Glial cell line-derived neurotrophic factor defines the path of developing and regenerating axons in the lateral line system of zebrafish

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How the peripheral axons of sensory neurons are guided to distant target organs is not well understood. Here we examine this question in the case of the posterior lateral line (PLL) system of zebrafish, where sensory organs are deposited by a migrating primordium. Sensory neurites accompany this primordium during its migration and are thereby guided to their prospective target organs. We show that the inactivation of glial cell line-derived neurotrophic factor (GDNF) signaling leads to defects of innervation and that these defects are due to the inability of sensory axons to track the migrating primordium. GDNF signaling is also used as a guidance cue during axonal regeneration following nerve cut. We conclude that GDNF is a major determinant of directed neuritic growth and of target finding in this system, and we propose that GDNF acts by promoting local neurite outgrowth.

axonal guidance | nerve regeneration | posterior lateral line | RET

Most sensory neurons of vertebrates are clustered in cranial or dorsal root ganglia and innervate sense organs that may be far away. How peripheral axons are guided to their target organs is not well understood. In the case of the posterior lateral line (PLL) system of fish, individual mechanosensory organs (neuromasts) are derived from a cranial primordium that migrates along the horizontal myoseptum to the tip of the tail (1, 2). In zebrafish, the embryonic PLL primordium, primI, migrates between 20 and 40 h postfertilization (hpf). During its migration, the primordium deposits five groups of cells that later become neuromasts L1 to L5 (reviewed in ref. 3), and forms two to three terminal neuromasts at the tip of the tail. primI also deposits a continuous stripe of interneuromast cells (Fig. S1). Those are maintained in a dormant state by the presence of glial cells along the nerve (4, 5) and become reactivated later during larval development to form intercalary neuromasts (6). A second set of neuromasts (LII) is deposited during early larval life by a second primordium, primII (ref. 7 and Fig. S1).

It was first suggested in amphibians (1), and later confirmed in zebrafish, that sensory neurites extend into the primordium before the onset of migration (8), accompany the primordium during its migration (9, 10), and eventually innervate the deposited neuromasts. Sensory axons serve in turn as a guiding pathway for neural crest-derived glial cells (11). The molecular mechanism underlying the comigration of primordium and of sensory growth cones is not known.

Glial cell line-derived neurotrophic factor (GDNF) was first identified as a potent survival-promoting factor for midbrain dopaminergic neurons (12). GDNF signaling has been implicated in the specification of subpopulations of dorsal root sensory neurons (reviewed in ref. 13), as a guidance cue for autonomic neurons (reviewed in ref. 14), and has been shown to support the survival of peripheral neuronal populations in vitro (15). Genes coding for various components of the GDNF signaling system are expressed in many developing peripheral sensory systems.

In zebrafish, the gene that codes for GDNF is expressed in the embryonic primordium (16). Two receptors are known for GDNF: GFRalphal and GFRalpha2, both of which interact with the RET receptor tyrosine kinase to initiate the signaling cascade that mediates GDNF activity (17–20). The two genes that code for GFRalphal, *gfra1a* and *gfra1b*, are both expressed in the PLL ganglion, as is *ret1*, the gene that codes for the RET kinase (16). The gene coding for the alternative GDNF receptor, GFRalpha2, is not detectably expressed in the PLL system. The observation that GDNF is produced by migrating primordium cells, and that GFRalphal receptors are present on sensory neurons, suggests that GDNF signaling may play a role in the tracking of primordium cells by sensory axons. Previous experiments showed that the inactivation of *ret1* by morpholino-antisense oligonucleotides or the simultaneous inactivation of *gfra1a* and *gfra1b*, does not affect PLL development or the innervation of neuromasts at concentrations that produce major effects on the enteric neurons, another target of GDNF signaling (16). Here we reexamine the role of GDNF in the association of afferent growth cones and migrating primordium cells during normal development and in the guidance of regenerating axons after nerve damage.

Results

Using the *Huc:kaede, Et20:gfp* line (Fig. 1*A*), where neurons express the photoconvertible protein Kaede (21) and neuromasts express GFP (22), we confirmed that the inactivation of various components of the GDNF pathway has at best a minor effect on innervation (Table S1, lines 1–5), even using relatively high morpholino concentrations (1.5 mM). Interestingly, however, the double inactivation of *gdnf* and of either *gfra1a* or *gfra1b* increases the frequency of truncated nerves up to about 25%, and the double inactivation of *gdnf* and of the gene coding for its ultimate target, *ret1*, results in 65% nerve stops (Table S1, lines 6–8, and Fig. 1*B*, asterisk). Nerve arrest was always at the level of a neuromast, usually L2 (Fig. 1 *B* and *C*).

The *gdnf*-MO1 and *ret1*-MO1 oligonucleotide sequences contain only 32% and 36% GC, respectively, instead of the 40% recommended by GeneTools. Because low GC morpholino oligonucleotides work better at lower temperatures (GeneTools, personal communication), we examined whether a treatment at 25 °C would result in stronger phenotypes. Under this condition, treatment with either *gdnf*-MO1 or *ret1*-MO1 alone resulted in more than 60% nerve stops (Table S1, lines 9–10). Mismatch morpholino grown up at 25 °C caused no arrest in PLL nerve in 228 embryos, even at the very high concentration of 5 mM (*gdnf*-mismatch: 1.25 mM, $n = 42$; 2.5 mM, $n = 28$; 5.0 mM, $n = 31$ and *ret1*-mismatch: 1.25 mM, $n = 50$; 2.5 mM, $n = 42$; 5.0 mM, $n = 35$).

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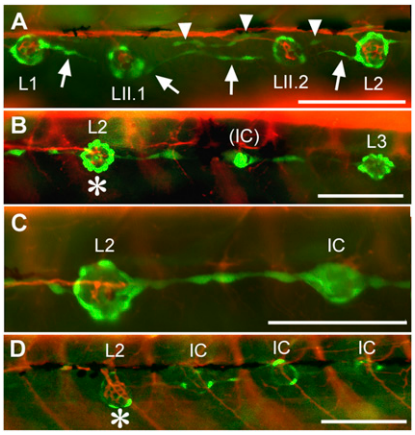

Fig. 1. Innervation defects in *Huc:kaede, Et20:gfp* early larvae. (*A*) Normal pattern in a control larva at 4 dpf. Nerve branches extend to and arborize in each neuromast. Interneuromast cells deposited by the primordium between L1 and L2 (arrows) have moved ventrally ahead of the primII-derived neuromasts, LII.1 and LII.2. Interneuromast cells deposited by primII can also be observed close to the myoseptum (arrowheads). (*B*) Peripheral afferent axons stop at L2 (asterisk) in a 3-dpf *gdnf, ret1*-MO1 fish. An intercalary neuromast (IC) is beginning to develop between L2 and L3. The red fibers posterior to L2 belong to motor axons innervating the somitic muscles. (*C*) A higher magnification photograph to show that no nerve branch extends beyond L2, in a 4-dpf double morphant fish. (*D*) Nerve stop at L2 (asterisk) in a 4-dpf *gdnf*-MO2 fish. Intercalary neuromasts have formed on all somitic borders posterior to the nerve arrest. Anterior is *Left* in all panels. (Scale bar, 100 mm.)

We also examined a second set of *gdnf* and *ret1* morpholino oligonucleotides (MO2) designed to have a higher GC content. *gdnf*-MO2 embryos showed nerve stop around somite 10 in 90% of the cases at a concentration of only 0.6 mM (Fig. 1*D* and Table S1, line 11), and *ret1*-MO2 embryos showed essentially no nerve extension at concentrations as low as 0.15–0.3 mM (Table S1, lines 12–16). Morphant embryos show no developmental delay, no mortality, and no morphological abnormalities.

Precocious nerve stop could be due, not to a defect in axonal extension, but to axon retraction. We examined this question in *gdnf, ret1*-MO1 double morphant embryos, where the nerve usually extends up to L2 or L3. We observed in all cases the presence of intercalary neuromasts distal to the last innervated neuromast (Figs. 1 *B* and *D* and 2 *A*–*C*). Intercalary neuromasts form precociously when glial cells are absent (4, 5), and their presence beyond the position where the nerve stops indicates that afferent axons (which guide the glial cells) never extended beyond this point.

We attempted to visualize nerve detachment from the migrating primordium in *cldnb:gfp* embryos, where all cells of the primordium fluoresce in green (23). The frequency of nerve arrests in *gdnf, ret1*-MO1 double morphant *Huc:kaede, cldnb:gfp* embryos is much lower than in *Huc:kaede, Et20:gfp* embryos (10% instead of 60%), possibly because migration is slowed down in the *cldnb:gfp* line (6). A slower primordium may leave more time for reduced GDNF signaling to act on the growing neurites or may allow slower neurites to maintain contact. We could nevertheless observe cases of nerve arrest in *Huc:kaede, cldnb:gfp* embryos treated with *gdnf, ret1*-MO1 with neurite-free primordia migrating beyond the point where the peripheral axons stalled (Fig. 2*D*). We also performed video time-lapses in *nbt:dsred, cldnb:gfp* embryos, where neurons fluoresce in red without need to photoconvert (ref. 24 and Movie S1). Movie S2 illustrates a *gdnf, ret1*-MO1 *nbt:dsred, cldnb:gfp* embryo where axons accompanied the primordium up to the deposition of L3. The movie shows that the nerve neither extends beyond L3 nor retracts over the following 10 h. The situation did not change over the next 7 d.

Because of the reduced frequency of nerve stops in the *cldnb:gfp* background, we confirmed nerve detachment from migrating primordia using the *cxcr4b:red* reporter line where a membrane version of mCherry (RFP) is driven by a minimal 139-bp *cxcr4b* promoter (25), resulting in red fluorescence in the migrating primordium. Injection of 0.15 mM *ret1*-MO2 in *Huc:kaede, cxcr4b:red* embryos results in a high frequency (89%, $n = 44$) of nerve stops, although somewhat lower than the same injection in *Huc:kaede, Et20:gfp* (Table S1). We observed again neurite-free primordia (Fig. 2*E*), and we conclude that the nerve truncations observed in morphant fish reflect a reduced ability of sensory growth cones to comigrate with the primordium.

GDNF has been shown to be essential for the proliferation, differentiation, and survival of many types of peripheral neurons (reviewed in ref. 26). It might be, therefore, that the inactivation of GDNF signaling affects the formation of afferent neurons, rather than their primordium-tracking capability. Nerve stops might then be a secondary effect of a reduction in the number of afferent neurons. We assessed this possibility by counting the number of neurons in 2.5 mM *ret1*-MO1 embryos grown at 25 °C, where nerve stop is observed in 80% of the cases ($n = 20$). We counted the number of neurons, and the position reached by the PLL nerve, at 3 dph. We found no significant difference between the numbers of neurons in normal (23.2 ± 3.3) and morphant (23.9 ± 3.4) embryos. Furthermore, there is no correlation between the number of afferent neurons and the position reached by the PLL nerve among morphant embryos (Fig. 3). Thus a global reduction in the number of afferent neurons does not seem to be responsible for nerve arrest in *ret1*-MO1 embryos.

Using the more efficient *ret1*-MO2 oligonucleotide, however, we observed a systematic albeit small reduction in neuronal number, even at early times (30 hpf) and at the low concentration of 0.15 mM (17.1 ± 3.3, $n = 7$ vs. 21.2 ± 1.3, $n = 5$, in controls). This difference is considered significant under unpaired t test, Welch corrected ($P = 0.018$). It is conceivable that this reduction reflects a specific defect in the leading afferent neurons. The leading axons that arborize extensively within the migrating primordium, and later innervate the posteriormost neuromasts (8), are extended by the earliest differentiating afferent neurons (27). A simple explanation for precocious nerve arrest would therefore be a deficit in those early neurons. We assessed this possibility by examining whether the presence of early differentiating, leading neurons is affected in morphant embryos.

For this experiment we used the *ret1*-MO2 extreme phenotype, where nearly all embryos show a severe defect in nerve extension (Table S1). We relied on somite number, which is an accurate measure of developmental age, to asses the timing of afferent development. The earliest PLL differentiating neurons, as visualized in the *Huc:kaede* reporter line, can be detected around the 18-somite stage (27). We examined the presence of fluorescent cells in the PLL ganglion of 0.15 mM *ret1*-MO2, *Huc:kaede* embryos from the 18- to 20-somite stage. The results are shown in Table 1. We observed no difference between control and morphant embryos, indicating that the leading neurons are not preferentially affected in morphant embryos.

Nerve arrest could be due to a decreased affinity of the outgrowing axons for the migrating primordium cells. We examined the extent of neurite arborization in *Huc:kaede, cldnb:gfp* embryos and observed that sensory neurons show extensive arborization under the primordium in normal embryos (refs. 8 and 10 and Fig. 2*F*). In contrast, the arborization is much reduced in *gdnf, ret1*-MO1 morphant embryos (Fig. 2*G*), showing that sensory neurites have a decreased affinity for primordium cells in the double morphant conditions. As expected, this phenotype is more pronounced with the more extreme *ret1*-MO2 oligonucleotide, with afferent neurites having abandoned the primordium before L1 deposition (Fig. 2*H*).

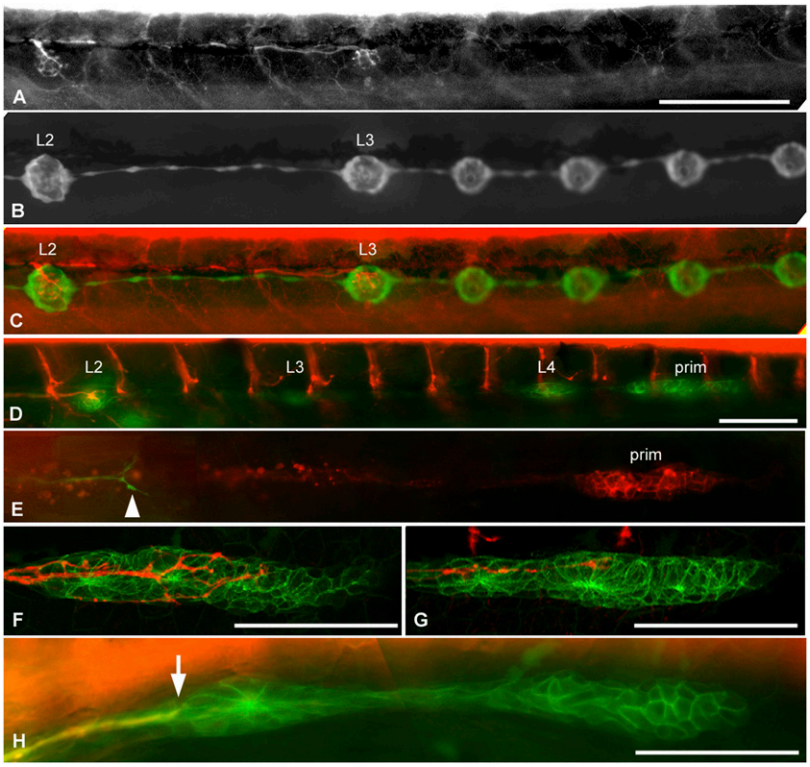

Fig. 2. Innervation defects are due to precocious nerve arrest. (*A–C*) Nerve interruption at L3, in 4-dpf *gdnf, ret1*-MO1 double morphant fish. Nerve arrest (*A*) is associated with the formation of precocious intercalary neuromasts distal to the interruption (*B* and *C*). (*D* and *E*) Nerve interruption in double morphant embryos is manifest at a time when the primordium is still migrating in photoconverted *Huc:kaede, cldnb:gfp* (*D*) or in *Huc:kaede, cxcr4b:rfp* (*E*, arrowhead marks the nerve ending) embryos. (*F* and *G*) Arborization of sensory neurites accompanying the migrating primordium at 35 hpf in a control (*F*) and a *gdnf, ret1*-MO1 double morphant embryo (*G*). (*H*) Nerve lagging behind the primordium (arrow) in an *nbt:dsred, cldnb:gfp* embryo injected with 0.15 mM *ret1*-MO2. L1 is about to be deposited. *E* and *H* are composite pictures assembled from two consecutive planes in Z-stacks. Anterior is left in all panels, and the primordium is migrating to the right in *D–H*. (Scale bar, 100 mm.)

We next examined nerve regeneration in the *foxd3:gfp* line, where glial cells are fluorescent (11). When the PLL nerve of a 3-day postfertilization (dpf) larva is cut directly posterior to the ganglion, the axons posterior to the cut degenerate over the next 4–8 h (Fig. 4 *A–C* and Movie S3). New axons regenerate along the glial cells, presumably using them as guidance cues, both in control and in *gdnf, ret1*-MO1 embryos (Fig. 4 *C–F* and Movie S3). Schwann cells have been shown to provide guidance to regenerating axons in mice (28). Glial cells do not detectably express *gdnf*, and regeneration along the glial chain is not affected in *gdnf, ret1*-MO1 conditions, suggesting that axonal guidance along glia does not involve GDNF signaling and may depend on the same recognition mechanism that allows glial cells to migrate along axons in the first place (11).

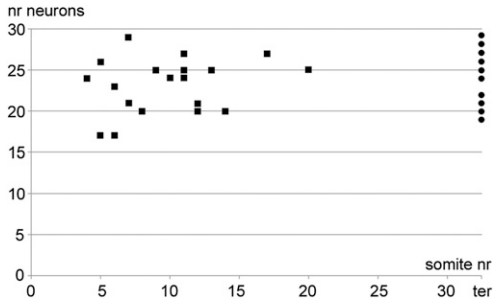

Fig. 3. Partial inactivation of *ret1* induces nerve stops, but no neuronal loss, in *nbt:dsred, Et20:gfp* embryos. The plot represents number of neurons (ordinate) vs. the position reached by the nerve (abscissa) in 19 *ret1*-MO1 embryos raised at 25 °C (squares). Only one side was examined for each embryo. In control embryos (dots) the nerve always reached the terminal neuromasts. (Scale bar, 100 mm.)

We then examined the behavior of regenerating axons in the absence of glia, by ablating the nerve (axons and glia) between L1 and L2, the first two neuromasts of the embryonic line, in 3-dpf *foxD3:gfp* larvae. The axons had regenerated on the next day, as revealed by the presence of new glial cells between L1 and L2. Whereas the normal nerve path follows the myoseptum, the pathway followed by the regenerating axons was more ventral. This difference is very obvious at 6 dpf (Fig. 5*A*): the path of the regenerated nerve (arrowheads) is festooned and ventral to the horizontal myoseptum, rather than straight and aligned with the myoseptum as is normally the case. This pattern resembles that of interneuromast cells (Fig. 5*B*, arrowheads). At 6 dpf the garland of interneuromast cells has been pushed ventrally by a second wave of neuromasts deposited by a second primordium (ref. 29 and Fig. S1) and assumes the festooned pattern shown in Fig. 5*B*.

Table 1. Blocking GDNF signaling does not change the time of appearance of the earliest PLL neurons

	18 som	19 som	20 som
ctrl	50% (*n* = 18)	95% (*n* = 19)	100% (*n* = 18)
ret1-MO2	65% (*n* = 20)	93% (*n* = 27)	100% (*n* = 18)

Huc:kaede embryos were injected with 0.15 mM *ret1*-MO2, and the presence of fluorescence in the PLL ganglion was assessed between 18 and 19 hpf.

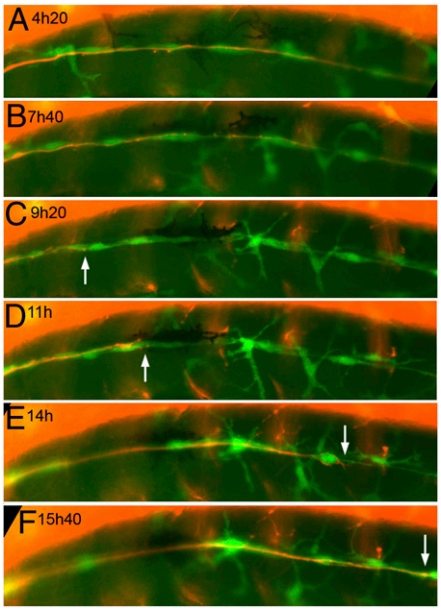

Fig. 4. Still panels from Movie S3. The nerve was cut just posterior to the ganglion at time 0 in a 3-dpf, *nbt:dsred, foxd3:gfp* embryo. (*A*–*C*) Progressive axonal degeneration; (*C*–*F*) progress of the regenerating growth cones (white arrows). (Scale bar, 100 mm.)

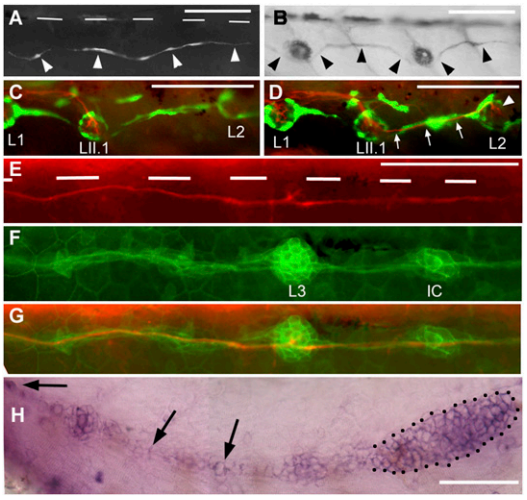

Fig. 5. Regenerating axons can be guided by interneuromast cells. (*A*) After ablation of glial cells in the *foxd3:gfp* line, new glial cells extend posteriorly (arrowheads). (*B*) Their path resembles that of interneuromast cells (arrowheads) as visualized by alkaline phosphatase labeling. (*C* and *D*) After glial ablation between L1 and L2 in 3-dpf *Huc:kaede, Et20:gfp* fish, axons regrow along the myoseptum up to LII.1 (*C*) and follow the interneuromast cells (arrows) up to L2 (arrowhead in *D*). (*E*) Regenerating axons in a 54-hpf *Huc:kaede, cldnb:gfp* fish where the nerve had been cut at 30 hpf. White bars indicate the position of the horizontal myoseptum. (*F*) Trail of interneuromast cells in the same embryo; (*G*) merge. (*H*) Expression of *gdnf* in the primordium (dotted outline) and in the interneuromast cells (arrows) as revealed by in situ hybridization. (Scale bar, 100 mm.) *A* and *H* were assembled from two consecutive levels in Z-stacks.

The similarity between Fig. 5 *A* and *B* led us to ask whether, in the absence of glia, axons might be guided by the trail of interneuromast cells, much as normal axons are guided by the primordium itself. To test this idea, we repeated the nerve ablation between L1 and L2 in 3-dpf *Huc:kaede, Et20:gfp* fish, where interneuromast cells fluoresce in green. We observed regenerating axons on the next day in all cases ($n = 10$). In 8 out of 10 cases, regenerating neurons followed the trail of interneuromast cells. Fig. 5*C* illustrates a case where, 24 h after ablation, the regenerating axons had reached LII.1, the first neuromast deposited by primII, just posterior to L1. After another 14 h, the neurites had followed the interneuromast cells to reach and innervate L2 (Fig. 5*D*).

The ectopic course followed by the regenerating axons might have been due to laser-induced damage to the myoseptum, causing regenerating axons to avoid the scar. To preclude potential damage to the myoseptum, we cut the nerve just posterior to the ganglion in 30-hpf *Huc:kaede, cldnb:gfp* embryos, a stage when the primordium has reached somite 6–8 approximately. Due to the nerve cut, axons do not follow the primordium any more, and consequently glial cells will be present up to this level, but no further, because they need the axons as substratum for their own migration. We examined the treated larvae 1 d later and observed that axons had regenerated posteriorly in the absence of glial guidance. In all cases, however ($n = 5$), the axons did not follow their normal pathway along the horizontal myoseptum (white bars, Fig. 5*E*) but they regenerated along the trail of interneuromast cells (Fig. 5*G* and Movie S4).

To determine whether guidance of the regenerating nerve along interneuromast cells recapitulates the association between growing axons and migrating primordium, and relies on GDNF signaling, we first examined whether *gdnf* is expressed by interneuromast cells. We observed a low but distinct expression (Fig. 5*H*) at a level similar to that observed in neuromasts and trailing cells of the primordium. We also confirmed expression of the genes coding for GDNF receptors GFRa1a, GFRa1b, and RET1 in the ganglia of the anterior and posterior lateral line (ref. 16 and Fig. S2), but not in the primordium or interneuromast cells.

We determined the effect of impairing GDNF signaling on regeneration in the same conditions as in the previous regeneration experiment: cutting the nerve just posterior to the ganglion in 30-hpf *Huc:kaede, cldnb:gfp* embryos that were doubly morphant for *gdnf* and *ret1*. We observed on the next day nerve regeneration along the horizontal myoseptum over the first eight somites on average (7.8 ± 4.2, $n = 10$; Table 2), consistent with the idea that glia can serve as a guiding cue for regenerating neurites, independently of GDNF signaling. Once the axons reached the end of the glial trail, however, they stalled (Movie S5 in a *nbt:dsred, foxd3:gfp* embryo). No further regeneration occurred over the following 5 d in 9 out of 10 morphant larvae (Table 2), suggesting that nerve regeneration cued by interneuromast cells is prevented in the absence of GDNF/RET signaling. In contrast, axons had fully regenerated up to the tip of the tail 3 d after ablation in control fish.

Knowing that regeneration is complete 3 d after nerve cut in control embryos, we repeated the experiment and recorded the position reached by the nerve at the time of nerve cut, and 3 d later. This was done in either the *gdnf, ret1*-MO1 condition, or in 2.5 mM *ret1*-MO1, the same condition that was used to count the

Table 2. Interfering with GDNF signaling blocks nerve regeneration in *Huc:kaede, cldnb:gfp* fish

	1 dpa	2 dpa	3 dpa	5 dpa	*n*
Control	15.4 ± 1.1	26.2 ± 4.1	>30		5
gdnf-MO1, *ret*-MO1	7.8 ± 4.2	8.5 ± 5.0	8.8 ± 5.2	8.9 ± 5.2	9

The nerve was cut just behind the ganglion in 30- to 32-hpf embryos. The somite reached by the regenerating nerve was examined 1, 2, 3, and 5 d postablation (dpa). Greater than 30 (>30) indicates that the nerve has reached the tip of the body and reinnervated the terminal neuromasts. Out of 10 *gdnf, ret1*-MO1 fish, 1 showed nearly normal regeneration and has not been included in the data.

number of neurons (Fig. 3). The results are shown in Fig. 6. In nonmorphant embryos, the nerve had regenerated to the tip of the body in all cases (n = 11). On the contrary, the regenerated axons stopped close to the position that the nerve had reached at the time of the cut in 23 out of 24 *gdnf, ret1*-MO1 and in 13 out of 15 2.5-mM *ret1*-MO1 embryos (Fig. 6). Taken together, these three experiments show complete regeneration in 18 out of 18 nonmorphant embryos and truncated regeneration in 45 out of 49 morphant embryos. We conclude that GDNF signaling can lead regenerating axons along the trail of interneuromast cells, much as it secured the close association between growing axons and migrating primordium cells during embryogenesis.

Discussion

Our experiments show that GDNF signaling plays a key role in the affinity of sensory growth cones for primordium cells, during embryonic development. This affinity endows PLL afferent axons with the ability to track their migrating target and to innervate neuromasts as they are deposited by the primordium. We observed that, in the absence of glial cells, regenerating axons also depend on GDNF signaling to grow along the trail of interneuromast cells deposited by the embryonic primordium. Taken together, these results indicate that GDNF signaling plays a major role in the innervation of the lateral line system both during normal development and during regeneration.

The partial inactivation of *gdnf* and of *ret1* by the MO1 morpholino oligonucleotides revealed an interaction phenotype whereby the proportion of nerve arrests is much higher in *gdnf, ret1*-MO1 double morphant fish than in either *gdnf* or *ret1* morphants. The presence of such a "compound" phenotype has classically been interpreted as indicating a direct interaction between the products of the two genes involved (30). Interestingly, Hirschsprung disease, where another target of GDNF signaling (enteric neurons) is affected, involves the presence of mutations in both GDNF and in RET, similar to the interaction that we observed here (31, 32).

We noted that partial inactivation of GDNF signaling in *ret1*-MO1 embryos did not reduce the number of neurons even at the high concentration of 2.5 mM, where it induces 80% nerve stops at 25 °C, whereas *ret1*-M02 induced significant neuronal loss even at low concentration. This suggests that neuron survival depends on some trophic factor released by neuromast cells, possibly including GDNF itself: in *ret1*-MO2 embryos, nerve detachment occurs before any neuromast is deposited, and therefore the neurons will have no supply of trophic factor at all, whereas in *ret1*-MO1 embryos nerve detachment usually takes place at L2 or L3, providing the afferents with a limited, but seemingly sufficient, supply.

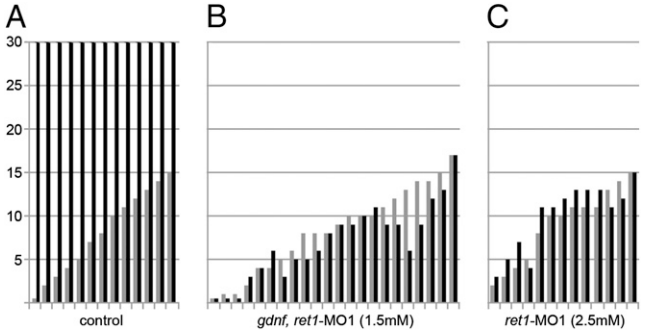

Fig. 6. Position reached by the axons at the time the nerve was cut posterior to the ganglion (gray bar) and 3 d later (black bar) in control (*A*), 1.5 mM *gdnf, ret*-MO1 double morphant (*B*), and 2.5-mM *ret1*-MO1 embryos (*C*). (Scale bar, 100 mm.)

A simple interpretation of our results is that the comigration of primordium cells and of afferent growth cones reflects a local increase in neurite outgrowth as a response to GDNF signaling. This would necessarily lead to afferent extension along the primordium, a process that could be described as primordium tracking by afferents, or as axon towing by the primordium, or even as contact guidance. We observed in different morphant conditions that the nerve stop phenotype was always less severe in *cldnb:gfp* background, where the primordium migrates at about 60% of its normal speed. Although nerve stalling phenotype was particularly weak in *gdnf, ret1*-MO1 *cldnb:gfp* embryos, regeneration along interneuromast cells was efficiently blocked in the same embryos, supporting the notion that the severity of the stalling phenotype depends on the speed of migration and not on some other property of the *cldnb:gfp* line. Slower primordium migration would make it easier for axons with reduced outgrowing capability, due to disabled GDNF signaling, to remain in contact with migrating cells for a longer period.

The growing axons are intimately associated with the cells providing GDNF, both during normal development and during regeneration. This close association suggests that, in the case of the embryonic PLL, GDNF acts at extremely short range. It will be interesting to see to what extent GDNF signaling is also involved in later steps of PLL development, for exemple, for the innervation of intercalary neuromasts, which form at a distance from the PLL nerve.

Materials and Methods

Fish Care and Fish Strains. Fish were kept under standard conditions as described by Westerfield (33). Embryos were raised in fish water (300 mS, pH 7, 28.5 or 25 °C) and staged by hpf or dpf using standard morphological criteria. For imaging, embryos were anesthetized with Tricain (3-amino benzoic acid ethylester, Sigma A-5040) 4 mg/mL in 20 mM Tris pH 7, and mounted in 0.7% agar in fish water. Sensory neurons including their neurites were visualized in *Huc:kaede* fish (21), where the initially green fluorescent Kaede protein was photoconverted to red by illuminating the embryo with UV light for 1 min using a fluorescent microscope equipped with a 100 W HBO lamp and a 10× objective or in *nbt:dsred* fish (24), where all neurons show red fluorescence. When *Huc:kaede* was used in combination with *cxcr4b:red* the larvae were not photoconverted before examination. Neuromasts and interneuromast cells were visualized in the *Et20:gfp* (22) or *cldnb:gfp* (23) lines, and the primordium in either *cldnb:gfp* or *cxcr4b:red* embryos (25). Glial cells were visualized in *foxd3:gfp* fish (11).

Morpholino Injections. Antisense MOs were obtained from GeneTools. MOs were diluted in sterile filtered water containing 5% Phenol Red solution (Sigma). Approximately 2 nL of the MO solution was pressure injected into one- or two-cell stage zygotes. The sequences used to design the morpholinos are as follows:

gfra1a: 5′ CGCTTTTATCCGTGGTAAGTTCGCT 3′
gfra1b: 5′ TCATCGTCGCTTTATTCAGATCCAT 3′
ret1-MO1: 5′ GTCAATCATAAGTGTAATGTCACAA 3′ (anti-start, 32% GC)
ret1 5-base mismatch: 5′ GTgAATgATAAcTGaAATcTCACAA 3′;
ret1-MO2: 5′ ACACGATTCCCCGCGTACTTCCCAT 3′ (anti-start, 56% GC)
gdnf-MO1: 5′ TGTCCCATAACTTCATTTTAGACTC 3′ (anti-start, 36% GC)
gdnf 5-base mismatch: 5′ TcTCgCATAAgTTCATTTTAcAgTC 3′
gdnf-MO2: 5′ TGCACGCTCACTGACTTACATTGTT 3′ (anti-splice, 44% GC).

GDNF Probe and in Situ Hybridization. The *gdnf* probe was prepared as follows: the primers TTTCTGAAGCTCCGGTCTGT and TCCTGTAACCCAAGTCCAGG were used to amplify a 540-bp fragment of the *gdnf* ORF. The PCR product was cloned in the pCRII–TOPO vector (Invitrogen) and used as a template to transcribe the antisense digoxigenin-labeled RNA probe using SP6 RNA polymerase (Roche). Other probes were obtained from D. Raible (University of Washington, Seattle). In situ hybridization for *gdnf*, *gfra1a*, *gfra1b*, and *ret1* was performed according to described protocols (33). The hybridized probe was detected by an alkaline phosphatase-coupled anti-digoxigenin antibody (1:2,000).

Laser Ablation. A Micropoint laser system (Photonic Instruments) was used on a Zeiss Axioplan 2 microscope, using coumarin 440 nm, 5 mM in methanol, as a laser medium. The laser power was attenuated to the appropriate level with neutral density filters. Nerve cut was achieved under a 63× water immersion

objective. Ablation of the nerve between L1 and L2 was done either in *foxd3:gfp* fish, where the glial cells are directly visualized, or in *Huc:kaede* fish, on the basis of the position of the axons.

Time-Lapse Imaging. Time-lapse movies were done on an XYZ motorized Zeiss Axioimager equipped with a stage-heating device. Pictures were captured with a Coolsnap camera and processed with Axiovision or ImageJ software. Images were taken at various intervals using water immersion, long-distance 10×, 20×, and 40× objectives. The movies were done on embryos heterozygous for *nbt:dsred* and *cldnb:gf*, rather than on *Huc:kaede, cldnb:gfp* embryos, to avoid the need to photoconvert Kaede before each frame.

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