Molecular and Cellular Neuroscience 42 (2009) 296–307

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Axonal and neuromuscular synaptic phenotypes in *Wld*^{S}, *SOD1*^{G93A} and *ostes* mutant mice identified by fiber-optic confocal microendoscopy

Frances Wong [a,b], Li Fan [a], Sara Wells [b], Robert Hartley [a], Francesca E. Mackenzie [b], Oyinlola Oyebode [a], Rosalind Brown [a], Derek Thomson [a], Michael P. Coleman [c], Gonzalo Blanco [b,1], Richard R. Ribchester [a,*,1]

[a] *Euan MacDonald Centre for MND Research, The University of Edinburgh, George Square, Edinburgh, EH8 9JZ, UK*
[b] *Mouse Neuromuscular Genetics Group, MRC Mammalian Genetics Unit, Harwell, OX11 0RD, UK*
[c] *Laboratory of Molecular Signalling, The Babraham Institute, Babraham, Cambridge, CB22 3AT, UK*

ARTICLE INFO

Article history:
Received 7 May 2009
Revised 16 July 2009
Accepted 4 August 2009
Available online 14 August 2009

ABSTRACT

We used live imaging by fiber-optic confocal microendoscopy (CME) of yellow fluorescent protein (YFP) expression in motor neurons to observe and monitor axonal and neuromuscular synaptic phenotypes in mutant mice. First, we visualized slow degeneration of axons and motor nerve terminals at neuromuscular junctions following sciatic nerve injury in *Wld*^{S} mice with slow Wallerian degeneration. Protection of axotomized motor nerve terminals was much weaker in *Wld*^{S} heterozygotes than in homozygotes. We then induced covert modifiers of axonal and synaptic degeneration in heterozygous *Wld*^{S} mice, by N-ethyl-N-nitrosourea (ENU) mutagenesis, and used CME to identify candidate mutants that either enhanced or suppressed axonal or synaptic degeneration. From 219 of the F1 progeny of ENU-mutagenized BALB/c mice and *thy1.2-YFP16*/*Wld*^{S} mice, CME revealed six phenodeviants with suppression of synaptic degeneration. Inheritance of synaptic protection was confirmed in three of these founders, with evidence of Mendelian inheritance of a dominant mutation in one of them (designated CEMOP_S5). We next applied CME repeatedly to living *Wld*^{S} mice and to *SOD1*^{G93A} mice, an animal model of motor neuron disease, and observed degeneration of identified neuromuscular synapses over a 1–4 day period in both of these mutant lines. Finally, we used CME to observe slow axonal regeneration in the ENU-mutant *ostes* mouse strain. The data show that CME can be used to monitor covert axonal and neuromuscular synaptic pathology and, when combined with mutagenesis, to identify genetic modifiers of its progression *in vivo*.

Introduction

Many neurodegenerative diseases, ranging from Amyotrophic Lateral Sclerosis (ALS) to Alzheimer's Disease, remain intractable despite more than a decade of research using mutants and transgenic models in mice and other species (Bruijn and Cleveland, 1996; Bruijn et al., 2004; Gotz et al., 2007; Gotz and Ittner, 2008; Jackson et al., 2002). However, discovery of novel pathways for neuroprotection or enhancement of regeneration in animal models continues to be important for identifying new targets for treatment of disease. One approach is through genome-wide mutagenesis, a powerful methodology that has generated new animal models and provided insight into cell biological mechanisms of development and normal physiological functions.

Screening for mutations generated by N-ethyl-N-nitrosourea (ENU) in mice is one proven method for discovery of novel gene functions. For instance, mutant phenotypes identified by ENU-mutagenesis screens have led to better understanding of diseases ranging from complex behavioural dysfunctions to autoimmune disorders (for recent reviews see Acevedo-Arozena et al., 2008; Cook et al., 2006; Godinho and Nolan, 2006; Gondo, 2008). ENU screens are required to achieve sufficient throughput to identify mutants within a relatively short overall time frame. They should also be non-invasive, or minimally-invasive, since phenodeviant founders identified in a screen are the only resource for breeding and testing of inheritance of the deviant phenotype(s). Standardized protocols such as SHIRPA (Nolan et al., 2000) readily expose overt neurological phenotypes in ENU screens. However, these methods are less suitable for identifying *covert* phenotypes that may be protective, or otherwise beneficial, and thus producing no overt neurological signs. Such phenotypes will normally be missed in primary screens that are restricted to detection of external or behavioural anomalies.

The most compelling precedent for discovery and importance of covert, neuroprotective phenotypes was the serendipitous finding of slow Wallerian degeneration in the spontaneous mutant *Wld*^{S} mouse. In these mice, orthograde degeneration of distal axons after nerve injury is delayed by 1–4 weeks (Lunn et al., 1989; Lyon et al., 1993;

* Corresponding author.
E-mail address: Richard.Ribchester@ed.ac.uk (R.R. Ribchester).
[1] These authors contributed equally to the design and management of this study.

1044-7431/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.mcn.2009.08.002

Perry et al., 1990b). Once this remarkable phenotype had been established, systematic molecular genetic analysis was then used to show that the mutation underlying it encodes a chimeric Ube4b–Nmnat1 protein that, when expressed in transgenic mice, reproduces the slow degeneration of axons and neuromuscular synapses observed in the natural mutant (Coleman et al., 1998; Conforti et al., 2000; Mack et al., 2001). Subsequent detailed phenotypic analysis showed that axon protection in peripheral nerves of *Wld*^{S} mice following nerve section is very strong: distal axons are preserved for 21 days or more after the nerve injury (Lunn et al., 1990; Perry et al., 1990a,b, 1992). The amount of axon protection also varies in a 'gene-dose' dependent fashion: that is, the number of preserved axons in a low stringency assay performed at 3 days after nerve section showed variation among transgenic mouse lines as a function of their Wld^S protein expression (Mack et al., 2001).

Interestingly, Western-blot analysis of brain and quantitative measurements of immunostaining for Wld^S protein in brain and spinal cord show expression levels in heterozygotes at about half the level in homozygotes. However, the level of axon protection in *Wld*^{S} heterozygotes is virtually the same as in homozygotes (Beirowski et al., 2005; Mack et al., 2001; Wilbrey et al., 2008). Despite this, protection of axons by the *Wld*^{S} gene in both the native mutant and in the transgenic equivalents is stronger than that of motor nerve terminals, suggesting that degeneration of motor nerve terminals may be regulated differently from axons (Gillingwater and Ribchester, 2001; Gillingwater et al., 2002). Synaptic protection further declines with age: thus, in *Wld*^{S} mice aged over about 8 months very few intact motor nerve terminals are present by 3 days after nerve injury (Gillingwater et al., 2002; Perry et al., 1992; Ribchester et al., 1995). Subsequent cross-breeding of *Wld*^{S} mice with mouse models of neuropathy and neurodegenerative disease, showed that *Wld*^{S} mitigates onset and progression of disease signs in some but not others, including mouse models of familial ALS (Ferri et al., 2003; Fischer et al., 2005; Kariya et al., 2008; Rose et al., 2008; Samsam et al., 2003; Vande Velde et al., 2004). This neuroprotective weakness may result from the age-dependent or gene-dose-dependent decline in the capacity of Wld^S protein to adequately protect synapses (Gillingwater et al., 2002).

Thus, the fortuitous discovery of the *Wld*^{S} phenotype is highly instructive in several respects. First, it proves that beneficial, neuroprotective mutations may be undetectable using conventional screening methods based on clinical observation of behaviour. Second, it establishes a compelling and urgent case for systematic screening, in order to identify additional mutants with covert, neuroprotective phenotypes. Third, the benefits of one type of neuroprotective mutation may not be transferable to all forms of neurodegenerative disease, so one goal of systematic screening is to find mutations that are more effective in mitigating disease onset or progression than *Wld*^{S}. Finally, new methods are clearly required to carry out such systematic screening effectively if potentially-beneficial phenotypes are to be recognized unequivocally, in the absence of overt clinical signs.

Our principal aim in the present study was to find genetic modifiers that would enhance synaptic protection in *Wld*^{S} mice. In order to identify these modifiers (phenodeviants), we first developed a method for screening axonal and neuromuscular synaptic phenotypes in living mice based on fiber-optic confocal microendoscopy (CME; Pelled et al., 2006; Vincent et al., 2006). We then induced genomic mutations using ethylnitrosourea (ENU) and screened for mutations that affected (enhanced or suppressed) axonal or synaptic degeneration in *Wld*^{S} mice using CME. Specifically, we crossbred ENU-mutagenized BALB/C mice with *thy1.2-YFP16*/*Wld*^{S} transgenic mice that express yellow fluorescent protein (YFP) in motor neurons (Bridge et al., 2007; Feng et al., 2000) and employed CME to assess the preservation of morphology of axons and neuromuscular junctions following nerve section in the adult F1 offspring. Once the breeding pipeline for mutagenized mice had been established, the screen had a relatively high throughput (that is, it enabled *Wld*^{S}-modifiers to be identified on a shorter time scale than more conventional ENU-screening methods in mice). We identified several phenodeviants that were subsequently tested for inheritance, including re-assessment of axonal and neuromuscular synaptic protection using CME. We also extended the CME methodology to investigation of covert synaptic pathology in the well-established transgenic *SOD1*^{G93A} mouse model of ALS, which shows early onset of disease signs in motor axons and NMJs (David et al., 2007; Frey et al., 2000; Gurney et al., 1994; Pun et al., 2006; Schaefer et al., 2005; Turner and Talbot, 2008). Finally we applied CME to the *ostes* mutant mouse, which was identified from a previous ENU-mutagenesis screen using standard SHIRPA screening protocols (Mackenzie et al., 2009), and which shows defects in axon regeneration (Mackenzie et al., 2007). Together, the results prove that CME and genomic mutagenesis can be combined in a powerful, flexible and versatile approach to investigation of neuromuscular biology and pathology at a cellular level *in vivo*.

Results

Neuromuscular synapses degenerate more rapidly in Wld^S *heterozygotes than homozygotes*

We showed previously that in young adult (1–2 month old) homozygous *Wld*^{S} mice most neuromuscular synapses degenerate within 4–10 days of axotomy (Bridge et al., 2007; Gillingwater et al., 2002; Mack et al., 2001). Electrophysiological recording and analysis of neuromuscular synaptic transmission in double-homozygous *thy1.2-YFP16:Wld*^{S} mice also showed no difference in the rate of synaptic degeneration following axotomy in these mice compared with those that did not express the YFP reporter (Bridge et al., 2007). Conventional confocal microscopy of muscles in *thy1.2-YFP16:Wld*^{S} mice supported these previous findings but additionally revealed striking differences in neuromuscular synaptic protection in *Wld*^{S} homozygotes compared with heterozygotes (Figs. 1A–F). In this cohort, the rate of synaptic degeneration in *Wld*^{S} heterozygotes was virtually indistinguishable from that in wild-type mice: 24–48 h after sciatic nerve section, motor endplates were vacant, uncovered by motor nerve terminals in both genotypes (Fig. 1G). By 3 days there was therefore a clear distinction between almost complete preservation of motor nerve terminals in *Wld*^{S} homozygotes, in contrast to almost complete synaptic degeneration in *Wld*^{S} heterozygotes. This distinction was evident despite indistinguishable levels of protection in the axotomized tibial nerve axons in homozygotes and heterozygotes at this time point (see below).

In light of the striking difference in synaptic protection in *Wld*^{S} heterozygotes and homozygotes, we envisaged using young adult heterozygous *Wld*^{S} mice as a sensitized background for seeking modifiers of axonal and synaptic degeneration. We reasoned also that a basal protective level of Wld^S protein, mitigating Wallerian degeneration of axons, would facilitate detection of protective mutations that might selectively suppress degeneration of synapses. In other words, normal Wallerian degeneration might obscure weak synaptic-protection phenotypes but *Wld*^{S} would delay this. Likewise, using *Wld*^{S} as a sensitized background also provided a more prolonged time-window for detecting mutations that might accelerate Wallerian degeneration of axons.

CME distinguishes intact from degenerating axons and synapses

We developed a method for screening ENU mutants, based on direct *in vivo* imaging of axons and neuromuscular junctions using fiber-optic confocal microendoscopy (CME). To validate this method, we first used CME to visualize intact axons and NMJs in the tibial nerve on one side in *Wld*^{S} and wild-type mice, employing a