Neuromuscular Junction in Health and Disease Mini Symposium 5

Neuromuscular Junction in Motor Neuron Disease: SMA and ALS

28th Nov 2011

Paper 1: Presented by Laura Murray

Murray LM, Comley LH, Thomson D, Parkinson N, Talbot K, Gillingwater TH. Selective vulnerability of motor neurons and dissociation of pre- and post-synaptic pathology at the neuromuscular junction in mouse models of spinal muscular atrophy. Hum Mol Genet. 2008 Apr 1;17(7):949-62

**BBQ:** What are the mechanistic differences within and between Fasyn and Desyn muscles in the different SMA mouse models (including ectopic synapse formation) and how do these confer susceptibility to Smn?

**Aim:** Provide insight into the disease mechanisms underlying SMA by understanding the pathological events occurring in NMJs and skeletal muscle fibres

**Methods:** Two different mouse models, Smn−/−; SMN2 (type 1 SMA) and Smn−/−; SMN2; Δ7 (less severe phenotype, lacks exon 7). Used immunohistochemistry, quantitative fluorescence, confocal microscopy, and electron microscopy.

**Conclusions:** Muscle fibre-type and body location are important factors in regulating synaptic vulnerability in SMA. Presynaptic pathology occurs early on in disease course. SMNΔ7 transgene attenuates pre-synaptic pathology. Pre- and post-synaptic changes can occur independently of one another. FaSyn motor neurons may be particularly vulnerable in SMA.

Paper 2: Presented by Amanda Swan


**BBQ:** What is the role of motor unit size in the selectivity of degeneration/compensation in motor pools in SOD1 mice and is the selectivity of YFP expression helpful for establishing this?

**Aim:** To understand how loss and growth occur at individual NMJs and see whether the two types of behaviour of die-back and compensatory growth, occur in different branches of single neurons or, alternatively, whether entire motor units are of one type or the other.

**Methods:** G93A SOD1 mice were bred to transgenic mice in which all motor axons were labelled with yellow fluorescent protein. They used in vivo time-lapse imaging and the AChRs were labelled with αBTX.

**Conclusions:** Degenerative versus regenerative changes are largely confined to distinct populations of neurons within the same motor pool. Degenerative branches are easily distinguishable from those undergoing compensatory reinnervation.
Paper 3: Presented by Laura Wagstaff


BBQ: Method, model, mechanism and relevance: how does this study of I/R in SOD1 mice measure up?

Aim: Test whether motor terminals in G93A-SOD1 mouse model of familial ALS and more vulnerable than WT and when this occurs as well as if there is a difference between fast and slow muscle innervating fibres.

Methods: Tourniquet-induced ischemia/reperfusion analysed by confocal microscopy.

Conclusions: Fast muscles more vulnerable than slow muscles to injury from I/R oxidative stress. This early vulnerability appears about 1 month before disease is symptomatic and so might be a sign of impending motor terminal degeneration. Notable, regeneration occurs if the animal is allowed to recover from its injury.

Paper 4: Presented by Jilly Hope


BBQ: What are the limitations of the technique (CME/YFP) and methodology (ENU mutagenesis) and why is WldS protection gene-dose dependent?

Aim: To use fibre optic confocal microendoscopy (CME) to analyse the axonal and neuromuscular patterns seen in three mouse models of neuromuscular degeneration, WldS, SOD1G93A and ostes.

Methods: Confocal microendoscopy, a new method which allows the pathology of synapses and axons to be monitored in vivo, along with YFP marking and transgenic mice strains.

Conclusions: ENU mutagenesis and CME allow for excellent imaging and identification of degenerative phenotypes in vivo. It is diagnostically valuable because it allows the phenotype of mutants to be imaged in vivo in situations that would have previously been impossible. This is useful because of the idea that malfunction may already be occurring in axons and their terminals long before symptoms of this appear.
Selective vulnerability of motor neurons and dissociation of pre- and post-synaptic pathology at the neuromuscular junction in mouse models of spinal muscular atrophy


Spinal muscular atrophy (SMA)

- Alpha motor neurons in ventral horn of spinal cord targeted
- Results in denervation and atrophy of muscles in the limbs and trunk
- Caused by low levels of a protein produced by the survival motor neuron (SMN) gene
- SMN gene has two near identical copies: SMN1 – loss or disruption reduces protein levels
  SMN2 – copy number determines disease severity

Aims and hypothesis

Aim: Provide insight into the disease mechanisms underlying SMA by understanding the pathological events occurring in NMJs and skeletal muscle fibres

Hypothesis: There is significant disruption of neuromuscular synapses in SMA

Methods

Used two different mouse models:

- Smn−/−; SMN2 (type 1 SMA)
- Smn−/−; SMN2; Δ7 (less severe phenotype)

Techniques:

- Immunohistochemistry
- Quantitative fluorescence
- Confocal microscopy
- Electron microscopy

Motor nerve terminal loss, neurofilament accumulation and muscle fibre shrinkage in Smn−/−;SMN2 mice

TVA = transversus abdominis (slow-twitch, short nerve stumps)
LAL = levator auris longus (fast-twitch, short nerve stumps)
Lumbrical muscles (fast-twitch, long nerve stumps)

TVA more affected than LAL and lumbricals and has significant shrinkage

Intact ultrastructure but abnormal neurofilament accumulation in Smn−/−;SMN2 mice

A – Intact myelin sheath and neurofilaments
B – Intact myofibrils
C – Neurofilament whorls
D – Abnormal neurofilament accumulations

Suggests involvement of mechanism distinct from Wallerian degeneration

Black arrow = intact myelin sheath
White arrow = neurofilaments
Synaptic pathology at the NMJ is underestimated when based on occupancy counts alone

At P6 most synapses are polyneuronally innervated, need to look at number of inputs rather than just % of fully occupied endplates

Smn-/-;SMN2 mice have a significant reduction in average number of inputs compared to WT

Could reduced number of synaptic inputs also result from defective synapse formation?

Motor terminal loss, neurofilament accumulation and muscle fibre shrinkage in Smn-/-;SMN2;Δ7 mice

- Synaptic pathology in all muscle groups
- LAL less affected than the TVA
- Attenuation of pre- and post-synaptic pathology differently modulated by the SMNΔ7 transgene

Post-synaptic endplate shrinkage and pre-synaptic terminal loss can occur independently at the NMJ

- Significant reduction in endplate area in P6 Smn-/-;SMN2 TVA compared to WT
- No correlation between endplate area and occupancy
Endplate shrinkage does not occur as a direct result of pre-synaptic pathology

Selective vulnerability of a subpopulation in the LAL

Higher levels of nerve terminal loss in the thinner, caudal band (B) of the muscle than the rostral band (A)

Patterns of innervation in the LAL of WT mice

- Distinct subpopulations of motor neurons innervate the rostral and caudal bands of the LAL, with no overlap
- Preferential loss of innervation at caudal band likely to represent a selective vulnerable population of motor neurons

Time-course analysis of synaptic pathology in the TVA and LAL of Smn-/-;SMN2 mice

C – Onset and progression of presynaptic pathology in the LAL and TVA of Smn-/-;SMN2 mice.
D – Pre- but not post-synaptic pathology present in TVA.
E – Both pre- and post-synaptic pathology absent in LAL.

Synaptic pathology progressively worsened with time and was always more severe in the TVA than the LAL.
Selective vulnerable motor neurons in the caudal band of LAL conform to a FaSyn phenotype

Motor neurons with FaSyn-like characteristics may be more likely to be vulnerable to SMA-induced synapse loss.

Conclusions

1. Muscle fibre-type and body location are important factors in regulating synaptic vulnerability in SMA
2. Presynaptic pathology occurs early on in disease course
3. SMNΔ7 transgene attenuates pre-synaptic pathology
4. Pre- and post-synaptic changes can occur independently of one another
5. FaSyn motor neurons may be particularly vulnerable in SMA

Future Studies

- Development of synaptoprotective treatments for SMA using whole-mount TVA and LAL muscles (lack of human material – limitation)
- Is SMN gene activity required in both neuronal and muscle tissue in order to alleviate the pathological phenotype?
  - Repeat current set of experiments in mouse models in which SMN levels have been reduced in muscle or nerve individually
- Is there a causal link between FaSyn characteristics and selective vulnerability?
  - Genetic tools to experimentally manipulate FaSyn and DeSyn characteristics not yet available (limitation)
- The mechanisms through which motor neurons and their synapses are specified to be, and develop into, FaSyn or DeSyn motor units
  - May provide information into vulnerability of motor neurons in SMA

A Compensatory Subpopulation of Motor Neurons in a Mouse Model of Amyotrophic Lateral Sclerosis

Annaliese M. Schaefer, Joshua R. Sanes and Jeff W. Lichtman
(2005)

Presented by Amanda Swan

Background

- Electrophysiology: Some motor axons attempt to compensate and result in enlarged axonal arbors.
- Histology: During the course of disease some axonal branches die back

Aim

- To understand how loss and growth occur at individual NMJs.
- To see whether the two types of behaviour of die-back and compensatory growth occur in different branches of single neurons or, alternatively, whether entire motor units are of one type or the other.
1. The mouse model

- G93A SOD1 mice were bred to transgenic mice in which all motor axons where labelled with yellow fluorescent protein.

2. Disease Progression

- **Presymptomatic**: indistinguishable from WT littermates. P41-77
- **Symptomatic**: hind limbs trembled and pulled in on raising the tail. P81-119
- **End stage**: hind limb paralysis. P123-145

3. Imaging NMJs and axons

- Laser scanning confocal microscope.
- Muscles: neck, diaphragm and hind limb.
- YFP: Axonal morphology
- αBTX: AChRs
- Antibody: Neurofilament 150
- Is YFP appropriate?

3.1 YFP a representative marker of axonal morphology

- Green: YFP staining
- Red: Neurofilament antibody staining
- Blue: AChRs labelled with α-BTX

Control mouse

3.2 Imaging NMJs and axons

P125-144: end stage with complete limb paralysis.

<table>
<thead>
<tr>
<th>Range of abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1: Completely denervated with normal AChRs (2/3)</td>
</tr>
<tr>
<td>A2: AChRs abnormal (1/3)</td>
</tr>
<tr>
<td>A3: Normal</td>
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* Thin axons probably represent reinnervating axons.
4. Relationship between these features to disease processes

- Fragmented tips resembled Wallerian Degeneration
- Thin smooth branches resembled naturally occurring synapse elimination

4.1 In vivo time-lapse imaging

- Mice anaesthetised
- 2% of AChRs labelled with αBTX
- Superficial junctions imaged using standard epifluorescence microscope.

4.2 Time course: in vivo time-lapse imaging

A1/2: No significant change between P41-77.

B1/2 symptomatic stage. Can now see denervation and reinnervation between the time points.

Loss and growth have distinct morphological signatures

<table>
<thead>
<tr>
<th>First Look</th>
<th>Second Look</th>
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<tbody>
<tr>
<td>Normal</td>
<td>Fragmented Degeneration</td>
</tr>
<tr>
<td>Normal</td>
<td>Denervated Degeneration</td>
</tr>
<tr>
<td>Fragmented</td>
<td>Thin smooth axon Reinnervation</td>
</tr>
<tr>
<td>Normal</td>
<td>Thin smooth axon Degeneration /</td>
</tr>
<tr>
<td></td>
<td>Reinnervation</td>
</tr>
<tr>
<td>Thin smooth axon</td>
<td>Increased occupancy of the site</td>
</tr>
<tr>
<td></td>
<td>Reinnervation</td>
</tr>
</tbody>
</table>
Important Bit

- None of the thin smooth axons became fragmented or absent at the second view.
- Indicates these axons are in the process of reinnervation rather than being lost.

6. How are branch loss and growth apportioned within single motor units?

6.1 The mouse model

- G93A SOD1 mice were bred to transgenic mice in which a small subset of motor neurons where labelled with yellow fluorescent protein.
- Anti-GFP antibody to enhance the fluorescent signal.

6.2 Reconstruction of motor units

- Normal

- Fragmented branches but no thin reinnervating axon

- Reinnervating (thin smooth) branches but few or no fragmented branches.

6.3 Fragmented terminal axon branches intermixed with normal terminal branches
Complete motor unit that lacks a single normal junction.

Earliest morphological changes are at the most distal parts if the axon branches.

6.4 The winter tree

6.5 Statistical analysis

- Monte Carlo analysis: P<10^{-13}
- Distribution of fragmented and thin terminal branches was highly skewed and not due to chance

Separate motor units are either denervating or reinnervating

Next steps

- Shorten the time period between imaging sessions for in vivo time-lapse.
- Whether these 2 groups show variability within them.
- Do the “compensators” eventually switch to becoming “losers” and what changes to cause this switch
- Motor neuron variability in SOD1 expression

Conclusions

- Degenerative versus regenerative changes are largely confined to distinct populations of neurons within the same motor pool
- Degenerative branches are easily distinguishable from those undergoing compensatory reinnervation.

Weaknesses

- Graphs but no statistical analysis
- S100 immunostaining.
- Was there any change in the tSC?
- Only stated no increase in end stage, what about the other stages.

- In vivo time-lapse imaging
- Better method for reconstructing the motor units
- How reliable analysing parts of motor units?
What is the role of motor unit size in the selectivity of degeneration/compensation in motor pools in SOD1 mice and is the selectivity of YFP expression helpful for establishing this?

- Thought that small caliber axons persist over larger caliber axons, actually new thin axons.
- Selectivity of YFP facilitates tracing axons to reconstruct motor units.

**SUMMARY**

- Mouse models of fALS vulnerable to oxidative stress in vitro
- Does this occur in the nerve terminal?
- Tourniquet induced ischemia/reperfusion
- G93A-SOD1 and WT
- YFP motor neurons
- Fast muscles more vulnerable than slow

**BACKGROUND**

**ALS**

- Amyotrophic lateral sclerosis (ALS)
- Degeneration of neurons located in the ventral horn and of the cortical neurons that provide their efferent input
- Fatal

**OXIDATIVE STRESS**

- Imbalance of production and clearance of reactive oxygen species (ROS)
- Toxic – free radicals and peroxides
- Damages cells through protein and DNA
SOD1 MICE
• Superoxide dismutase 1
• Bind copper and zinc
• Destroys superoxide radicals
• Models overexpress SOD1
• YFP in motor neurons

AIMS
• Test whether motor terminals in G93A-SOD1 mouse model of familial ALS and more vulnerable than WT
• When this occurs
• If there is a difference between fast and slow muscle innervating fibres

HYPOTHESIS
• Fast muscle innervating motor terminals will be more effected in the G93A-SOD1 mouse

METHODS
• Tourniquet-induced ischemia/reperfusion
• Confocal microscopy

Motor terminals in EDL of SOD1-G93A mice are highly sensitive to I/R

I/R causes dramatic reductions in endplate occupancy in SOD1-G93A mice
SOD1-G93A mice motor terminals innervating fast muscles are more sensitive to I/R injury than slow muscle innervating motor terminals.

Increases exposure to ischemia leads to a decrease in endplate occupancy, EDL more vulnerable than SOL.

Axotomy does not duplicate the effects of I/R.

SOD1-G93A EDL degenerates rapidly post I/R.

**SUMMARY**

- Mouse models of fALS vulnerable to oxidative stress *in vitro*
- Does this occur in the nerve terminal?
- Tourniquet induced ischemia/reperfusion
- G93A-SOD1 and WT
- YFP motor neurons
- Fast muscles more vulnerable than slow
METHOD, MODEL, MECHANISM AND RELEVANCE: HOW DOES THIS STUDY MEASURE UP?

METHOD

• Motor neurons undergo oxidative stress due to anaerobic activity caused by reduced blood flow.
• They reduce the blood flow to motor neurons

MODEL

• SOD1 mouse:
• No human cases are due to overexpression of SOD1
• Neither knockout nor overexpression of normal mSOD1 in mice produces ALS
• No convincing treatments that ‘rescue’ hSOD1 mice or substantially delay onset or progression of disease
• Reported benefits treatments in hSOD1 mice usually ineffective or harmful in human clinical

MECHANISM

• I/R does not kill all EDL neurons
• Reinnervated
• Repeated stress
• Disease progression

RELEVANCE

• Correlates with exercise effecting disease
• Protection of vulnerable motor neurons

**A reminder about WldS mice...**

- WldS homozygotes show enhanced axonal and synaptic protection compared to wild type mice.
- WldS heterozygotes show the same enhanced axonal protection, but reduced synaptic protection compared to homozygotes.
- Therefore, synaptic protection is gene-dose dependent.

**Aim and main methodology**

**Aim:** To generate mutant mice which show enhanced synaptic protection, additional to the protection provided by the WldS gene.

**Methods:**
- Crossed ENU exposed BALB/c mice with WldS-thy1.2-YFP-16 double homozygous mice. Examined 219 F1 progeny using CME under anesthesia.
- NMJ examined – Tibial nerve and muscles it innervates. Following sciatic nerve section.
- Also examined axons and synapses in WldS, SOD1-G93A and osteos mice using CME.

**Repeated imaging using CME**

- Imaged same NMJs at different time points.
- Used SOD1-G93A and WldS mice.
- To view degeneration of motor units over time.
Ostes Mice

- Discovered in a previous ENU mutagenesis screen
- Display slow regeneration of motor axons following nerve injury
- This axonal regeneration is gene-dose dependent

Main findings

- Found seven mice in F1 which showed changes in axonal or synaptic phenotype – six provided increased synaptic protection and one provided reduced axonal protection
- CME can be used to view the same NMJs at different time points
- CME can be used to view the slow regeneration of axons in ostes mice

Conclusions

- ENU mutagenesis can be used in combination with CME to identify mutations which alter axon and synapse degeneration and regeneration.
- This discovery could have implications in the treatment of neurodegenerative disease
- CME can be used to view degeneration of motor units over time
- Slow regeneration of motor axons in ostes mice is gene-dose dependent, like the synaptic protection phenotype in WldS mice
### Future experiments

- Do the ENU mutagenesis and crossing experiment using SOD1 mice instead of WldS mice to see if mutants can be generated which modify degeneration of axons and synapses
- Modify CME technique to allow analysis of NMJs in humans
- Find out which genes are mutated in the seven F1 mice which were generated

(CME/YFP) and methodology (ENU mutagenesis) and why is WldS protection gene-dose dependent?

- CME – mostly advantages mentioned, but it does say in the paper that it has low spatial resolution.
- ENU mutagenesis – cannot visually identify mutations associated with the NMJ, as they have no overt phenotypes. Therefore, must combine with a technique such as CME to screen for phenotypes.
- WldS gene-dose-dependent – Need two copies of chimeric gene for “full protection”. Chimeric gene interacts with other proteins