Properties of skeletal muscle in the teleost *Sternopygus macrurus* are unaffected by short-term electrical inactivity

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Güth R, Chaidez A, Samanta MP, Unguez GA. Properties of skeletal muscle in the teleost *Sternopygus macrurus* are unaffected by short-term electrical inactivity. *Physiol Genomics* 48: 699–710, 2016. First published July 22, 2016; doi:10.1152/physiolgenomics.00068.2016.—Skeletal muscle is distinguished from other tissues on the basis of its shape, biochemistry, and physiological function. Based on mammalian studies, fiber size, fiber types, and gene expression profiles are regulated, in part, by the electrical activity exerted by the nervous system. To address whether similar adaptations to changes in electrical activity in skeletal muscle occur in teleosts, we studied these phenotypic properties of ventral muscle in the electric fish *Sternopygus macrurus* following 2 and 5 days of electrical inactivation by spinal transection. Our data show that morphological and biochemical properties of skeletal muscle remained largely unchanged after these treatments. Specifically, the distribution of type I and type II muscle fibers and the cross-sectional areas of these fiber types observed in control fish remained unaltered after each spinal transection survival period. This response to electrical inactivation was generally reflected at the transcript level in real-time PCR and RNA-seq data by showing little effect on the transcript levels of genes associated with muscle fiber type differentiation and plasticity, the sarcomere complex, and pathways implicated in the regulation of muscle fiber size. Data from this first study characterizing the acute influence of neural activity on muscle mass and sarcomere gene expression in a teleost are discussed in the context of comparative studies in mammalian model systems and vertebrate species from different lineages.

skeletal muscle atrophy; fiber types in teleost fish; spinal cord transection; muscle inactivation; muscle transcriptome

SKELETAL MUSCLES ARE COMPOSED of a heterogeneous population of muscle fibers with different morphological, biochemical, and physiological properties (13, 14). That many of these muscle fiber properties can be altered by changes in nervous system input has been demonstrated through cross reinnervation, chronic stimulation, and electrical inactivation studies. Together, these studies suggest that an important property of neural input affecting muscle fibers is the electrical activity of innervating motor neurons (20, 43, 58, 67). The molecular mechanisms that link electrical activity to changes in muscle gene expression have been the object of intensive investigation. Although the signaling pathways that transduce electrical muscle activity into distinct muscle gene expression programs are not well understood, studies in mammals, mainly rodents, have shown that removal of nerve-dependent electrical activity can lead to extensive changes in muscle fiber size, fiber types, and associated sarcomere gene expression that occur largely via transcriptional mechanisms. These changes have been reported to occur as early as 2 days after electrical inactivation (21, 31, 46, 50, 53, 59, 74). Several observations have suggested that transcription factor families including members of the MyoD, Mef2, and NFAT families, which are essential in myogenesis, also function as mediators through which neural activity regulates the types of transcripts and proteins expressed in a muscle fiber (4, 11, 12, 15, 16, 22, 34, 52, 70, 71).

This speculation is supported by studies showing that changes in electrical activity in skeletal muscles of adult animals are coupled to alterations in the levels of these transcriptional regulators, which in turn lead to modifications in the transcription of contraction-associated genes (1, 16, 47, 54). However, the role that Mef2, NFAT, and MyoD families play in regulating muscle properties other than changes in contractile protein isoforms has not been fully determined. In addition, the role of innervation on muscle properties in nonmammalian vertebrate model systems has been greatly overlooked. Hence, little is known about how neural activity may regulate the skeletal muscle phenotype via conserved signaling pathways that transduce electrical muscle activity into distinct muscle gene expression programs across diverse vertebrate taxa.

Here, we report that the skeletal muscle fibers of the electric fish *Sternopygus macrurus*, a freshwater species of knife fish native to South America, do not change their size or their myosin heavy chain (MHC)-based fiber type composition even after a 5-day electrical inactivation period. We informed these morphological studies with gene expression analysis of different muscle protein systems, focusing primarily on the sarcomeric complex and the protein synthesis and degradation pathways and using real-time PCR and deep RNA sequencing (RNA-seq) to identify comprehensively genes that are differentially expressed after electrical inactivation and facilitate our investigations aimed at elucidating gene networks involved in electrical activity-dependent plasticity. The mRNA levels of genes associated with the sarcomere complex or the members of the Mef2, NFAT, and MyoD transcription factor families were unaffected. There was a transient upregulation in the expression of proteolytic pathway components after only 2 days of inactivation, and transcript profiles of genes in the IGF-Akt protein synthesis pathway were unaltered after 2 and 5 days of inactivity. The present results demonstrate that activity-independent mechanisms remain important sources of the control of the differentiated muscle phenotype in *S. macrurus*. Data from this first study characterizing the neural influence on muscle fiber size and sarcomere gene expression in a teleost are discussed in the context of comparative studies in mammalian model systems and vertebrate species from different lineages.
Materials and Methods

Animals and Spinal Transection Surgery

Adult Sternoptygus macrurus were obtained commercially from Ornamental Fish (Miami, FL). Fish used in this study measured about 30 cm in length and were of undetermined sex. Fish were housed individually in 15- to 20-gallon tanks, fed three times weekly, and maintained in aerated aquaria at temperatures of 25–28°C. A total of 21 fish were separated into three groups: a control unoperated group (n = 8) that received no treatment and two spinal transected (ST) groups from which tissues were harvested at 2 (2-day ST, n = 6) and 5 (5-day ST, n = 7) days after surgery. Fish that underwent ST surgery were anesthetized with 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO) in tank water (1.0 ml/l). A dorsal incision (~3 cm long) was made at approximately midlength of the fish’s body and followed by a partial dorsal laminectomy. The exposed spinal cord was transected with scissors, and complete transection was verified under a stereoscope by a clear separation of two spinal cord segments (Fig. 1). Following ST, the skin was sutured and treated with a topical antibiotic (nystatin and triamcinolone acetone ointment USP; E. Fougera & Co., Melville, NY). Fish were immediately returned to their tanks and monitored until fully recovered from anesthesia, and STRESS COAT (Aquarium Pharmaceuticals, Chalfont, PA) was added to the tanks as an additional anti-infection agent. To validate the success of a complete ST, we monitored fish 10–15 min daily for the absence of muscle movement caudal to the ST site throughout the 2- and 5-day ST periods. All animal treatment and handling procedures used in this study complied with the American Physiological Society Animal Care Guidelines and were approved by the Institutional Animal Care and Use Committee at the New Mexico State University.

Tissue Dissection

At 2 and 5 days after ST surgery fish were reanesthetized to harvest ventral skeletal muscle tissues and tail segments posterior to the ST site (Fig. 1). For transcript analyses, a segment (~3 cm) of ventral muscle was excised, skinned, blotted dry, and immediately immersed in RNAlater (Thermo Fisher Scientific, Waltham, MA) from control (n = 5), 2-day ST (n = 6), and 5-day ST (n = 4) fish. All quantitative transcript analyses carried out with real-time PCR used a minimum of the same four fish samples. The sample of ventral muscle from a control animal used to obtain a complete transcriptome with RNA-seq was not used for real-time PCR analysis. Morphological and immunolabeling analyses were performed on portions of ventral muscle caudal to those taken for quantitative RNA studies. Specifically, the tail segment (~2 cm) immediately posterior to the ST site from control (n = 3) and 5-day ST (n = 3) fish was blotted dry, embedded in Tissue-Tek O.C.T. embedding compound (Sakura Finetek, Torrance, CA) on cork, and flash-frozen by immersion in liquid nitrogen-cooled isopentane (Sigma-Aldrich). All ventral muscle samples were stored at ~80°C until further analysis.

RNA Isolation

Samples stored in RNAlater were blotted dry, weighed, pulverized in liquid nitrogen, and homogenized in TRIzol reagent (Thermo Fisher Scientific), and total RNA was extracted following manufacturer’s instructions. Total RNA concentration and purity (A260/A280) were determined spectrophotometrically on a NanoDrop 2000 (Thermo Fisher Scientific) before and after removal of residual genomic DNA with DNase I, amplification grade (Thermo Fisher Scientific).

Real-time RT-PCR Determination of Transcript Abundances and Statistical Analysis

Total RNA samples isolated from control, 2-day ST, and 5-day ST fish were DNased and used for cDNA synthesis in triplicate using the SuperScript First-Strand Synthesis kit (Thermo Fisher Scientific) as per manufacturer’s instructions. Additionally, one no-RT reaction that omitted the reverse transcriptase enzyme was performed for each sample to ensure absence of DNA contamination in subsequent PCR reactions. cDNA and no-RT samples were diluted 80-fold in nuclease-free water and stored at ~20°C. Gene-specific primers (Table 1) were designed using Primer3 (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and evaluated with NetPrimer (http://www.premierbiosoft.com/netprimer). Annealing temperature and primer concentration were optimized for each primer pair. Real-time PCR reactions were performed using Perfect SYBR Green Fastmix (Quanta Biosciences, Gaithersburg, MD) on cDNA and no-RT samples on an Opticon2 DNA Engine (Bio-Rad, Hercules, CA) running Opticon Monitor software (v.3.1.32, Bio-Rad). PCR conditions were as follows: initial template denaturation and polymerase activation for 30 s at 95°C followed by 40 cycles of 1 s denaturation at 95°C, 15 s primer annealing at a temperature optimized for each primer pair (range: 57–62°C), 30 s extension at 72°C, and fluorescence signal reading. To validate primer specificity, we performed the following: 1) the PCR reactions were immediately followed by melting curve analysis (65–95°C in 0.2°C steps with a holding time of 2 s per step) to ensure the presence of a single sharp peak, and 2) PCR products were visualized on SYBRSafe-stained (Thermo Fisher Scientific) agarose gels. Raw fluorescence data were exported and analyzed with the real-time PCR Miner tool (http://www.miner.eiwindup.info; Ref. 76) to determine C, values and amplification efficiencies for each primer pair. C, values were exported into Microsoft Excel and analyzed using primer pair efficiency adjustment and geometric mean normalization against rps11, cct5, and snrpb to derive fold expression differences between control and ST muscle samples. Fold changes were assessed in R for significance using one-way ANOVA and Benjamini-Hochberg P value adjustment for multiple testing. Genes with P values that retained significance (P < 0.05) following multiple testing adjustment were subsequently tested for pair-wise group differences between 2-day ST vs. control and 5-day ST vs. control groups using the “contrasts” function in R.

Illumina HiSeq2000 Sequencing and Transcriptome Processing

The assembly, annotation, and analysis of transcriptomic data-sets obtained from ventral muscle of S. macrurus used in this study have been described previously, comparing the gene expression profiles of skeletal muscle and the myogenic electric organ (EO) in control adult fish (44). These transcriptomic datasets also included one muscle sample each from control, 2-day spinally transected, and 5-day spinally transected fish. In the present study, we used the
DESeq2 package as per the program vignette (35) for normalization of transcript counts and to identify differentially expressed genes instead of the EBSeq package that was used in the transcriptomic analysis comparing muscle and EO of control fish (44).

Heat-map Plotting

For plotting of transcriptomic data in heat-map format the “heatmap.2” function within the “gplots” package (66) was used in R (60). Gene lists for pathways associated with regulating muscle fiber size used for heat-map plotting were assembled either manually based on literature searches (IGF pathway, myostatin pathway) (8, 49, 51) or based on KEGG pathways retrieved from the “pathview” package (36) in R with manual addition of differently expressed genes.

Immunolabeling

Longitudinal and cross sections (20 mm thick) from fish tail segments adjacent to the ST site were cut with a CM3050 cryostat (Leica Microsystems, Buffalo Grove, IL) and mounted on glass slides for immunolabeling processing as described in Unguez and Zakon (62). In brief, tissue cryosections were air-dried, rehydrated in 0.1 M PBS (pH 7.4) (5 min), fixed in 2% paraformaldehyde (10 min), and incubated in blocking solution (1% normal horse serum in PBS plus 0.1% Triton X-100) (1 h). Tissue sections were rinsed with PBS twice (5 min each) before incubation at room temperature with primary antibody in blocking solution overnight. Primary antibodies used included rabbit anti-laminin (Sigma-Aldrich, 1:20 dilution), mouse anti-slow and anti-fast MHC antibodies N2.261 (1:10) and A4.74 (1:2), respectively (Developmental Studies Hybridoma Bank, Iowa City, IA). Sections incubated with anti-slow and anti-fast MHC antibodies were coinmunolabeled with anti-laminin antibody. Following primary antibody incubation, tissue sections were washed three times with PBS (5 min each) and incubated with anti-mouse AlexaFluor488 and anti-rabbit AlexaFluor564 (Molecular Probes, Eugene, OR) diluted 1:200 each in blocking solution for 1 h at room temperature. Sections were washed twice in PBS (5 min each) before being mounted in Fluoromount (Sigma-Aldrich) and coverslipped. Immunolabeling of target antigens was detected, and tissue sections were imaged with a Zeiss DFC365 FX camera on a Zeiss Axioskop (Carl Zeiss Microimaging, Thornwood, NY) interfaced with a personal computer running the Leica Application Suite (v.3.1.0).

Quantification of Fiber Type Distribution and Fiber Size in Ventral Muscle and Statistical Analysis

In S. macrurus, as in most teleosts, ventral muscle is organized into myofibers, or fascicular bundles, of muscle fibers enveloped by thick connective tissue sheaths. In S. macrurus, individual myofibers were readily identifiable by their myofibrillar outlines, which were immunolabeled with anti-laminin antibody (Fig. 2). These myofibers are arranged in pairs (left and right), and the number of myofiber pairs decreases in the posterior direction along the tail (Fig. 2). Tail portions distal to the ST site generally contained six or seven myofibers (Fig. 2). Within a tail cross section, myofibers were labeled in ascending order from ventral to dorsal surface. The second pair of myofibers defined as the most dorsally located myofibers contained a centrally located population of type I (anti-slow MHC-positive) fibers along the dorsoventral region (Fig. 2). In control fish, the myofiber pairs that contained muscle fiber populations with the least variability in fiber type distribution and cross-sectional area were #3 and #4, and therefore, our analyses on muscle fiber type distribution and cross-sectional area used myofiber pairs 3–4 from both control (n = 3) and 5-day ST (n = 3) fish. The myosepta around myofibers pairs 3 and 4 were traced to obtain the total area for each myofiber, and the myomere area containing type I fibers were outlined separately. Individual images for each fluorescence emission channel were assembled as a panorama using Adobe Photoshop Elements 5.0 (Adobe System, San Jose, CA), and the assembled images were then merged and loaded into Adobe Photoshop Elements 5.0 to manually trace outlines of myofibers and type I and II (anti-fast MHC-positive) muscle fibers. NIH ImageJ (v. 1.43) was used to quantify the areas occupied by type I and type II fibers as well as total myomere areas. Measurements for each of

Table 1. Oligonucleotide primers used for quantitative RT-PCR

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<tr>
<th>Gene Name</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Length, bp</th>
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<tr>
<td>cct5</td>
<td>5'-AGATCGGAGATGAGCAGACTCTG-3'</td>
<td>5'-TGTCACATCGATCGATCGAAG-3'</td>
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<tr>
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<td>5'-TGTCACATCGAAG-3'</td>
<td>248</td>
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Sequences for sense and antisense primers as well as amplicon length are shown. Official gene names are used.
Electrical Inactivation Effects on Teleost Skeletal Muscle

RESULTS

Electrical Inactivation for 5 Days Does Not Result in Atrophy of S. macrurus Ventral Muscle

To determine the effects of electrical inactivation on the size of muscle fibers in skeletal muscle of S. macrurus we measured the MFD for type I and type II fibers before and after the 5-day ST period. Using MFD measurements, we mathematically derived corresponding values for CSAs. In control fish, the mean CSA of type I fibers (73.0 ± 9.5 μm²) was more than 10-fold smaller than that of type II fibers (809.0 ± 16.1 μm²) (Fig. 3A). After the 5-day ST period, the average CSAs of type I fibers (5-day ST, 102.2 ± 11.1 μm²; Fig. 3A) and type II fibers (5-day ST, 804.3 ± 20.9 μm²; Fig. 3B) were not significantly different from control (type I fibers, P = 0.12; type II fibers, P = 0.89). These data showed that 5 days of electrical inactivation by ST resulted in no measurable reductions in cell size in type I and type II muscle fibers.

Effects of Electrical Inactivation on Muscle Fiber Type Composition and Distribution

Spatial distribution of muscle fiber types I and II. In every myomere analyzed, type I fibers were located most peripherally and adjacent to the skin, whereas type II fibers were located medial to the type I fibers (Fig. 2). In the three ventral-most myomeres (Fig. 2: L-R1, L-R2, L-R3), clusters of contiguous type I fibers were also found adjacent to the midline. This clear separation between fiber type populations was found in all control S. macrurus fish tails, and this pattern was unchanged following 5 days of electrical inactivation by ST (data not shown). Similar to control fish, we also did not observe any muscle fibers that were colabeled with antibodies against both slow and fast MHCs in 5-day ST fish (data not shown). The fiber type composition in ventral muscles of control and 5-day ST fish was established by measuring the area occupied by type I fibers relative to the total myomere area. In control and ST fish, the myomere area occupied by type I fibers was smaller than that occupied by type II fibers (Fig. 4). Figure 4 shows the average myomere areas occupied by type I fibers in myomeres of three control (means ± SD: 11.0 ± 2.0%, 10.0 ± 1.8%, 18.9 ± 2.1%) and three 5-day ST fish (11.3 ± 1.5%, 15.2 ± 1.3%, 11.9 ± 1.2%). The mean type I fiber proportions for the control (means ± SE: 13.3 ± 2.8%) and 5-day ST (12.8 ± 1.2%) groups were not found to differ significantly between these groups (P = 0.89). These data suggest that muscle fiber type composition and distribution in S. macrurus were not affected by 5 days of electrical inactivation.

Expression of the Muscle Program Is Largely Unaffected by Electrical Inactivation

Myogenic transcription factors. In mammals, elimination of nerve-induced electrical activity triggers changes in the expression of muscle-specific transcription factors including the family of myogenic regulatory factors (MRFs). Changes in MRF expression precede alterations in structural muscle proteins providing a link with a likely transcriptional mechanism by which electrical activity may exert its effect on contractile properties in muscle (9, 17, 18, 48, 68). Our RNA-seq data showed that electrical inactivation had little effect on the expression levels of MyoD (myod), myogenin (myog), and...
were also unaffected by ST (Fig. 5A). The calcium-activated transcription factors belonging to the MeF2 (mef2c and mef2d) and the NFAT (nfatc1 to nfatc4) families did not show differences in transcript levels before and after ST (Fig. 5A). Other transcription factors involved in myogenesis, i.e., six1, eya1, sox6, and srf, also showed no changes in their gene expression levels before and after ST (Fig. 5A). Interestingly, one transcription factor that showed increased mRNA levels with ST and remained elevated compared with controls 5 days after ST was prdm1a. In the teleost D. rerio, the prdm1 transcription factor has been reported to induce type I muscle fiber differentiation by repressing the expression of sox6 (29). In S. macrurus, the increase in prdm1 transcript levels after ST was detected in the absence of changes to its target sox6 (Fig. 5A) or changes in the proportion of type I fibers in ventral muscle (Fig. 4).

**Contraction-related genes.** A well-characterized response of muscle properties to nerve-dependent activity is the change that takes place in sarcomere gene expression following removal of neuronal activity. Our transcriptome provided extensive coverage of genes that form components of the contraction complex, i.e., the contractile sarcomeres. In total, only nine of 62 sarcomeric genes identified in our transcriptome were flagged as differentially expressed at the 2-day ST time point compared with control (Fig. 5B). Among these nine genes, two (trim63a, ampd3b) were found to be upregulated. In contrast, tmod1, mybpc1, ampd3a, capn3, tnn1a, mybpc3, and myoz2b were downregulated. At the 5-day ST time point only one of the 62 genes was found to be differentially expressed (upregulated) compared with control. In contrast to what has been reported in some mammals after electrical inactivation, our transcriptome data showed that electrical inactivation of ventral muscle for up to 5 days had little effect on the expression of key muscle transcription factors and genes that make up the entire contractile sarcomere unit.

**Proteolytic Pathways Implicated in the Regulation of Fiber Size Are Only Transiently Induced Following Electrical Inactivation**

Electrical inactivation-induced reduction in muscle fiber size in mammals is often accompanied by the activation of proteolytic mechanisms that include the ubiquitin-protea-
Fig. 5. Heat map of transcript abundance ratios of muscle transcription factor and sarcomeric genes between control and ST fish. Normalized transcript abundance ratios were determined for 2-day ST/control and 5-day ST/control muscle samples and the log$_2$-transformed ratios were visualized as heat maps using the “heatmap.2” function in the “gplots” package in R for muscle transcription factors (A) and sarcomere genes (B). Transcription factors were ordered by the fiber type specificity of their target genes, whereas sarcomere genes were sorted by expression pattern using the default row clustering method in the “heatmap.2” function. Genes flagged as differentially expressed by DESeq2 are indicated by including the unadjusted $P$ value overlaying the appropriate color cell.
some system, autophagy, and cathepsins (26, 37, 41, 75).
Our quantitative expression analysis using real-time PCR of 19 mammalian orthologs of proteolytic genes in *S. macrurus* skeletal muscle showed an upregulation of eight of 14 proteasome-associated genes 2 days after ST (Fig. 6). The eight genes were the muscle-specific U3 ubiquitin ligases *trim63* (a.k.a. MuRF1) and *fbxo32* (atrogen-1), the ubiquitin-conjugating enzyme *ube2a*, the ubiquitylated protein shut-

gling factor *zfand5*, and the proteasome components *psemc2*, *psmc5*, *psmd3*, and *psmd12*. Increase in mRNA levels for four of five autophagy-related genes, i.e., *becn1*, *bnip3*, *gabarapa*, and the cathepsin-like *ctsll*, were also observed after a 2-day ST period (Fig. 6). However, the inactivity-induced increase in mRNA levels for most of these proteolytic genes was not maintained at the 5-day ST period. After 5 days of ST, the ubiquitin ligase *trim63* was significantly decreased, while the proteasome-associated *zfand5* and the autophagy regulator *bnip3* remained upregulated compared with control levels (Fig. 6).

**Transcriptomic Analysis Confirms Transient Induction of Proteolytic Pathways**

We complemented our real-time PCR data (*n* = 4) with next-generation RNA-seq analyses on ventral muscle tissue obtained from one control, 2-day ST, and 5-day ST fish. We found that of the 12 genes flagged as differentially expressed by real-time PCR, DESeq2-based assessment found six genes (*fbxo32*, *trim63*, *psemd3*, *zfand5*, *bnip3*, and *ctsll*) to be differentially expressed. The remaining gene (*foxo3b*) identified by DESeq2 as differentially expressed was found to be significantly changed (*P < 0.05*) by real-time PCR only before we adjusted its *P* value for multiple testing. In sum, DESeq2 analysis was consistent with real-time PCR data but was more conservative at flagging genes as differentially expressed (Fig. 6, Table 2).

Genes annotated to belong to pathways involved in regulating cell size were identified with the “pathview” package in R as well as manual literature searches. Changes in gene expression levels found at the 2-day and 5-day ST survival periods relative to control levels were visualized as heat maps created from the transcriptomic data. These data showed that several components related to the ubiquitin-proteasome pathway were transiently upregulated at the 2-day ST time point (Fig. 7). Six ubiquitylation-related genes were found to be upregulated after 2 days of ST, and these included the muscle-specific E3 ubiquitin ligases *fbxo32* and *trim63*. At the 5-day post-ST time point only two genes were found to be upregulated, including *fbxo32*. For the proteasome, only one gene was found to be differentially expressed at each of the two time points. However, the expression of all proteasome components showed an overall induction at both time points post-ST, which appeared stronger at the 2-day ST time point. Few genes involved in the regulation of autophagy and the lysosome system were found to be differentially expressed at either time point, and no overall trend was discernible. A pattern of transient induction similar to that of ubiquitin-proteasome components was also observed for some members of the forkhead box O (FoxO) family of transcription factors, specifically *foxo1a*, *foxo3b*, and *foxo4-like* (Table 3). In contrast, transcript levels of genes in the IGF-Akt and myostatin pathways that are involved in regulating protein synthesis showed little evidence of altered gene expression with no genes flagged as differentially expressed at either time point post-ST (data not shown), a response to electrical inactivity unlike that reported in mammalian skeletal muscle (27, 31, 50, 55, 74).

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**Fig. 6.** Quantification of transcript abundance ratios of selected genes of interest between spinally transected and control ventral muscle using real-time PCR (qPCR). Primers listed in Table 1 were used to amplify transcripts of interest. Abundance ratios determined by qPCR (*n* = 4) for 2-day ST/control and 5-day ST/control were normalized using 3 internal references genes (*psmd11*, *snrpb*, *cct5*) as described in MATERIALS AND METHODS. Results are shown with means ± SE. *Statistically significant results with *P* < 0.05 after multiple testing adjustment. +Genes with statistical significance (*P* < 0.05) before, but not after, multiple testing adjustment.
Table 2. Comparison of transcript quantification between real-time PCR (qPCR) and RNA-seq

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<th>Gene Name</th>
<th>Pathway</th>
<th>2-day ST/control</th>
<th>5-day ST/control</th>
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<tr>
<td>fbxo32 (atrogin-1)</td>
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<td>4.948#</td>
<td>5.184 ± 0.923*</td>
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<td>3.453#</td>
<td>2.576 ± 0.635*</td>
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<td>3.316 ± 0.311*</td>
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<td>psmd3</td>
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<td>7.985 ± 0.572*</td>
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<td>psmd4b</td>
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<td>psmd7</td>
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<td>11.195 ± 1.770*</td>
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<td>autophagy-related</td>
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<tr>
<td>ctld</td>
<td>autophagy</td>
<td>6.761#</td>
<td>4.298 ± 0.561*</td>
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Data for qPCR are the same as shown in Fig. 6. Corresponding RNA-seq ratios based on sample size of n = 1 for control, 2-day spinally transected, and 5-day spinally transected ventral muscle were selected based on the transcript sequence amplified by qPCR primers. Results for qPCR are shown as means ± SE with statistically significant results indicated (*, +) as in Fig. 6. Results for RNA-seq data are shown and those genes flagged as differentially expressed by DESeq2 are indicated (#).

DISCUSSION

Our results show that morphological and biochemical properties of skeletal muscle in adult S. macrurus fish remained largely unchanged after 2 and 5 days of electrical inactivity by spinal transection. Specifically, the distribution of type I and type II muscle fibers and the differences in CSAs between these fiber types observed in control fish remained after each spinal transection survival period. This response to electrical inactivation was generally reflected in the RNA-seq data by showing little effect on the transcript levels of genes associated with muscle fiber type differentiation and plasticity, the sarcomere complex, and proteolytic pathways implicated in the regulation of muscle fiber size. Although spinal transection induced an upregulation in mRNA levels of some of these genes after 2 days of inactivity, this response was only transient, as gene expression profiles at 5 days after spinal transection were similar to those in control unoperated fish. These data expand on a previous study that tested the long-term effects of neural input on skeletal muscle plasticity in S. macrurus (62). Given that no dramatic changes in fiber type composition and fiber size were observed after 2 and 5 wk of electrical inactivation (62), our results suggest an absence of both short-term and chronic activity-induced cellular plasticity in skeletal muscle of S. macrurus.

Muscle Fiber Type Is Unaffected by Changes in Neural Input

A prominent effect of electrical inactivation of hindlimb muscles in rodents and feline species is the conversion of fiber type identity whereby type I fibers suppress the expression of type I sarcomeric gene isoforms and induce the expression of type II isoforms (3, 17, 21, 46, 58). The lack of support for fiber type conversion taking place in S. macrurus after 2 and 5 days of inactivity was evident in the fiber type distribution analysis and in the absence of any hybrid muscle fiber expressing type I and type II sarcomeric MHCs based on immunolabeling. Our whole muscle tissue transcriptomes also revealed little to no changes in sarcomere isoform transcripts associated with type I or type II muscle fibers after either electrical inactivation period (Fig. 5B). Furthermore, transcription factors sensitive to electrical activity changes in mammalian muscle like the MRFs and the calcium-activated transcription factors belonging to the Mef2 (mef2c and mef2d) and the NFAT (nfatc1 to nfatc4) families were unaltered in S. macrurus. Interestingly, the expression of prdm1a, a transcription factor implicated in the induction of slow type fiber genes, was induced at both inactivation time periods in S. macrurus (Fig. 5A). However, induction of the prdm1a gene was insufficient to result in any detectable fiber type change.

Resistance of Muscle Size to Change after Elimination of Neural Activity

Electrical inactivation consistently leads to a decrease in fiber size in skeletal muscles of mammals, particularly in the hindlimb muscles of rodents (7, 21, 25, 38, 39, 46). In rodents, muscle fibers can atrophy 20% or more depending on the fiber type following 5 days of electrical inactivation (21, 46). In contrast, electrical inactivity did not lead to a significant decrease in size for either type I or type II fibers in S. macrurus. This lack of response of muscle size to neural inactivity in S. macrurus resembles that reported in some hibernating mammalian species including several species of ground squirrels, bears, and bats. These hibernating animals experience extensive periods (3–9 mo) of muscle inactivity and yet show little to no atrophy in their hindlimb muscles compared with those of other mammalian species following nonhibernation inactivity (19, 28, 32, 40, 57, 61). Even more striking is the finding that denervation of ankle
muscles in hibernating bears for up to 11 wk has little effect on muscle properties (33).

In hibernating mammals, the suppressed atrophy response of inactive muscle has been linked to a lack of induction of proteolytic pathways (23, 32, 40, 64, 72). This is in contrast to the activation of two proteolytic mechanisms, the ubiquitin-proteasome and autophagy-lysosome systems, in hindlimb muscles of nonhibernating mammals after removal of electrical activity (5, 7, 46, 50, 63, 65, 74, 75). Our expression analyses showed an upregulation of genes associated with these pathways at 2 but not 5 days after spinal transection (Fig. 7). Hence, the response of S. macrurus muscle to electrical inactivity is somewhere between the opposite responses observed in hindlimb muscles of hibernating mammals.

Table 3. Transcript abundances of FoxO

<table>
<thead>
<tr>
<th>Ratios (RNA-seq)</th>
<th>2-day/Con</th>
<th>5-day/Con</th>
</tr>
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<tr>
<td>foxo1a</td>
<td>2.453</td>
<td>1.204</td>
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<tr>
<td>foxo1b</td>
<td>0.860</td>
<td>0.861</td>
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<td>foxo3a</td>
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<td>1.260</td>
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<td>foxo4-like</td>
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<td>1.140</td>
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</table>

DESeq2-derived fold changes in expression for 2-day spinally transected (ST)/control and 5-day ST/control ventral muscle are shown for each FoxO transcript variant detected in our transcriptome. The expression ratio of foxo3b for 2-day ST/control was flagged as differentially expressed and is indicated (*).
nating and nonhibernating mammals. The identity of the factors responsible for suppressing the atrophy response in S. macrurus ventral muscle is unclear at this point. Passive stretch of electrically inactivated muscles and fibers has been suggested to be sufficient to induce compensatory hypertrophy in electrical-inactivated muscles in at least one human patient and experimentally in chicken (6, 56). In this study, fish continued to move freely in their aquatic housings following spinal transection surgery using the muscles proximal to the transection site. The swimming movements in S. macrurus consist of synchronized undulations of the ventral muscle tissue. While segments distal to the transection site appeared immobile, we cannot completely exclude the possibility that passive muscle stretches were nonetheless present and influenced the muscle fiber response in this study.

A Shout-out for Comparative Studies

While inactivity-induced fiber type transitions and atrophy are typical of hindlimb muscles in rodents and feline species (3, 58), it is important to note that the skeletal muscle response to inactivity is not the same across muscle types even within mammals. For example, in rats the diaphragm muscle undergoes limited morphological and fiber type changes after weeks of electrical inactivity by denervation (24, 73) compared with limb skeletal muscle (30, 39, 42). Similarly, inactivity effects have been found to differ between muscles following spinal cord transection in rodent limb muscles (21, 38). Few studies have examined the role of the nervous system on muscle properties in adult teleosts. Studies in piscine species report small changes in metabolic and contractile protein content in carp (69) and few changes in MHC fiber type conversion in zebrafish (2, 45) after a 14-day denervation period. In the common carp (Cyprinus carpio), a 2 wk denervation period resulted in small changes in metabolic markers and contractile protein content (69). A similar 2 wk denervation of adult zebrafish (Brachydanio rerio) muscle led to few changes in fiber type conversion, but not in all muscle fiber types (2, 45). Together, these data underscore the need for investigations using vertebrate species and muscle groups other than those most commonly used to date (particularly hindlimb muscles in rodents) to more rigorously test the prevailing premise that nerve-muscle interactions and muscle groups other than those most commonly used to date (particularly hindlimb muscles in rodents) to more rigorously test the prevailing premise that nerve-muscle interactions and muscle properties in adult teleosts.

REFERENCES

ELECTRICAL INACTIVATION EFFECTS ON TELEOST SKELETAL MUSCLE


