Assembly of Trigeminal Sensory Ganglia by Chemokine Signaling

Holger Knaut,[1,4,*] Patrick Blader,[2] Uwe Strähle,[3] and Alexander F. Schier[1,4,*]
[1]Developmental Genetics Program
Skirball Institute of Biomolecular Medicine
and Department of Cell Biology
New York University School of Medicine
New York, New York 10016
[2]Centre de Biologie du Developpement
bat 4R3, Universite Paul Sabatier
118 route de Narbonne
31062 Toulouse
France
[3]Institut für Toxikologie und Genetik
Forschungszentrum Karlsruhe
Postfach 3640
76021 Karlsruhe
Germany

Summary

Sensory neurons with related functions form ganglia, but how these precisely positioned clusters are assembled has been unclear. Here, we use the zebrafish trigeminal sensory ganglion as a model to address this question. We find that some trigeminal sensory neurons are born at the position where the ganglion is assembled, whereas others are born at a distance and have to migrate against opposing morphogenetic movements to reach the site of ganglion assembly. Loss of Cxcr4b-mediated chemokine signaling results in the formation of mispositioned ganglia. Conversely, ectopic sources of the chemokine SDF1a can attract sensory neurons. Transplantation experiments reveal that neuron-neuron interaction and the adhesion molecules E- and N-Cadherin also contribute to ganglion assembly. These results indicate that ganglion formation depends on the interplay of birthplace, chemokine attraction, cell-cell interaction, and cadherin-mediated adhesion.

Introduction

The vertebrate nervous system is composed of hundreds of different cell types. This diversity of neurons is generated during development and is essential for the plethora of functions that the nervous system performs. Two predominant principles seem to govern neuronal organization. In some parts of the nervous system, such as the cerebral cortex, neurons are organized in layers of cell types. In other regions, such as motor pools or sensory ganglia, neurons with related functions are clustered in nuclei or ganglia (Ramon y Cajal, 1894). While our understanding of neuronal generation and diversification is advanced, little is known about the processes organizing neuronal subtypes into discrete layers or clusters.

Prominent examples of neuronal organization are the sensory ganglia of the peripheral nervous system. Each pair of bilateral ganglia harbors a specific set of neurons that provide sensory information (Romer and Parsons, 1986). Sensory neurons are born at different times and places and, therefore, face the challenge of assembling with their counterparts into a ganglion. For example, the trigeminal sensory ganglion (TgSG) is composed of neural crest and placodal cells that ultimately form a compact cluster of neurons on either side of the head between eye and ear (Davies, 1988). The trigeminal sensory neurons (TgSNs) extend peripheral axons underneath the skin of the head, to detect mechanical, chemical, and thermal stimuli, and central axons into the hindbrain, to communicate these inputs to the central nervous system (Baker and Bronner-Fraser, 2001; Hamburger, 1961).

Although it is unclear how the trigeminal, or any other, ganglion is assembled, several of the initial specification events have been delineated on a molecular level (Bertrand et al., 2002). Neural crest and placodal cells are induced at the neural plate border by a combination of diffusible factors (Meulemans and Bronner-Fraser, 2002). Lateral inhibition then singles out a subset of cells to adopt a neuronal fate, a process that requires atonal-related bHLH transcription factors such as Neurogenin (Ngn) (Bertrand et al., 2002). ngn is expressed in sensory neurons and loss of ngn function leads to loss of sensory ganglia in mouse and zebrafish (Andermann et al., 2002; Cornell and Eisen, 2002; Fode et al., 1998; Ma et al., 1998a, 1999). Conversely, misexpression of Ngn induces the formation of ectopic neurons (Blader et al., 1997; Kim et al., 1997; Ma et al., 1996), indicating that Ngn is necessary and sufficient for neurogenesis.

In contrast to the mechanisms underlying precursor specification and neurogenesis, it is unclear how TgSNs are assembled into a ganglion. Here, we combine genetic, embryological, and in vivo imaging approaches in zebrafish to address this question. Our results suggest that four processes contribute to the assembly of TgSNs into ganglia. First, neurons are born at or nearby the site of ganglion assembly. Second, neurons are attracted to and retained at the site of ganglion assembly by Cxcr4b-mediated chemokine signaling. Third, neuron-neuron interactions influence ganglion location. Fourth, E- and N-Cadherin contribute to the correct positioning of TgSNs. These results define a multicomponent process for ganglion assembly.

Results

Trigeminal Sensory Neurons Migrate and Cluster
By 36 hr post fertilization (hpf), zebrafish TgSNs are tightly packed into bilateral ganglia located halfway be-

*Correspondence: knaut@saturn.med.nyu.edu (H.K.); schier@saturn.med.nyu.edu (A.F.S.)
[4]Current address: Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138
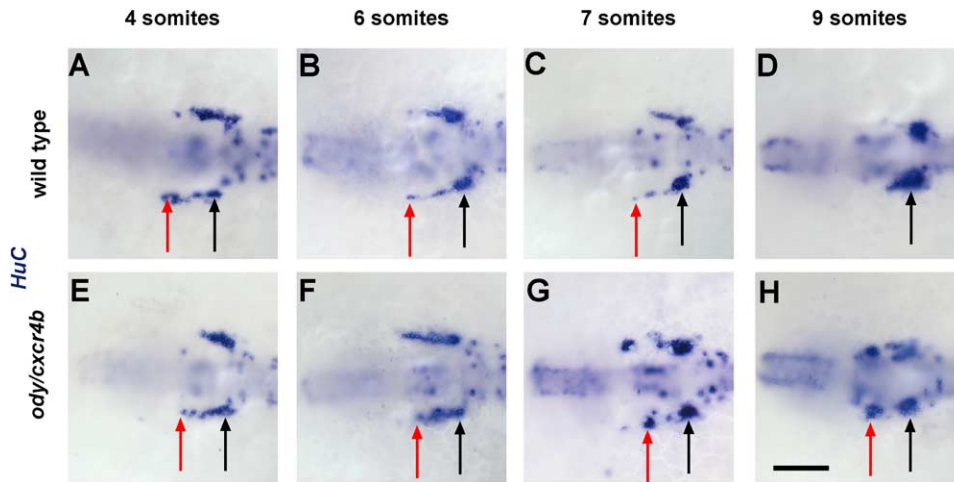
Figure 1. Formation of Trigeminal Sensory Ganglia in Wild-Type and *ody/cxcr4b* Mutant Embryos

Embryos were hybridized with *HuC* antisense probe to visualize TgSNs. Dorsal view; anterior to the left; arrows point to neurons located on the left side of the embryo. In wild-type embryos (A–D), TgSNs are arranged in small clusters along the anterior-posterior axis at the four-somite stage. (A) During subsequent development, anterior TgSNs (red arrow) move posteriorly such that at the six- and seven-somite stage, progressively fewer TgSNs are found at anterior positions ([B] and [C], respectively). At the nine-somite stage, all TgSNs have moved to a posterior position (black arrow) at the level of the mid-hindbrain boundary and form a compact cluster (D). In *ody/cxcr4b* mutant embryos (E–H), TgSNs are arranged in small clusters along the anterior-posterior axis at the four-somite stage (E). This arrangement is indistinguishable from that in wild-type embryos. At the six-somite stage, anterior TgSNs (red arrow) fail to move to more posterior positions (F), and at the seven-somite stage, anterior (red arrow) and posterior (black arrow) TgSNs coalesce into two separate clusters (G). At the nine-somite stage, TgSNs are clearly divided into an anterior and a posterior cluster (H). During the four- to nine-somite stage, TgSNs only slightly increase in number (Table S1). Red arrows indicate anteriormost and black arrows indicate posteriormost TgSNs. Embryos were fluorescently costained with anti-Vasa serum to confirm the wild-type or *ody/cxcr4b* mutant phenotype (data not shown). Scale bar, 100 μm.

tween eye and ear at the midbrain-hindbrain boundary (Andermann et al., 2002). To determine how these neurons assemble into a ganglion, we first analyzed TgSG development in wild-type embryos using *HuC*, a marker for neuronal progenitors (Kim et al., 1996). TgSNs are born as individual, sometimes scattered, cells that often form small clusters and are located along the anterior-posterior axis lateral to the midbrain and midbrain-hindbrain boundary (tailbud to four-somite stages; Figure 1A; Table S1). Hence, some TgSNs are initially located anterior to the site of ganglion assembly, whereas others are located at the site of ganglion assembly. This stripe-like arrangement changes when anterior TgSNs shift toward and condense with more posterior neurons next to the midbrain-hindbrain boundary (six- to seven-somite stage; Figures 1B and 1C), which results in the formation of a compact cluster at the nine-somite stage (Figure 1D).

These observations suggested that anterior TgSNs actively move to join more posterior TgSNs. To test this idea, we performed a time-lapse analysis using a transgenic line that expresses GFP from the *ngn* promoter (Blader et al., 2003) and faithfully recapitulates *HuC* expression in TgSNs. We found that TgSNs are motile and move posteriorly to form one compact cluster (Figures S1A–S1E and Movies S1A and S1B in the Supplemental Data available with this article online). Tracking individual cells showed that anterior TgSNs move toward the midbrain-hindbrain boundary, where they meet posterior neurons to assemble into a compact cluster (Figures S1F–S1J and Movie S1B). Occasionally, a sin-

gle neuron leaves the cluster, but frequently rejoins it later (see the cell labeled with a green asterisk in Movie S2B). These cells often extend a long protrusion that maintains a connection with cells in the cluster. In addition to these cell-cell interactions, neurons at the edge of the trigeminal cluster display cellular protrusions that extend and retract. Eventually, these protrusions cease to retract and begin to resemble outgrowing axons (Movies S2A and S2B, and data not shown). Taken together, these observations indicate that TgSNs are motile and that some are born at the site of ganglion assembly, whereas others form more anteriorly and aggregate into small clusters as they move posteriorly to assemble into a ganglion.

Cxcr4b Chemokine Signaling Is Essential for Trigeminal Sensory Ganglion Positioning

The analysis of wild-type development indicated that anterior cells move posteriorly to cluster with other TgSNs. We investigated the role of Cxcr4b in this process, because of its known function in guided cell movements in zebrafish and other systems (Kunkel and Butcher, 2003; Schier, 2003; Tran and Miller, 2003). Cxcr4b is a G protein-coupled receptor that is activated by the chemokines SDF1a and SDF1b. It is expressed in trigeminal ganglia and mediates the migration of germ cells and another placodal-derived sensory organ, the posterior lateral line primordium (Chong et al., 2001; David et al., 2002; Doitsidou et al., 2002; Gilmour et al., 2004; Knaut et al., 2003; Li et al., 2004). TgSNs start to express *cxcr4b* shortly after their birth at tailbud
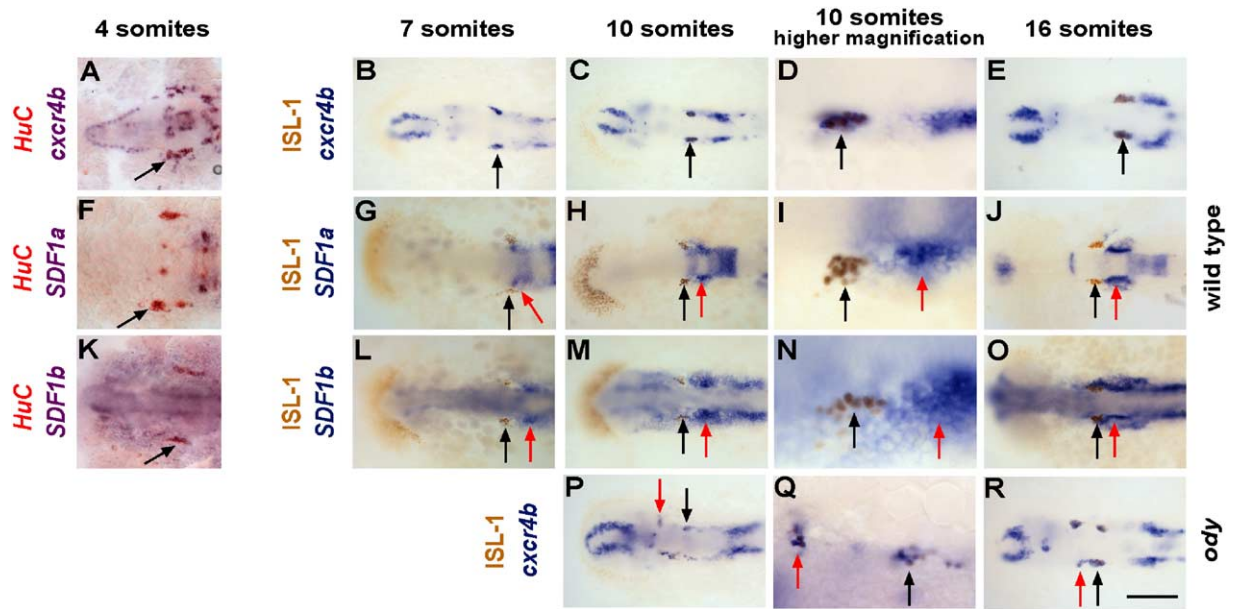
Figure 2. Expression Patterns of *cxcr4b*, *SDF1a*, and *SDF1b* during Trigeminal Sensory Ganglion Assembly

Whole-mount in situ hybridization against *cxcr4b* (A–E and P–R), *SDF1a* (F–J), and *SDF1b* (K–O) (blue), and staining of TgSNs by *HuC* in situ (red) (black arrow, [A], [F] and [K]), and by ISL-1 antibody staining (brown) (black arrows, [B–E], [G–J], [L–O], and [P–R]). TgSNs express the chemokine receptor *cxcr4b* during TgSN development and ganglion formation in wild-type (A–E) and *ody/cxcr4b* mutant embryos (P–R). TgSNs are in the vicinity of a more posteriorly located *SDF1a* expression domain (red arrow in [G–J]) and adjacent to a ventral and posterior *SDF1b* expression domain (red arrow in [L–O]). High magnifications of ISL-1 positive TgSNs show that these neurons are in the vicinity of the two sources of *SDF1* (red arrow, [I] and [N]) and indicate that TgSNs express the chemokine receptor *cxcr4b* (black arrow, [D]), but not its *SDF1* ligands (black arrow, [I] and [N]). Scale bar in (R) corresponds to 250 μm in all panels except (D), (I), (N), and (Q), where it corresponds to 50 μm, and (A), (F), and (K), where it corresponds to 200 μm.

stage and continue to express it until late somitogenesis (Figures 2A–2E). Sectioning of embryos indicates that all TgSNs express *cxcr4b* at the 16-somite stage (Figures S2A–S2J).

Rhombomere 1 is the tissue closest to the TgSNs that expresses the Cxcr4b ligand *SDF1a*, starting at the two-somite stage and persisting until late somitogenesis. At the seven-somite stage, a region lateral to rhombomere 1 and posterior to the TgSNs also starts to express *SDF1a* (Figures 2F–2J). This *SDF1a* expression domain is located about 100 μm from the most anterior TgSNs. *SDF1b*, the other Cxcr4b ligand, is expressed in the anterior endoderm throughout somitogenesis (data not shown). At the seven-somite stage, a domain lateral to rhombomere 1 and posterior to the TgSG starts to express *SDF1b* (Figures 2K–2O). This *SDF1b* expression domain partly overlaps with the expression domain of *SDF1a* and is also about 100 μm distant from the most anteriorly located TgSNs.

The proximity of the *SDF1a* and *SDF1b* expression domains to the *cxcr4b*-expressing TgSNs suggested a potential role for Cxcr4b-mediated chemokine signaling in TgSG development. To test this idea, we analyzed wild-type and *odysseus* (*ody*) mutant embryos. The *ody* mutation introduces a premature stop codon, deleting the carboxy terminus of Cxcr4b thought to be essential for G protein-coupled receptor signaling (Knaut et al., 2003). Using HNK-1 as a neuronal marker at the 16-somite stage, we found that TgSNs form two clusters in *ody/cxcr4b* mutant embryos. One cluster of TgSNs

is normally positioned, but often abnormally elongated. The other cluster of TgSNs is located ectopically. This ectopic cluster is located more anteriorly and medially than wild-type neurons (Figures 3E and 3A, respectively, and Table S2). The ectopically located neurons in *ody/cxcr4b* mutant embryos extend axons and arborize in a manner similar to the TgSNs that are positioned correctly (Figures 3C and 3G).

To determine the fate of the ectopically positioned neurons, we analyzed *ody* mutants until 5 dpf. Similarly sized ectopic clusters of approximately six cells (6 ± 3 [mean ± SD], n = 16 embryos) can be detected until 36 hpf, when clusters of ectopic TgSNs start to gradually become smaller in number; by 5 dpf, they have completely disappeared (data not shown). Accordingly, the total number of TgSNs in *ody/cxcr4b* mutant embryos is initially similar to that in wild-type, but decreases as the ectopic neuronal cluster becomes smaller and ceases to exist (Table S1). Injection of morpholinos against *cxcr4b* leads to a similar displacement of TgSNs, as observed in *ody/cxcr4b* mutant embryos (Figures 3K and 3J, respectively). These results reveal that *cxcr4b* is required for the correct positioning of TgSNs.

Since the chemokines *SDF1a* and *SDF1b* are expressed posteriorly to *cxcr4b*-expressing TgSNs (Figure 2) and act through Cxcr4b in other contexts (David et al., 2002; Doitsidou et al., 2002; Klein et al., 2001; Knaut et al., 2003; Li et al., 2004; Ma et al., 1998b; Reiss et al., 2002; Zhu et al., 2002; Zou et al., 1998), we re-
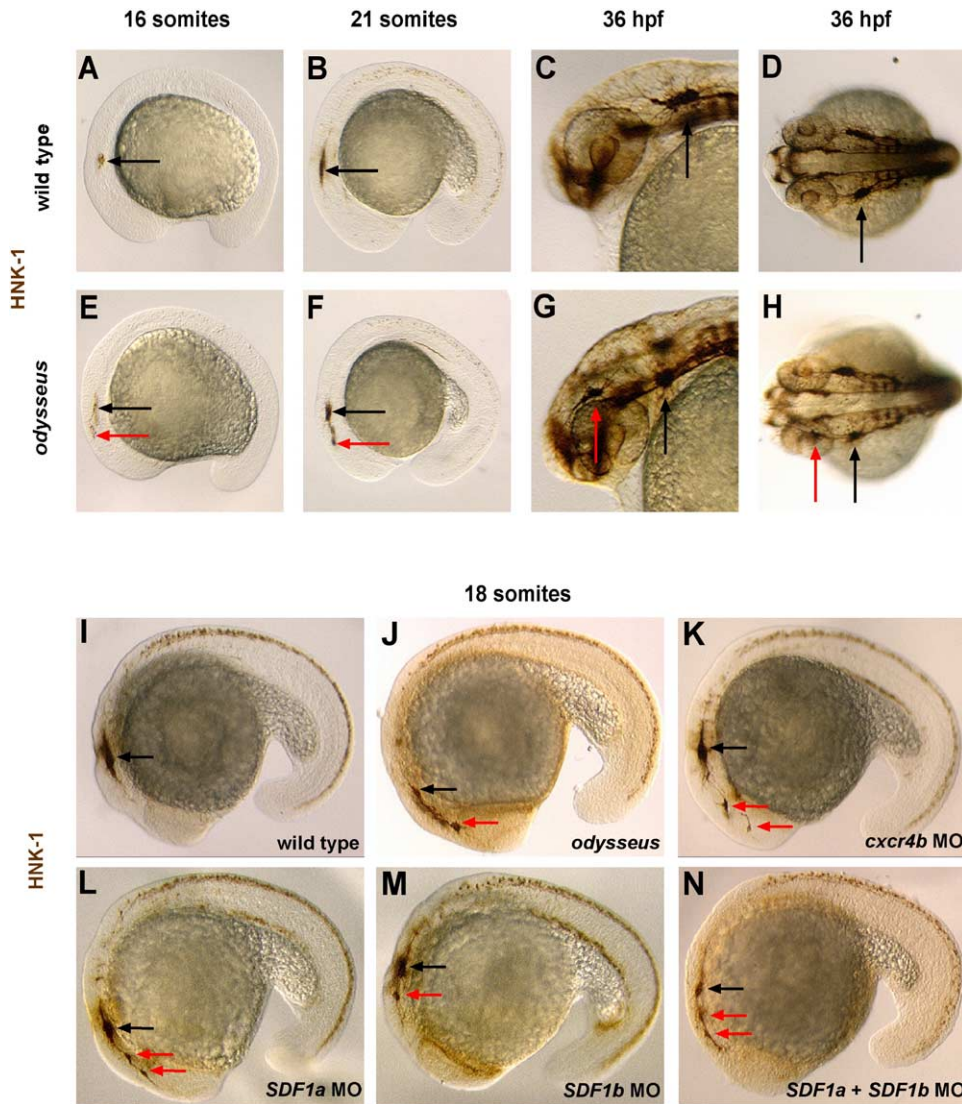
Figure 3. Cxcr4b Signaling Is Required for Trigeminal Sensory Neuron Positioning

Wild-type (A–D and I), *ody/cxcr4b* mutant (E–H and J), and morpholino-injected embryos (K–N) are stained with HNK-1 to visualize TgSNs. (A–C, E–G, and I–N) Lateral views with dorsal up and anterior to the left; (D and H) dorsal views. In wild-type embryos, TgSNs (black arrow) are positioned equidistantly between the eye and ear (D). At the 16-somite stage, the HNK-1 antibody starts to label the cell body of TgSNs (A) and, as axons mature, it starts to also highlight the major axonal projections (B–D). In *ody/cxcr4b* mutant embryos, TgSNs do not form one compact cluster as in wild-type embryos, but extend more anteriorly and are often divided into two distinct clusters (E–H). At the 16-somite stage, an ectopic TgSN cluster (red arrow, [E]) is frequently visible anterior to the correctly positioned trigeminal sensory cluster (black arrow, [E]). These two clusters remain separate (black and red arrows, [F]), and anteriorly displaced TgSNs are detectable at 36 hpf (black and red arrows, [G] and [H]). Reducing levels of Cxcr4b, SDF1a, SDF1b, or SDF1a and SDF1b by morpholino-mediated knockdown results in one cluster at the wild-type position (black arrow, [K–N], respectively) and one or more ectopic clusters at more anterior positions (red arrows, [K–N], respectively), similarly to impairing Cxcr4b function genetically (J).

duced the activity of *SDF1a* and *SDF1b* by morpholino injection and analyzed trigeminal development using HNK-1 as a neuronal marker. At the 18-somite stage, *SDF1a* and *SDF1b* morphants frequently showed anteriorly displaced TgSNs (Figures 3L and 3M, respectively, and Table S2). Coinjection of both morpholinos increased the frequency of ectopic clusters (Figure 3N and Table S2), while injection of control morpholinos against *SDF1a* and *SDF1b* bearing five mismatch mutations did not affect the positioning of the TgSNs (Table

S2). These results suggest that SDF1a and SDF1b signaling through Cxcr4b is essential for the correct positioning of TgSNs.

To determine whether the ectopically located neurons might represent a specific subset of TgSNs, we analyzed the expression of various marker genes. All ectopically located TgSNs express *cxcr4b* as do correctly positioned TgSNs (Figures 2P–2R and Figures S2K–S2T). Moreover, markers that are expressed in correctly located neurons are also expressed in the
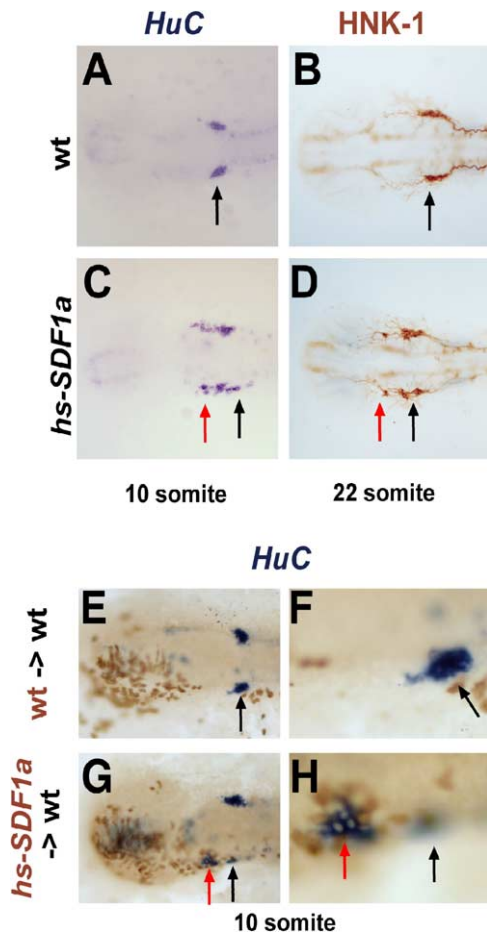
HuC   HNK-1

Figure 4. Misexpression of SDF1a Perturbs Trigeminal Sensory Neuron Positioning/Ectopic Expression of SDF1a Displaces Trigeminal Sensory Neurons

Wild-type embryos and embryos carrying the *heatshock*-SDF1a transgene were heat-shocked at the six-somite stage for 1 hr and stained for *HuC* (A and C) and for HNK-1 at the 22-somite stage (B and D). In heat-shocked wild-type embryos, TgSNs form a compact cluster at the ten- and 22-somite stage (black arrow in [A] and [B], respectively; n = 22 of 22 embryos). In heat-shocked embryos carrying the *heatshock*-SDF1a transgene, TgSNs do not coalesce into a compact cluster at the ten-somite stage (black and red arrows in [C]; n = 21 of 21 embryos) and form—in addition to a correctly positioned cluster (black arrow, [D])—one or more anteriorly displaced clusters at the 22-somite stage (red arrow in [D]; n = 14 of 23 embryos).

One-cell stage wild-type or transgenic embryos carrying the *heatshock*-SDF1a transgene were injected with rhodamine-dextran and biotin-dextran, and at the 1000-cell to sphere stage, approximately 20 donor cells were transplanted into recipient embryos of an equivalent stage (E–H). Recipient embryos were heat-shocked for 1 hr at the six-somite stage or not subjected to a heat shock and, at the ten-somite stage, stained with *HuC* to visualize TgSNs (blue). Donor-derived cells are biotin-dextran-positive (brown).

Wild-type cells transplanted into wild-type host embryos do not perturb TgSN positioning after heat shock (black arrow in [E] and [F]; n = 19 of 19 embryos). Similarly, *heatshock*-SDF1a transgenic cells transplanted into wild-type host embryos that have not been heat-shocked do not affect TgSN positioning (data not shown; n = 16 of 16 embryos). In contrast, in heat-shocked wild-type embryos with clones of *heatshock*-SDF1a transgenic cells, frequently some TgSNs are found at positions close to ectopic sources of SDF1a (red arrow in [G] and [H]), while others are correctly positioned next

mispositioned TgSNs (HNK-1, *HuC*, ISL-1, acetylated tubulin, and *P2X3*, data not shown) (Inoue et al., 1994; Kim et al., 1996; Norton et al., 2000; Piperno and Fuller, 1985; Trevarrow et al., 1990; Wilson and Easter, 1991). Since TgSNs originate from both neural crest and placodal precursors, we analyzed whether ectopic TgSNs are derived from one of these precursor populations or represent a mixture of the two. We found that TrkA, a potential neural crest marker, and TrkC, a potential placodal marker (Lindsay, 1996), are expressed in a subset of TgSNs in both the ectopically and the correctly positioned cluster (arrow, Figures S3C, S3D, S3G, and S3H). Moreover, genetically eliminating the neural crest contribution using *narrow-minded* (*nrd*), a mutation in the transcription factor *blimp1* (Artinger et al., 1999; Hernandez-Lagunas et al., 2005; Roy and Ng, 2004), reduces the number but does not affect the positioning of TgSNs at ectopic or correct locations (arrow, Figure S4). These observations suggest that ectopically and correctly positioned TgSNs are composed of both neural crest and placodal precursors.

A Local Source of SDF1 Is Required and Sufficient for Positioning of Trigeminal Sensory Neurons

The expression of SDF1a and SDF1b posterior to TgSNs suggested that the two chemokines might guide migrating TgSNs by providing a localized source of attractants. To test this idea, we disrupted the restricted expression of the chemokines by elevating SDF1a levels in all tissues at the onset of TgSN migration. We heat-shocked transgenic embryos expressing SDF1a under the control of a heat-shock promoter at the six-somite stage. Analysis with the neuronal markers *HuC* and HNK-1 revealed that TgSNs form two or more clusters in these embryos, but not in controls (Figures 4A–4D). These results indicate that a local source of chemokines is important for correct positioning of TgSNs.

To determine if SDF1 is sufficient to recruit migrating TgSNs, we created a local source of SDF1a at a more anterior position than the endogenous chemokine source. Specifically, we transplanted wild-type and *heatshock*-SDF1a transgenic cells into wild-type and *heatshock*-SDF1a transgenic host embryos and heat-shocked the mosaic embryos at the six-somite stage. Using the neuronal marker *HuC* we found that ectopic sources of SDF1a in wild-type embryos frequently recruit some TgSNs to positions anterior to their correct position (Figures 4G and 4H, n = 16 of 22 embryos). In contrast, wild-type cells transplanted into wild-type hosts do not perturb TgSN positioning (Figures 4E and 4F, n = 19 embryos). Similarly, wild-type or *heatshock*-SDF1a transgenic cells transplanted into *heatshock*-SDF1a transgenic hosts are not associated with the position of anteriorly displaced TgSNs after heat shock (n = 13 embryos and n = 11 embryos, respectively; data not shown). The total number of TgSNs does not change

to the endogenous source of SDF1a and SDF1b (black arrow in [G] and [H]; n = 16 of 22 embryos). Importantly, wild-type or *heatshock*-SDF1a transgenic clones in *heatshock*-SDF1a transgenic host embryos did not correlate with ectopic TgSNs after heat shock (n =13 and n = 11, respectively; data not shown).

when the neurons are challenged with an ectopic source of SDF1a-expressing cells in comparison with an ectopic clone of wild-type cells (24.4 ± 2.2 neurons/side, n = 15, and 25.2 ± 4.2 neurons/side, n = 12, respectively). These results indicate that ectopic SDF1a sources can compete with the endogenous SDF1 sources and recruit TgSNs to ectopic sites.

Cxcr4b Chemokine Signaling Attracts and Retains Trigeminal Sensory Neurons

Two models could explain the formation of an ectopic trigeminal cluster when chemokine signaling is disrupted. The ligands SDF1a and SDF1b might normally act as long-range attractants that recruit an anterior subpopulation of TgSNs to a more posterior position. In the absence of chemokine signaling, the anterior subpopulation of TgSNs would remain in place and form a second ectopic trigeminal cluster. Alternatively, SDF1a and SDF1b might act as short-range retention signals for a subpopulation of TgSNs to ensure that these neurons stay at a posterior position. Failure to perceive the retention signal may expose this subpopulation of TgSNs to other cues, causing them to move to a more anterior position. Either model could account for the formation of an ectopically located anterior and a normally positioned posterior cluster.

To distinguish between the attraction and retention models, we analyzed trigeminal development in *ody/cxcr4b* mutant embryos. Using the neuronal marker *HuC*, we found that TgSNs in *ody/cxcr4b* mutants initially have the same scattered distribution as that in wild-type embryos (Figures 1F and 1B, respectively). During subsequent development, however, some TgSNs fail to join the posterior neurons (Figure 1G). This results in the formation of two clusters of TgSNs in *ody/cxcr4b* mutants (Figure 1H), even though the total number of TgSNs is similar to that in wild-type (Table S1).

These observations suggested that *ody/cxcr4b* mutant anterior TgSNs fail to migrate posteriorly. To test this prediction, we followed TgSNs in *ody/cxcr4b* mutants that harbored the *ngn*-GFP transgene (Blader et al., 2003). As observed in wild-type, *ody/cxcr4b* mutant TgSNs were motile (compare Figures 5A–5L to Figures 5M–5X and Movies S3A and S3B to Movies S4A and S4B). However, anterior neurons failed to cluster with the posterior neurons and instead formed an ectopic anterior cluster (Figures 5M–5P and 5S–5V). This cluster moved more anteriorly as development progressed, separating it further from the correctly positioned posterior cluster (Figures 5Q, 5R, 5T, and 5X). As in wild-type, single *ody/cxcr4b* mutant TgSNs sometimes left the posterior cluster, but—in contrast to wild-type TgSNs—did not rejoin the cluster. In both wild-type and *ody/cxcr4b* mutant embryos, escaping neurons were initially connected to the neuronal cluster by cellular protrusions (see the cell labeled with a green asterisk in Figures 5H, 5I, and 5V–5X and Movies S2B, S3B, and S4B), but in *ody/cxcr4b* mutants, these protrusions sometimes appeared broken and the neurons had moved anteriorly (see cell labeled with green asterisk in Figures 5V–5X, Movie S4B, and data not shown). Taken together, these results suggest that both attraction and retention mechanisms are disrupted in *ody/cxcr4b* mu-

tant embryos. First, chemokine signaling attracts anterior TgSNs to a more posterior position lateral to the midbrain-hindbrain boundary. Second, chemokine signaling retains TgSNs at their correct position by preventing single neurons from permanently leaving the cluster.

Trigeminal Sensory Neurons Withstand the Morphogenetic Movements of Neighboring Tissues

Our phenotypic analysis of *ody/cxcr4b* mutants indicates that anterior TgSNs fail to be attracted posteriorly and then aberrantly move anteriorly. Preliminary time-lapse analysis of *ngn*-GFP embryos colabeled with rhodamine dextran-positive cell debris suggested vigorous anteriorly directed streaming of tissues surrounding the TgSNs (data not shown). This observation raised the possibility that the aberrant placement of TgSNs in *ody/cxcr4b* mutant embryos may be due to neurons being carried anteriorly with neighboring cells. To test this possibility, we labeled anterior TgSNs and neighboring cells in wild-type and *ody/cxcr4b* mutants. *Ngn*-GFP transgenic embryos were injected with caged fluorophores and raised to the four-somite stage. Groups of five to ten cells in the region of the GFP-positive TgSNs were labeled by laser-induced liberation of caged fluorescein and rhodamine (black arrow, Figures 6A and 6D). The location of labeled cells was analyzed 16 hr later at 28 hpf. In wild-type embryos, a striking separation of TgSNs and neighboring skin and mesenchymal cells was observed (Figures 6B and 6C). The neurons had stayed in place while most neighboring cells had streamed anteriorly. Similarly to wild-type, non-neural cells in *ody/cxcr4b* mutants had moved anteriorly; however, in dramatic contrast to wild-type embryos, some TgSNs had also moved anteriorly (Figures 6E and 6F). These observations suggest that TgSNs require Cxcr4b chemokine signaling to withstand displacement by morphogenetic movements of neighboring tissues.

Neuron-Neuron Interactions Influence the Position of Trigeminal Sensory Neurons

The *cxcr4b* gene might be required cell-autonomously in TgSNs or non-cell-autonomously in surrounding tissues. The findings that *cxcr4b* is expressed in TgSNs, encodes a receptor, and is required within germ cells for chemotaxis suggested that it may also act cell-autonomously in TgSNs. To test this idea, we generated genetic chimeras. Upon transplantation into *ody/cxcr4b* mutant or wild-type hosts, *ody/cxcr4b* TgSNs populated both ectopic and normal positions (ect = 26% and 24%, respectively; transplanted neurons, n = 46 and n = 97, respectively; Figures 7E and 7F and Figures 7G and 7H, respectively; Table S3). As expected, wild-type TgSNs transplanted into wild-type hosts rarely populated ectopic positions (ect = 3%, n = 75; Table S3 and Figures 7A and 7B). These findings reveal that a wild-type environment cannot suppress the requirement for *cxcr4b* in ganglion positioning and are consistent with a cell-autonomous role for *ody/cxcr4b* in TgSNs. Surprisingly, however, 13% of wild-type TgSNs in *ody/cxcr4b* mutant embryos populated ectopic positions (n = 85; Table S3 and Figures 7C and 7D). This observation suggested an additional, non-cell-autonomous role
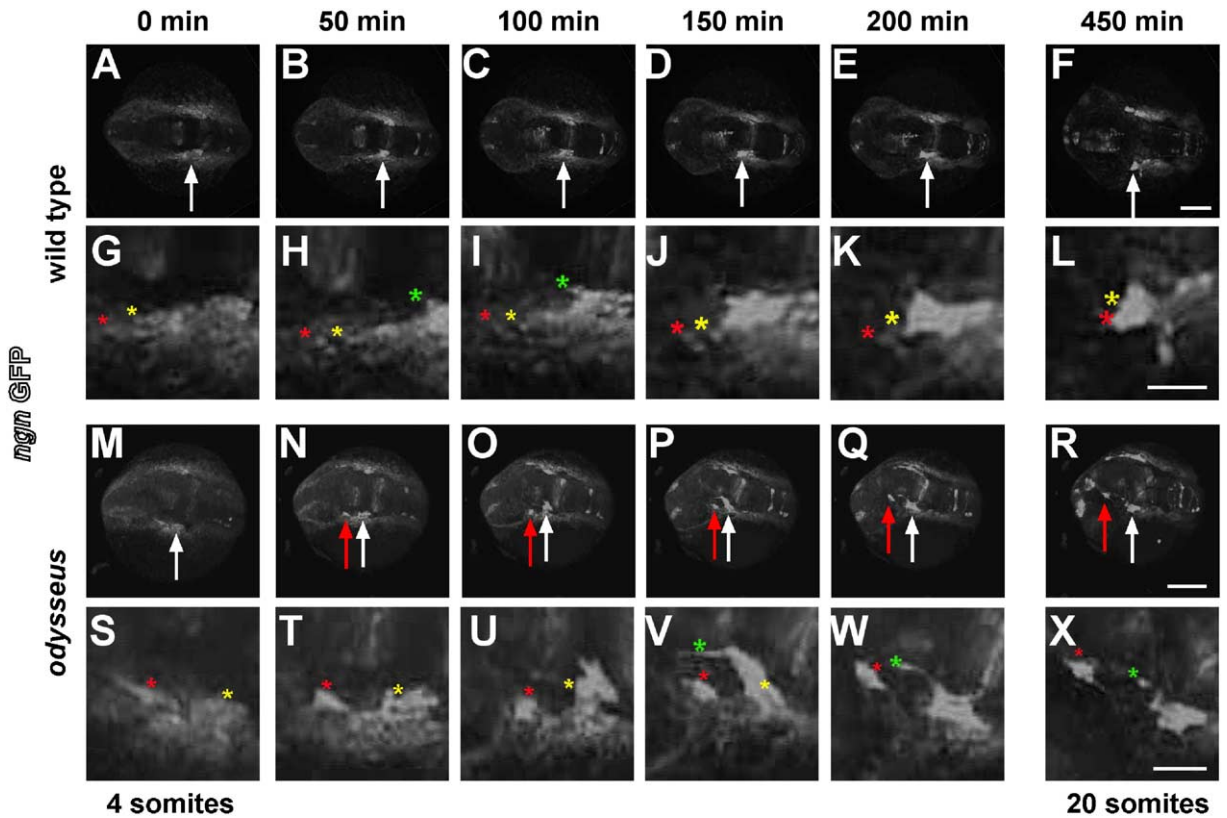
Figure 5. Time-Lapse Analysis of Trigeminal Sensory Ganglia Formation in Wild-Type and *ody/cxcr4b* Mutant Embryos

Wild-type and *ody/cxcr4b* mutant embryos carrying the *ngn*-GFP transgene were time-lapsed starting at the four-somite stage for 450 min. Dorsal view; anterior to the left; arrow points to neurons located on the left side of the embryo. In wild-type embryos TgSNs are motile and eventually form one compact cluster at the level of the midbrain-hindbrain boundary (A–F). Tracking the position of individual cells at higher magnification shows that they move posteriorly (cells labeled with red and yellow asterisks in [G]–[L]). Sometimes, individual cells leave the cluster and return. Such cells often extend cytoplasmic protrusions that remain connected to the cluster (cells labeled with green asterisk in [H] and [I]). In *ody/cxcr4b* mutant embryos, TgSNs are also motile, but anterior neurons fail to move posteriorly and do not join posterior TgSNs at the correct site of ganglion assembly next to the midbrain-hindbrain boundary. Instead, anterior neurons form an additional, displaced cluster of TgSNs (M–R). Tracking the position of individual cells at higher magnification confirms that anterior cells do not move posteriorly, but shift to more anterior positions, while posterior cells remain at the correct location (anterior cell labeled with red asterisk and posterior cell labeled with yellow asterisk in [S]–[X]). Occasionally, individual cells leave the cluster but extend cytoplasmic protrusions that maintain contact with the cluster. These cytoplasmic protrusions sometimes seem to break and the escaping cell separates farther from the main cluster (cell labeled with green asterisk in [V]–[X]). Asterisks are above and slightly to the left of tracked cells. Scale bars, 150 μm in (F) and (R) and 50 μm in (L) and (X).

for *ody/cxcr4b* in addition to its cell-autonomous requirement in TgSG assembly.

There are two possible explanations for the apparently non-cell-autonomous requirement of *ody/cxcr4b* in ganglion development. Cxcr4b signaling might be required in the cells neighboring the TgSNs to facilitate their migration to the wild-type position. Alternatively, cell-cell interactions among TgSNs might cause wild-type neurons to acquire the same ectopic position as *ody/cxcr4b* mutant neurons. The latter scenario was suggested by the cell-cell contacts observed between TgSNs (Figure S1, Figures 1 and 5 and Movies S1–S4). To distinguish between these two possibilities, we determined how wild-type neurons behaved in an *ody/cxcr4b* mutant host that lacked TgSNs. In such embryos any potential effects of mutant TgSNs on their wild-type counterparts should be abolished. To this end, we placed wild-type TgSNs in *ody/cxcr4b* mutant

hosts whose endogenous TgSN development had been blocked by *ngn* morpholinos. In these embryos, wild-type TgSNs populated the normal ganglion position (ect = 1%, n = 132; Table S4 and Figures 7K and 7L). Placing wild-type TgSNs into wild-type embryos lacking TgSNs did not affect their positioning (ect = 3%, n = 158; Table S4 and Figures 7I and 7J). Similarly, placing *ody/cxcr4b* mutant TgSNs into wild-type or *ody/cxcr4b* mutant embryos lacking TgSNs did not change their arrangement (ect = 26% and 33%, respectively; n = 172 and n = 198, respectively; Table S4 and Figures 7O and 7P and 7M and 7N, respectively). These results reveal that the cause of the abnormal positioning of wild-type neurons in *ody/cxcr4b* hosts is the presence of mutant TgSNs. Taken together, these observations suggest that *ody/cxcr4b* acts cell-autonomously and that interactions among TgSNs can contribute to ganglion assembly.
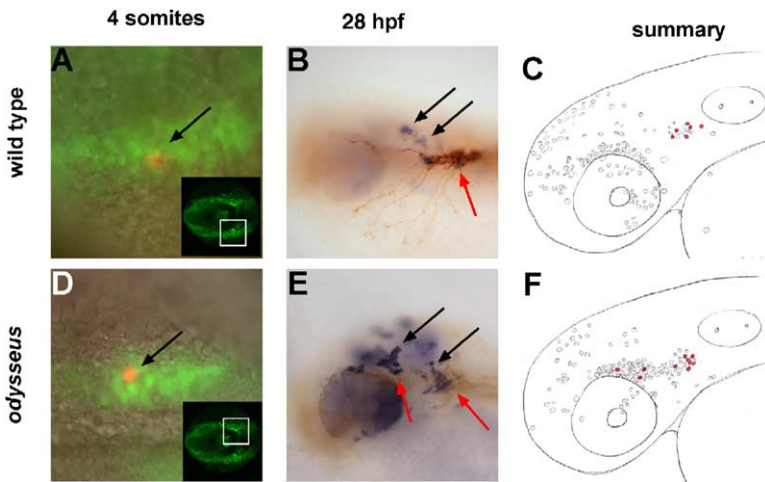
Figure 6. Trigeminal Sensory Neurons in *ody/cxcr4b* Mutant Embryos Move Anteriorly along with Non-Neuronal Cells

(A and D) Dorsal view, (B, C, E, and F) lateral view, anterior is to the left. A small cluster of cells that neighbor anterior TgSNs was marked by liberating caged rhodamine- and fluorescein-dextran with a laser beam at the three- to four-somite stage in *ngn*-GFP transgenic wild-type or *ody/cxcr4b* mutant embryos (black arrows in [A] and [D], respectively). TgSNs (green) and uncaged cells (red) are superimposed with a DIC (differential interference contrast) image. For overview, the insets in (A) and (D) show a similarly staged *ngn*-GFP transgenic embryo at a lower magnification. The white rectangle in the inset indicates the position shown in higher magnification in (A) and (D). The location of uncaged cells was recorded at 28 hpf by antibody staining against uncaged fluorescein (purple), (black arrow in [B] and [E]) and costained with HNK-1 antibody (brown) to visualize the TgSNs (red arrow in [B] and [E]). At 28 hpf, uncaged cells are found at more anterior positions in both wild-type and *ody/cxcr4b* mutant embryos (B and E, respectively) and—in the case of *ody/cxcr4b* mutant embryos—colocalize with ectopic TgSNs (E). Superimposing the positions of uncaged cells at 28 hpf shows that cells neighboring anterior TgSNs generally move anteriorly and dorsally in both wild-type and *ody/cxcr4b* mutant embryos (uncaged embryos analyzed, shown in [C] and [F]: n = 11 and n = 20, respectively). Open black circles indicate the position of uncaged cells, and closed red circles indicate the position of uncaged TgSNs.

E- and N-Cadherin Contribute to the Positioning of Trigeminal Sensory Neurons

In *ody/cxcr4b* mutant embryos, a quarter of the TgSNs form ectopic clusters (ect = 24%, n = 196 at 28 hpf) while the remaining neurons populate the wild-type ganglion position (Table S2). This indicates that a subset of TgSNs does not require Cxcr4b signaling for normal positioning and might rely on additional mechanisms. Cell adhesion molecules belonging to the cadherin family are candidates for contributing to ganglion formation. E- and N-cadherin are expressed in TgSNs (Figure S5; Liu et al., 2003), and *N-cadherin* appears to be required for the maintenance of the ganglion at later stages of development (48 hpf) (Kerstetter et al., 2004). We therefore analyzed *cadherin* function in TgSNs by mosaic analysis. We used this assay because interference with *E-cadherin* function results in early embryonic lethality (Kane et al., 2005; Montero et al., 2005) and blocking of *N-cadherin* activity has multiple pleiotropic effects (data not shown and Babb and Marrs, 2004; Lele et al., 2002). We transplanted cells from *E-* and *N-cadherin* morpholino-injected donors into wild-type hosts. These embryos undergo normal morphogenesis, but harbor *E-* and *N-cadherin*-deficient TgSNs. These TgSNs still formed a compact cluster at the correct position of the ganglion (Table S5), indicating that loss of *E-* and *N-cadherin* function does not impair TgSG positioning in this context.

The lack of a phenotype upon knockdown of *E-* and *N-cadherin* indicates that E- and N-cadherin do not act upstream of Cxcr4b signaling. Otherwise, loss of cadherin-mediated adhesion would result in a similar phenotype as loss of Cxcr4b signaling. Alternatively, chemokine attraction and cadherin function might act in parallel, and Cxcr4 signaling may be sufficient to position TgSNs even upon loss of *E-* and *N-cadherin*. This model makes three predictions. First, impairing Cxcr4b signaling in *E-* and *N-cadherin*-deficient TgSNs should uncover a role for cadherins. Second, TgSNs lacking E- and N-cadherin should be misplaced by neighboring *ody/cxcr4b* mutant neurons to ectopic positions more readily than wild-type neurons. Third, E- and N-cadherin expression should not be regulated by Cxcr4 signaling. To test the first prediction, we transplanted cells that were both mutant for *ody/cxcr4b* and morphant for *E-* and *N-cadherin* into *ngn* morphants. This generated embryos in which all TgSNs were impaired for *N-cadherin*, *E-cadherin*, and *cxcr4b* function. In such embryos, 36% (n = 236) of the TgSNs populated ectopic positions compared to 26% (n = 172) in control embryos whose TgSNs lacked only Cxcr4b function (Table S5). This result suggests a supporting role for cadherins in neuronal positioning.

To test the second prediction, we determined how TgSNs impaired in *E-* and *N-cadherin* function behave in *ody/cxcr4b* mutant embryos. Remarkably, these neurons populated ectopic positions with a much higher frequency than did wild-type TgSNs (ect = 30% versus 13%, n = 201 and n = 85, respectively; Figures 8C and 8D; Table S5). This displacement of *E-* and *N-cadherin*-deficient TgSNs depends on *ody/cxcr4b* mutant neurons since *E-* and *N-cadherin*-deficient TgSNs are not displaced in *ody/cxcr4b* mutant embryos lacking endogenous TgSNs (Table S5, Figures 8A, 8B, 8E, and 8F). Hence, *ody/cxcr4b* mutant TgSNs displace neurons much more efficiently to ectopic positions if these neurons are depleted of *E-* and *N-cadherin*. These results are consistent with the idea that Cxcr4 signaling and cadherin-mediated adhesion act in parallel.

To test the third prediction, we asked whether Cxcr4b signaling affects E- and N-cadherin expression levels. Analysis in wild-type and *ody/cxcr4b* mutant embryos showed that E- and N-cadherin are expressed at comparable levels in TgSNs in the ganglion of wild-type em-
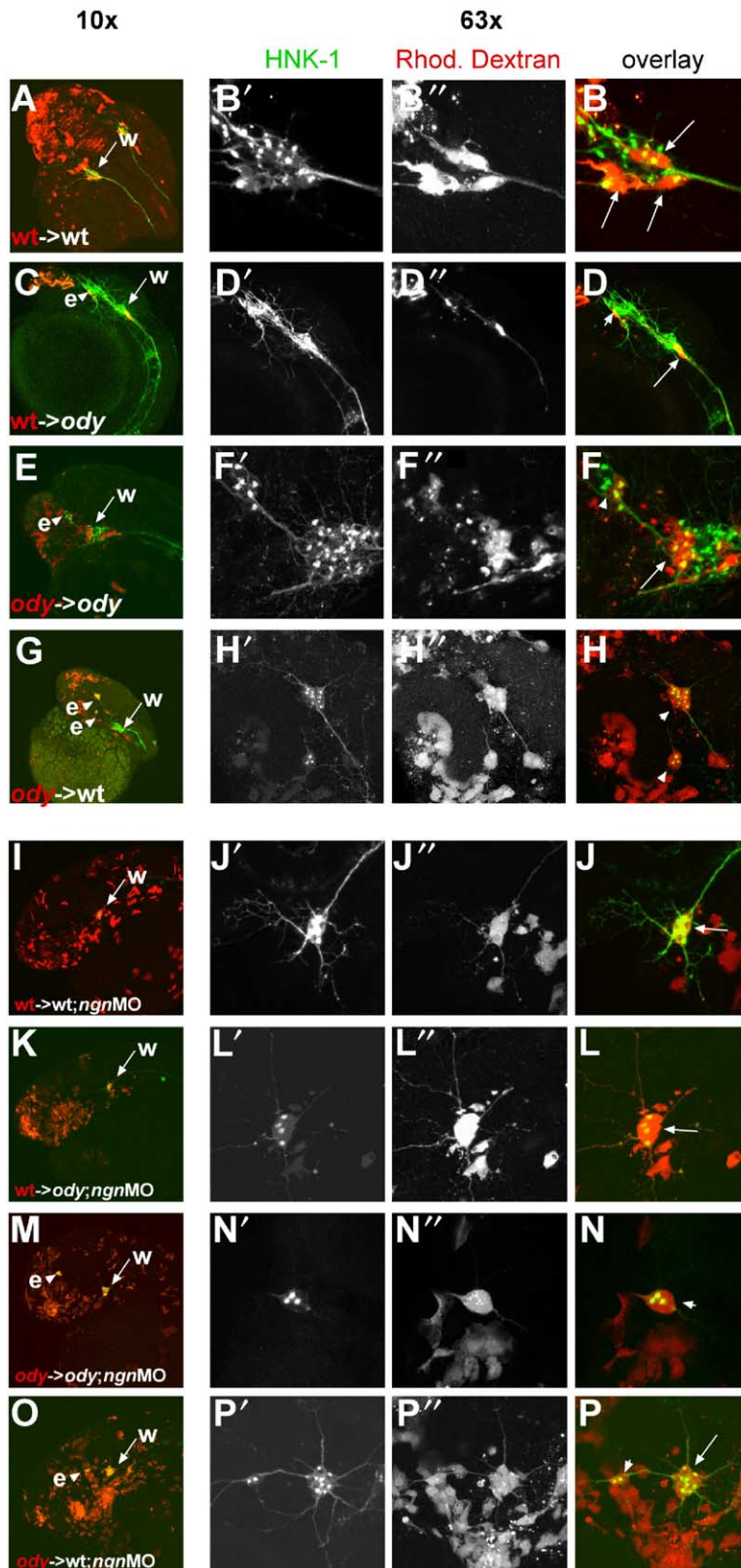
Figure 7. Mosaic Analysis of *cxcr4b* Requirement in Trigeminal Sensory Ganglion Assembly

One-cell stage wild-type or *ody/cxcr4b* mutant donor embryos were injected with rhodamine-dextran, and at the 1000-cell to sphere stage, approximately 100 donor cells were transplanted into recipient embryos of an equivalent stage. At 28 hpf, recipient embryos were stained with HNK-1 to visualize TgSNs (green). Donor-derived cells are rhodamine-dextran-positive (red). Wild-type TgSNs transplanted into wild-type host embryos contribute exclusively to the wild-type TgSN cluster (arrow labeled with "w" in [A] and arrows in [B]). Wild-type TgSNs transplanted into *ody/cxcr4b* mutant host embryos can contribute to both the anteriorly displaced (arrowhead labeled with "e" in [C] and arrowhead in [D]) and the wild-type TgSN cluster (arrow labeled with "w" in [C] and arrow in [D]). *Ody/cxcr4b* mutant TgSNs transplanted into *ody/cxcr4b* mutant host embryos can be found anteriorly displaced (arrowheads labeled "e" in [E] and arrowheads in [F]) compared to the correctly positioned cluster (arrow labeled with "w" in [E] and arrow in [F]). Similarly, *ody/cxcr4b* mutant TgSNs transplanted into wild-type host embryos can be found anteriorly displaced (arrowheads labeled "e" in [G] and arrowheads in [H]) in comparison with the correctly positioned cluster (arrow labeled with "w" in [G]). Wild-type TgSNs transplanted into *ngn* morphants are positioned correctly (arrow labeled with "w" in [I] and arrow in [J]). Similarly, wild-type TgSNs transplanted into *ody/cxcr4b* mutant host embryos injected with *ngn* morpholino are positioned correctly (arrow labeled with "w" in [K] and arrow in [L]). *Ody/cxcr4b* mutant TgSNs transplanted into *ody/cxcr4b* mutant host embryos injected with *ngn* morpholino are anteriorly displaced (arrowhead labeled with "e" in [M] and arrowhead in [N]) and/or positioned correctly (arrow labeled with "w" in [M]). Similarly, *ody/cxcr4b* mutant TgSNs transplanted into *ngn* morphants are anteriorly displaced (arrowhead labeled with "e" in [O] and arrowhead in [P]) and/or positioned correctly (arrow labeled with "w" in [O] and arrow in [P]).
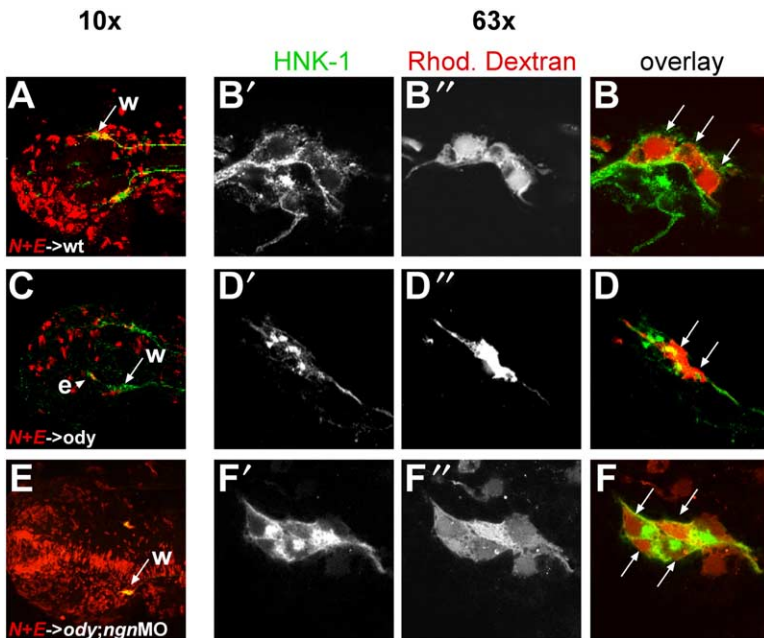
10x   **63x**

Figure 8. Mosaic Analysis of *E-* and *N-Cadherin* Function in Trigeminal Sensory Ganglion Assembly

One-cell stage wild-type donor embryos were injected with rhodamine-dextran and *E-* and *N-cadherin* morpholinos, and at the 1000-cell to sphere stage, approximately 100 donor cells were transplanted into recipient embryos of an equivalent stage. At 28 hpf, recipient embryos were stained for HNK-1 to label TgSNs (green). Donor-derived cells were identified by rhodamine-dextran (red). TgSNs with reduced levels of E- and N-cadherin transplanted into wild-type host embryos contribute exclusively to the wild-type TgSN cluster (A and B), while TgSNs with reduced levels of *E-* and *N-cadherin* transplanted into *ody/cxcr4b* mutant host embryos contribute to the anteriorly displaced (arrowhead labeled with "e" in [C] and arrows in [D]) and the wild-type TgSN clusters (arrow labeled with "w" in [C]). TgSNs with reduced levels of *E-* and *N-cadherin* transplanted into *ody/cxcr4b* mutant host embryos injected with *ngn* morpholinos are positioned correctly (arrows labeled with "w" in [E] and arrows in [F]).

bryos (arrow, Figures S5A, S5B, S5E, and S5F) and in both the ectopic (arrowhead, Figures S5C, S5D, S5G, and S5H) and the correctly positioned clusters in *ody/cxcr4b* mutant embryos (arrow, Figures S5C, S5D, S5G, and S5H). Taken together, these results suggest that Cxcr4b signaling and E- and N-cadherin act in parallel to position the TgSG.

Discussion

We used the zebrafish TgSG as a model system to study the positioning and assembly of neurons into compact clusters. Our results suggest that four processes contribute to ganglion formation. First, neurons are born locally, placing them nearby or at their site of assembly. Second, neurons are attracted by localized sources of chemokines and migrate posteriorly to join other neurons. Third, cell-cell interactions can influence the assembly of neurons. Fourth, cadherin-mediated adhesion contributes to ganglion positioning.

The Role of Chemokine Signaling in Ganglion Positioning

TgSNs are initially scattered in groups of loosely clustered cells extending along the anterior-posterior axis. During early segmentation stages, anterior neurons move posteriorly and aggregate with more posteriorly located neurons into one compact cluster. Our results reveal that this process is, in part, guided by the chemokine receptor Cxcr4b, which is expressed in TgSNs, and its ligands SDF1a and SDF1b, which are expressed posteriorly to the final position of the ganglion. Upon disruption of Cxcr4b signaling, anteriorly located neurons do not migrate posteriorly and posteriorly positioned neurons can escape anteriorly, resulting in the formation of an ectopic anterior cluster. This phenotype seems to be further augmented by the anteriorly di-

rected morphogenetic movement of cells surrounding TgSNs. Hence, chemokine signaling is required to correctly position TgSNs. Conversely, global expression of SDF1a also disrupts TgSG assembly. Moreover, an ectopic source of SDF1a can recruit TgSNs to ectopic positions. Analogously, dorsal root ganglion (DRG) neuron precursors, a cell type related to TgSNs, are attracted in vitro to a local source of SDF1 (Belmadani et al., 2005). These results indicate that Cxcr4 signaling is not simply permissive, but acts as a guidance cue to position the TgSG. The expression patterns of *SDF1a* and *SDF1b* with respect to the position of TgSNs indicates that chemokine signaling is both long-range, attracting the most anterior neurons over a distance of more than 100 μm, and short-range, retaining the most posterior neurons at the correct position.

Interestingly, all TgSNs express *cxcr4b*, but only a subset of neurons are mispositioned upon perturbing its function. This result raises the possibility that there are two subclasses of TgSNs, only one of which requires chemokine signaling to be positioned correctly. Indeed, both neural crest and placodal cells give rise to the TgSG. However, in *ody/cxcr4b* mutant embryos, placodal and neural crest marker gene expression do not differentiate between ectopic and correctly positioned TgSNs, and in double mutant embryos that lack Cxcr4b activity and neural crest cells, TgSNs are reduced in number but still form two clusters. These results indicate that anterior and posterior TgSNs are derived from both neural crest and placodal cells. Thus, both neural crest- and placodal-derived TgSNs seem to use similar mechanisms to reach their target site in the embryo.

Comparative studies have suggested that the TgSG evolved as a fusion of two distinct ganglia—the profundal and the trigeminal sensory ganglia (Schlosser and Northcutt, 2000). It is tempting to speculate that the absence of Cxcr4b-mediated chemokine signaling may

revert the trigeminal complex to its ancestral separation into two separate ganglia. There is no marker gene known to be expressed specifically in either the profundal or trigeminal ganglion, and we have not found any differences in marker gene expression between *cxcr4b*-dependent and -independent neurons. Thus, there is currently no known genetic difference between anterior and posterior neurons other than their positioning during TgSG formation. It might simply be this difference in initial positioning that imposes distinct behaviors in the absence of chemokine signaling. For example, the anteriorly directed morphogenetic movements of tissues surrounding the TgSNs might be more vigorous anteriorly than posteriorly. In the absence of Cxcr4b signaling, anterior TgSNs might fail to withstand these tissue movements and so are swept to more anterior positions.

The role of Cxcr4 chemokine signaling in TgSG formation parallels its role in other systems (Kunkel and Butcher, 2003; Schier, 2003; Tran and Miller, 2003). For example, chemokine signaling is employed during the positioning of germ cells in zebrafish (Doitsidou et al., 2002; Knaut et al., 2003) and of external granule layer (EGL) cells in the mouse cerebellum (Klein et al., 2001; Ma et al., 1998b; Reiss et al., 2002; Zhu et al., 2002; Zou et al., 1998) and of DRG neuron precursors in mouse (Belmadani et al., 2005). In each case, *SDF1* is expressed in a restricted pattern whereas the receptor is expressed in the migrating cells. However, there are also intriguing differences in the way that Cxcr4 guides different cell types. In contrast to TgSNs, germ cells are not born locally, but develop at several very distant positions (Knaut et al., 2002; Weidinger et al., 1999, 2002; Yoon et al., 1997). Moreover, they always seem to be in direct association with a constantly moving *SDF1* expression domain (Reichman-Fried et al., 2004). In contrast, most TgSNs are initially located at a distance from, and are not found directly adjacent to, a stable *SDF1* expression domain. This suggests that short-range chemokine signaling may be sufficient for germ cell migration, whereas long-range signaling is involved in TgSN positioning.

Although the movement of EGL cells has not been analyzed in live embryos, it appears that these *cxcr4*-expressing neurons are located next to *SDF1*-expressing meningeal cells (Klein et al., 2001; Ma et al., 1998b; Reiss et al., 2002; Zhu et al., 2002; Zou et al., 1998). *SDF1* and *cxcr4* function are required to retain EGL cells at this position until a later stage when they migrate ventrally. Hence, the role of chemokine signaling in EGL formation resembles the retention of TgSNs, but might not involve the long-range attraction that is required to position the TgSG. The finding that Cxcr4 signaling is involved in the formation of both neuronal layers and ganglia suggests that it might be a general tool to assemble neural ensembles in vertebrates.

An intriguing difference between chemokine signaling in the aforementioned cases and in TgSN positioning is the observation that TgSNs are not in direct contact with *SDF1*-expressing tissues. This could be due to TgSNs becoming less responsive to Cxcr4b signaling as they approach the SDF1 source. However, when challenged with ectopic sources of SDF1a, TgSNs frequently are found directly adjacent to chemokine-expressing cells. We therefore favor the alternative hypothesis that TgSNs encounter a physical (or molecular) barrier that hinders their posterior migration onto the SDF1 source. Taken together, these observations indicate that the SDF1/Cxcr4 signaling system can be differentially employed to assemble various neural ensembles in vertebrates.

A Role for Cadherins in Ganglion Formation
Our observation that a subset of TgSNs is positioned correctly in *ody/cxcr4b* mutant embryos points to the existence of additional mechanisms that cooperate with Cxcr4b-mediated chemokine signaling in neuronal positioning. Our results suggest that E- and N-cadherin act together with Cxcr4b chemokine signaling in the positioning of TgSNs. In particular, we find that the abnormal positioning of neurons in *ody/cxcr4b* mutants is enhanced if they have reduced levels of E- and N-cadherin. Three lines of evidence suggest that Cxcr4 signaling and E- and N-cadherin act in parallel. First, loss of Cxcr4 activity alone induces a phenotype, whereas loss of only E- and N-cadherin does not. This result argues against an essential role for E- and N-cadherin upstream of Cxcr4 signaling. Second, E- and N-cadherin expression levels are not regulated by Cxcr4 signaling. This observation argues against the idea that Cxcr4 signaling is upstream of E- and N-cadherin. This result and the finding that the cell types in the ectopic and correctly positioned ganglia are indistinguishable also argue against models that suggest that the phenotypes are caused by differential adhesion and sorting out. Third, loss of E- and N-cadherin enhances the Cxcr4 null phenotype. This result is consistent with parallel roles for chemokine signaling and cadherin activity in sensory neuron positioning.

A role for cadherins in neuronal assembly in the spinal cord has also been described. Functionally related motor neurons organize into discrete clusters, termed motor pools. In this system, the differential expression of type II cadherins appears to define specific motor pools by homophilic interactions among neurons (Price et al., 2002). In contrast, our studies do not reveal any subdivision of the TgSG by Cadherins, but implicate Cadherins in the positioning of TgSNs. It remains unclear, however, which cells these neurons adhere to. E- and N-cadherin are expressed widely, suggesting that TgSNs may interact with many surrounding tissues. In this scenario, chemokine signaling would guide cells to their correct location, where they then adhere to the surrounding tissue. Alternatively, neurons might employ cadherin-mediated substrate interactions to reach their target, as is the case for border cell migration in the *Drosophila* egg chamber (Niewiadomska et al., 1999).

Sensory Neurons Interact with Each Other
Our imaging and mosaic analyses uncovered a role for neuron-neuron interactions that might play a role in ganglion assembly. First, tracking of cells revealed dynamic cell-cell contacts between neurons and the formation of compact neuronal clusters. Second, *ody/cxcr4b* mutant neurons can misplace wild-type neurons to ectopic positions when the latter are transplanted into *ody/cxcr4b* mutants. This effect can be sup-

pressed by blocking the formation of *ody/cxcr4b* mutant neurons. These neuron-neuron interactions might serve several potential functions. For example, they might assist chemokine attraction in the positioning of neurons. However, we have observed that single neurons assume a correct position even in the absence of their siblings (H.K. and A.F.S., unpublished data). We therefore suggest that these neuron-neuron interactions might promote the compact clustering required for ganglion formation in wild-type. Initially, these cell-cell interactions may trigger TgSNs to assemble into local clusters, as seen even in the absence of Cxcr4 signaling. Chemokine attraction then recruits TgSNs to their correct posterior position and brings them in closer proximity. This may allow for further neuron-neuron interaction that will finally result in the assembly of one compact cluster. Thus, cell-cell interaction may assemble TgSNs into clusters, while Cxcr4-mediated guidance is instrumental in positioning these clusters correctly. The molecular basis for the observed cell-cell interactions is not yet known, but our E- and N-cadherin ablation studies suggest that these molecules might not be involved in this process.

In summary, our findings implicate four processes in the assembly of neurons into a TgSG: local birth, chemokine attraction, neuron-neuron interaction, and adhesion. We suggest that these disparate mechanisms might have evolved not only to allow the precise positioning of sensory neurons but also to counter other morphogenetic movements. TgSNs face extensive tissue rearrangements as the embryo elongates. Neighboring cells do not remain adjacent to TgSNs but move to more anterior positions. Withstanding morphogenetic movements might thus be an essential step to allow neuronal assembly in a dynamic environment.

Experimental Procedures

Zebrafish Strains
Embryos were staged as described (Kimmel et al., 1995). *ody*[JI0049] homozygous embryos were generated by inbreeding homozygous adults or crossing homozygous adults with heterozygous adults to obtain 50% wild-type and 50% *ody/cxcr4b* mutant embryos for synchronized development. In the latter case, wild-type and *ody/cxcr4b* mutant embryos were distinguished by fluorescent antibody staining for the germ cell marker Vasa (Knaut et al., 2000). *nrd/blimp1;ody/cxcr4b* double mutants were generated by inbreeding adults heterozygous for *nrd/blimp1* and *ody/cxcr4b*. Transgenic zebrafish carrying the zebrafish heatshock promoter (Halloran et al., 2000) driving SDF1a expression were generated as described (Thermes et al., 2002).

Whole-Mount In Situ Hybridization and Antibody Staining
Preparation of RNA probes and in situ hybridizations were performed as described (Ober and Schulte-Merker, 1999). For double in situ hybridizations, RNA probes against *cxcr4b*, *SDF1a*, *SDF1b*, and *pax-2* were labeled with DIG (Roche) and the second probe against *HuC*, with DNP (Mires) and detected with anti-DIG antibody (Roche) and NBT/BCIP (Roche) and anti-DNP antibody (Mires) and NBT/BCIP or INT/BCIP (Roche), respectively. Antibody staining against HNK-1 was performed as described (Trevarrow et al., 1990). For antibody in situ hybridization costainings, in situ hybridization was performed as above, followed by ISL-1 antibody staining (39.4D5; Developmental Studies Hybridoma Bank, University of Iowa) as described (Ericson et al., 1992; Holley et al., 2000). For costainings against N-cadherin (Transduction Laboratories), E-cadherin (Transduction Laboratories), TrkA (Santa Cruz Biotechnology), or TrkC (Santa Cruz Biotechnology) and HNK-1 embryos were processed as described (Trevarrow et al., 1990). Antibodies against N-cadherin, E-cadherin, TrkA, and TrkC were diluted 1:100 and detected using an anti-mouse antibody conjugated to HRP (Jackson Immunolab) and the Cy3-tyramid system (NEN Life Science). The embryos were incubated with 100 mM glycin, (pH, 2.2) to remove the primary antibodies and stained for HNK-1 as described (Trevarrow et al., 1990), using anti-mouse Alexa 488 secondary antibodies (Molecular Probes). For sectioning embryos were embedded as described (Knaut et al., 2000).

Morpholino Injections
Morpholinos were injected into one-cell stage embryos. *Cxcr4b* and *cxcr4b* mismatch morpholinos (Knaut et al., 2003) were injected at a concentration of 0.2 mM and a volume of 1 nl; *SDF1a* and *SDF1a* mismatch morpholinos (Doitsidou et al., 2002), at a concentration of 0.5 mM and a volume of 2 nl; *SDF1b* and *SDF1b* mismatch morpholinos (Knaut et al., 2003), at a concentration of 2 mM and a volume of 2 nl; *ngn* morpholino (5′-cgatctccattgttgataacctta-3′) (Genetools), at a concentration of 0.5 mM and a volume of 1 nl; *N-cadherin* morpholino MO1 (Lele et al., 2002), at a concentration of 0.1 mM and a volume of 2 nl; and *E-cadherin* morpholino (5′-ATCCCACAGTTGTTACACAAGCCAT-3′), at a concentration of 0.1 mM and a volume of 2 nl.

Mosaic Analysis
One-cell stage donor embryos were injected with lysine-fixable rhodamine-dextran (Molecular Probes). At the 1000-cell to sphere stage, approximately 100 donor cells were transplanted into recipient embryos of an equivalent stage. At 28 hpf, TgSNs were identified by HNK-1 antibody staining as described above with the modification that HRP activity was detected with fluorescein-tyramide (NEN Life Sciences) and donor-derived cells were identified by rhodamine-dextran. Donor and recipient embryos were either wild-type or *ody/cxcr4b* mutant and not injected or injected with either *ngn* and/or *N-cadherin* or *ngn* and/or *E-cadherin* morpholino, as indicated (Tables S2–S4).

SDF1a Misexpression
For ubiquitous misexpression of SDF1a, wild-type and *heatshock*-SDF1a transgenic embryos were heat-shocked at the six-somite stage for 1 hr in a 37°C water bath, raised at 28°C to the ten- or 22-somite stage and stained for *HuC* or HNK-1, respectively, as described above.

For local misexpression of SDF1a, one-cell stage wild-type or transgenic embryos carrying the *heatshock*-SDF1a transgene were injected with rhodamine-dextran and biotin-dextran, and at the 1000-cell to sphere stage, approximately 20 donor cells were transplanted into recipient embryos of an equivalent stage. Recipient embryos were heat-shocked at the six-somite stage for 1 hr in a 37°C water bath or not subjected to a heat shock, raised at 28°C to the ten-somite stage and stained for *HuC* and biotin-dextran. HuC staining was done as described above. Biotin-dextran-labeled cells were detected using HRP-coupled streptavidin (Vectashield ABC Kit, Vector) and DAB (Roche). The genotype of transgenic embryos was confirmed by staining for *SDF1a*.

Cell Fate Mapping
Neighboring cells of TgSNs were fate mapped in *ngn*-GFP transgenic wild-type and *ody/cxcr4b* mutant embryos by laser-induced liberation of lysine-fixable caged fluorescein-dextran at the three-to four-somite stage as described (Carmany-Rampey and Schier, 2001) with the modification that embryos were coinjected with caged rhodamine-dextran (Molecular Probes) to visualize uncaged cells in the background of GFP-positive neurons. Embryos were raised until 28 hpf, fixed with 4% paraformaldehyde in PBS and stained for HNK-1 as described above. Uncaged cells were detected by incubation with anti-fluorescein antibody conjugated to alkaline phosphatase (1:5000; Roche) and stained with NBT/BCIP (Roche).

Time-Lapse Recordings

For time-lapse analysis, embryos were prepared as described (Knaut et al., 2002). Migration of TgSNs was analyzed in wild-type and *ody/cxcr4b* mutant embryos expressing GFP from the *ngn* promoter (Blader et al., 2003). *ody/cxcr4b* mutant and *ngn*-GFP transgenic embryos were generated by crossing adults heterozygous for *ody/cxcr4b* and *ngn*-GFP with homozygous *ody/cxcr4b* mutant adults. Embryos with the desired genotype were identified by injection of *GFP* RNA fused to the *vasa* 3′UTR at the one-cell stage to visualize germ cell mismigration in *ody/cxcr4b* mutant embryos (Wolke et al., 2002). TgSN migration was followed using a Zeiss LSM 510 laser scanning microscope equipped with a heated stage set to 28°C. Image stacks and sequences were processed using Zeiss LSM 510 software and Quicktime Pro. Stacks of ten to 15 sections were recorded every 5 min.

Supplemental Data

Supplemental data include five figures, five tables, and eight movies and are available with this article online at http://www.neuron.org/cgi/content/full/cgi/47/5/653/DC1.

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