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ACTIVITY-DEPENDENT DEGENERATION OF AXOTOMIZED NEUROMUSCULAR SYNAPSES IN *WLD*^{S} MICE

R. BROWN, A. HYNES-ALLEN, A. J. SWAN, K. N. DISSANAYAKE, T. H. GILLINGWATER AND R. R. RIBCHESTER *

Euan MacDonald Centre for Motor Neurone Disease Research, Hugh Robson Building, University of Edinburgh, George Square, Edinburgh EH8 9XD, UK

Abstract—Activity and disuse of synapses are thought to influence progression of several neurodegenerative diseases in which synaptic degeneration is an early sign. Here we tested whether stimulation or disuse renders neuromuscular synapses more or less vulnerable to degeneration, using axotomy as a robust trigger. We took advantage of the slow synaptic degeneration phenotype of axotomized neuromuscular junctions in flexor digitorum brevis (FDB) and deep lumbrical (DL) muscles of Wallerian degeneration-Slow (*Wld*^{S}) mutant mice. First, we maintained *ex vivo* FDB and DL nerve-muscle explants at 32 °C for up to 48 h. About 90% of fibers from *Wld*^{S} mice remained innervated, compared with about 36% in wild-type muscles at the 24-h checkpoint. Periodic high-frequency nerve stimulation (100 Hz: 1 s/100 s) reduced synaptic protection in *Wld*^{S} preparations by about 50%. This effect was abolished in reduced Ca^{2+} solutions. Next, we assayed FDB and DL innervation after 7 days of complete tetrodotoxin (TTX)-block of sciatic nerve conduction *in vivo*, followed by tibial nerve axotomy. Five days later, only about 9% of motor endplates remained innervated in the paralyzed muscles, compared with about 50% in 5 day-axotomized muscles from saline-control-treated *Wld*^{S} mice with no conditioning nerve block. Finally, we gave mice access to running wheels for up to 4 weeks prior to axotomy. Surprisingly, exercising *Wld*^{S} mice *ad libitum* for 4 weeks increased about twofold the amount of subsequent axotomy-induced synaptic degeneration. Together, the data suggest that vulnerability of mature neuromuscular synapses to axotomy, a potent neurodegenerative trigger, may be enhanced bimodally, either by disuse or by hyperactivity. **© 2015 The Authors. Published by Elsevier Ltd. on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).**

Key words: neuromuscular junction, axotomy, synaptic plasticity, activity.

INTRODUCTION

Maintenance and degeneration of synapses are thought to depend on their use or disuse. For instance, activity may influence the rates of synaptic and neuronal degeneration both during normal aging and in neurodegenerative conditions in which cognitive decline or progressive impairment of muscle function is associated with early signs of synaptic dysfunction and demise (Frey et al., 2000; Selkoe, 2002; Swaab et al., 2002; Fischer et al., 2004; Frick and Benoit, 2010; Power et al., 2010; Valdez et al., 2010; Li et al., 2011; Punga and Ruegg, 2012; Stern, 2012; Shors et al., 2012). Altering some forms of neuromuscular activity may also influence progression of disease. For example, imposing moderate levels of activity in mouse models of amyotrophic lateral sclerosis (ALS) delays onset and slow progression of disease signs, reducing premature mortality (Kirkinezos et al., 2003; Veldink et al., 2003; Liebetanz et al., 2004; Deforges et al., 2009; Gordon et al., 2010; de Almeida et al., 2012). By contrast, intensive activity has also been reported to accelerate disease progression, both in mouse models and in sporadic forms of human ALS, perhaps through excitotoxicity or enhancing vulnerability to reactive oxygen species (Carri et al., 2003; Bruijn et al., 2004; Carrasco et al., 2004; Mahoney et al., 2004; Al-Chalabi and Leigh, 2005; Chio et al., 2005; Boillee et al., 2006; Pun et al., 2006; David et al., 2007; Bell and Hardingham, 2011; Alvarez et al., 2013; Mehta et al., 2013).

Despite speculation and debate on the influence of use or disuse of synapses on synaptic degeneration, there is as yet no compelling, direct evidence that connects normal axonal activity to synaptic maintenance, or abnormal axonal activity to the vulnerability of synapses to neurodegenerative triggers. By contrast, there is compelling evidence that some forms of synaptic remodeling or withdrawal are highly sensitive to activity. For instance, the rate of postnatal synapse elimination, a controlled process of presynaptic withdrawal that has been well characterized at developing neuromuscular

*Corresponding author. Address: Euan MacDonald Centre for MND Research, School of Biomedical Sciences, University of Edinburgh, Hugh Robson Building, Edinburgh EH8 9XD, UK. Tel: +44-0131-650-3257.
E-mail address: Richard.Ribchester@ed.ac.uk (R. R. Ribchester).

Abbreviations: ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; CME, confocal microendoscopy; DL, deep lumbrical muscle; EPP, endplate potential; FDB, flexor digitorum brevis muscle; MEPP, miniature endplate potential; MPS, mammalian physiological saline; NAD, nicotinamide adenine dinucleotide; NMJ, neuromuscular junction; NMN, nicotinamide mononucleotide; Nmnat, nicotinamide mononucleotide adenyl transferase; QC, quantal content; Sarm1, sterile alpha and TIR motif-containing protein-1; SOD, superoxide dismutase; TTX, tetrodotoxin; WD, Wallerian degeneration; Wld^S, Wallerian degeneration-Slow mutant; WT, wild-type; YFP, yellow fluorescent protein.

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junctions (NMJs) or following nerve regeneration in adults, is readily modifiable and strongly influenced by activity (Brown et al., 1976; Thompson et al., 1979; Betz et al., 1980a; Ribchester and Taxt, 1983; Taxt, 1983; Thompson, 1983; Barry and Ribchester, 1995; Costanzo et al., 2000; Keller-Peck et al., 2001; Gillingwater and Ribchester, 2003; Walsh and Lichtman, 2003; Personius et al., 2007; Leslie and Nedivi, 2011; Favero et al., 2012; Turney and Lichtman, 2012; Caroni et al., 2014). Reactive growth (sprouting) and enhancement of synaptic function also occurs in adults in response to imposed inactivity or activity (Betz et al., 1980b; Ribchester and Taxt, 1984; Tsujimoto et al., 1990; Dorlochter et al., 1991; Ribchester, 1993; Fahim, 1997). We therefore asked in the present study whether variations in axonal or synaptic activity either before or after a neurodegenerative trigger might influence the resistance of neuromuscular synapses to pathological stimuli for synaptic degeneration in adult muscles as well.

An accessible, reliable and readily controllable model of synaptic degeneration is the Wallerian-like breakdown of motor nerve terminal structure and function that occurs after nerve injury (axotomy) or following functional disruption of axonal transport (Slater, 1966; Miledi and Slater, 1970; Winlow and Usherwood, 1975, 1976; Hudson et al., 1984; Wang et al., 2000; Gillingwater and Ribchester, 2001; Coleman and Freeman, 2010). This process is now thought to have mechanisms in common with several forms of neurodegenerative disease (Conforti et al., 2014). Considerable insight into axotomy-induced degeneration of axons and synapses has been obtained through study of the Wallerian degeneration-Slow mutant (Wld^S) mouse mutant, in which axonal and synaptic degeneration are profoundly retarded by overexpression of a stable, aberrant isoform of the nicotinamide adenine dinucleotide (NAD)-synthetic enzyme nicotinamide mononucleotide adenyl transferase (Nmnat)-1 (Lunn et al., 1989; Ribchester et al., 1995; Mack et al., 2001; Wang et al., 2001; Gillingwater et al., 2002; Gillingwater et al., 2004; Gillingwater et al., 2006; Coleman and Freeman, 2010). This isoform substitutes for a more labile axoplasmic form, Nmnat-2, whose levels decline steeply after axotomy, an event which normally is sufficient to trigger fragmentation and subsequent degeneration of axons (Beirowski et al., 2005; Beirowski et al., 2009; Babetto et al., 2010; Gilley and Coleman, 2010; Gilley et al., 2013; Milde et al., 2013; Di Stefano et al., 2014).

Interestingly, the protective influence of the Wld^S protein is modifiable in several ways. For instance, the strength of axonal or neuromuscular synaptic protection in Wld^S mice is sensitive to: housing environment and husbandry of the mice; neuronal maturity, reduced mutant gene copy-number (“gene dose”); disrupted targeting to nuclei or intracellular organelles; and interaction with other genes, including Sarm-1 (Perry et al., 1990; Mack et al., 2001; Gillingwater et al., 2002; Beirowski et al., 2009; Conforti et al., 2009; Wong et al., 2009; Babetto et al., 2010; Osterloh et al., 2012; Massoll et al., 2013). Moreover, motor axons and their terminals are less well protected from axotomy-induced degeneration in Wld^S mice than those of sensory neurons (Brown et al., 1994; Oyebode et al., 2012). There are several plausible explanations for this but a key difference is that sensory endings and their axons continue to be active in response to natural orthodromic stimulation, including touch, pressure, nociceptive and proprioceptive stimuli (Oyebode et al., 2012) whereas distal axons and motor nerve endings, while remaining competent to conduct action potentials and evoke transmitter release, require continuity with their initial segments and motor neuron cell bodies for orthodromic activation, which is interrupted upon axotomy (Tsao et al., 1994; Ribchester et al., 1995; Gillingwater et al., 2002; Beirowski et al., 2009; Brown et al., 2014).

Thus, taking together the indirect evidence for an influential role of activity in normal aging or neurodegenerative disease; the strong influence of activity on the rate of natural remodeling processes like synapse elimination; and the enhanced persistence of sensory endings and their axons observed following axotomy in Wld^S mice, we enquired whether activity also influences Wallerian-like degeneration of axotomized neuromuscular synapses. If this were the case, then we would predict that systematically altering or imposing neuronal activity patterns should change the rate of synaptic degeneration after axotomy.

We tested our hypothesis by controlling axonal and neuromuscular synaptic activity in Wld^S mice, in three ways. First, we measured the effect of continuous stimulation of isolated and cultured nerve-muscle preparations on the rate of synaptic degeneration *ex vivo*. Second, we preconditioned axons by chronic nerve conduction block *in vivo*, then measured the effect of this disuse-priming on synaptic degeneration over several days after axotomy. Finally, we enriched the environment of mice for up to one month, by providing them with running wheels, thus encouraging increased levels of volitional activity, before cutting axons and measuring the subsequent levels of neuromuscular synaptic degeneration. Surprisingly, the data suggest a bimodal response: both inactivity and intense stimulation appear to increase the vulnerability and rate of synaptic degeneration in both Wld^S and wild-type (WT) mice, while moderate levels of activity were beneficial or without adverse effect. We discuss possible implications of the data for unified views of links between Wallerian-like degeneration of neuromuscular synapses, developmental synapse elimination, and neurodegenerative diseases in which synaptic dysfunction or degeneration are early phenomenological or clinical signs (Gillingwater and Ribchester, 2001; Raff et al., 2002; Gillingwater and Ribchester, 2003).

EXPERIMENTAL PROCEDURES

Ethical approval

All experiments reported in the present paper were approved by the University of Edinburgh College of Medicine and Veterinary Medicine Local Ethics Committee and conducted under the terms of a Project Licence and Personal Licences from the UK Home

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