Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man
Fisher LR, et al. 2004
Jamie Loan

ALS overview
• Background
• Aims
• Results and inference
• Strengths and Weaknesses
• Further work
• BBQ

ALS overview
• Progressive neurodegenerative disease that selectively affects UMN and LMN
• 2% due to mutations in Zn/Cu-superoxide dismutase (SOD1) coding gene
• Intracellular aggregates
• Microgliosis and astrocytosis
• Excitotoxicity
• Widespread caspase-mediated cell death

Background
• Most previous work in spinal cord
• In Schaefer AM, et al. paper they claim that they noted dying back in 2002. Couldn’t find this paper.
• No loss of motor neurones until 80-90 days in SOD1 mouse — Chiu et al. 1995
• Deficits in retrograde axonal transport ⇒ Pooling of toxic factors in distal neurone?
  Could degeneration be distal in origin?
Aims

– To quantify “... the numbers of spinal motor neurones, axons in the nerve roots and the degree of denervation at NMJs...” over time

• Thus temporal and spatial description of ALS

Results

• In Brief...
  – Mice lost co-ordination and balance at day 78
  – Progressive centripetal motoneuron degeneration starting at the end plate ≈ 47 days
  – Regeneration of small fibres noticeable in later stages
  – Case Report

Onset of signs

• Decreased rotorod performance first reported on day 85
• Weight an unreliable measure of disease onset or progress
Neuropathological findings:

NMJ

• 28 days most endplates innervated
• From day 47 progressive loss of endplates
• No explanation for error bars

Neuropathological findings: Ventral Root

• Pathological changes first seen day 80

Innervated, Intermediate and Denervated NMJs

("intermediate" counts as terminal axon still present but no connection to end-plate)
Neuropathological findings: Ventral Root

- Pathological changes first seen day 80
- Decreased motor axon density
- Large diameter axons preferentially affected
- After 80 days total number of axons increased: by day 120 significantly more than days 80 or 100
Neuropathological findings: Ventral Root

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- After 80 days total number of axons increased: by day 120 significantly more than days 80 or 100
  ⇒Regeneration?

Neuropathological Findings: Ventral Horn of Spinal cord

- Signs of active degeneration (vacuolation) first seen day 80 [same as with ventral root]
- Number of large caliber MNs first seen decreased was on day 100

From: “Neuroscience for Kids” website. URL: http://faculty.washington.edu/chudler/spinal.html

Neuropathological Findings: Ventral Horn of Spinal cord

- Quantitative analysis
  - α-motoneurones decreased by 40% from 80-100 days
    - α-motoneurones defined as: Neurones having a cross-sectional area ≥250μm²
  - Total neurone number also decrease by 40% over this period
Neuropathological Findings:
Ventral Horn of Spinal cord

- Few active astrocytes at 47 days and increased progressively until death
  - Not quantified
- Indistinct microglial infiltration. Did not precede pathology in ventral roots or NMJ (other spinal pathology?)

Neuropathological Findings: Ventral Horn of Spinal cord

Case Report

- 58 male. 6 month Hx – weakness and wasting
- Examination:
  - Diffuse fasciculations
  - Muscle atrophy (diffuse?)
  - Normal to brisk reflexes (where?)
  - FVC 57% of predicted (FEV,?)
- EMG: Acute and chronic denervation. Upper and lower extremities and paraspinal muscles
- Died 2 weeks later in minor surgery (presumably unrelated)
### Case Report – Neuropathology

- Grouped atrophy
- Fibre-type grouping
- Ventral roots – little axonal degeneration
- Spinal cord normal at all levels with no astrocytosis or microgliosis.
- Motor cortex and corticospinal tracts normal
- NMJ?

### Case Report – Neuropathology

- Mice appeared to show pathological changes occurring centripetally for all measures
- Case report seemed to concur with this as patient died quite early in disease

### Inferences

- Compensatory changes as increase in proportion small calibre motor neurones and number of axons in ventral roots rises despite ongoing degeneration
- Hypothesised that glial activation would correlate with CNS pathogenesis. But no firm evidence that abnormalities inside MN soma result in gliosis.

### Hypotheses for “dying back”

- Longest axons most susceptible – but then why bulbar and focal onset sometimes rather than “glove and stockings”
- Sublethal insult to cell body causes undernourishment for distal cell.
- Accumulations of aggregates (SOD1 complexes or neurofilament)
  - Anterograde/Retrograde axonal transport
  - Dynactin – autosomal dominant MND. "Trophic factors.
- Chronic glutamate toxicity disrupt maintenance of axon
- Disrupted axon may cause degeneration
  - In Progressive Motoneuropathy mice prevention of axonal degeneration prolongs neuron lifespan. So degeneration due to disrupted axon
Weaknesses

• Don’t include control data
• Only looked at mice at 28, 47, 80, 100 and 120 day old mice – quite big gaps
  – Other studies reported physiological changes at day 40. Kennel et al. 1996.
  – Couldn’t distinguish between ventral root and horn onset.
• Case report of limited use but...

Strengths

• Case study of this kind hard to come by. Was referenced in future paper (can’t find!)
• Study identified and started to address a gap in knowledge
• Quantitative analysis using computers
• Pilot counting study to determine number of sections of lumbar spine to get 100-200 MNs

Future research

• Bjornskov et al (1984) study on post-mortem humans showed compensatory reinnervation
  ⇒ Possible to get non-end stage tissue
  – Look at NMJs to see if this really is the start-point
• This paper helped lay the foundation for the next three papers to be presented.
  • Also further papers on the effect of myofibril type (Hegedus D, et al. 2008), I/R injury in different muscle types (Baxter B, et al. 2008)
• Do spinal MNs also degenerate in a distal-proximal manner?
  • In vitro work suggests that this is the case (King AE, et al. 2007)
• Review article that I couldn’t access but looks good:

BBQ

“What is the link between dying-back, astrocytic and microglial activation and rapid motor neurone degeneration/loss?”
“What is the link between dying-back, astrocytic and microglial activation and rapid motor neurone degeneration/loss?”

- Elements of dying-back phase of ALS can be seen for a long time presymptomatically
- Astrocytosis is a relatively late feature
- This paper, and papers to follow (I/R injury, and muscle specific SOD1) show that MN degeneration and loss can be induced proximal changes that also induce dying back.
- Therefore a reasonable hypothesis:
  - A trigger induces dying back of axons and fragmentation of MN terminals. This causes neuron loss and hence reactive astrocytosis/microgliosis

References


Introduction

- ALS is a progressive, fatal, neurodegenerative disease caused by the degeneration of motor neurons, the nerve cells in the central nervous system that control voluntary muscle movement
- For patients without a family history of the disease, which includes ~5-10% of cases, there is no known cause for ALS
- Superoxide Dismutase (SOD1) enzyme is associated with approximately 20% of familial ALS
Introduction

Whether the two types of behaviours 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 used for all analyses
- G93A SOD1 crossed with thy1-YFP mice
- Tissue staining of sternomastoid, cleidomastoid and clavotrapezius in the neck, the diaphragm, and the extensor digitorum longus (edl) and soleus in the hindlimb
- Motor units and neuromuscular junctions were imaged on a laser scanning confocal microscope (Olympus FV500 or Bio-Rad 1024, and CA with an Olympus BX50WI microscope)

Results

- Dissected muscles analysed by Monte Carlo analysis to determine the likelihood fragmented axons were randomly distributed across all motor units and results were compared against randomly generated datasets that maintained the total number of motor units and their size
- Mice were anesthetised with a subcutaneous injection and a superficial incision was made in the ventral neck. Approximately 2% of the acetylcholine receptors in the sternomastoid muscle were labeled
- Superficial Junctions imaged with standard epifluorescence microscopy and a QImaging Retiga EXi cooled CCD camera
- This procedure was repeated for subsequent imaging sessions
Results

Results

Results

Results
Conclusion

- The main conclusion is that in a genetically valid mouse model of ALS, individual motor pools contain two distinct types of motor neurons, "losers" (which contain fragmented, degenerating branches) and "compensators" (which contain thin, reinervating branches).
- A subset of neurons within a motor neuron pool is refractory is a novel finding. The results show that new growth occurs in the form of thin branches and that these thin branches emanate primarily from axons, as opposed to terminal sprouts.

Further Research

- Mechanisms by which neurones are susceptible to become either "losers" or "compensators"
- Why motor units in muscles are affected earlier or later in the disease
- How SOD1 cellular mechanisms leads to ALS
- How similar are the mechanisms of sprouting from the axon in ALS to developmental?

BBQ

- What confers differential susceptibility (eg physiological differences?) to degeneration and is sprouting neuroprotective?
- Essential random fluctuations in some quality may reach a threshold value that starts some motor neurons on an irreversible path to their demise. Such mechanisms have been invoked in cell fate choices during development and aging (Herndon et al., 2002) and could also help explain the course of slowly progressive diseases such as ALS.
- Relevant variation is external to the motor neuron itself. Recent studies of chimeric mice have shown that some effects of SOD1 on motor neuron survival are non-cell-autonomous.
- Motor neurons might vary in the level of SOD1 they express.
- Motor neurons differ in some endogenous quality that is related to their normal function but also affects their susceptibility to disease. Such qualities could be known distinctions such as motor unit size, fiber type, or expression of markers such as tropic factor receptors or transcription factors.
- Sprouting is neuroprotective to a certain extent as it slows the progression of ALS and other neurodegenerative disorders, however it ultimately it is a difficult to target as a therapeutic tool.

"…not only our understanding of the fundamental mechanisms of synaptic transmission: such analysis could ultimately lead the way to novel therapeutics, in the ongoing war of attrition against those psychiatric or neurodegenerative disorders whose present, imperfect treatment taxes human societies physically, emotionally and economically”

Questions?

• Introduction and Background
• Aims of the study
• Methods
• Results
• Conclusions and Implications
• Strengths and Weaknesses
• Burning question
• Future Work

Introduction

• ALS is a distal axonopathy with more extensive peripheral than central morphological damage (Fisher et al., 2004)
• This has led to the development of a hypothesis that distal damage can accelerate the disease progression of ALS (in mice and rabbits).
• Therefore, are motor terminals subject to stresses that other synapses and the perikaryon are not?
  — Ischaemia during (anaerobic) exercise? Ischaemia / reperfusion injury following decrease in blood flow?
• **Ischaemia**
  – inadequate blood flow to a part of the body caused by constriction or blockage of the blood vessels supplying it.

• **Reperfusion**
  – restoration of blood flow to a tissue or organ following a period of ischaemia.

• **Reperfusion injury**
  – tissue damage that occurs following reoxygenation. Inflammation and the induction of oxidative stress occurs due to the return of blood flow (and thus leukocytes and inflammatory mediators) to the area.

• **Slow-twitch muscle (eg, Soleus)**
  – Type I fibres
  – Slow oxidative with dense capillary structure, and high fibre-to-capillary ratio
  – High number of mitochondria

• **Fast-twitch (eg, EDL)**
  – Type II fibres (a and b)
  – Type IIa: similar to slow-twitch in composition; high number of mitochondria, fatigue-resistant, has some glycolytic capacity
  – Type IIb: fatiguable, glycolytic

(Ouedek et al, 2004; Murakami et al, 2010)

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**Oxidation Pathways**

**Introduction – Previous Work.**

• Study by Makitte and Teravainen (1977) showed swelling of mitochondria in nerve terminals after ischaemic stress.

• Tombol et al (2002) showed that following 2 hours of ischaemia there were no changes in muscle fibres, axons or Schwann cells but signs of degeneration in motor nerve terminals.

• Eastlack et al (2004) showed that the NMJ is a major site of I/R injury.

• Sharps et al (2005) Peripheral nerve injury is shown to accelerate the degeneration in SOD1 mice.
Aims
• To test if motor terminals in the G93A-SOD1 ALS mouse model are more vulnerable to I/R injury than wild-type (WT) mice.
• To see when this vulnerability manifests in comparison to WT
• To compare different muscle fibre types and if they show a differing sensitivity to I/R injury
• Following I/R injury, whether motor terminals are capable of regeneration in SOD1-G93A mice

Method
• I/R – tourniquet ischaemia by application to hindlimb with no direct pressure on any muscle tested in the study. Ischaemia lasted 15 to 60 minutes. One hindlimb underwent I/R injury while the contralateral hindlimb acted as control.
• SOD1-G93A mice expressed YFP in motor neurons
• Mice (both WT and mutant) were assayed at several ages, both pre- and post-symptomatic. (See results)
• α-BgTx was used to label acetylcholine receptors (AchR)

Results

Results - EDL Motor Terminals in SOD1 Mice are Sensitive to I/R Injury both pre- and post-symptomatically

• WT and SOD1 Mice at pre-symptomatic ages (P31-33) underwent I/R injury – 30 min stress with 6 h reperfusion.
• Non-stressed SOD1 mice endplates were fully innervated, showing that motor terminal degeneration does not occur at this age.
• Following I/R stress,
  • YFP-only endplates remained innervated
  • SOD1-YFP mice showed widespread denervation
Results – Fast muscles are more sensitive to I/R injury

• Evidence has shown that fast IIb fibres degenerate faster than other muscle fibre types
• Hypothesised that type IIb fibres (as in the EDL) would show increased vulnerability to I/R injury compared to slow fibres (as in the soleus).
• Non-stressed SOD1-YFP mice show a decreasing endplate occupancy with age in EDL but not Soleus
• Stressed SOD1-YFP endplate occupancy fell dramatically with age in EDL but not soleus
• Stressed and non-stressed YFP-mice showed consistently high endplate occupancy in EDL and soleus.

Results – Fast muscles are more sensitive to I/R injury

• Measured mean endplate occupancy in the Soleus and EDL muscles as a function of age in both stressed and non-stressed WT and SOD1 mice.
• Occupancy decreased with age in EDL but not soleus.
• Stressed SOD1 mice EDLs showed a sharp decrease in occupancy with age.
• YFP-Mice showed >70% occupancy in both muscle types and at all ages.
Results – Fast muscles are more sensitive to I/R injury

- Increased vulnerability to I/R injury occurs weeks before significant denervation in the contralateral muscle.
- EDL is not unique as a fast muscle in being sensitive to I/R injury.
  - Plantaris also affected in a similar way
  - I/R injury denervated 90% of EDL endplates, 90% of plantaris and 7% of soleus in one mouse. In another mouse, 22% of EDL, 33% of plantaris and no endplates in soleus were denervated
  - What accounts for this difference?

Results – SOD1 I/R denervation is not replicated by axotomy at tourniquet site

- Sciatic nerve innervating one hindlimb was cut and the contralateral limb underwent tourniquet-induced ischaemia.
- Axotomised hindlimb EDL still had high endplate occupancy in comparison to the high level of denervation in the contralateral EDL
- Ergo, post I/R denervation of EDL endplates was not due to axonal injury.
Results – Denervation of EDL endplates develops rapidly following I/R injury

Figure 5

• Figure 5Bc shows that the greatest loss of terminal innervation (as demonstrated by decrease in fluorescence) was between ~80 and 120 minutes.
• Post I/R terminal degeneration in the in vivo experiments was likely to be complete within this time as SOD1 endplate occupancy showed no correlation with in vivo reperfusion times.

Results – EDL motor terminals in SOD1 can reinnervate following I/R injury

• A 65 day old mouse underwent I/R injury and 10 days reperfusion.
• Almost all endplates in the EDL were innervated when examined following I/R injury suggesting reinnervation has occurred.
• Nerve sprouts were found that extended beyond the endplate region, again suggesting reinnervation.
Results – EDL motor terminals in SOD1 can reinnervate following I/R injury

• Examined two older mice (89 days old) to measure their reinnervation capacity.
• Fraction of fully occupied endplates was 47 +/- 5.5% in stressed limb and 93 +/- 1.4% in non-stressed limb.
• Sprouts were present in 14 +/- 1.9% in stressed limb and 5.3 +/- 1.8% in the non-stressed limb.

Conclusions

• SOD1 mutant mice show a decrease in endplate occupancy following I/R injury.
• Fast-type show an increased vulnerability to I/R injury from an early age.
  — See Burning Question
• Post I/R injury, denervation of terminals is rapid following reperfusion, occurring between ~80-120 minutes.
• SOD1 mice show some capacity to reinnervate endplates following injury but this decreases with age.

Implications ?

• Oxidative stress as a mechanism that accelerates ALS?
• Motor terminals as a site of significant damage during ALS, therefore a preferential target for therapeutics?
• Some reinnervation capacity is seen even at late-stages of the disease, is there some way to increase and maintain this reinnervation?
• Is vigorous exercise a potential risk factor for ALS?

Burning Question: Could the difference in the vulnerability to ischemia be due to increased production of free radicals? What is the relationship to ALS in humans?

• Reperfusion generates Reactive Oxygen Species (ROS).
• Fast (type IIb) muscles in SOD1 mice seem especially vulnerable to ROS in comparison to slow muscle type.
  — Reason ? Invasion of more muscle fibres ? Lower basal blood flow ? Fast muscles producing more H2O2 ? Greater production of NO and thus ROS ?
• Type IIb fibres have no mitochondria and altered SOD1 in mutant mice, so, is there the ability to deal with increased ROS impaired, leading to the distal damage of motor terminals?
  — Evidence that mutant SOD1 actually increases ROS production in fibres, with mitochondrial swelling and activation of NO synthase. (Martin et al, 2007)
• So, increasing ROS via SOD1 damages mitochondria and increases cell vulnerability. (Rizzardi et al 2005)
  — However, only an increase in vulnerability, evidence has also shown that apoptotic pathways are blocked in SOD1 mice so controlled cell death may be lost to the gradual ‘dying back’ degeneration seen in this type of ALS. (Martin et al, 2007)
Burning Question: Could the difference in the vulnerability to ischemia be due to increased production of free radicals? What is the relationship to ALS in humans?

- How relevant is I/R injury to human ALS?
  - Under what circumstances could this actually occur in humans?
  - Method in this study allows for the creation of ROS and shows the vulnerability of fast type motor terminals but could these levels of free radicals ever occur in human disease? Is it possible that short, cumulative damage of ROS is responsible for MN damage in human ALS?
  - Are the results of this study merely an artefact of an artificial experimental method or does it elucidate on features of human SOD1 ALS?

Strengths

- Approaches ALS from a new perspective with an interesting method.
- Clear approach with good verification.
- Good quantification of data.
- Raises interesting questions regarding the role of oxidative stress in ALS pathogenesis.

Weaknesses

- Inconsistent use of mice with respect to age.
  - Experimental results compromised by older SOD1 mice who are already showing denervation due to ALS rather than I/R injury.
- "Motor nerve terminals may be subjected to stresses not normally experienced by the parent motor neuron, such as transient partial ischemia during intense (anaerobic) muscle activity or I/R injury following temporary interruption of limb blood supply."
- Specific data is often difficult to find, ie, number of mice used, statistical significance.
- The study did little to show that fast type fibres in general are affected and the results they do have show great divergence in denervation between two mice.
- Relevance to human disease?

Exercise as a risk factor for ALS?

- Could intense anaerobic exercise be a risk factor for ALS?
- Conflicting evidence:
  - Mahoney et al (2004) have shown that intense exercise in SOD1 mice accelerates disease progression (but Kirkinezos et al (2003) have shown that moderate exercise slows progression).
- Rather than a matter of repeated spikes in oxidative stress, is ALS in athletes due to repeated head trauma?
  - Gavett et al, 2010
Future Work

- Does I/R injury actually accelerate disease progression?
- Is oxidative stress an initiating component of ALS pathogenesis or a consequence that arises from other factors (ER stress, protein misfolding, excitotoxicity, etc.)?
- What cellular mechanisms give rise to this vulnerability to ROS? Is it a possible therapeutic target?
- Does muscle have a significant contributory factor to ALS (and oxidative stress)?

References


Skeletal muscle-restricted expression of human SOD1 causes motor neuron degeneration in transgenic mice

Margaret Wong & Lee J. Martin, Human Molecular Genetics (2010)

Terms:
- ALS – Amyotrophic Lateral Sclerosis
- SOD1 – Superoxide Dismutase-1
- tSOD1 – Human SOD1
- tSOD1 – tg mice, bred from different strains, with muscle restricted hSOD1

Introduction

ALS – progressive and fatal neurodegenerative disease

SOD1 – metalloenzyme that maintains intracellular free radical O$_2^-$ by the following catalytic activity

$$\text{Superoxide as substrate}$$

<table>
<thead>
<tr>
<th>SOD1</th>
<th>( \text{D}^{\text{O}} \text{O}_2^- )</th>
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<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>( \text{H}_2\text{O} + \text{O}_2 )</td>
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SOD1 – mutants appear to gain toxic property rather than reduced detoxification action
Introduction

Skeletal Muscle – pathology in skeletal muscle occurs in both sporadic ALS and familial ALS in humans, though it has been generally assumed up until this point that these symptoms are unrelated to the pathogenesis mechanisms of the disease.

This leads to the question…

"Does skeletal muscle itself contribute to the degeneration of MNs in ALS?"

Hypothesis:

“skeletal muscle is a primary site of disease in ALS”

Methods

hSOD1 mutant transgenic mice

• tg mice, derived from either SOD1 mutants (G93A or G37R) or wildtype, were created with hSOD1 restricted to skeletal muscle in all cases

• tests were performed to determine that there was indeed hSOD1 expression in skeletal muscle and no expression elsewhere in the body

Analysis of pathology

• mouse phenotype was assessed using a hang-time test and voluntary activity wheel

• histological and immunohistochemical analysis of skeletal muscle pathology

• evaluation of loss of cells in skeletal muscle and spinal cord

• MN cell body volume was measured for 25-50 cells in each mouse

• NMJs of mice were analysed to determine the degree of denervation
**Methods**

**Data analysis**
- Group means and variances were statistically analysed using ANOVA and Student’s t-test.

**Photography**
- Images were captured digitally by either a SPOT or Nikon digital camera and various software.

**Results**

**tg mice show neurologic phenotype**

**Results**

**tg mice develop skeletal muscle pathology**

**Results**

**tg mice show oxidative modification of proteins**

- Proteins in mitochondria-enriched fraction showed significant modification.
- hSOD1 gains toxic properties involving mitochondrial function?
Results
tg mice show oxidative modification of proteins
- Cytochrome c itself plays role in apoptosis and has been shown to interact with other cytochrome c oxidases (COX4I2)

Results
tg mice develop NMJ abnormalities and MN distal axonopathy

Results
tg mice have degeneration of MNs
- NeuN is a protein found in mice α-MNs

Results
tg mice have degeneration of MNs
- In addition, remaining MNs show structural, cytoskeletal and other intracellular abnormalities consistent with spinal MN degeneration in an apoptotic-like fashion
Conclusions

“skeletal muscle expression of hSOD1 is sufficient to cause MN disease in mice”

What the authors conclude:

• that disease in skeletal muscle is a causative factor for the development of ALS
• that skeletal muscle disease or injury can trigger MN degeneration
• that hSOD1 might acquire toxic properties through alteration of mitochondrial function
• that denervation of skeletal muscle and axonopathy may be a result of damage to NMJs
• that their mouse model is possibly a more relevant model for studying sporadic ALS

Strengths & Weaknesses

Strengths

• a lot of different experiments performed with a huge range of data collected
• very informed and insightful results with a lot of diagrams and tables
• generation of new mouse model for ALS

Weaknesses

• tedious to read and difficult to get through
• methods were very clear, but it was unclear why certain methods were used
• conclusions seem to get ahead of the results

How does the onset and progression of pathology in skeletal muscle compare with that of motor neurones? Does the caspase-3 pathway play a role?

• astrocytes have been implicated in MN pathology
• similar SOD1 mutations affect MNs the same as skeletal muscle

Skeletal muscle pathology induces pathology in associated MNs

BBQ

How does the onset and progression of pathology in skeletal muscle compare with that of motor neurones? Does the caspase-3 pathway play a role?

![Graph showing Caspase 3 activity in muscle tissue]{322x322}
Questions