Skeletal Muscle Transformation into Electric Organ in S. macrurus Depends on Innervation

Graciela A. Unguez,1 Harold H. Zakon2

1 Department of Biology, New Mexico State University, Foster Hall, Las Cruces, New Mexico 88003
2 Section of Neurobiology School of Biological Sciences, University of Texas, Austin, Texas 78712

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ABSTRACT: The cells of the electric organ, called electrocytes, of the weakly electric fish Sternopygus macrurus derive from the fusion of mature fast muscle fibers that subsequently disassemble and downregulate their sarcomeric components. Previously, we showed a reversal of the differentiated state of electrocytes to that of their muscle fiber precursors when neural input is eliminated. The dependence of the mature electrocyte phenotype on neural input led us to test the hypothesis that innervation is also critical during formation of electrocytes. We used immunohistochemical analyses to examine the regeneration of skeletal muscle and electric organ in the presence or absence of innervation. We found that blastema formation is a nerve-dependent process because regeneration was minimal when tail amputation and denervation were performed at the same time. Denervation at the onset of myogenesis resulted in the differentiation of both fast and slow muscle fibers. These were fewer in number, but in a spatial distribution similar to controls. However, in the absence of innervation, fast muscle fibers did not progress beyond the formation of closely apposed clusters, suggesting that innervation is required for their fusion and subsequent transdifferentiation into electrocytes. This study contributes further to our knowledge of the influence of innervation on cell differentiation in the myogenic lineage. © 2002 Wiley Periodicals, Inc. J Neurobiol 53: 391–402, 2002

Keywords: electric organ; regeneration; muscle transdifferentiation; myogenesis; neural influence of electrocyte phenotype

INTRODUCTION

Electric organs (EOs) derive from a variety of skeletal muscles in different lineages including tail, axial, and even oculomotor muscles (Bennett, 1961, 1971; Bass, 1986). Despite their independent origins, EOs share a number of features. For example, in all independently evolved species of electric fish known to date, the excitation–contraction coupling process of muscle is disabled so that EOs can discharge without simultaneous muscle contraction. Further, in most electric fish, maturation of the EO results in the disassembly and/or degradation of the myofilamentous structures in parallel with significant alterations in the morphology of myogenic cells (Bennett, 1971; Fox and Richardson, 1978; Unguez and Zakon 1998a). How the developmental program for striated muscle is altered so strikingly to give rise to a distinct specialized tissue remains unclear.

Our previous studies began to address this question by first identifying the process of muscle transdifferentiation into electrocytes, i.e., the current-producing cells of the EO, during regeneration of the EO in the weakly electric fish Sternopygus macrurus. S. macrurus has the ability to regenerate its spinal cord, skin, connective tissue, vertebrae, muscle, and EO (Patterson and Zakon, 1993, 1997). Further, the process of
EO regeneration appears to recapitulate that of early developmental events (Kirschbaum and Westby, 1975; Kirschbaum, 1977). Based on ultrastructural and immunohistochemical analyses of regenerating EO following tail amputation, we found that mature muscle fibers fuse with one another to give rise to electrocytes (Unguez and Zakon, 1998a). Moreover, the muscle fibers that undergo fusion express fast myosin heavy chain (MHC) (Unguez and Zakon, 1998a). The fusion of differentiated fast muscle fibers accounts for the much larger cross-sectional area of electrocytes (up to 11-fold) compared to that of muscle fibers (Unguez and Zakon, 1998a).

What drives the phenotypic switch from fast muscle fiber phenotype to electrocyte? One hypothesis is that neural input is required for this process. Support for this idea comes from previous data indicating the importance of neural input in the maintenance of the mature electrocyte phenotype (Unguez and Zakon, 1998b). In S. macrurus, mature electrocytes are innervated by electromotoneurons (EMNs). Removal of EMN input to electrocytes by denervation or spinal transection results in a partial reversal of the electrocyte phenotype to an earlier developmental stage of its myogenic lineage. Specifically, immunohistochemical analyses show that 2–5 weeks after removal of neural input, electrocytes reexpress sarcomeric proteins including fast MHC and tropomyosin, and form sarcomere clusters de novo (Unguez and Zakon, 1998b). In contrast, differentiated muscle fiber types appeared unaltered by the absence of neural input. These data clearly demonstrate that electrocytes depend on innervation to maintain their phenotype. Studies in other vertebrates, including birds and mammals, have also shown the importance of neural input in phenotypic plasticity within the myogenic lineage (Pette and Vrbova, 1985; Schiaffino et al., 1998; Efimie et al., 1991; Gunning and Hardeman, 1991).

The maintenance of the mature electrocyte phenotype by neural input led us to test the hypothesis that innervation is also critical for the development of electrocytes from skeletal muscle fibers. We used immunohistochemical analyses to examine the development of skeletal muscle and EO during regeneration in the presence and absence of innervation. Our data showed that innervation is critical for the fusion and subsequent transdifferentiation of fast muscle fibers to give rise to electrocytes in S. macrurus. Differentiation and spatial distribution of distinct muscle fiber types, although fewer in number, appeared unaltered by the absence of neural input. In contrast, the process of electrocytogenesis did not progress beyond the formation of fast muscle fiber clusters after denervation. Fast muscle fibers did not appear to fuse and undergo subsequent phenotypic conversion to give rise to electrocytes in the absence of neural input.

**METHODS**

*S. macrurus* is a fresh-water species of knifefish native to South America and was obtained commercially from various fish importers. Adult fish of both sexes, 20–35 cm in length, were housed individually in 15 to 20-gallon aerated aquaria maintained at 25–28°C and fed three times weekly. For all surgical procedures, fish were anesthetized using 2-phenoxy ethanol (1:1500 in tank water), and then returned to their tanks and monitored until they recovered fully from anesthesia. All surgical wounds were sutured immediately and treated with Woundex® (a topical fungicide/antibiotic). All procedures used in this study followed the American Physiological Society Animal Care Guidelines and were approved by the Animal Use Committees at New Mexico State University and the University of Texas at Austin, TX.
Surgical Manipulations

Elimination of Neural Input. Figure 1 illustrates the surgical procedure performed in this study. An incision was made through the skin on the dorsal surface of the distal third of the tail. A partial laminectomy was performed to expose the spinal cord. To eliminate all motoneuronal influence on the electric organ, we removed the distal-most portions (15–18 segments) of the spinal cord. Extraction of spinal motoneurons results in the degeneration of their distal axons, leaving target electrocytes and muscle fibers denervated (Fig. 1B). In sham-operated fish (n = 5), a partial laminectomy was performed, but the spinal cord was not removed. Care was taken to minimize bleeding and injury to the surrounding tissues. The fish were immediately returned to their tanks and monitored until they were fully recovered from anesthesia.

Because only distal segments were denervated, all fish continued to produce an electric organ discharge (EOD) by using the remaining intact EO (proximal to the site of spinal cord removal). Hence, EOD was not used as a criterion to assess the extent of denervation of electrocytes throughout their survival period. Instead, the absence of spinal cord and neurofilaments innervating the electric organ and muscle fibers was confirmed immunohistochemically (see below) and used to determine the success of denervation. These denervation procedures caused no detrimental effects to the health of the fish. In general, fish maintained normal feeding behavior and motility up to 5 weeks after spinal cord removal.

Tail Transection. The tail was transected distal to the spinal cord removal site, at the caudal end of the anal fin. At this level of the tail, electric organ predominates, but skeletal muscle, connective tissue, spinal cord, and skin are also present (Patterson and Zakon, 1993). Fish had their tails amputated at two different times relative to denervation (Fig. 2). In group 1 (n = 5), tails were amputated immediately after spinal cord removal. In group 2 (n = 10), tails were amputated and allowed to regenerate for 10 days before spinal cord removal. Following a 2-5-week survival period, the regenerating blastema of all experimental and sham-operated fish were removed, mounted on cork, frozen in liquid nitrogen-cooled isopentane, and stored at −80°C for immunohistochemical analyses.

Immunohistochemical Analysis

Serial cryostat cross sections (12-μm thickness) of control and experimental adult tails were cut at −20°C, mounted on glass slides, and air dried at room temperature for immunohistochemical analysis. Tissue sections were rehydrated in phosphate-buffered saline (PBS) for 5 min, incubated in blocking solution (PBS, 2% bovine serum albumin (BSA), and 5% horse serum) for 30 min, and subsequently incubated overnight at 4°C in the appropriate dilution of specific monoclonal antibodies raised against neurofilament-associated protein (3A10), all sarcomeric MHC (MF20), I/Ila MHC (N2.261), and developmental MHC (Dev). Antibodies 3A10, MF20, and N2.261 were purchased from the Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA. Anti-Dev antibody was obtained from Novocastra Laboratories (Vector Laboratories, Burlingame, CA). The specificity of each antibody used in this study has been determined previously for S. macrurus (Unguez and Zakon, 1998a). Table 1 shows the structures stained by each antibody in S. macrurus. Specifically, all neurofilaments were labeled with antibody 3A10. Antibody MF20 labels all muscle fibers, N2.261 recognizes slow (type I) muscle fibers, and Dev recognizes fast (type II) muscle fibers in S. macrurus (Unguez and Zakon, 1998a). Sections incubated without primary antibody were used as controls to visualize nonspecific staining.

Primary antibodies were visualized using a biotinylated secondary antibody (Vectastain ABC kit, Vector Labs), and a horseradish peroxidase (HRP) reaction was run to amplify the signal by use of diaminobenzidine (DAB) and hydrogen peroxide (H₂O₂). A brown precipitate indicated a positive label. Images of immunolabeled tissue sections were captured using a Zeiss Axioskop epifluorescence microscope connected to a Zeiss 35-mm camera. Final images were scanned on an HP Scanjet 5470c (Hewlett Packard), and rendered using Adobe Photoshop (version 6.0; Adobe Systems, Inc.).

Muscle Fiber Number Analysis

Muscle fiber numbers were obtained from cross-sections near the mid-region of the regenerating blastema from sham control (n = 3) and denervated fish tails from group 2 (n = 3) that were immunoreacted with antibodies MF20,
RESULTS

Muscle and Electric Organ Regeneration in Control Adult Fish

Adult *S. macrurus* can fully regenerate their spinal cord, muscle, EO, vertebrae, and skin following tail amputation (Patterson and Zakon, 1993; Unguez and Zakon, 1998a). Figure 3 shows a control tail from adult *S. macrurus* [Fig. 3(A)] in comparison to a regenerated blastema 2 weeks [Fig. 3(B)] and 3 weeks [Fig. 3(C)] after tail amputation. On average, 2-week regenerated blastemas measured 7 mm (n = 5; range, 5–10 mm) and 3-week blastemas measured 15 mm (n = 5; range, 12–21 mm) in length. Immunohistochemical analysis of regenerating blastema showed a spatial and temporal pattern of muscle fiber type differentiation similar to that observed in a previous study (Unguez and Zakon, 1998a). Figure 4 shows serial cross-sections taken from the mid-level of a 2-week regenerating blastema immunolabeled with antibodies MF20 [Fig. 4(A)], N2.261 [Fig. 4(B)], and Dev [Fig. 4(C)]. Slow fibers (N2-261-positive, Dev-negative) were located peripherally and closer to the skin whereas fast fibers (N2-261-positive, Dev-negative) were located more centrally and farther away from the skin.

Denervation of Muscle and Electric Organ after Spinal Cord Removal

In all control groups, 3A10 immunolabeling showed the presence of a spinal cord within the vertebral column [Fig. 5(A)], whereas fish that underwent denervation had no evidence of a spinal cord present [Fig. 5(B)]. 3A10 also labeled bundles of neurofilaments throughout the blastema at all stages of regeneration. Within blastemas, positive label by 3A10 was demonstrated by filamentous structures with a brown coloration, whereas melanocytes have a black coloration and temporal pattern of muscle fiber type differentiation similar to that observed in a previous study (Unguez and Zakon, 1998a). Figure 4 shows serial cross-sections taken from the mid-level of a 2-week regenerating blastema immunolabeled with antibodies MF20 [Fig. 4(A)], N2.261 [Fig. 4(B)], and Dev [Fig. 4(C)]. Slow fibers (N2-261–positive, Dev-negative) were located peripherally and closer to the skin whereas fast fibers (N2-261–positive, Dev-positive) were located more centrally and farther away from the skin.

Table 1 Specificity of Antibodies in *S. macrurus*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Species</th>
<th>Structure Labeled</th>
</tr>
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<tbody>
<tr>
<td>3A10</td>
<td>neurofilament-associated protein</td>
<td>chicken</td>
<td>neurofilaments</td>
</tr>
<tr>
<td>MF20</td>
<td>all sarcomeric myosin heavy chain</td>
<td>chicken</td>
<td>all muscle fibers</td>
</tr>
<tr>
<td>N2.261</td>
<td>I, IIA myosin heavy chain</td>
<td>mouse</td>
<td>slow fibers</td>
</tr>
<tr>
<td>Dev</td>
<td>embryonic/neonatal heavy chain</td>
<td>rat</td>
<td>fast fibers</td>
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"Storey et al. (1992); "Bader et al. (1982); "Hughes and Blau (1992); "Talmadge et al. (1995); "Unguez and Zakon (1998a).
tion [Fig. 5(C)–(D)]. Figure 5(C) shows the presence of neurofilaments in a 2-week regenerated blastema. In contrast, absence of 3A10 labeling in fish that had their spinal cord removed, confirmed the success of the spinal cord removal at each of the postsurgery time periods analyzed [Fig. 5(D)]. Further, based on 3A10 labeling, no neurofilaments were observed in blastema that regenerated without innervation [Fig. 5(D)].

**Growth of Regenerating Blastemas Is Arrested after Removal of Neural Input**

Regeneration of blastemas following concurrent tail amputation and spinal cord removal surgeries was significantly different from that observed in control groups (Fig. 6, compared to Fig. 3). In two fish, no blastema regenerated. The amputated site was covered with epidermis without a blastema forming inside it throughout the entire 2-week survival period. In the remaining three fish, initial generation of a blastema was observed during the first 5 days postsurgery. These blastemas were generally no longer than 1–2 mm, and no further regeneration was seen even after 5 weeks postamputation. Figure 6 illustrates a regenerated blastema representative of the latter three fish that had their tails amputated and their spinal cords removed at the same time. Note that the blastemal growth is predominant on the dorsal surface of the distal stump.

**Distinct Muscle Fiber Phenotypes in the Absence of Neural Input**

The aim of this study was to test the role of neural input on the development of muscle fibers and electrocytes. However, we found a relative absence of blastemal growth when amputation and denervation were performed at the same time (see above). Thus, we also performed denervations at a regeneration stage when myogenesis had begun. In *S. macrurus*, the first immunohistochemical indication of myogenesis during regeneration occurs between days 6 and 8 after tail amputation (Patterson and Zakon, 1997). By day 10, the first muscle fibers begin to fuse to give rise to electrocytes (Patterson and Zakon, 1997). Thus, in a separate group of fish, a 5–6 cm segment of the spinal cord proximal to the regenerating blastema was removed 10 days after tail amputation. On average, 10-day regenerated blastema were 3 mm long (range: 3–3.5 mm; n = 5). Immunohistochemical analyses were carried out 2 (n = 5) and 5 (n = 5) weeks postdenervation surgery.

The effect of denervation on the proportion of muscle fibers during regeneration was determined based on fiber counts obtained from cross-sections near the mid-region of the blastema from sham control (n = 3) and denervated (n = 3) fish tails that were immunoreacted with antibodies MF20, N2.261, and Dev. Because little blastemal growth occurred when spinal cord removal and tail transection were performed at the same time, we estimated the muscle fiber distribution from denervated 10-day regenerated blastemas. The number of muscle fibers in sham con-
trol tails (329 ± 38) was significantly greater than that in denervated 10-day regenerated blastemas (136 ± 67). The mean numbers of fibers of a particular type in sham control tails were 285 (±21) and 44 (±17), for slow and fast fibers, respectively. Denervated 10-day regenerated blastema had means of 96 (±45) and 50 (±8) for slow and fast fiber types, respectively.

Figure 7 shows the presence of slow [Fig. 7(B)] and fast [Fig. 7(C)] fibers 2 weeks after denervating a 10-day blastema. Although fewer muscle fibers were present in denervated tails, the spatial distribution of slow and fast muscle fibers was similar to that found in control, regenerated tails (Fig. 4). Figure 7(B) shows a partial cross-section stained with N2.261, and this shows positively labeled fibers close to the epidermis (Ep in the figure). A cross-section serial to that in Figure 7(B) was stained with antibody Dev, and this labeled fibers that were more centrally located than slow fibers, i.e., farther from the epidermis [Fig. 7(C)]. However, Dev staining did not reveal the pres-
ence of electrocytes. In contrast, control regenerated tails contain developing electrocytes at a similar stage of regeneration (Fig. 7A). Figure 7(A) shows a larger portion of a cross-section from a control regenerated tail that was stained with MF20 and shows all muscle fibers and developing electrocytes.

Electric Organ Formation from Muscle Transdifferentiation Does Not Occur after Denervation

In *S. macrurus*, the first immunohistochemical indication of electrocyte formation from muscle fiber fusion is observed on the tenth day of regeneration (Patterson and Zakon, 1997). When denervation was performed 10 days after tail amputation, we observed the beginning stages of electrocyte formation from muscle fiber fusion (Fig. 8). Figure 8 shows partial cross-sections of 10-day regenerated blastemas that were denervated and immunolabeled with Dev antibody 2 weeks [Fig. 8(A)] and 5 weeks [Fig. 8(B)] after spinal cord removal (Fig. 2, group 2). As shown in Figure 8(A), some fast muscle fibers were present in closely apposed clusters 2 weeks after denervation of 10-day regenerated blastemas. The absence of further electrocyte formation persisted even after a 5-week survival period [Fig. 8(B)]. Thus, even after 5 weeks of regeneration, the most advanced stage of electrocyte differentiation observed without neural input was the beginning stages of fast muscle fiber fusion. There was no evidence that subsequent stages of muscle transdifferentiation took place. Specifically, the fusion of fast muscle fibers followed by the disassembly of sarcomeres and absence of MHC expression that characterizes the complete transdifferentiation of fast fibers into electrocytes was not observed in the absence of neural input by spinal cord removal. Although not shown in Figure 8, slow muscle fibers were present in these blastemas. These slow fibers were not found in clusters.

**DISCUSSION**

Our results demonstrate that innervation plays a key role in the regeneration and cell differentiation processes in adult *S. macrurus*. Specifically, this work indicates that the regenerative response of the blastema to denervation varies between different stages of regeneration. Further, once some regeneration has occurred, the transdifferentiation of skeletal muscle to electric organ is stunted in the absence of innervation, i.e., the fusion of fast fibers followed by the disassembly of sarcomeres and downregulation of sarcomeric proteins does not occur. Together with previous studies, we conclude that the electrocyte phenotype in *S. macrurus* shows considerable dependence in its differentia-
Innervation Is Necessary for Blastema Formation in *S. macrurus*

The regenerative process after tail amputation in adult *S. macrurus* has been previously described (Patterson and Zakon, 1993, 1997). When the tip of the tail is amputated, a stage of active cell proliferation follows and wound closure occurs within 24 hours. The high proliferation stage of limb regeneration is consistent with the formation of a cone-shaped blastema consisting of undifferentiated ependymal and mesenchymal progenitor cells. Cell proliferation continues throughout the regeneration process, allowing the blastema to grow in length. During the second week of regeneration, blastemal cells begin to differentiate into peripheral skeletal muscle and a centrally located EO (Patterson and Zakon, 1997; Unguez and Zakon, 1998a).

This study demonstrated that the response of the blastema to denervation varies between different stages of regeneration. Specifically, concurrent tail amputation and denervation resulted in the formation of a wound epidermis at the amputation site. However, little to no growth of a blastema was observed. The requirement of neural input for the initiation and early progression of regeneration is consistent with observations in urodele amphibians (Schotte and Butler, 1944; Singer and Craven, 1948; Singer, 1952; Brockes, 1987) and other te-}

leost fish (Geraudie and Singer, 1979, 1985). Adequate innervation by peripheral nerves has been shown to be an essential requirement for the high cell-proliferation stage that immediately follows limb amputation (Globus et al., 1983; Brockes, 1984; Olsen and Tassava, 1984). We speculate that, similar to other systems, early denervation suppresses mitosis in all tissue precursor cells (reviewed in Mescher and Tassava, 1975; Mescher, 1984; Nüesch, 1985; Wang et al. 2000). Bromodeoxyuridine labeling studies have shown that tail amputation in *S. macrurus* results in the activation of cell proliferation in the distal stump regions (Patterson and Zakon, 1993). Thus, it is likely that putative neuronal factors similar to those found in amphibians are also present in *S. macrurus* and these factors support cell proliferation during the early stages of regeneration.

In contrast to what we found when complete denervation was performed at the same time as spinal cord removal, denervation after 10 days of blastemal regeneration resulted in additional blastemal growth, although the blastema was smaller (average length = 3 mm) compared to that in controls (average length = 7 mm). These data are in agreement with reports from amphibian studies showing that denervation of blastemas at later stages of regeneration results in correctly patterned, but abnormally small, regenerates (Schotte and Butler, 1944). Our results underscore the possible conservation of cellular and molecular mechanisms of nerve dependency that underlie the different stages of regeneration of lost body parts in urodele
amphibians. Future studies in *S. macrurus* could significantly contribute to our knowledge of the relationship between regeneration of different tissue types and their neural control.

**Distinct Fiber Types Are Formed in the Absence of Innervation**

In *S. macrurus*, regeneration following tail amputation proceeds by a mechanism that gives rise to a pattern of structurally and functionally distinct fiber type distributions (Unguez and Zakon, 1998a). We have previously shown that cells located more peripherally continue to mature into muscle cells, while those located more centrally (farthest from the skin) show a number of changes as they convert from muscle fibers to electrocytes. Differentiation of muscle fiber types takes place with the emergence of slow fibers (more peripherally) preceding the differentiation of fast fibers (more centrally) (Unguez and Zakon, 1998a) — a process similar to that observed in most other teleosts (Devoto et al., 1996; Johnston, 2001). The differentiation of the two fiber types and their temporal and spatial patterning was not affected when removal of the spinal cord was performed 10 days after tail amputation, i.e., after the onset of cell proliferation, even after a 5-week survival. This spatial distribution of slow and fast muscle fibers was observed despite the lower than normal number of muscle fibers present in denervated blastemas.

Myogenesis in *S. macrurus* normally begins between days 6 or 8 after tail amputation (Patterson and Zakon, 1997). Because blastemas were denervated on day 10, it is likely that some regenerating axons were present during the early stages of muscle fiber differentiation. Thus, we cannot rigorously exclude the possibility that innervation influenced initial fiber type differentiation in the blastema. Our data indicate that fiber number was affected by changes in neural input during regeneration. However, our results show that the emergence of slow and fast fiber phenotypes, and their maintenance up to 5 weeks of regeneration, is not dependent on the continuous presence of neural input. These data concur with results from studies on mammals and birds demonstrating that the formation and survival of some primary and secondary muscle fibers require innervation (reviewed in Miller and Stockdale, 1987; Condon et al., 1990; Fredette and Landmesser, 1991).

We have also shown that muscle fiber type distribution, morphology, and MHC expression is unaltered when the mature adult tail is denervated (Unguez and Zakon, 1998b). The absence of phenotypic changes in skeletal muscle was observed up to 5 weeks without neural input. In light of these data, we conclude that, at least within the resolution of our studies, innervation affects muscle fiber number, but it does not play a major role in the differentiation of muscle fibers during regeneration, or in the maintenance of the muscle fiber phenotype in *S. macrurus*.

**Absence of Muscle Fiber Fusion and Transdifferentiation into Electrocytes under Aneural Conditions**

The formation of the electric organ in *S. macrurus* is a well-regulated process through which cells acquire specialized characteristics (Unguez and Zakon, 1998a). In particular, cell recognition among fast muscle fibers must occur before the formation of fiber clusters, followed by membrane breakdown to give rise to the much larger electrocyte cells. Under aneural conditions, 10-day blastemas showed the differentiation and persistence of fast fiber phenotypes, and the recognition among some of these fibers to form an apparent fiber cluster (Fig. 8). However, in the absence of ultrastructural analysis, we cannot conclude whether or not membrane breakdown was initiated along the closely apposed fibers within each cluster.

Electrocyte formation is strikingly similar to the formation of muscle fibers from the fusion of myoblasts during myogenesis (see Knudsen, 1992). Identification of molecules that inhibit myoblast fusion in vertebrate tissue culture systems has provided some biochemical insight into the fusion process. Essential components include, for example, cell adhesion molecules, calcium and calmodulin, phospholipases, lipids, and others (Wakelam, 1985; Knudsen, 1992). Studies in humans have also indicated that myoblasts must express particular types of ion channels for fusion to occur (Bijlenga et al., 2000; Fischer-Loughheed et al., 2001). The present data provide an excellent basis for experiments that identify and investigate the function of analogous factors involved in the fusion of mature fast muscle fibers in this system. Further, elucidating the extent to which fusion of fibers to form an electrocyte is determined by the number of fast muscle fibers present is warranted.

The close association that exists between nerves and muscle precursors throughout development has been a focus of investigations on the neural regulation of early events during myogenesis in both vertebrate and invertebrate species. In general, these studies have demonstrated differences in the level of neural control of myogenesis at different stages of development. For example, in mammals and birds, primary muscle fibers appear to largely diversify in the absence of nerves (Crow and Stockdale, 1986; Condon
et al., 1990; Fredette and Landmesser, 1991), whereas formation of secondary muscle fibers is more nerve dependent (reviewed in McLennan, 1994). Similar findings have been reported in studies of neural control on myogenesis in insects. During embryogenesis of *Drosophila melanogaster*, myoblasts fuse normally to form syncytiotubules in the absence of motor nerves, suggesting a nerve-independent process of myogenesis (Broadie and Bate, 1993). However, the formation of muscle fibers during metamorphosis of the hawkmoth *Manduca sexta* appears to require the nerve (Consoulas and Levine, 1997). In the latter study, myoblasts remained as dispersed cells or as unfused aggregates in the absence of innervation. Comparable to these studies are our present observations of aggregates of unfused fast muscle fibers in denervated blastemas. It is feasible that some mechanisms that underlie the differences in the nerve dependence of myogenesis at different stages of development in other species may also be conserved during regeneration in *S. macrurus*.

**Comparison with Other Studies**

In *S. macrurus*, EMNs constantly drive the EO at frequencies between 50–200 Hz, depending on the sex of the fish (Mills et al., 1992). On the other hand, somatomotoneurons in teleost fish activate skeletal muscle fibers intermittently and at frequencies lower than 8 Hz (Rome et al., 1992, 1996). In a study by Patterson and Zakon (1997), removal of electrical activity by high spinal transections did not prevent the formation of electrocytes, at least during the first 2 weeks of regeneration. In contrast, we observed that muscle fibers failed to transform into electrocytes in the absence of synaptic innervation. The occurrence of electrocyte formation during tail regeneration in the absence of electrical activity may be due to a combination of (1) an incomplete (over a 2-week period) loss by EMNs to induce the electrocyte phenotype, and/or (2) an incomplete loss (over a 2-week period) of fast muscle fibers to respond to neural cues by EMNs that induce the electrocyte phenotype.

This would explain why electrocytes develop when they are innervated by EMNs that are rendered electrically silent (Patterson and Zakon, 1997), yet fail to develop when innervation by EMNs is completely removed (present study). If this hypothesis is correct, we predict that in spinal-transected fish, newly formed electrocytes will revert back to a muscle phenotype after regeneration periods longer than 2 weeks. Consistent with this hypothesis are our previous observations on the effects of denervation and spinal transection on the mature electrocyte phenotype (Unguez and Zakon, 1998b). Specifically, 60% of mature electrocytes reexpress fast MHC after a 2-week denervation period. By comparison, only 30% of mature electrocytes reexpress fast MHC 2 weeks after spinal transection. Interestingly, all electrocytes in both experimental groups reexpress fast MHC after a 5-week survival period (Unguez and Zakon, 1998). The lower incidence of electrocytes found to reexpress fast MHC in 2-week, but not 5-week spinal-transected fish could be due to a gradual loss of the capacity by EMNs to maintain the electrocyte phenotype. Alternatively, these data could also be explained by a gradual loss by mature electrocytes to respond to an EMN-derived signal. Both scenarios would be consistent with the hypothesis proposed above.

It is noted that in the study by Unguez and Zakon (1998b), denervation of electrocytes was performed by removing the distal-most segments of the spinal cord as done in the present study, leading to the removal of all synaptic contact with electrocytes. In contrast, spinal cord transections were performed at the level of the pectoral fins rendering all electrocytes electrical inactive without disrupting their neural synaptic contacts, as was done in the study by Patterson and Zakon (1997). Thus, elimination of electrical activity in the adult induces a partial dedifferentiation of electrocytes into a fast muscle fiber-like phenotype (Unguez and Zakon, 1998b), but it does not entirely prevent the formation of electrocytes during regeneration (Patterson and Zakon, 1997). It will be interesting to determine the extent to which specific patterns of electrical activity influence the formation of electrocytes, and whether the site of action of this activity pattern is in the motoneuron, the target cells (i.e., muscle fibers or electrocytes), or both.

Differences in the robustness of the effects obtained in spinal transections and denervation studies in the formation of electrocytes during regeneration (Patterson and Zakon, 1997, present study) and in the temporal dedifferentiation of electrocytes in the adult (Unguez and Zakon, 1998b) indicate that influence exerted by nonactivity neuronal trophic factors are also likely to play a role. To date, we are not aware of any nonactivity dependent neuronal factors that affect muscle cell fusion and the contractile protein system of skeletal muscle fibers. It is critical for future experiments to determine the attributes of the regulation by EMNs on electrocyte phenotype formation and maintenance.

Electric organs have evolved multiple times in fish. Do electrocytes from each independently evolved group depend on innervation, as electrocytes from *S. macrurus* appear to be? A study on a mormyrid weakly electric fish (*Pollimyurus isidori*, Szabo and
Kirschbaum, 1983) found that EO development was not impeded by the removal of axonal inputs during early development. This led the authors to conclude that the presence of neuronal input was not required for EO differentiation. It is noted that the criteria used to assess the extent of denervation based on absence of nerve fibers in the developing EO in this study were not rigorously defined. Replication of this study incorporating a more accurate confirmation of denervation, such as the use of nerve-specific antibody as done in the present study is warranted. Nevertheless, it is possible that in mormyrids, the differentiation and maintenance of electrocyte phenotype is independent of innervation making these electrocytes more similar to muscle fibers.

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