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WLD^S CAN DELAY WALLERIAN DEGENERATION IN MICE WHEN INTERACTION WITH VALOSIN-CONTAINING PROTEIN IS WEAKENED

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Abstract—Axon degeneration is an early event in many neurodegenerative disorders. In some, the mechanism is related to injury-induced Wallerian degeneration, a proactive death program that can be strongly delayed by the neuroprotective slow Wallerian degeneration protein (Wld^S) protein. Thus, it is important to understand the Wallerian degeneration mechanism and how Wld^S blocks it. Wld^S location is influenced by binding to valosin-containing protein (VCP), an essential protein for many cellular processes including membrane fusion and endoplasmic reticulum-associated degradation. In mice, the N-terminal 16 amino acids (N16), which mediate VCP binding, are essential for Wld^S to protect axons, a role which another VCP binding sequence can substitute. In *Drosophila*, the Wld^S phenotype is weakened by a similar N-terminal truncation and by knocking down the VCP homologue ter94. Neither null nor floxed VCP mice are viable so it is difficult to confirm the requirement for VCP binding in mammals *in vivo*. However, the hypothesis can be tested further by introducing a Wld^S missense mutation, altering its affinity for VCP but minimizing the risk of disturbing other aspects of its structure or function. We introduced the R10A mutation, which weakens VCP binding *in vitro*, and expressed it in transgenic mice. R10AWld^S fails to co-immunoprecipitate VCP from mouse brain, and only occasionally and faintly accumulates in nuclear foci for which VCP binding is necessary but not sufficient. Surprisingly however, axon protection remains robust and indistinguishable from that in spontaneous Wld^S mice. We suggest that either N16 has an additional, VCP-independent function in mammals, or that the phenotype requires only weak VCP binding which may be driven forwards *in vivo* by the high VCP concentration.

Key words: axon, axon degeneration, VCP, ter94, neuroprotection.

Wallerian-like degeneration of axons plays a critical role in many neurological disorders ranging from multiple sclerosis to neurotrauma. It is considered an auto-destructive programme, similar in principle to apoptosis but distinct in molecular terms (Raff et al., 2002; Coleman et al., 2005; Koike et al., 2007). Understanding the mechanism should lead towards new therapeutic strategies for neurodegenerative disorders.

Wallerian degeneration can be delayed about tenfold by the slow Wallerian degeneration protein (Wld^S) in mice, rats and flies (Lunn et al., 1989; Mack et al., 2001; Adalbert et al., 2005; Hoopfer et al., 2006; MacDonald et al., 2006). The protein acts cell-autonomously and dose dependently (Mack et al., 2001). Although abundant in nuclei, its efficacy is enhanced significantly by shifting towards the cytoplasm where it associates with several membranous organelles (Beirowski et al., 2009). Wld^S alleviates axon pathology, and sometimes symptoms, in diverse models of neurodegeneration (Wang et al., 2001, 2002; Ferri et al., 2003; Samsam et al., 2003; Gillingwater et al., 2004; Sajadi et al., 2004; Mi et al., 2005; Howell et al., 2007; Beirowski et al., 2008), although there are some exceptions (Kariya et al., 2008; Rose et al., 2008). This indicates a potential for neuroprotective therapy and establishes a role for Wallerian-like degeneration in pathogenesis. As its protective effect is compartment-specific (Deckwerth and Johnson, 1994; Gillingwater and Ribchester, 2001; Adalbert et al., 2006) Wld^S can also be used to test whether neuronal cell death is secondary to axon loss in specific neurodegenerative diseases (Ferri et al., 2003; Beirowski et al., 2008).

Wld^S is a chimeric protein composed of the N-terminal 70 amino acids of the multiubiquitination factor Ube4b (N70) followed by a 18 amino acid linking region (Wld18) and the entire sequence of Nmnat1, a key enzyme of nicotinamide adenine dinucleotide (NAD^+) biosynthesis (Mack et al., 2001). The mechanism is only partially understood. Strong overexpression of Nmnat 1 or 3 is sufficient to delay axon degeneration *in vitro* and in *Drosophila* (Araki et al., 2004; Wang et al., 2005; MacDonald et al., 2006; Sasaki et al., 2006; Avery et al., 2009) and Nmnat enzyme activity is necessary in every system where axons have actually been cut (Araki et al., 2004; Conforti et al., 2007, 2009; Avery et al., 2009; Yahata et al., 2009). However, removing N70 completely abolishes the phenotype in mice (Conforti et al., 2007; Yahata et al., 2009) and weakens it in *Drosophila* (Avery et al., 2009) and primary culture (Conforti et al., 2007; Watanabe et al., 2007). This indicates a role for N70 sequences in boosting the effectiveness of Nmnat for axon protection.

An essential sequence within N70 has been defined as the N-terminal 16 amino acids (N16). Removing N16 abolishes the axon protection phenotype in mice (Conforti et al., 2009) and weakens it in flies, while fusing N16 directly

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Abbreviations: FDB, flexor digitorum brevis; NMJs, neuromuscular junctions; UPS, ubiquitin proteasome system; VCP, valosin-containing protein.

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to Nmnat1 without the intervening sequences enhances the effect of Nmnat1 in flies (Avery et al., 2009). The only known biochemical function of N16 is direct binding to valosin-containing protein (VCP/p97) (Laser et al., 2006; Morreale et al., 2009), an essential protein with multiple roles regulating membrane trafficking, ER-associated degradation and other events (Wang et al., 2004; Meyer, 2005; Wojcik et al., 2006; Ballar and Fang, 2008). Previous data suggest that this function is important for the Wld^S phenotype, as an alternative VCP binding sequence can substitute for N16 in mice (Conforti et al., 2009) while in *Drosophila* reducing the expression of the VCP homologue ter 94 weakens the phenotype (Avery et al., 2009). Moreover, the ability of VCP binding to influence fine localization of Wld^S (Wilbrey et al., 2008) suggests that subcellular targeting could be part of the mechanism. The nuclear component of Wld^S accumulates in foci in some neurons and cultured cells but becomes homogeneous when N16 is removed or mutated to reduce VCP binding affinity, or when VCP levels are substantially reduced (Wilbrey et al., 2008). N16 does not alter the distribution of Wld^S between nucleus and cytoplasm (Conforti et al., 2009), but its influence on intranuclear distribution is likely to be replicated in cytoplasm, targeting Wld^S to VCP-rich regions. The low concentration of Wld^S in cytoplasm (Beirowski et al., 2009) makes this difficult to study, but a very abundant protein such as VCP (Wang et al., 2004) would be expected to drive forward the binding interaction.

We aimed to test the VCP hypothesis further in mice by introducing the missense mutation R10A into Wld^S, which greatly weakens VCP binding *in vitro* and abolishes the formation of nuclear foci (Wilbrey et al., 2008; Morreale et al., 2009). This mutation leaves the predicted secondary structure unaltered in this region, an α-helix, and is therefore far less likely than the N-terminal truncation to alter other aspects of protein folding or protein stability. Moreover, the detrimental effect of downregulating (Wojcik et al., 2004, 2006; Nan et al., 2005; Sasagawa et al., 2007) or deleting VCP (Muller et al., 2007) makes it difficult to be sure that VCP knockdown acts by blocking a key step in the Wld^S mechanism rather than having a less specific effect on axon survival, although uninjured *Drosophila* axons remain viable after the knockdown (Avery et al., 2009). The lack of viable VCP null or conditional null mice also makes it impossible to test whether Wld^S confers the phenotype in the absence of VCP. Here we characterize R10AWld^S transgenic mice with greatly weakened interaction between Wld^S and VCP and compare the rate of Wallerian degeneration to that of native Wld^S mice.

EXPERIMENTAL PROCEDURES

Generation, crossbreeding and genotyping of transgenic mice

The transgene construct designed for expression of point mutated R10AWld^S under β-actin promoter control in pHβ-Apr1 vector was generated as previously reported (Wilbrey et al., 2008) and linearized using EcoRI and NdeI restriction enzymes. The R10A point mutation was verified by sequencing (Cogenics Lark, UK). Generation and identification of transgenic mice was performed as described recently (Beirowski et al., 2009). Transgenic founders with medium to high copy number integrations as assessed on Southern blots were selected to generate transgenic lines 1, 2, 5–7 and crossed to homozygous YFP-H mice (Jackson Laboratories, Bar Harbour, ME, USA) to breed mice hemizygous for the R10AWld^S and YFP transgene. Subsequently, these mice were intercrossed to obtain mice homozygous for the R10AWld^S and positive for the YFP transgene. Mice positive for the YFP transgene were identified by Southern blotting using a ^{32}P-labelled YFP cDNA probe.

Immunoprecipitation

Immunoprecipitation using nuclear extracts (pooled from six mouse brains per experimental group) was performed as previously described with minor modifications (Laser et al., 2006). Rabbit polyclonal Wld18 serum was used to precipitate variant Wld^S proteins and VCP. Nuclear extracts and washed immunoprecipitates from each experimental group were further processed for SDS PAGE and western blotting.

Western blotting

Western blotting of mouse brain and spinal cord homogenates for assessment of variant Wld^S expression levels was performed as previously described (Beirowski et al., 2009). The variant Wld^S proteins were detected using rabbit polyclonal antiserum Wld18 (1:2000) and mouse monoclonal anti-β-actin (1:3000, Abcam, Cambridge, MA, USA, ab8226) was used as loading control. Mouse monoclonal anti-VCP (1:1000, BD Transduction Laboratories, San Jose, CA, USA, 612182) was used for western blots of brain nuclear extracts and purified immunoprecipitates.

Measurement of Nmnat enzyme activity

Sagittally-divided half mouse brains were flash-frozen in liquid nitrogen immediately post mortem and stored at −80 °C until use. Nmnat enzyme activity was determined as described previously (Conforti et al., 2007, 2009; Beirowski et al., 2009).

Immunofluorescence staining

For immunofluorescence detection of variant Wld^S protein and VCP on brain and lumbar spinal cord tissue obtained from perfusion fixed mice (4% PFA in 0.1 M PBS, pH 7.4), 20 μm cryostat sections on poly-L-lysine coated glass slides (VWR SuperFrost Plus, VWR International, West Chester, PA, USA) were incubated overnight in citrate buffer (pH 6.0) at 50 °C or treated with 0.05% citraconic anhydride solution (pH 7.4) for 45 min at 98 °C (Namimatsu et al., 2005). Subsequent incubation steps for double immunofluorescence staining with primary rabbit polyclonal Wld-18 (1:500), mouse monoclonal anti-VCP (1:500, BD Transduction Laboratories, 612182) and secondary Alexa fluorophore coupled antibodies were carried out as described previously (Beirowski et al., 2009). In some experiments sections were counterstained with primary mouse monoclonal anti-neuronal class βIII-tubulin (1:500, Covance, Princeton, NJ, USA, MMS-435P). Nuclear staining was performed with DAPI or Hoechst 33258. Samples were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

The percentage of neurons showing nuclear Wld^S and Atx3Wld^S foci colocalized with VCP immunoreactivity were quantified in layer two-thirds of the mouse primary motor cortex on brain sagittal sections. 100 cortical neurons were counted per mouse.

Confocal imaging and fluorescence intensity quantification

Immunostained brain and spinal cord sections and vital dye labeled muscle preparations were imaged on a Zeiss LSM 510 Meta