Passive transfer of Lambert-Eaton syndrome to mice induces dihydropyridine sensitivity of neuromuscular transmission.

*Flink et al. (2002)*
Background

P/Q type $\text{ca}^{2+}$ channels regulate release of Ach at mammalian NMJ

- AP arrives
- depolarisation opens P/Q type channels
- $\text{ca}^{2+}$ triggers the release machinery
- docked vesicles fuse to membrane
- release of Ach
Lambert-Eaton myasthenic syndrome (LEMS)

Antibodies against P/Q type $\text{ca}^{2+}$ channels:
- disrupt active zone organisation
- block or eliminate the P/Q type $\text{ca}^{2+}$ channels
- reduced $\text{ca}^{2+}$ influx
- decrease in no. of quanta released

- MEPP not changed.
Passive transfer of LEMS to mice:

• reduces the amplitude of the P/Q-type $\text{ca}^{2+}$ current
• induces the appearance of a dihydropyridine (DHP) sensitive L-type $\text{ca}^{2+}$ current

AIM:

To determine whether $\text{ca}^{2+}$ influx through this L-type $\text{ca}^{2+}$ channel contributes to Ach release
METHOD

• 3 groups of mice differed in the duration of exposure to the LEMS or control plasma:
  • 1. 2h
  • 2. 24h
  • 3. 30 days
Acute treatment studies

- Killed mice + removed diaphragm muscle and attached phrenic nerve
- Incubated tissue for 2h or 24h with LEMS or control plasma.
- prepared tissue for electrophysiological recording
- muscle was cut either side of the main intracellular nerve branch – prevented muscle contraction
- stimulated nerve + recorded the EPP and MEPP
- Quantal content = mean EPP amplitude
  mean MEPP amplitude
Chronic Treatment studies

• Injected mice with cyclophosphamide – suppress immune response

• Mice injected once daily for 30d with 1.5ml of LEMS or control plasma

• Killed the mice + removed diaphragm muscle with attached phrenic nerve + prepared it for electrophysiological recording

• Recorded EPP, MEPP + quantal content
RESULTS

At low frequency stimulation:

<table>
<thead>
<tr>
<th>Control treatment</th>
<th>RMP$^{a,b}$ (mV)</th>
<th>MEPP amplitude (mV)</th>
<th>EPP amplitude (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days</td>
<td>−41.4 ± 1.9</td>
<td>0.46 ± 0.04</td>
<td>9.90 ± 1.20</td>
</tr>
<tr>
<td>24 h</td>
<td>−43.7 ± 1.7</td>
<td>0.42 ± 0.05</td>
<td>9.60 ± 0.84</td>
</tr>
<tr>
<td>2 h</td>
<td>−42.9 ± 2.1</td>
<td>0.43 ± 0.05</td>
<td>9.30 ± 0.92</td>
</tr>
</tbody>
</table>

$^a$Values are the mean ± S.E.M. of at least four preparations of each control treatment. $^b$Preparations were ‘cut’ to prevent muscle contraction, resulting in depolarised preparations due to the release of K$^+$ from cut fibres. Extracellular [K$^+$] was reduced to 2.5 μM; an equiosmolar increase in [NaCl] was made.

duration of exposure to control plasma did not affect neuromuscular transmission from diaphragm preparations
RESULTS

values = % of quantal content from LEMS-plasma treated preparations to that of control-plasma treated preparations

Significant reduction in quantal content in LEMS-treated mice in comparison with control-treated mice.

45.1% of control value.
Acute incubation with LEMS plasma caused a significant reduction in quanta content.
RESULTS

In preparations treated chronically with LEMS plasma there was a significantly larger reduction in quantal content than that seen in preparations which were incubated with LEMS plasma for 2 hr.
RESULTS

High frequency (40Hz) stimulation of nerve:

3 control treatment paradigms:
Reduction in EPP amplitudes

3 LEMS treatment paradigms:
Facilitation of EPP amplitudes
RESULTS

Compared the nerve-stimulated release of Ach before and after the addition of nimodipine:

3 control groups:
Nimodipine had no effect on EPP

Diaphragm preparations incubated for 2h and 24h with LEMS plasma: nimodipine had no effect on EPP

Diaphragm preparations incubated for 30 days with LEMS plasma: EPP amplitude reduced following application of nimodipine
Acute incubation with LEMS plasma:
No significant difference in quantal content after addition of nimodipine

Chronic incubation with LEMS plasma:
Quantal content was significantly reduced after addition of nimodipine
SUMMARY OF RESULTS

Both chronic + acute passive transfer of LEMS to mice caused the typical electrophysiological features seen in LEMS:

• reduced quantal content
• facilitation of EPP amplitude at high freq. Stimulation

Mice treated chronically with LEMS plasma had a significantly greater reduction in quantal content than mice treated for 2h with LEMS plasma.
L-type channels were only shown to become involved in synaptic transmission when mice had been treated chronically with LEMS plasma.
Why do L-Type calcium channels become involved in synaptic transmission only after chronic treatment with LEMS plasma?

- enough time for protein synthesis resulting in insertion of L-type Ca 2+ channels at active zone

- could reflect time required for L-type channels already present to be relocalised to the active zone.
may be due to reduction in $K_{(Ca)}$ current at motor nerve terminal:

- $K_{(Ca)}$ channels are located close to the P/Q type Ca channels in the active zone
- $Ca^{2+}$ activates these $K_{(Ca)}$ channels and $K^+$ flows out
- $K_{(Ca)}$ channels limit duration of depolarisation
- In mice treated chronically with LEMS plasma there is reduction in Ca influx.
- Less activation of $K_{(Ca)}$ channels
- Duration of depolarisation is prolonged.
- Enhanced depolarisation could prolong the activation of L-type $Ca^{2+}$ channels allowing them to become involved in synaptic transmission.
Functional repair of motor endplates after botulinum neurotoxin type A poisoning: Biphasic switch of synaptic activity between nerve sprouts and their parent terminals

De Pava et al (1999)
Botulinum Toxin

- Botulinum toxin is one of the most poisonous naturally occurring toxins
- Causes serious paralysis and can lead to death
- Blocks ACh transmission at NMJ via cleavage of SNAP-25 at the presynaptic terminal and therefore exocytosis
- There is eventual recovery of transmission and muscle use
- Seen to promote nerve sprouting – not sure of role in regaining synaptic transmission
Aims

• To see what affect the BoTx/A induced sprouting has on recovery
• To look at the alterations in the morphology and location of the synaptic-vesicle turnover during and after paralysis
Methods

• Neck muscle (sternomastoid) NMJs studied in mice
• Nerve endings were stained with either 4-di-2-ASP or in a solution of 4-di-2-ASP with FM1-43, along with a Krebs-Ringer solution
• 4-di-2-ASP is a fluorescent dye which stains mitochondria (green)
• FM1-43 is a exoendocytotic marker as incorporates into the synaptic vesicles (red)
• Immunolaballing carried out looking at exocytotic proteins and postsynaptic nAChRs
• Use CCD camera and Image Pro Plus imaging
• BoTx/A administered by injecting into muscle about 2mm from the endplate
Criteria for FM1-43 for Exoendocytosis

- Needed to confirm that the measurement of stimulated endocytosis reflected the blockade of exocytosis by BoTx/A
- Control experiments showed that when depolarised there was increased staining compared to no stimulation or when BoTx/A was administered
- Shows that BoTx/A impairs endo as well as exocytosis in the nerve endings
Results - Numbers of Sprouts

Table 1: Botox/A induced terminal sprouts visualized with F of 273F in living mice

<table>
<thead>
<tr>
<th>Sprout criteria</th>
<th>d2</th>
<th>d4</th>
<th>d7</th>
<th>d14</th>
<th>d28</th>
<th>d42</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of sprouts</td>
<td>0</td>
<td>1.2 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>2.6 ± 0.7</td>
<td>3.9 ± 1.3</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>Average sprout length, μm</td>
<td>0</td>
<td>4.3 ± 5.8</td>
<td>9.9 ± 5.2</td>
<td>28.4 ± 9.6</td>
<td>70.2 ± 17.7</td>
<td>150.6 ± 20.1</td>
</tr>
<tr>
<td>No. of branches</td>
<td>0</td>
<td>1.7 ± 0.5</td>
<td>2.5 ± 0.9</td>
<td>6.8 ± 0.2</td>
<td>11.6 ± 3.4</td>
<td>16.2 ± 3.4</td>
</tr>
</tbody>
</table>

- Muscle paralysis seen after administration
- Muscle use regained at d28
- By d4 sprouts occurring
- Increase in size and number of sprouts up until d42
Production of Sprouts

- d3 to d7 – reduction in uptake of stain in parent terminal and increase in sprout uptake
- Sprouts maintain the stain until d42 and parent terminals keep a low level of staining
 Imaging of Sprout Formation

- **d0** - yellow staining so both dyes incorporated
- **d14** - green staining at the parent terminal and therefore no uptake of FM1-43 and no exoendocytosis. Sprouts have some yellow staining towards the ends
- **d28** - sprouts have more yellow stain as exoendocytosis is occurring more and therefore transmission can occur
Exocytotic Related Proteins

- Image (e) is stained for SNAP-25, which is involved in exocytosis, to detect sprouts
- Image b is stained for synaptobrevin, also involved in exocytosis
- Arrows show presence of these proteins in sprouts, away from the parent terminal and where there are no nAChR seen
Exocytotic Related Proteins

- v-SNARE (image c) and t-SNARE (image f) imaging was carried out at d28
- nAChR can be seen abutting these sprouts
- Therefore all machinery (presynaptic proteins and postsynaptic arrangement) in place ready for transmission
Recovery of Muscle

- At d28 recovery of muscle is seen
- Must be occurring via sprouts as there is no transmission seen in the parent terminals
Recovery of Muscle

- Graph shows that at d42 when muscle is recovered, FM1-43 uptake is not at normal levels
- Takes until d97 to regain full stain uptake
- Therefore further alterations must occur at the synapse after muscle control is regained
Reduction in Sprout Number

- After d42 sprout number and size begins to decrease
Loss of Sprouts

• At d42 can see a lot of yellow stain so therefore exoendocytosis is occurring in the sprouts and there is not much occurring at the parent terminal. Synaptic transmission is at these sites.
• At d63 a reduction of yellow staining is seen in the sprouts and an increase in seen in the parent terminal, suggesting that transmission is occurring at the parent terminal
Loss of Sprouts

- At d63 can see a reduction in yellow staining
- At d91 see an almost complete retraction of all sprouts. Size of the terminal is similar to before the experiment and pattern of staining is beginning to return to normal
Results Summary

• The sprouts are the only sites undergoing vesicle turnover once muscle use is regained at d28
• Sprouts therefore form to release ACh and induced synaptic transmission after paralysis at the NMJ
• A complete recovery of function to the parent terminals is seen by d91
Discussion

• This is not the remodelling that occurs at all synapses in the body and after all types of injury

• If a peripheral nerve is crushed then the nerve degenerates rather than remodels
  – However sprouts do form, BUT from adjacent terminals resulting in polyneuronal innervation

• Sprouts are triggered via intrinsic and extrinsic factors

• Muscle activity induced by the sprouts induce remodelling at the parent terminal so there is complete functional repair
Burning Questions

• Why don't junctions compensate effectively, by remodelling their form and function in myasthenia gravis?
• What mechanisms maintain compensatory nerve sprouts, e.g. after botulinum toxin-induced paralysis?
Pre and post synaptic abnormalities associated with impaired neuromuscular transmission in a group of patients with “limb-girdle” myasthenia.

Slater et al., 2006
Limb-girdle Myasthenia (LGM)

- Term introduced by McQuillen 1966
- Rare
- Incidence – less than 200,000 cases in USA
- Young onset of impaired neuromuscular transmission
- Proximal limb weakness
- Little/no impairment of facial, ocular or bulbar muscles
- Fluctuations of weakness – timescale of weeks
- Waddling gait – proximal muscle weakness of lower limbs
- [http://uk.youtube.com/watch?v=TKL5krEsiVI](http://uk.youtube.com/watch?v=TKL5krEsiVI)
Case Studies

- 1971 – 1991
  - 27 cases
    - 12 autoimmune
    - 2 congenital myasthenia with episodic apnoea (familial infantile myasthenia)
    - 1 ocular congenital myasthenia
    - 1 severe early form
    - 10 fluctuating muscle weakness
  - 6 patients most classic to LGM syndrome

- Many had pseudomyopathic presentation
Case Study

• Cases
  – 6 from initial group
  – 2 others
  – Control group

• Aim
  – Categorise structural and function defects in NMJ in LGM
  – Determine pre/post synaptic effect
  – Rule out genetic mutations in several candidate genes
  – Relate to LGM phenotype
Methods

Diagnosis
- Full history
- Clinical examination and features
- Neurophysiological studies
  - Nerve conduction studies
  - Electromyography
  - Repetitive nerve stimulation
  - Single fibre EMG
- Motor point muscle biopsy
  - Intracellular recordings
  - Light and Electron microscopy
  - Functional analysis
  - Immunocytochemistry
- Biochemical tests e.g. AChR antibodies and creatine kinase
- DNA Analysis – mutations and single strand conformational polymorphisms (SSCP)
  - AChR subunit genes
  - RAPSYN
  - COLQ
  - CHAT
  - MUSK
Results

• Clinical features
  – Onset between 6 months to 9 years
  – Severe walking impediment age 20
  – Signature stance and gait
  – Progressive and fluctuating muscle weakness
    • Variation weeks to months; long term compared to Myasthenia gravis
    • Not as severe as autoimmune
    • Exercise induced
    • Seemingly random pattern, not muscle specific (not oculomotor, only slight in facial muscles)
    • No other neurological problems
  – No significant muscle wastage (except in disabled cases, due to disuse)
  – Absence of AChR antibodies
  – Normal levels of creatine kinase

• Confirmed diagnosis
Neurophysiological studies

• Motor and sensory nerve conduction
  – Normal

• Repetitive nerve stimulation
  – Decrement of Compound Muscle Action Potential (CMAP) (3Hz)
    • Greater in proximal muscles
      – 41% decrement in ANC and DELT
      – 19% decrement in ADM

  – Post-Activation potentiation
    • Decreased decrement after exercise and Edrophonium
    • Edrophonium also Increased CMAP
EMG Studies

Single fibre EMG (SFEMG)
- Abnormal jitter
- Impulse blocked in 5-100%

Macro EMG studies
- Median macro motor unit potential amplitude mostly normal (2 out of 8 reduced)
- Fibre density mostly normal (1 out of 8 increased)
- Therefore motor unit size is remaining constant
In Vitro Electrophysiology

Impaired neuromuscular transmission
- Mean EEP amplitude reduced by 46% to that of controls
  - Significantly correlated to fraction of abnormal muscle fibre pairs in SFEMG
  - Reasoning for muscle weakness in vivo
In Vitro Electrophysiology

• Reasoning
  – Both reduced quantal content (59% of control), and reduced efficiency of quantal released (mean mEPP 68% of control)
In Vitro Electrophysiology

EPC amplitude reduced BUT mEPCs not

- AChR abundance?
- Influenced by increase muscle fibre diameter in LGM patients, leading to reduced resistance to input

mEPCs show slight increase in decay time BUT EPC decay time constant

- Still dependent on membrane potential and same reversal potential
- Suggesting presence and kinetics of AChR normal
- AChE must also have normal abundance and activity as no evidence of prolonged synaptic currents
Light Microscopy

- General appearance of muscle normal
- Proportion of fibre types normal, apart from there being slightly fewer type IIb fibres
- LGM muscle fibres diameter 14% larger (Fibres I and IIb)
- Only 1 case of tubular aggregates
Ultrasonic Structure

• Increased fraction of NMJs innervated by axon collateral branches
  – Functional terminal innervation ratio was increased compared to control as a result

• Smaller and less compact varicosities

• Normal distribution and abundance of AChRs
  – Though seemingly LGM binding was 40% of that of the controls

• Intensity of staining for AChE activity equal to control, but area reduced to 55% in LGM (compared to control)
  – Though no sign of increased length along muscle fibre as seen in AChE deficient pathology
Electron Microscopy

• Similar general appearance
• But
  – Folding
    • Reduced in LGM
    • Two measurements
      – Total length of folded membrane (FoldL)
      – Increase in post synaptic length caused by folding (Folding Index) (6D,F)

Both reduced 50-60% in LGM patients

• Again, no substantial evidence for tubular aggregates
• H/I – LGM/Control – AChR stain
• 6D vs. F – variation of NMJ between patients
• 6F vs. G – variation within patient
• 6E and 6G - aggregates

• A/B – control – abundant folding
• 6B – folded membrane outside region of nerve contact
• C/D – LGM – reduced folding
Functional Significance

• Neurotransmitter release
  – Quantal content proportional to synaptic area
  – Rule applies to LGM patients too
  – No significant difference between LGM and control group
  – So decrease in quantal content is matched by decrease in synaptic area (ratio still the same)
Further Studies

- Protein immunocytochemistry
  - AChE
  - s-Laminin
  - AchR subunits
  - Rapsyn
  - Na$_v$1
  - AnkyrinG
  - Utrophin
  - Dystrophin
  - Syntrophin
  - β-dystroglycan
  - β-spectrin
  - Agrin
  - β-dystroglycan

- All substantially increased in NMJ compared to non functional regions of the muscle (Both LGM and control)
Further Studies

• DNA Analysis
  – Genes common to myasthenia syndromes
  – Coding exons, untranslated regions 5’ to ATG initiation codon
    • AChR subunits (α, β, ε)
    • Rapsyn
    • ColQ (end portion of AChE)
    • Cholinacetyltransferase
    • MuSK
  – No mutations found
Response to Treatment

- To try to
  - Improve muscle strength
  - Reduce decrement on the CMAP during repetitive stimulation
- No consistent pattern of response in patients
  - Pyridostigmine
  - 3,4-diaminopyridine
  - Ephedrine

<table>
<thead>
<tr>
<th></th>
<th>Pyridostigmine</th>
<th>3,4-diaminopyridine</th>
<th>Ephedrine</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGM2</td>
<td>x</td>
<td>x</td>
<td></td>
<td>Good effect, only when combined</td>
</tr>
<tr>
<td>LGM5</td>
<td>x</td>
<td>x</td>
<td></td>
<td>Moderate improvement, but intolerable cholinergic side effects</td>
</tr>
<tr>
<td>LGM3</td>
<td></td>
<td></td>
<td>x</td>
<td>Improved, but deteriorated on Pyridostigmine</td>
</tr>
</tbody>
</table>
Discussion

• Normal
  – Quantal release per unit area of junction
  – Size and kinetics of mEPCs
  – Genes and proteins common to disease and junction

• 2 main abnormalities
  – Reduced size of NMJ
  – Reduced post-synaptic folding
    • Leading to an increased threshold for firing an AP, which concurs with phenotype of inefficient transmission, and muscle weakness
Conclusions

• NMJ formation main factor
• Both sides of synapse
• Suggestion that complex protein interaction maintain junctional folds
  – Different proteins maybe?
  – Were they decreased in LGM patients?
Limitations and points of discussion

- Very small sample size
- Control group – why not healthy subjects?
- As AChE activity was measured by area/volume plane of cut might have been an influence on final figures
- Further work maybe into tubular aggregates
- Don’t compare protein levels between Control and LGM?
- Heterogeneous aetiology of LGM – difficult to define
- Some LGM do have antibodies raised against AChRs
- Tubular Aggregates
  - Non-specific appearances and unclear role
  - Calcium homeostasis?