Loss of translation elongation factor (eEF1A2) expression in vivo differentiates between Wallerian degeneration and dying-back neuronal pathology

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Abstract

Wallerian degeneration and dying-back pathology are two well-known cellular pathways capable of regulating the breakdown and loss of axonal and synaptic compartments of neurons in vivo. However, the underlying mechanisms and molecular triggers of these pathways remain elusive. Here, we show that loss of translation elongation factor eEF1A2 expression in lower motor neurons and skeletal muscle fibres in homozygous Wasted mice triggered a dying-back neuropathy. Synaptic loss at the neuromuscular junction occurred in advance of axonal pathology and by a mechanism morphologically distinct from Wallerian degeneration. Dying-back pathology in Wasted mice was accompanied by reduced expression levels of the zinc finger protein ZPR1, as found in other dying-back neuropathies such as spinal muscular atrophy. Surprisingly, experimental nerve lesion revealed that Wallerian degeneration was significantly delayed in homozygous Wasted mice; morphological assessment revealed that ~80% of neuromuscular junctions in deep lumbrical muscles at 24 h and ~50% at 48 h had retained motor nerve terminals following tibial nerve lesion. This was in contrast to wild-type and heterozygous Wasted mice where < 5% of neuromuscular junctions had retained motor nerve terminals at 24 h post-lesion. These data show that eEF1A2 expression is required to prevent the initiation of dying-back pathology at the neuromuscular junction in vivo. In contrast, loss of eEF1A2 expression significantly inhibited the initiation and progression of Wallerian degeneration in vivo. We conclude that loss of eEF1A2 expression distinguishes mechanisms underlying dying-back pathology from those responsible for Wallerian degeneration in vivo and suggest that eEF1A2-dependent cascades may provide novel molecular targets to manipulate neurodegenerative pathways in lower motor neurons.

Key words axon; neuromuscular junction; neuropathology; synapse; Wasted mice.

Introduction

Pathways regulating neuronal vulnerability in vivo are of critical importance to our understanding of a wide spectrum of neurodegenerative disorders from Alzheimer’s disease to motor neuron disease. A significant body of evidence now suggests that the maintenance of neuronal viability is compartmentalized within neurons, as cell soma, axons and synapses are all capable of independent regulation. One significant consequence of this compartmentalization is that distal neuronal compartments such as axons and synapses are particularly sensitive to perturbations of neuronal homeostasis (Gillingwater & Ribchester, 2001; Coleman, 2005; Gillingwater et al. 2006; Wishart et al. 2006; Bettini et al. 2007; Saxena & Caroni, 2007; Baxter et al. 2008).

Several apparently distinct cellular pathways are known to be capable of bringing about the degeneration of axons and synaptic terminals, including dying-back pathology and Wallerian degeneration (WD). For example, several different motor neuron diseases and sensory neuropathies are thought to occur primarily via dying-back pathways (Schmalbruch et al. 1991; Frey et al. 2000; Cifuentes-Diaz et al. 2002; Fischer et al. 2004; Keswani et al. 2006; Murray et al. 2008), whereas conditions such as multiple sclerosis and degeneration after traumatic nerve injury are more commonly associated with WD (Ferguson et al. 1997; Perry & Anthony, 1999; Gillingwater & Ribchester, 2001). Morphologically, WD is characterized by rapid axonal and synaptic fragmentation associated with disruption and loss of organelles and plasma membranes, breakdown of the axonal myelin sheath, and phagocytosis of synaptic and axonal debris by cells including Schwann cells and invading macrophages (for review see Gillingwater & Ribchester, 2001). At the
neuromuscular junction (NMJ), this process is characterized by an early depletion of synaptic vesicles, swollen and burst mitochondria, a breakdown of pre-synaptic plasma membranes and terminal Schwann cell processes penetrating into the synaptic cleft (Miledi & Slater, 1970; Winlow & usherwood, 1975; Gillingwater et al. 2003). By contrast, dying-back neuropathies are characterized by a wave of degeneration beginning at, and progressing retrogradely from, the distal extremities of the neuron. Here, the early withdrawal/retraction of synaptic terminals at the NMJ occurs via a process devoid of the gross fragmentation associated with WD and more akin to a progressive reabsorption of synaptic and distal axonal organelles and plasma membranes back into the parent axon (for review see Gillingwater & Ribchester, 2003). Despite these clear morphological differences little is known about the extent to which their underlying molecular mechanisms converge or diverge (Coleman, 2005; Hoopfer et al. 2006). However, evidence has been presented suggesting that morphologically distinct degeneration pathways can share common mechanistic links (Coleman, 2005; Mi et al. 2005). More detailed knowledge of the cellular and molecular mechanisms that regulate and perturb viability in distal neuronal compartments is therefore of significant importance for our understanding of the healthy and pathological nervous system.

Here, we detail neuropathological changes occurring in the peripheral nervous system of mice carrying a spontaneous mutation that abolishes the gene expression of eEF1A encoding the translation elongation factor eEF1A2 [Wasted (Wst); Shultz et al. 1982; Chambers et al. 1998]. eEF1A (of which there are two variant forms, i.e. eEF1A1 and eEF1A2) is the second most abundant protein in non-proliferating cells, constituting 1–2% of total protein and playing an integral role in the elongation stages of protein synthesis during which the polypeptide chain is assembled (Condeelis, 1995). Alongside important roles in protein synthesis, eEF1A proteins have also been postulated to have roles in non-canonical pathways including modification of the cytoskeleton (Condeelis, 1995), the heat shock response (Shamovsky et al. 2006) and synaptic plasticity (Giustetto et al. 2003). Expression patterns of the two variant forms (eEF1A1 and eEF1A2) are mutually exclusive in all cells and tissues examined. The eEF1A2 variant is only expressed in mature, terminally differentiated neurons and muscle (Pan et al. 2004). Its post-natal appearance in nerve and muscle coincides with loss of eEF1A1 expression. eEF1A1 is expressed ubiquitously at the time of birth but its expression in nerve and muscle declines from the first post-natal week onwards, being absent by post-natal day (P)20–21 (Pan et al. 2004). During this period of declining eEF1A1 expression in nerve and muscle, its role is normally replaced by correlated increasing expression levels of eEF1A2 (Chambers et al. 1998; Khalyfa et al. 2001), with the latter being the sole translation elongation factor by P21.

It has previously been shown that loss of eEF1A2 results in the degeneration of lower motor neurons in Wst mice, characterized by vacuolation and neurofilament accumulation in neuronal soma and denervation of skeletal muscle fibres (Newbery et al. 2005). Importantly, the onset of degeneration correlates precisely with the switch to reliance on eEF1A2 expression (Newbery et al. 2005) and it has been conclusively demonstrated that the specific loss of eEF1A2 function is solely responsible for these events (Newbery et al. 2007). Preliminary observations of the morphological correlates of degeneration at the NMJ in homozygous Wst mice (Newbery et al. 2005) highlighted potential similarities to neuropathological events previously described in other mouse models of dying-back neuropathy (e.g. Frey et al. 2000; Cifuentes-Diaz et al. 2002; Fischer et al. 2004; Murray et al. 2008). However, dying-back pathways were not conclusively demonstrated in this study (Newbery et al. 2005). We have therefore undertaken a detailed qualitative and quantitative analysis of synaptic and axonal pathology in Wst mice to determine whether dying-back pathways are indeed instigated following the loss of eEF1A2 expression in vivo. We show that the absence of normal post-natal eEF1A2 expression in Wst mice leads to early-onset degeneration of motor nerve terminals, resulting in a dying-back pathology distinct from classic WD. Quantitative protein expression experiments showed that dying-back pathology in Wst mice correlated with reductions in expression levels of the zinc finger protein ZPR1. Surprisingly, a parallel set of nerve lesion experiments in homozygous Wst mice revealed delayed WD of distal axons and synaptic terminals, demonstrating that eEF1A2 is required for the normal initiation and progression of WD pathways. These experiments demonstrate that loss of eEF1A2 expression distinguishes mechanisms underlying dying-back pathways from those responsible for WD in vivo.

Materials and methods

Mouse maintenance and surgery

Pairs of heterozygous Wst mice were kindly provided by Dr Cathy Abbott (University of Edinburgh) and a breeding colony established. Litters contained homozygous and heterozygous mice as well as wild-type littersmates. Heterozygous and wild-type littersmates were used throughout as controls. Breeding colonies of YFP–H mice (Feng et al. 2000) were already established in animal care facilities at the University of Edinburgh and were crossed with Wst mice in order to obtain homozygous and heterozygous Wst mice, as well as wild-type littersmates, endogenously expressing yellow fluorescent protein (YFP) in a subset of neurons. Smn–/–;SMN2 mice (Monani et al. 2000) were obtained from existing breeding colonies in Edinburgh. All mice were housed in a semi-barrier facility and were fed a standard chow diet. Wst, Wst;YFP–H and Smn–/–;SMN2 mice were genotyped using standard polymerase chain reaction techniques, as described previously (Newbery et al. 2005; Murray et al. 2008). The YFP status was ascertained by examining ear punches for evidence of YFP-labelled neurons. Mice were killed by overdose of isofluorane via
inhaling or cervical dislocation. Wst mice and wild-type litters-
mates were killed at P20 for early-symptomatic data and P24–P27
for late-symptomatic data. All surgical procedures were per-
formed under general anaesthesia (inhaling of isofluorane;
2% in 1:1 N2/O2) as described previously (Gillingwater et al. 2003).
All breeding and surgical procedures were carried out with the
licensed authority of the UK Home Office.

Immunohistochemistry
Muscles were immunohistochemically labelled to allow quantifi-
cation of neuromuscular innervation as described previously
(Murray et al. 2008). Briefly, muscles were immediately dissected from
recently killed mice and dissected in oxygenated mammalian
physiological saline before labelling post-synaptic acetylcholine
receptors with α-bungarotoxin conjugated to tetramethylrhoda-
mine isothiocyanate (5 μg mL⁻¹ for 10 min, Molecular Probes, USA).
Muscles were then fixed in 4% paraformaldehyde in phosphate-
buffered saline (PBS) for 1–2 h. Non-YFP-expressing muscles were
then blocked in 4% bovine serum albumin and 1% TritonX in
buffered saline (PBS) for 1–2 h. Non-YFP-expressing muscles were
studied Hybridoma Bank, USA) or 150 kDa neurofilament proteins
and the synaptic vesicle protein SV2 (both 1:200, Developmental
antibodies raised against either 165 kDa neurofilament proteins
(Chemicon, USA) and visualized with sheep anti-mouse fluorescein
isothiocyanate-conjugated secondary antibodies (1:200; Diagnostics
Scotland, UK) or swine anti-rabbit fluorescein isothiocyanate-conju-
gated secondary antibodies (1:40; DAKO, USA), respectively.
Muscles were then whole-mounted in Mowoil® (Calbiochem) on
glass slides and coverslipped for subsequent imaging.

FM4–64FX labelling of neuromuscular synaptic function
Freshly dissected levator auris longus (LAL) muscles from Wst;YFP-
H mice were loaded with the styryl dye FM4–64FX using a high K⁺
stimulus. Muscle preparations were exposed to a fixable form of
the styryl dye FM4–64 (FM4–64FX, 2 mg mL⁻¹; Molecular Probes) in
high K⁺ Krebs’ solution sparged with 95% : 5% O₂ : CO₂ (102 mm
Na⁺, 50 mm K⁺, 2 mm Ca²⁺, 2 mm Mg²⁺, 132 mm Cl⁻, 23.8 mm HCO₃⁻,
0.4 mm H₂PO₄⁻, 5 mm D-glucose, 5.5 mm HEPES, pH 7.2–7.4) for
10 min. After rigorous washing, muscles were fixed in 4% para-
formaldehyde/PBS solution (Electron Microscopy Science, PA,
USA) and post-synaptic acetylcholine receptors were labelled with
α-bungarotoxin conjugated to alexa-647 (5 μg mL⁻¹ for 10 min,
Invitrogen, USA). Muscles were whole-mounted in Mowoil®
(Callbiochem) on glass slides and coverslipped for subsequent imaging.

Imaging and quantification
Immunohistochemically-labelled NMJs and YFP-expressing muscles
and nerves were imaged using either a standard epifluorescence
microscope equipped with a chilled charge-coupled device camera
(40× objective: 0.8 NA; Nikon IX71 microscope; Hamamatsu
C4742-95; Improvision Openlab Software) or a laser scanning
confocal microscope (40× objective: 0.8 NA; Radiance 2000,
BioRad, Hemel Hempstead, UK; BioRad Lasersharp 2000 Software).
Between 50 and 200 endplates, selected at random, were quanti-
fied from each muscle preparation. Wherever possible, all analysis
was performed without the operator knowing the status of the
material. For basic occupancy counts, the occupancy of individual
NMJs was evaluated by categorizing endplates as either fully
occupied (neurofilament/SV2 partially covers endplate) or vacant
(neurofilament/SV2 overlies endplate).

Montages and reconstructions
Reconstructions of immunohistochemically-labelled and YFP-H-
labelled muscle and nerve preparations were produced using
Adobe Photoshop software by layering and combining multiple
individual micrographs.

Electron microscopy
Nerve and muscle preparations were prepared for electron microscopy
as described previously (Gillingwater et al. 2003). Briefly, preparations
were fixed in 0.1 m phosphate buffer (pH 7.4) for 2–3 h at 4°C. The fix-
ations were washed in PBS (0.1 m, 5 m, pH 7.4) for 30 min before incubation
overnight in primary antibodies raised against either 165 kDa
neurofilament proteins and the synaptic vesicle protein SV2 (both 1:200; Developmental
Studies Hybridoma Bank, USA) or 150 kDa neurofilament proteins
(Chemicon, USA) and visualized with sheep anti-mouse fluorescein
isothiocyanate-conjugated secondary antibodies (1:200; Diagnost-
cs Scotland, UK) or swine anti-rabbit fluorescein isothiocyanate-conju-
gated secondary antibodies (1:40; DAKO, USA), respectively.
Muscles were then whole-mounted in Mowoil® (Calbiochem) on
glass slides and coverslipped for subsequent imaging.

Quantitative fluorescent (Li-COR) western blots
Total protein was isolated from the spinal cord of late-symptomatic
Wst mice and control littersmates as well as late-symptomatic
(P5/P6) Smm-1/-;SMN2 mice and control littersmates (N = 3 mice
per genotype; see Murray et al. 2008) and quantitative western
blots were performed as described previously (Wishart et al.
2007). Briefly, protein was separated by sodium dodecyl sulphate-
polyacrylamide gel electrophoresis on 4–20% pre-cast NuPage 4–
12% Bis Tris gradient gels (Invitrogen) and then transferred to a
polyvinylidene difluoride membrane. For basic protein detection,
membranes were blocked using Odyssey blocking buffer (Li-COR
Biotechnology) and incubated with primary antibodies as per the manufacturer’s
instructions (tubulin, Abcam; SMN1, BD Bioscience; ZPR1, BD Bio-
science). Odyssey secondary antibodies were added according to
the manufacturer’s instructions (goat anti-rabbit IRDye 680 and
goat anti-mouse IRDye 800). Blots were imaged using an Odyssey
Infrared Imaging System (Li-COR Biosciences). The scan resolution
of the instrument ranged from 21 to 339 μm and in this study
blots were imaged at 169 μm. Quantification was performed on
single channels with the analysis software provided. Bands were
identified according to their relative molecular weight and deline-
ated using Odyssey software and the arbitrary fluorescence
intensity calculated by the software. For each membrane, scans
were carried out at three different intensities in order to minimize
possible user error in determining correct scan intensities or over-
saturation of the membrane. The average of these three separate
scans (giving an N = 1 per membrane) was used for analysis.

Results
Loss of eEF1A2 expression triggers a dying-back
europathy: synaptic pathology
In order to investigate the cellular pathway(s) through which
lower motor neurons degenerate following perturbations
in expression of the translation elongation factor eEF1A2 in Wst mice, we used confocal and electron microscopy to examine the detailed morphological correlates of neuromuscular pathology in two muscle groups previously shown to be affected in Wst mice, i.e. transversus abdominis from the anterior abdominal wall and deep lumbrical muscles from the hind-paw (Newbery et al. 2005). In both of these muscle groups, immunohistochemical labelling of distal axons and pre-synaptic motor nerve terminals confirmed previous reports of a significant loss of innervation at the NMJ in late-symptomatic (P24–P27) homozygous Wst mice, characterized by partially occupied and vacant endplates (Fig. 1). Partial occupancy of endplates by overlying motor nerve terminals is a morphological correlate associated with dying-back pathways, distinct from the rapid in-situ fragmentation occurring at the NMJ during WD (Miledi & Slater, 1970; Winlow & Usherwood, 1975; Fischer et al. 2004; Murray et al. 2008).

We extended these observations to include other muscle groups, finding evidence for similar synaptic pathology in the LAL muscle from the dorsal aspect of the head and flexor digitorum brevis muscle from the plantar aspect of the hind-paw (Fig. 2). In keeping with previous reports of a rostrocaudal progression of motor neuron degeneration in Wst mice, pathology was more severe in neurons supplying the LAL than those innervating the flexor digitorum brevis (cf. Fig. 2 in the current paper with Fig. 6 in Newbery et al. 2005). Closer examination of the distinct rostral and caudal bands of the LAL muscle, known to be differentially affected by dying-back pathology in mouse models of spinal muscular atrophy (SMA) (Murray et al. 2008), showed similar levels of synaptic pathology in each band (data not shown).

We next examined the ultrastructure of degenerating NMJs in late-symptomatic Wst mice (Fig. 1D and E). In all preparations examined (> 200 NMJs in total), we never observed any of the classical ultrastructural markers of WD (Miledi & Slater, 1970; Winlow & Usherwood, 1975). Motor nerve terminal plasma membranes remained intact, even during the process of retraction (as shown by partially occupied motor endplates; Fig. 1D and E). Similarly, nerve terminal organelles such as mitochondria and synaptic vesicles could clearly be identified and terminal Schwann cells were never observed engulfing or phagocytosing nerve terminals (Fig. 1D and E, and H and I). One other conspicuous morphological feature of Wst motor nerve terminals and distal axons was a conspicuous accumulation of neurofilaments (Fig. 1F–H), once again appearing very similar to previous reports of other dying-back neuropathies (Cifuentes-Diaz et al. 2002; Murray et al. 2008).

As mitochondrial fragmentation and synaptic vesicle depletion are two of the major hallmarks of WD (Miledi & Slater, 1970; Winlow & Usherwood, 1975), we undertook a more detailed ultrastructural investigation of these organelles. The vast majority of nerve terminal mitochondria remained intact (Fig. 1I) with the numbers of mitochon-
Fig. 1 Synaptic loss at the NMJ in Wst mice occurs by a pathway morphologically distinct from WD. (A) Confocal micrograph showing a normal, control NMJ in an immunohistochemically-labelled transversus abdominis (TVA) muscle preparation from a P21 wild-type littermate mouse [green, 165 kDa neurofilaments and SV2; red, post-synaptic acetylcholine receptors labelled with α-bungarotoxin conjugated to tetramethylrhodamine isothiocyanate (TRITC-α-bungarotoxin)]. (B) Electron micrograph showing a normal, control NMJ in a lumbrical muscle preparation from a P25 wild-type littermate mouse. (C) Confocal micrograph showing two partially occupied NMJs in an immunohistochemically-labelled TVA muscle preparation from a P21 Wst mouse (green, 165 kDa neurofilaments and SV2; red, post-synaptic acetylcholine receptors labelled with TRITC-α-bungarotoxin). Partial occupancy of endplates is a morphological characteristic associated with nerve terminal retraction (e.g. dying-back) processes rather than WD. Electron micrographs showing partially occupied NMJs (identified by regions of vacant post-synaptic folds; blue arrows) in the flexor digitorum brevis (D) and lumbrical (E) muscles from late-symtomatic (P25) Wst mice. Note how the remaining motor nerve terminal boutons show none of the characteristic ultrastructural signs of WD; mitochondria, synaptic vesicles and pre-synaptic plasma membranes were all present and intact, and there was no evidence of phagocytosis by the terminal Schwann cell. (F) Confocal micrograph showing characteristic neurofilament accumulation, alongside interspersed thinning, in distal axons (blue and yellow arrows respectively) and motor nerve terminals from a P25 Wst mouse lumbrical muscle. (G) Electron micrograph showing a cross-sectional profile of an axon from the intramuscular region of an intercostal nerve supplying the TVA muscle in a P26 Wst mouse. Note the presence of large neurofilament accumulations in the axonal cytoplasm (cf. F) and the absence of any degenerative characteristics; the myelin sheath and axonal membranes remain intact. (H) Electron micrograph showing a NMJ in the flexor digitorum brevis muscle from a P26 Wst mouse. Note the presence of large neurofilament accumulations in the motor nerve terminal (blue arrows) but the lack of any morphological characteristics of WD (see above). (I) High-power electron micrograph showing three mitochondria in a motor nerve terminal from the flexor digitorum brevis muscle of a P26 Wst mouse. Note how the mitochondrial membranes and cristae are preserved (disruption of mitochondrial morphology is one of the earliest signs of WD). (J) Bar chart (mean ± SEM) showing significant increases in synaptic vesicle densities in motor nerve terminals from the lumbrical muscles of Wst mice at P20 (early-symptomatic) and P26 (late-symptomatic) compared with wild-type littersmates (**P < 0.01 for both ages, ANOVA with Tukey’s post-hoc test; N = 24 nerve terminals and n = 6694 vesicles in P20 wild-type, N = 20 and n = 3540 in P20 Wst, N = 26 and n = 8071 in P26 Wst). Synaptic vesicles are normally depleted from motor nerve terminals during WD. Scale bars: 10 μm (A), 15 μm (C), 0.75 μm (B, D and E), 20 μm (F), 0.75 μm (G), 1 μm (H), 0.1 μm (I).
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Individual YFP-expressing axons were then imaged and reconstructed over a minimum distance of 1 mm (Fig. 4A and B). In all nerves examined, we never observed any signs of axonal degeneration. All individual YFP-labelled axons were intact along their entire length, with no evidence of axonal breaks, axon fragmentation or spheroid formation (Fig. 4A–C; cf. Beirowski et al. 2005). To confirm these observations, we also examined semi-thin toluidine-blue-stained sections of intercostal (n = 11 sections, N = 4 nerves) and tibial (n = 10 sections, N = 2 nerves) nerves from late-symptomatic P24 Wst mice (Fig. 4D). In all sections examined < 0.5% of axons were classified as having a ‘degenerative’ morphology (e.g. disrupted myelin sheath and/or axonal profile). Ultrastructural analyses of proximal and intramuscular axons from end-stage (P27) Wst mice again showed no evidence of gross axonal pathology (Fig. 4E). These data therefore reveal that synaptic loss occurs significantly in advance of axonal pathology in Wst mice, supporting the hypothesis that synaptic compartments of neurons are particularly sensitive to altered levels of eEF1A2.

Synapse loss occurs asynchronously within single motor units in Wst mice

As WD is known to bring about a relatively synchronous and rapid (i.e. within several hours) degeneration of NMJs (Miledi & Slater, 1970), whereas dying-back neuropathies are characterized by a progressive asynchronous loss of nerve terminals, we examined patterns of synaptic pathology occurring within the entire cohort of motor nerve terminals in single YFP-labelled motor units of Wst;YFP-H mice. We reconstructed and analysed synaptic cohorts of single motor units supplying LAL muscles from five late-symptomatic P25 Wst;YFP-H mice. Branching diagrams produced by tracing individual labelled motor units (N = 5) revealed a common response, with all motor neurons seemingly affected to a similar level (i.e. all motor units exhibited clear evidence of synaptic pathology). However, each motor unit displayed a heterogeneous range of synaptic morphologies, including intact synapses (fully occupied) and synapses at various stages of dismantling (partially occupied). Comparison of

Fig. 2 Synaptic pathology extends to the flexor digitorum brevis (FDB) and levator auris longus (LAL) muscles in symptomatic Wst mice. Confocal micrographs showing NMJs in immunohistochemically-labelled LAL (A) and FDB (B) muscle preparations from P25 Wst mice (green, 165 kDa neurofilaments and SV2; red, post-synaptic acetylcholine receptors labelled with α-bungarotoxin conjugated to tetramethylrhodamine isothiocyanate). Partially occupied (white arrows in B) and vacant (white arrow in A) endplates were present throughout both muscle groups, as previously observed in transversus abdominis (TVA) and lumbrical muscles (Fig. 1 in the current paper; Newbery et al. 2005). Occasionally, remnants of synaptic terminals and distal axons withdrawing from post-synaptic endplates were observed (‘retraction bulbs’; blue arrow in A). (C) Bar chart (mean ± SEM) showing the percentage of fully occupied endplates, partially occupied endplates and vacant endplates in LAL muscles from wild-type mice at P20 (WT P20; black bars; N = 2 mice), Wst mice at P20 (→ P20; N = 3 mice) and Wst mice at P25 (→ P25; N = 4 mice). Note how the percentage of fully occupied endplates declines and the percentage of vacant endplates increases with advancing age in Wst mice. Similar loss of pre-synaptic innervation was observed in FDB muscles at P25, albeit to a lesser extent than in LAL or TVA (as in neighbouring lumbrical muscles; ~30–40% of NMJs showed disrupted morphology; data not shown). Scale bars: 20 μm (A), 15 μm (B).
the innervation states of neighbouring synapses that shared a single branch point showed that synapses were being lost progressively and asynchronously in Wst mice. This finding indicates that local mechanisms, resident at individual synapses, are likely to play an important role in determining an individual synapse’s response to the effects of a loss of eEF1A2. Taken together with the other data presented above, these experiments demonstrate that disruption of eEF1A2 in lower motor neurons and skeletal muscle fibres triggers dying-back pathways.

Molecular correlates of dying-back neuropathy in Wst mice: a role for ZPR1?

Although eEF1A2-specific mutations are not thought to be directly responsible for any human neurodegenerative conditions, the gene and its protein product share notable molecular linkages with other proteins known to be involved in the pathogenesis of other dying-back neuropathies such as the childhood motor neuron disease SMA. For example, eEF1A proteins assemble into complexes with the survival motor neuron protein (SMN1; where mutations abolish expression and cause SMA) and a zinc finger protein (ZPR1) (Gangwani et al. 1998; Mishra et al. 2007). Interestingly, the dying-back pathology observed following disruption of eEF1A2 in Wst mice closely mirrors morphological correlates of lower motor neuron pathology occurring in mouse models of SMA and also in ZPR1-deficient mice (Monani et al. 2000, 2003; Doran et al. 2006; Murray et al. 2008). Therefore, we quantified levels of Smn protein in the spinal cord of late-symptomatic Wst mice (P25) and littermate controls using fluorescent (Li-COR) western blots to test whether the dying-back pathology in Wst mice could simply be accounted for by reduced levels of Smn protein resulting from decreased eEF1A2 expression. Smn levels remained relatively stable in homozygous Wst mice, showing a slight trend towards increased rather than decreased expression levels (Fig. 6). This was in stark contrast to the significant reduction in Smn levels observed in the spinal cord of late-symptomatic Smn−/−;SMN2 mice (an established mouse model of severe SMA) (Monani et al. 2000; Murray et al. 2008). Thus, the dying-back pathology observed in Wst mice could not be simply attributed to downstream changes in Smn protein levels. Next, we asked a similar question but this time focusing on levels of ZPR1 protein expression. Interestingly, ZPR1 protein levels were found to be consistently reduced in Wst mice, to a level similar to that found in Smn−/−;SMN2 mice (Fig. 6). As reduced ZPR1 levels have been suggested to contribute to dying-back pathology in SMA (Gangwani et al. 2001; Helmken et al. 2003), a similar reduction in Wst mice may provide evidence for a common regulatory role of ZPR1 in regulating dying-back pathways in both Wst and Smn−/−;SMN2 mice.

**Fig. 3** Functional loss precedes structural loss at NMJs undergoing dying-back pathology in Wst mice. Confocal micrograph (merge of all three channels in top panel with separate channels shown below) of NMJs from a P25 Wst;YFP-H mouse LAL muscle. Motor nerve terminals were loaded with FM4–64FX using a depolarizing high-potassium solution leading to the selective labelling of functionally-active terminals (i.e. with the retained ability to recycle synaptic vesicles). In the preparation shown, two NMJs with motor nerve terminals containing YFP can be seen but only one of these motor nerve terminals demonstrated retained functional ability (red arrow; note the presence of strong FM4–64FX label in the bottom panel). The junction indicated by the white arrow was present morphologically (as shown by the YFP label) but had lost its functional capacity to recycle synaptic vesicles and hence had not taken up the FM4–64FX label. Thus, synaptic function was lost before morphological retraction occurred in Wst mice. By contrast, in control preparations from YFP-H wild-type littermates, all motor nerve terminals showed strong FM4–64FX labelling (data not shown). Scale bar, 35 μm.
**eEF1A2** is required for the normal initiation and progression of axotomy-induced WD

As we found strong evidence that eEF1A2 is an important regulator of dying-back pathways in vivo, we next asked whether deficiencies in eEF1A2 also exert any influence on WD pathways by studying responses to nerve injury in homozygous Wst, heterozygous Wst and wild-type littermate mice. For these experiments we examined lumbral muscles as the synaptic pathology resulting from dying-back pathology was significantly less in these muscles compared with more rostral muscle groups (Figs 1 and 2) (Newbery et al. 2005), thereby minimizing the complexity of distinguishing disease-induced changes from those induced by nerve injury. Late-symptomatic (P24) Wst and Wst;YFP-H mice (as well as wild-type and heterozygous Wst littermates) were subjected to a unilateral tibial nerve cut under general anaesthesia. Following recovery and resumption of normal behaviour, mice were killed 24 h later (P25) and their lumbral muscles and tibial nerves distal to the site of lesion were removed.

As expected, nerve lesion in wild-type littermate mice resulted in a complete loss of neuromuscular innervation at 24 h after surgery, via classical WD pathways characterized by rapid breakdown and fragmentation of pre-synaptic motor nerve terminals and distal axons (Fig. 7A and F). Surprisingly, the vast majority (~80%) of motor nerve terminals and their intramuscular axon collaterals remained intact in lumbral muscles from homozygous Wst mice at 24 h after nerve lesion (Fig. 7B and F). Similarly, the tibial nerve distal to the site of nerve lesion showed no signs of degeneration in homozygous Wst mice. Examination for early axonal ultrastructural indicators of WD (swelling and damage of mitochondria) (Miledi & Slater, 1970; Winlow & Usherwood, 1975) revealed subtle but widespread changes in wild-type nerves (~50% of axons examined showed mitochondrial changes or disruption of the myelin sheath; Fig. 7D) but the almost complete
absence of any early markers of WD in Wst nerves (only one out of ~200 axons showed signs of disrupted mitochondria; Fig. 7E). Importantly, we were able to rule out the possibility that the genetic background of the mice was influencing the WD process by comparing homozygous Wst mice with heterozygous and wild-type littermates (Fig. 7F). In order to confirm that the significant inhibition of WD found in homozygous Wst mice extended beyond the 24 h period that we initially examined, we quantified degeneration in mice subjected to a unilateral tibial nerve cut at P23 followed by killing 48 h later (P25). Even at 48 h post-axotomy, approximately 50% of motor nerve terminals and their intramuscular axon collaterals remained intact in lumbrical muscles from homozygous Wst mice (Fig. 7G). This contrasts with the situation in wild-type and heterozygous littermates where almost all motor nerve terminals (> 95%) had undergone degeneration after 24 h (Fig. 7F).
It was not possible to extend the time-course of our investigations much beyond this 48 h period because the operated mice did not live beyond P26 (due to the severity of dying-back pathological changes in the more severely affected rostral muscle groups) and mice could not be operated earlier than P23 in order to ensure a complete loss of eEF1A1 expression (cf. Pan et al. 2004). However, these experiments demonstrate that eEF1A2 is required for the normal initiation and progression of WD. These data also further support the conclusion that the dying-back pathways instigated following disruption of eEF1A2 in Wst mice are mechanistically distinct from WD, as WD pathways are actually inhibited (rather than triggered) in Wst mice.

Discussion

The experiments reported here raise four important conclusions. First, they demonstrate that the spontaneous loss of eEF1A2 expression that occurs in Wst mutant mice triggers dying-back pathways in lower motor neurons in vivo, where synaptic loss at the NMJ is an early event. This supports the hypothesis that synaptic compartments of neurons are particularly sensitive to perturbations in neuronal homeostasis (Gillingwater & Ribchester, 2001; Wishart et al. 2006) and shows that eEF1A2 expression is essential for the prevention of pre-synaptic degeneration in vivo. Second, they show that eEF1A2 expression is required for the normal initiation and progression of WD following nerve injury in vivo. This finding adds significant support to previous experimental studies suggesting that WD is an active, genetically-regulated process, rather than a simple passive consequence of disconnection from the parent cell body (Mack et al. 2001; Coleman & Perry, 2002). Third, the experiments show that degeneration of axons and synapses can be regulated by at least two mechanistically-divergent pathways. Specifically, dying-back pathology can be caused by deficiencies in eEF1A2, whereas WD requires...


**eEF1A2 expression. This finding contradicts, to some extent, an emerging view that many axonal and synaptic degenerative pathways converge onto common underlying mechanisms (cf. Coleman, 2005). Fourth, they suggest that eEF1A2-dependent molecular cascades, potentially acting via ZPR1-dependent pathways, may be capable of modifying the type and/or severity of neurodegenerative pathways instigated in lower motor neurons. The current study therefore highlights the significant potential that exists for further investigations into the molecular pathways acting downstream of eEF1A2 to provide novel insights into neurodegenerative cascades resident in axonal and synaptic compartments of lower motor neurons.**

**Can deficiencies in protein synthesis explain the neurodegenerative phenotype in Wst mice?**

The most parsimonious explanation for the current findings is that defects in protein synthesis, resulting directly from a loss of eEF1A2 expression and function, are responsible for triggering dying-back pathways and for the delay in WD. There is little doubt that deficiencies in eEF1A proteins lead to disruption of protein synthesis (Shultz et al. 1982; Chambers et al. 1998; Pan et al. 2004). Moreover, the protein synthesis machinery is known to influence axonal and synaptic form and function in neuronal cells (Piper & Holt, 2004; Grossman et al. 2006; McCann et al. 2007), and eEF1A is known to be important for local protein synthesis determining synaptic stability and function (Giustetto et al. 2003). However, although protein synthesis has been shown to influence neurodegeneration of axons and synapses in vitro (e.g. Stavisky et al. 2003), the influence of protein synthesis on neurodegenerative mechanisms resident in axons and synapses in vivo remains unclear.

It is not currently possible to directly visualize or measure de-novo protein synthesis in distal axonal and/or neuromuscular synaptic compartments with any accuracy, ruling out the possibility of definitively linking the induction of dying-back pathways to deficiencies in protein synthesis. Similarly, the current data do not allow us to directly link the inhibition of WD with deficiencies in local protein synthesis. However, Wst mice are likely to provide an ideal model system within which to further investigate downstream molecular mechanisms once more sensitive techniques to measure local neuronal protein synthesis become available. Its attraction as an experimental model is highlighted by the fact that eEF1A2 deficiencies are restricted to neuronal cells and muscle, and are only instigated post-natally.

**Could non-canonical roles of eEF1A2 be responsible for the neurodegenerative phenotype in Wst mice?**

Alternative hypotheses explaining the potential mechanisms underlying modifications in dying-back pathways and WD in Wst mice can be proposed in the light of other known non-canonical functions of eEF1A proteins. For example, several studies have implicated eEF1A proteins in assembly and stability of the cytoskeleton, including roles in actin binding and microtubule severing (Yang et al. 1990, 1993; Shiina et al. 1994). It is possible therefore that the dying-back pathways instigated in Wst mice occur as a direct result of perturbations in the stability of the axonal and synaptic cytoskeleton. Moreover, a loss of ability to transport the products of cytoskeletal disruption down the distal axon may explain the inhibition of WD pathways. If the latter were found to be true, it would provide experimental support for the hypothesis that WD is triggered by a signal that passes from the site of injury down to the nerve terminal (Miledi & Slater, 1970). Alternatively, it is possible that perturbations in synaptic plasticity pathways, known to be modulated by eEF1A levels (Giustetto et al. 2003), lead to destabilization of the NMJ and induction of dying-back pathology. However, it should be noted that it has yet to be demonstrated that the eEF1A2 variant has the same non-canonical roles previously attributed to non-variant-specific eEF1A protein. It therefore also remains possible that eEF1A2 has as yet unidentified roles that are responsible for the phenotypes described in the current study.

It should also be noted that it is by no means certain that the same downstream effects resulting from loss of eEF1A2 in Wst mice are responsible for the dual effects on dying-back pathways and WD pathways. Further mechanistic experiments, beyond the scope of the current study, are required to distinguish the specific characteristics or molecular targets of eEF1A2 required to prevent a dying-back neuropathy and/or inhibit the initiation and progression of WD.

**Implications for the stability and vulnerability of lower motor neurons in human motor neuron disease**

Although eEF1A2 mutations are not currently thought to be directly responsible for any human neurodegenerative conditions, the gene and its protein product are known to interact with other proteins that regulate dying-back pathology in human neurodegenerative conditions such as the childhood motor neuron disease SMA. eEF1A proteins assemble into complexes with the survival motor neuron protein (SMN1; mutations in which cause SMA) and a zinc finger protein (ZPR1) (Gangwani et al. 1998; Mishra et al. 2007). The finding that dying-back pathology occurring in Wst mice closely resembles lower motor neuron pathology occurring in mouse models of SMA and in ZPR1-deficient mice (Monani et al. 2000, 2003; Doran et al. 2006; Murray et al. 2008) prompted us to examine whether the pathways modified by loss of eEF1A2 converged onto those involving SMN and ZPR1. We found that dying-back pathology in Wst mice cannot be attributed to corresponding reductions in Smn protein levels, suggesting
that dying-back pathology in Wst and Smn−/−;SMN2 mice is not triggered by the same molecular cues. However, our observation that ZPR1 protein levels were modestly reduced in Wst mice, to a level similar to that found in Smn−/−;SMN2 mice, raises the possibility of shared downstream mechanistic pathways focused around ZPR1. As decreasing ZPR1 levels have been shown to correlate with disease severity in SMA patients (Helmken et al. 2003) and ZPR1 is known to be required for the normal maintenance and localization of Smn protein (Gangwani et al. 2001), it is tempting to speculate that ZPR1 is capable of playing an important role in regulating lower motor neuron vulnerability in SMA and in Wst mice. Further investigations into the expression and function of ZPR1 in Wst and Smn−/−;SMN2 mice may therefore provide novel insights into cellular and molecular cascades regulating the vulnerability of motor neurons in disease. Moreover, as the current data suggest that eEF1A2 is required to prevent pre-synaptic degeneration via a dying-back neuropathy, it may also be worth investigating the possibility of increasing levels of eEF1A2, on its own or in combination with ZPR1, as a potential neuroprotective strategy in dying-back neuropathies, such as SMA.

Conclusions

In this study we have shown that loss of expression of the translation elongation factor eEF1A2 triggers a dying-back neuropathy characterized by early loss of motor nerve terminals at the NMJ. In sharp contrast, in the same motor neurons the process of WD was significantly delayed following an experimental nerve lesion. This suggests that dying-back pathology and WD are regulated by fundamentally different mechanisms in vivo.

Acknowledgements

We thank Dr S. Parson, Dr C. Abbott, Dr H. Newbery, T. Chater and members of the laboratory of T.H.G. for helpful assistance with and advice on this study. This study was supported by grants to T.H.G. from Medical Research Scotland, BDF Newlife and the Anatomical Society of Great Britain and Ireland. L.M.M. is the recipient of a PhD Studentship from the Anatomical Society of Great Britain and Ireland.

References


level decreases the amount of its interacting partners and intron2-beta1. 


Supporting information
Additional Supporting Information may be found in the online version of this article:

Fig. S1 Additional branch diagram reconstructions of single motor units undergoing dying-back pathology in homozygous Wst mice.

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