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Development of the Myotomal Neuromuscular System in Embryonic and Larval Angelfish, *Pterophyllum scalare*

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ABSTRACT—To reveal the developmental sequence of the myotomal neuromuscular system in a teleost angelfish, *Pterophyllum scalare*, we investigated the differentiation and axonal outgrowth of the somatic spinal motoneurons as well as the differentiation of the axial muscles by means of anatomical and histochemical methods. Acetylcholinesterase histochemistry and retrograde labeling with HRP revealed two large motoneurons in each spinal hemisegment in the late embryos. To clarify the posthatching change of the motoneuron number, the number of axons in the anal-level ventral root was counted, since ChAT-immunohistochemical labeling of cholinergic spinal neurons and electron microscopic observation in the adult showed that the ventral roots around the anal level contained only somatic motor axons. We found that 15 primary motoneurons in each spinal hemisegment participated in the muscle innervation in just-hatched larvae. The motor axons rapidly increased in number beyond the adult level within three days posthatching, and then decreased to reach the adult level within a few weeks. The result suggests that competition among the motoneurons for their target muscles takes place. To reveal the temporal sequence of differentiation of the myotomal muscle fibers, in addition to electron microscopic observation of the muscle, a fluorescent mitochondrial marker dimethylaminostyrylethylpyridiniumiodine (DASPEI) was used to detect red muscle fibers. In the late embryo, immature white muscle fibers subserving the twitching movement of the animal in the egg capsule were observed. Differentiation of the red muscle was not evident until day 10. The present results show that a complete set of the axial muscle motoneurons differentiates before the differentiation of the multiple muscle fiber types in the angelfish.

INTRODUCTION

The aim of the present study is to elucidate the developmental changes of the neuromuscular system subserving the swimming behavior in the angelfish, *Pterophyllum scalare*. In mammalian nervous systems, proliferation of somatic motoneurons is generally completed during the embryonic period. In contrast, in fishes, situations are different depending on species examined. In some elasmobranch fishes, it has been reported that new motoneurons are added as the fishes grow (Leonard *et al*., 1978; Fox and Richardson, 1982; Mos and Williamson, 1986). On the other hand, in a teleost zebrafish, *Danio rerio*, the number of spinal motoneuron has been shown to reach the adult level in an early larval stage before the differentiation of muscle fiber types in the myotomes completes (van Raamsdonk *et al*., 1983), although the precise number of motoneurons, except for the primary ones, in the developing larvae is not known. In European eel, *Anguilla anguilla*, the numbers of axons in the ventral roots (VR) are very similar among individuals with various body sizes and developmental stages, whereas the number of muscle fibers increases substantially in relation to body length (Smit *et al*., 1991). In the eel at the early larval stage, in which the neuromuscular development is marked, however, developmental changes of the spinal motoneuron number have not been examined in detail.

Developmental changes of the spinal motoneurons innervating the axial muscles in posthatching angelfish has been investigated by means of retrograde labeling with horseradish peroxidase (HRP) (Yoshida *et al*., 1999). They have shown that the axial muscle motoneurons are composed of two classes of cells; primary and secondary. The primary motoneurons are relatively large and already present in lar-

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vae just after hatching. On the other hand, the secondary motoneurons begin to be backfilled later. It has been suggested that in angelfish increase in number of axial muscle motoneurons cease within a few days after hatching (Yoshida *et al.*, 1999). However, it is difficult to label all the motoneurons using the retrograde labeling method. Thus, in the present study, to elucidate precisely the temporal sequence of the development of the spinal motoneurons innervating the trunk muscles, we utilized multiple anatomical and histochemical methods, including retrograde labeling with HRP, detection of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) and electron microscopy. Histochemical methods for detection of AChE (Tago *et al.*, 1986; Kuchiiwa, 1990; Stoiber *et al.*, 1998) and ChAT immunohistochemistry (Brantley and Bass, 1988; Schmidt, 1995) have been used to stain cholinergic neurons including somatic motoneurons.

The organization of the myotomal muscles in fish is relatively simple. The myotomal muscle consists of discrete sets of histochemically defined muscle fibers that, in many cases, form distinct layers in the trunk (Fetcho, 1987; Koumans and Akster, 1995; Mascarello *et al.*, 1995). In mammals and birds, it is well established that the increase in the number of muscle fibers stops at, or shortly after, birth (Goldspink, 1972; Campion, 1984). In contrast, in fishes, the hyperplastic growth of the trunk musculature and differentiation of multiple muscle fiber types occur during considerably long period of the posthatching development (van Raamsdonk *et al.*, 1982; Koumans and Akster, 1995; Ramírez-Zarzosa *et al.*, 1995; Rowlerson *et al.*, 1995). It is important to reveal temporal sequence of both the axonal outgrowth of the spinal motoneurons and the differentiation of multiple muscle fiber types for understanding to what extent the motoneurons determine the differentiation and growth of the muscle fibers. Most of the studies on the differentiation of the muscle fiber types have been done by using histochemical methods including immunostaining with anti-myosin antibody and staining for ATP-ase activity (van Raamsdonk *et al.*, 1980; van Raamsdonk *et al.*, 1982; Mascarello *et al.*, 1995; Ramírez-Zarzosa *et al.*, 1995; Devoto *et al.*, 1996; Stoiber *et al.*, 1998). In the present paper, for a rough estimation of the timing of the muscle differentiation, we introduced a new convenient method to detect functionally differentiated red muscle fibers .

A styryl dye, dimethylaminostyrylethylpyridiniumiodine (DASPEI), has been known to stain mitochondrial membranes in live cells (Bereiter-Hahn, 1976). The aerobic slow red muscle fibers contain large number of mitochondria compared with the anaerobic fast white fibers (Veggetti *et al.*, 1993; Luther *et al.*, 1995; Ramírez-Zarzosa *et al.*, 1995), and therefore they may well be detected by examining the axial muscles treated with the dye. Thus, in order to reveal the temporal and spacial sequence of the differentiation of the red muscle, we examined the trunk muscle by staining with the fluorescent mitochondrial marker DASPEI followed by observation with confocal laser scanning microscope (CLSM) as well as transmission electron microscopy (TEM).

MATERIALS AND METHODS

Animals

Embryos and larvae of the angelfish, *Pterophyllum scalare*, used in the present experiments were obtained from laboratory-reared adult pairs that bred spontaneously at 26.5°C. The embryos and the larvae were kept as described by Yoshida *et al.* (1996). Twelve embryos and 164 larvae ranging from 10 hr before hatching to 30 days old were used for the following experiments. After about 60 hr prehatching embryonic period, the fish hatch out of egg capsule. The first 24 hr of posthatching larval period is defined as day 1, next 24 hr is day 2, and so on. In some experiments, including choline acetyltransferase (ChAT) immunohistochemistry and EM observations, seven adult angelfish of 8-10 cm in body length were also used.

ChAT immunohistochemistry

Spinal cells containing ChAT were immunohistochemically labeled to reveal the localization of cholinergic neurons in the spinal cord of adult angelfish (n=3). The adult angelfish was deeply anesthetized with tricaine methanesulphonate (MS-222) in water (200 ppm), and paralyzed by an intraperitoneal injection of d-tubocurarine chloride (1–2 μg/g body weight) in saline. The animal was then fixed by transcardial perfusion of the vascular system with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) which was preceded by perfusion with heparinized saline (10 mg/ml saline). Whole spinal cord was cut out of the animal and postfixed with the same fresh fixative overnight at 4°C. After fixation the tissue was washed thoroughly in phosphate buffered saline (PBS) containing 0.4% Triton X-100 (PBST) and embedded in 6% agar (gelling temperature, 30–31°C) dissolved in distilled water (DW). The preparation was then cooled to 4°C in order to facilitate the gelling of the agar. Transverse sections of the spinal cord with 50–100 μm in thickness were cut using a microslicer (Dosaka EM, Kyoto, Japan). During sectioning, the tissue was kept soaked in PBST. The sections were washed 3 times 1 hr each in PBST at 4°C and incubated with a 10% solution of normal goat serum in PBST to block non-specific binding of anti-ChAT antibody in the tissues. All the subsequent steps except for the diaminobenzidine (DAB) reaction were performed at 4°C with gentle agitation. The sections were incubated with rabbit anti-ChAT polyclonal antiserum (Chemicon, CA, USA) (1:7500 dilution) for 96 hr. After washing in PBST, the sections were incubated with a biotinylated anti-rabbit secondary antibody (Seikagaku Kogyo, Tokyo, Japan) for 2 hr. The sections were then washed in PBST and incubated with a peroxidase-conjugated streptavidin (Seikagaku Kogyo, Tokyo, Japan) for 2 hr. After washing in PBST, the sections were rinsed in 0.1 M Tris-HCl buffer (TB: pH 7.4), and incubated in 0.05% DAB (Nacalai Tesque, Kyoto, Japan) in 0.1 M TB for 10 min at room temperature (RT). The preparation was then incubated in the fresh DAB solution in the presence of 0.03% H_2O_2 in the dark for 5 min at 20–22°C. After washing in DW, the preparation was placed on a glass slide, coverslipped and examined under a microscope. Stained sections, as well as control ones, were photographed and drawn using a camera lucida.

When the primary antibody was substituted with antiserum diluent, no labeled cells were observed in any part of the cord (n=3).

ChAT-immunoreactive (ChAT-IR) cells in the motor column of the spinal cord were considered to be somatic motoneurons and were subjected to later analyses. To avoid overestimation of the number of labeled motoneurons, the number of labeled motoneurons with cell nuclei were counted. The number of presumable somatic motoneurons per spinal hemisegment were determined by dividing the total number of motoneurons in several spinal hemisegments around the anal level with the number of segments examined.

AChE histochemistry

Localization of acetylcholinesterase (AChE) in the embryos and

larvae were examined histochemically according to the method of Kuchiiwa (1986) with slight modifications. The embryos and the larvae at various developmental stages, under cold anesthesia, were fixed in 2% PFA and 2% glutaraldehyde (GA) in 0.1 M PB for 2 hr at 4°C. After washing in PBS, the preparation was incubated in 0.05 M acetate buffer solution (pH 5.0) containing 4 mM acetylcholine iodide (Nacalai Tesque, Kyoto, Japan), 10 mM glycine and 7.5 mM copper sulfate for 1 hr with occasional agitation at 37°C. The following procedures were carried out at RT with periodic agitation. The preparation was rinsed five times in DW, and incubated in 1.25% sodium sulfate in PB for 3 min. After washing in DW, the preparation was then incubated in 1% solution of silver nitrate for 45 min. The preparation was subsequently rinsed in DW and treated with 1% sodium thiosulfate for 10 min. Then the preparation was rinsed in DW, postfixed in 1% OsO_4 for 2 hr, dehydrated and embedded in resin (Quetol-812, Nisshin EM, Tokyo, Japan). Serial semithin sections with 1–2 μm in thickness (transversal, horizontal or sagittal) were cut with a glass knife and stained with 1% toluidine blue. The sections were examined and photographed with a light microscope. Ultrathin sections with 60 nm in thickness were cut transversely using a diamond knife (Diatome, Nisshin EM, Tokyo, Japan) and stained with uranyl acetate and lead citrate. The sections were examined and photographed with a TEM (H-600A, HITACHI).

Retrograde labeling of spinal motoneurons with HRP

To identify the ventral root containing the spinal motoneurons innervating the trunk muscles in the larvae at day 6 or younger stages, we retrogradely labeled the motoneurons with HRP according to the method of Yoshida *et al.* (1999). After visualization of the HRP with DAB, the preparation was dehydrated and embedded in Quetol-812 resin. For light microscopic observation, serial semithin sections of the spinal cord with 1–2 μm in thickness were cut with a glass knife and stained with 1% toluidine blue. The angle of the plane of sectioning was adjusted to obtain the cross-sectioned profiles of the axons running in the VR. For electron microscopic observation, serial ultrathin sections with 60 nm in thickness were cut with a diamond knife, stained with uranyl acetate and lead citrate and examined and photographed with a TEM. In the larvae older than day 6, the VR was obviously identified based on its morphological features in semithin sections without labeling the motoneuron axons with HRP.

On examining the VR in the adult, a part of the spinal cord with the VR attached around the anal level was isolated after the perfusion fixation, and subjected to both light and electron microscopic examinations.

For statistical analyses of the number of axons running in the VR, we used one-way analysis of variance (ANOVA) using a software package “Stat View” on an Apple Macintosh computer. Values were considered to be significantly different from one another when $p<0.05$.

Electron microscopic observation of the muscle

Two embryos and twenty seven larvae at various developmental stages were fixed in 2% PFA and 2% GA in 0.1 M PB for 2 hr at 4°C under cold anesthesia. The preparation was dehydrated and embedded in Quetol-812 resin. Ultrathin sections of the trunk region around the anal level were cut transversely. Ultrastructures of the axial muscles were examined using a TEM.

Staining with a fluorescent mitochondrial marker, DASPEI

To examine the temporal sequence of the differentiation of the red muscle, we used a fluorescent mitochondrial marker, dimethylaminostyrylethylpyridiniumiodine (DASPEI, Sigma, MO, USA). Fifty-seven larvae at various developmental stages were first killed by decapitation under MS-222 anesthesia. The trunk of the animal was embedded in 6% low-gelling-temperature (30–31°C) agar in DW at 30–40°C. Immediately after embedding, the preparation was cooled to 4°C to facilitate the gelling of the agar. The preparation was cut into 200 μm cross sections using a microslicer, and incubated with 2 μM DASPEI in saline for 10 min in the dark at RT. After washing in saline, the preparation was placed on a glass slide and examined and photographed with a conventional fluorescent microscope or a CLSM (MRC-600, Bio-Rad)

RESULTS

ChAT immunohistochemistry

ChAT immunoreactivity in the rostral (1st–3rd segments),

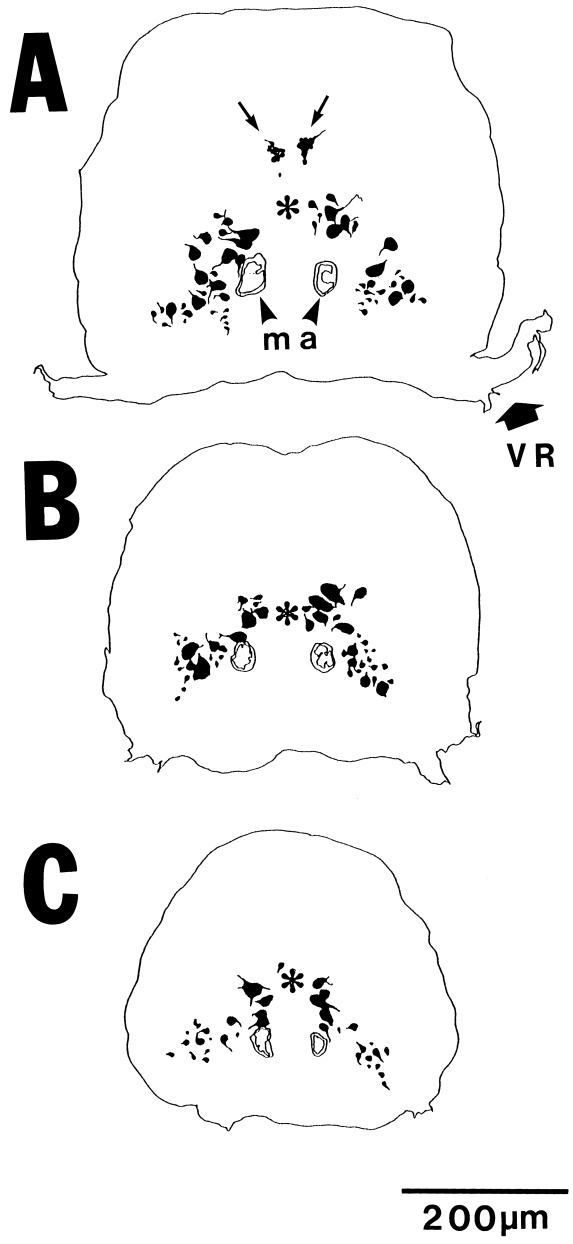

Fig. 1. Camera-lucida drawings of transverse sections of the spinal cord of the adult angelfish showing choline acetyltransferase-immunoreactive cells. (**A**) Rostral (2nd segment). (**B**) Anal level (11th segment). (**C**) Caudal (20th segment). Clusters of small immunoreactive cells (small arrows in **A**) are observed only in the rostral part of the spinal cord. Asterisks mark the central canals. ma, Mauthner axon; VR, ventral root.

mid (10–12th segments, anal level) and caudal (19–21th segments) parts of the spinal cord in adult angelfish was examined. Relatively large ChAT-immunoreactive (ChAT-IR) cells were located in the motor column in all three parts of the spinal cord (Fig. 1). Judging from their location and size, they were apparently the cell bodies of motoneurons (Figs. 1, 2).

In the rostral part of the cord, small ChAT-IR cells were clustered bilaterally forming distinctive longitudinal cell columns dorsolateral to the central canal (Figs. 1A, 2). These cell clusters obviously consisted of preganglionic sympathetic neurons (Funakoshi *et al.*, 1995; Funakoshi *et al.*, 1996). No such cluster of ChAT-IR cells was observed in the mid and caudal segments (Fig. 1), indicating that the VRs around the anal level exclusively consist of somatic motor axons.

From the transverse sections immunostained for ChAT, the total motoneuron number per spinal hemisegment at the anal level (10–12th segment) was estimated. We found that the average number of the motoneurons per spinal hemisegment was 178.8±21.3 (mean±SD, n=6 hemisegments from 3 different animals). Since we cannot exclude the possibility of double-counting the same motoneurons whose nuclei were in the plane of sectioning, the number of motoneurons per spinal hemisegment could be less than the above estimation.

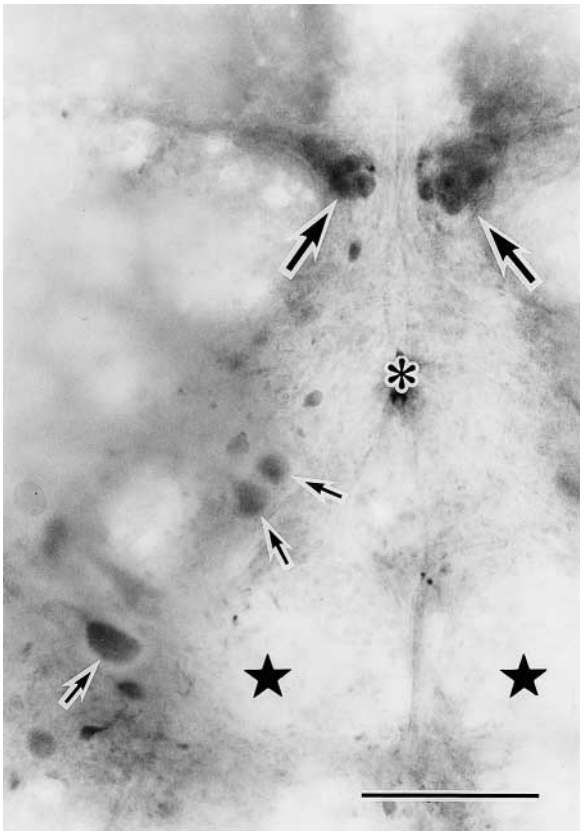

Fig. 2. Photomicrograph showing choline acetyltransferase-immunoreactive cells in the adult rostral cord (2nd segment). Clusters of presumable preganglionic sympathetic neurons (large arrows) and motoneurons (small arrows, for example) show choline acetyltransferase-immounoreactivity. Asterisk marks the central canal. Stars mark the Mauthner axons. Scale bar=100 μm.

In the larvae, ChAT immunohistochemistry failed to label any spinal cells. Thus, we applied AChE histochemistry, which is another method for labeling cholinergic neurons, to the embryos and the larvae.

AChE histochemistry

Spinal cells having AChE activity were examined histochemically in the embryos and the larvae at various developmental stages. Fig. 3 shows a horizontal section at the level of motor column of the spinal cord in the embryo at 50 hr after fertilization. In prehatching embryos and larvae just after hatching, two large AChE-positive cells were found to be located in the ventrolateral part of each hemisegment of the spinal cord near the exit of the VR (n=10) (Fig. 3). These cells were identified as motoneurons even prior to hatching based on their characteristic oval shapes and ventrolateral positions in the spinal cord. These “paired” motoneurons were frequently observed to be situated side by side with each other near the VR. The paired motoneurons were also identifiable in the larvae 6 hr after hatching (n=6, see Fig. 5 for HRP labeling). However, in the larvae at 18 hr after hatching, it was difficult to distinguish these neurons from other motoneurons that differentiated later during the early posthatching period.

Eight animals, from 50 hr after fertilization to day 6 in their stage, were subjected to TEM observation after AChE detection in whole-mount preparations. In all cases, AChE activity was found to be associated with the membranous structures in the cytoplasm and the nuclear membrane in the motoneurons (Fig. 4).

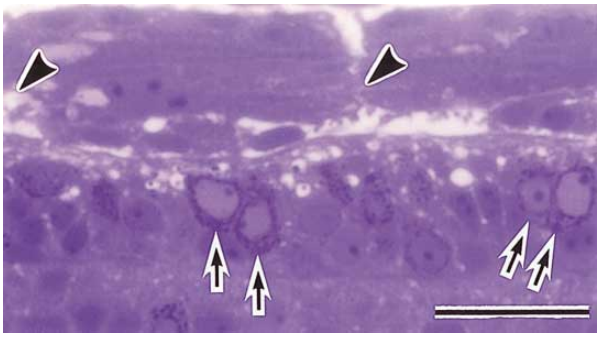

Fig. 3. Photomicrograph of a toluidine blue-stained semithin horizontal section of the spinal cord at the anal level in the embryo 50 hr after fertilization (10 hr before hatching) stained histochemically for acetylcholinesterase. Each spinal hemisegment contains large paired motoneurons (arrows). Arrowheads indicate the myoseptums. Rostral to the right. Scale bar=20 μm.

HRP labeling and EM observation of motoneurons

The morphology and the location of the spinal motoneurons retrogradely labeled with HRP from the axial muscles in the larvae in the present experiment were consistent with the findings by Yoshida *et al.* (1999). In addition, we found that the paired motoneurons tended to be labeled more intensely

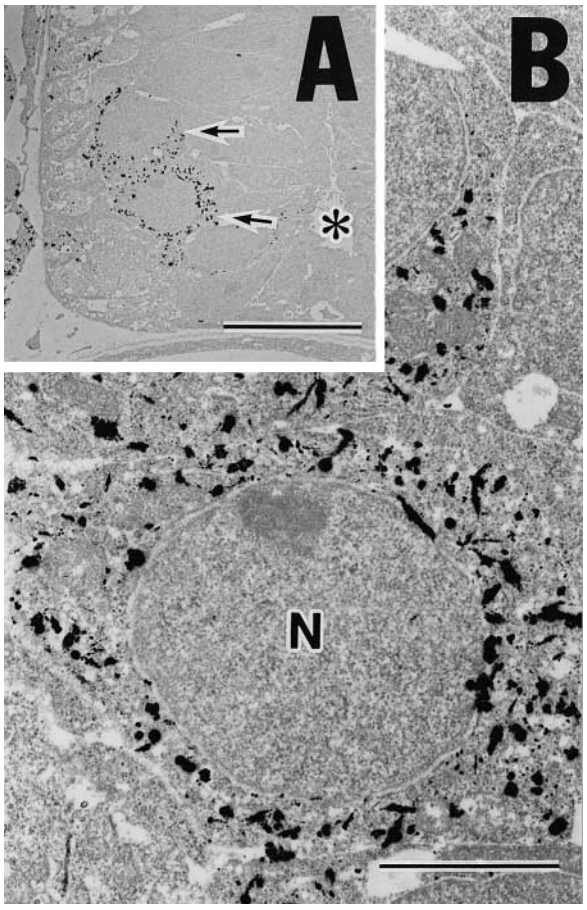

Fig. 4. Transmission electron micrographs of the anal-level transverse section of the spinal cord in the larva 6 hr after hatching. (**A**) Large AChE-positive paired motoneurons (arrows) in the spinal cord. Scale bar=10 μm. Asterisk marks the central canal. (**B**) Higher magnification of one of the motoneurons in (**A**), showing AChE-activities are observed in the cytoplasm. N, nucleus. Scale bar=2 μm.

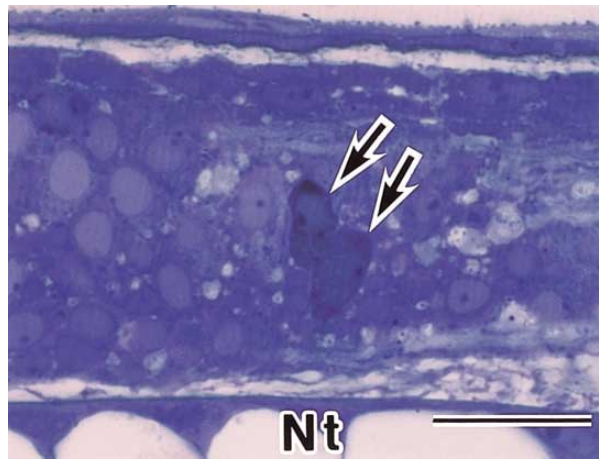

Fig. 5. Photomicrograph of a toluidine blue-stained semithin sagittal section of the spinal cord at the anal level of the larva 6 hr after hatching. Arrows indicate HRP-labeled paired-motoneuron somata. Nt, notochord. Scale bar=20 μm.

than the other motoneurons. Furthermore, in all prehatching embryos (50 hr after fertilization) examined (n=4), only the paired motoneurons were found to be filled, suggesting that the other primary-type motoneurons do not extend their axons in the embryonic stages. Thus, together with the results in AChE histochemistry, only the paired motoneurons seem to contribute to the axial muscle innervation. The paired motoneurons were closely associated with each other and the major part of the motoneurons was found to be occupied by large nucleus. By tracing the axons of the HRP-labeled motoneurons in serial sections (Fig. 5), we could identify the VR (Fig. 6A), which is usually difficult to find in hatched larvae without HRP filling. In the larvae at 6 hr after hatching, the number of axons was 15 in 3 VRs in 3 different animals and 14 in 1 VR in 1 animal (Figs. 6A, 7A). In the angelfish, each motoneurons appear to possess a single axon that exits via a single VR (Yoshida *et al.* 1999), and hence the number of axons running in the VR should correspond to the motoneuron number per hemisegment of the spinal cord. Thus, the number of primary motoneurons seems to be 15.

Thirty-two VRs in 32 animals, 4 animals from each of 8 different posthatching stages including day 1 (6 and 18 hr after hatching), day 2, day 3, day 6, day 10, day 20 and adult, were examined electron microscopically. The number of the axons running in the VRs at the anal level was markedly increased within a few days after hatching (Figs. 6 and 7). Fig. 6B shows an electron micrograph of a VR in the day 3 larva. Many small axons are probably those of secondary motoneurons. Several axons with large diameter are likely to be of primary motoneurons. However, it was difficult to identify the axons of primary motoneurons only from the axon size. Mean axon number in the VR was 206.8±2.4 (mean±SD, n=4) at 60 hr after hatching (Figs. 6B, 7B). Thus, nearly 200 secondary motoneurons had differentiated and extended their axons to the VR within the short period after hatching (Fig. 7). Hypertrophic growth of the axons was also obvious in the population of relatively large axons, whereas many small axons did not show apparent thickening until day 10 (Fig. 7). In day 20, some thick axons were found to be clearly myelinated (Fig. 6C). In the adult VR, all axons were surrounded by myelin sheath (Fig. 6D).

After reaching its peak in day 3, the number of axons decreased gradually by day 20. The number of VR axons in day 20 was 120.5±16.9 (mean±SD, n=4) which was significantly smaller than that in day 3 ($p<0.05$). There was no difference in the number of axons between day 20 larvae and the adults (122.8±8.5) (mean±SD, n=4). The number of axons running in the VR in the adults almost corresponds to the motoneuron number per spinal hemisegment estimated by ChAT immunohistochemistry (see above).

Differentiation of the axial muscles

In the embryos at 50 hr after fertilization (10 hr before hatching), presumptive white muscle cells already contained immature myofibrils (n=2) (Fig. 8A). Nucleus of the cells were found to be located near the center of each cell (Fig. 8A). These morphological features are typical of myotube. TEM

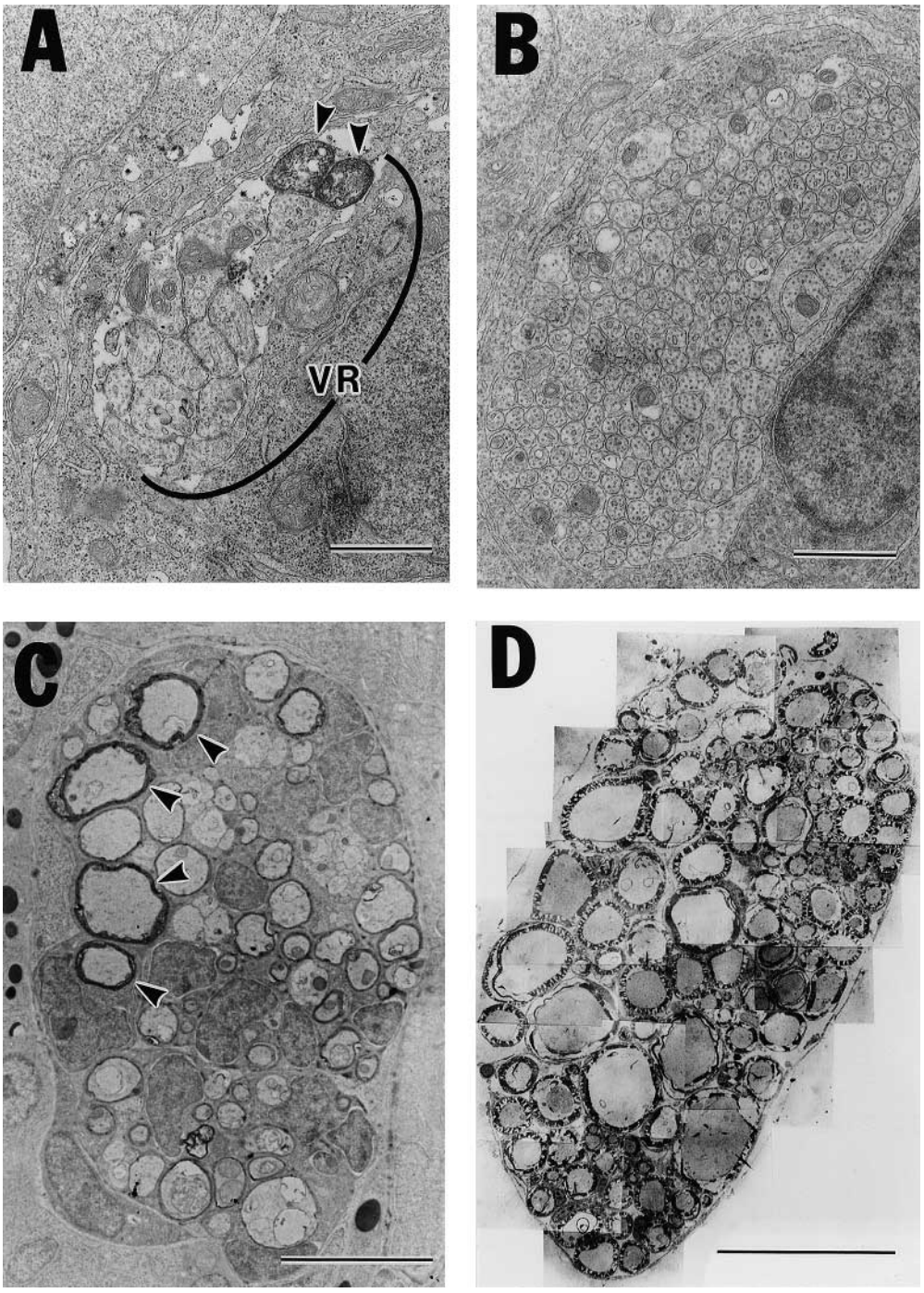

Fig. 6. Transmission electron micrographs of the anal-level transverse sections of the ventral roots at various developmental stages. (**A**) Six hours after hatching (day 1). The ventral root is bracketed by the curved lines (VR). Arrowheads indicate the axons of HRP-labeled paired motoneurons (see text). Scale bar =1 μm. (**B**) Sixty hours after hatching (day 3). Scale bar =1 μm. (**C**) Day 20. Many large myelinated axons are observed (arrowheads, for example). Scale bar =5 μm. (**D**) Photographic montage of the ventral root in the adult angelfish. All the axons in the ventral root are myelinated. Scale bar=25 μm.

observations of the white muscle fibers in day 5 larvae revealed that the cytoplasm of the myocytes was filled with the myofibrils, and their nuclei were located superficially in the cells (n=5) (Fig. 8B). This observation indicates that the white muscle fibers almost mature before the start of free swimming, which occurs at late day 5 or day 6 (Yoshida *et al.*, 1996). However, differentiation of the aerobic red muscle fibers was not evident before the onset of free swimming.

In 20-days-old larvae, the aerobic red muscle fibers were clearly found electron microscopically in the peripheral region of the trunk muscle (Fig. 8C). As known in other teleost species, the red muscle fibers contained a large number of mito-

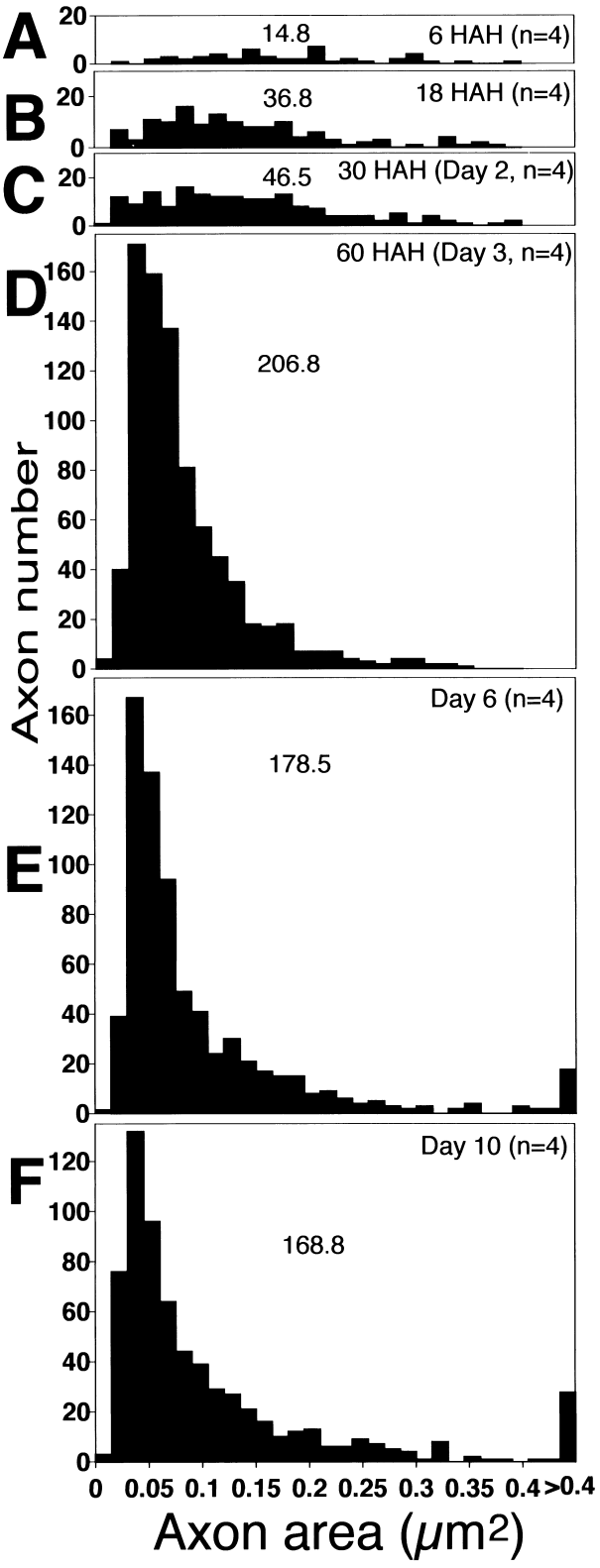

Fig. 7. Histograms showing the areas of the axons (μm^2) running in the ventral root 6 hr (**A**), 18 h (**B**), 2 days (**C**), 3 days (**D**), 6 days (**E**) and 10 days (**F**) after hatching. The averages of 4 ventral roots at the anal level in each of the 6 developmental stages are shown. The numerical values in the middle of each graph indicate the averages of the total axon number per ventral root.

chondria (Fig. 8C), while the white muscle fibers contained relatively few mitochondria (Fig. 8B). Therefore, in order to reveal the temporal sequence of the differentiation of the red muscle fibers, we applied a fluorescent mitochondrial marker DASPEI to the trunk muscle to label the mitochondria-rich red muscle fibers in the larvae and juveniles.

We found no DASPEI-labeled mitochondria-rich muscle fibers in day 5 larvae (n=5) (Fig. 9A). This result is consistent with the TEM observation which showed that no red muscle fibers were differentiated in day 5 larvae yet. In all the fish examined, DASPEI-fluorescence was also observed in the spinal cord (Figs. 9A–C). The fluorescence in the cord was probably due to numerous mitochondria contained in the nerve cells in the spinal cord (Figs. 9A–C).

It was not until day 10 larvae that the DASPEI-labeled muscle fibers were detected. In day 10 larvae, DASPEI-labeled muscle fibers were first observed to be scattered beneath the skin of the trunk around the horizontal septum at the level of anus (n=6) (Fig. 9B). Thus, functional differentiation of the aerobic red muscle fibers seem to occur around day 10. This result suggests that the hovering behavior of the larvae, which lasts for considerable long period during daytime and needs continuous movement of the trunk, is controlled by the white muscles until at least day 10. In all larvae on day 12 (n=4), DASPEI-labeled superficial red muscle layer was obvious (Fig. 9C). In the larvae at day 30, the red muscle fibers were massively labeled as a superficial layer around the horizontal septum (n=3). No fluorescence was apparent in the trunk region of the day 12 larvae without DASPEI treatment (n=3), indicating that the fluorescence observed in the spinal cord and the trunk muscles in the present experiments was not autofluorescence but DASPEI fluorescence.

DISCUSSION

Yoshida *et al.* (1999) have suggested that there are about 15 primary-type motoneurons in each spinal hemisegment in angelfish larvae. In the present study, we demonstrated that there are 15 primary motoneurons and they can be subdivided into two subclasses based on the timing of the differentiation. One class consists of two motoneurons that differentiate before hatching are juxtaposed each other and have a similar location in different segments, and hence we call them paired motoneurons. The other consists of 13 motoneurons which differentiate around hatching and have similar morphology to the paired motoneurons. In zebrafish embryos, 3 or 4 primary motoneurons have been identified in each spinal hemisegment (Eisen *et al.*, 1986; Eisen *et al.*, 1990). These primary motoneurons are uniquely identifiable as individuals by their stereotyped soma positions and peripheral branching patterns even in the adult (Westerfield *et al.*, 1986). The paired motoneurons identified in the embryonic and early larval angelfish in the present study might be functionally equivalent to the zebrafish primary motoneurons. In the zebrafish, it has been shown that one of the primary motoneurons first leave the spinal cord and act as a “pioneer” in establishing the VR

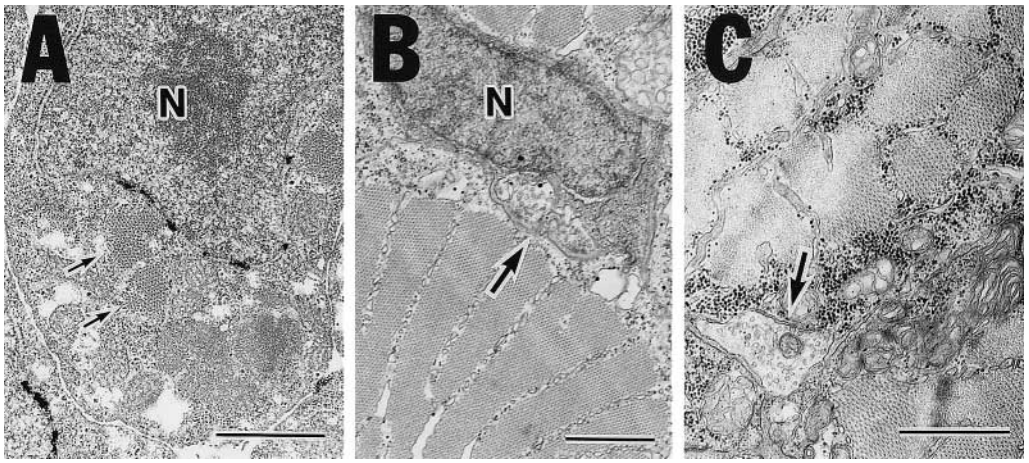

Fig. 8. Transmission electron micrographs of the anal-level transverse section of the trunk muscles. (**A**) Presumptive white muscle cells in the embryo 50 hr after fertilization. Arrows indicate the myofibrils in the myocyte. N, nucleus. (**B**) The deep white muscle fibers in the day 5 larva. Cytoplasm of the myocyte is filled with myofibrils. Nucleus (N) is located near the surface of the cell. Arrow indicates the nerve terminal. (**C**) The superficial red muscle fibers in the day 20 larva. They contain large number of mitochondria and are abundant in glycogen (dark dots). Arrow indicates the nerve terminal. Scale bar=1 μm.

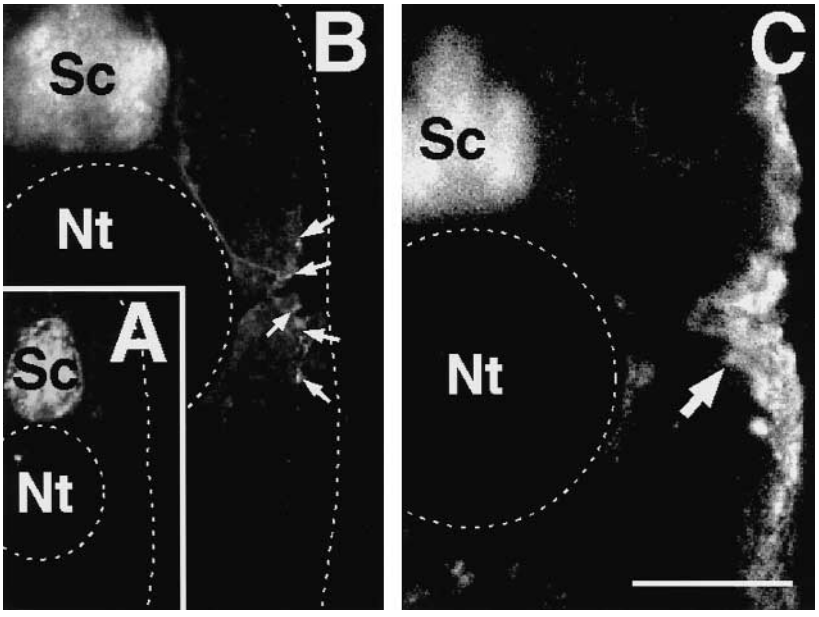

Fig. 9. Confocal laser scanning images of the DASPEI-stained trunk muscles at the anal level in day 5 (**A**), 10 (**B**) and 12 (**C**) larvae. (**A**) In the day 5 larva, there is no DASPEI-labeled muscle fibers in the trunk. (**B**) In the day 10, small number of muscle fibers (arrows) beneath the skin of the trunk around the horizontal septum are labeled with DASPEI. (**C**) In the day 12, DASPEI-labeled superficial red muscle layer is obvious (arrow). Dotted lines indicate the trunk surfaces and notochords. Sc, spinal cord; Nt, notochord. Scale bar=200 μm.

(Eisen *et al.*, 1986). We do not know at this point whether one or both of the paired motoneurons in the embryonic angelfish can act as pioneer.

In contrast to the primary motoneurons in the zebrafish, the paired motoneurons are difficult to identify individually in the older larvae only by their soma positions. It might be possible to identify the paired motoneurons in the older larvae or adults by examining their branching patterns in the trunk muscle. The paired motoneurons tended to be retrogradely labeled with HRP more intensely than the other primary motoneurons. The paired motoneuron axons may have higher affinity to HRP than the others, and hence they are filled with larger amount of HRP. Otherwise, the paired motoneurons may project to the trunk muscle more extensively from early ontogenic period than the other cells.

Angelfish larvae offer several advantages in studying neuronal development underlying the development of swimming behavior. Their translucent body is suitable for whole-mount observation of histological preparation and their accessibility for electrophysiological recording of neuronal activity facilitates investigation of physiological basis of the behavioral development. Yoshida *et al.* (1996) have reported

that the wriggling movement of early posthatching larvae are controlled by rhythmic motor activity consisting of brief bursts of motoneurons, and each burst consists of only a few spikes. The paired motoneurons might be the neurons controlling the twitching or wriggling movements observed in prehatching embryos and larvae just after hatching. Thirteen other primary motoneurons might be recruited to exert high frequency movement which is another pattern of motor activity in young larvae and consists of relatively longer motoneuron bursts with multiple spikes (Yoshida *et al*., 1996). The number of motoneuron impulses and the duration of the burst in each cycle during the spontaneous rhythmic VR activity increase during larval development (Yoshida *et al*., 1996). In the present study, we found that the number of axons running in single VR peaks at day 3, and the number is over 200. However, since the maximum number of the spinal motoneurons per hemisegment backfilled from the muscle was about 30 (Yoshida *et al*., 1999), it is unlikely that all the axons in the VR contribute to the innervation of the axial muscles. The number of motor axons tends to decrease after day 3, suggesting that there are competitions among the motoneurons for their target muscles. In the zebrafish, it has been shown that the stereotyped pattern of innervation by the primary motoneurons is due to initial selection of the appropriate pathway, rather than elimination of incorrect projections (Eisen *et al*., 1986). However, we suggest that, at least among the population of secondary motoneurons, motoneuron death during the first several days after hatching may contribute to the maturation of the neuromuscular system in the angelfish.

In the adult goldfish, *Carassius auratus*, the VRs always contained a few (8–12) relatively large axons, which are probably of the primary motoneurons, and such large axons in the most VRs were grouped together (Fetcho, 1986). Similarly, in the European eel, *Anguilla anguilla*, a few particularly large axons have been observed in the VRs, and a few distinctive large motoneurons are located similarly in different segments (Smit *et al*., 1991). Situations are also similar in the angelfish. Several axons with relatively large diameter were found to be grouped in the adult VRs (see Fig. 6D). The number of the large axons is similar to that of the primary motoneurons. The large size of the primary motoneuron axon may reflect the large size of their field of innervation compared with that of secondary motoneurons (Westerfield *et al*., 1986).

The present results show that preganglionic sympathetic neurons are clustered bilaterally to form distinct longitudinal cell column just dorsolateral to the central canal in the rostral spinal cord of the adult angelfish. No preganglionic sympathetic neuron was found in the mid (anal level) and caudal (segment 19–21) spinal cord. It has been shown in the filefish, *Stephanolepis cirrhifer* that such cell columns, the central autonomic nucleus (CAN), consisting of preganglionic sympathetic neurons extend from the level of the posterior rootlet of the first spinal nerve to the level of the third nerve in the spinal cord (Funakoshi *et al*., 1995). In the angelfish, the present results suggest that CAN is also situated in the similar position of the spinal cord to that in filefish. CAN neurons in the angelfish seem to project their axons to only a few rostral spinal nerves. Therefore, all the ChAT-IR cells in the anal level spinal cord of the angelfish are probably somatic motoneurons. In addition, since teleost axial musculature dose not contain muscle spindles, all motoneurons labeled in the present experiment can be said to be of α-type (Bone, 1978; Yoshida *et al*., 1999). Another evidence which supports our view that the number of VR axons correspond to the motoneuron number is that the number of ChAT-IR cells is very similar to that of VR axons. In the European eel, *Anguilla anguilla*, no unmyelinated axons in the VRs of the caudal cord have been observed, and all of them are considered to be motor axons (Smit *et al*., 1991).

In prehatching embryo (10 hr before hatching), presumptive white muscle fibers containing immature myofibrils are already present in the trunk. EM observation of the nerve terminals in the muscle indicates that white fibers are functional in early day 1 larvae. The nerve terminals are probably of the paired motoneurons. Occasional twitching movement of the embryo in the egg capsule is apparently produced by the contraction of the immature muscle fibers innervated by the paired motoneurons. The differentiation of the axial-muscle motoneurons appeared to complete before the actual start of free swimming, which occurs at late day 5 or day 6 (Yoshida *et al*., 1996). Furthermore, the present study revealed that the differentiation of the red muscle fibers occur after the start of swimming.

In the present study, to label the red muscle fibers in the trunk muscle tissue, we used an *in situ* mitochondrial marker DASPEI. Since the red muscle fibers are rich in mitochondrion for the high oxidative metabolic capacity, we can detect the functional red muscle fibers and follow their developmental sequence. Labeling with DASPEI showed that the superficial red muscle fibers differentiate in day 10-12, although the precursors of the red muscle fibers might emerge in slightly earlier stage. Immunohistochemical examination of the trunk muscle using anti-myosin antibody or histochemical analysis of ATPase activity (van Raamsdonk *et al*., 1980; van Raamsdonk *et al*., 1982; Mascarello *et al*., 1995; Ramírez-Zarzosa *et al*., 1995; Devoto *et al*., 1996; Stoiber *et al*., 1998) is needed to elucidate precisely the timing of differentiation of the multiple muscle fiber types. We propose, however, that staining musculature with DASPEI followed by observation with CLSM is a convenient method to estimate the muscle fiber types and the differentiation of red muscle fibers.

In the zebrafish, it has been shown that the adult red fibers appears at 2–3 weeks after fertilization and their increase in number contributes to the improvement of the slow steady swimming pattern (van Raamsdonk *et al*., 1982). In the angelfish, on the other hand, soon after detaching the substrate and transition to the free swimming life, the larvae show continuous hovering behavior using the pectoral and tail fins. Since aerobic red muscle fibers are not evident until day 10, the anaerobic white muscle fibers in the larvae may have different metabolic capabilities from that in the adult.

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