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***Sternopygus macrurus* electric organ transcriptome and cell size exhibit insensitivity to short-term electrical inactivity**

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Abstract

Electrical activity is an important regulator of cellular function and gene expression in electrically excitable cell types. In the weakly electric teleost fish *Sternopygus macrurus*, electrocytes, i.e., the current-producing cells of the electric organ, derive from a striated muscle lineage. Mature electrocytes are larger than muscle fibers, do not contain sarcomeres, and are driven continuously at frequencies higher than those exerted on muscle cells. Previous work showed that the removal of electrical activity by spinal cord transection (ST) for two and five weeks led to an upregulation of some sarcomeric proteins and a decrease in electrocyte size. To test whether changes in gene transcription preceded these phenotypic changes, we determined the sensitivity of electrocyte gene expression to electrical inactivity periods of two and five days after ST. Whole tissue gene expression profiles using deep RNA sequencing showed minimal alterations in the levels of myogenic transcription factor and sarcomeric transcripts after either ST period. Moreover, while analysis of differentially expressed genes showed a transient upregulation of genes associated with proteolytic mechanisms at two days and an increase in mRNA levels of cytoskeletal genes at five days after electrical silencing, electrocyte size was not affected. Electrical inactivity also resulted in the downregulation of genes that were classified into enriched clusters associated with functions of axon migration and synapse structure. Overall, these data demonstrate that unlike tissues in the myogenic lineage in other vertebrate species, regulation of gene transcription and cell size in the muscle-like electrocytes of *S. macrurus* is highly insensitive to short-term electrical inactivity. Moreover, together with data obtained from control and long-term ST studies, the present data suggest that neural input might influence post-transcriptional processes to affect the mature electrocyte phenotype.

Graphical abstract

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Keywords

electric organ; spinal cord transection; cell size regulation; electrical inactivity; muscle transcriptome; contractile gene expression

1. INTRODUCTION

Electric fish are unique in that they possess an electric organ (EO) that is specialized for the production of an electric field outside the body. This electric field is essential for navigation, communication, and mate selection behaviors (reviewed in Albert and Crampton, 2005; Bennett, 1971). Each EO is composed of many electrogenic cells called electrocytes, which in most electric fishes derive from a myogenic lineage (Bennett, 1971; Kirschbaum and Schwassmann, 2008; Schwassmann et al., 2014). However, unlike striated muscle cells, mature myogenically-derived electrocytes do not contain sarcomeres, are not contractile, yet continue to express some muscle genes associated with the striated muscle program including cytoskeletal, sarcomeric, myogenic transcription factor and neuromuscular junction proteins (reviewed in Albert et al., 2008). Furthermore, in many weakly electric gymnotiforms, these electrocytes are electrically activated by spinal motor neurons continuously day and night throughout the life of the fish at frequencies that range from 50 Hz to well over 1 kHz (Bennett, 1971) – activation patterns that are strikingly different than those known to be exerted on skeletal muscle cells (Bellemare et al., 1983; Schiaffino and Reggiani, 2011). Although numerous studies have already established that neural input is an important regulator of muscle function and gene expression (Batt et al., 2006; Kostrominova et al., 2005; Liu et al., 2005; Patterson et al., 2006), the role that nerve-dependent electrical activity plays on generating and maintaining the phenotypic properties of electrocytes remains largely unknown. We have begun to address this question by studying the activity-dependent coordination of subsets of muscle protein systems, i.e., sarcomeric, sarcolemmal, and size-related genes, in the non-contractile, current-producing cells of the weakly electric fish *Sternopygus macrurus*.

S. macrurus is a freshwater gymnotiform commonly known as longtail knifefish that is widely distributed in rivers throughout South America. Like other electric fish, mature

electrocytes of *S. macrurus* are innervated by specialized electromotoneurons (EMNs) in the spinal cord, and these are in turn controlled by pacemaker neurons (PMNs) located in the brainstem (Figure 1) (Bass, 1986; Bennett, 1971). Ultrastructural and immunolabeling studies show that fully differentiated electrocytes in *S. macrurus* are significantly larger in size than muscle fibers, lack sarcomeres, continue to produce some, but not all, “muscle-specific” proteins including desmin, titin, α -actin, and α -actinin, and, like their myogenic precursors, they are innervated by cholinergic neurons and are multinucleated (Cuellar et al., 2006; Kim et al., 2008, 2004; Patterson and Zakon, 1997; Unguez and Zakon, 1998a). This incomplete muscle-like phenotype is retained even though *S. macrurus* electrocytes maintain a transcriptome profile that is over ninety percent comparable to that of striated muscle fibers and includes virtually all transcripts associated with the sarcomere and their regulating transcription factors at levels similar to those detected in skeletal muscle cells (Gallant et al., 2014; Pinch et al., 2016). Since electrocytes are electrically activated by electromotoneurons at a continuous rate of 50-200 Hz (Mills et al., 1992), and muscle fibers are activated with tonic and phasic signals of frequencies <20 Hz by a different population of spinal motor neurons (Bellemare et al., 1983; Johnston and Altringham, 1988; Schiaffino and Reggiani, 2011), one question concerns whether impulse activity frequency exerted on the EO regulates properties of the striated muscle program in different ways, and secondly, to what extent the myogenic program is affected by activity-dependent versus activity-independent influences in electrocytes.

We previously showed that removing electrical activation of mature electrocytes by eliminating the supraspinal input to electromotoneurons and rendering the electrocytes electrically silent for two to five weeks led to an upregulation of the sarcomeric protein myosin heavy chain throughout the cytosol that was accompanied by the appearance of small sarcomere-like clusters in electrically inactive electrocytes (Unguez and Zakon, 1998b). These data suggested that electromotoneuron activation patterns trigger transcriptional and/or translational mechanisms within mature electrocytes to upregulate the myogenic program. However, we did not determine the extent of transcriptional changes. Moreover, robust transcriptional responses by electrically excitable cells like neurons and muscle cells to increases or decreases in nerve-dependent activity have been shown to occur almost immediately in vertebrates (Abe, 2008; Raffaello et al., 2006; Ryge et al., 2010; Vazdarjanova et al., 2002). The purpose of the present study, therefore, was to determine the sensitivity of muscle gene expression in electrocytes to short-term (two and five days) electrical inactivity periods. To characterize the time-dependent influence of neural activity on the gene expression profile of mature electrocytes, we performed whole tissue expression analyses using quantitative RT-PCR and deep RNA sequencing of transcriptomes of EO from control fish and fish that had undergone two and five days of electrical inactivity via spinal cord transection (ST). These gene expression analyses showed that: (1) two and five days of ST had a minimal effect on the mRNA levels of sarcomeric and muscle transcription factor genes in mature electrocytes; (2) short-term inactivity resulted in a transient upregulation of genes associated with pathways that induce cell atrophy despite the absence of any significant effects on cell size; and (3) genes associated with the “common toolkit” of electrocytes across electric fish species (Gallant et al., 2014) were essentially unaltered. The present data demonstrate a high level of persistence of the myogenic transcription program

in electrocytes and its cell morphology after short-term electrical inactivity. These findings are in contrast to the electrical activity dependence of many properties of the skeletal muscle program in other vertebrate systems. Moreover, together with data obtained from control and long-term ST studies, the present data suggest that neural input might influence post-transcriptional processes to affect the mature electrocyte phenotype.

2. MATERIAL AND METHODS

2.1. Animals and ST surgery

Adult *Sternopygus macrurus* were obtained commercially from Ornamental Fish (Miami, FL). Fish used in this study measured about 30cm in length and were of undetermined sex. Fish were housed individually in 55- to 75-liter 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 that received no treatment (n=8) and three ST groups from which tissues were harvested at two (2-day ST, n=5), five (5-day ST, n=5), and fourteen (14-day ST, n=3) days after surgery. Fish that underwent ST surgery were anesthetized with 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO) in tank water (1.0mL/L). A dorsal incision (~3cm long) was made at approximately mid-length 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 gap in the spinal cord (Figure 1). Following ST, the skin was sutured and treated with a topical antibiotic (Nystatin and Triamcinolone Acetonide Ointment USP, E. Fougera & Co., Melville, NY). Fish were immediately returned to their tanks, monitored until fully recovered from anesthesia, and Stress Coat® (Aquarium Pharmaceuticals, Inc., Chalfont, PA) added to the tanks as an additional anti-infection agent. To validate the success of a complete ST, fish were monitored for the absence of active muscle movement caudal to the ST site throughout the 14-day ST periods. In addition, the strength of EO discharges were recorded from locations anterior and posterior to the surgery site for each fish prior to and throughout the 14-day ST period to ensure that EO discharge magnitude was absent posterior to the surgery site (Figure 2). 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.

2.2. Tissue dissections

At two, five, and fourteen days after ST surgery fish were re-anesthetized to harvest EO tissues posterior to the ST site (Figure 1). EO tissues used for transcript analysis by quantitative RT-PCR and RNA-seq were collected by excising the caudal appendage, which was subsequently skinned, blotted dry, and immediately immersed in *RNAlater*® (Life Tech., Carlsbad, CA) for RNA isolation or flash frozen in liquid nitrogen for protein analyses. All quantitative transcript analyses carried out with quantitative RT-PCR used a minimum of four samples from different fish. For electrocyte cell size analyses, 3-cm tail segments located immediately anterior to the caudal appendage were isolated from fish and immediately 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, St. Louis, MO). All samples were stored at -80°C until further analysis.

2.3. RNA isolation and transcript quantification by quantitative RT-PCR and RNA-seq

The methods for extraction of total RNA from samples and use of these samples in quantitative RT-PCR (qPCR) and RNA-seq used in this work were the same as those published previously (Pinch et al., 2016). EO tissues were sampled from a total of three fish – with one fish sampled at each experimental time point (control, 2-day, and 5-days post-ST). All sampling and analysis techniques were performed as described previously (Pinch et al., 2016) with one exception: normalized expression ratios of transcripts between ST and control EO tissues were determined using the DESeq2 package (Love et al., 2014) instead of the EBSeq package.

qPCR reactions were performed on EO samples taken from control (n=4), 2-day (n=5), and 5-day (n=5) post-ST fish using the iCycler iQ™ Multicolor Real-Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA) running the iCycler iQ™ software (v. 3.1.7050). cDNA preparation, internal reference gene selection, and data analysis were performed as described previously (Pinch et al., 2016). Primer pair sequences, amplicon sizes, and annealing temperatures are included in Table 1.

2.4. DAVID analysis of transcriptomic data

The online tool DAVID (Huang et al., 2009a, 2009b) was used to investigate which biological aspects of the EO are influenced by electrical activity. To do so, we submitted lists containing the *Danio rerio* Ensembl gene ID annotations of genes flagged by DESeq2 as differentially expressed between 2-day ST and control EO (848 genes) or 5-day ST and control EO (483 genes) to DAVID (v.6.7). A list consisting of the *D. rerio* Ensembl gene IDs of all 16,889 genes annotated in our reference transcriptome was also submitted and used as a background for the Functional Annotation Tool analysis to identify KEGG pathways, Gene Ontology (GO) terms, and protein domains of potential interest. Functional annotation clustering was performed using the default settings, and the enrichment scores of each cluster were determined to be significant if they were > 1.3 , a cut-off established by the authors of the DAVID tool.

2.5. Heatmap plotting of transcriptomic data

For plotting of transcriptomic data in heatmap format the ‘heatmap.2’ function within the ‘gplots’ package (Warnes et al., 2015) was used in R (Team, 2014). Gene lists for pathways associated with regulating muscle fiber size used for heatmap plotting were assembled either manually based on literature searches (IGF pathway, myostatin pathway) (Bonaldi and Sandri, 2013; Rodriguez et al., 2014; Sandri, 2008) or based on *D. rerio* KEGG pathways (ubiquitylation, proteasome, regulation of autophagy, lysosome) retrieved from the ‘pathview’ package (Luo and Brouwer, 2013) in R with manual addition of further components. For visualization, fold changes in transcript abundances between ST and control conditions provided by DESeq2 were $\log_2$ -transformed and represented on a 100-step red-green color scale using the appropriate option for the ‘heatmap.2’ function.

2.6. Cell size measurements

In *S. macrurus* the EO consists of cigar-shaped large electrocytes that are arranged in several parallel arrays along the rostrocaudal axis of the fish (Figure 1). To measure electrocyte cell sizes the cross-sectional areas of electrocytes were determined in control (n=3), 5-day ST (n=3), and 14-day ST (n=3) fish. Cross-sections (20µm thick) from fish tail segments just anterior to the caudal appendage were cut using a CM3050 cryostat (Leica Microsystems, Buffalo Grove, IL) and mounted on glass slides for immunolabeling processing. Briefly, tissue cryosections were air-dried, re-hydrated in 0.1 M PBS (pH 7.4) (5min), fixed in 2% para-formaldehyde (10min), and incubated in blocking solution (1% normal horse serum in PBST (PBS plus 0.1% Triton™ X-100) (1hr). Tissue sections were rinsed with PBS twice (5min each) before incubation at room temperature with primary antibody against laminin (rabbit anti-laminin L9393; Sigma-Aldrich; 1:20 dilution) in blocking solution overnight. Following primary antibody incubation, tissue sections were washed three times with PBS (5min each) and incubated with anti-mouse Alexa Fluor™ 488 (Molecular Probes, Eugene, OR) diluted 1:200 in blocking solution for one hour at room temperature. Sections were washed twice in PBS (5min each) before mounting in Fluoromount™ (Sigma-Aldrich) and coverslipping. Immunolabeled tissue sections were imaged with a Zeiss DFC365 FX camera on a Zeiss Axioskop (Carl Zeiss Microimaging, Thornwood, NY) interfaced with a PC running the Leica Application Suite (v. 3.1.0). The electrocyte outlines were visualized by the anti-laminin immunofluorescent staining since laminin is an integral component that anchors electrocyte cells to the basement membrane. Images of electrocytes for each tail were assembled as a panorama using Adobe Photoshop Elements 5.0 (Adobe System, San Jose, CA), and the assembled images were then used to manually trace outlines of the electrocyte cell membranes. NIH ImageJ (v. 1.43) was used to measure the cross-sectional area of each intact electrocyte. Electrocytes have been described to have a cigar-like shape with tapered anterior and posterior faces, which are discernible by the presence of a large number of membrane invaginations. Care was taken to exclude such cells from cell size measurements. Statistical analysis of the electrocyte cell size data was performed as described under ‘Statistical Procedures’.

2.7. Statistical Procedures

Statistical analyses were performed on the data produced for cell size quantification, qPCR, and RNA-seq. Cell size data were analyzed using the Welch’s Two Samples t-test for unpaired samples in R. Cell size data from 5-day and 14-day ST fish were analyzed separately because the electrocytes sampled at different time periods were taken from different tail regions distal to the transection site and electrocytes in these regions showed significant differences in size among control fish (p=0.017 using Welch’s Two Sample t-test for unpaired samples). qPCR data were analyzed using one-way ANOVA. Post-hoc tests were performed using Tukey’s HSD to determine if significance was reached in the 2-day ST/control or 5-day ST/control comparisons. In all analyses, p-values<0.05 were considered to be statistically significant. For transcript expression ratios obtained by RNA-seq, the DESeq2 package was used to determine differential expression calls for the comparisons between 2-day ST vs. control and 5-day ST vs. control samples. Ratios flagged with an unadjusted p-value<0.05 were considered to be differentially expressed.

3. RESULTS

3.1. Transcriptional response of myogenic genes in electrocytes to ST

3.1.1. Myogenic transcription factors—The transcription of sarcomeric genes is driven by a large set of transcription factors, some of which are specifically expressed in the skeletal muscle lineage known as myogenic regulatory factors. Previous studies have reported changes in mRNA levels of many of these transcription factors as early as two days after removal of nerve-induced electrical activity (Buonanno et al., 1992; Rana et al., 2009; Wiberg et al., 2015). In contrast, our RNA-seq data showed that ST resulted in little effect on the expression levels of 36 transcription factors known to play a role in the induction, plasticity and maintenance of the striated muscle phenotype (Figure 3A). Figure 3A shows plots of the log₂-transformed ratio of mRNA levels for these 36 transcription factors in electrocytes from unoperated control to ST fish. Only four transcription factors were significantly altered in their expression (Figure 3A). Specifically, transcript levels for *myf5*, *foxo3b*, and *nfatc2.2* were significantly different from those in electrocytes from unoperated control fish at only one of the two time periods after ST. Only transcript levels of *prdm1a* were found to be upregulated after both two and five days of electrical inactivity (Figure 3A). The insensitivity of these transcription factors to short-term inactivity is consistent with the minimal effect observed on downstream sarcomeric gene targets discussed below.

3.1.2. Expression of sarcomere and contraction-related transcripts—Shown in Figure 3B are heatmaps for all 62 sarcomere genes found in the *S. macrurus* transcriptomes and the normalized transcript abundance ratios determined for 2-day ST/control and 5-day ST/control samples. Of these 62 genes, only five genes were differentially expressed in electrocytes after two (*tpm4*, *trim63a*, *tncc1*, *myoz2b*, and *ampd3a*) and five (*tpm4*, *tnnt2d*, *ampd3b*, *tnnt2e*, and *mybpc3*) days of electrical inactivity compared to those in unoperated control fish (Figure 3B). These genes belong to different sarcomeric substructures including Z-disks (*myoz2b*), thin filaments (*tpm4*, *tnnc1*, *tnnt2d*, *tnnt2e*), thick filaments (*mybpc3*), and M lines (*trim63a*, *ampd3a*, *ampd3b*). ST led to an upregulation of *tpm4* (tropomyosin 4) at both time periods and of *trim63a* (an E3 ubiquitin ligase that localizes to the Z-line and M-line lattices of myofibrils) at two days but not five days post-ST. A similar transient change in mRNA levels, but in the opposite direction, was observed for genes *tncc1*, *myoz2b*, and *ampd3a* at the 2-day period only. Downregulation of two troponin transcripts (*tnnt2d* and *tnnt2e*) and *mybpc3* was observed after five days of inactivity only. These data show that ST had little effect and did not result in a consistent upregulation of contractile gene expression in electrocytes.

3.2. Effects of electrical inactivity on size properties of electrocytes

3.2.1. Electrocyte cross-sectional area—The mean electrocyte cross-sectional area was not affected after either five or fourteen days of electrical inactivity (Figure 4). Electrocyte size, however, differed along the tail distal to the ST site. For example, in control and 5-day ST fish, mean electrocyte cross-sectional areas in the tail region immediately adjacent to the transection site (Region “R1”, Figure 4) were $13\,200 \pm 4\,400\mu\text{m}^2$ and $17\,200 \pm 4\,600\mu\text{m}^2$, respectively ($p=0.54$). By comparison, electrocytes located in the tail region distal to region R1, and slightly more distal to the transection site (Region “R2”, Figure 4)

were larger in both control ($61\,800 \pm 6\,900\mu\text{m}^2$) and 14-day ($70\,700 \pm 700\mu\text{m}^2$) animals ($p=0.33$). In tails from control animals, the difference in mean cross-sectional areas between electrocytes in regions R1 and R2 was found to be statistically significant ($p=0.0040$).

3.2.2. Molecular pathways implicated in the regulation of cell size—Pathways implicated in the regulation of cell size include protein synthesis mechanisms such as IGF signaling and the myostatin pathway and those regulating proteolysis including the ubiquitin-proteasome and autophagy processes (Bodine et al., 2001b; Glass, 2003; Rommel et al., 2001; Trendelenburg et al., 2009; Zhao et al., 2007). RNA-seq analysis of EO transcriptomes at two and five days of electrical inactivity showed essentially no change in expression of gene components in IGF and myostatin pathways that regulate protein synthesis (Figure 5, Suppl. Figure S1). The general trend observed in proteolysis related pathways was a transient change in mRNA levels of few genes after two days of inactivity with no differences in expression after five days (Figure 5, Suppl. Figure S1). For example, in the ubiquitinylation process the E3 ubiquitin ligases *fbxo32* (atrogin-1) and *trim63a* (MuRF1), as well as *zfand5b*, *sqstm1*, and *socs3b* showed an increase in mRNA levels from control values at the 2-day ST period, but only *socs3b* was differentially expressed at the 5-day ST period (Suppl. Figure S1). Similarly, in the autophagy pathway, the abundance of transcripts for *bnip3lb* and *bnip3* genes was increased at the 2-day period, but their levels return to those in control electrocytes at the 5-day period. Ten genes associated with the proteasome were flagged as significantly induced at two days, but not five days after ST. In sum, short-term electrical inactivity of electrocytes did not result in detectable adaptations of their size properties according to morphology measurements and gene expression profiles of signaling pathways that affect cell size (Figure 5; Suppl. Figure S1).

3.3. Genes differentially expressed in 2-day and 5-day ST compared to control EO

Using the DAVID functional annotation tool we found that of 16 889 genes, 848 genes were flagged as differentially expressed at the 2-day ST period compared to controls. Specifically, 477 genes were upregulated and 371 were downregulated (Figure 6). Many of the 477 upregulated genes were sorted into 45 functional annotation clusters by DAVID. Of these 45 clusters, eight were significantly enriched. Similar DAVID analysis showed that many of the 371 downregulated genes were grouped into 33 functional annotated clusters, eleven of which were significantly enriched (Figure 6). At the 5-day ST period, 483 genes were differentially expressed compared to controls with 332 genes being upregulated and 151 genes being downregulated (Figure 6). The upregulated genes were sorted into 30 functional annotation clusters, nine of which were significantly enriched, while the downregulated genes were sorted into five functional annotation clusters, with only one being significantly enriched (Figure 6).

3.3.1. Upregulated gene clusters—At the 2-day ST period, the two most highly enriched clusters (Clusters 1 and 2) contained genes associated with the proteasome and proteolysis (Figure 6; Suppl. Table T1). We note that not all protein degradation genes are included in Clusters 1 and 2, as Cluster 15 also contained genes related to protein degradation yet it was not found to be significantly enriched (Figure 6; Suppl. Table T1). Protein degradation (Cluster 8) was also one of the nine significantly enriched functional

annotation clusters found at the 5-day ST period (Figure 6; Suppl. Table T1). At the 5-day ST period, we also identified a significantly enriched cluster associated with intermediate filaments and the cytoskeleton (Cluster 6) (Figure 6; Suppl. Table T1) that contained several keratin-related and neuronal intermediate filament genes.

3.3.2. Downregulated gene clusters—At two days post-ST, eleven of 33 enriched clusters were found to be significantly enriched (Figure 6; Suppl. Table T1). Genes within these eleven clusters have functions associated with axonal migration and synapse structure including *thrombospondin1* (*thbs1*) (DeFreitas et al., 1995; Mendus et al., 2014; Risher and Eroglu, 2012), *hemicentin 1* (*hmcn1*) (Vogel and Hedgecock, 2001), *peripherin* (*prph*) (Yan et al., 2007; Yuan et al., 2012), *ryanodine receptor 2a* (*ryr2a*) (Wu et al., 2011), and calcium channel, voltage-dependent, L type, alpha 1D subunit (*cacna1da*) (Sheets et al., 2012). The number of enriched clusters decreased at the 5-day ST period. Only five clusters were enriched with one being considered significantly enriched (Cluster 1; Figure 6), which contained contraction-associated genes including *cardiac myosin binding protein c* (*mybpc3*) (Moss et al., 2015) and obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF (*obscna*) (Perry et al., 2013).

3.3.3. Further genes and gene clusters of interest—Given our interest in studying the effect of short-term electrical inactivity on electrocyte size, it was interesting to find two clusters related to cell size that were non-significantly enriched at the 2-day ST period. Genes in these two clusters were upregulated and have functions related to cell junctions (Cluster 16) and intermediate filament/cytoskeleton genes (Cluster 22) (Figure 6; Suppl. Table T1). The latter contained several genes that are not associated with the skeletal muscle program: a glial intermediate filament, *glial fibrillary acidic protein* (*gfap*) (Yang and Wang, 2015), and an epithelium-specific keratin isoform (*krt5*) (Coulombe and Lee, 2012). At the 5-day ST period, we identified a significantly enriched cluster of cytoskeletal genes (described above), and a non-significantly enriched cluster associated with cell adhesion (Cluster 10). In addition, four non-significantly enriched clusters contained downregulated genes with functions related to cell excitation of electrocytes as proposed by Gallant and colleagues (2014).

We wanted to determine if the genes identified by Gallant and colleagues (2014) as important in differentiating electrocytes from skeletal muscle were affected by loss of electrical activity. According to Gallant et al., (2014), these 23 “electrocyte-associated” genes were grouped into the following categories of function: nuclear transcription factors (*six2a*, *hey1*, *hey1b*, *six4b*, and *myog*); genes that regulate cell excitation (*atp1a2a*, *atp1a3a*, *znfr2a*, *scn4aa*, and *fgf13a*); genes that regulate cell size (*igf2b*, *arhgef12a*, *pik3r3b*, *net-37* like, *fbxo40*); genes involved in contraction and excitation-contraction coupling (*smyd1a*, *smyd1b*, *hspb11*, and *cacna1sa*); and genes encoding proteins that surround individual electrocytes to provide insulation (*col14a1*, *col6a6*, *gylt1b*, and *dmd*). In this study, only two of these 23 genes were found to be responsive to short-term electrical inactivity by ST. The gene *znrf2a* was significantly downregulated at both the 2- and the 5-day ST periods ($p=0.02$ at both time points), whereas *col6a6* was significantly downregulated at the 2-day ($p=0.02$), but not at the 5-day ST period ($p=0.39$).

3.3.4. qPCR validation of gene expression—Previous work has validated gene expression data from our RNA-seq datasets using the control muscle and EO samples (Pinch et al., 2016), as well as electrically silenced muscle samples (Güth et al., 2016). These validations included numerous genes belonging to proteolytic pathways, and the data showed strong similarity in the expression patterns for these genes between qPCR and RNA-seq. Here, we performed qPCR validations specifically on the EO data of the RNA-seq dataset by selecting a small set of genes indicated by DESeq2 as differentially expressed. For this purpose we selected three genes (*ryr2*, *hmcn1*, and *cacna1da*) that were found in several significantly enriched functional annotation clusters and have associated functions in synaptic transmission and axon migration (Figure 6, summarized in Figure 7). Moreover, these genes are closely related to the ‘excitation’ functional category that was identified by Gallant et al. to be a distinguishing feature of EOs compared to skeletal muscle (Gallant et al., 2014). Similar to RNA-seq, the qPCR data indicated that expression of *ryr2* and *hmcn1* at both time points, and *cacna1da* at two days after ST was reduced, though these changes did not reach statistical significance (Table 2). This was despite the fold change difference in reduction of *hmcn1* after electrical inactivity being greater in qPCR than in RNA-seq.

4. DISCUSSION

Our results demonstrate that mature electrocytes are highly insensitive to short-term periods (two to five days) of electrical inactivity by ST (Figure 7). First, minimal changes were detected in the levels of sarcomeric and myogenic transcription factor transcripts after either ST period. Second, there was a transient upregulation of genes associated with proteolytic mechanisms at two but not at five days after ST and electrocyte cell size was not affected. The observed electrocyte response to short-term inactivity was unexpected given the continuous non-stop depolarization of electrocytes by electromotoneurons, the well-established response in skeletal muscle gene expression to acute (<24-hrs) and short-term changes in electrical activity patterns in other vertebrates, the myogenic lineage of electrocytes, and the retention of many phenotypic aspects of the striated muscle program in mature electrocytes.

Resistance of muscle gene expression to change after ST-induced electrical inactivity

The skeletal muscle program can respond rapidly to reductions in electrical activity by changing the expression of sarcomeric genes and by decreasing the muscle fiber size or atrophying (Calura et al., 2008; Cohen et al., 2009; Dupont-Versteegden et al., 1998; Talmadge and Paalani, 2007). In mammals, for example, muscle fiber cross-sectional area can atrophy 20% or more depending on the muscle fiber type after five days of electrical inactivity (Dupont-Versteegden et al., 1998; Raffaello et al., 2006). The expression of transcription factor types and levels, as well as that of some of their target sarcomere and acetylcholine receptor subunits are altered immediately following ST. Specifically, elimination of neural input results in upregulation of all members of the MyoD family of transcription factors within 24–48 hours (Voytik et al., 1993; Walters et al., 2000), with maximal levels of induction reached between three and 28 days depending on the muscle group (Hyatt et al., 2006, 2003).

In contrast, the present data demonstrate that the gene expression profile of many myogenic transcription factors including MyoD family members in mature electrocytes of *S. macrurus* is highly insensitive to short-term electrical inactivity (Figure 3A). This result was accompanied by a generally unchanged sarcomeric gene transcription pattern in mature electrocytes two and five days after ST (Figure 3B). The size of electrocytes in *S. macrurus*, based on mean cross-sectional area, also did not change after ST (Figure 4) even though our transcriptomic analysis showed a transient upregulation, i.e., increase at the 2-day but not 5-day ST period, of many genes involved in pathways regulating cell size and atrophy (Figure 5). Interestingly, the size of electrocytes along the length of the EO (Figure 4, R1 and R2) differed significantly in that electrocytes located in the most rostral region (R1) were significantly smaller than electrocytes located more caudally (R2). To our knowledge, this is the first study to report regional size differences in mature electrocytes. An ontogenetic study of *S. macrurus* by Kirschbaum and Schwassmann (2008) suggested the existence of two distinct electrocyte precursor populations. It is possible that electrocyte size is an inherent property linked to the specific precursor cell population, and this population “lineage” factor prevails over extrinsic factors such as electrical inactivity – at least in the short term – in determining the size of these cells.

At the 5-day ST period, we also identified a significantly enriched cluster of upregulated genes associated with intermediate filaments and the cytoskeleton (Cluster 6) (Figure 6; Suppl. Table T1). The upregulation of cytoskeletal genes may occur to offset the atrophy response that has been demonstrated in mammalian skeletal muscle tissue after electrical inactivity (Bodine et al., 2001a; Sacheck et al., 2007) by increasing the density of cytoskeletal proteins to maintain cell volume and shape. These genes may also function to maintain cell size by increasing the adhesion of the cell to the extracellular matrix through hemidesmosomes or similar structures, as the upregulated *krt5* gene is an important cytoskeletal component, and is involved in hemidesmosome formation in epithelial tissues (Seltmann et al., 2012). Lastly, we determined the effect of short-term electrical inactivity on the expression of 23 genes recently proposed to compose a “tool-kit” for EO differentiation (Gallant et al., 2014). Only two of these 23 genes were found to be responsive to ST. In conclusion, data from the present study suggests the existence of a yet-to-be identified mechanism, or mechanisms, by which mature electrocytes resist the removal of electrical activation over a short-term period to maintain their cellular characteristics.

Short term versus long term effects of electrical inactivity on electrocytes of *S. macrurus*

While the current data demonstrated that short-term electrical inactivity of mature electrocytes resulted in minimal changes in the expression of transcripts encoding sarcomeric and transcription factor genes, we previously showed that a strong induction of sarcomeric protein expression took place after longer, i.e., a 2- and 5-week, ST period (Unguez and Zakon, 1998b). Immunolabeling studies showed that after a 2-wk ST period, up to a third of electrocytes contained sarcomeric myosin heavy chain and tropomyosin proteins throughout their cytosol. After a 5-wk ST period, these two sarcomeric proteins were detected in practically all electrocytes. In addition, few short sarcomere assemblies with apparent T-tubules and sarcoplasmic reticulum structures were identified in ultrastructural studies at the longer ST period (Unguez and Zakon, 1998b). These changes in

sarcomere muscle protein expression and organization were concurrent with cell atrophy. A significant decrease in mean electrocyte cross-sectional area after the 2-wk ST period was observed and the extent to which electrocytes atrophied did not increase after a longer 5-wk ST period. Hence, electrocytes of *S. macrurus* exhibit a greater resistance to inactivity-induced atrophy than electrically inactive muscle cells in other vertebrates, which characteristically undergo rapid and sustained loss of size after ST (Dupont-Versteegden et al., 1998; Nagpal et al., 2012; Niederle and Mayr, 1978; Raffaello et al., 2006; Sacheck et al., 2007).

In lieu of the present data, electrocytes in *S. macrurus* may not be completely insensitive to electrical inactivity as their response characterized by upregulation of sarcomeric proteins and induction of sarcomere structure formation is merely delayed (Figure 7). It is important to note that the long-term ST study analyzed electrocytes located in the caudal appendage exclusively (Unguez and Zakon, 1998b). In contrast, the present study used electrocytes in the caudal appendage for transcript expression analyses and more rostrally located electrocytes for immunolabeling studies (Figure 1). We cannot exclude from our ST model the possibility that electrical inactivity might differentially affect gene expression and morphological properties of electrocytes in different regions of the tail. Since the long-term ST study did not assay changes at the mRNA level, we also cannot determine the extent to which upregulation of sarcomeric proteins in electrocytes are attributable to electrical inactivity effects on transcriptional, translational, or post-translational processes, all of which have been shown to influence muscle gene expression (Cox et al., 1991; Gunning and Hardeman, 1991; Honda and Epstein, 1990).

The level of gene expression at which changes in electrical activity may exert their effect is relevant given that electrocytes in control adult *S. macrurus* depend on post-transcriptional processes to regulate their myogenic program as they continue to transcribe practically all muscle-specific genes while only producing some sarcomeric proteins (Pinch et al., 2016; Gallant et al., 2013). Based on data from control (Pinch et al., 2016; Gallant et al., 2013), short-term (present data) and long-term (Unguez and Zakon, 1998b) ST studies, we propose that electromotoneuron-induced electrical activity influences the striated muscle program in electrocytes through post-transcriptional regulation.

Electrical inactivity effects on electrocytes in other electric fish

Our findings in *S. macrurus* are consistent with biochemical and ultrastructural data from a denervation study using adult *Torpedo* (Gautron, 1974). In the latter study, sarcomere-like structures were first detected 24 days after removal of neural input, a post-denervation period that coincided with a peak in total protein increase in *Torpedo* electrocytes. No striations or changes in electrocyte protein content were observed between one and twelve days after denervation. However, no immunolabeling studies assessing the time-dependent changes in contractile proteins were performed. To date, no other studies have looked at the effect of innervation on the morphological or muscle properties of mature EO. Nevertheless, data from *Torpedo* and *S. macrurus* demonstrate that neural input 1) plays a dominant role in the maintenance of the mature EO phenotype and 2) is involved in suppressing the striated muscle program in mature electrocytes in independently evolved electric fish species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EO	electric organ
ST	spinal cord transection

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Highlights

- Spinal cord transection has minimal effect on sarcomeric gene expression.
- Electrical inactivity caused a transient upregulation of proteolytic mechanisms.
- Electrocyte size did not change after spinal cord transection.
- Electrocyte-“specific” genes were unaffected by short-term electrical inactivity.

Figure 1. Schematic of the electromotor circuitry of *Sternopygus macrurus* and the site of ST Electrocytes of the EO in *S. macrurus* are innervated by a population of spinal motoneurons called electromotoneurons (EMNs) that receive electrical signals from pacemaker nuclei (PMN) in the brainstem. ST surgery was performed to electrically silence the EMNs and electrocytes they innervate in the region caudal to the surgery site. The portion of the EO found in the caudal appendage of the tail was harvested for transcript analyses, whereas portions of tail located more rostral were used for cell size analyses using immunolabeling. While skeletal muscle fibers in the ventral muscle were similarly affected by the ST surgery, and corresponding RNA-seq libraries were included in the construction of the reference transcriptome, as described in detail in Pinch et al. (2016), the current study reports only the effects of electrical inactivity on the EO.

Electric Organ Discharge After Spinal Cord Transection

Figure 2. EO discharge after ST

Shown is a representative recording (top) and magnified traces (bottom) of the EO discharge recorded from a *S. macrurus* fish following ST surgery. Electrodes for recording were placed next to the fish in body regions rostral and caudal to the ST cut site. Successful electrical inactivity of electrocytes was confirmed by a reduction in the signal amplitude (voltage) recorded from the body region caudal to the transection site compared to that in the rostral region. Recordings were performed daily up to fourteen days after surgery and only fish that showed no EO discharge signal caudal to the surgery site were used in the study.

Figure 3. Heatmap of transcript abundance ratios of muscle genes between control and ST EO Normalized transcript abundance ratios were determined for muscle genes in EO obtained from 2-day ST vs. control and 5-day ST vs. control fish. Expression ratios were $\log_2$ -transformed and visualized (red – upregulated; green – downregulated) as heatmaps showing transcription factors known to induce myogenesis (**A**) and all sarcomeric genes (**B**) found in our transcriptome. Genes were sorted by expression pattern using the default row clustering method in the ‘heatmap.2’ function in R. Genes flagged as differentially expressed by DESeq2 are indicated by including the unadjusted p-value overlaying the appropriate color cell.

Figure 4. Effect of ST on size of electrocytes in the EO of *S. macrurus*
Electrocyte (EC) cross-sectional areas (CSAs) and standard errors (S.E.M.) were determined (in μm^2) in tail cross-sections obtained from body regions located between the caudal appendage and the site of ST surgery, as indicated. Electrocyte CSAs in 5-day ST fish were measured in tail region ‘R1’, whereas CSA measurements in 14-day ST fish were obtained from electrocytes located in region ‘R2’. CSAs of electrocytes in control fish were determined in similar fashion and compared to corresponding ST fish. Experimental details and data summaries are shown for 5-day ST vs. control and 14-day ST vs. control experiments. Electrocyte CSAs were found to be larger on average in region ‘R2’ compared to ‘R1’ in control tails ($p=0.0040$). The region of EO analyzed by Unguez and Zakon (1998b) for changes in electrocyte CSAs after fourteen days of electrical inactivity is shown for comparison.

Figure 5. Changes in the expression of genes involved in cell size regulation between control and ST EO

Normalized transcript abundance ratios were determined for 2-day ST/control and 5-day ST/control samples of EO using RNA-seq, and genes belonging to pathways involved in the regulation of cell size identified as indicated on the left column and described in the methods. Indicated are the numbers of genes in each pathway that were flagged by DESeq2 as upregulated, not differentially expressed, and downregulated using unadjusted p-values. None of the six *foxo* homologs identified in our transcriptome were flagged as differentially expressed following two or five days of electrical silencing. Heatmap visualizations of these pathways can be found in the supplementary materials (Suppl. Figure S1).

Figure 6. Differentially expressed genes between 2-day and 5-day ST compared to control EO Of the 16 889 annotated genes, 848 were identified as differentially expressed at two days post-ST, and 483 as differentially expressed at five days post-ST, with each bar containing the number of differentially expressed genes that were either induced (red) or repressed (green) at each time point (center). Functional annotation clustering of differentially expressed genes was performed in DAVID, and the most relevant functional clusters with their enrichment scores are presented. Enriched functional clusters of induced genes 2-day (left) and 5-day post-ST (right) are in the top row, while those of repressed genes are in the bottom row. The dashed line in each graph represents the significance cut-off for enrichment clusters as defined by DAVID. Full lists of all functional annotation clusters for induced and repressed genes at each time point are included in Suppl. Table T1.

Figure 7. Summary of the influence of electrical inactivity on the electrocyte phenotype in *S. macrurus*

Following electrical inactivity, no significant changes in the expression of virtually all myogenic transcription factors and sarcomeric genes was observed. Protein degradation pathways, particularly the ubiquitin-proteasome system, were found to be transiently induced after two days of electrical inactivity. However, many of these genes had reverted back to control levels by five days post-ST. Correspondingly, measurements of cross-sectional areas revealed that electrocyte cell size was not altered after five or fourteen days of electrical inactivity. In contrast, the expression of genes related to intermediate filaments was found to be induced at five days post-ST, while genes related to cell adhesion exhibited a slight enrichment at both two days and five days post-ST. Genes that were found to be repressed following ST were associated with axonal migration and synapse structure at two days post-ST, with fewer genes remaining repressed at five days post-ST. Abbreviations: 2d – 2-day post-ST; 5d – 5-day post-ST; 14d – 14-day post-ST. The colors of the boxes next to functional categories represent the general regulation pattern observed (red – induction; yellow – minimal change; green – repression).

Table 1

Oligonucleotide primers used for quantitative RT-PCR determination of mRNA abundance ratios. Sequences for sense and antisense primers, amplicon lengths, and optimized annealing temperatures are shown. Official gene names are used where possible.

Gene name	Sense primer	Antisense primer	Length (bp)	T _{ann.} (°C)
<i>rps11</i>	5'-TACCCAGAATGAAAGGGCGTAT-3'	5'-CATGTTCTTGTGCCTCTTCTCG-3'	322	59
<i>cct5</i>	5'-AGATCGGAGATGGGACTACTGG-3'	5'-GTCCATGTCAGCTACGGTCAAG-3'	299	59
<i>snrpb</i>	5'-AAGATCAAGCCCAAAAATTCCA-3'	5'-CCTGAGGTGTCATTACCTGCTG-3'	289	59
<i>ryr2</i>	5'-GATACTACTGCCTCAAGGTTCCC-3'	5'-CCCAGCAATTCAGTACGAC-3'	246	60
<i>hmcn1</i>	5'-AAGAAGGTGGTGGTGGTAAAG-3'	5'-TACATCCGTGCTCTCATCCG-3'	285	60
<i>cacna1da</i>	5'-GCTACTGCTGTTCAAGGTGTGCTAC-3'	5'-AACTCTTCAAGGTGATGTTGTCCC-3'	260	60

Table 2

Quantification of transcript abundances using quantitative RT-PCR (qPCR) and RNA-seq. RNA-seq ratios are based on sample size of n=1 each for control, 2-day ST, and 5-day ST EO. qPCR data are based on sample sizes n = 4 for EO, as described in the methods. Results for qPCR are shown as mean $\pm$ standard error. None of the 2-day ST/control and 5-day ST/control comparisons performed on qPCR data were found to reach statistical significance. Transcript abundance ratios determined by RNA-seq are shown and those genes flagged as differentially expressed by DESeq2 are indicated (#).

Gene name	2-day ST EO/control EO		5-day ST EO/control EO	
	RNA-seq	qPCR	RNA-seq	qPCR
<i>ryr2</i>	0.12#	0.12 $\pm$ 0.03	0.22#	0.47 $\pm$ 0.16
<i>hmcn1</i>	0.26#	0.03 $\pm$ 0.02	0.69	0.09 $\pm$ 0.03
<i>cacna1da</i>	0.17#	0.27 $\pm$ 0.02	0.38#	1.08 $\pm$ 0.42