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# DEVELOPMENT AND REGENERATION OF THE ELECTRIC ORGAN

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## Summary

**The electric organ has evolved independently from muscle in at least six lineages of fish. How does a differentiated muscle cell change its fate to become an electrocyte? Is the process by which this occurs similar in different lineages? We have begun to answer these questions by studying the formation and maintenance of electrocytes in the genus *Sternopygus*, a weakly electric teleost. Electrocytes arise from the fusion of fully differentiated muscle fibers, mainly those expressing fast isoforms of myosin. Electrocytes briefly co-express sarcomeric proteins, such as myosin and tropomyosin, and keratin, a protein not found in mature muscle. The sarcomeric proteins are subsequently down-regulated, but keratin expression persists. We investigated whether the maintenance of the electrocyte phenotype depends on innervation. We found that, after spinal cord transection, which silences the electromotor neurons that innervate the electrocytes, or destruction of the spinal cord, which denervates the electrocytes, mature electrocytes re-express sarcomeric myosin and tropomyosin, although keratin expression persists. Ultrastructural examination of denervated electrocytes revealed nascent sarcomeres. Thus, the maintenance of the electrocyte phenotype depends on neural activity.**

Key words: electric organ, muscle, regeneration, electric fish, *Torpedo*, *Sternopygus*, myosin, sarcomere, motor neuron, denervation.

## Introduction

Nineteenth century biologists were familiar with strongly electric organs in taxonomically disparate species such as *Torpedo* and *Electrophorus*. They were also aware that electric organs are embryologically derived from striated muscle. Darwin (1859) commented on the significance of these observations in *The Origin of Species*. He noted that electric organs pose a potential problem for the theory of evolution by natural selection since it is difficult to account for the adaptive value of a muscle in a transitional state no longer serving a contractile function, but perhaps not yet being a fully functional electric organ. In addition, it raised the question as to whether the electric organ evolved from muscle by similar or different processes in each lineage. The questions raised by Darwin are still with us. A fruitful approach to understanding how systems evolve is understanding how they develop. Thus, we may paraphrase Darwin and ask: how do electric organs develop from muscle and how have they developed in each lineage?

With the discovery of weakly electric fish in the 1950s, it is now known that electric organs have evolved at least six times. Electric organs derive from different muscles in different lineages including tail muscles, axial muscles and even oculomotor muscles (Bass, 1986; Bennett, 1961, 1971). Despite their independent origins, electric organs share a number of features, although the developmental pathways to these common ends may differ. For example, in all lineages, the excitation–contraction coupling process of muscle is disabled so that electric organs can discharge without concomitant muscle contraction. Yet, there are likely to be different developmental ‘solutions’ to this problem because sarcomeres are present in the electric organs of some groups but not in those of others. A main goal, then, in a developmental analysis of the electric organ is to determine how the developmental program for striated muscle has been altered in each species.

The evolutionary development of the electric organ from muscle is only part of the process of evolution of the electromotor system. In all groups, the electrocytes, the cells of the electric organ, are innervated by specialized electromotor neurons, and these are in turn controlled by pacemaker neurons located in the brainstem. The electromotor neurons derive from spinal or cranial motor neurons, depending on the taxon. The origin of pacemaker neurons is less obvious. However, since most motor output pathways possess premotor pattern-generating circuitry, pacemaker neurons probably arise from some pre-existing circuit of this type. It is intriguing to consider how all three cell types (pacemaker neurons, electromotor neurons, electrocytes) became distinct from their cells of origin and evolved into a functional electromotor system.

We have begun to address these questions by identifying the factors that control the transformation of muscle cells into electrocytes during the regeneration of the electric organ in the gymnotiform genus *Sternopygus*. We use this model system because (1) the events underlying the regeneration of the electric organ appear to recapitulate early developmental events (Keynes, 1961; Kirschbaum, 1977; Kirschbaum and Westby, 1975) and (2) the genus *Sternopygus* is thought to be the most primitive gymnotiform and is therefore likely to have the least derived electric organ (Alves-Gomes et al., 1995; Maggo-Leccia, 1978).

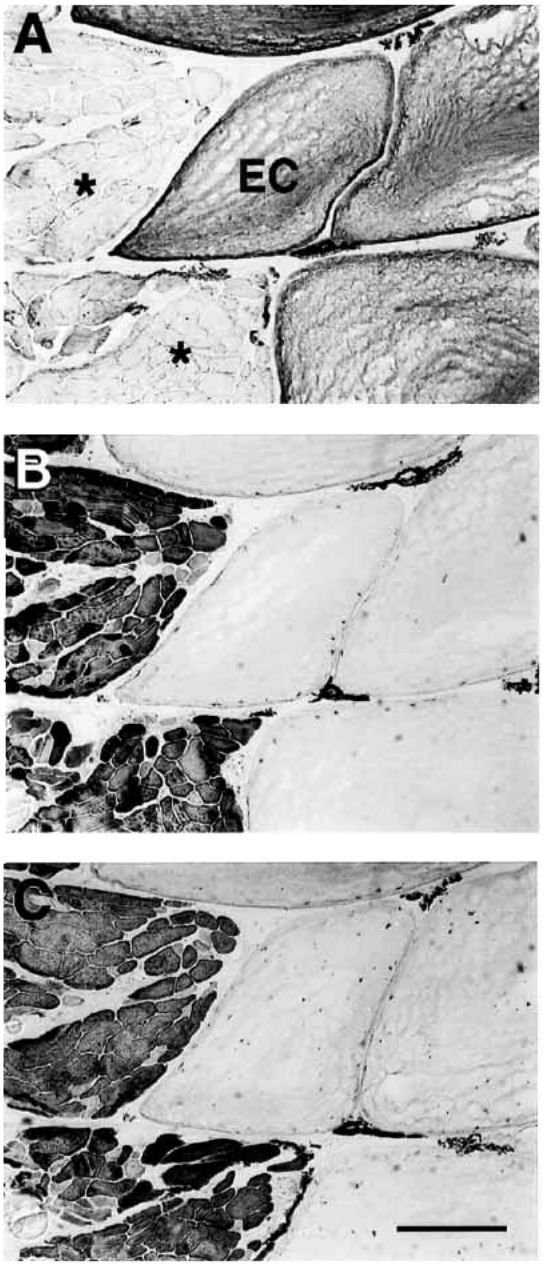


Fig. 1. Immunocytochemical profile of electrocytes (EC) and muscles (asterisks) in serial cross-sections. (A) Electrocytes, but not muscle fibers, label with an antibody against acidic keratin (AE1). Muscles, but not electrocytes, label with antibodies against (B) myosin heavy chain (MF20) and (C) tropomyosin (CH1). Sections cut at 12 μm thickness. Scale bar, 100 μm. Taken from Unguez and Zakon (1998b) with permission.

## Anatomy and immunocytochemical profile of the electric organ of *Sternopygus*

The electric organ of *Sternopygus* consists of large cylindrical electrocytes a few millimeters long and hundreds of micrometers in diameter (Mills et al., 1992). The elctrocytes are multinucleated, as befits a cell derived from muscle, but without sarcomeres, sarcoplasmic reticulum or T-tubules, and their cytoplasm is filled with a filamentous network. The electrocytes are oriented along the long axis of the fish and are innervated on their posterior face at an end-plate region that extends for a few hundred micrometers (Ferrari and Zakon, 1993).

Between the skin and the electric organ are small fascicles of striated muscle fibers. These are typical muscle fibers (Fig. 1), which are hundreds of micrometers long, tens of micrometers in diameter and possess well-organized sarcomeres, sarcoplasmic reticulum, etc. As in striated muscle in other species, the muscle fibers are of four different subtypes as defined by ATPase histochemistry and immunoreactivity to myosin heavy chain (MHC)-isoform-specific antibodies (Unguez and Zakon, 1998a). The fibers are arranged such that the small-diameter ‘slow’ MHC-expressing fibers are on the periphery of the fascicle and the large-diameter ‘fast’ myosin-expressing fibers are centrally located, as in the muscles of other species of fish (Devoto et al., 1996). The ‘fast’ MHC fibers are adjacent to the electrocytes. Not surprisingly, all muscle fiber types label with antibodies against other sarcomeric proteins such as tropomyosin and the muscle-specific intermediate filament protein desmin.

In contrast, the electrocytes do not label with antibodies against myosin or tropomyosin (Fig. 1). They do, however, express desmin throughout their cytoplasm and nicotinic acetylcholine receptors at their innervated end, which emphasizes their myogenic origin. In addition, they express keratin, a protein not observed in fully differentiated striated muscle fibers (Patterson and Zakon, 1996).

On the basis of these expression patterns, we conclude that mature electrocytes express some of the muscle phenotype, suppress other portions of it, but are more than just a partially surpressed muscle because they also express some proteins that muscles do not.

## Regeneration of the electric organ: the contribution of satellite cells

When the tip of the tail is amputated, epidermal cells at the wound margin rapidly proliferate and cover the wound within 24 h. Within a week, a blastema of apparently undifferentiated cells appears as a small cap on the remaining electric organ; it is visible as a swelling at the end of the tail. Over the next week, this swelling extends and, from it, a new tail develops complete

with a centrally located electric organ and peripheral muscles (Patterson and Zakon, 1997; Unguez and Zakon, 1998a).

To identify the source of the cells that contribute to the blastema, injections of bromodeoxyuridine (BrdU), a thymidine analog, were made into fish 24 h after amputation of the tail. When tail stumps were examined 3 h later, small BrdU-labeled cells were observed surrounding the muscles and electrocytes for a few hundred micrometers from the wound site. At the level of the electron microscope, these were identified as satellite cells, the source of regenerating muscle in mammals (Bischoff, 1980; Mauro, 1961). When tails were taken at daily intervals thereafter, BrdU-labeled satellite cells were observed to proliferate and migrate within the electric organ to the wound stump and form the blastema (Patterson and Zakon, 1993). A single injection of BrdU could label up to 25 % of all cells in the blastema. Nuclei within the intact electrocytes or muscle cells were never labeled with BrdU, showing that these cells do not dedifferentiate and participate in the formation of the regeneration blastema, as occurs in the regeneration of the newt limb (Lo et al., 1993). Eventually, BrdU-labeled nuclei were observed in newly developing muscle and electrocytes in the blastema. Thus, satellite cells are the precursors of regenerating muscle and electrocytes. It remains to be determined whether they form a single pluripotent class of cells or whether there are distinct satellite cells for muscle and electrocyte lineages.

### Regeneration of the electric organ: emergence of differentiated muscles and electrocytes

Regeneration proceeds posteriorly from the wound; the regions closest to the intact tail contain the most differentiated cells, whereas the cells are less and less differentiated towards the tip of the tail where the still mitotically active blastema is present. After 2–3 weeks, all the events of electric organ regeneration can be observed by examining the length of the regenerating tail from the wound site to the blastema. Within the blastema, clusters of cells indicate the first formation of muscle because the cells in these clusters express desmin, one of the earliest structural proteins expressed in developing muscle (Debus et al., 1983). At more proximal locations, a ring of desmin/myosin-expressing cells can be seen just below the skin encircling a central core of blastemal cells. More proximal still, the cells in this peripheral ring label with antibodies to other sarcomeric proteins in a ladder-like fashion typical of sarcomeres. When labeled with antibodies against different MHC isoforms, the pattern of peripherally located 'slow' fibers and more centrally located 'fast' fibers typical of differentiated teleost muscle emerges (Devoto et al., 1996; Unguez and Zakon, 1998a).

At this level, larger cells are observed in the central core, and these also label with antibodies to sarcomeric proteins. However, the sarcomeres in these cells are disrupted. Adjacent to these large cells within the core region, and sometimes appearing to push into them, are muscle fibers that label strongly with anti-myosin antibodies (Patterson and Zakon, 1997; Unguez and Zakon, 1998a) (Fig. 2). When examined in the electron microscope, muscle fibers can be observed fusing with one another and with nascent electrocytes (Unguez and Zakon, 1998a). These fusing muscle fibers and the electrocytes with which they are merging predominantly express 'fast' MHC, the isoform expressed in muscle fibers adjacent to the

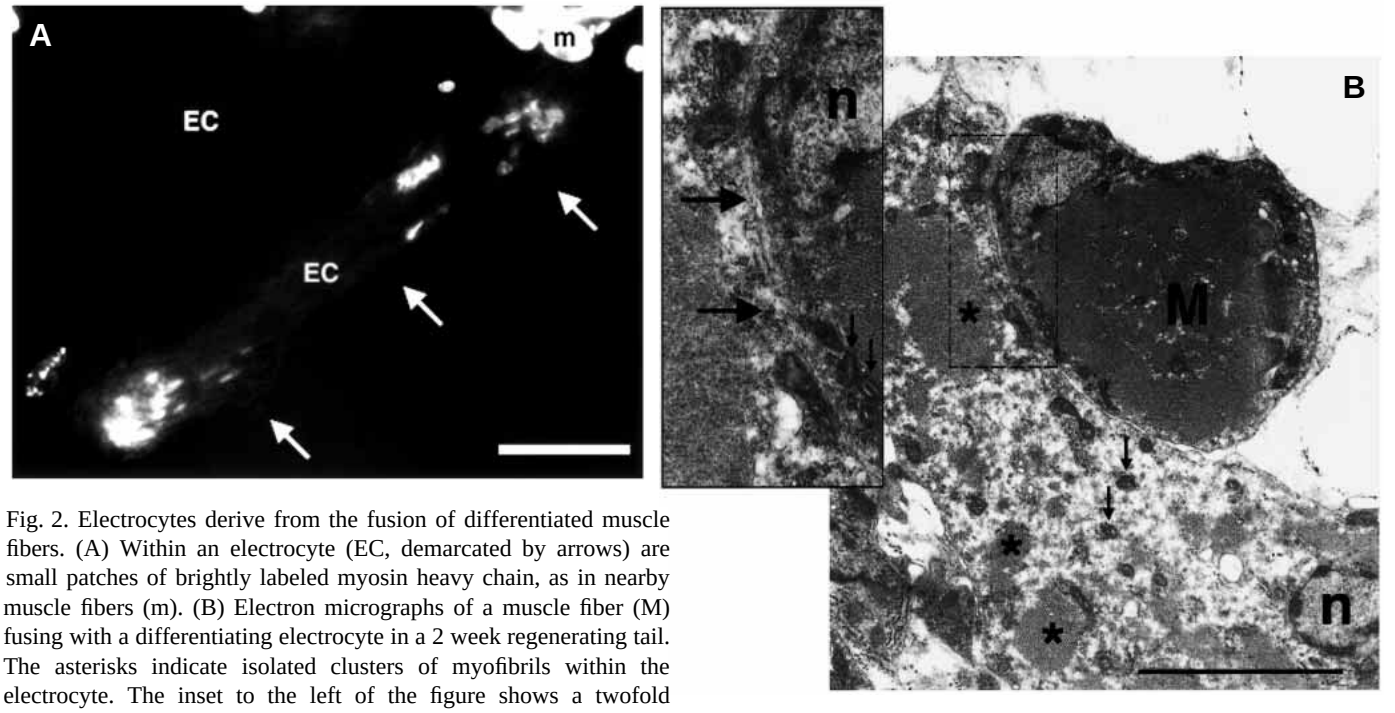


Fig. 2. Electrocytes derive from the fusion of differentiated muscle fibers. (A) Within an electrocyte (EC, demarcated by arrows) are small patches of brightly labeled myosin heavy chain, as in nearby muscle fibers (m). (B) Electron micrographs of a muscle fiber (M) fusing with a differentiating electrocyte in a 2 week regenerating tail. The asterisks indicate isolated clusters of myofibrils within the electrocyte. The inset to the left of the figure shows a twofold magnification of the region in the box. Note the close apposition of the muscle and electrocyte membranes (large arrows). n, nucleus; small arrows indicate mitochondria. Scale bar, 100 μm in A, 2 μm in B. Taken from Unguez and Zakon (1998a) with permission.

electric organ in the mature organ. Interestingly, these newly formed electrocytes also label with an antibody against the $Ca^{2+}$-ATPase specific to the sarcoplasmic reticulum of 'fast' muscle fibers. The myosin and sarcoplasmic-reticulum-protein labeling of nascent electrocytes is patchy and located mainly in the peripheral region of the cells, reinforcing the impression that muscle fibers fuse with the growing electrocyte.

Farther proximally, a clear distinction emerges between the peripheral layer of muscle fibers, now arranged into newly formed fascicles, and the much larger centrally located electrocytes. No small muscle fibers are observed around electrocytes. Near the wound site, electrocytes continue to express myosin throughout their cytoplasm, yet they neither contain organized sarcomeres nor express tropomyosin. These cells are, however, strongly positive for keratin (Patterson and Zakon, 1997). Farther proximally still, electrocytes no longer express myosin.

In sum, electrocytes develop from the fusion of differentiated muscle fibers that express 'fast' myosin. However, it is not clear whether it is solely the fiber identity, i.e. fast myosin, or its spatial location within the tail, i.e. more central, that earmarks these muscle fibers for phenotypic transformation. The fusion of differentiated muscle fibers, which is an unusual process, accounts for the large size of the electrocytes. The signals that instruct fully differentiated muscle fibers to fuse are not known. However, mechanisms similar to those underlying the fusion of myoblasts to one another or to differentiating myotubes early in myogenesis may also be at work in the formation of electrocytes. The developing electrocytes then lose their sarcomeres and pass through a transitional stage during which they co-express myosin and keratin, until myosin expression finally disappears; keratin expression persists, and the cell phenotype is that of a fully differentiated electrocyte.

### The role of innervation in development and maintenance of the electrocyte phenotype

What drives the phenotype switch from muscle to electrocyte? One hypothesis is that innervation plays a role in this process, given that innervation and neural activity patterns are influential in determining muscle phenotype in mammals (Eftimie et al., 1991; Gunning and Hardeman, 1991; Pette and Vrbova, 1985; Schiafinno et al., 1988). This is an attractive possibility since mature electrocytes are innervated by a distinct group of motor neurons from muscles (Unguez and Zakon, 1998a). Using an antibody to a neurofilament-associated protein, axons can be visualized in the blastema before differentiated muscles fibers appear and throughout further differentiation processes (Patterson and Zakon, 1997). However, without a specific marker for somatomotor neurons and electromotor neurons, it is impossible to know the identity of these nerve fibers.

Recent results indicate that innervation of the electrocytes, or the activation of those inputs, is critical for the development and maintenance of the adult electrocyte phenotype. Electrocytes fire constantly at regular frequencies (50–200 Hz depending on the sex of the fish; Zakon, 1996). The frequency at which the electric organ discharges is controlled by input from a medullary pacemaker nucleus and is conveyed to the motor neurons by the axons of neurons called relay cells. When the spinal cord is severed, the axons of the relay cells die (Schaefer and Zakon, 1996). When a spinal transection is performed in *Sternopygus*, the electromotor neurons are intact

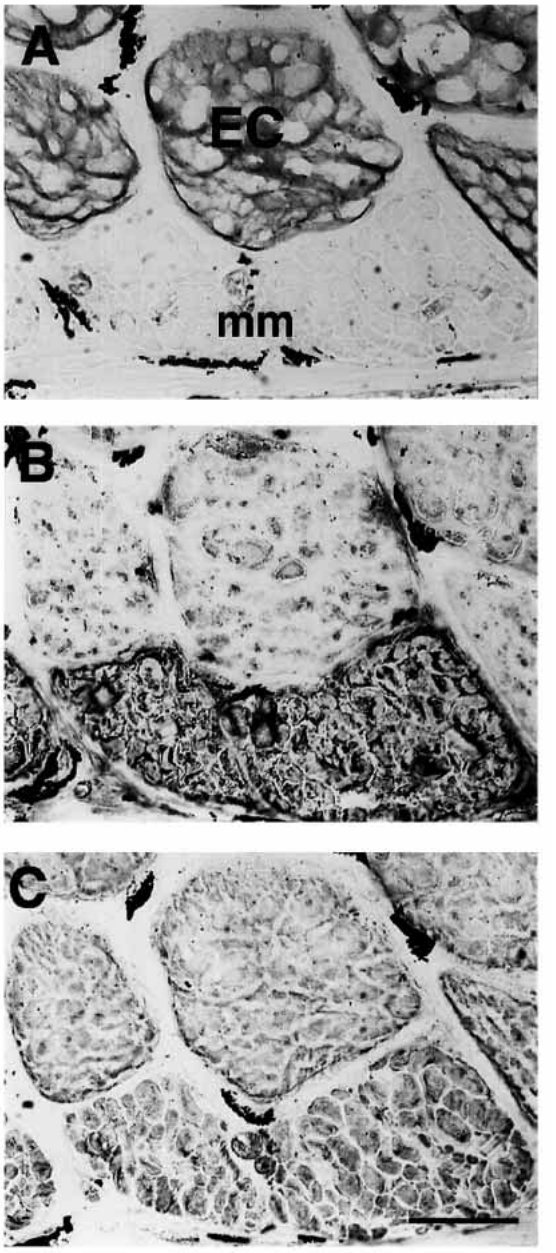


Fig. 3. The effects of 5 weeks of denervation on the immunolabeling pattern of serially sectioned electrocytes. (A) Acidic keratin is still expressed in the electrocytes (EC), and the labeling pattern reveals large pockets. It is not expressed in muscle (mm). Myosin (B) and tropomyosin (C) reappear in the electrocytes in patches approximately the size of differentiated muscle fibers in the fascicles below the electrocytes in this figure. Taken from Unguez and Zakon (1998b) with permission. Scale bar, 100 μm.

but inactive. The electrocytes may also be denervated by removal of a large segment of the spinal cord. The latter manipulation results in the elimination of the electromotor neurons and their axons, leaving the electric organ without innervation.

When the electric organ is either silenced or denervated and examined 2–5 weeks later, many electrocytes re-express 'fast' myosin and tropomyosin. These are not diffusely expressed throughout the cytoplasm, but are observed in small patches (Fig. 3). Keratin expression persists, however, and when keratin and myosin antibodies are used to label alternate sections, holes are found in the keratin matrix, presumably to accommodate the patches of myosin. In agreement with this observation at the level of the light microscope, small sarcomere clusters are evident in electron micrographs. In addition, small vesicles appear near the nascent sarcomeres that are likely to be part of the sarcoplasmic reticulum, and the membrane invaginates in a pattern reminiscent of the development of T-tubules (Unguez and Zakon, 1998b).

The muscle fibers in the fascicles overlying the electrocytes are also denervated. Unlike the electrocytes, denervated muscle fibers show no obvious morphological changes and continue to express the various isoforms of myosin in the correct gradient within the fascicle (Fig. 4).

These observations suggest that differentiated muscle fibers do not need innervation or activity to maintain the expression of their particular adult phenotype (i.e. 'fast' *versus* 'slow' MHC expression). However, the electrocytes depend on the electromotor neurons or their activity to maintain their phenotype. Once this is removed or silenced, the electrocyte begins to revert back into a muscle. We do not yet know whether electrocytes revert completely or remain as an electrocyte/muscle hybrid. For example, although myosin expression is strong after 5 weeks of denervation, keratin expression continues. Is this because keratin genes are no longer being transcribed but keratin remains refractory to degradation, or because the keratin gene is still active?

These results suggest that the nerve plays a role in the initial development of the electric organ. Our preliminary data indicate that this is so. If the spinal cord is transected to silence the electromotor neurons and the tail is cut to induce regeneration of a new electric organ, the development of the electrocytes appears normal for up to 2 weeks after spinal transection (Patterson and Zakon, 1997). Denervation has a more devastating influence on the electrocytes at this stage. When the spinal cord is removed at the same time as the tail is amputated, a small blastema forms, but it remains small and fewer muscle fibers develop compared with controls. Furthermore, when the spinal cord is removed 10 days after tail amputation, a blastema forms and muscle fibers develop. However, no electrocytes are evident even after 2 weeks of regeneration (G. A. Unguez, in preparation).

The latter experiments suggest that the nerve influences the transformation of muscle to electrocyte. Furthermore, denervation of adult electrocytes demonstrates that a cell is not irreversibly committed to an electrocyte phenotype since it can partially revert back to a phenotype similar to that of its precursor muscle fiber (Fig. 5). These results raise a series of questions. The muscle fibers are innervated throughout the transformation process. Are they initially innervated by somatomotor neurons, which become displaced by

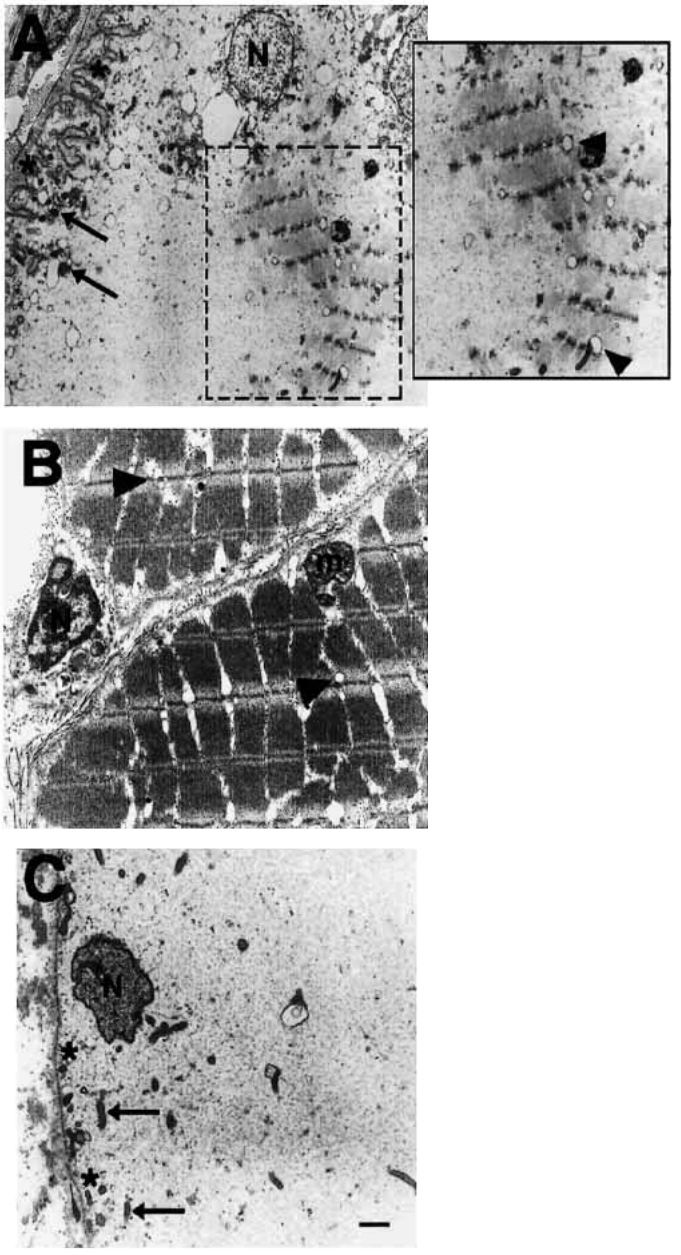


Fig. 4. Ultrastructure of a 5 week denervated electrocyte (A), a normal muscle (B) and an intact electrocyte (C). Note that the muscle contains well-developed sarcomeres and T-tubules (arrowheads in B). These organelles are absent in the electrocyte (C). However, they reappear in the denervated electrocyte (arrowheads in inset in A, which is a magnified version of the area bounded by dotted lines) along with deep membrane invaginations (asterisks) not normally present in electrocytes. N, nucleus; m, mitochondrion in B; arrows indicate mitochondria in A and C, Scale bar, 1 μm. Taken from Unguez and Zakon (1998b) with permission.

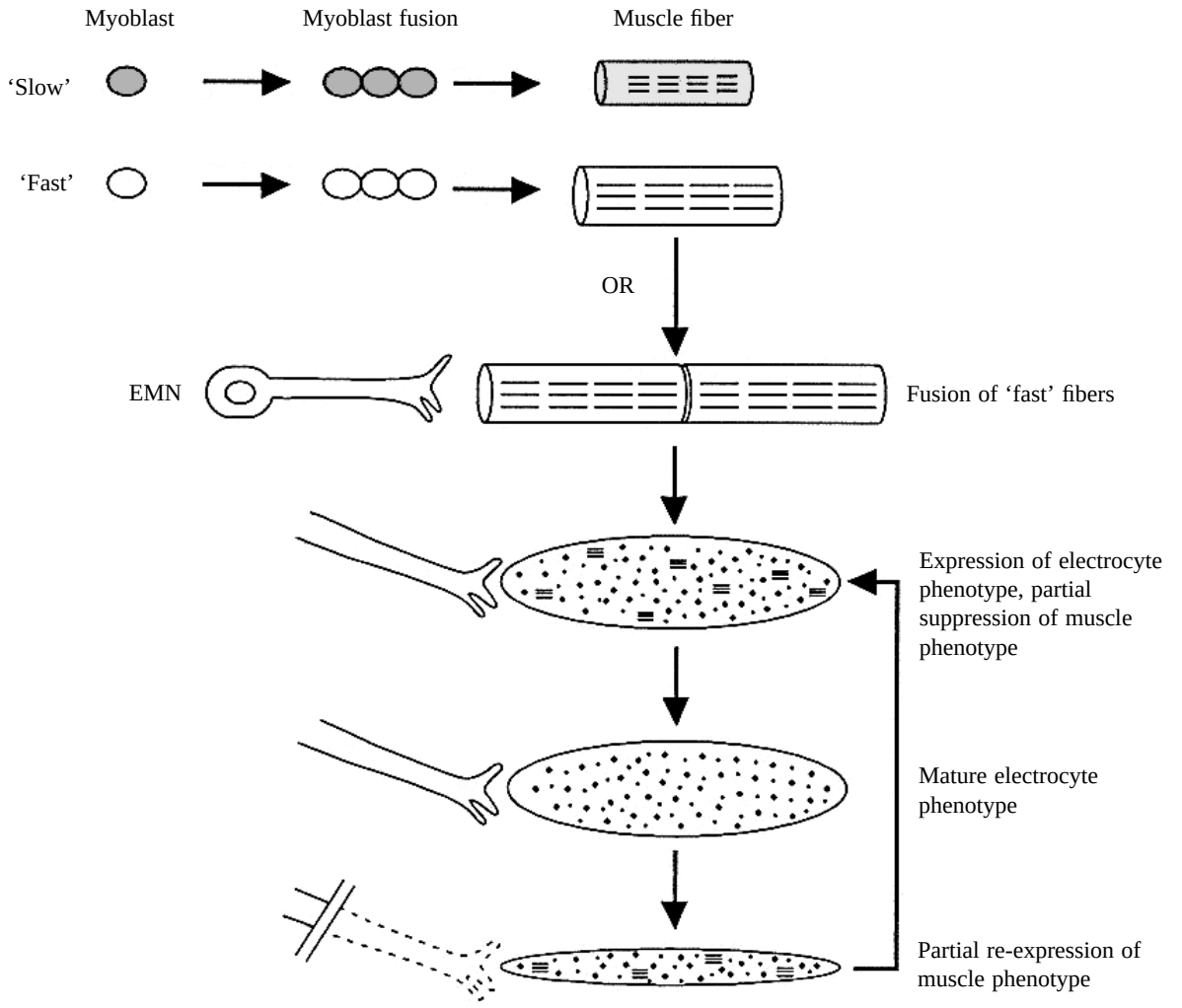


Fig. 5. Schematic diagram of the development of muscles and electrocytes in the electric organ of *Sternopygus*. On the basis of evidence from zebrafish (Devoto et al., 1996), it is likely that there are separate 'slow and 'fast' muscle precursor cell lineages. These eventually give rise to slow and fast muscles. Electrocytes arise from the fusion of 'fast' muscle fibers, presumably under the direction of the electromotor neurons. However, the electrocyte phenotype is not permanent and is at least partially reversed after denervation. EMN, electromotor neuron.

electromotor neuron axons, or are they only innervated by electromotor neurons but, nevertheless, differentiate into muscle cells initially? If the electromotor neurons are responsible for converting muscle into electrocytes, how did the electromotor neurons gain that ability during their own evolution from spinal somatomotor neurons and how did muscle gain the ability to respond to that signal in an appropriate way to form an electrocyte?

### Comparisons with other species

There are interesting parallels with the development of the electric organ in the strongly electric elasmobranch genus *Torpedo*. In this genus, the electrocytes also derive from muscle fibers whose sarcomeres disappear, and denervation of these electrocytes also causes a re-expression of myofibrils (Fox and Richardson, 1978, 1979; Gautron, 1974; Richardson et al., 1981). Yet, the electrocytes of *Torpedo* discharge only occasionally, in contrast to the constant high-frequency discharge of the *Sternopygus* electric organ. This suggests that, if activity patterns determine electrocyte phenotype, there must be different patterns in the different lineages. However, this may not be universal among electric organs since the electrocytes of mormyrids are reported to differentiate and appear normal after spinal transection or denervation (Denizot et al., 1982; Szabo and Kirschbaum, 1983).

It is important to note that the shape of electric organs shows much variation across species, even within the same lineage. For example, within the gymnotiforms, electrocytes are cigar-shaped in *Sternopygus* and *Eigenmannia*, cuboidal in pulse species such as the hypopomids and *Gymnotus*, and flattened in *Electrophorus*. Interestingly, the electrocytes of the hypopomid *Brachyhypopomus pinnicaudatus* are cigar-shaped in larval fish, as in mature *Sternopygus*, suggesting that the cuboidal shape of the electrocytes of pulse fish is a derived feature (Franchina, 1997). Similarly, the electrocytes of

mormyrids that possess complex cellular stalks are found in the more derived groups; these electrocytes also develop initially as a simple pancake-shaped cell in these species (Alves-Gomes and Hopkins, 1997). Thus, even if similar mechanisms are at work in the transformation of the electrocytes from muscle within a lineage, there must be additional factors that determine the shape of the electrocytes. Whether this information is also nerve-dependent, derived from the surrounding tissues or intrinsic to the nascent electrocyte is unknown.

## Conclusion and future directions

Electric organs and the neuronal control pathways that activate them have evolved multiple times in the evolution of fishes. This represents a paradigm case for how established organs and cell types become transformed by specific developmental processes to acquire new functions over evolutionary time and how this may occur independently in multiple lineages. Using the regeneration of the electric organ after amputation of the tail, we have shown that the large electrocytes of the weakly electric teleost *Sternopygus* derive from the fusion of numerous smaller muscle fibers. The newly formed electrocytes then down-regulate many muscle-specific proteins and organelles and become specialized for fine control of electrical excitability. Silencing or removing the neural input to the electrocytes causes them to re-express the muscle phenotype. A major quest for the future is understanding the co-evolution of the processes by which motor neurons produce, and muscle fibers respond to, specific signals for this phenotype transformation.

It is critical to determine what attributes of the electromotor neurons cause muscle fibers to transform into electrocytes. One way to analyze this is to replicate this phenomenon in culture. We predict that when myofibers are cultured with electromotor neurons, but not somatomotor neurons, some muscle fibers will be converted to electrocytes or at least there will be a down-regulation of myosin and a disruption of the sarcomeres in fibers contacted by electromotor neurons. It will be intriguing to test whether muscle fibers from other species of fish that do not produce electric organs, such as catfish or goldfish, or from unrelated lineages that have electric organs, such as mormyrids, have the capacity to respond to *Sternopygus* electromotor neurons. Eventually, manipulation of a culture system can also be used to determine whether there are diffusible factors involved or whether electromotor neurons must form synapses on muscle fibers, and whether transformation can occur in the absence of activity or with the imposition of particular activity patterns.

Ultimately, understanding how muscle fibers convert phenotype can only be answered by molecular methods. One line of attack would be to clone and sequence known muscle-specific transcription factors, such as myoD, myogenin, etc., and to compare their expression in muscle *versus* electric organ. This has been done for *Torpedo*, in which there are quantitative, but not qualitative, differences in the expression patterns of four of the five major muscle transcription factors (Neville and Schmidt, 1992). An approach that has the benefit of not being limited to searching for known sequences is using subtractive hybridization methods to screen for genes that are uniquely expressed in electric organ but not muscle. While some of these are likely to be structural genes, such as keratin, some may lead to unique regulatory elements.

Our studies of the development of *Sternopygus* electrocytes represent a modest beginning of a large-scale project that ultimately includes understanding how neuronal and muscle-derived elements of the electromotor system co-evolve and develop in a variety of unrelated species.

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