MINI-SYMPOSIUM II

Development and remodelling of the NMJ

Overview:
This symposium focused on the NMJ during development in both normal and disease-associated states. The four main articles presented each exposed different aspects of developmental changes at NMJs and, taken together, convey a picture of NMJ and neuronal plasticity in the maturation of these synapses. The first paper of the symposium centred on synaptic takeover, a natural occurrence in the development of mono-innervation at a synapse, and the axonal competition involved in this process. Our second paper was also focused on the topic of neuronal competition but looked at this phenomenon through the re-innervation of a muscle by regenerated axons. This study highlights the surprising lack of activity in axonal competition for synaptic space. The third article of the symposium provided a dramatic view of synapse elimination through the use of a SMA mouse model. This paper also exposed the physiological and developmental differences in motor neurones, and their respective susceptibility to disease. Finally, the fourth article presented dealt with an invertebrate system of synaptic elimination and dismantling. In this innovative study the process of NMJ elimination and remodelling during drosophila metamorphosis, was explored using a variety of practical techniques.
Synapse elimination is a normal part of development which allows abundant poly-innervation of muscle fibres to become pruned back to the more adult-like state of mono-neuronal innervation. It is thought that this process is based on competition between axons at the poly-neuronal endplate; that is a neuronal ‘fight’ in which the winner takes all, with or without associated territory expansion.

As stated by the title of this paper, this study presents a picture of the dynamic nature of synapse elimination though the use of in-vivo imaging. This was done by creating lines of transgenic mice expressing fluorescent proteins (GFP/YFP/CFP), resulting in differently coloured motor neurons. Through a screening method, suitable poly-neuronal NMJs were identified in transgenic mouse pups and were imaged over a period of time (time–lapse: lots of images are taken at certain time intervals) to observe the behaviour of the neurons at the NMJs. From the images taken at these junctions, the authors elucidated a common mechanism of synaptic takeover. It appeared that the process of elimination involved one input being lost, or withdrawing from the postsynaptic site, whilst the other axon took over this vacated territory. These actions are thought to be temporally coordinated as unoccupied postsynaptic sites were not found during this process. Competitive elimination did not necessarily occur smoothly, with one axon steadily gaining ground and the other losing out in a monotonic progression. Indeed in a number of NMJs imaged, certain ‘flip-flop’ events were noticed in which there was considerable flux in the neuronal behaviour. Terminals that occupied the larger proportion of synaptic space would retreat then expand once again, exhibiting a highly dynamic nature. This finding taken together with the apparent dynamism of both axon length and calibre also observed, underlines the flexibility of a young neuron in contrast to the more stable, more mature motor neurons in the older mouse. The article also points out that it was not possible to reliably predict which neuron would win the synaptic fight based on proportion of occupied ‘synapse space’. As discussed in the symposium, it would be brilliant to image whole neurons with their complete NMJ connections, not just at single junctions, to understand their behaviours as a whole.

Interestingly, a number of NMJs undergoing poly-neuronal elimination were found to become mono-neuronal but the vacated post-synaptic sites of the retreated axon had not been taken over by the remaining input. In these cases, the ‘naked’ ACh receptors rapidly disappeared from the post-synaptic space. These results indicate that territorial expansion is not an absolute requirement for synapse elimination.

This study concludes with the concept that synaptic takeover is a rapid method of acquiring a larger territory and hence increasing synaptic strength of an axonal input during NMJ development. The ability to adopt the vacated post-synaptic receptors allows the remaining input to quickly increase in strength whilst the leaving axon is weakened.
This study investigated the requirement of synaptic activity for competitive synapse elimination. The experimental procedure followed to examine this was using the fourth deep lumbrical muscle (4DL) in an adult rat, which is supplied by two nerves, the sural nerve (SN) and the lateral plantar nerve (LPN). The SN was crushed, which partially de-innervates the 4DL muscle as the SN axons degenerate. The intact LPN axons then go on to sprout new terminals and innervate the areas previously occupied by the SN. After some time the SN axons regenerate and seek to innervate the 4DL muscle once again. As a result, some of the inputs (motor endplates) of the 4DL are poly-innervated and subsequently must become mono-innervated through synapse elimination.

Thus at these sites it was possible to address the question of synaptic activity being an essential factor for competitive synapse elimination. By ceasing all activity in the regenerating SN it would result that the ‘silent’ SN would either be capable of displacing LPN axons at poly-neuronal endplates or it would not.

Activity requirement was tested in a series of experiments. Firstly by blocking all activity in the SN after regeneration of the axons and secondly, the 4DL muscle was also paralyzed, preventing any communication between the SN and the muscle. Blocking all synaptic activity was achieved by using α-bungarotoxin (blocks Ach receptors) postsynaptically and tetrodotoxin (blocks Na channels) presynaptically. In order to image the synapses to determine whether the SN axons were able to displace LPN axons, separate fluorescent dyes were used to identify the axons. SN axons were labelled green (FM1-43) and LPN axons were labelled orange (RH414). Fluorescence microscopy was then used to determine the innervation patterns of the SN and LPN.

The overall results of this study show that activity is not a requirement for competitive synaptic elimination in this model. SN axons per fused with TTX were capable of displacing LPN axons at poly-innervated NMJs, both with or without neuromuscular transmission (with/without α-bungarotoxin). Interestingly, compared with crush controls (no toxins applied) there were higher levels of resultant poly-neuronal endplates in paralyzed muscle configurations.

As raised in the symposium, there are some alternative suggestions for the factors influencing competitive synaptic elimination. These include possible competition for growth factors or neurotrophic factors. It has been suggested that the level of receptor expression in an axon may confer a competitive advantage and hence allow displacement of an opposing axon at a polyinnervated endplate.
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) is a degenerative disease of the lower motor neurons which results in denervation and atrophy of muscles of the upper and lower body. It is a childhood disease which can vary in severity depending on the particular type of SMA. In this study two mice models of SMA were used, one moderate (Smn -/-;SMN2;Δ7) and the other severe (Smn-/-;SMN2). The severity of SMA corresponds to the level of protein expressed by the SMN gene (survival motor neuron), which exists in two forms, at the telomere and at the centromere. Loss or disruption of the telomeric gene results in reduced (SMN gene product) protein levels but the number of copies (copy number) of the centromeric gene SMN2 determines the severity of the disease. This study addresses aspects of the molecular processes of synapse elimination at the NMJ in two mouse models of SMA. In order to develop potential therapeutic strategies for this terrible disease; it is crucial to examine the events occurring at the neuronal and the muscular level to identify target areas for treatment.

Firstly the investigators examined the neuromuscular pathology in both mouse models in both slow-twitch (TVA) and fast-twitch (LAL and lumbricals) muscles. In the most severe model (Smn-/-;SMN2), preparations from late-symptomatic mice (at days P5-P6) showed numerous empty or partially occupied endplates due to a loss of pre-synaptic motor neuron terminals. This loss of presynaptic nerve terminals was proposed to be a result of axon retraction rather than Wallerian degeneration due to the absence of markers associated with this process. Abnormal accumulations of neurofilament proteins, also observed in other mouse models of SMA, were found in pre-synaptic axon terminals. Significant reduction of muscle fibre diameter was also noted. Similar findings were presented for the less severe SMA phenotype, in the Smn-/-;SMN2;Δ7 mice. Interestingly in both models, although more pronounced in the less severe phenotype, the fast-twitch muscle (LAL) was seemingly less affected (ie showed less shrinkage) than the slow-twitch TVA muscle in SMA.

Further investigation additionally revealed a higher proportion of nerve terminal loss from the caudal end of the LAL muscle in both mouse models. Although analysis of muscle fibre shrinkage and endplate area reduction revealed no differences between the two ends of the LAL muscle (rostral vs caudal). As a note of interest, this provides evidence for the de-correlation of pre-synaptic and post-synaptic pathology in SMA, which was also further pursued in this work. By examining the motor units associated with the LAL, the authors discovered 2 distinct populations of innervating neurons; one rostral, one caudal. The rostral motor neuron group appear to have mainly DeSyn characteristics whereas those innervating the caudal LAL band have largely FaSyn character (based on their reactions to BotA toxin). These results would indicate that FaSyn synapses are more vulnerable than DeSyn synapses in SMA-associated synapse loss.
Distinct Presynaptic and Postsynaptic Dismantling Processes of Drosophila Neuromuscular Junctions during Metamorphosis.

Drosophila undergo a massive transformation from a larval to an adult form, via a pupal stage. During this pupation there is a massive remodelling of body structures, involving a variety of destructive processes including synapse elimination at NMJs. The thorough investigation presented in this article explores many new aspects of synapse remodelling in Drosophila by using a combination of confocal and electron microscopy, as well as live imaging techniques.

Firstly, the authors examined the elimination of NMJ synapses in the early stages of metamorphosis. Using GFP they were able to label postsynaptic markers such as K+ ‘Shaker’ channels and Dlg, a post-synaptic scaffold protein. This provided evidence of a destruction time-line; NMJ synapses at posterior abdominal segments were eliminated before more anterior NMJs.

Distinct staining methods also highlighted the different timings, and styles, of postsynaptic and presynaptic dismantling. Postsynaptic dismantling activity is distinguished by dispersion of Dlg protein and of glutamate receptors (although these do not share common elimination mechanisms). Seemingly, the signs of postsynaptic dismantling occur before similar presynaptic markers, and evidence suggests that post-synaptic dismantling occurs ∼1 before pre-synapse elimination. Presynaptic dismantling is characterized by the appearance of filopodial structures, followed by synaptic vesicle aggregation and retrograde transport of these vesicles, and finally retraction of the presynaptic membrane. Retrograde synaptic vesicle transport was elucidated in this study using time-lapse imaging and fluorescent dyes. An important finding of this article was observed during investigation of the presynaptic retrograde transport of synaptic vesicles. It was shown that blocking this transport mechanism (glued and Dhc64C mutants) inhibited presynaptic dismantling of the NMJs.

Ecdysone is a fundamental signalling hormone throughout the development of Drosophila and is considered to be the ‘master regulator’ of remodelling in the nervous system. Pre-synaptic blockade of ecdysone (by expression of negative ecdysone receptor isoforms) arrests the dismantling process in the presynaptic axon but does not affect postsynaptic activity. Preventing ecdysone expression in postsynaptic sites however stopped both pre and postsynaptic dismantling. Further experimentation revealed that blocking ubiquitination and apoptotic pathways postsynaptically prevented all synapse dismantling but no effect was observed if inhibition of these pathways occurred presynaptically. These findings suggest a dominant role for the postsynaptic side on instructing synapse elimination. The paper goes on to note that muscle histolysis in the Drosophila pupa and synapse elimination are tightly correlated and therefore there may be a signalling event arising from muscle destruction which triggers elimination of NMJs.

An unexpected finding of the study was the lack of glial cell involvement in NMJ synapse elimination.
In Vivo Time-Lapse Imaging of Synaptic Takeover Associated with Naturally Occurring Synapse Elimination
Mark K. Walsh and Jeff W. Lichtman*

Aims
- Background – competition between axons results in some axons retracting from synaptic connections while others are maintained or even expand to form more connections. The dynamics of this had not been directly observed.
- Aims – This study was designed to directly image this competitive process to determine the time course and structural changes that accompany axon elimination and see if structural alterations in the remaining input might accompany the elimination of a competitor.

Methods/techniques
- Investigation was carried out using a time lapse technique (24hrs – 1 week) beginning at the second post natal week.
- Used transgenic mice that expressed fluorescent proteins (CFP, YFP, GFP) in different subsets of neurons.
  - Mice that expressed either CFP or YFP in their motor axons were crossed with mice expressing GFP in motor axon subsets.
  - Created double transgenic progeny with motor axons expressing one fluorescent protein and a subset of motor axons expressing a different fluorescent protein.
- They used multiple colours to reduce confusion associated with axons that are closely juxtaposed and allow for individual identification of axons converging onto the same postsynaptic cell.
- This enabled them to study the dynamics of synaptic elimination in vivo.
Results

Obtained 25 mice with optically accessible and multiply innervated junctions. These were then successfully monitored and their junctions studied until they became singly innervated.

- Observed synaptic takeover of synaptic junctions which they said appeared closely coordinated as there was never a period of inoccupation at a post synaptic site.
- Observed that in some junctions it was the input that started off with the smaller proportion of terminal area that was ultimately maintained (surprising finding)
- Observed large variation in the speed of takeover
  - Fastest: 233% increase in area over 1 day
  - Average (median): 25% increase
  - Some cases almost no change at all.

Results continued

- Observed both monotonic progression of synaptic take over (Fig 2, 3A, 3B) and flip flop (Fig 3C-3F)
- Following complete withdrawal the axons never reformed synapses at that junction.
- Following synaptic takeover singly innervated junctions became stably maintained.
  - Led them to believe that axons are highly dynamic during synapse elimination
- Tested whether synaptic competition was the cause of the dynamic behaviour by examining singly innervated junctions and found that these were also highly dynamic.
- Finally they discovered synaptic elimination can take place in the absence of synaptic takeover
  - Indicates that when there is no take over by a remaining axon then the synaptic site rapidly becomes destabilised
  - To further investigate this finding they compared these results during synapse elimination with those from complete denervation.
- Discovered that denervation caused little change in AChR density
  - Led to the hypothesis that there must be some kind of ‘intersynaptic signalling permitting one axons synaptic sites to destabilize synaptic sites of competing axons’

Figure 1

- Can see expression of both the YFP and the CFP.
- Can also see AChRs labelled in red.

Fig 2 – synaptic takeover
Fig 3
A-B. Here we can see a dramatic reversal during synaptic takeover that occurs in a monotonic progression.

C-F. In these slides we can see an example of flip flop.

Fig 5 – Synaptic elimination without takeover

Key findings
• 1. Synaptic takeover does not affect the stability of the underlying postsynaptic apparatus.
• 2. Synapse elimination is not dependent on axon displacement at the synaptic site; based on observation that axons can withdraw from sites that are not subsequently taken over.
• 3. Occupied synaptic sites signal to unoccupied synaptic sites on the same postsynaptic cell to cause dismantling – inferred rather than proved.
• 4. Results suggest that synaptic takeover is due to high intrinsic dynamism of axons – young axons are continually exploring the postsynaptic territory which increases their availability to occupy newly vacant sites. Again this requires more research.
• 5. Territorial occupancy is not a valid indicator of which axon will be ultimately maintained.
• 6. Takeover can either progress monotonically or by flip flop.
• 7. Input withdrawal and synaptic takeover are intrinsically linked as a single developmental strategy that is highly co-ordinated.

Has the study satisfied the aims
• Aims – This study was designed to directly image this competitive process to determine the time course and structural changes that accompany axon elimination and see if structural alterations in the remaining input might accompany the elimination of a competitor.
• Yes, however a higher temporal resolution would provide a greater insight into the time course of axon elimination.
• No evidence that structural alterations in the remaining input directly influence the elimination of a competitor, simply that the two processes appear to be closely coordinated.
BBQ – *What are the significance and importance of non ‘monotonic’ synapse elimination at the NMJ?*

- The non monotonic progression of synaptic takeover describes a situation where an axon that was advancing was then seen to retreat.
- This phenomenon has been termed flip flop.
- They suggest that this may be due to competition for resources between neighboring axon branches that could influence the growth potential of a terminal branch in a positive or negative way over the time course of synaptic competition.
- Significance - indicates that it is not an intrinsic process and that the axons are able to adapt to surroundings and changing signals to ensure the appropriate connections.

**Analysis of the study**

**Strengths**
- Use of different fluorescent proteins
- Very useful images to convey results
- Consolidated existing knowledge and provided initiative for new studies.

**Weaknesses**
- Conclusions were largely based on assumption and scientific conjecture so although helpful, by no means definitive
- Despite multi fluorescent protein expression they had to concede that ‘exact temporal relationship between expansion and withdrawal could not be discerned un ambiguously’ because both axons expressed one of the fluorescent proteins’ therefore degree of overlap could not be determined.

**Further studies**
- Would be interesting to quantitatively examine the dynamics observed in this study at a higher temporal resolution to more accurately characterise events.
- Examine the importance of synaptic activity in competition (combined electrophysiology and dual colour imaging) - could also employ genetic techniques to silence transmission which could provide novel insight into effect of activity.
- Why does an axon retreat from a synaptic junction and why is this progression not always monotonic?

**References**
**Aim**

- To stringently test the hypothesis that there is an **absolute** requirement for **activity** in competitive synapse elimination in reinnervated muscle.

**Background**

- During embryonic and early postnatal life, the muscle fibers of vertebrates are polyinnervated. Subsequent pruning of these multiple inputs leads to the eventual mononeural innervations found ~2 weeks after birth. By the end of the first week after birth in this leg muscle, branches of at least two different motor axons can be seen innervating most neuromuscular junctions.

**Why is Activity So Important**

- The majority of evidence points to an activity dependent form of competition whereby equivalent inputs battle one another for synaptic resources.
- This arose from experiments in the 70’s and 80’s by where blockage of activity stopped elimination. (Duxson, 1982; Thompson et al., 1979). The culmination of these theories lead to the formation of two activity dependent models...
Why is Activity So Important

Model 1
- 1: Inputs compete for limited muscle derived factors. Less active inputs receive fewer resources and retract. More active inputs win more resources.

Model 2
- 2: Signalling molecules actively drive the process of elimination from less active neurons.

Examples
- This activity dependant form of synapse elimination was found to be influential in the formation of memories in the hippocampus whereby synapses follow a hebbian postulate, where activity of one synapse strengthens the post synaptic cell, whereas cells that don’t fire together lose connectivity.
- This forms the basis of LTP and LDP.

Experimental Design
- They took advantage of the fact that the 4th Deep Lumbral muscle in adult rats has a dual nerve supply.
  - The LPN (lateral planter nerve) supplies ~10 motor axons which account for 70% of muscle innervation
  - The SN (sural nerve) supplies 1-3 axons, and makes up ~30% of muscle inputs

Experimental Design
- Firstly
  - The 4DL muscle was partially denervated by crushing the Sural nerve, (SN).
  - Sprouting of the LPN terminal resulted in almost complete reinnervation of all muscle fibers in 14 days by the LPN.
  - On day 15, SN axons returned and polyinnervated the muscle fibers taken over by the LPN.

- Secondly
  - 14 days after crushing the SN, conduction of action potentials was blocked from the SN and 4DL muscle using TTX and α-bungarotoxin.
Experimental Design

- Styril dyes we used to label the SN, (green) and LPN motor nerve terminals, (orange).
- Fluorescent microscopy was then used to see how the innervation on single muscle fibers had occurred, establishing the proportion of SN innervation, LPN

Action of TTX and BTX

TTX-Derived from Puffer fish. It blocks Na channels and therefore stops the conduction of action potentials making the synapse electrically silent

BTX-derived from the venom of the Taiwanese krait snake. It is irreversible and competitively binds to AchR postsynaptically.

Hypothesis

- If activity was essential for competitive elimination, then paralyzed muscles should show no such elimination, i.e., SN and LPN inputs should coexist at a single muscle fiber and be polyinnervated, all muscle fibers reinnervated by the SN should also retain their sprouted LPN inputs
- If activity was not essential, then there would be evidence for exclusive innervation at endplates by the SN, despite complete paralysis.

Methods

- SN crushed using fine forceps on anaesthetized rats.
- 14 days later, a mini pump filled with TTX was placed in a cuff around the SN axon.
- Hind legs were tested daily for chronic block, showing no flexion withdrawal reflexes
- In the 2nd group, As well as TTX, α BTX

- SN and LPN isolated, and electrophysiology conducted.
- Muscles bathed in green dye first and then stimulated
- Then bathed in orange dye to stain the LPN boutons.
- Immunohistochemistry then used to label AchR’s, axons and terminals and proportions counted
Controls

• Correct interpretation of data depends on the following assumptions:
  • That LPN sprouting was complete before SN rejuvenation.
  • Complete and total paralysis
  • TTX and BTX had no toxic effect and didn’t affect overall results.

Results

• Paralysis did NOT prevent competitive elimination!
  • With TTX nerve block, 10% of fibers were polyinnervated by LPN, and regenerated SN terminals, (π).
  • A further 4.9% were reinnervated by SN alone.
  • The majority of fibers that were polyinnervated were more than 50% occupied by regenerating SN.

Results 2

• To test their hypothesis further, the same experiment was carried out on completely silent synapses using TTX/α-BTX to remove the possibility of spontaneous vesicle release.
  • In these silent muscles
    – 13% were polyinnervated
    – 4% exclusive to SN boutons

Staining using Styryl Dyes

• LPN terminals alone, (orange dye)
  – Polyinneravtion,
  – SN terminals alone (green dye)
  – Note-myelin tends to fluoresce yellow
Conclusions - Competition was clearly not affected by either form of paralysis

- Complete block and consequent paralysis of the 4DL actually promoted sprouting of LPN and the regeneration of SN axons and terminals.
- This allowed for SN to competitively displace LPN terminals.
- Thus, paralysis actually restored about half the original content of SN terminals pre crush.

Possible Explanations

- If activity is not critical, then it’s possible that synaptic or muscle derived molecules drive the process of competition.
- Perhaps regenerating terminals respond better to growth factors, due to up regulation of receptors at their membranes.
- Intact terminals don’t require constant gene expression and turnover of these receptors, so are caught out when a new nerve comes along.
- Regenerating terminals secrete proteases to directly attack competition.

Critique

Good Points
- Great care taken to make sure that the controls were rigorous and that it would not affect the outcome.
- The paper goes against the general trend of thinking whereby competition is activity dependent, therefore, revolutionary.
- Confirmation that reinnervation and competition occurs in silent synapses shown directly using dyes.

Not so Good Points
- Complete nerve block of a terminal is not often seen at a synapses, so observations show an unnatural response to injury.
- Antibodies used to detect AChR and axons/terminals for the IHC failed to penetrate deepest fibers, so only superficial.

Criticism

This work suggests that neither activation of postsynaptic muscle fibers nor asynchronous activity is required for competition to occur. However, the effects of paralysis induced in this study, (which can affect the growth and sprouting as well as withdrawal of inactive axons), complicate the interpretation of these experiments. Despite this limitation, this work highlights the important idea that activity may be a modulator, rather than a mediator, of competition.

ALTERNATIVE explanations

- Perhaps, original post synaptic sites are removed and replaced in the event of nerve trauma, and the new axon induces new sites.
- However, results found that total area of occupancy was same in the paralysis as in the crush.

- When AChRs in a small region of a junction were blocked by focal application of α-bungarotoxin, blocked receptors were observed to disappear over several days, and nerve terminals overlying the blocked AChRs were subsequently withdrawn. However, uniform blockade of all postsynaptic AChRs did not induce any loss of AChRs or nerve terminals. (Balice-Gordon and Lichtman 1994).

Work confirming hypothesis

- Zebra fish mutants lacking nAChR show signs of formation and elimination of terminals
- Ocular dominance bands in visual system form in the absence of retinal input.
  - removal of the eyes just prior to OD band formation in the ferret does not prevent OD band formation.

Wider Context

- If a period of synaptic silence in the NMJ is responsible to rejuvenation of the damaged synapse, then perhaps this might also occur in the NS as a whole. This could be important to nerve damage caused by stroke or damage to nerves following an accident.

Future Work

- Further experiments using paralyzed muscle to elucidate the molecular resource underlying competition. Possibly GDNF which has been show to cause reactive growth in adults
- Blocking all synaptic releaser using botulinum toxin which cleaves Snares preventing vesicle fusion and exocytosis.
  - However, their method of using dyes depends on vesicle recycling to fluoresce
- Recent data suggests that pre birth there is synchronized firing of axons which favors polyinnervation of muscle fibers,
- Then, later on, there is a sudden change to an asynchronous type of firing which activates synapse elimination and monoinnervation, characteristic of the adult neuromuscular junction.
BBQ: What characteristic other than activity influences competition?

- There is evidence that other factors probably participate in elimination, although their identity is unknown.
- It likely mediated through the postsynaptic side of the neuromuscular junction.
- Possible role for proteases (abundantly present at the neuromuscular junction together with protease inhibitors), or GDNF released from myofibrils, (Buffelli, M., 2004).

Key Points

- Activity of not an absolute requirement for synaptic competition in the even of nerve injury as was previously thought.
- When activity is completely absent, remodeling of the NMJ occurs.
- Paralysis actually induces more sprouting and competitive edge to regenerating neuron.

Bibliography

'Selective vulnerability of motor neurons and dissociation of pre- and post-synaptic pathology at the neuromuscular junction in mouse models of spinal muscular atrophy.'
SMA

• Spinal Muscular Atrophy is a degenerative childhood MN disease.
• Targets the lower α-MN’s in the spinal cord, leading to denervation and atrophy of trunk and limb muscles.
• Three main sub-types SMA I, II and III. Classification is based on age, severity and location of muscles most affected.
• Caused by low levels of a protein product expressed by the SMN gene (Survival Motor Neuron).
• Deficiency in this protein product results in hypotonia and muscle weakness.

Aim of the study?

• Further understanding of the role of the SMN gene in selective MN loss.
• To highlight the pathological events occurring at NMJs in SMA.
• Are pre- and post-synaptic pathology dependant/independent of one another?
• Are there intrinsic factors that exist, and ultimately do these have a role in MN susceptibility to SMA stimuli?

Methods and Techniques

• Use of two SMA mice models:
  - Smn-/-;SMN2 represented Type I SMA – most severe.
  - Smn-/-;SMN2Δ7 represented modified less severe phenotype.
• (Smn1: protein level, SMN2: severity)
• Immunocytochemistry to label the NMJ components.
• Fluorescent/Confocal microscopy, and TEM
• Muscle groups from different regions of the mouse: TVA (abdominal), LAL (fast-twitch, head surface) and deep lumbricals (fast-twitch from hind paw).
• Time-point analysis.
• Muscle fibre diameter, endplate occupancy counts, ...

NMJ pathology

• Identification of abnormalities at SMA NMJs (Smn-/-;SMN2Δ7/Confocal image of TVA).
• Analysis of endplate occupation by nerve terminal.
• Abnormal neurofilament accumulations.
• Muscle fibre diameter: most significant reduction in TVA
Analysis of Ultrastructure

- Using EM, identification of neurofilament accumulations ('whorls'), and immature NMJ.
- Differentiate from Wallerian degeneration
- SMA perhaps more a 'retractive' process

WT and Smn-/-:SMN2

- Effects of Smn's absence in SMA model shown by partial/vacant occupancy of endplate, and change in muscle fibre diameter.
- TVA always more severely affected – both in endplate occupation and average number of inputs per synapse.

Endplate occupancy and muscle fibre diameter: Smn-/-:SMN2Δ7 mice

C – P7 (early-mild) D – P14 (late symptomatic).
TVA in Δ7 model at P7 compared to P14 not significantly different. Modest pre-synaptic pathology in both muscle groups from P7 to P14.
P7 significant reduction in TVA muscle diameter, however P14 more significant shrinkage in both muscles.

Pre- and post-synaptic pathology

- Motor endplate areas found reduced in SMA I model (Smn-/-:SMN2).
- Analysed and compared occupancy of individual endplate areas with their area.
Distinct sub-populations?

- Caudal band of the LAL had higher levels of nerve terminal loss, than rostral - both models, all time points.
- Previously, shown that the LAL is composed of a homogenous fast-twitch population
- Mapping of innervation patterns in LAL muscles
- Found that each caudal and rostral were innervated by separate motor units.
- Selective vulnerability of synapses in caudal band of LAL.

FaSyn/DeSyn Phenotype?

- FaSyn/DeSyn differed according to AchR clustering, maturation and maintenance of the synapse.
- Analysed whether MN’s innervating caudal/rostral bands differed concerning FaSyn/DeSyn characteristics.
- Early studies showed DeSyn muscles undergo collateral sprouting and ectopic endplate formation when paralysed, with FaSyn largely unaffected
- Use of BoA to paralyse LAL.
- Revealed Caudal band to have little sprouting/ectopic endplate formation (like FaSyn) whilst rostral had >20% collateral sprouting and ectopic formation (like DeSyn).

FaSyn/DeSyn?

- Experiment revealed caudal band to be more FaSyn-like than rostral.
- Perhaps this concludes that FaSyn are more selectively vulnerable to synapse elimination in SMA than DeSyn?
- Variability among different MND S? (ALS, DeSyn susceptibility)

Key Findings

- TVA most severely affected – suggesting importance of muscle fibre type and location important determinants for vulnerability
- Differences in time course and severity of disease between two models.
- Dissociation between pre- and post-synaptic pathology; no correlation found.
- Identification of selectively vulnerable sub-population of MN’s that innervate caudal LAL band – leading to suggestion that FaSyn perhaps more vulnerable in SMA selectivity.
Other studies:

• Pre- and post-synaptic pathology in SMA models consistent with earlier studies.
• Finding that TVA (slow-twitch) is more affected than LAL (fast-twitch) contrasts with other studies on other adult MN disease.
• DeSyn is more susceptible than FaSyn in young adult mice with ALS.
• Onset of MN loss in one type of MN disease may be affected differently in other types of MN disease...muscle type, location.

Good points/Bad points?

Good:

• Allowed qualitative analysis of synaptic pathology using LAL and TVA whole mounts.
• Backed up qualitative analysis with quantitative – bar charts, scatter-plots, Mann-Whitney, t-tests...
• Used different muscle groups from different regions of the body.
• Blind assessment of models.
• Backed up evidence with other studies, and acknowledged their contrasting results with others.

Bad:

• Only analysed LAL muscle regarding sub-populations of MNs and FaSyn/DeSyn findings. Known that NMJs vary among muscle types.

The Future?

• Look at other muscles beside LAL to assess FaSyn and DeSyn and see if this finding is true across other muscle fibres.
• Is the SMN gene specific to both/either muscle or neuronal tissue? Precise role?
• Assess mouse models with reduced SMN protein levels, rather than complete absence.
• Apply methods in a tissue specific manner; in either muscle or nerve to test SMN role in each.
• Comparison of vulnerable sub-populations among other MN disease.
• What's the causal link between FaSyn characteristics and vulnerability?

BBQ: How compelling is the evidence that NMJs in FaSyn muscles are more vulnerable than those in DeSyn muscles; what are the criteria for 'vulnerability' and how do they come about? (e.g. endplate shrinkage)

LAL: Previous study showing evidence of DeSyn and FaSyn response to paralysis – with DeSyn more severely affected.

Confirmed this themselves by paralysing LAL muscle with BotA.

Find caudal less affected than rostral, indicating caudal as FaSyn phenotype. Since caudal is more selectively vulnerable in SMA, FaSyn perhaps targeted predominantly by SMA?

Vulnerability: SMA results in selective decrease in nerve terminal loss, post-synaptic endplate shrinkage and accumulation of neurofilament in nerve terminal supported by both SMA mice models and over varying time points.

Molecular and cellular mechanisms for how SMA specifically targets certain MNs as 'vulnerable' is still not fully understood.
Questions?

Distinct presynaptic and postsynaptic dismantling processes of *Drosophila* neuromuscular junctions during metamorphosis

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### Background Knowledge
- Much understood about mammalian NMJ
- Pruning of axons leads to death in mammalian neurons
- Pruning regulated by signalling pathways such as ecdysone and TGF-β
- Glial cells and phagocytosis also participate in the mammalian NMJ elimination process
- Metamorphosis in *Drosophila* involves the transition of larval physiology to adult physiology
- The *Drosophila* NMJ elimination mechanism is not well understood

### Aims/Hypothesis
- To understand more about the NMJ dismantling mechanism in *Drosophila*
- To examine synapse remodelling on both cellular and molecular levels
- To identify the pathways that facilitate the elimination process
**Methodology**

- Flies raised on standard media
- Pupal staging and dissection
- Immunohistochemistry
- NMJ quantifications
- Time-lapse imaging of synapse dismantling
- Electron microscopy of NMJs

**Findings**

- Elimination of NMJ synapses in early stage of metamorphosis
- Disc large (Dlg) and filopodial structures
- Retrograde axonal transport
- Ubiquitination pathway or apoptosis
- Ecdysone signalling
- Glial Cells

**Elimination of NMJ Synapses**

- NMJ 4 of abdominal segments A3 and A4 was easiest to visualise
- NMJs in different muscles were eliminated at different times
- Posterior abdominal segments went earlier than anterior
Dlg and Filopodial Structures

- Postsynaptic Dlg becomes more diffuse
- Presynaptic synaptic vesicles (SVs) aggregate
- Presynaptic filopodial structures also become more diffuse
- Presynaptic membrane retracts towards nerve-muscle contact
Retrograde Axonal Transport

- SVs are transported in a retrograde manner in the presynaptic cell
- Transportation is necessary for presynaptic elimination
- Blockade of this pathway prevents the elimination process

Ubiquitination Pathway

- Postsynaptic apoptosis results in elimination
- Presynaptic apoptosis does not cause synapse elimination
- This is evidence that the postsynaptic side directs elimination
**Ecdysone Signalling**

- Block of ecdysone pathway in the presynapse only affected presynapse
- Block in postsynapse affected both postsynapse and presynapse
- Postsynaptic side determined elimination of NMJ

**Glial Cells**

- Labelled with GFP marker
- Blockade of ecdysone signalling in glia and showed no effect on NMJ elimination
- Glial cells play no apparent role in NMJ synapse elimination

**Strengths and Weaknesses**

**Strengths**
- NMJ 4 allowed easy visualisation of dismantling process
- Examined mutants for presynaptic filopodia
- Data demonstrates for the first time the retrograde axonal transport

**Weaknesses**
- Some samples examined and compared under different conditions - 20°C versus 25°C
- All synapses examined were NMJ 4 from abdominal segment A3 or A4
- Drosophila are not ideal for understanding elimination process in mammals
Big Burning Questions

- How do pre- and post-synaptic mechanisms of degeneration differ?
- What are the triggers?
- Are they relevant to mammals and/or disease?

Answers to Big Burning Questions

- The presynaptic degeneration involves retrograde axonal movement and SV aggregation
- Formation of filopodial structures
- Retraction of its membrane towards nerve-muscle surface
- Boutons become larger and fewer
- In postsynaptic elimination, Dlg becomes diffuse and glutamate receptors become more distributed
- Triggers are likely to involve enzymes and caspases involved in apoptosis, but this is still largely unknown

Future Research

- Pruning and synapse elimination – are they dependent on each other?
- Glial cells – is there a possible role in synapse elimination in Drosophila?
- Postsynaptic apoptosis – how does this dictate synapse elimination?
- Neurodegenerative diseases – how are the mechanisms identified in Drosophila applicable to neurodegenerative diseases in mammals?

Follow-up Articles

- ‘Retrograde interactions during formation and elimination of neuromuscular synapses’ by Yang D and Mu-ming P.
- ‘Role of glia in the formation and maintenance of synapses’ by Frank W. Pfrieger
Conclusion

• Metamorphosis in *Drosophila* involves the elimination of NMJs
• These NMJs are eliminated by specific mechanisms and pathways
• The presynaptic side has a different mechanism from the postsynaptic side
• The postsynaptic side determines whether the synapse will be eliminated or not
• Future research could be directed towards investigating the mechanism involved in glia