SHORT COMMUNICATION

A rat model of slow Wallerian degeneration (WldS) with improved preservation of neuromuscular synapses

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Abstract

The slow Wallerian degeneration phenotype, WldS, which delays Wallerian degeneration and axon pathology for several weeks, has so far been studied only in mice. A rat model would have several advantages. First, rats model some human disorders better than mice. Second, the larger body size of rats facilitates more complex surgical manipulations. Third, rats provide a greater yield of tissue for primary culture and biochemical investigations. We generated transgenic WldS rats expressing the Ube4b/Nmnat1 chimeric gene in the central and peripheral nervous system. As in WldS mice, their axons survive up to 3 weeks after transection and remain functional for at least 1 week. Protection of axotomized nerve terminals is stronger than in mice, particularly in one line, where 95–100% of neuromuscular junctions remained intact and functional after 5 days. Furthermore, the loss of synaptic phenotype with age was much less in rats than in mice. Thus, the slow Wallerian degeneration phenotype can be transferred to another mammalian species and synapses may be more effectively preserved after axotomy in species with longer axons.

Introduction

Nerve injury induces Wallerian degeneration of distal axon stumps within 2 days and earlier loss of synapses (Miledi & Slater, 1970; Beirowski et al., 2004). A dominant mutation delays Wallerian degeneration ten-fold in slow Wallerian degeneration (WldS) mice (Lunn et al., 1989; Perry et al., 1990b), indicating that Wallerian degeneration is a regulated or active process controlled within neurons (Perry et al., 1990a; Glass et al., 1993; Buckmaster et al., 1995). The WldS gene encodes a nuclear Ube4b/Nmnat1 fusion protein (Conforti et al., 2000; Mack et al., 2001) that confers the axon phenotype through an indirect mechanism, probably involving NAD+ synthesis (Mack et al., 2001; Araki et al., 2004) and possibly altering ubiquitin proteasome metabolism (Zhai et al., 2003; Coleman & Ribchester, 2004).

WldS also delays dying-back degeneration in P0−/− and pmn mutants (Ferri et al., 2003; Samsam et al., 2003) and axonal spheroid pathology in gracile axonal dystrophy (Mi et al., in press). Vincreistine toxicity in vitro and neuropathology in transient global cerebral ischaemia are also attenuated (Wang et al., 2001; Gillingwater et al., 2004). It is important to extend these studies to other disorders, including many that are more effectively modelled, or better characterized, in rats. Spinal injury is often modelled in rats both for size reasons and because cavitation is less in mice (Guth et al., 1999). Diabetic neuropathy is usually studied in rats, and vincreistine-induced neuropathy, a dose-limiting complication of cancer chemotherapy, cannot be modelled as effectively in mice. White matter ischaemic injury can be induced by endothelin 1 in rats but not in mice (Hughes et al., 2003). Experimental autoimmune encephalomyelitis and experimental allergic neuritis are often studied in rats (Gold et al., 2000; Maurer & Gold, 2002) and amyotrophic lateral sclerosis can now be studied in SOD1 G93A rats as well as in mice (Howland et al., 2002). Finally, the larger size of rats facilitates stereotaxic injection, and lesion of optic or facial nerves and nerve roots.

Therefore, we have generated transgenic WldS rats. We confirmed axon and nerve terminal preservation in transected sciatic nerves and identified CNS neurons expressing WldS protein. The preservation of neuromuscular synapses was stronger in rats than in mice and was retained to an older age.

Methods

Generation of transgenic rats

A WldS mouse cDNA construct, exactly as previously described (Mack et al., 2001), was injected into rat embryo pronuclei (RCC, Füllinsdorf, Switzerland). Transgenic rats were generated and maintained on an outbred Sprague–Dawley background (stock IcoIbm: OFA).
**Sciotic nerve lesion**

Under anaesthesia with intraperitoneal 5 mg/kg Ketanest (Park Davis, Berlin, Germany) and 100 mg/kg Rompun (Bayer, Leverkusen, Germany) right sciatric nerves in 6–32-week-old rats were transected. Some rats were transcardially perfused 7–21 days later with 0.1 M PBS (2 min) then 4% paraformaldehyde (15 min). Others were used for electrophysiology. All the animal experiments reported were approved by the Stadt Köln Veterinaramt (licence K13,11/00).

**Light and electron microscopy**

Nerve segments 3–6 mm distal to the lesion were fixed in 0.1 M PBS/4% paraformaldehyde/2.5% glutaraldehyde, pH 7.4 (24 h). After an extensive wash in 0.1 M PBS, 2 h of postfixation (1% osmium tetroxide), and dehydration through an ascending ethanol series and propylene oxide, nerve segments were embedded in Durcupan resin (Fluka Chemie, Buchs, Switzerland) and cured (48 h at 60 °C). Transverse semithin sections (0.5 μm) were stained with toluidine blue and photomicrographed using a Zeiss Axioshot microscope and LM digital camera system (Universal Imaging Corp., Downingtown, PA, USA). Sections 60 nm thick were mounted on Formvar-coated copper grids, counter-stained with uranyl acetate and lead citrate, and examined with a Zeiss EM902 transmission electron microscope.

**Quantification of preserved axons**

In transverse semithin sections distal to a lesion, c. 1000 randomly chosen axons were counted. Survival criteria were normal myelin sheaths, uniform axoplasm and intact, unswollen mitochondria. Electron microscopy spotchecks confirmed intact cytoskeleton and healthy mitochondria. A two-tailed paired Student’s t-test was performed using SPSS for Windows.

**Neuromuscular junction (NMJ) staining**

Flexor digitorum brevis (FDB) or lumbrical muscle preparations for immunocytochemistry were fixed (4% paraformaldehyde/0.1 M PBS, 30–40 min) and acetylcholine receptors labelled (5 mg/ml TRITC-α-bungarotoxin, 20 min) (Molecular Probes, Inc., Eugene, OR, USA). After overnight blocking (4% bovine serum albumin, BSA) and permeabilization (0.5% Triton X/0.1 M PBS, 30 min) primary antibodies were applied overnight: 165-kDa neurofilament protein (WldS, C and TRITC- or FITC-conjugated anti-rabbit antibody (1:500, Wld-18 primary antibody (Samsam C), and synaptic vesicle protein SV2 (both at 1:200, Developmental Studies Hybridoma Bank, IA, USA), followed by a 30-min wash and 4 h in 1 : 200 FITC-sheep anti-mouse (Diagnostics Scotland, Edinburgh, UK).

**Fluorescence imaging**

Muscles were imaged on standard fluorescence microscopes (Olympus/Nikon) or a laser scanning confocal microscope (Bio-Rad Radiance 2000, Hemel Hempstead, UK). NMJ images were obtained using a 40× (0.8NA) or 60× (1.0NA) water-immersion objective and 543 nm excitation/590 nm emission (TRITC), 400–440 nm excitation/515 nm emission (FM1-43) or 488 nm excitation/520 nm emission (FITC), a Hamamatsu C5810 chilled CCD camera and Openlab (Improvision) imaging software. Confocal microscopy used 488-nm and 543-nm excitation laser lines. Confocal Z-series were merged using Lasersharp (Bio-Rad) software. Images were assembled using Adobe Photoshop.

**Electrophysiology**

For intracellular recordings 5–7 days after surgery, isolated tibial and plantar nerve/FDB preparations, dissected in Cologne, were couriered on the same day to Edinburgh in Hepses-buffered saline. Muscles were pinned in a Sylgard-lined bath and perfused with normal mammalian physiological saline (containing 134 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 0.4 mM Na2HPO4, 23.8 mM NaHCO3, 5.6 mM d-glucose), equilibrated with 5% CO2/95% O2. Muscle contractions were reduced/eliminated using 2.5 μM μ-conotoxin GIIIB (Scientific Marketing Associates, Barnet, UK) for 30–45 min. Twenty to 30 randomly chosen fibres per muscle were impaled using a glass microelectrode containing 4 M potassium acetate, activity recorded using a WPI M707 amplifier (WP Instruments, Inc., USA) and stored and analysed using WinWCP v3.0.8 software (Dr John Dempster, Strathclyde University, UK). For vital staining of recycling synaptic vesicles lumbrical muscles were immersed for 10 min in depolarizing physiological saline (50 mM potassium, 45 mM sodium) containing 8 μM FM1-43 (Molecular Probes), then washed (30–60 min) in normal physiological saline before imaging.

**Immunocytochemistry of spinal cord and brain**

Tissue from perfusion-fixed rats was postfixed for 3 h, sucrose-loaded and frozen in chilled isopentane for cutting 20-μm cryostat slices. For fluorescence staining, slices were blocked [4% BSA, 0.5% Triton-X (Sigma, St Louis, MO, USA) in PBS] followed by 1 : 500 Wld-I8 primary antibody (Samsam et al., 2003) overnight at 4 °C and TRITC- or FITC-conjugated anti-rabbit antibody (1 : 20; DAKO, Denmark) for 1 h at room temperature. Topro-3 (1 μM in PBS) was applied prior to mounting in Mowiol. For diaminobenzidine (DAB) immunostaining, endogenous peroxidase activity was quenched [0.5% hydrogen peroxide in methanol (Sigma), 30 min], and slices were blocked at overnight (4 °C) (0.5% BSA, 10% goat serum in PBS). Wld-I8 antibody (1 : 500) overnight at 4 °C was followed by biotinylated anti-rabbit antibody (1 : 100, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature and Vectastain Elite HRP solution (Vector Laboratories) for 1 h at room temperature. After washing in PBS, DAB solution (Vector Laboratories) was applied until colour developed (c. 4 min) followed by distilled water. Slices were mounted in Mowiol.

**Results**

**Generation of transgenic rats expressing WldS protein**

We used a mouse WldS transgene construct (exactly as in Mack et al., 2001) for pronuclear injection in rats because the Ube4b-derived domain has only two amino acid changes (A46R and M60T) and Nmnat1 enzymatic function is likely to remain effective in rats. Ninety-two offspring gave three founders, each with a multicopy domain has only two amino acid changes (A46R and M60T) and Nmnat1 enzymatic function is likely to remain effective in rats. Ninety-two offspring gave three founders, each with a multicopy

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Fig. 1. Protection of injured axons in WldS rats. Axon protection in the distal stump of sciatic nerve 7–21 days after transection. (a) The great majority of axons are structurally preserved with no signs of degeneration in the transgenic rat line 23, 7 days postlesion, whereas (b) in the wild-type rat all axons are lost by this timepoint. In the same transgenic line at 14 (c) and 21 days (d) following axotomy there is still a significant protection of axons similar to WldS and mutant mice (see Mack et al., 2001). (e and f) Electron microscopy (3000 and 12 000×) revealed good ultrastructural preservation of more than half of the transected axons even 14 days after lesion. The myelin sheaths, the cytoskeleton and the organelles are spared with no obvious signs of degeneration. (g) Quantitative analysis of axon preservation in transgenic lines 79 and 23. In both homozygous lines the axon protection is similarly strong. Means and standard deviations are shown (n = 3). Scale bars, 10 μm (a–d), 2.5 μm (e) and 0.5 μm (f).
Fig. 2.

Fig. 3.
WldS rats were studied below as homozygotes.

**Axon preservation after sciatic nerve lesion**

Structural preservation of axons was analysed 7, 14 and 21 days after unilateral sciatic nerve transaction in rats from lines 23 and 79 (n = 3) (Fig. 1). Almost all transected axons were morphologically and ultrastructurally preserved 7 days after lesion (Fig. 1a and g). After 2 and 3 weeks preservation rates were 62% and 23%, respectively (Fig. 1c–g). The phenotype was present in rats as young as P3 (data not shown). These results are similar to spontaneous and transgenic WldS mice, which express similar amounts of WldS protein (Supplementary material, Fig. S2). In line 88 hemizygotes, expressing less WldS protein, only 40% of axons were intact after 7 days (data not shown). Rats of both sexes were used, and there was no sign of any difference in phenotype.

**Structural and functional protection of axotomized motor nerve terminals**

We tested structural and functional preservation of the entire nerve–synapse–muscle fibre unit in axotomized neuromuscular synapses. Both lines, but especially line 79, showed strong persistence of functional innervation 5–7 days after lesion with almost 80% of motor nerve terminals in lumbrical muscles fully or partially occupying their motor endplates (n = 6) (Fig. 2b and c). Synaptic protection was age-dependent but less so than in mice. For example, at 11.5 weeks old and 5 days after axotomy, more than 90% of endplates were fully or partially occupied by nerve terminals (n = 3). At 8 months, an age by which WldS mice have completely lost their synaptic protection (Gillingwater et al., 2002, their figure 5), around 20% of endplates remained fully occupied by a motor nerve terminal and a further 4.4% were partially occupied (Fig. 2d). Thus, structural protection of axotomized neuromuscular synapses is strong in WldS rats, and appears to be less weakened with age than in WldS mice of a similar age.

Preserved nerve terminals of both lines retained competence to recycle synaptic vesicles for at least 5 days (Fig. 2a) compared with less than 24 h in wild-type rats (Miledi & Slater, 1970; Betz et al., 1992; Costanzo et al., 2000) and foot musculature contracted strongly upon stimulation of the tibial nerve stump during dissection. The presence of functional axons and synapses was confirmed by intracellular electrophysiological recording. Nerve stimulation produced action potentials and robust endplate potentials (EPPs) similar to unoperated muscles (Fig. 2e–i). Remarkably, in axotomized FDB muscles from two 11.5-week-old line 79 rats, 5 days after nerve section, 95% (19/20) of muscle fibres responded to nerve stimulation with robust EPPs (example in Fig. 2f). In one of these muscles, the remaining fibre showed spontaneous miniature EPPs (MEPPs) (Fig. 2g), indicating retention of a motor nerve terminal competent to release transmitter spontaneously. In a few fibres, we tested the responses to close-timed paired nerve stimulus pulses, which showed characteristics of normal synaptic facilitation (Fig. 2h), and with repetitive stimulus trains up to 50 Hz, which showed typical short-term synaptic depression (‘early tetanic rundown’). All these features indicate robust synaptic transmission qualitatively and quantitatively indistinguishable from intact NMJs in unoperated rats (data not shown). The robust nature of synaptic transmission was so remarkable that we felt compelled to double check we had dissected the lesioned leg, which we had.

Protection of synapses in line 23 was not quite as strong as in line 79, but was still good compared with WldS mice. In two 5-day lesioned FDB muscles of rats aged 7 and 11.5 weeks, 85% (17/20) and 70% (21/30) of muscle fibres showed functioning synapses (EPP and/or MEPP). Fewer synapses remained functional for 6 days (30–56%, 9/30 and 9/16) and 7 days (53.3%, 8/15). Thus, both lines show a high level of functioning synapses compared with WldS mice, where synaptic preservation rates are around 60% at 5 days and 30% at 7 days (Gillingwater et al., 2002).

Functional preservation of synapses was also retained better in older WldS rats than previously reported in mice. In FDB from two line 79 rats aged 4 months and 8 months, 70% (21/30) and 60% (12/20) of fibres, respectively, had functional synaptic activity after 5 days. In WldS mice at this age there was consistently little or no functional preservation (see figure 5 of Gillingwater et al., 2002). In summary, both structural and functional synaptic protection in both transgenic rat lines was very strong compared with WldS mutant and transgenic mice, and was retained better with age.
WldS protein expression pattern

To identify CNS regions that might show a WldS phenotype, we determined the regional expression pattern of WldS protein in line 23 (Fig. 3). WldS protein was particularly abundant in neurons from mid and lower layers of cortex (layers 3–6), in basket cells of cerebellum, in motoneurons of spinal cord and in dorsal root ganglia. A low level of expression was found in striatum, with only few cells showing faint staining, and hippocampus with faint nuclear staining in some dentate gyrus cells. CA1 and CA3 cells lacked obvious staining (data not shown).

Discussion

We report the generation of transgenic rats expressing high levels of WldS protein in many CNS and PNS neuronal subtypes. Structural and functional preservation of transected axons in two highly expressing lines is similar to those in WldS mice, indicating a successful replication of the phenotype in rat. Axon terminals were more strongly preserved than in mice, particularly in line 79, where 95% of NMJs remained functional 5 days after nerve lesion.

The stronger synapse preservation could be caused by the longer distal nerve stump of rats, especially as the WldS protein expression level is similar to that in mice (Supplementary material, Fig. S2). The protective effect of nerve stump length on nerve terminal degeneration was first demonstrated in wild-type rat diaphragm, where each additional centimetre of distal nerve stump delayed degeneration by 1–2 h (Miledi & Slater, 1970). In WldS mice lengthening the distal nerve stump delayed degeneration by 1–2 days per centimetre (Ribchester et al., 1995). Rat nerve stumps are clearly longer and, importantly, human axons are ten-fold longer still, raising the intriguing possibility that the effectiveness of WldS in humans is underestimated in rodent studies. Moreover, synapses (but not axons) of WldS mice around 4 months old revert to wild-type (Gillingwater et al., 2002; Gillingwater & Ribchester, 2003), limiting protection by WldS in neurodegenerative disorders (Samsam et al., 2003; Mi et al., in press). Rats retain the synaptic phenotype at least 4 months longer. This may reflect the stronger starting point in younger rats, or a slower reduction with age.

Eight independent WldS rodent lines have now been studied: the original mouse mutant, four transgenic mouse lines (Mack et al., 2001) and three rat lines. The only obvious aberrant effect is apparent male infertility of rat line 88, probably caused by insertional inactivation of another gene. Double homozygous mice (WldS plus line 4836 transgene) expressing a high dose of WldS protein are also healthy (our unpublished observations). Thus, WldS could be effective and harmless in a range of mammalian species. Recent in vitro data indicate that Nmnat1 enzyme activity is sufficient to induce the phenotype (Araki et al., 2004). If this holds in vivo, there is no obvious reason why overexpression of Nmnat1 in any mammal should not confer a WldS phenotype. However, earlier in vivo data indicated that the N-terminal Ube4b domain is required in vivo (Coleman & Perry, 2002). In this case it is important to note the high degree of conservation of this domain in mammals (identical in mice and humans and 97% identical between rats and mice) (Mahoney et al., 2002).

WldS rats can now be used to study a wider range of disease models (see Introduction). Other benefits of a rat model have also become apparent. The yield of tissues for in vitro or biochemical procedures is increased and longer axons will be useful to extend recent studies of the progressive nature of Wallerian degeneration (B. Beirowski et al., unpublished observations). Particularly important, however, is the opportunity for cross-species replication of any molecular change proposed to mediate the WldS phenotype. We investigated one such proposal, based on observations that the inflammatory mediator CD200 is increased in WldS mice (Chitnis et al., 2003) but we found no change in rat spinal cord (Supplementary material, Fig. S3), suggesting that CD200 does not mediate the WldS phenotype.

In summary, we have transferred the slow Wallerian degeneration phenotype between two mammalian species and found that synapses are preserved more strongly in rats than in mice. Disease studies can now take place in rat, overcoming some limitations of the mouse in addressing additional models.

Supplementary material

The following supplementary material may be found on: http://www.blackwellpublishing.com/products/journals/suppmat/EJN3833/EJN3833sm.htm

Fig. S1. Generation of WldS transgenic rats.

Fig. S2. Expression of WldS protein in rat and mouse.

Fig. S3. Investigation of the WldS protective mechanism.

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Abbreviations

EPP, endplate potential; FDB, flexor digitorum brevis; MEPP, miniature EPP; NMJ, neuromuscular junction; Nmnat1, nicotinamide mononucleotide adenyltransferase; P0, mice deficient in the peripheral myelin component P zero; pmm, progressive motoneuronopathy mouse model; Ube4b, ubiquitination factor E4b; WldS, slow Wallerian degeneration gene, mutation, mouse, rat or phenotype; WldS, slow Wallerian degeneration protein.

References


