First publ. in: Developmental Biology 235 (2001), 1, pp. 86-97

Function of Neurolin (DM-GRASP/SC-1) in Guidance of Motor Axons during Zebrafish Development

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Neurolin (zf DM-GRASP), a transmembrane protein with five extracellular immunoglobulin domains, is expressed by secondary but not primary motoneurons during zebrafish development. The spatiotemporally restricted expression pattern suggests that Neurolin plays a role in motor axon growth and guidance. To test this hypothesis, we injected zebrafish embryos with function-blocking Neurolin antibodies. In injected embryos, secondary motor axons form a broadened bundle along the common path and ectopic branches leave the common path at right angles. Moreover, the formation of the ventral and the rostral projection of secondary motor axons is inhibited during the second day of development. Pathfinding errors, resulting in secondary motor axons growing through ectopic regions of the somites, occur along the common path and in the dorsal and rostral projection. Our data are compatible with the view that Neurolin is involved in the recognition of guidance cues and acts as a receptor on secondary motor axons. Consistent with this idea is the binding pattern of a soluble Neurolin-Fc construct showing that putative ligands are distributed along the common path, the ventral projection, and in the area where the rostral projection develops.

Key Words: **axon guidance; immunoglobulin superfamily; Danio rerio; Zebrafish; motoneuron; pathfinding.**

INTRODUCTION

Skeletal muscles are innervated in a precise pattern to ensure coordinated body movement. During development, growth cones of specific motoneurons follow stereotyped pathways to reach their target muscles (reviewed in Eisen, 1998). In zebrafish embryos, motor axons in the spinal cord are segmentally iterated into peripheral nerves that innervate dorsal, medial, and ventral muscle blocks. The pathways of the peripheral nerves are pioneered by three primary motoneurons: the caudal primary (CaP), the middle primary (MiP), and rostral primary (RoP) motoneuron (Eisen *et al.*, 1986; Myers *et al.*, 1986; Westerfield *et al.*, 1986). Axons of the primary motoneurons leave the spinal cord through the ventral root and extend along a common path toward the horizontal septum (Eisen *et al.*, 1986). At this point, all primary motoneuron growth cones pause before continuing along their cell-specific pathways (Fig. 1A).

During embryonic development, axons of secondary motoneurons are progressively added along the paths of the primary motor axons (Myers, 1985). Secondary motoneurons whose cell bodies lie close to CaP or MiP somata track the path of the primary motoneurons to the different regions of the myotome (Pike *et al.*, 1992; Fig. 1A), suggesting that the growth cones of the secondary motoneurons might require the axons of the primary motoneurons for proper pathfinding (reviewed in Eisen, 1991). Indeed, if CaP is ablated, secondary motor axons are delayed in the region of the horizontal septum and form ectopic branches, but a normal ventral nerve does form later in development (Pike *et al.*, 1992). Thus, primary motoneurons may facilitate the outgrowth of secondary motor growth cones, but there is apparently enough guidance information along the motoneuron pathway to allow secondary motoneurons to find their targets without them.

Many molecular components are involved in motor axon pathfinding (reviewed in Eisen, 1998), including surface recognition molecules of the immunoglobulin superfamily (IgSF) (Brümmendorf and Rathjen, 1995). We have studied the role of Neurolin during motor axon guidance in zebrafish embryos. Neurolin (Paschke *et al.*, 1992) is the fish

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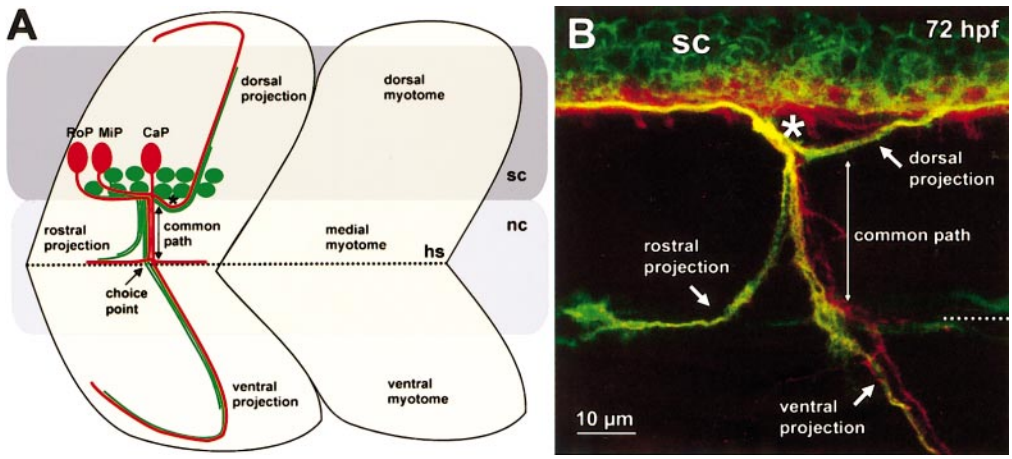

FIG. 1. Projection pattern of primary and secondary motoneurons in zebrafish. (A) Schematic drawing of a spinal hemisegment showing the axonal projections of primary (red) and secondary (green) motoneurons toward their target muscle regions in the myotome (lateral view). The primary motoneurons CaP, MiP, and RoP extend along the common path to a choice point at the horizontal septum (hs, dotted line) before they diverge into cell-specific projections that innervate the ventral, dorsal, and medial myotome, respectively. Secondary motor axons follow this pattern and form a ventral, rostral, and dorsal projection. The asterisk marks a cell which is weakly anti-Neurolin-positive and which lies directly caudal to the ventral root and close to the area where the dorsal projection develops. (B) Lateral view confocal scan of a 72-hpf embryo whole-mount stained with antibodies against Neurolin (green) and E587 antigen (red). The cell bodies of secondary motoneurons in the spinal cord are labeled here and in subsequent figures. Neurolin-expressing secondary motor axons extend toward the ventral, medial, and dorsal myotome in ventral, rostral, and dorsal projections. In contrast to the selective expression of Neurolin on seconday motor axons, E587 antigen is expressed by both primary and secondary motoneurons. hs, horizontal septum; nc, notochord; sc, spinal cord. All subsequent figures are lateral views and oriented accordingly with dorsal at the top and rostral to the left.

homologue of DM-GRASP/BEN/SC-1/ALCAM of birds and mammals (Burns *et al.*, 1991; Tanaka *et al.*, 1991; Pourquie *et al.*, 1992; Bowen *et al.*, 1995). It consists of five Ig-domains, a transmembrane segment, and a short cytoplasmic domain (Kanki *et al.*, 1994; Laessing *et al.*, 1994). Zebrafish DM-GRASP is recognized by the zn-5 antibody and is transiently expressed on the axons of secondary, but not primary, motoneurons (Fashena and Westerfield, 1999). In birds and mammals, DM-GRASP mediates cell adhesion and promotes axon growth and fasciculation (Burns *et al.*, 1991; Tanaka *et al.*, 1991; Pourquie *et al.*, 1992; Pollerberg and Mack, 1994; DeBernardo and Chang, 1995). In goldfish, the optic disk-directed growth of retinal ganglion cell axons is disturbed after Neurolin blockage (Ott *et al.*, 1998; Leppert *et al.*, 1999), suggesting that Neurolin's prime function in this system is that of receptor or coreceptor for a guidance component.

We examined Neurolin's effects on motor axon pathfinding by blocking Neurolin function with antibody injections. We found that Neurolin is required for pathfinding along the common path and along the different projections of secondary motor axons.

MATERIALS AND METHODS

Animals

Zebrafish embryos of the golden strain were obtained from our laboratory colony and maintained at 28.5°C. Embryos were staged by hours postfertilization (hpf) or, between 24 and 40 hpf, according to Kimmel *et al.* (1995) by the position of the posterior lateral line primordium. Dechorionated embryos were anesthetized in 0.03% aminobenzoic acid ethyl ester (MS222; Sigma-Aldrich, Steinheim, Germany) prior to injection and fixation.

Antibody Injections

Dechorionated embryos were oriented in a specialized sylgard matrix on a heated stage at 28.5°C, allowing for the efficient injection of 6 × 30 animals. Pulled 5-μl glass micropipettes were broken under optic control to short tips of 8 μm diameter and filled with solution using negative pressure. The pipette was manually inserted into the ventricle of the embryos and approximately 25 nl of solution was pressure injected in the direction of the spinal cord by using a microinjector (Eppendorf Transjector 5246, Hamburg, Germany). We injected either Fab fragments of a polyclonal serum against Neurolin (Neurolin Fabs, 10 mg/ml; Ott *et al.*, 1998), of a polyclonal serum against E587 antigen (E587 Fabs, 10 mg/ml; Weiland *et al.*, 1997), or 10 mM Tris buffer (pH 7.4). The specificity of the Fabs for their zebrafish antigens has been demonstrated by Western blots in earlier studies (Neurolin Fabs: Laessing *et al.*, 1994; E587 Fabs: Weiland *et al.*, 1997). Single injections were performed between 28 and 30 hpf, and embryos were fixed for immunocytochemistry at 36, 48, or 72 hpf. For repeated injections, embryos were removed from the matrix, revived, and kept at 28.5°C between the injection intervals. Embryos were double injected between 28 and 30 hpf and 38–40 hpf and fixed at 48 hpf. Fourfold injections were performed between 28 and 30 hpf, 38–40 hpf, 48–50 hpf, and 58–60 hpf, and embryos were fixed at 72 hpf.

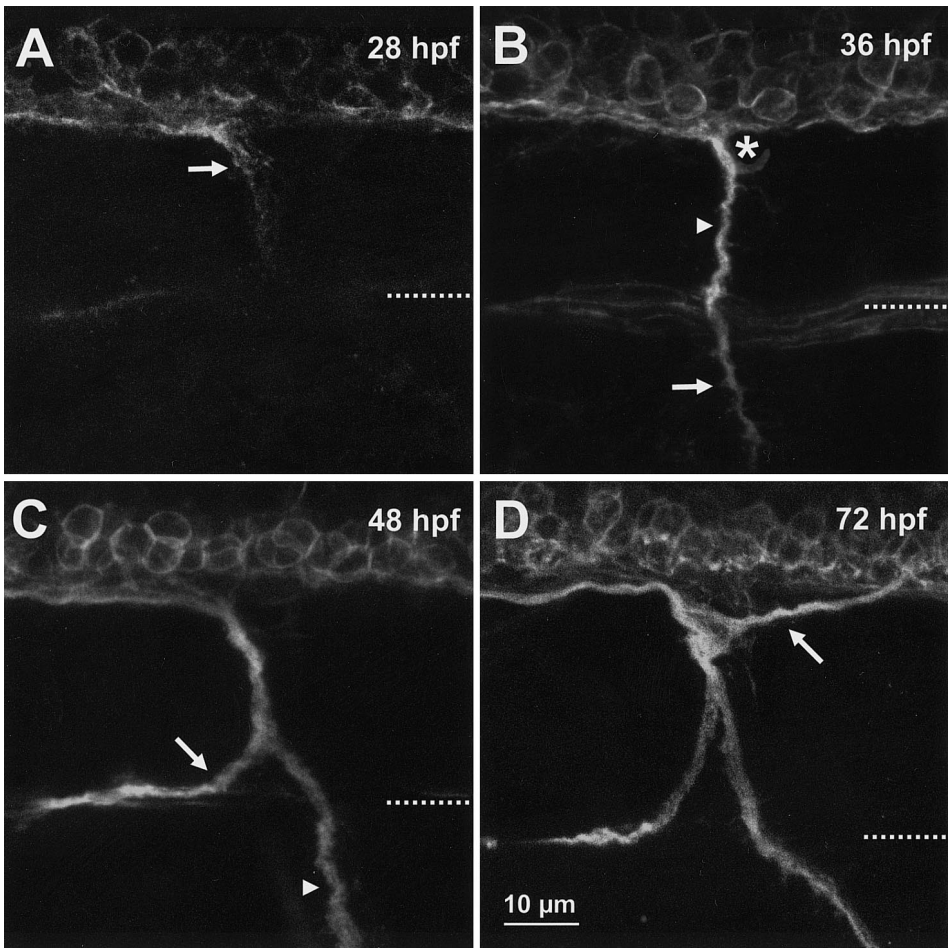

FIG. 2. Neurolin expression during the development of secondary motoneuron projections. (A–D) Lateral view confocal scan of segment 10 in embryos stained with Neurolin antibodies (dotted line indicates the horizontal septum). (A) At 28 hpf, the first secondary motor axons exit the spinal cord and grow along the common path (arrow). (B) At 36 hpf, more secondary motor axons join the common path (arrowhead). The first axons cross the horizontal septum and form the ventral projection (arrow). The asterisk marks a Neurolin-positive cell (compare Fig. 1) directly caudal to the ventral root. (C) At 48 hpf, further axons join the ventral projection (arrowhead). The rostral projection (arrow) extends along the horizontal septum. (D) At 72 hpf, the dorsal projection of secondary motor axons (arrow) originates at the ventral root.

Antibody Staining

Zebrafish embryos were processed for whole-mount immunohistochemistry as previously described (Weiland *et al.*, 1997). Briefly, embryos were fixed for 4 h at 4°C in 4% paraformaldehyde in 1× fixation buffer (Westerfield, 1994) and permeabilized by exposure to acetone (−20°C for 2–3 min). After incubation in blocking buffer (PBS with 1% BSA, 1% DMSO, 2% goat serum, 2% donkey serum) for 1 h at 37°C, they were exposed to primary antibodies overnight at 4°C. To visualize zebrafish motor axons, a monoclonal antibody against Neurolin (Mab N518, 25 µg/ml; Leppert *et al.*, 1999), a monoclonal antibody against E587 antigen (Mab E17, 25 µg/ml; Weiland *et al.*, 1997), a monoclonal antibody against the polysialated form of NCAM (Mab 735, 25 µg/ml; kindly provided by R. Gerardy-Schahn), a polyclonal serum against Neurolin (N581, 20 µg/ml), and a polyclonal serum against zebrafish Tag-1 (Lang *et al.*, 2001) were applied. Embryos were rinsed in wash buffer (PBS with 1% BSA, 1% DMSO) and incubated with secondary antibodies [Alexa-488-coupled goat anti-mouse (2 µg/ml; Molecular Probes, Eugene, OR) and cyanin-3-coupled donkey anti-rabbit (2 µg/ml; Dianova, Hamburg, Germany)] for 1 h at 37°C. After three 15-min rinses in wash buffer, they were stained with DAPI (0.5 µg/ml; Sigma-Aldrich) to visualize cell nuclei and stored in PBS at 4°C. For microscopy, yolk sacs were removed and embryos were embedded in Mowiol (containing n-propylgallate as an antifading agent) between two coverslips.

Neurolin-Fc Staining

The sequence coding for the extracellular domain of Neurolin was amplified by PCR and cloned into the pIgplus vector (kindly provided by P. Doherty, London) in frame with the sequence of human IgG1-Fc. The expression construct was transfected into Cos

cells by using DEAE-dextran as described (Ausubel *et al.*, 1994) and the soluble Neurolin-Fc fusion protein purified from the cell culture medium with a protein A sepharose column. Staining of zebrafish whole mounts with Neurolin-Fc protein was done essentially as described above for antibody staining. Briefly, zebrafish embryos were fixed in 4% paraformaldehyde in fixation buffer for 4 h and permeabilized with acetone. The embryos were incubated with Neurolin-Fc (20 μg/ml in 1% BSA/PBS) for 2 days at 4°C, washed with PBS, and incubated overnight with rhodamine-coupled goat anti-human antibody (Dianova) at 4°C. After extensive washing, embryos were embedded in Mowiol and analyzed. Control embryos were treated with human Fc-fragments and secondary antibodies and did not show any staining.

Analysis of Stained Embryos

Stained embryos were analyzed with a fluorescence microscope (Axiophot, Zeiss, Goettingen, Germany) for the quantification of aberrant projections. Selected embryos were further analyzed with a confocal microscope (LSM 510; Zeiss, Jena, Germany) equipped with a high aperture lens (C-Apochromat 40×/1.2 W; Zeiss, Goettingen, Germany) and the appropriate lasers. To visualize the pathways of secondary motor axons, serial optical sections were flattened into projections. All images were further processed with Adobe Photoshop 5.02 software.

RESULTS

Axial muscles in the trunk and tail of zebrafish embryos are divided into myotomes and are innervated by specific subpopulations of motoneurons as indicated in Fig. 1A. The primary motoneurons CaP, MiP, and RoP innervate the ventral, dorsal, and medial myotomes, respectively, prior to the differentiation of secondary motoneurons (Fig. 1). Both primary and secondary motoneurons express the L1-related IgSF member E587 antigen (Weiland *et al.*, 1997), but only the secondary motoneurons express Neurolin (Fig. 1B).

Development of Secondary Motoneuron Projections

Secondary motoneurons express Neurolin on their cell bodies and on their axons at about 24 hpf in rostral segments. The axons travel straight toward their exit point, the ventral root, leave the spinal cord at a 90° angle, and grow ventrally along the common path established by primary motoneurons (Pike *et al.*, 1992). This exit point is characterized by a weakly Neurolin-positive cell which lies directly ventral to the spinal cord and always caudal to the exit point near the area where the dorsal projection will later develop and is detectable by 36 hpf (asterisks in Figs. 1, 2, and 4). The first Neurolin-positive secondary motor axons exit the spinal cord in rostral segments at 28 hpf (Fig. 2A, arrow). By 36 hpf, more axons join the common path (Fig. 2B, arrowhead). At the horizontal septum, they are confronted with their first cell type-specific pathway decision. The first group of axons that arrive at this "choice point" grow ventrally and form the ventral projection (Fig. 2B, arrow). At 48 hpf, additional axons join the ventral

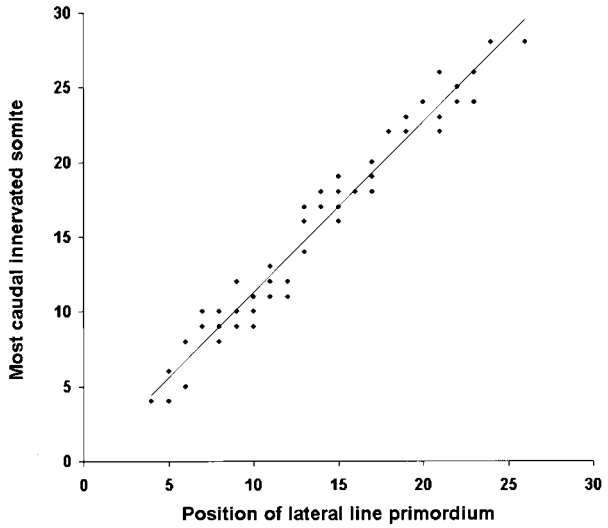

FIG. 3. Secondary motor axon outgrowth follows a rostral-to-caudal sequence. Data points represent the most caudal somite with Neurolin-expressing secondary motor axons plotted as a function of the lateral line primordium position in 24- to 36-hpf embryos. The outgrowth of secondary motor axons generally occurs in a rostral-to-caudal sequence, similar to, but 6–7 h later than, the axogenesis of primary motor axons (Hanneman and Westerfield, 1989).

projection (Fig. 2C, arrowhead), while a second group grows rostrally along the horizontal septum (Fig. 2C, arrow) to form the rostral projection to the medial muscles. The third projection of secondary motor axons (dorsal projection) originates close to the ventral root and is first observed in the most rostral segments at 48 hpf. It forms a dense fascicle with caudo-dorsal orientation at 72 hpf (Fig. 2D, arrow).

To analyze the temporal development of secondary motor axon outgrowth, the most caudal somite in which Neurolin-positive axons are visible was determined in embryos between 24 and 36 hpf. Like the development of primary motor axons (Hanneman and Westerfield, 1989), secondary motor axon outgrowth follows a general rostral-to-caudal sequence (Fig. 3). It proceeds at about 2 somites/h and occurs 6–7 h later than the outgrowth of primary motoneurons. The sequential development of the ventral, rostral, and dorsal projections of secondary motor axons also proceeds from rostral to more caudal segments.

Neurolin Blockage Induces Aberrant Branches along the Common Path (36 hpf)

To analyze the function of Neurolin during the development of secondary motor axon projections, Fab fragments of a polyclonal rabbit serum against Neurolin (Neurolin Fabs; Laessing *et al.*, 1994; Ott *et al.*, 1998) were injected into the ventricle of 28–30 hpf zebrafish. Embryos were fixed 7–10 h later, immunolabeled with a monoclonal antibody against Neurolin, and analyzed. Embryos labeled with anti-rabbit

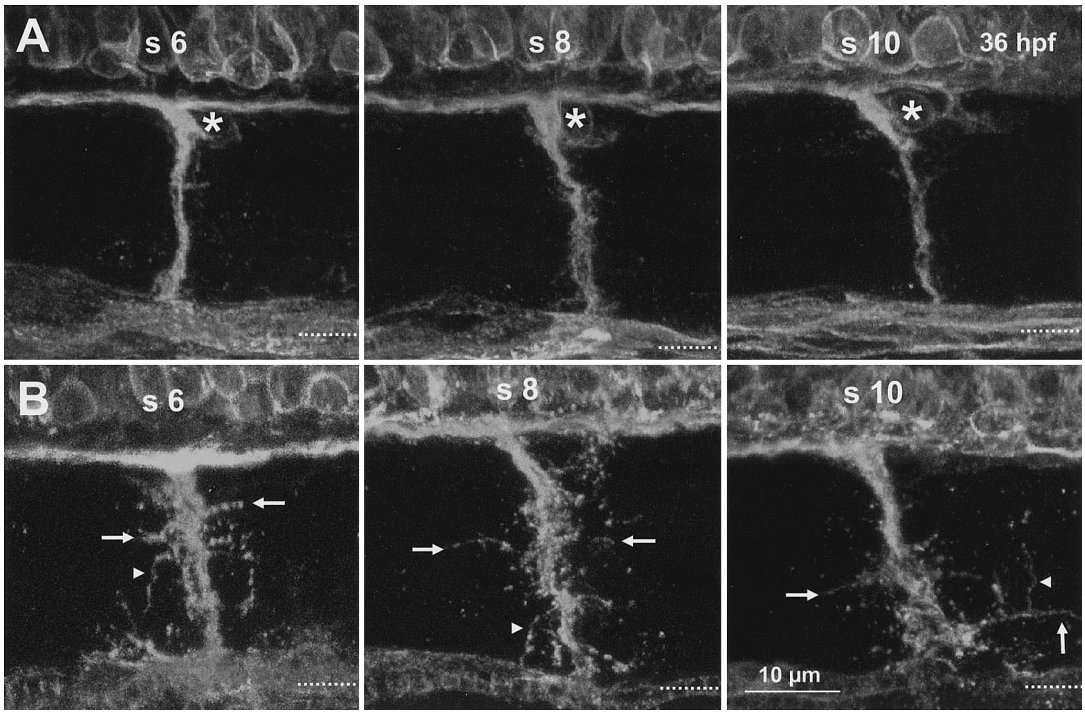

FIG. 4. Neurolin blockage induces aberrant branching along the common path. Lateral view confocal scans of segments (s) 6, 8, and 10 in control-injected (A) and Neurolin Fab-injected (B) embryos (36 hpf). (A) In control embryos, secondary motor axons extend along the common path in a tight bundle toward the horizontal septum (dotted line). The asterisk marks a Neurolin-positive cell which lies directly caudal to the ventral root. (B) After Neurolin blockage, secondary motor axons form a broadenend bundle along the common path and give off aberrant branches (arrows). These aberrant branches leave the common path at right angles (arrows) and grow toward or away (arrowheads) from the horizontal septum.

secondary antibodies showed that the injected Fabs initially spread throughout the entire nervous system and became concentrated along Neurolin-positive fiber tracts and neurons including secondary motor axons in all spinal cord segments (not shown). Control embryos received injections of Fab fragments of a polyclonal serum against the L1-like E587 antigen (Weiland *et al.*, 1997), buffer injections, or no injections.

In Neurolin Fab-injected embryos, the timing of secondary motor axon outgrowth was not affected, but the growth pattern was altered. In control-injected embryos, secondary motor axons grow in a tightly fasciculated bundle from their point of exit from the spinal cord to their choice point at the horizontal septum (Fig. 4A, Table 1). In Neurolin Fab-injected embryos, these axons form a broadened bundle and aberrant axonal processes leave the common path at right angles (Fig. 4B, arrows, Table 1). Since these processes are thin and do not seem to possess growth cones, we interpret them as side branches. Aberrant side branches project in rostral and caudal directions and some branches then redirect their growth toward or away from the horizontal septum (Fig. 4B, arrowheads).

It has previously been shown that Neurolin is restricted to the fascicles (fasciculated part) of secondary motor axons (Fashena and Westerfield, 1999). Thus, the aberrant branches observed could be newly formed or already existing branches that are visible because the blocked Neurolin diffused onto them. We therefore used as an independent marker an antibody against the polysialated form of NCAM (NCAM-PSA) that specifically labels secondary motor axons (Bastmeyer *et al.*, 1999). In control-injected embryos ($n = 14$), no branches were observed with either anti-Neurolin (Fig. 5A) or anti-NCAM-PSA (Fig. 5B) immunostaining. After Neurolin blockage, however, aberrant branches were consistently visualized with anti-Neurolin (Fig. 5C, arrows) and anti-NCAM-PSA (Fig. 5D, arrows) antibodies ($n = 18$). Neurolin blockage clearly causes aberrant growth of secondary motor axons off the common path. Branches stray into territories which they normally would not invade.

Another result of Neurolin blockage is that fewer secondary motor axons reach and cross the horizontal septum. To quantify this effect, we measured the diameter of secondary motor axon bundles stained by NCAM-PSA in somite ten 5 μm ventral to the exit point of the spinal cord and at the horizontal septum (Figs. 5B and 5D, arrowheads). At the ventral root, the fascicle diameter was 1.63 ± 0.22 μm in E587 Fab-injected control embryos ($n = 13$) and 1.65 ± 0.15

TABLE 1
Aberrant Branches along the Common Path after Neurolin Blockage

	Neurolin-Fabs	E587-Fabs	Tris	No injection
Analyzed embryos (36 hpf)	109	126	94	56
Embryos with aberrant branches	69 (63%)	0	0	0
Analyzed embryos (40 hpf)	40	46	45	—
Embryos with aberrant branches	20 (50%)	0	0	—
Analyzed embryos (48 hpf)	56	65	63	44
Embryos with aberrant branches	20 (36%)	0	0	0
Analyzed embryos (72 hpf)	19	21	21	20
Embryos with aberrant branches	0 (0%)	0	0	0

Note. Embryos received a single injection at 30 hpf and were analyzed after different survival times. Neurolin blockage-induced branches along the common path decrease during further development. No abnormal branches were observed in controls.

μm in Neurolin Fab-injected embryos ($n = 15$). At the horizontal septum, the fascicle diameter is significantly reduced from 1.07 ± 0.17 μm in controls to 0.89 ± 0.18 μm after Neurolin blockage ($P = 0.05$, Student's t-test). This indicates that Neurolin blockage prevents a proportion of secondary motor axons from reaching and crossing the horizontal septum at 36 hpf and thereby blocks growth into the ventral projection. This initial pertubation was transient and no longer detectable in embryos analyzed at 48 hpf.

Neurolin Blockage Causes Aberrant Rostral Projections (48 hpf)

In embryos that received a single injection at 28–30 hpf and were then analyzed at 40, 48, and 72 hpf, we observed fewer of the Neurolin Fab-induced defects (Table 1). This could be explained by a decrease in Fab concentration during further development. Therefore, Neurolin Fabs were injected twice (at 28–30 hpf and at 38–40 hpf) and embryos were analyzed at 48 hpf. As an independent marker, we used a polyclonal serum against zebrafish TAG-1 (Lang *et al.*, 2001) that labels primary and secondary motor axons at that age. In Neurolin Fab-injected embryos, broadening of the bundle and aberrant branching from the common path also occurred (Figs. 6C–6F). Most striking, however, was the altered formation of the rostral pathway. In control embryos, secondary motor axons depart from the common path and project rostrally in a distinct fascicle and then along the horizontal septum toward the medial muscles (Figs. 6A and 6B, arrows). After Neurolin Fab injection, this rostral fascicle is missing in the most severe cases (Figs. 6C–6F). There are branches in the direction in which the axons normally would turn but they apparently fail to grow along their predetermined pathway (Figs. 6C–6F, arrows). The region where the rostral fascicle would normally develop is broadened, as if axons are stalled upon arrival in this zone. These defects were observed in the majority of Neurolin Fab-injected embryos but not in all spinal hemisegments. A quantification of the Neurolin-induced defects is given in Table 2.

In summary, blockage of Neurolin appears to prevent axons from perceiving the appropriate cues that would allow them to grow into the rostral projection.

Neurolin Blockage Causes Aberrant Dorsal Projections (72 hpf)

Since the dorsal projection of secondary motor axons develops between 48 and 72 hpf, Neurolin Fabs were injected four times (at 28–30 hpf, 38–40 hpf, 48–50 hpf, and 58–60 hpf) and embryos were analyzed at 72 hpf. In control embryos receiving either no injections or injections of buffer or E587 Fabs, the dorsal projection of secondary motor axons develops normally. This projection originates near the ventral root and extends caudally toward the dorsal muscles in a distinct bundle (Fig. 7A, arrow). In Neurolin Fab-injected embryos, this projection is markedly disordered (Fig. 7B). Axons seem to cluster around the region where they make their dorsal pathway decision. Some grow aberrantly in a ventral direction (arrows Fig. 7B) and fail to grow to the dorsal muscles (arrowhead Fig. 7B). This is compatible with the view that axons are no longer perceiving the appropriate guidance cues for dorsal growth. A quantification of the defects in the dorsal projection that are induced by Neurolin blockage is given in Table 3.

In these 4-fold-injected embryos, aberrant growth of secondary motor axons also occurs along the common path and the rostral pathway in that the normally coherent axon bundle is divided in two or more fascicles in abnormal orientations (Figs. 7C and 7D and Table 3). In the rostral projection, secondary motor axons diverge and grow rostrally through fast muscle tissue (arrows Fig. 7C). Along the common pathway, aberrant branches grow laterally through fast muscle tissue (arrow, Fig. 7D) and contact slow muscle fibers of the medial myotome (not shown).

This demonstrates that Neurolin contributes to the navi-

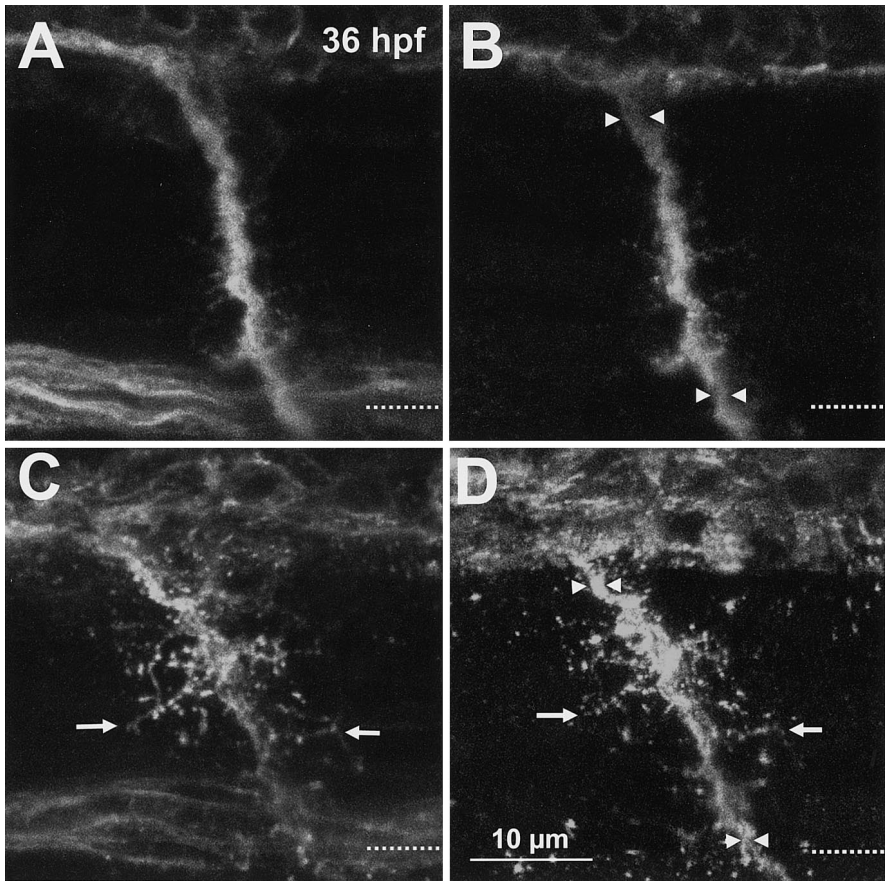

FIG. 5. The formation of the ventral projection is reduced after Neurolin blockage. Lateral view confocal scans of segment 10 in control (A, B) and Neurolin Fab-injected (C, D) 36-hpf embryos stained with Neurolin (A, C) and NCAM-PSA (B, D) antibodies. (A, B) At 36 hpf, secondary motor axons enter the common path at the ventral root (B, arrowheads) and most cross the choice point at the horizontal septum (B, arrowheads near the dotted line), forming the ventral projection in controls. (C, D) After Neurolin blockage, fewer secondary motor axons cross the horizontal septum towards the ventral projection (D, arrowheads near the dotted line), although the general outgrowth from the spinal cord is not affected (D, arrowheads). Instead, secondary motor axons frequently branch (C, D, arrows) along the common path region after Neurolin blockage.

gation of secondary motor axons to and beyond the choice point at the horizontal septum and at the ventral root where specific pathfinding decisions occur.

Neurolin Ligands

These results indicate that Neurolin acts as a receptor or as part of a receptor complex that is crucial for secondary motor axon guidance. A Neurolin-Fc fusion protein, consisting of the five Ig-like domains and the Fc part of human IgG, was used as a receptor affinity probe to search for candidate ligands and their distribution. Control embryos that were treated with human Fc fragments and secondary antibodies did not show any staining. At 32 hpf, Neurolin-Fc labels the pathway of secondary motor axons along the common path and the ventral projection (Fig. 8A, arrowheads). No binding is detected on the Neurolin-positive cell bodies and axons of secondary motoneurons in the spinal cord, indicating that Neurolin-Fc does not bind to Neurolin itself and therefore not to secondary motoneurons. Consistent with this view is the binding pattern of Neurolin-Fc throughout the CNS that does at no developmental age resemble a Neurolin-antibody staining pattern (not shown). At 53 hpf, the common path and the ventral projection are still labeled (Fig. 8B, arrowheads) and the area where the rostral projection originates is now characterized by a diffuse Neurolin-Fc staining (Fig. 8B, open arrowhead). In addition, we observed a Neurolin-Fc-positive cell latero-caudal to the ventral root (Fig. 8B, arrow). This cell has a dorsally and ventrally directed process and most probably represents the first differentiating dorsal root ganglion cell (DRG).

Thus, Neurolin-Fc staining shows that putative ligands are distributed along the predetermined ventral and rostral pathways of secondary motoneurons.

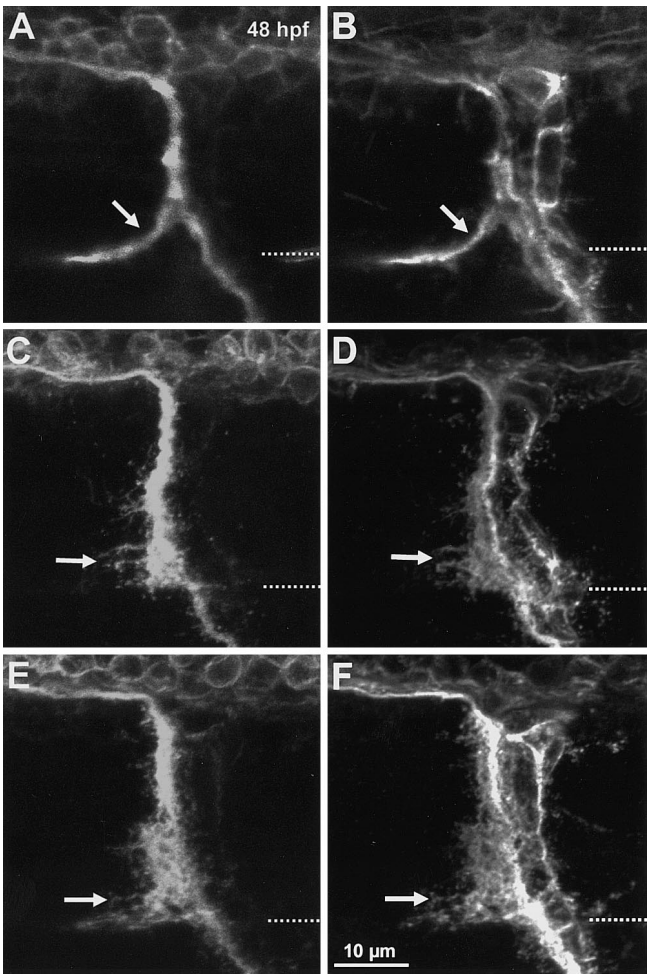

FIG. 6. Neurolin blockage inhibits the formation of the rostral projection. Lateral view confocal scans of segment 10 in control (A, B) and Neurolin Fab-injected (C–F) embryos stained with Neurolin (A, C, E) and TAG-1 (B, D, F) antibodies. TAG-1 antibodies served as an independent control and label in addition to motor axons (arrow in B) DRGs and migrating neural crest cells. (A, B) At 48 hpf, the rostral projection is formed by a distinct fascicle of secondary motor axons (arrows) that extend along the horizontal septum (dotted line) in control embryos. (C–F) After Neurolin blockage, secondary motor axons grow in a broadened front and form branches (arrows), but the formation of the rostral projection is inhibited.

DISCUSSION

The present study shows that Neurolin is involved in the growth and guidance of secondary motor axons. Neurolin blockage induced mainly three defects: (1) a broadening of the bundle and the induction of ectopic sidebranches along the common path, (2) inhibition of the turning into the rostral and dorsal projection and consequently a delayed formation of these projections, and (3) pathfinding errors in the rostral and dorsal projections. These findings indicate that axon-associated Neurolin is required for pathway choices and the directed growth of secondary motor axons and suggest that Neurolin is a receptor involved in guidance of secondary motoneurons. Consistent with this view is the staining pattern of a soluble Neurolin-Fc construct which binds along the common path and at the origin of the ventral projection but not to Neurolin itself.

That the observed effects after Neurolin Fab injections are specific is substantiated by controls done here and in an earlier study (Weiland *et al.*, 1997), which showed that buffer injections or injections of preimmune Fabs caused no defects. Furthermore, the overall development of the fish is not influenced and only Neurolin-positive axons are affected after Neurolin blockage, whereas injections of E587 Fabs caused perturbations in different E587-positive axon tracts (Weiland *et al.*, 1997). Most of the defects observed after Neurolin blockage are transient and decrease during further development, suggesting that additional guidance components with similar functions might compensate for the loss of Neurolin function.

A variety of molecules are involved in motor axon guidance and target recognition (reviewed in Eisen, 1998). In zebrafish, studies have concentrated mainly on the molecular and cellular cues that guide primary motor axons. Overexpression of Semaphorin Z1b inhibits outgrowth of primary motor axons (Roos *et al.*, 1999) and the removal of CSPG results in ectopic sidebranches along the ventral projection (Bernhardt and Schachner, 2000). Ablation of muscle pioneers causes transient overshooting of CaP and MiP axons as well as aberrant branching of primary motor axons along the common path (Melancon *et al.*, 1997). Zebrafish mutants with motoneuron phenotypes have recently been described, but they are unlikely to be defected in the neurolin gene. The *diwanka* gene was identified as a somite-derived cue that delineates the common path prior to axonogenesis (Zeller and Granato, 1999), and, in *stumpy* mutants, axons of primary and secondary motoneurons do not progress properly past intermediate targets and do not branch properly (Beattie *et al.*, 2000). It has been suggested that secondary motor axons follow the pathways that have been pioneered by primary motor axons. However, ablation experiments have demonstrated that secondary motor axons can navigate to their appropriate targets in the absence

TABLE 2
Aberrant Rostral Projections after Neurolin Blockage

	Neurolin-Fabs	E587-Fabs	Tris
Analyzed embryos (48 hpf)	15	16	14
Analyzed somites (5–20)	240	256	224
Aberrant rostral projections	43 (18%)	19 (7%)	15 (7%)

Note. Embryos were injected twice (at 30 hpf and at 40 hpf), and hemisegments 5–20 were analyzed at 48 hpf. Aberrant rostral projections were defined as either missing or reduced.

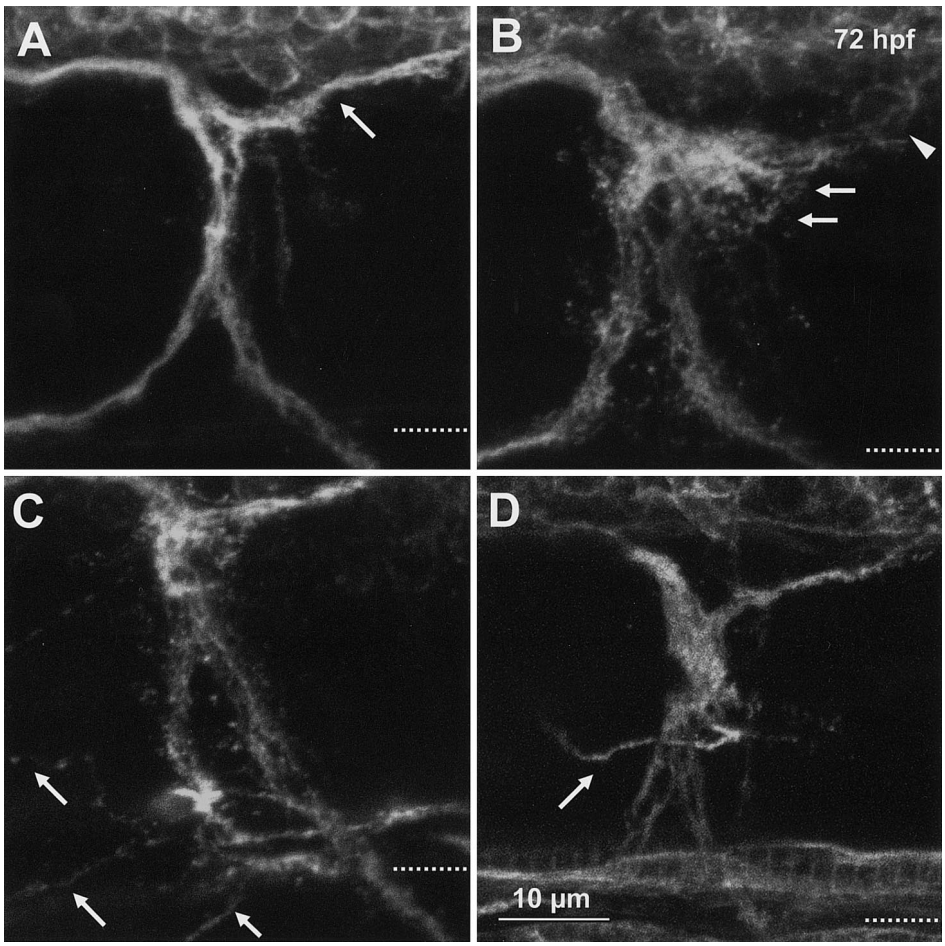

FIG. 7. Neurolin blockage induces aberrant dorsal, rostral, and common path projections. Lateral view confocal scans of control (A) and Neurolin Fab-injected (B–D) embryos. (A) At 72 hpf, the dorsal projection of secondary motor axons (arrow) consists of a tight fascicle. (B) After Neurolin blockage, secondary motor axons diverge at the ventral root (arrows) and few, if any, axons (arrowhead) extend into the dorsal projection. (C, D) Secondary motor axons commit pathfinding errors toward the rostral projection (arrows in C) and form aberrant projections along the common path (arrow in D). Furthermore, the fascicles of secondary motor axons are less tightly bundled (B–D) than in control embryos (A).

of primary motoneurons (Pike *et al.*, 1992). In the present study, we show that Neurolin is involved in the guidance of secondary motor axons.

By using a soluble Neurolin-Fc construct we were able to demonstrate that putative Neurolin ligands are distributed along the common path and the ventral projection. The staining pattern indicates that Neurolin-Fc does not bind to the cell bodies or axons of secondary motoneurons, i.e., Neurolin itself. This is consistent with the finding that Neurolin-Fc fails to bind Neurolin expressed on the surface of transfected CHO cells (H.D., unpublished results). The distribution of Neurolin-Fc at 32 hpf, however, correlates with the pathway of the CaP axon, suggesting that Neurolin ligands are either expressed in the pathway or on the surface of the main axon but not on the branches of this primary motoneuron. Putative Neurolin ligands are therefore present when Neurolin-positive secondary motor axons navigate along this pathway. These axons are affected through Neurolin blockage, form ectopic branches along the common path, and transiently fail to grow into the ventral projection.

Consistent with the view that Neurolin ligands are expressed by the CaP axon is the outcome of ablation experiments of primary motoneurons. Following CaP ablation, secondary motor axons form aberrant branches and the formation of the ventral projection is delayed (Pike *et al.*, 1992). The defects in the ventral projection are transient and experimental nerves appeared normal in the larval stage. The observed similarities after Neurolin blockage and ablation of primary motoneurons indicate that Neurolin on secondary motor axons binds to a ligand expressed by primary motor axons. Heterophilic binding was demon-

TABLE 3

Aberrant Dorsal Projections after Neurolin Blockage

	Neurolin-Fabs	E587-Fabs	Tris	No injection
Analyzed embryos (72 hpf)	29	26	25	26
Analyzed somites (5–20)	464	416	400	416
Aberrant dorsal projections	77 (17%)	29 (6%)	30 (8%)	20 (5%)
Aberrant rostral projections	11 (2.4%)	0	1	0
Aberrant common path projections	12 (2.6%)	0	0	0

Note. Embryos were injected four times (at 30, 40, 50, and 60 hpf) and analyzed at 72 hpf in both hemisegments. Aberrant projections were defined as either missing or as axons projecting into ectopic areas.

strated between DM-GRASP and NgCAM (DeBernado and Chang, 1996) and between ALCAM and the scavenger receptor CD6 (Bowen *et al.*, 1995, 1997). Primary motor axons in zebrafish do express the E587 antigen (Weiland *et al.*, 1997; this study) which is, like NgCAM, a member of the L1 family. However, since the distribution of the E587 antigen during zebrafish development differs considerably from the Neurolin-Fc binding, it is unlikely that this antigen represents a ligand of Neurolin. Whether primary motor axons in zebrafish express a homologue of the scavenger receptor CD6 is not known.

The development of the rostral projection of secondary motor axons differs from the RoP pathway in two aspects: (1) secondary motor axons project only rostrally at the horizontal septum, whereas RoP forms both rostral and caudal branches, and (2) secondary motor axons branch off the common path before reaching the horizontal septum and turn rostrally, whereas RoP grows to the horizontal septum and then turns at right angles (Pike and Eisen, 1990). Therefore, the development of the rostral projection cannot be explained by a mechanism of selective fasciculation whereby secondary motor axons just follow the RoP axon. That Neurolin-Fc binding was not observed along the RoP pathway is consistent with this idea. Instead, we detected a diffuse Neurolin-Fc labeling in the common path at the origin of the rostral projection, the same area where defects induced by Neurolin blockage occur. This area is populated by neural crest cells that migrate into this region

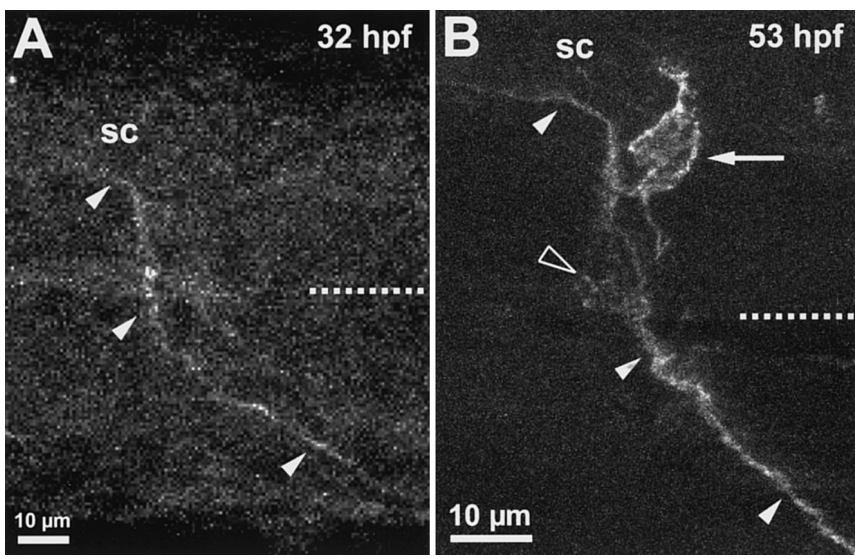

FIG. 8. Distribution of putative Neurolin ligands along the path of secondary motor axons. Lateral view confocal scans of embryos labeled with a soluble Neurolin-Fc fusion protein. (A) At 32 hpf, cell bodies and axons of secondary motoneurons are not stained, but the pathway of primary and secondary motor axons from their exit point at the spinal cord (sc) along the common path and the ventral projection (arrowheads) is labeled. (B) At 53 hpf, the ventral projection is still labeled (arrowheads) and the area where the rostral projection forms is characterized by a diffuse Neurolin-Fc staining (open arrowhead). A Neurolin-Fc-positive DRG (arrow) with a dorsal and a ventral process is found latero-caudal to the ventral root. The dotted line marks the horizontal septum.

and later differentiate into Schwann cells (Eisen and Weston, 1993; Fig. 2D).

In embryos analyzed at 72 hpf, Neurolin blockage induced pathfinding errors in the dorsal projection. Near the ventral root, secondary motor axons misproject ventrally and fail to grow dorsally. As a consequence, the dorsal projection of secondary motor axons was missing or significantly reduced. The ventral root region is characterized by a Neurolin-Fc-positive cell that probably represents the first differentiating dorsal root ganglion (DRG) cell. This cell is within filopodial reach of the growth cones in the dorsal projection and is probably involved in the specification of dorsal guidance. Defects different than those induced by Neurolin blockage were observed in the dorsal projection after MiP ablation (Pike *et al.*, 1992). The formation of the dorsal projection by secondary motor axons was transiently delayed and secondary motor axons projected dorsally through the myotome instead of along its boundary, forming a dorsal projection in an incorrect location. These different defects in the dorsal projection suggest that Neurolin is not involved in the dorsally directed guidance of secondary motor axons along the MiP axon. Instead, Neurolin might play a role in encouraging secondary motor axons to leave the common path caudally toward the dorsal projection.

In addition to the above described pathfinding errors, Neurolin blockage caused the formation of ectopic sidebranches and a broadening of the bundle of secondary motor axons along the common path. In principle, these defects can be explained by the blockage of homophilic interactions of Neurolin on the surfaces of secondary motor axons. In birds and mammals, DM-GRASP/BEN/SC-1 mediates cell adhesion through homophilic interactions and promotes axon growth and fasciculation (Burns *et al.*, 1991; Tanaka *et al.*, 1991; Pourquie *et al.*, 1992; Pollerberg and Mack, 1994; DeBernardo and Chang, 1995). However, all experiments performed so far with various antibodies, native and recombinant protein and fasciculation assays with axons provide no evidence for homophilic binding capacity of Neurolin (Ott *et al.*, 1998; Leppert *et al.*, 1999). It is unclear whether Neurolin interacts in a heterophilic manner with other IgSF proteins and thus contributes to axon fasciculation, which in turn can cause defasciculation (i.e., broadening of the bundle) in blocked embryos. In parallel with broadening of bundles, ectopic branches invaded territories where they normally would not project. One might speculate that Neurolin-blocked axons no longer receive the proper guidance cues and extend into "forbidden" territories and no longer restrict their ventral path to one tight bundle.

Since secondary motor axons pioneer the ventral root in the absence of all primary motor axons (Pike *et al.*, 1992), additional guidance cues must delineate the common path for secondary motor axons. Migratory neural crest cells and sclerotome cells share the common path and sclerotome ablation causes a retardation of the CaP axon that exhibits unusal branches (Morin-Kensicki and Eisen, 1997). Thus, the sclerotome might similarly influence secondary motor axons, but the influence of migratory neural crest cells on the pathfinding of spinal motor axons is not known. The *diwanka* gene was identified as a somite-derived cue that delineates the common path prior to axonogenesis (Zeller and Granato, 1999). *Diwanka* mutant embryos exhibit abnormal projections of primary motor axons along the common path, which could be rescued through the transplantation of adaxial cells.

In summary, the results presented in this study show that Neurolin is involved in secondary motor axon guidance. Most likely, Neurolin acts as a receptor, or as part of a receptor complex, on the surface of secondary motor axons that interacts with guidance cues distributed along the path of these axons. Since secondary motor axons in all three projections are affected after Neurolin blockage, additional receptors for cell type-specific pathway decisions are necessary.

ACKNOWLEDGMENTS

We thank M.A. Cahill for critically reading the manuscript, A.-Y. Loos for taking care of the zebrafish breeding colony, and M. Wiechers and U. Binkle for technical assistance. This work has been supported by grants of the Deutsche Forschungsgemeinschaft (DFG) (For 216/3) and the Fond der Chemischen Industrie (to C.A.O.S. and M.B.). M.B. is a Heisenberg fellow of the DFG.

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Submitted for publication January 29, 2001
Revised March 22, 2001
Accepted March 23, 2001
Published online May 30, 2001