BBQ - Do the ‘size principle’ and ‘minimal wiring diagram’ principles apply to the interscutularis muscle? What are the variables in the microanatomy?

**What did they do?** This study generated a complete wiring diagram of a peripheral neuromuscular circuit, to allow analysis based on direct observation rather than statistical inference, as done in previous studies.

**What did they find?** 1) The anatomical underpinnings of the ‘graded tensions in the size principle’ (that motor units are recruited from small>large number of muscle fibres activated as contraction increases) as postulated by Henneman, had not been previously proven. There was a predominance of small motor units over larger ones, a distribution pattern paralleled with previous studies on twitch tensions (muscle contraction), allowing the investigators to verify the theory. 2) Relationships between axonal calibre (A), arbour length (L) and motor unit size (M) were established. \[ A=\sqrt{M}, L=A, L=\sqrt{M} \]. These results support the idea that the principle determinant of axonal cross-sectional area is the energy cost associated with the axonal membrane. 3) When studying the axonal structural microanatomy, the preterminal branching was found to be highly variable and no two neurones or NMJ’s were found to look the same. This contrasts with the stereotypy (repetitive behavior) of neuromuscular innervation in invertebrates. 4) Axonal trajectories did not adhere to the minimisation of wiring length principle proposed by Cajal, in fact a superfluous 25% of wiring length was measured adding metabolic load to cell. This is insignificant however; the metabolic load of muscle contraction far exceeds that of axonal conduction. Sub-optimality does not imply irrelevance of the optimisation principle, rather suggesting that other factors play a significant role.

**Paper 2 – The Overall Morphology of Neuromuscular Junctions as revealed by Scanning Electron Microscopy, Amy Higgs.**

BBQ - What are the technical and quantitative limitations of this study and what is the significance of variability in NMJ morphology between species?

**What did they do?** This paper examined the NMJ’s from three different species of animal. It used electron microscopy after removal of connective tissue obstructing the view of the synaptic depressions by HCl hydrolysis. The surface features of the NMJ’s and organisation of the subsynaptic sarcolemma were imaged and detailed.

**What did they find?** The morphological variability between animal species was observed, and is described in the abstract and within the paper. The new technique using HCl allows the nerve ending to be easily pulled away from the muscle surface, allowing the investigators to obtain an ‘en face’ view of the synaptic depressions, in which the most striking inter-species differences were observed.

**Paper 3 – Macromolecular Connections of Active Zone Material to Docked Synaptic Vesicles and Presynaptic Membrane at Neuromuscular Junctions of Mouse, Katie Mitchell.**

BBQ - What molecular components of the AZM structure affect the probability/physiology of primary/secondary vesicle exocytosis and transmitter release in different species?
ACTIVE ZONES – The presynaptic plasma membrane of axon terminals, where initial events in impulse transmission take place. Comprise of three prominent structures, the AZM, docked synaptic vesicles, and macromolecular aggregates including trans-membrane Ca\(^{2+}\) channels.

**What does it do?** - Function of synaptic vesicles and Calcium channels well understood: depolarisation of presynaptic membrane > Ca\(^{2+}\) channels open > Ca\(^{2+}\) entry into terminal > protein mediated fusion of docked vesicles > exocytosis > neurotransmitter acts on postsynaptic cell (circa 1970). AZM, only recently studied (2001/04) in frogs using electron tomography (ET). They found;

**Active Zone Material (AZM)**

- **What is it?** - Dense aggregates of cytoplasmic macromolecules which are attached to the membrane.
- **Function** – Helps dock synaptic vesicles on presynaptic membrane, strategically anchors Ca\(^{2+}\) channels in the membrane, involved in vesicle fusion with membrane during synaptic transmission.
- **How can we check this?** – By identifying comparable relationships between AZM macromolecules, docked vesicles, and presynaptic membrane macromolecules at synapses where the active zones have different gross topography from that at frog NMJ’s.
- **Hence this study** – To determine by ET whether there were such relationships at NMJ’s of mouse muscles, where the Active Zones are smaller, and the AZM has different distribution relative to docked vesicles, than at frog NMJ’s.

**What did they find?** – The AZM of mouse is arranged in 2 parallel bands attached to the cytoplasmic surface of presynaptic membrane. Each band overlies one of the double rows of macromolecules containing Ca\(^{2+}\) channels. Mouse AZM is much smaller than of frog, and has a bilateral arrangement. Frog has unilateral arrangement. The ‘Beam, Rib and Peg’ analogy corresponds to the AZM arrangement in both species, and the number of connections made between ends of AZM to vesicles is similar. However these connections to the AZM in mouse are distributed over 2 hemispheres to form opposed bands, but in frog over single hemisphere to form single band. The investigators hypothesised the SDVs are manifestations of an early stage in the recycling of vesicle membrane after PDVs have fused.

### Paper 4 - Preferred Sites of Exocytosis and Endocytosis Colocalize during High- But Not Lower-Frequency Stimulation in Mouse Motor Nerve Terminals, Georgia Taylor.

**BBQ** - What is the physiological significance of the appearance, disappearance and co-localisation of hot-spots obtained at the 40/100 Hz stimulation frequencies used in this paper? Are the differences in kinetics of the reporter dyes significant?

**What did they do?** They used two fluorophores (spH and FM4-64) to examine simultaneously the sites of exocytosis and endocytosis in mouse motor nerve terminals.

**What did they find?** In a previous paper the investigators had shown nerve stimulation caused spH fluorescence hot spots, marking exocytic sites. It was shown in this study that nerve stimulation in the presence of FM4-64 caused hot spots of FM4-64 fluorescence, with similar features to that of the spH spots except the rate of disappearance was considerably slower. This is due to 'bulk endocytosis' – uptake of large cisternae or endosomes, which slowly bud synaptic vesicles. This occurs during intense stimulation by high frequency trains of action potentials. At 40Hz, exocytic and endocytic spots did not colocalise, but at 100Hz strong colocalisation of spH and FM4-64 occurred. The study concluded this represented important components of the synaptic vesicle cycle during high-frequency stimulation.
The Interscutularis Muscle Connectome
Ju Lu, Juan Carlos Tapia, Olivia L. White, Jeff W. Lichtman
2009

• The *interscutularis* is the muscle joining the base of the ear to the middle of the skull.
• Connectome: a complete connectional map of a neural circuit, e.g., the interscutularis muscle and all the nerves which innervate it.
• The "pooling data, probability" methods assume that "connectional specificity at the level of classes of cells suffices to account for properties of circuits … within a class each neurons connectivity is established independently. Oversimplified, some say "doomed to failure", therefore our new method of analysis.
• Observational method hopes to see stochastic aspects which will allow further probability analysis or specific organisation which requires observation.
• Motor unit - the number of NMJs innervated by one axon.

Aim: to generate the complete wiring diagram of a peripheral neuromuscular circuit.

• Previous connectomes described in nematodes such as C. Elegans have been valuable in further neuronal analysis, there for it is hoped that this connectome analysis may have implications.
• The "interscutularis" is a full set of motor axons and the complement of fibres they innervate was visualised using transgenic technologies and automated optical microscopy.
• The NMJ was chosen because it is peripheral, highly studied and has discrete innervation. Study of the functional organisation of the NMJ is what lead to the discovery of the "size principle".
• Size principle: motor neurones are recruited in order of increasing "twitch tension" (muscle contraction, fibre contraction, twitch force).

Methods

• Mice were anaesthetized and interscutularis muscle with a segment of nerve were removed fixed, mounted and squeezed with magnets to flatten.
• Muscles were fibre typed by blocking then incubating with myosin 1 and 2 antibodies, and then secondary antibodies.
• YFP-16 was used to stain 100% of the neurones.
• All imaging was done using a confocal laser scanning microscope.
• TED is the minimal number of operations (insertions, deletions and node relabeling) required to transform one "tree" into another.

Images showing colour coded axons before and after extramuscular neural bifurcation. All branches entered the same muscle as an end point and there was no observed tendency to branch at any particular point, refuting any functional role possibilities.

As xons of the left and right connectomes of the interscutularis muscle colour coded according to the size of their motor units. L1 and R1 are the largest axonal trees and L15 and R14 are the smallest, numbers underneath each represent motor unit size.

The white squares represent overlapping images of YFP labelled axons in an interscutularis muscle taken by confocal microscopy. Each image was sliced across the XY axis and the cross-sections were used to colour code the axons and then reassembled in 2D.
Figure A shows motor unit size is skewed toward the smaller end of the spectrum in all connectomes examined. Twitch tension distribution is similar to motor unit size distribution. Figure B demonstrates the decreased segment length of the axon, the more it branches. Figure C shows the linear relationship between the normalised cross-sectional area (A) of the axon and the square root of the motor unit size (M). Figure D is the linear relationship between the axonal arbor length (L) and the normalised cross-sectional area (A). Figure E: Linear relationship to the square root of M.

Symmetry

Topologies

A. The largest motor units represented as a topological map to show symmetry
B. Ten equally sized motor units (1/10) from a different muscle
C. TED (minimum number of changes to make trees symmetrical) analysis of the largest motor units in the 6 muscles, no significant difference
D. TED analysis of medium size axons (as in B), no significant difference

An example of the non-application of the theory of minimising neural wiring length
**Conclusion**

- Next: now that the connectome of a small muscle has been elucidated, more mammalian neural analysis of more complex structures, maybe even the brain one day may be possible. The inferences of microanatomy may be applied to the rest of the physiology of mammals.

- Four new principles found:
  1. Motor units recruited are skewed towards the smaller end of the scale.
  2. Quantitative relationships between axonal calibre arbor length and motor unit size.
  3. Each motor neuron is unique. If defined by size principles then no similarity, topological differences even though identical genetic background within one animal and presumable same environment, this is unique to mammals.
  4. Minimalizing wiring length hypothesis is not correct, activity dependant reorganisation is the method of wiring set up.

**INTRODUCTION TO PAPER**

- **AIM:**
  - To study the overall morphology of the NMJ
    - Surface features of muscle and nerve
    - Surface features of NMJ
    - Organization of subsynaptic sarcolemma
  - To compare NMJ morphology in different vertebrate animal species

**METHODS AND TECHNIQUES**

- Modified version of HCl-collagenase procedure (omitting digestion with collagenase)
  - Digestion of extracellular connective tissue to prevent impairment of ultrastructural analysis

- Scanning electron microscopy

**BACKGROUND**

- Basic morphology of the Neuromuscular Junction

(Fig. 1) Surface view of the NMJs in the sartorius muscle of the frog

**THE OVERALL MORPHOLOGY OF NEUROMUSCULAR JUNCTIONS AS REVEALED BY SCANNING ELECTRON MICROSCOPY**

JUNZO DESAKI and YASUO UEHARA

Fig. 2  Frog nerve endings showing small lateral projections

Fig. 3  Synaptic gutters and junctional folds of frog NMJ after detachment of nerve endings from muscle surface

Fig. 4  Surface view of the NMJs in the posterior latissimus dorsi muscle in the zebra finch

Fig. 5  Varicose swellings at ramifying nerve endings of finch NMJ

Fig. 6  Synaptic depressions in sarcolemma of finch NMJ

Fig. 7  Surface view of NMJ in Chinese hamster

Fig. 8  Synaptic depressions of Chinese hamster NMJ

**WHAT NEXT?**

- Quantitative study of NMJ morphology
- Comparison of single ‘en plaque’ NMJs and multiple ‘en gruppe’ NMJs
CONCLUSION

- This study provides a detailed view of NMJ morphology, demonstrating its variation between species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Nerve Cell</th>
<th>Schwann cell</th>
<th>Sub synaptic specialisations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frog</td>
<td>Thin threadlike strands 2-5μm wide and 20-30μm long, not perforated, no radials.</td>
<td>Small irregularly shaped regions. Small, round holes or wide, irregularly shaped regions, enclosed by irregular membranes.</td>
<td>Multiple punctate regions of differing sizes.</td>
</tr>
<tr>
<td>Zebra Finch</td>
<td>Identifiable rows of radial elements 2-5μm in diameter.</td>
<td>Identifiable rows of radial elements 2-5μm in diameter.</td>
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</tr>
<tr>
<td>Chinese Hamster</td>
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Macromolecular Connections of Active Zone Material to Docked Synaptic Vesicles and Presynaptic Membrane at Neuromuscular Junctions of Mouse

By SHARUNA NAGWANEY, MARK LEE HARLOW, JAE HOON JUNG, JOSEPH A. ZIETLE, DAVID MISS, JING XU, ROBERT M. MARSHALL, AND UEL JACKSON MCMAHAN.
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Background

Active zones are made up of:
- AZM
- Docked synaptic vesicles
- Aggregates of macromolecules

Synaptic Transmission:
- Incoming nerve impulse causes depolarisation
- Ca^{2+} channels open allowing influx of Ca^{2+}
- Fusion of docked vesicles
- Exocytosis of neurotransmitter

Methods

- **Aim:** to use electron tomography to view the active zone material (AZM) at mouse neuromuscular junction.
- **Hypothesis:** the AZM is a multifunctional organelle that helps dock synaptic vesicles on the presynaptic membrane, anchors Ca^{2+} channels in the membrane at a particular distance from each other and is involved in fusion of vesicles with the synaptic membrane during synaptic transmission.

- **Figure 1:** shows the paired double rows of membrane macromolecules at a mouse NMJ.
Figure 2: shows a 3D model of the active zone.

Figure 3: shows the filamentous macromolecules which connect the synaptic vesicles to the AZM.

Figure 4: shows the active zone including the position of the secondary docked vesicle beyond the ends of the AZM bands.

Figure 5: shows the areas of direct contact between primary and secondary docked vesicles and the presynaptic membrane.

Figure 6: shows the distribution of connection sites of AZM filaments on the hemispheres of primary docked vesicle.

Figure 7: shows the position of the beams and ribs relative to the AZM. The beams run the full length of the AZM band, orthogonal to the ribs.
Figure 8: Shows a comparison of neuromuscular arrangement in mouse and frog. The mouse has a bilateral arrangement whereas the frog has a unilateral arrangement.

Further Study

- What allows higher fusion rate in mouse – Ca²⁺ channels?
- What is the exact function of the secondary docked vesicles?
- Repeat study in other animals.

Preferred Sites of Exocytosis and Endocytosis Colocalize During High-But Not Lower-Frequency Stimulation in Mouse Motor Nerve Terminals.

Gaffield M.A, Tabares L., and Betz W.J.

Hypothesis: hot spots of endocytosis, possibly in the form of bulk uptake, occur at of very near to highly active exocytic sites during high-frequency stimulation.

Aim: Find the degree of colocalization of endocytic and exocytic sites in living motor nerve terminals during different stimulation intensity levels.

Found that there exists a multiple of similarities between the sites of exocytic and endocytic regions, including size, density and rate of appearance of the fluorescence markers.

Methods

- Imaging by synaptopHluorin (spH) expressing mice and FM4-64 dyes that both fluoresce under different physiological conditions. Enabling the imaging and tracking of exocytosed and endocytosed vesicles.
- spH fluoresces was used to signify exocytosis sites at the nerve terminals. FM4-64 highlighted sites of endocytosis.
- FM4-64 is most often used for labelling Bulk endocytosis, which is known to occur during times of intense activity, this form of endocytosis is characterised by a lot of membrane being added to the surface via vesicles.
- Nerve terminal was electrically stimulated, and the changing degree of fluorescence intensity for each dye was recorded along with the distribution, size and occurrence of the spots.

More on the Fluorescence dyes

- spH – genetically modified GFP that is pH-sensitive. Fuses to the luminal site of a vesicle at an acidic pH (within the vesicle), spH is non-fluorescent. Upon exocytosis and the spH dye is exposed to the neutral extracellular medium and therefore fluoresces.
- FM4-64 – FM dyes are mostly used to label endosomes but can also be used to reveal sites of exocytosis. The dye will only fluoresce significantly when in a hydrophobic environment (a lipid-rich membrane). FM4-64 was present in the extracellular solution.

Found labelled membrane at the surface, added by exocytotic vesicles, absorbs the FM dye.

On the basis that spH fluoresce will be quenched whenever within a vesicle due to the acidic environment, FM dye remains fluorescent following endocytosis and is therefore used to track the endocytosed vesicle.

Results – Figure 1

A) Shows that the FM4-64 dye fluorescence increases greatly as it intertines within the surface membrane. Results taken at the cells resting potential.
B) Fluorescence image of FM4-64 following stimulation (100Hz for 30seconds).
C) The increase of fluorescence intensity for spots compared to the whole terminal following stimulation.
Figure 2

Results highlight the similarities between FM4-64 and spH spots occurring following stimulation.

A) Shows the occurrence of spots in two different preparations following 100Hz stimulation for 30sec.

B) Graph shows both the similarity of the size of the spots and that they are significantly smaller than randomly placed pixels.

C) Plot of the same data presented in figure 1C but including data for spH spots. Shows the similarity of the increased fluorescence of both dyes over time.

Figure 3

Highlights the continued fluorescence of FM4-64 following internalisation of the endosome.

A) Shows that the dispersal of fluorescence for FM4-64 happens much more slowly (spH decay dotted line). The non-spot areas shows the fluorescence of FM4-64 outside vesicles.

B) Data shows that the majority of vesicles exocytosed during stimulation are internalised by the end.

Figure 4 and 6

Image 4) shows the close proximity of spH and FM4-64 spots. Image taken following 100Hz stimulation for 30sec with both dyes present in the same terminal.

Image 6) Much less overlap of spots. Image shows the fluorescence change in response to a 30sec, 40Hz stimulation. Overlap in third columns and is depicted by yellow colouring.

Figure 5

Quantification of spH and FM4-64 spot overlap.

A) Shows the nearest neighbouring spots for FM4-64 compared to a randomly generated distribution of spots.

B) Nearest neighbour distances for FM4-64 and spH spots.

C) Total fluorescence change in each pixel was determined. Degree of overlap is significantly greater than chance, depicted by dashed line.

Conclusion

The data presented in the results showed that:

- Similarities exist between the size, intensity, distribution and rate of appearance of the spots.
- Sites of exocytosis and endocytosis occurred in relatively close proximity, but only at high frequencies.
- Answered hypothesis by showing that there is a high degree of overlap for spots representing endocytosis and exocytosis.