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Notochord alters the permissiveness of myotome for pathfinding by an identified motoneuron in embryonic zebrafish

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SUMMARY

During zebrafish development, identified motoneurons innervate cell-specific regions of each trunk myotome. One motoneuron, CaP, extends an axon along the medial surface of the ventral myotome. To learn how this pathway is established during development, the CaP axon was used as an assay to ask whether other regions of the myotome were permissive for normal CaP pathfinding. Native myotomes were replaced with donor myotomes in normal or reversed dorsoventral orientations and CaP pathfinding was assayed. Ventral myotomes were permissive for CaP axons, even when they were taken from older embryos, suggesting that the CaP pathway remained present on ventral myotome throughout development. Dorsal myotomes from young embryos were also permissive for CaP axons, however, older dorsal myotomes were non-permissive, showing that permissiveness of dorsal myotome for normal CaP pathfinding diminished over time. This process appears to depend on signals from the embryo, since dorsal myotomes matured in vitro remained permissive for CaP axons. Genetic mosaics between wild-type and *floating head* mutant embryos revealed notochord involvement in dorsal myotome change of permissiveness. Dorsal and ventral myotomes from both younger and older *floating head* mutant embryos were permissive for CaP axons. These data suggest that initially both dorsal and ventral myotomes are permissive for CaP axons but as development proceeds, there is a notochord-dependent decrease in permissiveness of dorsal myotome for CaP axonal outgrowth. This change participates in restricting the CaP pathway to the ventral myotome and thus to neuromuscular specificity.

Key words: axonal pathfinding, genetic mosaics, tissue transplantation, zebrafish, *floating head*

INTRODUCTION

Communication between the nervous system and muscles in the body is mediated by centrally located motoneurons whose axons extend out of the spinal cord to innervate the appropriate muscle cells. During development, motor growth cones undergo a process referred to as pathfinding, in which they typically traverse significant distances along distinct pathways to reach their correct muscle targets. These pathways are thought to be defined by molecular cues and are crucial for formation of proper neuromuscular connectivity. An important and unresolved issue is when and how these pathways are established during development.

In both insects and vertebrates, growth cones follow distinct pathways resulting in reproducible axonal patterns (reviewed by Goodman and Shatz, 1993). This precision arises, at least inpart, because only particular regions of the embryo are permissive for axonal pathfinding (see Tosney, 1992). In the *Drosophila melanogaster* wing, transplanted heterologous neurons extend growth cones along the third wing vein, the normal pathway for growth cones of endogenous sensory neurons, while avoiding the rest of the wing (Blair et al., 1987). This particular region, therefore, appears to delineate a pathway that can support the directional extension of growth cones from many types of neurons. In the chick limb, surgically re-routed axons from inappropriate types of neurons follow the existing motor axonal pathway (Lewis et al., 1983) and motor axons project along their normal routes even after small dislocations of the spinal cord (Lance-Jones and Landmesser, 1980). Thus pathways, in contrast to the regions surrounding them, are especially permissive for growth cones. These and other experiments demonstrate that permissive pathway regions exist, but how they are set up during development remains unclear. Pathways may be established in an autonomous manner or they could be induced by surrounding tissues.

Primary motoneurons of embryonic zebrafish provide an excellent model to address this issue because they can be individually identified and their initial axon projections are relatively simple. Every spinal cord hemisegment contains 3 or 4 individually identified primary motoneurons, each of which sends a growth cone out of the spinal cord to innervate a particular region of the myotome (Eisen et al., 1986, 1990; Myers et al., 1986). Initially all of the growth cones extend along a common pathway; later the growth cones diverge and extend along cell-specific pathways. The most caudal primary motoneuron, CaP, extends its axon along the medial aspect of ventral myotome ultimately innervating all the muscle cells in this region (Westerfield et al., 1986). Previous studies demonstrated that each specific pathway is recognized uniquely by

the primary motoneuronal growth cone that normally traverses that pathway (Gatchalian and Eisen, 1992). Thus, the initial pattern of neuromuscular connectivity in the embryonic zebrafish involves single axons extending along unique myotome regions.

To address how these axonal pathways are established during development, we used the CaP growth cone as an assay for the presence or absence of its pathway on different myotome regions. Our data show that initially both dorsal and ventral myotome are permissive for CaP axonal outgrowth. As development proceeds, however, the dorsal myotome undergoes a notochord-dependent loss of permissiveness that contributes to localization of the CaP pathway to the ventral myotome.

MATERIALS AND METHODS

Fish

Zebrafish embryos were obtained from the University of Oregon Zebrafish Facility and maintained as described by Myers et al. (1986). Embryos grown between 25.5-28.5°C were staged by counting the number of somites and converted to standard hours postfertilization at 28.5°C (h; Kimmel et al., 1995). Homozygous *floating head*^{n1} mutant embryos (Talbot et al., 1995) were obtained by heterozygote matings.

Donor myotomes

Donor embryos were labeled at the 2-4 cell stage with 5% lysinated rhodamine dextran (10×10^3 M_r; Molecular Probes) injected into the yolk, as described by Ho and Kane (1990). At the time of the experiment, skin was removed along the trunk using fine forceps. Embryos were mounted in 1.2% agar on a microslide and visualized at 40× using Nomarski (DIC) optics. The dorsal aspect of the myotomes was labeled with 0.5 μm fluorescein isothiocyanate (FITC)-conjugated microspheres (Polysciences) by pressure injection from a micropipette broken off to a diameter of 5 μm. To obtain isolated myotomes, labeled donor embryos were incubated in 5× pancreatin (625 mg/ml; GibcoBRL) in physiological saline until tissues began to separate (approximately 0.5-1 minute); they were then triturated with a small bore Pasteur pipette. Myotomes containing the fluorescent microspheres were collected and transferred to Leibowitz's L15 (GibcoBRL) medium containing 10% fetal bovine serum for 3-5 minutes to inactivate the pancreatin. Myotomes were kept in L15 medium without serum until transplanted.

Host embryos

The skin of host embryos was removed along the trunk with fine forceps. Embryos were mounted in 1.2% agar on a microslide and visualized at 40× using Nomarski (DIC) optics. Two myotomes from axial levels 6-9 were removed by aspiration using a micropipette broken off to a diameter of 10-20 μm attached to a 2 ml micrometer syringe.

Transplantations

To assay ventral myotome, myotomes were taken from the right side of the donor and transplanted into the right side of the host. To assay dorsal myotome, myotomes were taken from the left side of the donor, rotated about the dorsoventral axis, and transplanted into the right side of the host, preserving the anterioposterior and mediolateral axes.

Heterochronic transplants: host embryos were 15.5-16.5 h (13-15 somites); donor embryos were 19-20 h (20-22 somites).

Isochronic transplants: both host and donor embryos were 15.5-16.5 h.

In vitro heterochronic transplants: host embryos were 15.5-16.5 h; donor myotomes were removed from 15.5-16.5 h embryos and incubated in Leibowitz's L15 medium at 28.5°C for 2.5-3.0 hours followed by transplantation into host embryos.

***floating head* heterochronic transplants:** genetic mosaics were created by transplanting myotomes from 19-20 h *floating head* embryos into 15.5-16.5 h wild-type host embryos or by transplanting 19-20 h wild-type myotomes into 15.5-16.5 h *floating head* mutant host embryos.

Uninnervated heterochronic transplants: CaP, VaP and MiP motoneurons were ablated from 16-17 h donor embryos. After developing for 2.5-3.0 hours at 28.5°C, myotomes overlying the ablated motoneurons were isolated and transplanted into 15.5-16.5 h host embryos.

After transplantation, host embryos were allowed to develop for 6-8 hours in Leibowitz's L15 medium containing 50 units penicillin and 5 μg streptomycin at 28.5°C. Embryos were fixed in 4% paraformaldehyde and processed for immunohistochemistry. The success rate of the experimental transplants was 22.5±11.0%; an experiment was deemed unsuccessful if the embryos died posttransplantation, if the transplanted myotomes did not remain in place, if the embryo developed a kink in its body resulting in poor transverse sections or if there was no evidence of a CaP axon extending out of the spinal cord. We interpreted this last result to mean that either the myotome or CaP was damaged or that the transplanted myotome was not placed close enough to the spinal cord to support axonal extension.

Immunohistochemistry

Embryos were cut into 16 μm transverse serial sections. The monoclonal anti-Hu antibody (1:400; Marusich et al., 1994) was used to recognize neuronal cell bodies in the spinal cord and the monoclonal antibody znp-1 (1:1000; Trevarrow et al., 1990) was used to detect axons (Melancon, 1994). A FITC-conjugated goat anti-mouse secondary antibody was used to detect both primary antibodies. Antibodies were diluted in buffer containing 0.5 M NaCl, 0.01 M PO_4 pH 7.3, 3% bovine serum albumin, 0.3% Triton X-100 and 2.5% goat serum. To ensure that all host myotome cells were removed, sections were also incubated with the DNA intercalating dye, Hoechst-33258 (Calbiochem). Donor myotome cells were recognized because they were labeled with both rhodamine fluorescence and Hoechst dye. Host myotome cells, on the other hand, were only labeled by the Hoechst dye. All embryos were analyzed in this way to ensure complete removal of host myotomal cells.

To make camera lucida drawings, sections were reprocessed for observation under bright-field optics. Sections were re-incubated with the anti-Hu and znp-1 antibodies. After using the peroxidase anti-peroxidase antibody system (Sternberger Monoclonals Inc.), sections were incubated in diaminobenzidine (DAB; 0.5 mg/ml) and peroxidase (0.001%) until a brown precipitate formed. Measurements were

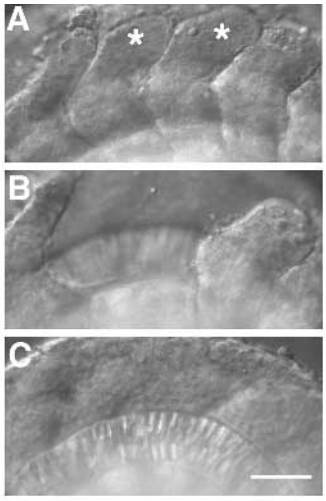

Fig. 1. Myotomes can be removed from zebrafish embryos. (A) Lateral view of a live 16 h embryo with asterisk marking myotomes 7 and 8 (anterior to left; dorsal to top). (B) Lateral view of the same embryo after removal of myotomes 7 and 8. (C) The same field as above but focused on the underlying spinal cord and notochord. Scale bar, 50 μm.

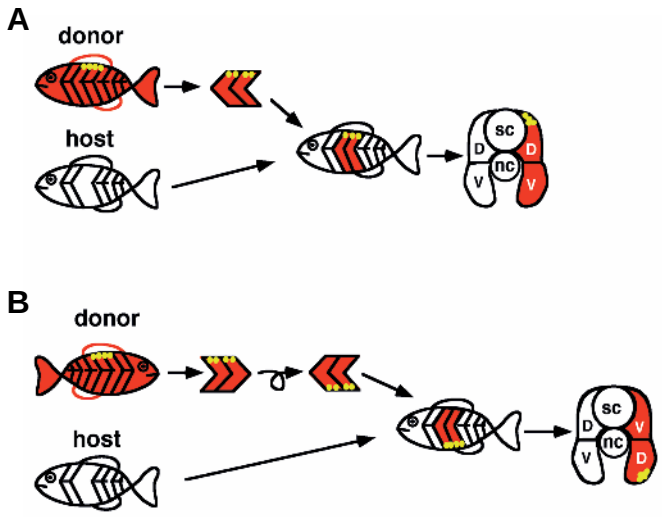

Fig. 2. Transplant procedure. Donor embryos were labeled at the 2- to 4-cell stage with lysinated-rhodamine dextran. At the time of the transplant, the dorsal aspect of donor myotomes was labeled with FITC-conjugated microspheres (yellow dots) and the embryos were enzymatically dissociated. Contiguous pairs of labeled donor myotomes were gathered and transplanted into host embryos from which two myotomes had been removed. After 7 hours, the embryos were sectioned and analyzed in cross section (see Materials and Methods for details). The donor myotome region being assayed replaces the host ventral myotome. (A) To assay ventral myotome, donor myotomes were transplanted in the correct dorsoventral orientation. (B) To assay dorsal myotome, donor myotomes were transplanted in the reversed dorsoventral orientation. sc, spinal cord; nc, notochord; V, ventral myotome; D, dorsal myotome.

taken of a control and an experimental axon in the same section. If no control axon was present in the same section, the axon in the next serial section was used.

Identification of CaP

At present, there are no antibodies that exclusively recognize CaP; therefore, CaP was identified by its cell body position in the spinal cord. To aid in this process, we determined the morphology of the CaP cell body in transverse sections by labeling individual CaPs intracellularly with fluorescent dye as described by Eisen et al. (1989). After serial sectioning and antibody labeling, CaP could be distinguished from other neuronal cell bodies, including other primary motoneurons, by its shape and location in the spinal cord. In addition, camera lucida reconstructions allowed the CaP axon, which is in the same plane of section as its cell body, to be traced in its entirety, including its connection with the cell body. These criteria were used in all experiments to ensure that we were exclusively analyzing the CaP axon.

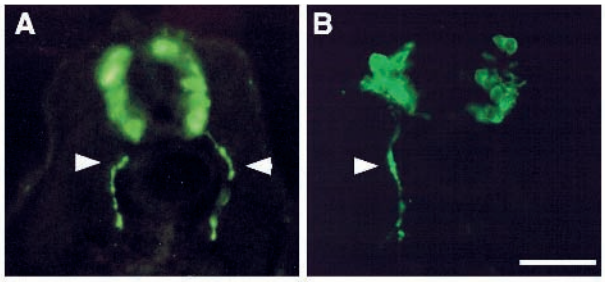

Fig. 3. Myotome is required for CaP growth cone extension. (A) Cross section through a 23 h embryo showing neurons in the spinal cord and CaP axons extending along their normal pathway (arrowheads). (B) 23 h embryo 7 hours after myotome removal showing a normal CaP axon extending along the ventral myotome on the left (arrowhead). When the myotome is removed (right side) no CaP axon is present (n=16). Both sections were labeled with a monoclonal antibody against Hu, a protein present in all differentiated neurons, and znp-1, a zebrafish monoclonal antibody that recognizes axons. Scale bar, 50 μm.

RESULTS

Strategy

We designed a transplantation paradigm to learn how the CaP axonal pathway becomes established on the ventral myotome during development. To this end, we examined the permissiveness of dorsal and ventral myotomes for CaP axonal outgrowth at different stages of development. We chose two time points; 16 h, a time before the initiation of axonal outgrowth and 19 h, a time when the CaP axon is leaving the

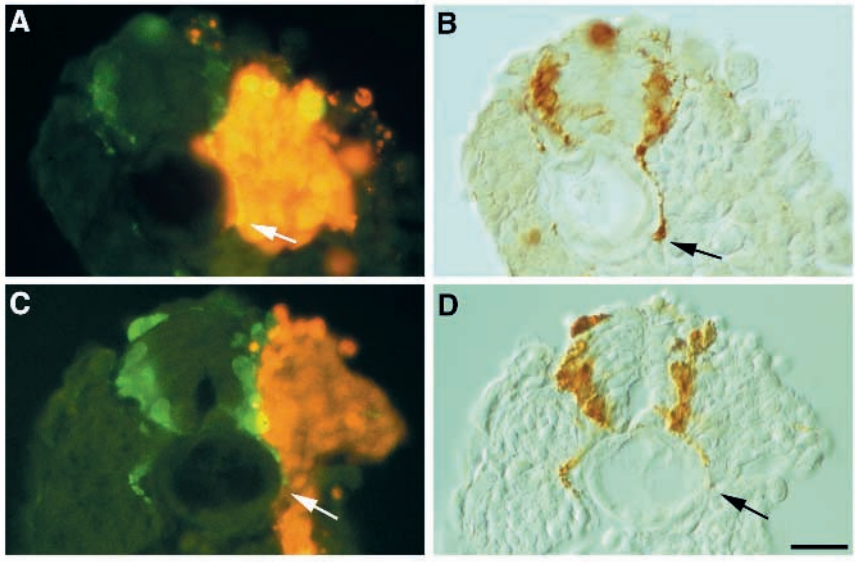

Fig. 4. CaP pathfinding on ventral myotomes. (A,C) Fluorescence double exposure and (B,D) bright-field photomicrographs of cross sections through two different host embryos containing transplanted donor myotomes (red); neuronal antibody labeling is in green or brown (B,D). Arrows point to the axon on the transplanted side in all panels. Left panel and adjacent right panel show the same section. (A,B) 16 h donor myotomes were transplanted in the correct dorsoventral orientation into a 16 h host embryo. (C,D) 19 h donor myotomes were transplanted in the correct dorsoventral orientation into 16 h host embryos. These data show that CaP can extend a normal axon along both 16 h and 19 h ventral myotomes. Scale bar, 25 μm.

common pathway and extending along its cell-specific pathway. Myotomes were removed from unlabeled host embryos before CaP axogenesis making sure that the underlying spinal cord was not damaged (Fig. 1). Donor myotomes were labeled throughout with rodamine-dextran, to mark them as donors, and dorsally with fluorescent beads, to enable their orientation to be determined once they were removed from the embryo. Labeled myotomes were removed from donor embryos and transplanted into unlabeled host embryos where native myotomes had been removed. To assay for the presence of the CaP axonal pathway on the ventral myotome, donor myotomes were transplanted in the correct dorsoventral orientation (Fig. 2A). To assay for the presence of the CaP axonal pathway on the dorsal myotome, donor myotomes were transplanted in a reversed dorsoventral orientation without altering the anterioposterior or mediolateral orientations (Fig. 2B). We first confirmed that CaP growth cone extension was dependent on the myotome, as previously suggested (Eisen and Pike, 1991), by removing myotomes before CaP axogenesis. Under these conditions, CaP did not extend an axon out of the spinal cord (Fig. 3).

Ventral myotome is permissive for CaP axonal outgrowth

The CaP growth cone extends along the myotome starting around 17 h (Myers et al., 1986), thus we would expect the pathway to be present by this stage. To confirm this for experimental myotomes, we transplanted 16 h donor myotomes into 16 h host embryos in the correct dorsoventral orientation and found that CaP projected a normal axon comparable in length to CaP axons on native ventral myotomes (Fig. 4A,B; Table 1b). To learn whether the pathway persisted at later stages, we replaced 16 h myotomes with 19 h myotomes in the normal orientation. Again, we found that CaP extended a normal axon along the transplanted ventral myotome (Fig. 4C,D; Table 1c). Thus, experimental ventral myotomes are permissive for CaP axonal outgrowth throughout the period of normal CaP axonal extension.

Dorsal myotome loses permissiveness for the CaP axon during development

If the CaP axonal pathway is present exclusively on ventral myotome, we would predict that other myotome regions would be non-permissive for CaP axons. To address this issue, we assayed whether dorsal myotomes of 16 h embryos were permissive for CaP axonal outgrowth by transplanting donor myotomes into host embryos in a reversed dorsoventral orientation. Under these conditions, CaP extended a growth cone along the dorsal myotome now located ventrally (Fig. 5A, B) resulting in a normal CaP axon comparable in length to those extending on native ventral myotomes (Table 2a). These results demonstrate that dorsal, as well as ventral, myotomes are permissive for CaP axons at this stage of development.

To learn whether the dorsal myotome remained permissive for CaP axons later in development, 19 h dorsal myotomes were assayed by transplanting donor myotomes into host embryos in the reversed dorsoventral orientation. In over 70% of the cases (8/11), CaP extended a short ventral process that often went laterally, failing to extend along the dorsal myotome now situated ventrally (Fig 5C,D; Table 2b). In less than 30% of the cases, CaP extended a growth cone ventrally along the dorsal myotome resulting in a normal CaP axon. These results suggest that while the dorsal myotome is initially permissive for CaP axonal outgrowth, as development proceeds it becomes non-permissive.

Table 1. CaP pathfinding on ventral myotomes

Transplanted myotome region assayed	*n*	exp/ct
a. Control no transplant	7	0.97±0.14
b. 16 h ventral	5	0.84±0.10
c. 19 h ventral	4	0.92±0.11

Axons were measured from camera lucida reconstructions and the results expressed as the ratio of the length of the axon on the experimental side (exp) over the axon length on the control side (ct) ± s.d. *n*, number of experiments.

Embryonic signals change the permissiveness of dorsal myotome for CaP axonal outgrowth

Loss of permissiveness for the CaP axon could be myotome-autonomous or it could require signals from other tissues. If this process is myotome-autonomous, then dorsal myotomes isolated from embryonic signals should still become non-permissive for CaP axons. Conversely, if embryonic signals are necessary, then dorsal myotomes isolated from the embryo should remain permissive for CaP axons. To distinguish between these possibilities, we removed 16 h myotomes from donor embryos and allowed them to develop for 2.5-3.0 hours in vitro before transplanting them into 16 h hosts. This experiment mimics the timing of the heterochronic transplants except that the myotomes mature in vitro instead of in the embryo, and thus are isolated from embryonic signals.

We found that dorsal myotomes matured in vitro remained permissive for CaP axonal outgrowth. CaP axons extending on these transplanted myotomes were comparable in length to those extending on native ventral myotomes (Fig. 5E,F; Table 2c). This result is what we would expect if changes in myotome permissiveness for CaP axons were non-autonomous. Thus, we conclude that embryonic signals cause the dorsal myotome to lose permissiveness for CaP axons.

The notochord is involved in changing the permissiveness of dorsal myotome for CaP axons

To identify the source of embryonic signals that cause the dorsal myotome to lose permissiveness for CaP axonal outgrowth, we examined the possible roles of notochord and motor innervation, both of which have been shown to influence muscle development. In chick, the notochord promotes myogenesis in vitro (Buffinger and Stockdale, 1994; Munsterberg and Lasser, 1995; Stern and Hauschka, 1995) and under some conditions in vivo (Rong et al., 1992; Teillet and Le Douarin,

Table 2. CaP pathfinding on dorsal myotomes

Transplanted myotome region assayed	*n*	exp/ct
a. 16 h dorsal	6	0.97±0.20
b. 19 h dorsal	8	0.35±0.09
c. 19 h in vitro dorsal	6	0.87±0.15
d. 19 h *flh*$^{-}$ dorsal	6	0.93±0.14
e. 19 h uninnervated dorsal	3	0.42±0.11

Axons were measured from camera lucida reconstructions and the results expressed as the ratio of the length of the axon on the experimental side (exp) over the axon length on the control side (ct) ± s.d. n, number of experiments.

1983). In zebrafish, the notochord is adjacent to the medial myotome where motoneuronal axons grow. To address whether notochord-derived signals play a role in establishing axonal pathways, we created genetic mosaics between *floating head* (*flh*) mutants which lack notochords (Talbot et al., 1995; Halpern et al., 1995) and wild-type embryos. We asked whether *flh*$^-$ dorsal myotomes remained permissive for CaP axons later than wild-type dorsal myotomes.

We first established that ventral myotomes of 19 h *flh*$^-$ embryos were permissive for CaP axons. We transplanted 19 h *flh*$^-$ myotomes in their normal orientation into wild-type embryos and found that CaP axons extended in a manner indistinguishable from their extension on 19 h wild-type ventral myotomes (data not shown). We then tested dorsal myotomes from 19 h *flh*$^-$ embryos and found that they were also permissive for CaP axons (Fig. 5G,H; Table 2d). This is in contrast to similarly staged wild-type dorsal myotomes that were non-permissive for CaP axons (compare with Fig. 5C,D. Table 2b). Thus, in the absence of notochord, dorsal myotomes remain permissive for CaP axons.

Innervation has also been implicated in muscle development in both vertebrates (Buller et al., 1960a,b; Gauthier et al., 1983) and insects (Currie and Bate, 1995; Lawrence and Johnston, 1986). In the 19 h myotomes used in this study, axons have already extended along the common pathway and the CaP and MiP axons have begun to extend along their cell-specific pathways. While these axons have not yet established synapses, they have been shown to release transmitter during elongation (Grunwald et al., 1988). In contrast, 16 h myotomes have not yet been contacted by axons. Thus, it was necessary to learn whether motor axon contact affected the permissiveness of dorsal myotomes for the CaP axon. To address this issue, we ablated primary motoneurons from several adjacent spinal cord segments before axonal outgrowth and allowed the embryos to develop to 19 h. We then assayed dorsal myotomes overlying these spinal cord segments by transplanting them in the reversed dorsoventral orientation. In the majority of cases (3/4; Table 2e), the axon on the transplanted side was approximately half the length of the control axon. In only one case did we observe a normal CaP axon along the previously uninnervated dorsal myotome. These results are comparable to those obtained from transplants using 19 h dorsal myotomes that had been contacted by motor axons (compare Table 2b and 2e) and suggest that axon contact does not influence the permissiveness of dorsal myotomes for CaP axons.

CaP axons can distinguish permissive from non-permissive myotomes

We tested directly whether myotome permissiveness contributed to pathway choice by juxtaposing permissive and non-permissive myotomes. Since *flh* mutants lack notochord (Talbot et al., 1995), it was possible to juxtapose the native, permissive, *flh*$^-$ myotome with a non-permissive, wild-type myotome at the midline of the embryo by transplanting 19 h wild-type donor myotomes in the reversed dorsoventral orientation into *flh* mutant embryos. Under these conditions, the CaP axon on the transplanted side crossed the midline (3/5) and grew along the ventral *flh*$^-$ myotome rather than extending directly along the dorsal wild-type myotome (Fig. 6). In the remaining cases (2/5), CaP extended a short ventral axon along the wild-type myotome. These results contrast with control experiments in which 19 h wild-type or *flh*$^-$ myotomes were transplanted in the normal dorsoventral orientation into *flh* mutants. Under these conditions, the CaP axon on the transplanted side grew along the transplanted ventral myotome (5/5; data not shown). Thus, the CaP axon can distinguish between permissive and non-permissive myotomes and selectively extends along the permissive myotome.

DISCUSSION

We have addressed how a particular motoneuronal pathway is established during development. Previous experiments demonstrated that the CaP pathway was localized to ventral myotome (Gatchalian and Eisen, 1992). Our approach was to determine whether the pathway was localized to this region throughout development or whether other regions were also permissive for the CaP axon. In addition, we addressed whether these events were myotome-autonomous or dependent on signals from the embryo. Our results suggest that early in development both dorsal and ventral myotomes are permissive for CaP axonal outgrowth. Later, however, dorsal, but not ventral myotomes, become non-permissive for CaP axons. When dorsal myotomes were allowed to mature isolated from the notochord, they remained permissive for CaP axons, suggesting that notochord-derived signals are required for the CaP pathway to become localized to the ventral myotome.

Notochord-derived signals pattern vertebrate somites

Midline tissues influence the developing somite. During avian somitogenesis, dorsoventral cell fates are initially plastic, but then become irreversibly specified to sclerotome, dermatome and myotome. Before specification, somite regions can develop either dorsal or ventral cell fates, depending on their exposure to different midline tissues (Aoyama and Asamoto, 1988; Ordahl and Le Douarin, 1992), suggesting that midline signals establish the dorsoventral polarity of the somite. In addition to patterning the somite, both in vivo and in vitro experiments have demonstrated that the notochord is necessary for the survival and differentiation of somitic derivatives such as myotomal cells (Teillet and Le Douarin, 1983; Rong et al., 1992; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995; Munsterberg and Lasser, 1995) and sclerotome (Brand-Saberi et al., 1993; Pourquie et al., 1993; Fan and Tessier-Lavigne, 1994). Some of these effects have been attributed to Sonic hedgehog (Johnson et al., 1994), a molecule expressed by notochord and spinal cord floor plate (Echelard et al., 1993; Krauss et al., 1993; Roelink et al, 1994). Molecules released from the notochord, therefore, play a role both in patterning the somite and in influencing the development of somitic derivatives.

Observations in zebrafish also indicate that notochord can influence the developing myotome. In zebrafish, the somitic derivative next to the notochord is myotome (Morin-Kensicki and Eisen, 1997), not sclerotome as in amniotes such as chick and mouse. Signals from notochord are important for patterning the myotome (Halpern et al., 1993; Currie et al., 1996; Weinberg et al., 1996); however, a differentiated notochord appears unnecessary for overt myogenesis in zebrafish. *flh* mutants, which lack a notochord, undergo myogenesis as

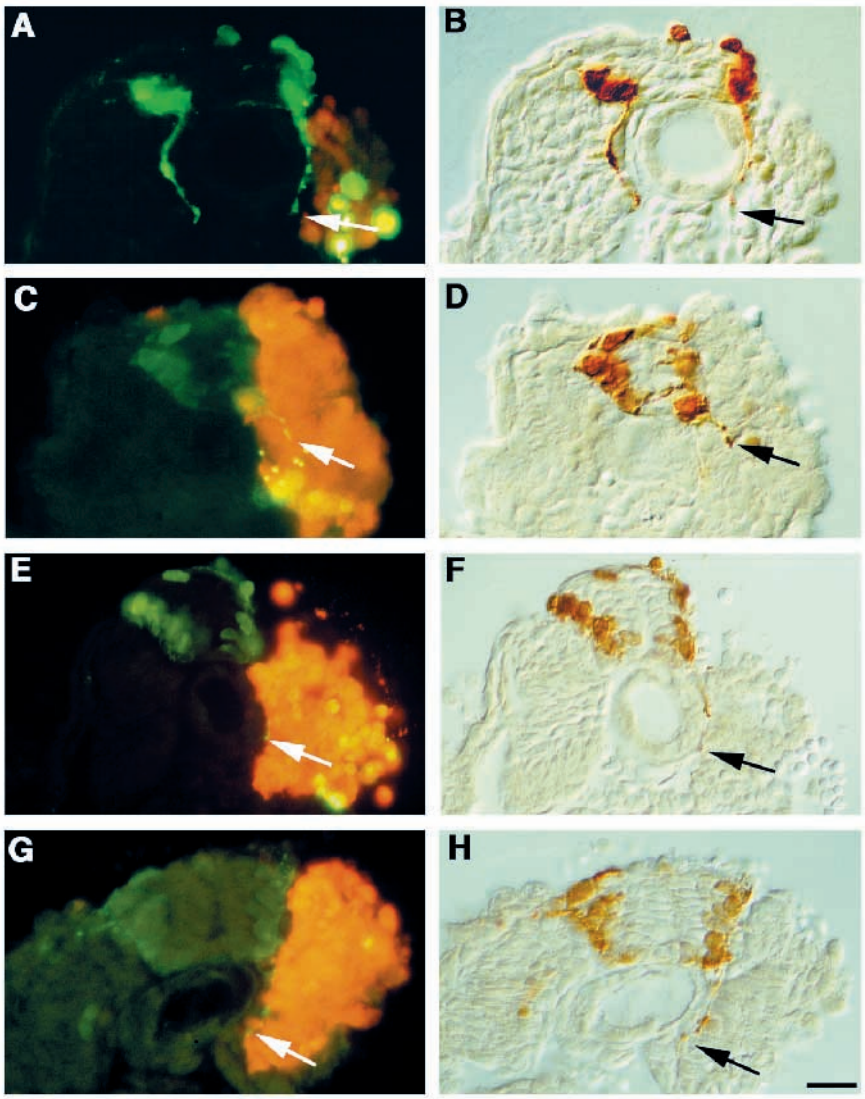

Fig. 5. CaP pathfinding on dorsal myotomes. (A,C,E,G) Fluorescence double exposure and (B,D,F,H) bright-field photomicrographs of cross sections through host embryos containing transplanted donor myotomes (red); neuronal antibody labeling is in green (A,C,E,G) or brown (B,D,F,H). Arrows point to the axon on the transplanted side in all panels. Left panel and adjacent right panel show the same section. (A,B) 16 h donor myotomes were transplanted in the reversed dorsoventral orientation into 16 h host embryos. (C,D) 19 h donor myotomes were transplanted in the reversed orientation into 16 h host embryos. (E,F) 16 h donor myotomes were incubated in culture for 2.5-3 hours then transplanted in the reversed dorsoventral orientation into 16 h host embryos. (G,H) 19 h donor myotomes from *flh*$^{-}$ embryos were transplanted in the reversed dorsoventral orientation into 16 h wild-type host embryos to create genetic mosaics. These data show that CaP can extend a normal axon along 16 h, but not 19 h, wild-type dorsal myotome. However, if donor myotomes matured in vitro or in embryos lacking notochords, 19 h dorsal myotomes remained permissive for CaP axons. Scale bar, 25 μm.

evidenced by expression of *myoD* (Halpern et al., 1995) and *tropomysin* (Talbot et al., 1995). The proximity of the notochord to the CaP growth cone pathway and the ability of signaling molecules expressed in the notochord to induce cell fates and to regulate gene expression in the myotome, suggest that the notochord could play a role in the development of the motoneuronal pathways on the medial myotome surface.

The notochord could effect establishment of axonal pathways in several ways. One possibility is that early in development both dorsal and ventral myotomes express cues that delineate motoneuronal pathways. As development proceeds, signals from the notochord might localize these cues to the appropriate region of the myotome; for example, CaP pathway-specific molecules would become restricted to ventral myotome. Without notochord, the molecules remain present throughout the myotome, making both dorsal and ventral

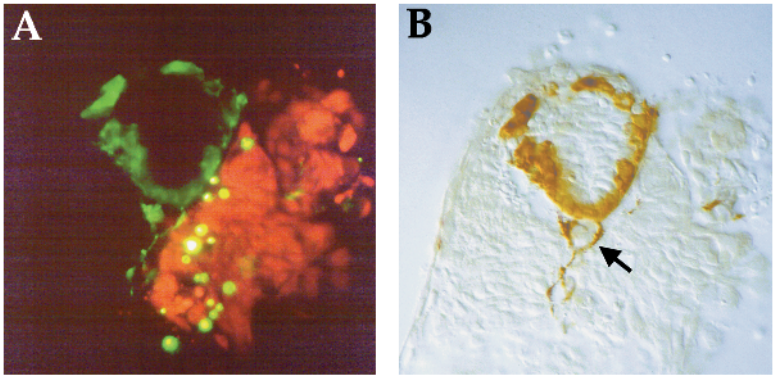

Fig. 6. CaP can choose between permissive and non-permissive myotomes. (A) Fluorescence double exposure and (B) bright-field photomicrograph of the same cross section through a *flh*$^{-}$ host embryo containing a transplanted wild-type donor myotome in the reversed dorsoventral orientation (red); neuronal antibody labeling is in green (A) or brown (B). The arrow points to the axon from the transplanted side. If the CaP axon has equal access to both a permissive and a non-permissive myotome, it will extend along the permissive myotome.

regions permissive for CaP axons. Identification of the molecules that determine the primary motoneuronal pathways and markers that conclusively differentiate between dorsal and ventral myotome will allow us to test this model.

Changes in pathway permissiveness affect pathway choice

Is restriction of the CaP pathway to the ventral myotome required for CaP pathfinding specificity? The dorsal myotome becomes non-permissive for CaP at about the same time that the CaP growth cone is leaving the common pathway and beginning to extend along its cell-specific pathway (Eisen et al., 1986; Myers et al., 1986), suggesting that changes in permissiveness could influence CaP pathway choice. However, even when both dorsal and ventral myotome remain permissive for the CaP axon, as in *flh* mutants, CaP axons extend ventrally. Thus, there must be other signals, in addition to permissiveness of the myotome, that are important in establishing the initial direction of outgrowth as motor axons leave the spinal cord. This idea is bolstered by transplantation experiments in which motor growth cones always extended directly out of the spinal cord in their normal direction independent of the orientation of the cell body (Eisen, 1991). However, permissiveness of the myotome does appear important in the specificity of CaP axonal extension. When CaP is experimentally given access to both a permissive and a non-permissive myotome, it extends along the permissive myotome even if there is already another CaP axon on this pathway. The two axons do not fasciculate, suggesting they are making independent pathway choices, as has also been observed in other cases in which two CaP axons were forced to extend along the CaP-specific axon pathway (J. S. E., unpublished data). Thus, pathway permissiveness appears important to enable the CaP axon to select a pathway and extend along it, even if its initial direction of extension is guided by other cues.

Our data suggest that the CaP axonal pathway is established in a stepwise fashion. First, both dorsal and ventral regions of the medial myotome are permissive for CaP axons. Later, the pathway becomes restricted to the ventral myotome in a notochord-dependent manner. The ability to generate mutations in zebrafish should identify the molecules involved in this process and reveal how they are regulated during development.

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