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Olfactory Sensory Axons Expressing a Dominant–Negative Semaphorin Receptor Enter the CNS Early and Overshoot Their Target

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Summary

Sensory axons extend from the chick olfactory epithelium to the telencephalon well before the maturation of their target, the olfactory bulb. During a waiting period of several days, olfactory axons arrive and accumulate outside the CNS while the bulb differentiates beneath them. Semephorin-3A is expressed in the telencephalon during this period and has been proposed to prevent their entry into the CNS. We show that the misexpression of a dominant–negative neuropilin-1 that blocks SEMA-3A-mediated signaling in olfactory sensory axons induces many of them to enter the telencephalon prematurely and to overshoot the olfactory bulb. These results suggest that chemorepellents can prevent the premature innervation of immature targets.

Introduction

Growing axons are guided to their targets by specific guidance cues located in their environment. These can act as either attractants or repellents, and either at a distance as diffusible molecules or on contact as extracellular matrix or cell surface molecules. Many of these guidance cues are likely to bind to receptors on the growth cone surface and stimulate signaling pathways that affect growth cone motility. The semaphorins are a large family of signaling molecules, at least some of which can function during development as axonal guidance cues (for reviews, see Kolodkin, 1998; Raper 1999). Class 3 semaphorins are secreted proteins of about 120 kDa (for review, see Yu and Kolodkin, 1999). The best-characterized member of this class is SEMA-3A (semephorin-3A, formerly known as chick collapsin-1, human sema-III, mouse sema-D), which acts as a repellent for growth cones from specific neurons in vitro. These include DRG neurons (Luo et al., 1993), sympathetic neurons (Adams et al., 1997; Koppel et al., 1997), motor neurons (Shepherd et al., 1996; Varela-Echavarria et al., 1997), sensory neurons from the cranial nerve ganglia V and VII (Kobayashi et al., 1997), olfactory sensory neurons (Kobayashi et al., 1997), cortical neurons (Bagnard et al., 1998), and hippocampal neurons (Chedotal et al., 1998).

The ability of SEMA-3A to collapse and repel growth cones in vitro has suggested that it plays a role as a chemorepellent for specific axon tracts during development in vivo. For instance, sensory neurons from DRGs send axons into the spinal cord where they form synapses with their appropriate targets. SEMA-3A expression in the dorsal spinal cord has been hypothesized to prevent sensory afferents from entering the cord prematurely (Shepherd et al., 1997). Sensory axons enter only after SEMA-3A expression becomes restricted to the ventral cord. Later, ventral expression of SEMA-3A may help prevent most sensory axons from invading ventral regions of the spinal cord (Messersmith et al., 1995). However, the analysis of *Sema3a* knockout mice has provided little or no evidence that SEMA-3A plays a role in patterning sensory trajectories in the spinal cord (Behar et al., 1996; Taniguchi et al., 1997). Based on the distributions of SEMA-3A in the developing CNS, several other pathways might be expected to be perturbed by its absence. These include cerebellar mossy fiber projections, thalamocortical pathways, basal forebrain projections, and motor nerves. Thus far, these pathways also appear to project normally in *Sema3a* knockout mice (Catalano et al., 1998). One possible explanation for these observations is that other guidance molecules, possibly other class 3 secreted semaphorins, have overlapping distributions and functional activities that compensate for the loss of SEMA-3A. One possible approach that might overcome this problem would be to express a dominant–negative receptor in vivo that simultaneously blocks the functions of multiple class 3 semaphorins.

The receptors for class 3 semaphorins are just now being fully defined. Neuropilin-1 is a SEMA-3A binding protein that is absolutely required for SEMA-3A function (He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997). Neuropilin-1 binds other class 3 semaphorins with approximately equal affinities (Feiner et al., 1997) and is required for SEMA-3C function as well (Nakamura et al., 1998; Renzi et al., 1999). A second neuropilin family member, neuropilin-2, binds SEMA-3C and SEMA-3F with high affinity but binds poorly, if at all, to SEMA-3A. Neuropilin-2 is required for the repulsive effects of SEMA-3C and SEMA-3F on sympathetic neurons in vitro (Chen et al., 1998; Giger et al., 1998; Nakamura et al., 1998). Neuropilins probably do not mediate ligand signaling on their own and appear to require additional receptor components for this purpose. Members of the plexin family of transmembrane proteins are likely candidates for these additional receptor components (Takahashi et al., 1999; Tamagnone et al., 1999). Plexin-1 forms stable complexes with either neuropilin-1 or neuropilin-2. SEMA-3A binds to plexin-1/neuropilin-1 complexes expressed in Cos cells and induces a change in their cell morphology analogous to growth cone collapse. A dominant–negative plexin-1 can block SEMA-3A-induced collapse of sensory growth cones in vitro (Takahashi et al., 1999). Semaphorin receptors are therefore likely to consist of one or both neuropilins that bind and present specific semaphorins to one or more plexins thereby initiating a biological response. The information

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that is currently available suggests that neuropilin-1 presents SEMA-3A, neuropilin-2 presents SEMA-3F, and neuropilin-1 and -2 together present SEMA-3C (Chen et al., 1998; Giger et al., 1998; Nakamura et al., 1998).

We have previously described a dominant–negative receptor strategy to block the function of class 3 semaphorins (Renzi et al., 1999). Sympathetic neurons are normally repelled by SEMA-3A, SEMA-3C, and SEMA-3F. When caused to express a truncated neuropilin-1 that is missing a specific extracellular domain, growth cones from sympathetic neurons are no longer responsive to SEMA-3A and SEMA-3C, but remain responsive to SEMA-3F. This truncated form of neuropilin-1 (dnNP-1) therefore acts as a dominant–negative receptor for specific secreted semaphorins. A dominant–negative approach to blocking semaphorin function might be advantageous since multiple family members with similar biological functions can be blocked all at once. This could be important if the loss of a single semaphorin is partially compensated for by other family members with similar functions.

In this paper, we report that expressing dnNP-1 in olfactory sensory axons alters their trajectories when they reach the telencephalon. Instead of pausing at the surface of the brain and waiting for their target, the olfactory bulb, to mature as they normally would; dnNP-1-expressing axons enter the brain prematurely and overshoot the area that will become their appropriate target. These results suggest that class 3 semaphorins act as repellents in vivo and prevent axons from entering their target prematurely.

Results

Transfection of Olfactory Sensory Neurons Using In Ovo Electroporation

The expression of dnNP-1 was induced in embryonic chicks by electroporating an appropriate eukaryotic expression plasmid in ovo. This method of misexpressing genes in the chick has several advantages over avian retroviral vectors. These include: (1) high levels of recombinant protein are produced within 8 hr of electroporation, (2) large recombinant proteins can be produced since larger coding inserts are better tolerated than with viral vectors, (3) there are no reported limits to the cell types that can be transfected, and (4) expression of recombinant proteins is restricted to transfected cells and their progeny. The transfection of olfactory neurons within the olfactory epithelium is possible since the olfactory placode that gives rise to the olfactory epithelium is derived from superficial ectoderm and is accessible to plasmid DNA delivered from outside the embryo.

A chimeric protein composed of human placental alkaline phosphatase (AP) fused to the transmembrane and cytoplasmic portions of chick NP-1 was used as a tracer construct to identify transfected cells and visualize their axonal processes. Expression of this AP-tagged marker does not affect semaphorin signaling in cultured sympathetic neurons (data not shown). It was cotransfected in a 1:10 ratio with either a plasmid carrying the β-galactosidase reporter gene as a control (AP+β-gal), a plasmid containing full-length neuropilin-1 (AP+NP-1), or a plasmid containing a myc-tagged dnNP-1 (AP+dnNP-1).

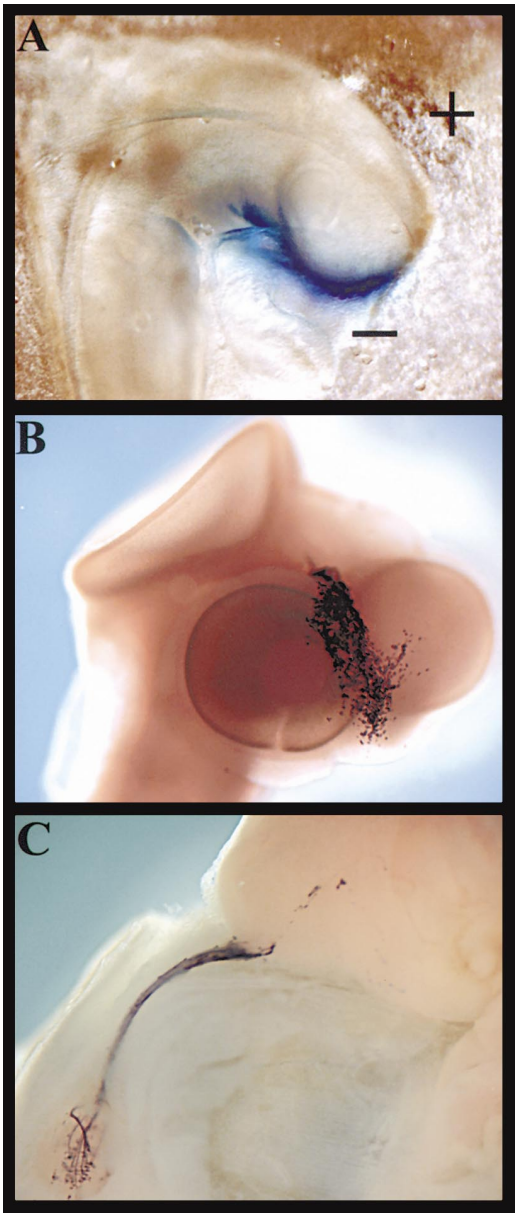

Figure 1. Transfection of the Olfactory Epithelium in the Embryonic Chick Using In Ovo Electroporation

(A) A stage 13 embryo marked with blue dye to show the injection site beneath the amniotic membrane and adjacent to the nasal pit. The electrodes (+, −) were placed 5 mm apart and positioned as shown.

(B) Distribution of transfected cells in E4 whole-mount embryo. AP-labeled transfected cells can be seen in and around the nasal pit.

(C) A whole-mount preparation of an E6 embryo bisected at the midline and viewed from the medial surface. Labeled olfactory axons leave the olfactory epithelium at lower left and project within the olfactory nerve to the nascent olfactory bulb.

Plasmid DNA was injected into the amniotic sac adjacent to the nasal pit of stage 13 embryos (E2) and electroporated with the electrodes oriented to force the plasmid toward the embryo (Figure 1A). Visualization of the

AP marker 2 days later showed transfection of ectodermal cells in and around the nasal pit and in the lens of the eye (Figure 1B). At later ages, the labeled axons of olfactory sensory neurons could be seen leaving the olfactory epithelium to form the olfactory nerve (Figure 1C). Other structures that are, at least in part, derived from the ectoderm were also transfected. These included the lens of the eye as well as the trigeminal, vagal, and glossopharyngeal ganglia (data not shown).

Electroporation resulted in a large amount of embryo mortality. In individual experiments, as many as 50% of the treated embryos died within 3 days of electroporation. The survival rate decreased further with time, falling to as low as 20% by 7 days post treatment. This high lethality is unlikely caused by electroporation itself or the incorporation of expression plasmids into embryonic tissues. Similar rates of lethality are observed with uninjected embryos electroporated with 0 volts. Surviving embryos were found to be normal upon gross inspection, and the axonal trajectories of olfactory sensory axons in embryos electroporated with control constructs appeared normal (see below).

Olfactory Axons Expressing dnNP-1 Overshoot Their Normal Target

The first olfactory axons exit the olfactory epithelium and reach the telencephalon by E5 where the vast majority of them halt for several days before entering the CNS (Figure 2A). As olfactory axons accumulate outside the CNS, the olfactory bulb evaginates from the telencephalon and differentiates beneath them (Kobayashi et al., 1997). Olfactory axons enter the bulb at E8—first projecting into the superficial olfactory nerve fiber layer (ONL), and then projecting into and making synaptic connections within the deeper glomerular layer. To determine if the expression of dnNP-1 in sensory axons interferes with their guidance, the trajectories of olfactory axons cotransfected with AP+βgal, AP+NP-1, or AP+dnNP-1 plasmids were compared in E7 embryos.

Olfactory axons normally make contact with the surface of the telencephalon without entering the CNS by E7 (Figure 2B). Olfactory axons transfected with AP tracer tag plus β-galactosidase (AP+β-gal) were seen to exit the olfactory epithelium, join the olfactory nerve, and extend to the telencephalon. At E7, the majority of these axons were found to terminate just outside of the rostral-most telencephalon where the olfactory bulb evaginates (Figures 3A, 3C, and 3E). In some of these control embryos, occasional axons were observed extending beyond the point where the rest of the olfactory axons had stopped (see Figure 3E). In embryos where the bulb had already begun to form, olfactory axons covered its surface but did not cross the border between the olfactory bulb and the forebrain (Figure 3C). Migrating cells transfected with AP+β-gal were observed migrating beyond the point at which sensory axons terminate. The majority of these cells were located along a specific pathway that extended dorsocaudally from the olfactory nerve for some distance before diving ventrally toward the midbrain.

Olfactory sensory axons cotransfected with AP tracer tag plus dnNP-1 (AP+dnNP-1), like controls, entered the olfactory nerve and projected to the telencephalon.

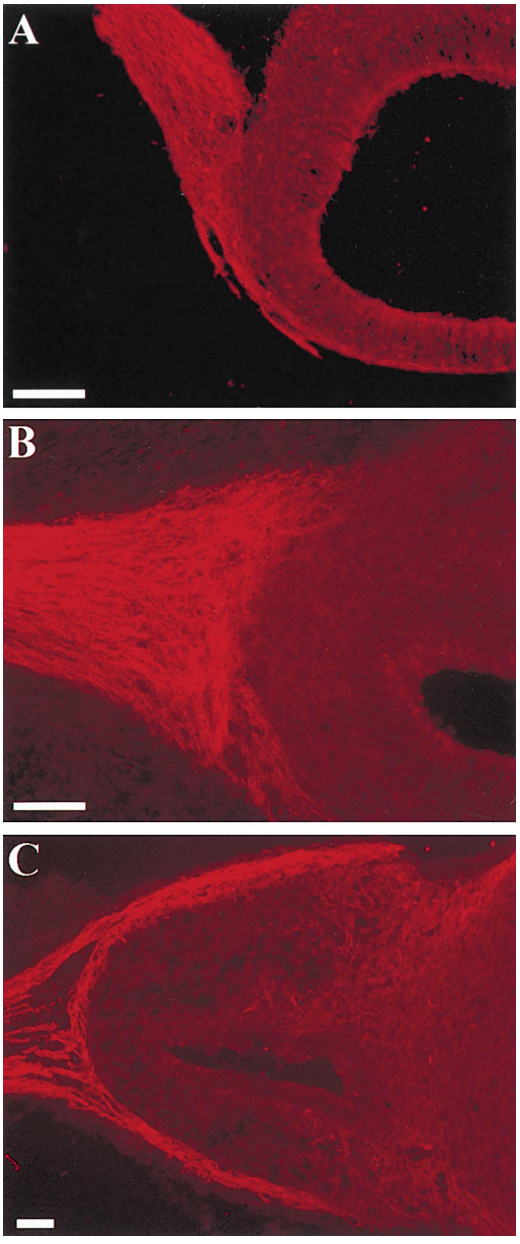

Figure 2. Progressive Development of the Olfactory Nerve in the Embryonic Chick

Coronal sections through the developing olfactory nerve and olfactory sensory axons were visualized using an anti-neurofilament antibody and a Cy3-conjugated secondary antibody.

(A) At E5, sensory axons have grown out of the olfactory epithelium and crossed the intervening mesenchyme to reach the telencephalon. At this stage, axons stop upon contact with the surface of the telencephalon and do not enter the CNS.

(B) E7 olfactory axons continue to project to and accumulate on the surface of the telencephalon.

(C) By E9, the olfactory bulb has formed and olfactory axons form the olfactory nerve layer.

Scale bar: 100 μm.

Their trajectories within the olfactory nerve appeared indistinguishable from those transfected with β-gal. Although the majority of AP+dnNP-1-transfected axons were found to terminate on the surface of the telenceph-

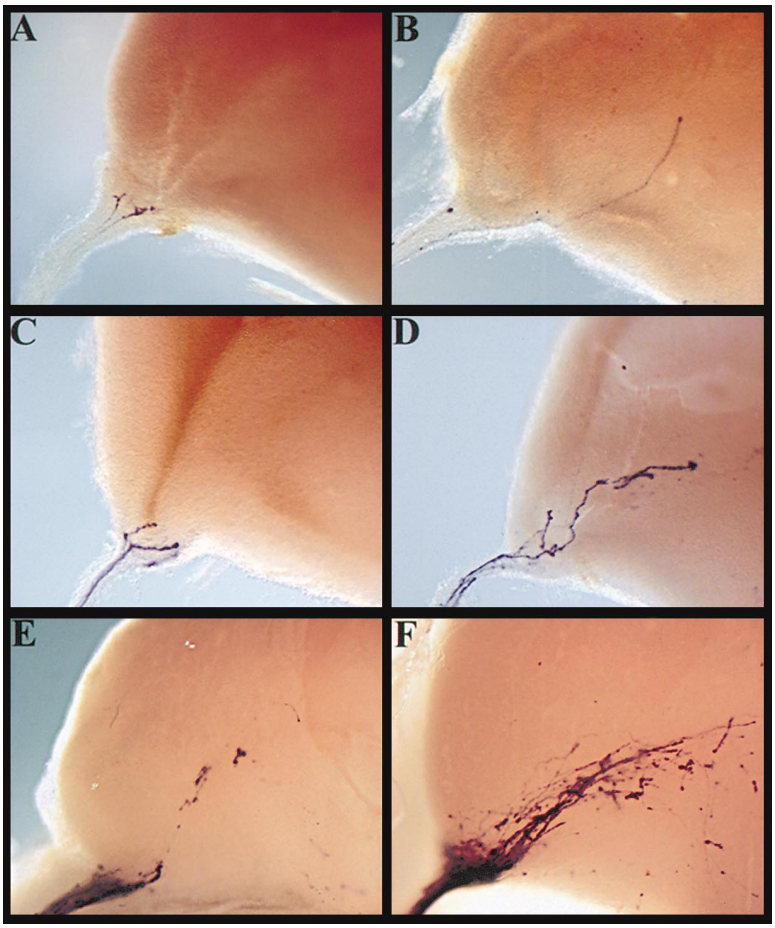

Figure 3. Olfactory Axons Expressing dnNP-1 Are More Likely to Overshoot their Target

Whole-mount images of E7 chick brains showing AP-labeled axons (seen in purple) extending in the olfactory nerve to the telencephalon. The majority of olfactory axons expressing AP+β-gal (A, C, and E) stop outside of the telencephalon and do not enter the CNS. Occasionally, a single axon expressing AP+β-gal grew past the surface of the telencephalon and into the CNS. Olfactory axons expressing dnNP-1 (B, D, and F) show an increased tendency to enter and extend within the telencephalon.

alon in their normal position, a greatly increased number as compared to controls was seen to overshoot this stopping point (Figures 3B, 3D, and 3F). When examined in whole mount, the overshooting axons appeared to extend on, or just below, the surface of the telencephalon. The trajectories of these escaping axons varied considerably, but the overwhelming majority grew on the medial side of the nascent olfactory bulb and projected caudally along the medial surface of the forebrain. In contrast, two embryos from a total of 16 each had a large fascicle of AP+dnNP-1-transfected axons that projected some distance outside of the lateral surface of the telencephalon. These fascicles of axons appeared to break off from the main olfactory nerve prior to contact with the telencephalon. Cells transfected with AP+dnNP-1 were found migrating in the telencephalon along a route similar to that seen in controls (Figure 3F).

Olfactory sensory axons cotransfected with AP tracer tag plus full-length neuropilin-1 (AP+NP-1) behaved just as control (AP+β-gal) axons did. The overexpression of full-length neuropilin-1 does not enhance or reduce the responsiveness of cultured sympathetic axons to SEMA-3A (Renzi et al., 1999). Since the dnNP-1 is a simple deletion construct made from full-length neuropilin-1, the overexpression of NP-1 on olfactory sensory axons should reproduce any effects that dnNP-1 expression might have beyond its ability to neutralize semaphorin signaling. The aberrant trajectories of olfactory sensory axons expressing dnNP-1 therefore most likely result from the blockade of semaphorin signaling.

To quantify dnNP-1-induced overshooting of the target, we compared the number of labeled axons overshooting in AP+βgal-, AP+NP-1-, and AP+dnNP-1-transfected embryos. Since more axons were observed to overshoot when many labeled axons arrived at the target, data are expressed as the number of overshooting axons in relation to the total number of labeled olfactory axons in each preparation (Figure 4). Transfected axons could be counted accurately due to their small numbers; however, it is possible that the number of arriving axons might occasionally have been underestimated when they fasciculated closely. In AP+β-gal-transfected embryos, no overshooting axons were observed when fewer than four transfected axons reached the telencephalon. The number of overshooting axons did not exceed two in any of the AP+β-gal-transfected embryos analyzed. Identical outcomes were observed for axons transfected with the full-length neuropilin-1 construct, although as many as three or four aberrantly projecting axons were observed in embryos with an unusually large number of transfected axons. In contrast, dnNP-1-expressing olfactory axons were observed to overshoot their target when as few as two of them reached the telencephalon. The number of overshooting axons increased dramatically as more transfected olfactory axons reached the telencephalon. An

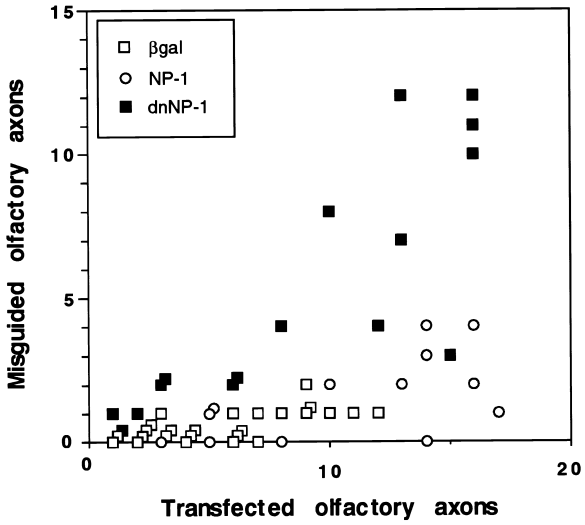

Figure 4. Quantification of Axon Guidance Errors in Transfected Olfactory Axons

The total number of misprojecting olfactory axons (y axis) is compared to the total number of AP-labeled axons (x axis) in embryos cotransfected with AP+β-gal (open squares), AP+NP-1 (open circles), or AP+dnNP-1 (closed squares). Regardless of overall transfection levels, axons transfected with dnNP-1 showed a substantially greater number of errors than did control axons. An ANOVA analysis indicates that dnNP-1-expressing axons are significantly misguided as compared to the combined controls at a level of significance of $p \leq 0.0001$.

ANOVA analysis of the data indicates that the frequencies with which β-gal-expressing and full-length neuropilin-1-expressing axons overshoot their target are not significantly different, but that overshooting of dominant–negative expressing axons is greater as compared to the two control conditions taken together at a significance level of $p \leq 0.0001$.

Overshooting Olfactory Axons Enter the Telencephalon Prematurely

AP+dnNP-1-transfected embryos were sectioned and counterstained with an anti-neurofilament antibody to determine if overshooting olfactory axons entered into the telencephalon or grew upon its surface. Three embryos were selected representing the usual experimental result in which AP-labeled, dnNP-1-expressing overshooting axons were defasciculated and extended largely on the medial side of the telencephalon. Labeled axons were found to extend within the CNS in these embryos. They grew in the most superficial layers of the telencephalon just beneath the pial surface (Figures 5B and 5C). A fourth embryo was selected to represent the two experimental cases in which AP-labeled, dnNP-1-expressing, overshooting axons were highly fasciculated and extended on the lateral surface of the telencephalon. The labeled axons in this embryo were found to extend outside the pial membrane on the surface of the brain. Labeled axons were bundled together with additional unlabeled axons that may have originated in the olfactory epithelium (Figures 5E and 5F).

dnNP-1-Induced Misprojection of Olfactory Axons Persists in E9 Embryos

We next examined the trajectories of olfactory axons in E9 embryos transfected with either AP+β-gal or AP+dnNP-1 to see if overshooting axons survived to later ages and/or converged on inappropriate secondary targets. Because survival to this late age was rare after transfection, only four embryos in each treatment group were analyzed. Olfactory axons transfected with AP+β-gal extended to the nascent olfactory bulb and terminated on its surface. No labeled axons were seen to extend past the caudal margin of the olfactory bulb and into the forebrain (Figures 6A and 7A). In contrast, all of the four E9 embryos transfected with AP+dnNP-1 contained at least one labeled axon that had overshot the olfactory bulb and extended into the forebrain (Figures 6B and 7D). One particularly dramatic aberrant projection appeared to extend the entire length of the forebrain, turning and branching multiple times.

dnNP-1-Expressing Axons Are Present in the Olfactory Nerve Fiber Layer

Once the olfactory bulb evaginates from the telencephalon, olfactory axons ramify to form the olfactory nerve fiber layer (ONL) (Figure 2C). To determine if olfactory axons-expressing dnNP-1 remain in this layer or instead enter inappropriate deeper layers, olfactory bulbs from two E9 embryos cotransfected with AP+β-gal and two E9 embryos cotransfected with AP+dnNP-1 were sectioned and costained with an anti-neurofilament antibody. Olfactory axons transfected with AP+β-gal were located within the ONL (Figures 7B and 7C). Serial sections through a single bulb showed that these axons were distributed throughout the ONL (data not shown). Olfactory axons transfected with AP+dnNP-1 were also located in the ONL. A section containing an overshooting olfactory axon shows that it extended in the deepest portion of the ONL (Figures 7E and 7F). In this limited sample size, we did not detect dnNP-1-expressing axons that projected abnormally into inappropriate deeper layers of the bulb.

SEMA-3A Is the Most Likely Candidate for a Repellent that Prevents Olfactory Axons from Entering the Telencephalon

Olfactory axons express neuropilin-1, and SEMA-3A induces the collapse of their growth cones in vitro. SEMA-3A expression in the telencephalon makes it an attractive candidate for a repellent that keeps these axons from entering the telencephalon prematurely (Kobayashi et al., 1997). To investigate the possibility that other class 3 semaphorins might have comparable repellent functions for olfactory axons, we determined whether SEMA-3C, SEMA-3D, or SEMA-3E is expressed in the telencephalon during the time period that olfactory axons are halted at its surface. Along with SEMA-3A, these represent all of the currently identified class 3 chick semaphorins.

When olfactory axons are just beginning to reach the telencephalon at E5, SEMA-3A expression is seen in the olfactory epithelium and in cells of the most superficial layer of the telencephalon. SEMA-3C and SEMA-3E expression are seen in deep layers of the telencephalon.

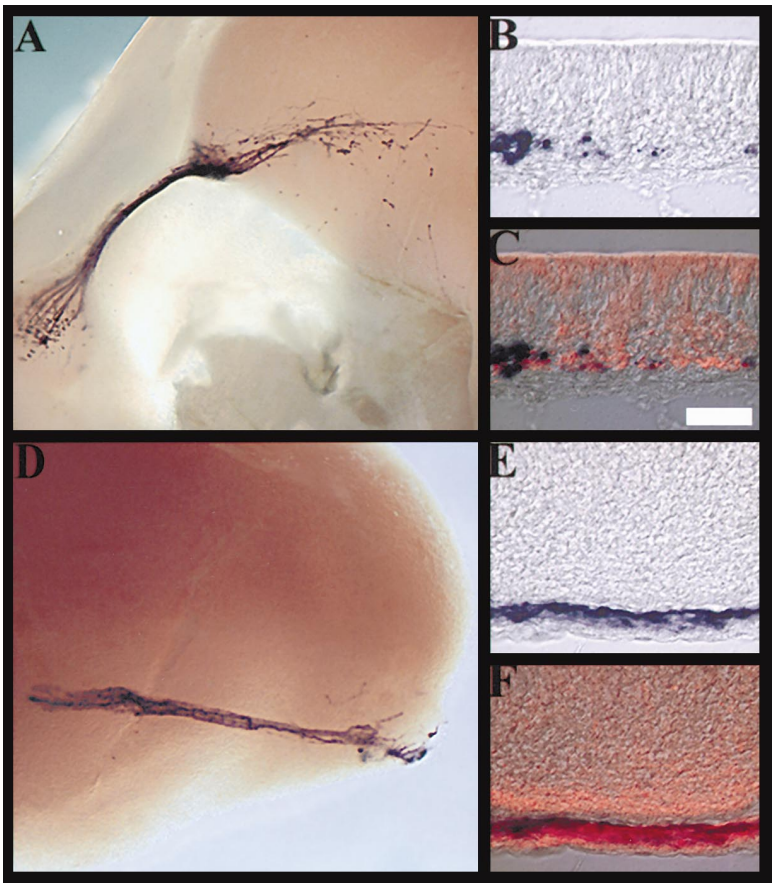

Figure 5. dnNP-1-Expressing Axons that Overshoot Their Target Grow into the Telencephalon

The trajectories of olfactory axons expressing dnNP-1 were reacted with AP histochemistry and examined in whole mounts at E7 (A and D). The brains were then sectioned and probed with anti-neurofilament antibodies to visualize axons within the CNS (C and F). The majority of overshooting axons extended on the medial side of the forebrain. In rare instances (2 of 16 embryos), a large bundle of axons overshot on the lateral side (D). Bright field (B), and a composite section of bright field and fluorescence (C), show axons overshooting on the medial side extend within the telencephalon just below the pial surface. A bright field (E) and a composite section (F) show axons overshooting on the lateral side extended outside the CNS. Scale bar for (B), (C), (E), and (F): 20 μm.

SEMA-3D expression is not detectable in the E5 telencephalon (data not shown). By E7, SEMA-3A expression is observed in superficial layers throughout the telencephalon including its point of contact with olfactory axons (Figure 8). SEMA-3C, SEMA-3D, and SEMA-3E are no longer expressed in the telencephalon, but all are expressed very weakly by cells located within the olfactory nerve itself (Figure 8). SEMA-3D is also expressed by a subset of cells in the olfactory epithelium (data not shown). By E9, olfactory axons ramify within the olfactory nerve layer in the bulb. At this age, SEMA-3A is no longer expressed on the surface of the bulb but is restricted to deeper layers (Kobayashi et al., 1997). SEMA-3C is expressed in a cluster of cells located adjacent to the olfactory nerve entry point, and SEMA-3D is expressed within or just below the developing ONL. No SEMA-3E expression was detected in the E9 bulb (data not shown). These results indicate that of these class 3 semaphorins, only SEMA-3A is expressed in the correct position at the appropriate time to provide a repellent signal that would prevent olfactory axons from extending into the telencephalon.

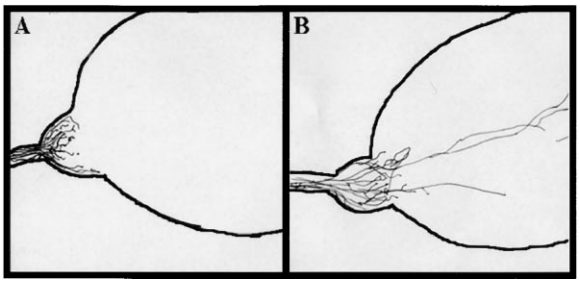

Figure 6. Overshooting Olfactory Axons Persist in E9 Embryos

The trajectories of olfactory axons from four E9 embryos cotransfected with AP+β-gal compared to those of four E9 embryos cotransfected with AP+dnNP-1.

(A) Composite sketch of labeled axon trajectories from four embryos transfected with AP+β-gal demonstrate that they project to the superficial layers of the nascent olfactory bulb. No labeled axons were found extending beyond the olfactory bulb/forebrain border.

(B) Axons expressing dnNP-1 covered the surface of the nascent olfactory bulb, and some of these axons overshot the bulb/forebrain border and grew extensively over the surface of the brain.

Discussion

The developing olfactory system is an ideal system in which to study the mechanisms that control axon guidance. It is made up of a relatively homogeneous population of sensory cells that project to a recognizable target, and its development has been extensively characterized in rats (Santacana et al., 1992), mice (Doucette, 1989), frogs (Byrd and Burd, 1993a, 1993b), and chickens (Drapkin and Silverman, 1999).

Primary sensory neurons are located in the olfactory epithelium that is derived from the olfactory placode. The olfactory placode invaginates from the surface of the chick embryo to form the nasal pit beginning at stage 18. The first olfactory axons begin to grow out of the

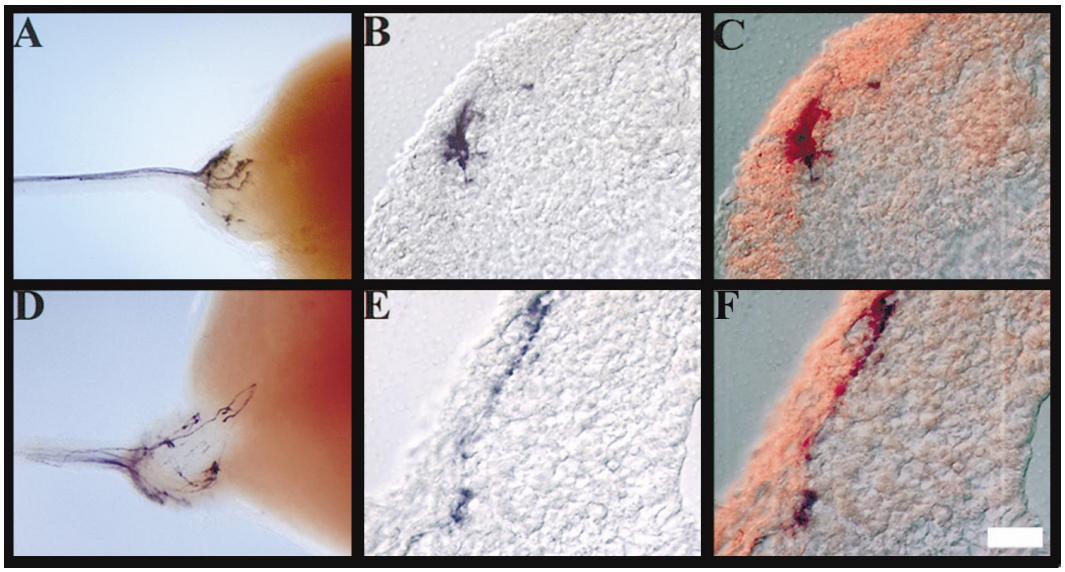

Figure 7. dnNP-1-Expressing Axons Are Confined to the Olfactory Nerve Fiber Layer

Whole-mount views of E9 olfactory axon projections in embryos cotransfected with AP+β-gal (A) and AP+dnNP-1 (D). Bright-field images (B and E) and composites of bright-field and fluorescent images (C and F) showing that both control and dnNP-1-expressing axons extended in the olfactory nerve layer. Scale bar for (B), (C), (E), and (F): 20 μm.

olfactory epithelium and into the adjacent mesenchyme by late stage 19, These axons have reached the surface of the telencephalon by E5 (Kobayashi et al., 1997; Drapkin and Silverman, 1999). The vast majority of olfactory sensory axons do not enter the CNS at this time but, instead, halt at the outside surface of the telencephalon where the olfactory bulb will form. A small number of axons do penetrate the telencephalon transiently, accompanied by cells that originate in the olfactory epithelium and migrate along the olfactory nerve. Olfactory axons continue to project from the olfactory epithelium and accumulate on the surface of the telencephalon. The bulb forms beneath them over the next several days in chicks and in other species (Doucette, 1989; Santacana et al., 1992; Byrd and Burd, 1993a, 1993b). Olfactory axons cover the surface of the nascent olfactory bulb to form the olfactory nerve fiber layer (ONL) by E9. They then leave the ONL to make connections in deeper layers of the bulb.

What keeps olfactory axons out of the CNS until their target, the olfactory bulb, has formed? There are several possible mechanisms: (1) a physical barrier might prevent olfactory axons from penetrating the telencephalon, (2) the telencephalon might not express molecules permissive for olfactory axon growth, or (3) the telencephalon might contain or secrete a repellent that prevents olfactory axons from entering the CNS.

Arguing against the presence of a physical barrier or the idea that the telencephalon is nonpermissive for olfactory axon growth is the observation that, during olfactory development in the chick, a small number of processes can be observed entering the telencephalon through small breaks in the basal lamina of the radial glial boundary (Drapkin and Silverman, 1999). Similar processes have been described in the mouse (Hinds, 1972; Doucette, 1989) and confirmed to be axons with electron microscopy. These axons project transiently into the nascent olfactory bulb, sometimes reaching as far as the ventricular surface before retracting and ending in their appropriate layers. The appearance of these axons coincides with the appearance of mitral cells, the major output cell of the olfactory bulb (Byrd and Burd, 1991). This observation has led to the suggestion that these early, transient incursions may be responsible for inducing the development of cells within the bulb (Byrd and Burd, 1993a, 1993b; Gong and Shipley, 1995). It has also been hypothesized that early invading axons may play a role in defining the boundary between the olfactory bulb and the forebrain in the rat (Santacana et al., 1992).

The presence of a repellent in the telencephalon is an attractive explanation for the failure of early arriving olfactory axons to enter the CNS. SEMA-3A is a likely candidate for this repellent. Olfactory sensory axons express neuropilin-1 and are sensitive to SEMA-3A-induced growth cone collapse (Kobayashi et al., 1997). SEMA-3A expression is detectable in superficial cells of the telencephalon as early as E5 when the olfactory axons first reach the CNS. High levels of SEMA-3A expression are maintained in the superficial half of the telencephalon through E7, the waiting period during which olfactory axons fail to enter the CNS (Kobayashi et al., 1997; this study). The invasion of olfactory axons into the olfactory bulb coincides with the restriction of SEMA-3A to deeper layers of the bulb. Thus, early expression of SEMA-3A in the telencephalon may act to keep olfactory axons from entering the CNS prematurely, and its later expression in deep layers of the bulb may function to prevent them from growing past their target layers. SEMA-3A has been hypothesized to play a similar role in preventing sensory axons from entering the spinal cord early (Shepherd et al., 1997) and, once

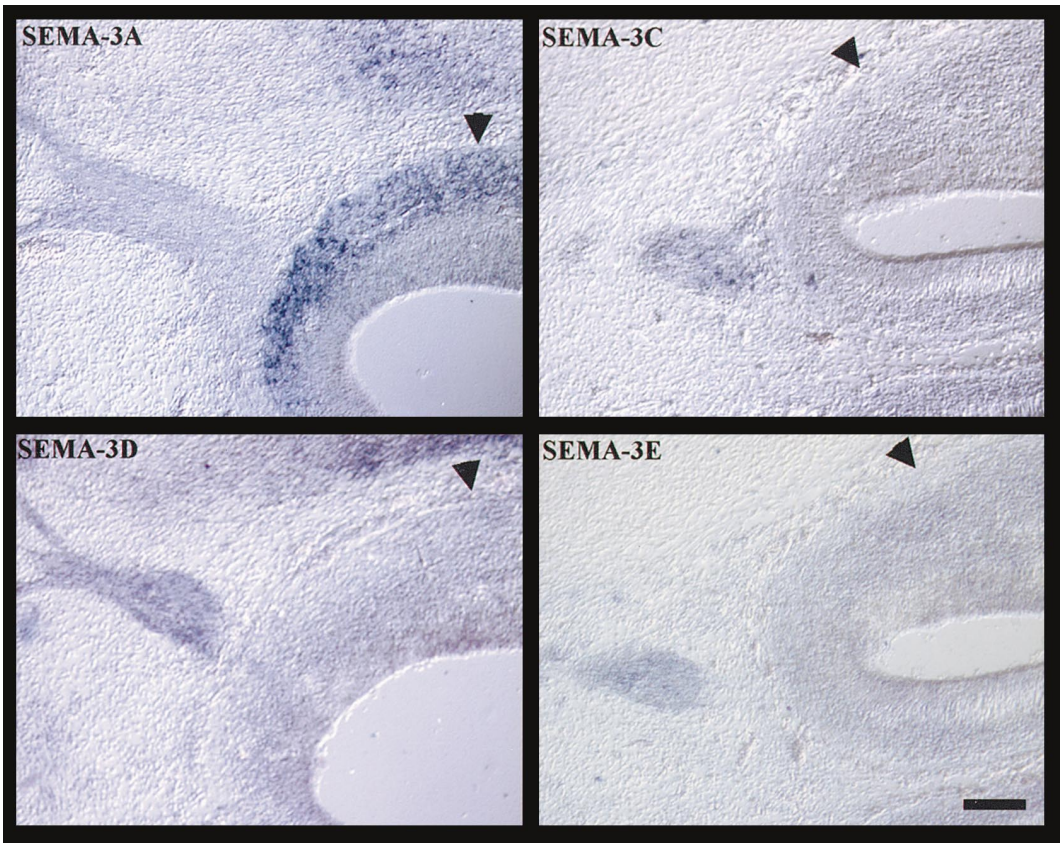

Figure 8. Expression of Class 3 Semaphorins in the Developing Olfactory System

Horizontal sections through the telencephalon and developing olfactory nerve of E7 embryos were incubated with DIG-labeled antisense RNA probes for chick SEMA-3A, SEMA-3C, SEMA-3D, and SEMA-3E. Arrowheads mark the outside edge of the CNS. SEMA-3A is expressed in superficial layers of the telencephalon, while SEMA-3C, SEMA-3D, and SEMA-3E are expressed weakly in the olfactory nerve. Scale bar: 100 μm.

they do enter, in preventing them from projecting into inappropriate layers of the cord (Messersmith et al., 1995).

We therefore expressed dnNP-1 in developing olfactory axons to test the hypothesis that a repellent semaphorin is responsible for preventing them from entering the telencephalon early. We found that olfactory axons expressing dnNP-1 are more likely to overshoot their target area than normal axons. These overshooting axons generally enter the CNS and extend in the most superficial layers of the telencephalon just below the pial surface. The dnNP-1 construct we used is known to block the function of some class 3 semaphorins but not others. The expression patterns of those we examined in the developing olfactory system suggests that SEMA-3A, but not SEMA-3B, -3C, or -3D, is expressed in the appropriate time and place to exclude olfactory axons from the telencephalon. These results support the hypothesis that an active repellent, expressed by the telencephalon, is responsible for preventing olfactory axons from entering the telencephalon early and that the most likely candidate for this repellent is SEMA-3A.

Although SEMA-3A is a very attractive candidate for this repellent activity, the possibility remains that the errors resulting from dnNP-1 expression could in principle reflect neuropilin-1's role in signaling events mediated by other semaphorins or even non-semaphorin-related ligands. The 165 amino acid (aa) isoform of vascular endothelial growth factor ($VEGF_{165}$) and placenta growth factor 2 (PlGF) are members of the VEGF family that bind to neuropilin-1. VEGF promotes cell migration, cell proliferation, and angiogenesis by signaling through one of the two receptor tyrosine kinases KDR/flk-1 or flt-1 (Waltenberger et al., 1994; Clauss et al., 1996). Neuropilin-1 is not required for VEGF signaling, but its presence in the receptor complex increases $VEGF_{165}$ binding to KDR and potentiates $VEGF_{165}$-mediated cell migration (Soker et al., 1998). PlGF also induces the migration of specific cell types (Clauss et al., 1996) and may also promote cell proliferation and angiogenesis (Park et al., 1994). Neuropilin-1 binds PlGF but the role it plays in PlGF signaling is unclear (Migdal et al., 1998). At present neither VEGF nor PlGF have been shown to function as repellents or as axon guidance cues, and they are therefore less attractive candidates than the semaphorins for an olfactory repellent produced by the telencephalon. Neuropilin-1 can also act as a cell adhesion molecule that binds unknown heterophilic partners (Shimizu et al., 2000). Alterations in olfactory axon guidance induced by the expression of dnNP-1 are unlikely to be caused by an overabundance of adhesive sites since the expression of full-length NP-1 does not affect olfactory axon guidance.

It may be functionally significant that SEMA-3A can

not only repel, but also paralyze, responsive growth cones. Under normal conditions, olfactory axons not only fail to enter the telencephalon, but accumulate outside it without crawling away. Olfactory sensory axons expressing dnNP-1 continue to extend when they would normally remain stationary outside the CNS. The paralytic activity of SEMA-3A may hold them in place near their target during the waiting period that precedes their entry.

We have seen some hints that dnNP-1-induced errors in olfactory axon pathfinding may be amplified by non-cell-autonomous effects. As previously mentioned, in two embryos transfected with dnNP-1, aberrant fascicles coursing outside the lateral surface of the olfactory bulb were found to contain AP tracer–tagged dnNP-1-expressing axons and a larger number of untagged neurofilament positive axons. Similar axon bundles were not observed in control embryos. One interpretation of these observations is that a small number of dnNP-1-expressing axons failed to halt at their normal location outside the nascent bulb and, instead, continued extending abnormally. These axons appear to have acted as an attractive pathway upon which additional, normal olfactory axons fasciculated and extended.

Olfactory axons overshoot their target at a low frequency in normal embryos. As described previously, a small number of transient projections have been observed entering the telencephalon prior to the formation of the olfactory bulb during the development of the olfactory system (Hinds, 1972; Doucette, 1989; Drapkin and Silverman, 1999). These axons then retract to end in their appropriate layers. The overshooting axons observed in our control experiments probably represent this population of axons because they are rare and, since they are absent in the older embryos, appear to be transient. The dnNP-1-expressing overshooting axons can persist through E9 and, therefore, may not be equivalent to the early entering, transient population of olfactory axons present in normal embryos.

It is instructive to consider olfactory pathfinding events that are not perturbed by the expression of dnNP-1. Expressing axons exit from the olfactory epithelium, enter the olfactory nerve, and project along a normal course to the telencephalon. SEMA-3A is expressed in the olfactory epithelium when olfactory axons exit (Kobayashi et al., 1997). SEMA-3A might have been thought to influence the initial direction in which these axons extend by repelling them out of, and away from, the olfactory epithelium. Our finding that axons expressing dnNP-1 exit the olfactory epithelium and enter the olfactory nerve as they normally would does not support this function for SEMA-3A. Our results also suggest that those class 3 semaphorins whose function is inhibited by NP-1 are not likely to play a very important role in the guidance of olfactory sensory axons while they grow in the olfactory nerve. It should also be noted that the expression of dnNP-1 in olfactory sensory neurons probably does not affect their cell fate or differentiated state, since their axonal processes behave normally until they reach the telencephalon.

A population of cells born in the olfactory epithelium migrate along the olfactory nerve and enter the telencephalon without any apparent pause in both our control and dnNP-1-expressing embryos. GnRH-expressing cells migrate along this route and ultimately populate anterior regions of the hypothalamus; however, they have been reported to pause before entering the telencephalon just as olfactory axons do (Mulrenin et al., 1999). If true, the migrating cells that enter the CNS without delay may represent some other cell type that is insensitive to the repellent activities of SEMA-3A.

Not all axons marked with the AP tracer tag project aberrantly. In fact, the majority behave normally even when they reach the telencephalon. There are several possible explanations for this. First, it is likely that AP-labeled axons express dnNP-1 at different levels, and some may express none at all. For this reason, the full null phenotype, corresponding to the total blockade of semaphorin function, can never be revealed by this approach. To address this issue, we constructed an AP-tagged dnNP-1 that combines the tracer and dominant–negative functions in a single construct. However, its relatively low level of expression did not permit us to trace dnNP-1-expressing axons with the precision and confidence provided by the AP tracer construct used in this series of experiments.

Second, dnNP-1 is unlikely to block the functions of all of the guidance cues that determine olfactory axon trajectories. Although dnNP-1 can block the function of more than one class 3 semaphorin, it is possible that other semaphorins with overlapping functions could compensate for their loss. For example, some class 3 semaphorin family members have been shown to act exclusively through neuropilin-2 (Chen et al., 1998; Giger et al., 1998), and they may play a role in this system. Although in situ hybridization experiments suggest that SEMA-3A is the most likely candidate for a telencephalic repellent activity, other semaphorins we did not examine, or other altogether unrelated guidance molecules, may help keep olfactory axons out of the CNS. Loss-of-function mutations in *C. elegans* and *Drosophila* often show partial penetrance and variable phenotypes due to the presence of additional guidance cues with partially redundant functions (Hedgecock et al., 1990; Wills et al., 1999).

A third possible explanation for the apparently normal behavior of the majority of dnNP-1-expressing olfactory axons is that only the earliest of them may be affected. Very early arriving axons that grow past the location where the olfactory bulb will form never have the opportunity to contact, recognize, and terminate in their appropriate target. But later arriving axons, even those expressing dnNP-1, could have that opportunity once the olfactory bulb has started to differentiate. The differentiated bulb may provide appropriate synaptic sites or other cues that actively encourage olfactory axons to stop growing and begin to make synapses.

In conclusion, our results suggest that an active chemorepellent is responsible for preventing olfactory axons from entering the telencephalon prematurely and further suggest that SEMA-3A is the most likely candidate for mediating this response. The establishment of long axonal projections is facilitated if neurons make their appropriate connections early while distances are short. The obvious disadvantage of this strategy is that axons may arrive at their destinations well before their appropriate targets are ready to be innervated. Chemorepellents may provide an active mechanism by which early arriving axons are prevented from entering a target that is not yet ready to receive them.

Experimental Procedures

DNA Preparation

PCR was used to generate truncated forms of neuropilin-1. A truncated form of neuropilin-1 that is missing its C domain and acts as a dominant–negative receptor component (dnNP-1) has been described previously (Renzi et al., 1999). A more severely truncated form of neuropilin-1 missing its entire extracellular domain (abc deletion) was made as a control construct and for tracing axonal trajectories. Standard PCR amplification was performed between ACCATCATAGCCATGAGTGCA and CAGAATTCTTACTCGGAAGCA TGA using oligonucleotide primers that placed a BglII restriction site 5′ and a NotI restriction site 3′ of the amplified sequence. The resulting fragment was cloned into the AP-PAG vector (Kobayashi et al., 1997), which added a signal sequence and a human placental alkaline phosphatase tag at the 5′ end of the clone. An expression plasmid containing the β-galactosidase reporter gene was received as a gift from Jeff Golden and was used as a control construct in expression experiments.

Electroporation

Eggs from white Leghorn chickens were incubated at 37°C until stage 13–14 (48–52 hr). The eggs were then windowed, and visualization of the embryo was aided by injecting a 1:10 dilution of pelican ink in PBS beneath the embryo for contrast. A glass microcapillary tube was pulled on a Flamin-Brown Electrode puller, attached to a 100 μl Hamilton glass syringe and filled with heavy mineral oil. The capillary was then loaded with 20 μl of DNA (1.5 μg/μl final concentration), suspended in TE (10 mM Tris-HCl [pH 8.0], 1 mm EDTA [pH 8.0]). Plasmid containing AP-abc-del neuropilin-1 (AP tracer tag) was diluted 1:10 with either plasmid containing the β-galactosidase reporter gene (AP+β-gal) for control transfections, plasmid containing full-length neuropilin-1 (AP+NP-1), or with plasmid containing dnNP-1 (AP+dnNP-1). The DNA mixture was then injected into the amniotic sac just rostral to the nasal pit (Figure 1A).

The electroporation apparatus consisted of a circuit designed to generate the electric field, a DC power source to supply the voltage, and a function generator to control the frequency of the pulses. The electric field was applied to the surface of the egg through platinum genetrodes (BTX industries). For the electroporation of the olfactory placode, the electrodes were placed on the surface of the egg as illustrated in Figure 1A. The electrodes were lowered to form a slight depression in the vitelline membrane that was then filled with 200 μl of sterile PBS. Three pulses of 25V at 20 Hz followed by three pulses of 25V at 10 Hz were then applied to the surface of the embryo. After electroporation, 200 μl of 10× Penicillin/streptomycin (Life Technologies) was added to the surface of the egg, the egg was sealed with tape, and it was placed back in the incubator.

Analysis of Whole Mounts

Chick embryos were sacrificed on the appropriate day and fixed in 4% paraformaldehyde in PBS for 2–4 hr at 4°C. Embryos were rinsed with PBS and then incubated in PBS at 65°C for 3 hr to inactivate endogenous alkaline phosphatase. After inactivation, the embryos were rinsed in AP reaction mix (0.5 mg $MgCl_2$, 0.3 mg NaCl, 5 ml 1M Tris-HCl [pH 9.5], 50 μl Tween 20) without substrate for 15 min–1 hr at 4°C, then incubated in AP reaction buffer with 0.33 mg/ml NBT and 0.17 mg/ml BCIP for 1–3 days at 4°C in the dark. The reaction was stopped by washing the embryos in acidic PBS (pH 5.0) containing 0.1% Tween 20 overnight at 4°C.

Embryos with AP-labeled cells in and around the nostril were identified (Figure 1B). To better visualize the olfactory nerve, these embryos were bisected sagitally through the midline of the head so that they could be viewed from the medial surface (Figure 1C). Embryos that had AP staining in the olfactory nerve were cleared in 80% glycerol overnight. The olfactory bulb was dissected out from the remaining half of the head. Cleared whole mounts were examined at high power through a dissecting microscope, and individual axons in the olfactory nerve near the telencephalon were counted, as were axons that had grown past the normal olfactory nerve stopping point (defined as the rostral-most end of the telencephalon in E5 and E7 embryos and the olfactory bulb/forebrain border in E9 embryos).

Analysis of Sections

Forebrains that had been dissected from transfected embryos were cryoprotected in 20% sucrose in PBS and imbedded in O.C.T. embedding compound. Those brains that had been cleared in glycerol were rehydrated in PBS overnight at 4°C prior to cryoprotection. Sections of 30 μm were cut on a cryostat (Leica) and collected on Superfrost Plus slides (Fischer). Sections were then washed in PBS, incubated in blocker (2% powdered milk in PBS) for 1 hr, and then incubated with anti-neurofilament antibody (4H6; Developmental Hybridoma Bank) diluted in blocker for 3 hr at room temperature. Neurofilament staining was visualized with a Cy3-conjugated secondary antibody.

In Situ Hybridization

Chick embryos were staged according to Hamburger and Hamilton (1951). Brain sections from E5, E7, and E9 embryos were prepared for in situ hybridization as follows. Embryos were sacrificed, and their heads were fixed in 4% paraformaldehyde in PBS at 4°C overnight. The following day, the heads from E5 and E7 embryos were cryoprotected in 20% sucrose in PBS at 4°C overnight. To section an E9 embryo, the forebrain and olfactory bulb were first dissected out of the E9 embryo then cryoprotected as described above. Tissue was then frozen in O.C.T. embedding media compound. Sections of 35 μm were cut on a cryostat (Leica) and collected on Superfrost Plus slides. Sections were washed in PBS, incubated in acetylation buffer (3.5 ml triethanolamine, 0.75 ml acetic anhydride in 300 ml sterile water), then permeabilized in PBT (PBS, 0.1% Triton X-100) and washed again in PBS, all at room temperature. Sections were prehybridized in hybridization buffer (50% formamide, 4× SSC, 1× Denhardt's reagent, 10% Dextran sulfate, and 0.5 mg/ml fish sperm DNA) for at least 1 hr at room temperature, then incubated overnight with the DIG-labeled probe, diluted to 400 ng/ml in hybridization buffer at 72°C. The next day, sections were washed in 0.2× SSC at 72°C, rinsed briefly in 0.2× SSC, and rinsed in PBS. Sections were blocked in blocker (2% powdered milk in PBS) for 1 hr and then incubated in an alkaline phosphatase anti-DIG antibody diluted 1:2500 in blocker for 3 hr at room temperature. Alkaline phosphatase was visualized by incubating the sections in AP reaction buffer without substrate for 5 min and then incubated overnight in AP reaction buffer containing 0.33 mg/ml NBT and 0.17 mg/ml BCIP.

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