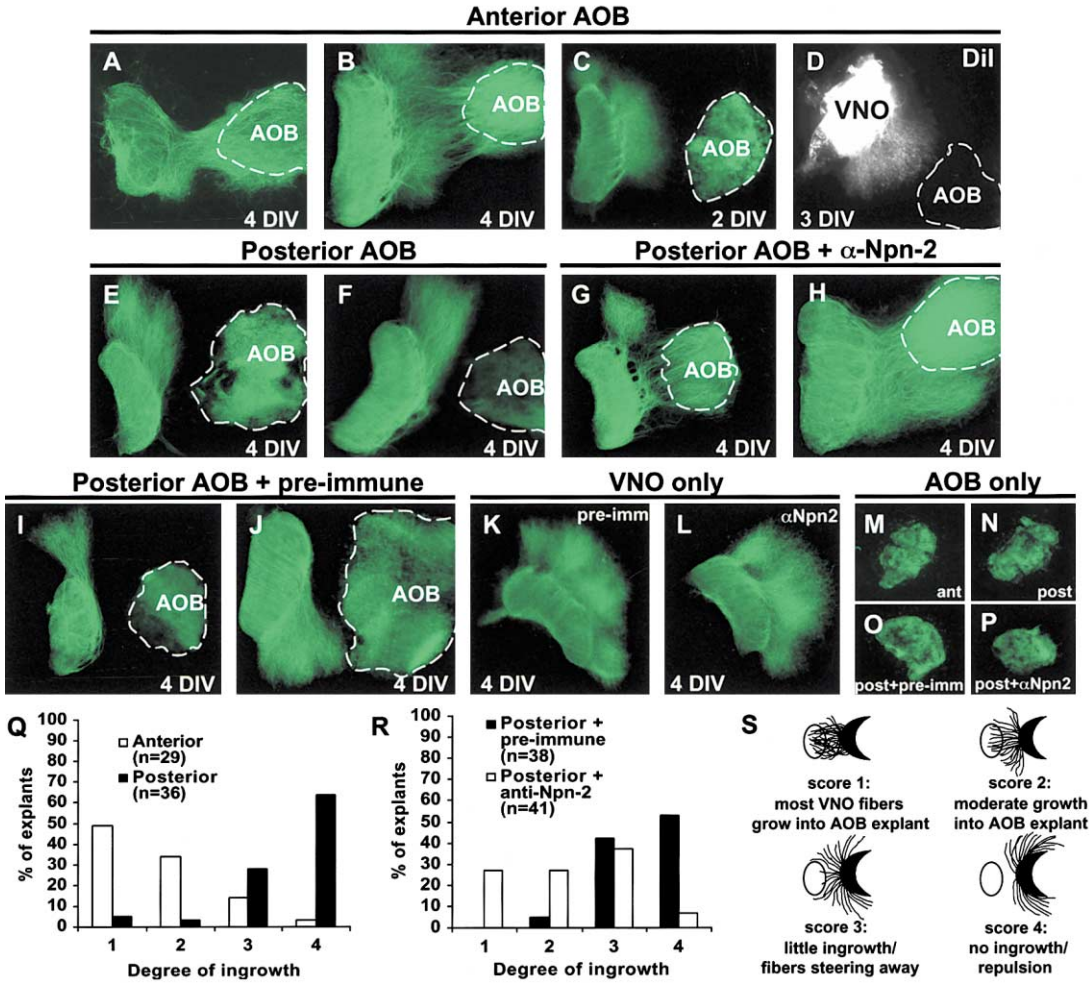


Figure 9. Axons of Vomeronasal Neurons Are Responsive to a Npn-2 Ligand Secreted by the Posterior Half of the Accessory Olfactory Bulb

(A–L) Vomeronasal epithelia isolated from E15 rat embryos were cocultured with pieces of the anterior (A–D) or posterior region (E–J) of the AOB isolated from P1–P3 rat brains. Axons emanating from the vomeronasal epithelia are repelled by factor(s) secreted by the posterior AOB explants (E, F, I, and J) but grow readily into anterior AOB explants (A and B). Axons emerging from the vomeronasal epithelium can be detected prior to reaching the AOB by labeling with DiI (D) or anti-tau-1 (C). Addition of anti-Npn-2 IgG (G and H), but not preimmune IgG (I and J), to the growth medium attenuates the repulsion of vomeronasal axons by the posterior AOB explants. Addition of anti-Npn-2 IgG did not affect the outgrowth of axons from the vomeronasal epithelia when compared to preimmune IgG (K and L), nor did it promote outgrowth of fibers from the posterior AOB (O and P). The extent of growth of vomeronasal neurons into the AOB explants were scored blind by two observers by assigning each explant a score of one to four, representing the degree of ingrowth into the AOB explant as depicted in (S). The percentage of explants assigned to each score is represented graphically in (Q) for the comparison between anterior and posterior AOB explants and in (R) for the comparison between preimmune and anti-Npn-2 treatments on repulsion by the posterior AOB. Differences between responsiveness to the anterior and posterior AOB ($p < 0.0001$ for each of two observers) and between preimmune and anti-Npn-2 is $p < 0.0001$ for each of two observers.

together, these results suggest that the posterior half of the AOB secretes a factor(s) capable of repelling Npn-2-positive vomeronasal neurons.

We next sought to determine whether the posterior AOB repellent acts in a Npn-2-dependent fashion. Thus, in another series of experiments, we grew vomeronasal epithelial explants in proximity to posterior pieces of the AOB in medium containing either preimmune IgG or anti-Npn-2 (Figures 9G–9J and 9R). Addition of anti-Npn-2 to the growth medium significantly attenuated the repulsive effect of the posterior AOB on vomeronasal neurons. Addition of anti-Npn-2 to the growth medium did not alter the extent of outgrowth of axons from the vomeronasal epithelia nor did it promote outgrowth of axonal projections from the posterior AOB (Figures 9K, 9L, and 9P). In combination, these results indicate that the posterior AOB secretes a factor(s) that normally repels vomeronasal neurons in a Npn-2-dependent manner.

## Discussion

Secreted semaphorins and their high-affinity receptors, the neuropilins, have been implicated in the development of the nervous system through their chemorepulsive effect on a wide variety of neuronal projections. Expression patterns of Npn-2 and its semaphorin ligands in the accessory olfactory system suggest that Npn-2 plays a role in guiding and patterning axons of

vomeronasal neurons. Consistent with this model, we have found that the secreted semaphorin Sema3F acts in vitro as a potent vomeronasal fiber repellent in a Npn-2-dependent manner. Importantly, in *npn-2*$^{-/-}$ mice, the vomeronasal nerve is defasciculated and some vomeronasal neurons ectopically innervate the MOB. Moreover, zonal segregation of axons from $G_{\alpha i2}$-expressing VNO neurons in the AOB is defective in *npn-2*$^{-/-}$ mice. Finally, the posterior AOB secretes a factor that repels axons of VNO neurons in a Npn-2-dependent manner. Taken together, our results show that Npn-2 and its secreted semaphorin ligands play multiple roles during the development of the accessory olfactory system, including regulating fasciculation of the vomeronasal nerve and zonal segregation of vomeronasal neuronal projections within the AOB.

### Npn-2 Is Required for Fasciculation of the Vomeronasal Nerve and for Segregation of the Accessory Olfactory System from the Main Olfactory System

Analysis of the vomeronasal nerve in *npn-2*$^{-/-}$ mice revealed that Npn-2 is required to maintain axons of vomeronasal neurons in a fasciculated state as they traverse the medial surface of the MOB. The defasciculation of the vomeronasal nerve observed in *npn-2*$^{-/-}$ mice might be due to the inability of Npn-2-deficient vomeronasal neurons to respond to a Npn-2 ligand expressed in the medial region of the MOB. Consistent with this idea, the medial side of the MOB repels vomeronasal fibers in a Npn-2-dependent fashion in vitro. In contrast, the lateral main olfactory bulb does not repel vomeronasal neurons. The Npn-2 ligand Sema3F is an excellent candidate medial MOB repellent because it is more highly expressed medially than laterally in the MOB, and in vitro Sema3F is a potent repellent for VNO neurons. Examination of the integrity of the vomeronasal nerve in mice harboring mutations in the genes encoding secreted semaphorins will establish the identity of the ligand(s) responsible for promoting fasciculation of the VN. How secreted semaphorins acting through Npn-2 promote fasciculation of the VN is still unclear. One possibility is that release of secreted semaphorins near the vomeronasal nerve forces VN bundling through surround repulsion. Alternatively, secreted semaphorins may regulate Npn-2-dependent adhesive properties of vomeronasal neuron axons.

The accessory and main olfactory systems are normally segregated, despite the fact that axons of their respective primary sensory neurons are in close proximity to one another as they extend through the cribriform plate toward their central targets in the AOB and MOB. How are the projections of the primary sensory neurons of the two olfactory systems kept separate? Our results indicate that Npn-2 is critical for preventing at least a subset of vomeronasal neuron axons from ectopically innervating the MOB. In *npn-2*$^{-/-}$ mice, some vomeronasal fibers were found to ectopically innervate the MOB. These aberrant fibers often appear to terminate in glomeruli, though further analysis is required to determine whether active synapses are being formed. Most of these errant fibers innervate the medial region of the MOB, although a minority of fibers were found to innervate more dorsal or ventral regions of the MOB. Sema3F expressed in the medial MOB is likely to prevent ectopic innervation of the MOB by vomeronasal fibers projecting to the AOB. Thus, our results support a model in which Npn-2 and its ligands are involved in maintaining the proper separation of the two olfactory systems.

Several studies have implicated the secreted semaphorin Sema3A, a Npn-1 ligand, in the guidance of main olfactory neurons (Kobayashi et al., 1997; Pasterkamp et al., 1999; Schwarting et al., 2000; Williams-Hogarth et al., 2000). Expression of a dominant-negative Npn-1 molecule in olfactory neurons in chick leads to overshooting of main olfactory neurons through *sema3A*-expressing structures during the early establishment of these projections (Renzi et al., 2000). Moreover, mice bearing a targeted deletion at the *sema3A* locus, though lethal at or near P1, show misrouting of Npn-1-positive fibers within the olfactory bulb in a pattern suggesting that Sema3A influences the establishment of these projections (Schwarting et al., 2000). Npn-1 and Npn-2 appear to be expressed in distinct populations of main olfactory receptor neurons based on the segregation of axons from Npn-1 and Npn-2-containing neurons in the MOB. Npn-2-expressing main olfactory neurons innervate the lateral region of the MOB at caudal levels, while Npn-1-expressing neurons more prominently innervate the medial region of the caudal MOB (Figure 3C; Pasterkamp et al., 1998; Nagao et al., 2000; Schwarting et al., 2000). We have also noted ectopic projections of olfactory marker protein (OMP)-expressing fibers in deeper layers of the MOB beyond the glomerular layer in *npn-2*$^{-/-}$ mice (our unpublished data). It is therefore probable that expression of a Npn-2 ligand in the medial MOB serves not only to prevent ectopic innervation of the MOB by vomeronasal neurons, but also to prevent innervation by a subset of Npn-2-expressing main olfactory neurons of the medial MOB. Combined with the observation that Sema3F can repel olfactory neurons in vitro (de Castro et al., 1999), it is likely that both Npn-1 and Npn-2 coordinately contribute to the proper wiring of the main olfactory system.

### Npn-2 Is Required for the Anterior-Posterior Zonal Segregation, but Not for Axonal Convergence into Glomerular Domains

Remarkably, as many as one-half of VN2- and VN12-expressing fibers were found to be misrouted to the posterior region of the AOB in *npn-2*$^{-/-}$ mutant mice. Yet, despite this incorrect segregation, fibers that were correctly targeted to the anterior AOB converge accurately into glomerular domains similar to those observed in wild-type mice. This is in marked contrast to the observation that axons of vomeronasal neurons lacking pheromone receptors segregate properly to the anterior half of the AOB but wander randomly in the anterior glomerular layer and do not form glomeruli (Belluscio et al., 1999; Rodriguez et al., 1999). Therefore, Npn-2 appears essential for proper segregation of $G_{\alpha i2}$-positive axons to the anterior AOB, but not for glomerular convergence. Interestingly, V1R-expressing apical VNO neurons mistargeted to the posterior AOB in *npn-2*$^{-/-}$ mutants appear to contact posterior AOB glomeruli in a nonrandom fashion, often exhibiting a striking pattern

of innervation that resembles a mirror image of that observed in the anterior AOB (Figure 8). Further analyses will address the suggestion from these data that a similar, Npn-2-independent, glomerular map is encoded in both zones of the AOB and whether or not V2R-expressing basal VNO neurons utilize related guidance cues to establish glomerular maps in the posterior AOB.

It is likely that other molecules provide guidance information within the AOB to direct axons to their appropriate targets. A recent report suggests that ephrins and Eph receptors contribute to guidance of vomeronasal projections (Knoll et al., 2001). Ephrin A3 and A5 are expressed in vomeronasal neurons, and EphB receptors are expressed in the AOB during embryogenesis. Mice harboring a deletion at the *ephrinA5* locus show some ectopic innervation of the posterior AOB by some VN12-containing vomeronasal neurons. Thus, semaphorins and ephrins may act in concert to restrict axon guidance of V1R-expressing neurons within the AOB. The nearly even distribution of V1R fibers to the anterior and posterior AOB in the *npn-2*$^{-/-}$ mutant strongly argues for Npn-2 playing a major role in this process.

The exact mechanism by which Npn-2 mediates segregation of axons of V1R neurons to the anterior half of the AOB remains unknown. There are several possibilities that could explain the mistargeting of these neurons to the posterior AOB in *npn-2*$^{-/-}$ mice. First, improper development or disorganization of the vomeronasal epithelium may be indirectly responsible for this defect. This seems unlikely, however, since in situ hybridization analyses on the VNO of *npn-2*$^{-/-}$ mice showed that $G_{\alpha i2}$- and $G_{\alpha o}$-positive neurons are properly restricted to the apical and basal zones of the VNO, respectively, and that secreted semaphorins and the V1R *tau-lacZ* marker also show identical distributions in the VNO in *npn-2*$^{+/+}$ and *npn-2*$^{-/-}$ mice (data not shown). A second possibility is that organization of fibers within the vomeronasal nerve itself could be critical for the ultimate segregation of axons in the AOB. Defasciculation of the vomeronasal nerve could lead to loss of this intrinsic organization that, in turn, could result in improper entry of fibers into the AOB and loss of zonal segregation of V1R axons. We do not favor this possibility either for several reasons. Immunostaining of coronal sections through the vomeronasal nerve of wild-type mice using BS lectin, OCAM, and $G_{\alpha o}$ antibodies at caudal levels of the MOB showed that $G_{\alpha i2}$- and $G_{\alpha o}$-positive neurons are not segregated into discrete bundles within the vomeronasal nerve (data not shown). Also, many V1R-expressing axons enter the AOB at the appropriate level, but they appear to make incorrect steering decisions within the AOB neuropil (see Figure 8). Furthermore, it appears that the entire vomeronasal nerve, including $G_{\alpha o}$-positive fibers, is defasciculated in *npn-2*$^{-/-}$ mice (Figure 5B). Despite this nerve defect, $G_{\alpha o}$-positive fibers appear to innervate only the posterior AOB in *npn-2*$^{-/-}$ mice; we did not observe loss of zonal segregation of these fibers. It is therefore unlikely that defasciculation of sub-bundles within the nerve is solely responsible for the incorrect segregation of *npn-2*$^{-/-}$ V1R-containing axons in the AOB.

Our results support a model in which segregation of V1R-expressing axons to the anterior region of the AOB is mediated by Npn-2 function on V1R-positive axons

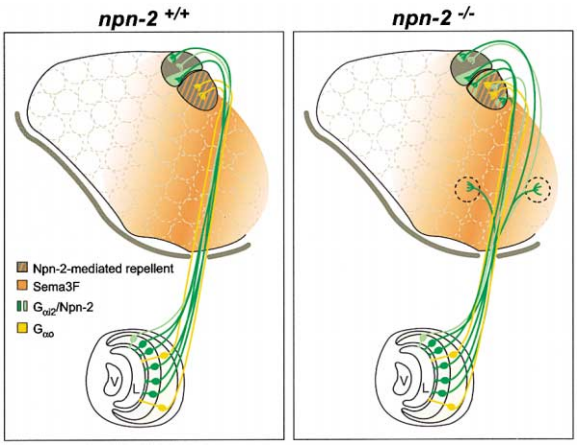


Figure 10. Npn-2 Is Required for Axonal Fasciculation and Targeting of Primary Accessory Olfactory Neurons

Representation of VNO projections in wild-type and *npn-2*$^{-/-}$ mice. $G_{\alpha i2}$-expressing (green) and $G_{\alpha o}$-expressing (orange) vomeronasal neurons project to the anterior and posterior halves of the AOB, respectively. Axons of vomeronasal neurons projecting to the AOB grow along the medial surface of the olfactory bulb. Npn-2 is required to maintain the nerve in a tightly fasciculated state. Expression of a chemorepellent Npn-2 ligand by the medial surface of the olfactory bulb may maintain the fasciculation of the VN. In addition, Npn-2 dependent responsiveness of vomeronasal neurons to this ligand prevents wandering of V1R-expressing neurons and ectopic innervation of the olfactory bulb by these neurons. By virtue of its expression pattern and in vitro repellent activity on vomeronasal neurons, the secreted semaphorin Sema3F (red shading) is a good candidate for regulating axonal fasciculation of the vomeronasal nerve. Axons of vomeronasal neurons innervate the AOB in a segregated fashion. Npn-2 is essential for the segregation of $G_{\alpha i2}$-expressing neurons to the anterior half of the AOB. A large proportion of axons from V1R neurons are misrouted to the posterior half of the AOB in *npn-2*$^{-/-}$ mice. Nevertheless, fibers correctly targeted to the anterior AOB converge accurately to form glomerular domains. In contrast, V2R-expressing neurons are properly targeted to the posterior half of the AOB in *npn-2*$^{-/-}$ mice. Potential molecular mechanisms regulating the segregation of vomeronasal neurons in the AOB are discussed in the text.

in the AOB (Figure 10). V1R axons express Npn-2, they respond to the Npn-2 ligand Sema3F, and as many as one-half of the V1R axons are mistargeted to the posterior half of the AOB in *npn-2*$^{-/-}$ animals. Importantly, in vitro coculture experiments indicate that the posterior AOB releases a chemorepellent ligand for Npn-2. What is the posterior AOB repellant? Our analysis of secreted semaphorin expression has so far not revealed any clear expression gradients in the mitral cell layer of the AOB that can obviously account for differential effects on neurons expressing Npn-2. Nonetheless, the possibility remains that a gradient of protein distribution for one or several secreted semaphorins exists. The lack of good immunological reagents to detect these proteins makes it difficult to address this possibility. An alternate possibility is that cells of the posterior AOB express a Npn-2 ligand other than Sema3F that repels V1R-positive axons.

While results of both in vivo and in vitro experiments strongly support a model in which Npn-2 function is required within V1R-expressing axons, we cannot rule out the possibility that Npn-2 on dendrites of mitral cells

of the AOB also contributes to segregation of axons within the AOB. Indeed, Npn-2 is more highly expressed in the mitral cell layer of the anterior AOB than posterior AOB during target innervation (Figure 4). Npn-2 on dendrites of anterior AOB mitral cells could facilitate ingrowth of axons of V1R neurons through sequestration of Npn-2 ligands within the glomerular layer of the anterior half of the AOB, which could, in turn, result in an anterior-posterior gradient of a repellent. Selective ablation of Npn-2 function in V1R-expressing neurons, as well as in target cells, will be needed to formally distinguish between these and other possibilities. Finally, since axons of V2R-expressing neurons do not express Npn-2, at least postnatally, and since they appear to properly innervate the posterior region of the AOB in *npn-2*$^{-/-}$ mice, a future challenge will be to identify guidance cues and their receptors that control zonal segregation of these projections.

Proper development of primary olfactory projections in the main olfactory system relies on the expression of odorant receptors in olfactory neurons and on their ability to respond to axonal guidance cues. It has now become clear that, as observed in the main olfactory system, establishment of connections in the primary accessory olfactory system is not only dependent on the expression of vomeronasal receptors in vomeronasal neurons, but also on their ability to process guidance information provided by their surroundings. Our results indicate that pheromone receptors and Npn-2 function independently and play complementary roles during patterning of primary accessory olfactory neuron projections.

## Experimental Procedures

### In Situ Hybridization and AP Fusion Protein Binding

Nonradioactive, dioxygenin-labeled cRNA probes with either sense or antisense orientation were synthesized by in vitro transcription. Probes synthesized included rat *sema3A* (Giger et al., 1996), mouse *sema3B*, mouse *sema3C*, rat *sema3F* (Giger et al., 2000), rat *npn-2* (Giger et al., 1998), and rat $G_{\alpha i}$ and $G_{\alpha o}$ (Jones and Reed, 1987). Sections from mouse embryos at E16 and E18 or postnatal mice at P5 and P15 were processed as previously described (Kolodkin et al. 1997). AP fusion protein binding to tissue sections was performed as described before (Giger et al., 1998).

### Immunohistochemical Procedures

Adult (3- to 4-month-old) mice were anesthetized and perfused transcardially with ice-cold PBS containing 4% paraformaldehyde. Brains were dissected, postfixed for 3 hr in perfusion solution, and cryoprotected overnight in PBS containing 30% sucrose. Cryosections (25 μm) were mounted on superfrost plus microscope slides, rinsed in TBS (50 mM Tris-HCl [pH 7.6] and 150 mM NaCl), and blocked for 2 hr in TNT (50 mM Tris-HCl [pH 7.6], 500 mM NaCl, and 0.5% Triton X-100) containing 10% fetal bovine serum (FBS). Sections were incubated overnight with primary antibody at 4°C in TNT/2% FBS using the following dilutions: anti-OCAM (1:1000) (a gift of Dr. Kensaku Mori), anti-β-galactosidase (1:500) (Sigma clone Gal-13); anti-GTP Binding Protein $G_{o\alpha}$ (1 μg/ml; Medical & Biological Laboratories Co.). After rinsing in TBS, primary antibody was detected with the appropriate oregon green or Cy3-conjugated secondary antibody (1:500; Molecular Probes) and either Fluorescein-Erythrina Cristagalli lectin (1:500; Vector Laboratories) or Rhodamine-Griffonia (Bandeiraea) Simplicifolia Lectin I (1:500; Vector Laboratories) in TNT containing 2% FBS. For anti-Npn-2 immunohistochemistry, E16 to 3-month-old tissue was processed as described above. Prior to the incubation with anti-Npn-2, sections were pretreated overnight at 60°C in 50% formamide, 2× SSC. Sections were rinsed several times in PBS and then blocked for 1 hr at room temperature in TBS containing 0.24% gelatin and 0.4% Triton X-100. Sections were incubated overnight at 4°C in blocking buffer containing purified Npn-2 IgG (2 mg/ml) diluted to 1:500. After several washes in PBS, sections were incubated for 2 hr at room temperature in blocking solution containing either goat anti-rabbit Ig-HRP (1:1000; Amersham) or alexa red conjugated goat anti-rabbit Ig (1:500, Molecular Probes).

### X-Gal Staining and Glomerular Mapping

Whole-mount X-Gal staining was performed as described (Belluscio et al., 1999).

### Collagen Gel Repulsion Assay

VNOs were dissected from rat E15 and mouse E14 embryos and incubated for 30 min on ice in a digestion solution prepared from a 1:1 mix of 0.25% trypsin dissolved in Versene and 10× pancreatin. The digestion was stopped by transferring the VNOs into L15 medium containing 50% FBS and incubating for 1 hr on ice. Vomeronasal epithelia were seperated from the surrounding mesenchymal tissue and kept on ice in L15 medium containing 5% FBS. Vomeronasal epithelia were cultured on glass cover slips coated with polylysine (50 μg/ml) and laminin (10 μg/ml) for 3–4 days, in close proximity to either 293T cell aggregates, mouse olfactory bulb slices, or pieces of rat AOB, as indicated in the figure legends. 293T cell aggregates were prepared as previously described. 300–400 μm olfactory bulb slices were prepared from P2 to P8 mouse brains using a vibratome. Slices of the rostral pole and most caudal part of the bulb were discarded. Of the remaining slices, dorsal-ventral and medial-lateral orientation was determined based on morphological criteria when the cocultures were assembled and embedded in collagen matrigel (5:1). For VNO-AOB coculture experiments, AOBs were dissected from P1 to P3 rat brains and cut into three pieces. The most anterior and posterior pieces were used in the experiments, while pieces containing the anterior-posterior border were discarded. Cultures were allowed to grow for 2–4 days in Neurobasal medium containing B27 and gentamycin before processing for immunostaining. The cultures were fixed in PFA (4%) for 1 hr, washed in PBS, and blocked in PHT (PBS containing horse serum [1%]; Triton X-100 [0.1%]). Primary antibodies were added to PHT containing Triton X-100 (0.3%) and incubated overnight at 4°C. Primary antibodies used included anti-tau-1(1:1000; Chemicon), anti-OCAM (1:1000), and anti-Npn-2 (1:500). The cultures were rinsed in PBS and then incubated with secondary FITC- or Cy3-coupled secondary antibody (1:1000) overnight at 4°C. The explants were rinsed in PBS and mounted in fluoromount. For DiI labeling of explants, cultures were fixed overnight in PFA (4%). Crystals of DiI were introduced into the vomeronasal epithelia and the cultures were incubated at 37°C for 3 days to allow diffusion of the tracer. Cultures were then rinsed in PBS and mounted in fluoromount. Confocal microscopy with z-stack analysis was performed to image the explants. For quantification of axon repulsion, each explant was given a score according to the degree of repulsion or ingrowth observed as represented in Figures 5D and 9A. This approach was used for scoring since the very small diameter of axons projecting from the vomeronasal epithelia prevented us from accurately tracing and counting/measuring individual neurons. The explants were scored blind by two independent observers and paired t test statistical analysis was performed.

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