

Proteomic Analysis of *Torpedo Californica* Electroplaque as a Model for the Neuromuscular Junction

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Abstract

The neuromuscular junction (NMJ) has been used as a model synapse, where nerve cells communicate with and activate muscle cells. Most effort has been dedicated to understanding electrical transmission and signal transduction, and the signals from the nerve that initiate and maintain the NMJ cellular specialization at the point of nerve/muscle contact. However little is known about the protein makeup of the NMJ. The *Torpedo* electroplaque, which is primarily comprised of NMJs, has been extensively used as a model to understand the electrical, biophysical, and molecular properties of the NMJ. In this study we used the *Torpedo californica* electroplaque to explore the proteome of the NMJ.

We have generated a partial proteome map of *Torpedo* electroplaque. Overall, the gel array contained more than 224 spots of which 56 were confidently identified using MALDI-TOF-TOF MS/MS and database search against NCBI nr protein database. All of these proteins have human or mice homologues. However, several proteins with distinct mass fingerprints did not match any of the known proteins in the database. These proteins were manually matched using Blast MS of short sequence tags. Additionally, *Torpedo* electroplaque was found to contain 8 isoforms for creatine kinase with different pI and molecular masses. Sequence homology between these isoforms was estimated to be around 40% as judged from partial *de novo* sequencing of the peptides obtained from these isoforms. LCM MS/MS analyses of the membrane fraction resulted in the identification of 70 proteins by database mining of homologous sequences in human and mice. Finally we generated a cDNA library from the *Torpedo* electroplaque and sequenced 513 clones. These sequences were then translated into 6 reading frames, *in silico* digested, and indexed as an in-house database.

Overall, the survey of the proteome of *Torpedo californica*, for which a genomic database does not currently exist, resulted in identification of a total of 105 unique proteins. A handful of known NMJ-associated proteins are within the obtained list. Defining molecular players associated with the *Torpedo* electroplaque may bring insight into proteins involved in the NMJs which are difficult to obtain due the relatively small size of NMJ in human or mice (30nm). Further functional characterization of these genes/proteins may expand our understanding of the factors vital to NMJ stability and function.

1. Background and Introduction

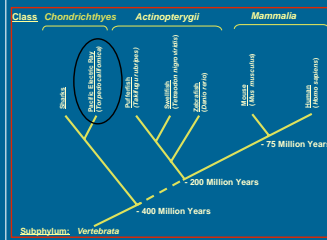


Figure 1.2 A cladogram showing distant relationship of *Torpedo* and other vertebrates. The common ancestor of shown species is subphylum vertebrate. *Torpedo californica* (encircled) like sharks and other rays are more ancient species that falls under the Chondrichthyes class (cartilaginous fish which lack "true bones"). Zebrafish, puffer fish, and swell fish belong to the Actinopterygii (ray-finned fishes) class. Human and mice belong to class Mammalia and are closely related to actinopterygii than chondrichthyes.

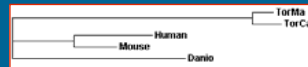


Figure 1.2 A cladogram showing distant relationship of *Torpedo* to human, mice and zebra fish based on amino acid sequences. Shown is the cladogram of *Torpedo californica* (Torca), *Torpedo marmorata* (Torma), human, mouse zebra fish (Danio). The cladogram was produced by analyzing the creatine kinase (CK) amino acid from these species. Both Torma and Torca are very distant to other vertebrates and even other fish (zebrafish).

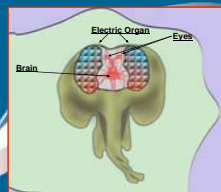


Figure 1.3 Schematic representation of *Torpedo californica*. Shown is the anatomical location of *Torpedo californica* electric organ. The electric organ is a kidney shaped modified muscle (electroplaque), located in the pelvis area.

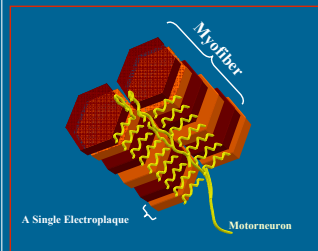


Figure 1.3 Schematic representation of *Torpedo californica* electric organ. Shown is the schematic view of electric organ. In *Torpedo californica*, the electric organ is modified gill muscle capable of producing electricity. Modified myofibers form electroplaques which are innervated on one side by motor neurons. Activation of electroplaques generates a series of depolarization which is the source or electropotential.

2. Research Design and Methods

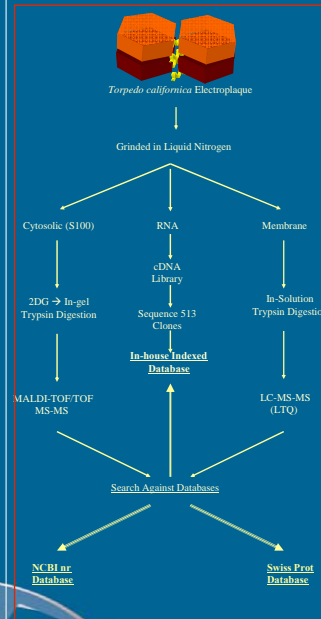


Figure 2.1 Methods. Frozen *Torpedo californica* electroplaque (150mg) was ground in liquid nitrogen. The (S100) cytosolic fraction (250ug) was then processed for 2-DG (17cm pH 3-11) and MALDI-TOF-TOF analysis of in-gel digested spots. The membrane fraction (200ug) was in-solution digested with trypsin. Resulting peptides were analyzed by LC-MS and MS/MS using nano-LC-packing system coupled to a Thermo-Finnigan LTQ instrument. The proteins were identified using Sequest and Mascot search engines.

3. Results

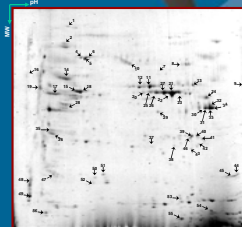


Figure 3.1 Cytosolic protein spots identified by 2-DG followed by MALDI-TOF-TOF-MS/MS. Protein spots (arrows 1-56) were isolated and subjected to in-gel protein digestion with trypsin. Digested proteins were then identified using mass peptide fingerprinting by MALDI-TOF and peptide sequencing by MS/MS. Asterisks represent creatine kinase.

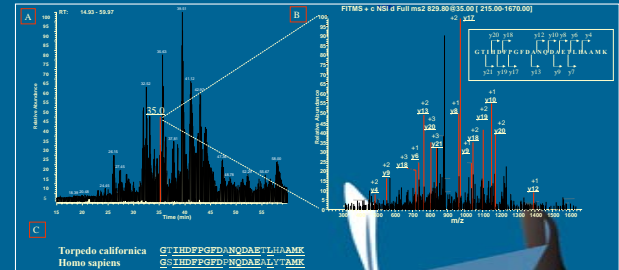


Figure 3.2 Peptide mass fingerprint of *Torpedo californica* annexin.

Panel A shows the peptide mass fingerprint of *Torpedo californica* annexin using LTQ (LTO) and and its subsequent sequencing by MS-MS (panel B). Peak 1107.70 was selected and sequenced by tandem mass spectrometry (MS/MS) where Y ions were positively identified (inset in panel B). *Torpedo californica* in-house indexed database (see Materials and Methods) was then searched to identify this protein as annexin with high XCorr (70.3).

Panel C shows the homology between the amino acid sequence for identified peptide between human and *Torpedo*. Using available databases (NCBI and SProt) would not identify this annexin.

GENE SYMBOLE	cDNA Clone #	PEPTIDE (XCORR)
ACbE	7	5
Rapsyn	5	1
α-AChR	2	2
γ-AChR	3	1
β-AChR	2	4
δ-AChR	4	2
Presynaptic Calcium Channel	1	
VAT-1	7	7
α NA ⁺ /K ⁺ ATPase	3	3
Chloride Channel	10	10
SITS-Binding Protein	7	7
Vimentin	4	4
Synuclein	2	2

Table 3.1 Known *Torpedo*, NMJ associated proteins

Name	Protein/GI ID	MS/MS Peptide #
		Membrane
Phosphoglycerate kinase	P51903	2
Tubulin beta-1 chain	Q9VHC3	7
Adenylate kinase	Q9R0V5	1
GAPDH	AAU 89484	2
Triosephosphate isomerase	P00940	6
Gamma Endase	P17183	3
Ubiquitin	C.A35999	1
Thioredoxin peroxidase	NP_035164	1
...ing factor, arginine/serine-rich 5	Q13243	1
6-phosphofructokinase	P47857	1
Plectin 1	Q14152	1
		S100

Table 3.2 Novel Proteins of *Torpedo californica* Electroplaque



Figure 3.3 Possible (8 different) post-translational modifications of creatine kinase (CK).

4. Results

- A total of 105 proteins were identified:
 - 35 Cytosolic proteins (MALDI-TOF-TOF-MS-MS)
 - 70 Membrane proteins (Nano-LC-MS-MS)
- 20 of 36 known *Torpedo* proteins were identified
 - 15 Known NMJ proteins
 - 10 Known NMJ proteins, previously unknown in *Torpedo*