Evidence of differential gene transcription between electric organs and skeletal muscle in the mormyrid *Brienomyrus brachyistius*

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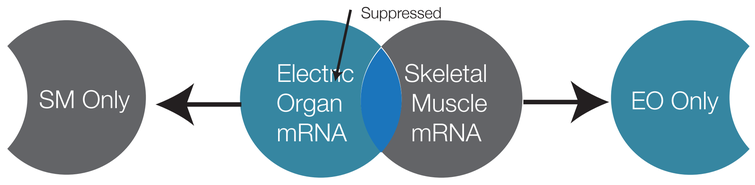
1. Background and Experimental Approach

Background: Weakly electric fish (gymnotiforms and mormyriforms) are a celebrated model system in neuroethology because of their remarkably convergent evolution on two continents (South America and Africa). Their adult electric organs (EOs) have remarkably similar physiological and behavioral functions, and both derive from differentiated skeletal muscle (SM). Despite these similarities, mormyrid and gymnotid EOs differ in their shape and biochemical properties. Specifically, mormyrid EOs are known to retain sarcomeric proteins after differentiation from SM, while gymnotids to not. Though much is known about the molecular biology of gymnotiform EOs, comparably little is known about mormyrid EOs. We present here a strategy we have used to identifygenes and proteins expressed uniquely in the electric organ.



Experimental Approach:

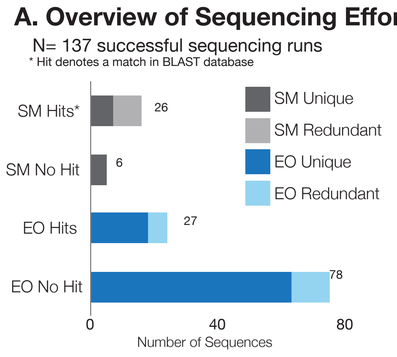
- Supressive Subtrative Hybridization (SSH) was performed on mRNA pooled from 5 individual *Brienomyrus brachyistius* from either SM or EO
- SSH product was cloned, DNA from clones were submitted for Sanger sequencing.
- DNA sequences were used to perform BLAST query against NCBI databases (blastx and blastn searches against nr database)

2. What genes are differentially expressed?

We **randomly selected** 143 EO library clones and 38 SM library clones for sequencing (average length = 647bp for all sequences)

A. Overview of Sequencing Effort

N= 137 successful sequencing runs
* Hit denotes a match in BLAST database



The majority of genes sequenced were not identified using BLAST. We presume the idenitiy of these sequences may be UTR regions of known genes.

B. Enriched in EO:

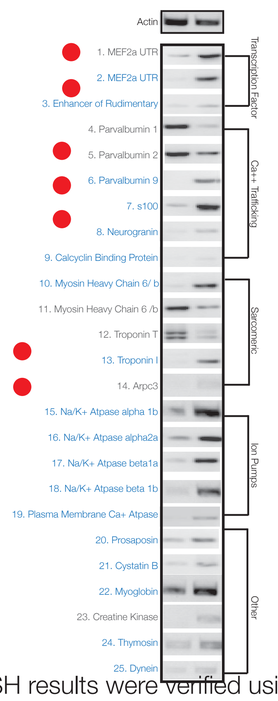
	Homologue Name	# Hits
● 2.	MEF2A UTR	1
3.	Enhancer of Rudimentary	1
● 4.	Parvalbumin "A"	1
7.	s100 Calcium Binding	3
8.	Neurogranin	1
9.	Calcyclin Binding Protein	1
● 10.	Myosin Heavy Chain	1
13.	Troponin I (tnn i2a.4)	1
14.	Na+/K+ transporting ATPase Alpha	
	Alpha 1 isoform	2
	Alpha 2 isoform	1
15.	Na+/K+ transporting ATPase Beta	3
16.	Plasma Membrane Ca++ Transporting ATPase 4	2
17.	Prosaposin	1
18.	Cystatin B	2
19.	Dynein Light Chain Roadblock-type 1	1
20.	Annexin a11	1
21.	Heat Shock Protein Beta-1	1
22.	Programmed Cell Death Protein 6	1
23.	Myoglobin	2

C. Enriched in SM:

	Homologue Name	# Hits
● 1.	MEF2A UTR	1
● 5.	Parvalbumin "B"	10
● 6.	Parvalbumin "C"	3
● 11.	Myosin Heavy Chain	9
12.	Troponin T (tnn t3b)	1
22.	Creatine Kinase-2	2

● Denotes **different isoforms** of the **same gene** type which were sometimes found in the EO and SM libraries. We further analyzed these sequences or performed additional cloning to understand seequence differences in (3) below.

C. RT-PCR of SSH Hits

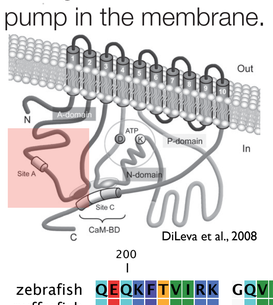


SSH results were verified using RT-PCR. Many of the hits were different in absolute or relative expression levels after 25 cycles.

3. What is different about genes in common?

Case Study #1: Non-Synonymous Substitutions in EO PMCA

Configuration of a PMCA pump in the membrane.

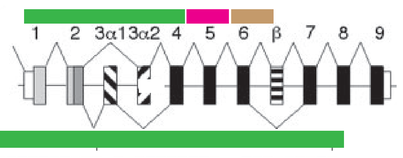


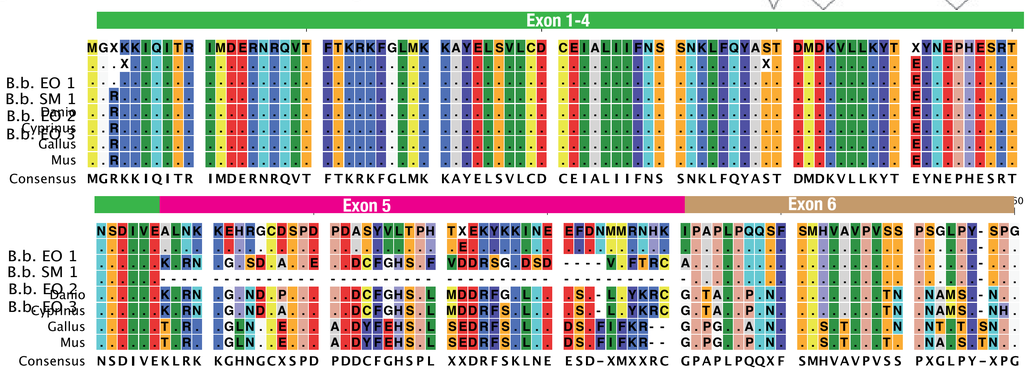
Four vertebrate genes encode plasma membrane calcium transporting ATPase (PMCA) . The EO specific transcript we detected shows non-synonymous substitutions (red arrows; synonymous in black) in an important domain for calmodulin binding (Site A).

Case Study #2: MEF2A Alternative Splicing in EO and SM

Myocyte Enhancing Factor 2A (MEF2a) is highly alternatively spliced. We have found evidence of differential alternative splicing between SM and EO in MEF2a. Below are consensus sequences (n=4 aligned sequences for each) for MEF2a cloned from EO and SM aligned to other vertebrate MEF2a genes (source: NCBI Protein)

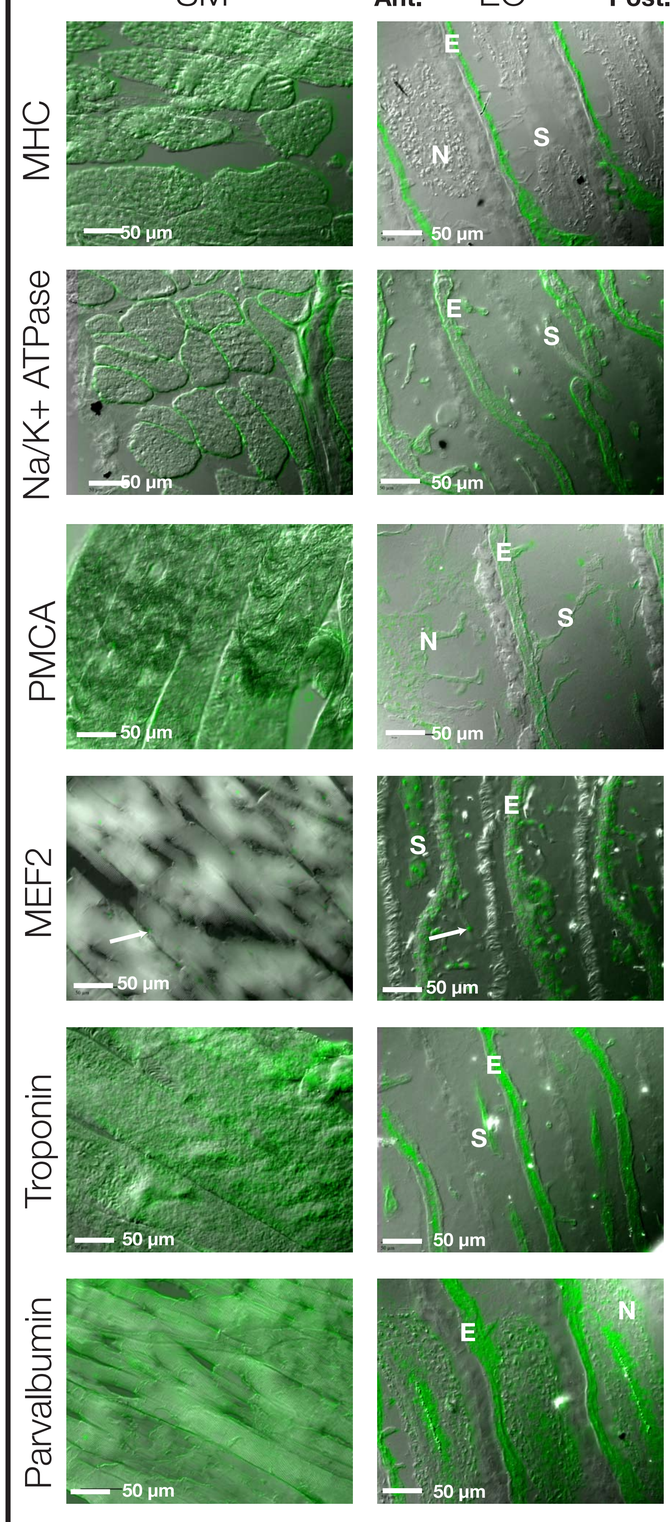
(Ramachandran et al, 2007)





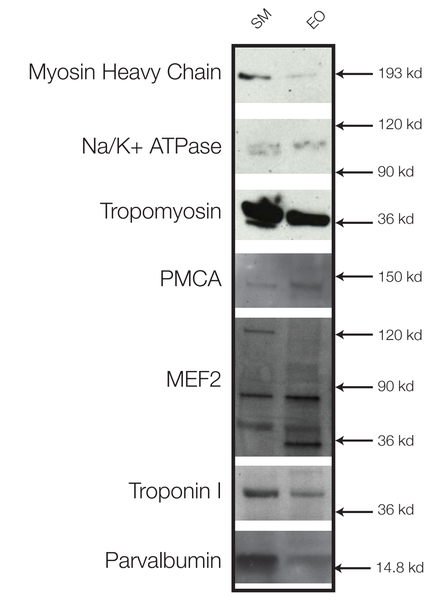
4. Are genes from SSH translated into protein?

A. Immunohistochemistry



- IHC images are superimposed DIC + fluorecence for selected antibodies representing proteins detected with SSH
- Proteins are spatially distributed in similar ways in both tissues (e.g. MEF2, NaKatpase)
- 6/6 antibodies show that proteins are not differentially expressed - antibodies less sensitive than SSH at detecting different gene/protien isoforms
- Western blots indicate approximate amount of protein per 10µg of sample & verify proper epitope is detected in IHC
- WB & IHC show genes detected in SSH screen are translated.
- Protein data + SSH data suggest different isoforms of these proteins in EO and SM (e.g. MEF2)

B. Western Blots



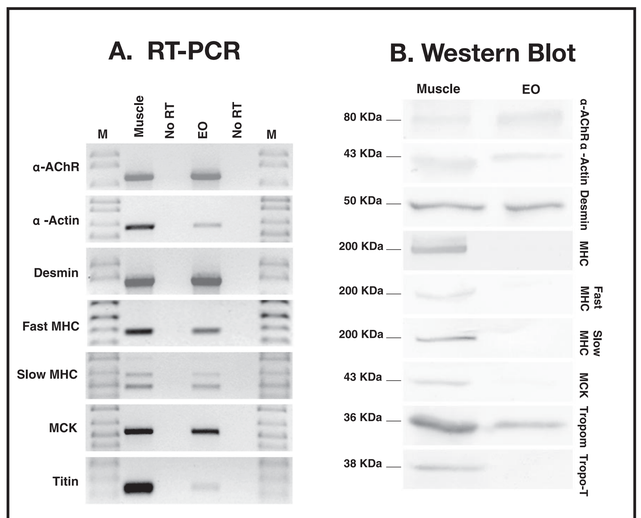
C. Antibodies & Concentrations

Antigen	Name	Host	WB	IHC	Expected Size
MEF2	C-21	Rabbit	1:50	1:50	40-65 kd
Myosin Heavy Chain	MF20	Mouse	1:5000	1:1000	200 kd
Na/K+ ATPase alpha	a5	Mouse	1:100	1:10	113 kd
Parvalbumin	MAB1572	Mouse	1:1000	1:50	12kd
Tropomyosin	CH1	Mouse	1:200	N/A	36kd
Troponin I	MAB1691	Mouse	1:100	1:100	27.6kd
PMCA	5f10	Mouse	1:1000	1:500	149kd

IHC: Primary antibody concentrations are listed in above table, secondary antibody used was a Alexa488 conjugated anti-host (1:200)
WB: Primary antibody concentrations are listed in above table, secondary antibody used wsas a HRP-conjugated anti-host (1:5000) with ECL detection (GE Biosciences).

5. Discussion

- In South American electric fish (Gymnotidae), the transition between skeletal muscle and electric organ is associated with the down-regulation of sarcomeric proteins, and the up-regulation of EO-specific proteins (Patterson and Zakon, 1997). African electric fish (Mormyridae), unlike gymnotids, retain sarcomeric proteins during this transition, histologically appearing more “muscle like”
- Little is known about the mechanisim that “induces” SM to transform into EO. Denervation studies have shown that the nerve is not required for EO formation in mormyrids (Szabo and Kirshbaum, 1983).
- There is considerable evidence (e.g. Unguez and Zakon, 1998; 2002) that innervation is required for electric organ development in gymnotid electric organ. Denervated gymnotid EOs “revert” to SM in the absence of innervation, expressing SM proteins.
- Recent work (Cuellar et al., 2006) suggests that innervation may post-transcriptionally repress protein translation in EO, while mRNAs are still expressed.



Data representing similar effort in *Sternopygus (gymnotid)* figures from : Cuellar et al. (2006)

Our work shows no evidence of post-transcriptional regulation of protein in mormyrids. Taken with Szabo and Kirshbaum’s (1983) findings that innervation is not required for normal EO development, this suggests these convergent EOs may develop using different mechanisims. **Our data suggests that differences between EO and SM in mormyrids may be due to mechanisms such as alternative splicing and/or neofunctionalization of gene duplicates (e.g. Zakon et al., 2006).**

6. Acknowledgements

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References: Cuellar et al (2006). FASEBJ v.20(14) pp. 2540; Zakon et al (2006). PNAS v. 103(10) pp. 3675; Unguez and Zakon (1998) J. Neurosci. v18(23) pp. 9924; Unguez and Zakon (2002) J. Neurobiol. v53(3) pp. 391; Patterson and Zakon (1997) Dev. Biol v 186(1) pp115.; Szabo and Kirshbaum (1983) in *The Physiology of Excitable Cells* pp. 451-460

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