‘Sprouting and regression of neuromuscular synapses in partially denervated mammalian muscles’

(Brown and Ironton, 1978)

Image from:
http://neurobio.drexel.edu/SonWeb/Media/CurrentProjects/Compensatory.jpg
Background to nerve sprouting

- It has been established since 1940s (work by various groups) that muscle partially denervated will almost completely regain muscle tension within a few weeks – even if damaged axons do not regenerate

- Confirmed by Van Harreveld (1945) that hypertrophy of fibres still innervated is not of sufficient magnitude to account for recovery

- Workers (eg Hoffman) in 1950s – gold/silver impregnations of partially denervated muscle preparations observed:
  - Both collateral (nodal) and terminal sprouting of intact axons
Continued

- Major development from then til the time of 1st paper: accumulation of evidence that induction of sprouting due to a signal from inactive muscle fibres
- 3 possible mechanisms suggested:
  - a) Nerves intrinsically tend to grow, signal is a diffusible inhibitor and sprouting = disinhibition due to reduced signalling
  - b) Nerves will grow if a substrate (muscle signal) is present. Sprouting= permission to grow due to release of signal
  - c) Nerves require a constant factor for maintenance. Sprouting= excess production of this factor resulting in growth
- Papers discussed today should shed light on which of these mechanisms actually occurs
Aims

• To use the technique of partially denervating a muscle to gain further insights into the phenomenon of nerve sprouting
• Specifically:
  • Quantify the extent of sprouting in motor neurons of different sizes/types
  • Re-examine which sites sprouts arise from and where they grow to
  • Analyse the effects of re-innervation by regenerating axons
Methods

• Peroneus tertius m from white mice was predominantly used (soleus m also in some experiments). Reasons:
  • Supplied by axons from L4 & L5 – easy to conduct partial denervation, survives well in vitro, motor units vary widely in size, easy to visualise muscle twitch
  • % innervation of muscle = max nerve evoked tension/directly evoked tension
  • Histological sections taken to visualise end-plates & nerve terminals with the light microscope
  • Zinc iodide/Osmic acid staining was used
Key findings
1. Extent of sprouting

- Initial motor unit size had no influence – all units increased in size ~ proportionally
- Mean increase was 2.8x
- If not restricted by availability of muscle fibres = 5.3x which reflects intrinsic maximum sprouting capacity of axons
Text-fig. 6. Variation of the logarithm of unit twitch tension (expressed as a percentage of the whole muscle twitch tension) with axon conduction velocity for sprouted units from partially denervated muscles where re-innervation had not occurred. ●, units from muscles which had been partially denervated 10 or more days previously and which had become 100% innervated again by the intact axons. ○, units from muscles partially denervated for 10 or more days which had not become 100% innervated by the intact axons. The continuous line (---) is the regression line of log % twitch tension upon conduction velocity ($r = 0.508$, $n = 67$, $P < 0.001$) for all the units. The dashed line (-----) is the regression line of log % twitch tension upon conduction velocity ($r = 0.558$, $n = 11$, $P = 0.05$) for the units from muscles not 100% innervated. The dotted line (.........) is the regression line for normal units as shown in Text-fig. 2.
Key findings
2. Site of origin of sprouts

- Examined end plates from 10 partially denervated peroneus tertius ms:
- 252 end-plates innervated by unmyelinated branches (sprouts)
- 48% terminal sprouts, 21% collateral, 31% undeterminable
- From 6 partially denervated soleus ms:
  - 85% arose terminally
- In addition was observed that most successful sprouts targeted pre-existing end-plates
Key findings

3. Effects of re-innervation

- 1\textsuperscript{st} signs appeared at 14 days following crush/19 days following cut
- Regenerating axons also target old end-plates
- When >3 motor units remain intact a much greater proportion of twitch is seen to be due to sprouting
Continued

- An overlap between regenerating axons and sprouts was observed
• Despite no natural tendency for sprouts to be lost a reduction in functional terminals fields of sprouts follows re-innervation.
Continued

• Post re-innervation the mean motor unit size was significantly reduced (7.646 ± 0.623%, normal = 10.631 ± 1.084%)
• Therefore there is only partial restoration of innervation
• Shrinkage of sprouts (withdrawal of innervation) varied with the number of returning axons
• Implying that some form of competition is occurring between the two
Conclusions

- Ability of axons to sprout is independent of motor unit size
- Sprouting *in mice* is highly effective at restoring innervation – if >3 motor units are saved almost 100% re-innervation of fibres by 10 days
- Shown clearly that regenerating axons will re-innervate end-plates already occupied by sprouts
- A mechanism for this competitive process is postulated: based on activity & continual movement of nerve terminals allowing one terminal to displace another
Relevant Burning Qs

• Are axons guided back to their original end-plates and if so, how?
Limitations

• Visualisation of sprouts: resolution of light microscopy a limiting factor – resulted in high proportion (31%) of sprouts with an unidentified origin

• Staining technique more effective at displaying nerve terminals than silver/gold impregnation but not as good for nodal areas

• Therefore may bias towards a higher ratio of terminal:collateral sprouts than present in vivo

• Potential inaccuracies in estimation of overlapping innervations:
  • Stimulation could possibly spread from spinal root to the next which would over-estimate degree of overlap but at the same time could have been under-estimated if there were synapses with low quantal content (which would not have been detected)
Continued

• Although sprouting is highly successful in the mouse (and the peroneus tertius m in particular)

• This may be due to the small size of the muscle and the low number of muscle fibres per motoneurone (<30 fibres per neuron)

• Sprouting less successful in higher mammals (larger muscle diameters, > fibres per neuron)
• Therefore findings are less relevant for human/other animal disease intervention
Further work

- Is the proposed mechanism for competition correct? Is this the same process which occurs during elimination of π in post-natal development?
- Results suggest more mature sprouts have greater resistance to elimination – confirm/refute hypothesis
- Investigate the differences in the degree of terminal/collateral sprouting in different muscles: Is this due to the size of muscle/ type of fibres etc?
- What factors mediate the targeting of sprouts (and regenerating axons) to existing end-plates? Suggested role for endoneural sheath – developed in paper 2: action of Schwann cells which produce myelin sheath
Competition at silent synapses in reinnervated skeletal muscles

Costanzo et al. 2000
Introduction

- Polyneuronal innervation – present during neurodevelopment
- Mononeuronal innervation – occurs after neurodevelopmental stage due to decrease in number of connections that each neuron/target cell receives (convergence)
- Those changes – neither spontaneous nor random
- Driven by competition – selective growth of some synapses and elimination of others
- Nerve injury – ‘similar’ changes as those observed during loss of polyneuronal innervation
- Polyneuronal innervation -> mononeuronal innervation: delayed by NM paralysis, resume when activity is restored
- More active convergent synapses are more advantageous when it comes to competitiveness
Introduction

• Suggested: competitive NM synapse elimination -> strongly influenced by endogenous activity
• BQ: How does activity regulate innervation?
• Activity – strictly necessary for the induction expression of synapse elimination?
• Counterarguments:
  – Organisation of connections in visual system (no need for neither visual experience nor neural activity)
  – Hippocampal neurons: strengthening of group of synapses – locally at electrically stimulated sites OR at nearby, not stimulated sites
  – Polynervonal junctions – persist in the presence of activity
  – Sometimes inactive synapses have a competitive advantage over the active ones
Hypothesis

• There is an absolute necessity for activity in competitive synapse elimination during re-innervation of the muscle.
Experimental design

• Fourth deep lumbrical (4DL) muscle in adult rat – dual motor nerve supply:
  – Lateral planar nerves (LPN): ~10 motor axons innervating >70% of 4DL muscle fibres
  – Sural nerves (SN): 1-3 motor axons innervating <30% of 4DL muscle fibres

• Injury of SN – minor partial denervation of 4DL muscle
  – Little reactive sprouting by LPN axons required for complete collateral re-innervation of SN de-innervated muscles (1wk of SN crush -> almost all fibres innervated by LPN)
  – Very small fraction of the re-innervated muscle – terminals supplied by both nerves (become mononeurally innervated through the process of competitive synaptic elimination)
Q: Are there any re-innervated 4DL muscle fibres that would become mainly/exclusively supplied by regenerating SN motor axons when all neuromuscular activity is blocked?

• If activity is necessary:
  – no synapse elimination in paralysed muscles
  – all muscle fibres innervated by SN should keep their sprouted LPN inputs

• If activity in not necessary:
  – Exclusive/majority of SN innervation of the endplates despite complete neuromuscular paralysis
Results

• Assessment of re-innervation
  – Staining with vital fluorescent dyes:
    • SN: FM1-43
    • LPN: RH414
  – Conducted after 2wk of continuous, complete nerve conduction block (with or without additional neuromuscular block)
  – Regenerating SN synapses: competitively displaced LPN synapses from most polyneurally innervated muscle fibres
Results

• Control experiments
  – LPN sprouting: nearly complete by the time regenerating axons returned
  – Chronic paralysis: complete throughout the period of SN regeneration
  – No direct toxic effect of either TTX or α-BTX on motor nerve terminal
Results: Chronic nerve block

- Block with TTX:
  - ~10% of muscles polyinnervated by both SN and LPN terminals
  - ~5% of muscles re-innervated exclusively by regenerating SN terminals alone
  - ~15% of total muscle fibres re-acquired SN input (~ half the number in most unoperated muscles)
Results: Chronic nerve block

- Area occupied by SN motor nerve terminal at the polyneuronal endplates – significantly greater than that of intact LPN terminals supplying the same junction
Results: Chronic nerve block

- Total area of polyneuronally innervated motor endplates – not detectably different from that of mononeurally innervated junctions
- Majority of fibres innervated both by LPN and SN (>50% occupied by regenerating SN terminals)
- Independent of the overall sizes of the endplate

Sum up: Chronic nerve block did not prevent synapse elimination

* Open circles: SN; Filled circles: LPN
Results: Synapse elimination

• Spontaneous quantal/non-quantal release and consecutive action of the neurotransmitter (ACh) at the endplate – possible!
• Result: involvement in competition between intact and regenerating synaptic terminals
• Solution: blockage of all Ach-induced activity at 4DL NMJ → TTX + α-BTX
Results: Synapse elimination

- 15% of the motor endplates – reinnervated by regenerating SN motor axons
- ~13% - polyneuronal innervation
- ~4% of fibres – exclusively supplied by regenerating SN synapses
Results: Synapse elimination

- Distribution of motor nerve terminal areas on polyneurally innervated muscle fibres: \( \sim \) equal
Results: Synapse elimination

- Distribution of percentage occupancies – not distinguishably biased towards any of the nerves
- Mean fractional occupancy by SN terminals at polyneurally innervated junctions – significantly smaller than that of TTX-only or crush-only groups
Results: Synapse elimination

• Thus
  – Complete neuromuscular block may have caused a slight additional delay in competitive synapse elimination
  – Still, competition is NOT prevented
Results: Synapse elimination

- Crush-only control:
  - ~5% of total muscle fibres – re-innervated by the SN by 30 days
  - Few polyneurally innervated fibres
  - ~half of the endplates – supplied by regenerated SN axons (mononeurally innervated)
  - Distribution of fractional occupancies in the small number of polyneurally innervated junctions – similar to that of TTX-only group
Results: Synapse elimination

• Sum up:
  – Nerve conduction and neuromuscular transmission block:
    • promotes sprouting of the LPN
    • promotes regeneration of the SN motor axons and their terminals
  – Muscle paralysis: ↑ proportion of muscle fibres that become re-innervated by the regenerating SN motor axons (restoring ~50% of the original complement of the SN motor nerve terminals
  – Synapse elimination continued at a significant subset of NMJ despite chronic nerve conduction & NM block
Results: Effectiveness of Sprouting and Paralysis

- Assumptions for correct data interpretation
  1. Endplates: fully/mostly occupied when the regenerating axons return
  2. Paralysis of the muscles: complete during re-innervation by the SN
  3. No direct toxicity/mechanical trauma caused by TTX or α-BTX administration
Results: Effectiveness of Sprouting and Paralysis

1. Endplates
   - Does the regenerating axons merely re-occupied junctions that failed to become innervated by sprouts from LPN axons at their terminals? i.e. check for synapse competition
   - Staining with FM1-43 and TRICT-α-BTX 14 days after SN was cut
   - No examples of unoccupied endplates – almost all fully occupied by LPN terminals
Results: Effectiveness of Sprouting and Paralysis

- Staining with neurofilament/SV2 (immunostaining) and TRITC-α-BTX (styryl dyes: stain nerve sprouts poorly)
- Confirmed vital staining: LPN sprouting was nearly completed within 2 weeks from SN injury
Results: Effectiveness of Sprouting and Paralysis

2. Paralysis of muscles:
   - Test on isolated preparations with daily behavioural test of neuromuscular function in vivo – almost all endplates blocked
   - Low-impedance NaCl-filled micropipette probing for residual synaptic currents in TTX/α-BTX paralysed muscles:
     - No spontaneous activity
     - No nerve-evoked synaptic currents
     - Strong endplate signals evoked from the endplate region of reinnervated controls
     - Spontaneous activity (contraction) due to fibrillation – absent from chronically blocked muscles (no fibrillation potential detected)
Results: Effectiveness of Sprouting and Paralysis

- TRITC-α-BTX staining:
  - absent from endplates on the side that received daily unstained α-BTX injections
  - receptors on the control side: heavily stained
- Chronically paralysed muscles:
  - Poor staining even at a saturating level of TRITC-α-BTX (faint punctuate patches)
  - All endplates in control: heavily stained
Results: Effectiveness of Sprouting and Paralysis

3. Toxicity/mechanical trauma
   - Techniques used to block nerve conduction neither mechanically nor chemically injured the motor axons
   - α-BTX injections
     - Subcutaneous rather than intramuscular
     - Minimises possibility of damage to motor nerve terminal
   - Group control:
     - SN crush
     - TTX after 2 weeks + resection of SN axons
     - No uninnervated receptor patches detected
   - All endplates: fully/mostly occupied by LPN motor nerve terminals – consistent with absence of traumatic injury of axons & terminals during toxin administration
Discussion

- Motor endplates – most fully occupied by SN motor terminals due to competition between intact LPN axons and regenerating inactive terminals despite unresponsiveness of the postsynaptic sites to the Ach (electrically silenced)
- Almost all endplates occupied in 80% by the time regenerating axons returned
- Polyneuronal innervation: controls < chronically paralysed muscles
  - Sprouting stimulus released by inactive muscle fibres promotes regenerating axon growth
  - Stimulus from paralysed muscle fibres – inhibits/prevents synaptic elimination
Discussion

• More fibres reinervated in cases with blocked conduction than in controls as well as when LPN is injured – presence of LPN motor nerve terminals at endplates inhibits/represses synapse formation by regenerating SN axons (active or not)
• Proportion of regenerating axons re-innervated endplates is greater after blocking conduction alone – spontaneous activity at endplate may give slight competitive advantage
• Activity – NOT required for competitive elimination of mammalian NM synapses
Further work

• Paralysed, re-innervated muscles:
  – May establish primary molecular resources for NM synaptic competition
  – Find if those mediate consumptive or spatial form of competition
  – ? GDNF (glial-cell derived neurotrophic factor):
    • Sustains high levels of polyneuronal innervation
    • Enhances synaptic transmission

• Looking for alternative mechanism – direct interference between competing synapses
Age-dependent synapse withdrawal at axotomised neuromuscular junctions in Wld<sup>+</sup> mutant and Ube4b/Nmnat transgenic mice

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By Giles Hockridge
Introduction

• Nerve lesion results in degeneration
• In Wld$^s$ mutant mouse axotomy-induced degeneration is delayed
• Degeneration mechanisms are compartmentalised within neurones
Introduction

- Wld mutation affects axons and synapses differently
- After axotomy – Distal axons persist for up to 3 weeks.
- Nerve terminals persist for 4-10 days
Introduction

• Discrepancy…in 1995...

‘Wld\textsuperscript{s} phenotype is lost with age, so by 6 months mutant mice exhibit normal rates of axon degeneration.’
– Ribchester

OR

‘In wls\textsuperscript{s} mutants, axons and synapses are well protected at all ages up to 16 months’ – Crawford

• This study aims to solve this discrepancy…
Age independence of axon protection and Wld gene expression

- Examined levels of expression of the Wld gene in Wld<sup>s</sup> mice of different ages.
- Western blot method

- Preservation of severed distal axons in 4 days axotomised nerves from 2 month and 7 month old mice.
- Counts of myelinated axons
• Did not look at possible axon degeneration at different times – only after 4 days...

• Did not look at possible functional degeneration – morphological analysis results assume all is well…
Progressive loss of synaptic terminals in juvenile Wld<sup>s</sup> mice

- Looked at axotomised NMJs in 2 months old Wld<sup>s</sup> mice at 3-7 days post-axotomy
- Immunocytochemical staining of neurofilaments (NF and SV2) and Ach receptors (TRITC-α-BTX).
- Electron microscopy
- Electrophysiological recording to measure functional withdrawal of synaptic terminals.
• (A) 3 days post axotomy

• (B) 6 days post axotomy – retention of lower terminal, but retraction at top (arrow = retraction bulb)

• (E - G) Electron micrographs of NMJ 3, 4 and 3 days post axotomy respectively

(C) 4 days post axotomy – retraction bulb swelling

(D) 6 days post axotomy – occupied and vacant endplates

(H-J) Intracellular recordings 5 days post axotomy showing robust transmission, weak transmission, and loss of transmission respectively
Time course of synapse withdrawal in 2-month-old Wld<sup>s</sup> mice
A. No effect of endplate size and occupancy on time course of synaptic withdrawal – morphological analysis
Rapid degeneration of Wld\(s\) synaptic terminals in mature mice

- Looked at axotomised NMJs in 4, 7 and 12 month old Wld\(s\) mice at 3-7 days post-axotomy.
- Same analyses used to determine degeneration state as in young mice – Ultrastructural, immunocytochemical and electrophysiological.
• (A) Electron micrograph of 2 synaptic boutons of 4 month old Wld\textsuperscript{s} NMJ, 3 days post axotomy – left has disrupted mitochondria

• (B+C) NMJs from 7 month old Wld\textsuperscript{s} mouse, 2 days post axotomy. Swollen mitochondria, membrane disruption (B) and Schwann cell phagocytosing fragmented nerve terminal (C).

4 months old mice

2 months old mice
Recapitulation of synaptic withdrawal ar reinnervated Wld$^s$ muscles

- Is the transformation in the axotomy reaction of synaptic terminals due to ‘age’ or ‘maturity’
- Nerve crushed in mice aged 7-12 months
- Regeneration allowed – synaptic activity regained
- Then nerve lesioned again and state of synaptic terminals monitored immunocytochemically and electrophysiologically
(B) Original lesion, 3 days post axotomy – last occupied endplate + 3 vacated

(C) Regenerated synapse, 5 days post axotomy – all endplates occupied

(D) Electrophysiological recording of 14 month old fibre with reinnervated connections, 5 days post axotomy

(E) Percentage of fibres with innervations
IM=immunological
EM=electrophysiological
Age dependence of synaptic protection in Wld transgenic mice

• Could the age dependent synaptic protection be due to regulation by its endogenous promoter?

• Two transgenic mice created – 4836 and 4830. In both expression controlled by β-actin promoter. 4836 line expresses Wld protein more strongly than 4830.

• Measured Wld expression levels (independent of age in both lines)

• Axon preservation after axotomy measured by retention of neurofilament heavy chain retention and myelinated axon count.

• Synapse retention measured by morphological and electrophysiological examination.

Source: Konrad Bishop, BSE Inquiry, London, 2000
(B) Western blot of heavy chain neurofilaments 3 days post axotomy—age independent axon retention in 4836 line

(C) Immunocytochemically labelled synapse of 4836 line 5 days post axotomy

(D) Retained synaptic bouton of 4836 line 5 days post axotomy

(E) Time course of decline in synaptic activity in 2 month old 4836 line

(F) Age dependence of synapse loss in both transgenic lines
Age dependence of synaptic protection in Wld transgenic mice

- Axon preservation independent of age in both lines
- Homozygous 4836 line (more Wld expressed) had greater synaptic preservation at 4 months following axotomy
- Hemizygous 4836 and Homozygous 4830 mice showed same age dependence in synaptic response to axotomy as Wld₃ mice.
- Level of Wld expression may mediate synaptic preservation
Protection of axons and synapses expressing fluorescent protein by Wld gene

- Mice available which express cyan fluorescent protein under control of a thy1 promoter
- Used to visualise axon and synaptic protection
- These mice were crossed with WldS mice
- Expression of CFP did not interfere with protection of axons and synapses conferred by Wld gene in young mice

Suggests possibility of visualising axotomy-induced synapse withdrawal in real time
Summary of results and discussion

- Lesions on the peripheral nerve in mice induce one of at least two independent modes of synaptic degeneration in Wld-expressing mice, depending on the maturity of the synapses that are axotomised.

- In young Wld$^s$ mice – synapses progressively withdrawn

- In older mice - rapid degeneration of synaptic terminals, although axons are still preserved.

- In older mice with reinnervated nerves - synaptic withdrawal response following axotomy reinstated
Discussion

- Synapse withdrawal in young mice shares features of synapse elimination during development
  - Partial occupancy
  - Retraction bulbs
  - Synaptic efficacy decline precedes loss of terminals
  - Accumulation of neurofilaments

- Further work – appraise further these similarities
  - Continuous visualisation
  - Observe patterns of retraction in different Wld\textsuperscript{s} boutons of same motor unit
  - Answer to whether withdrawal due to intrinsic or extrinsic factors
Discussion

• Age – no effect on Wld expression
• Crawford support – axonal loss independent of age
• Ribchester support - synaptic preservation is age-dependent or moreover ‘maturity’ dependant
• Is this due to
  • altered biochemical state following regeneration?
  • Recapitulation of gene expression patterns?
• Consequences for experimental design – the age factor
Discussion

- Further support for compartmentalised degenerative mechanisms

- Newly established Wld\textsuperscript{s} neurones retain molecular mechanisms for retraction?
- Synapse elimination during development – self axotomy?!
- ‘Mouthwatering’ prospect of CFP Wld\textsuperscript{s} mouse
- Wld protein mechanism…ubiquitination may be important for synaptic plasticity