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Expression of the neuroprotective slow Wallerian degeneration (WldS) gene in non-neuronal tissues

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Abstract

Background: The slow Wallerian Degeneration (WldS) gene specifically protects axonal and synaptic compartments of neurons from a wide variety of degeneration-inducing stimuli, including; traumatic injury, Parkinson’s disease, demyelinating neuropathies, some forms of motor neuron disease and global cerebral ischemia. The WldS gene encodes a novel Ube4b-Nmnat1 chimeric protein (Wld S protein) that is responsible for conferring the neuroprotective phenotype. How the chimeric WldS protein confers neuroprotection remains controversial, but several studies have shown that expression in neurons in vivo and in vitro modifies key cellular pathways, including; NAD biosynthesis, ubiquitination, the mitochondrial proteome, cell cycle status and cell stress. Whether similar changes are induced in non-neuronal tissue and organs at a basal level remains to be determined. This may be of particular importance for the development and application of neuroprotective therapeutic strategies based around WldS-mediated pathways designed for use in human patients.

Results: We have undertaken a detailed analysis of non-neuronal WldS expression in WldS mice, alongside gravimetric and histological analyses, to examine the influence of WldS expression in non-neuronal tissues. We show that expression of WldS RNA and protein are not restricted to neuronal tissue, but that the relative RNA and protein expression levels rarely correlate in these non-neuronal tissues. We show that WldS mice have normal body weight and growth characteristics as well as gravimetrically and histologically normal organs, regardless of WldS protein levels. Finally, we demonstrate that previously reported WldS-induced changes in cell cycle and cell stress status are neuronal-specific, not recapitulated in non-neuronal tissues at a basal level.

Conclusions: We conclude that expression of WldS protein has no adverse effects on non-neuronal tissue at a basal level in vivo, supporting the possibility of its safe use in future therapeutic strategies targeting axonal and/or synaptic compartments in patients with neurodegenerative disease. Future experiments determining whether WldS protein can modify responses to injury in non-neuronal tissue are now required.
Background
Degeneration of axonal and/or synaptic compartments of neurons is an early and pathologically important process in many disorders of the human nervous system, ranging from Alzheimer’s disease and motor neuron disease [1-8]. Therapies designed to specifically delay or halt the progression of axonal and synaptic degeneration are therefore actively being sought for a wide range of neurological disorders.

The most robust delay in axonal and synaptic degeneration reported to date in animal models of neurological disorders has been generated by the introduction of the slow Wallerian degeneration (WldS) gene. To date, the WldS gene has been shown to significantly modify disease onset and/or progression in animal models of traumatic axonal injury [9,10], Parkinson’s disease [11,12], demyelinating neuropathies [13], some forms of motor neuron disease [14] and cerebral ischemia [15]. These experiments highlight the potential for using the WldS protein and/or its downstream molecular interactions to generate novel therapeutic approaches for the treatment of neurological disorders. Importantly, the ability to successfully deliver the WldS gene and confer robust neuroprotection using gene therapy approaches [16,17] has opened up the possibility of directly delivering WldS-related therapies to human patients.

The chimeric WldS gene occurred as the result of a spontaneous mutation in the C57BL/6 line of mice (originally termed C57BL/6/Ola [9]), resulting in a tandem triplication of a region already present on the distal region of chromosome 4. Mice carrying the WldS mutation are otherwise indistinguishable from their C57BL/6 strain mates in genotyping of more than 50 microsatellite markers and restriction fragment length polymorphisms (RFLPs) [18-20]). The triplicated region contains sequences coding for Nmnat1, Rbp7 and Ube4b [21]. The boundaries within the triplicated region result in 2 copies of a fusion gene comprising the N70 terminal amino acids of Ube4b and the entire coding region of Nmnat1 (C Terminal 285 amino acids), linked by 18 amino acids from the 5′ untranslated region of Nmnat1 which are not normally expressed [18,21,22]. The chimeric portion of the triplication (i.e. the N-70 Ube4b/Nmnat1 C-303 chimera) has been shown to be sufficient to recapitulate the WldS phenotype through the generation of transgenic lines in mice, rats and drosophila [23-25].

Although the WldS gene has obvious therapeutic potential, its mechanism of action remains unclear. However, several studies have shown that expression of WldS protein in neurons in vivo and in vitro induces changes in core cellular pathways, including: NAD biosynthesis, ubiquitination, the mitochondrial proteome, cell cycle status and cell stress [17,26-28]. Even though the extent to which each of these modifications contribute to the neuroprotective phenotype remains unclear, the fact that WldS modifies key cellular pathways raises potential problems for its use as a therapeutic agent that have yet to be investigated. For example, it is conceivable that changes in NAD biosynthesis pathways and/or cell cycle pathways in non-neuronal organs and tissues may alter their form and/or function.

Here, we have undertaken the first detailed study of the effects of WldS expression in non-neuronal tissues in vivo. We show that WldS protein is expressed at differing levels in a range of non-neuronal organs in WldS mice. Systemic expression of WldS did not affect overall body weight or growth. Gravimetric and histological analysis of a wide range of organs and tissues confirmed no changes in WldS mice. We also demonstrate that previously reported alterations in cell cycle and cell stress proteins reported in WldS brain tissue are a neuronal-specific response not observed in non-neuronal organs in vivo.

Results
WldS expression is not limited to neuronal tissue in vivo
Despite numerous reports in the literature concerning the therapeutic potential of the WldS gene (see background above), no previous studies have undertaken a rigorous assessment of the consequences of WldS expression in non-neuronal tissue. We first examined the expression of WldS protein and RNA levels in a variety of organs from WldS mice and wild-type controls and compared expression levels to those observed in the cerebellum [29,30]. Organs examined included liver, kidney, heart, lung and spleen. We used quantitative fluorescent western blotting techniques to assess and compare protein expression levels using a proven antibody for the WldS protein (Wld-18 antibody [23,30,31]; Figure 1). Example bands are shown in Figure 1A for liver (which had little to no WldS protein expression), spleen (intermediate levels of expression) and the cerebellum (strong expression).

These experiments showed that WldS protein expression was not limited to neuronal tissue, but that it was not strongly expressed in all organs examined (Figure 1B). For example, the liver had the lowest expression at 0.06 ± 0.09 (mean ± SEM) fold increase compared to wild-type background signal, the spleen showed a 6.27 ± 1.05 fold increase, whereas the cerebellum showed a 17.82 ± 0.96 fold increase. We also examined RNA expression in WldS mice (Figure 1C) and found that RNA expression varied greatly between different organs and did not necessarily correlate with protein expression. For example, the liver (which showed the lowest protein expression of the organs examined) had relatively high RNA expression levels, approaching those observed in the cerebellum. Taken
together, these findings demonstrate that Wld<sup>S</sup> protein and RNA are present in a range of non-neuronal organs at differing levels, but also demonstrate that the presence of RNA does not always indicate that Wld<sup>S</sup> protein will also be present at comparable levels.

The observation that Wld<sup>S</sup> protein is strongly expressed in non-neuronal tissues led us to investigate whether there would be sufficient protein in tail tips to facilitate accurate genotyping of Wld<sup>S</sup> mice using western blotting. We found that quantitative western blotting techniques easily distinguished between wild-type, heterozygous and homozygous Wld<sup>S</sup> mice (Figure 2).

Systemic expression of Wld<sup>S</sup> did not affect gross appearance or growth rates

In order to test whether systemic Wld<sup>S</sup> expression affected total body weight, mice were bred from parents hetero-
zygous for the Wld\textsuperscript{S} mutation (see methods). Litters from this heterozygote cross contained mice which were null for Wld\textsuperscript{S} (WT), mice which were heterozygous for Wld\textsuperscript{S} (Het) and mice which were homozygous for Wld\textsuperscript{S} (Wld\textsuperscript{S}). All experimental comparisons were carried out within litters to remove any potential effects of background strain. There were no obvious qualitative differences in the size or behaviour of mice within litters. Figure 3A shows 5 female mice from the same experimental litter (containing a mixture of WT, Het and Wld\textsuperscript{S} mice), indistinguishable from one another. Mice were weighed at regular intervals post weaning up to 2 months of age. There were no significant differences between the body weights of WT, Het and Wld\textsuperscript{S} mice at any time-point examined (P > 0.2, ANOVA; Figure 3B; Het data not shown; N = 7 WT, N = 5 Wld\textsuperscript{S}).

**Wld\textsuperscript{S} expression did not affect gravimetrics or histopathology of non-neuronal tissue**

Detailed necropsies were carried out by an experienced rodent pathologist (DGB) on WT, Het and Wld\textsuperscript{S} littermates to determine if non-neuronal expression of Wld\textsuperscript{S} protein had any affects on tissue gravimetrics (Table 1) or histopathology (Figure 4). Organs examined for gravimetrics included kidneys, liver, spleen, thymus, heart and whole brain. In order to minimize the effects of natural biological variability within litters data were expressed as organ weight per grams/body weight. No significant differences were observed between mice from any of the 3 genotypes (Table 1). Mice were also examined for alterations in body fat deposits (interscapular, pelvic, subcutaneous and mesenteric), with no evident differences (data not shown).

Histopathological assessments were carried out on the following organs and tissues: kidney, heart, lungs, mediastinum, liver, gallbladder, urinary bladder, vagina, uterus, ovary, spleen, pancreas, lymph nodes, salivary gland, pituitary gland, adrenal gland, stomach, intestines (6 levels), calvarial bone, femur, tibia, knee joint, spine and spinal cord (4 levels, transverse sections), eyes, head, various sympathetic and parasympathetic ganglia, brain (transverse sections at 250 \( \mu \)m intervals). No significant differences were observed between any of the genotypes. Example sections for comparison are shown in Figure 4.

**Cell cycle and cell stress alterations are specific to neuronal tissue**

We have previously reported alterations in proteins involved with cell cycle and cell stress in non-injured neuronal tissue from Wld\textsuperscript{S} mice in vivo and in vitro [26,27]. In order to determine whether similar changes were instigated in non-neuronal organs and tissues expressing Wld\textsuperscript{S} protein we examined expression levels of the cell stress marker antiphosphohistone H2Ax (H2Ax) and a marker of cell cycle progression acetylated histone H3 (H3) in liver, kidney, heart, lung, spleen and cerebellum from wild-type and Wld\textsuperscript{S} mice. Example bands for both of these proteins are shown in Figure 5A. As previously demonstrated there was a significant (P < 0.001; unpaired t-test) increase in expression of both cell stress and cell cycle markers in Wld\textsuperscript{S} cerebellum compared to wild-type (Figure 5). However, no changes in expression of either of these proteins were found in any of the non-neuronal organs examined (Figure 5). Thus, previously reported dif-
ferences in cell cycle and cell stress status appear to be a neuronal-specific response to expression of WldS at a basal level.

**Discussion**

Here we have shown that WldS RNA and protein are expressed at differing levels in a range of non-neuronal organs in WldS mice but did not influence overall body weight or growth. Gravimetric and histological analysis of a wide range of organs demonstrated that the presence of WldS RNA and/or protein had no overt influence on non-neuronal tissues at a basal level. We have also shown that previously reported alterations in cell cycle and cell stress proteins reported in WldS brain tissue are neuronal-specific and are not observed in non-neuronal organs in vivo. It will now be of interest to establish whether WldS protein can modify responses to injury in non-neuronal tissues and organs, where it is expressed. Expression data in the current study show that the spleen has a comparatively high level of protein, suggesting that this organ may be useful for such studies.

These data demonstrate that downstream consequences of WldS expression, incorporating pathways including NAD biosynthesis, ubiquitination, the mitochondrial proteome, cell cycle status and cell stress [17,26-28], do not have any major adverse effects on non-neuronal organs. This suggests that the utilization of WldS-based therapeutics for treating neurodegenerative conditions can be considered safe for other body systems, should the treatment spread beyond the confines of the nervous system (e.g. unintentionally or as a result of systemic administration). Studies reporting that WldS-mediated neuroprotection (as well as many of its downstream effectors such as Nmnat pathways) can be successfully delivered to the nervous system using approaches such as viral gene delivery suggest that WldS-based therapeutics are a realistic possibility [16,17].

Our experimental data have also revealed nervous system-specific effects of WldS expression. The finding that modifications in cell cycle and cell stress status previously reported in neurons expressing WldS [27] are not replicated in non-neuronal tissues suggests that neurons may have distinct intrinsic responses to the presence of WldS. One possible explanation for this is that neurons are terminally differentiated cells whose cell cycle status is markedly different from many non-neuronal cells (for review see [32]). Whether or not this contributes directly to the neuroprotective phenotype remains to be determined. Nevertheless, these data support the hypothesis that WldS acts by targeting a specific step(s) in degenerative pathways intrinsic to neurons [33].

**Table 1:** WldS protein expression did not affect gravimetrics of non-neuronal organs (bwt: body-weight)

| Organ          | WT Mean | WT SEM | WldS Mean | WldS SEM | t test |
|----------------|---------|--------|-----------|----------|--------|
| Bwt (g)        | 25.46   | 1.39   | 23.56     | 1.61     | ns     |
| Kidneys (g/bwt)| 14.20   | 2.23   | 17.37     | 0.62     | ns     |
| Liver (g/bwt)  | 62.16   | 1.66   | 57.06     | 2.51     | ns     |
| Spleen (g/bwt) | 3.73    | 0.25   | 4.24      | 0.44     | ns     |
| Thymus (g/bwt) | 3.03    | 0.34   | 3.66      | 0.35     | ns     |
| Heart (g/bwt)  | 6.59    | 0.33   | 6.63      | 0.32     | ns     |
| Brain (g/bwt)  | 20.65   | 1.36   | 21.08     | 0.86     | ns     |

**Figure 4**

WldS protein expression did not affect histopathology of non-neuronal organs. H & E staining of tissues showed no obvious qualitative differences between wild type (left), heterozygous (data not shown) or homozygous WldS mice (right) for liver (A), kidney (B), and spleen (C). The selected organs shown represent non-neuronal organs with low (liver), medium (kidney) and high (spleen) levels of WldS protein expression. Scale bar = 100 μm (A&B), 200 μm (C).
Conclusions

We conclude that expression of WldS protein has no adverse effects on non-neuronal tissue at a basal level in vivo, supporting the possibility of its safe use in future therapeutic strategies targeting axonal and/or synaptic compartments in patients with neurodegenerative disease.

Methods

Mouse breeding

Natural mutant C57Bl6/WldS (WldS) mice and C57Bl/6 (wild-type) mice were obtained from Harlan Olac Laboratories (Bicester, UK) and housed within the animal care facilities in Edinburgh. All animal experiments were carried out in accordance with the guidance and rules of, and under license from, the UK Home Office. Breeding pairs made up of one WldS mouse and one wild-type mouse were used to generate heterozygous WldS mice. These mice were then bred to produce litters which contained mice null for the WldS mutation (wild-type), mice heterozygous for the mutation (Het) and mice homozygous for the mutation (WldS). All data were obtained from tissue harvested from 1-2 month old mice. For body weight measurements, mice were weighed every 2 days post weaning. A minimum of 3 mice were used per group for all experiments.

Genotyping

Mice were bred as detailed above, ear-notched and assigned individual identifying numbers which were used so the experimenter remained blind to the genotype of individual mice throughout. Mice were genotyped post mortem and their genetic status was only assigned after data collection and analysis was complete. Mice were initially genotyped by quantitative western blotting for WldS protein expression levels using protein extracted from tail tips using similar methodology described below (see Figure 2). These genotypes were all validated by real-time PCR as previously described [34].

Necropsy

Mice were killed with carbon dioxide gas and immediately weighed. Selected organs were weighed and a standard panel of organs were immersion-fixed in 10% neutral-buffered formalin for histopathology. Fixed organs were embedded in paraffin, sectioned at 4 mm, and stained with hematoxylin and eosin. All analyses were undertaken with the investigator blind to the genotype of each animal.

RNA extraction & qRT-PCR

Cerebellum, liver, spleen, thymus, lung, and heart from 6-week-old female WldS mice were flash frozen on dry ice and mRNA was extracted using the RNAEasy Kit (Qiagen, Valencia, CA). Messenger RNA from all tissues was transformed into cDNA using the Superscript III kit (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (qRT-PCR) was performed to examine WldS gene expression using a Sybr-Green '1-step qRT-PCR kit' (Invitrogen) on an ABI PRISM 7700 Instrument (Applied Biosystems, Foster City, CA). The following primer sequences were used:

WldS-726F TGTGCCCAAGGTGAAATTGC

Figure 5

Cell cycle and cell stress pathway alterations were specific to neuronal tissue. A - Representative examples of bands obtained from quantitative fluorescent western blots from wild-type and WldS mouse spleen and cerebellum probed with antibodies against cell stress (H2Ax) and cell cycle (H3) proteins (beta actin is shown as a loading control). Spleen was chosen because it had the highest WldS protein expression levels of all the non-neuronal tissues examined (Figure 1). Note the increases in H2Ax and H3 in WldS cerebellum compared to wild-type mice, but no change in the expression of either protein in the spleen. B - Bar chart showing cell cycle (H3) and cell stress (H2Ax) protein levels in a range of organs from WldS mice expressed as fold change in protein relative to wild-type. Both proteins examined showed only very minor fluctuations in expression levels in non-neuronal tissue (left hand side of dotted line). The magnitude of change in non-neuronal tissue did not begin to approach those observed in the cerebellum of WldS mice (right hand side of dotted line). A minimum of 3 mice per genotype were used for each experiment.
β-actin was used as a control gene. To verify that there was minimal genomic DNA contamination, we also performed qRT-PCR analysis of selected extracted mRNA samples prior to conversion to cDNA, which demonstrated a negligible genomic DNA presence (10,000× less).

**Protein extraction & quantitative western blotting**

Mice were killed by cervical dislocation and organs for examination rapidly removed. Protein was extracted from tail tips, cerebellum and organs including the kidney, liver, heart, lung and spleen of age- and sex-matched mice in RIPA buffer with 10% protease inhibitor cocktail (Sigma) as previously described [26,27]. 30 μg of protein per lane was separated by SDS/Polyacrylamide gel electrophoresis on 4-20% pre-cast NuPage 4-12% Bis Tris gradient gels (Invitrogen) and then transferred to PVDF membrane overnight. The membranes were then blocked using Odyssey blocking buffer (Li-COR) and incubated with primary antibodies as per manufacturers instructions (anti acetyl Histone H3 - Lake Placid Biologicals; antiphosphohistone H2Aax - Upstate; anti beta actin and anti beta-III-tubulin - Abcam). Wld-18 antibodies were a kind gift from Dr Michael Coleman and were used as previously described [30]. Odyssey secondary antibodies were added according to manufacturers instructions (Goat anti rabbit IRDye 680 and Goat anti mouse IRDye 800). Blots were imaged using an Odyssey Infrared Imaging System (Li-COR Biosciences). Scan resolution of the instrument ranges from 21-339 μm and in this study blots were imaged at 169 μm. Quantification was performed on single channels with the Li-COR analysis software provided, as previously described [26,27].

**Authors’ contributions**

TMW participated in the design of the study, carried out experiments, analysed data and drafted the manuscript. DGB carried out necropsy experiments and analysed data. DT carried out experiments. AM1/KMB/JWT carried out QPCR experiments. THG conceived of the study, participated in its design and coordination, analysed data and drafted the manuscript. All authors read and approved the final manuscript.

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