Neurobiology of Disease 49 (2013) 57–67

Contents lists available at SciVerse ScienceDirect

Neurobiology of Disease

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

Defects in neuromuscular junction remodelling in the $Smn^{2B/-}$ mouse model of spinal muscular atrophy

Lyndsay M. Murray [a], Ariane Beauvais [a], Kunal Bhanot [a], Rashmi Kothary [a,b,c,*]

[a] Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, ON, Canada K1H 8L6
[b] Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada K1H 8M5
[c] Department of Medicine, University of Ottawa, Ottawa, ON, Canada K1H 8M5

ARTICLE INFO

Article history:
Received 23 July 2012
Revised 17 August 2012
Accepted 22 August 2012
Available online 30 August 2012

Keywords:
Neuromuscular junction
Spinal muscular atrophy
Remodelling
Sprouting
Endplate
Schwann cell

ABSTRACT

Spinal muscular atrophy (SMA) is a devastating childhood motor neuron disease caused by mutations and deletions within the survival motor neuron 1 (*SMN1*) gene. Although other tissues may be involved, motor neurons remain primary pathological targets, with loss of neuromuscular junctions (NMJs) representing an early and significant event in pathogenesis. Although defects in axonal outgrowth and pathfinding have been observed in cell culture and in lower organisms upon Smn depletion, developmental defects in mouse models have been less obvious. Here, we have employed the $Smn^{2B/-}$ mouse model to investigate NMJ remodelling during SMA pathology, induced reinnervation, and paralysis. We show that whilst NMJs are capable of remodelling during pathogenesis, there is a marked reduction in paralysis-induced remodelling and in the nerve-directed re-organisation of acetylcholine receptors. This reduction in remodelling potential could not be attributed to a decreased rate of axonal growth. Finally, we have identified a loss of terminal Schwann cells which could contribute to the defects in remodelling/maintenance observed. Our work demonstrates that there are specific defects in NMJ remodelling in an intermediate SMA mouse model, which could contribute to or underlie pathogenesis in SMA. The development of strategies that can promote the remodelling potential of NMJs may therefore be of significant benefit to SMA patients.

Introduction

Spinal muscular atrophy (SMA) is a childhood motor neuron disease caused by anomalies in the survival motor neuron (*SMN*) 1 gene (Lefebvre et al., 1995). SMA is characterised by loss of lower motor neurons and atrophy of the associated skeletal musculature (Monani, 2005). Furthermore, the use of a growing number of mouse models has identified neuromuscular junctions (NMJs) as early and significant pathological targets (Murray et al., 2008a). Reported defects include denervation, poor terminal arborisation, neurofilament accumulation, abnormal expression of synaptic proteins and delayed post-synaptic maturation alongside a disruption in synaptic vesicle release and calcium homeostasis causing defects in motor neuron excitability (Cifuentes-Diaz et al., 2002; Dachs et al., 2011; Jablonka et al., 2007; Kariya et al., 2008; Kong et al., 2009; Ling et al., 2011; Michaud et al., 2010; Murray et al., 2008a; Ruiz et al., 2010; Torres-Benito et al., 2011). Although the availability of human samples is more limited, similar structural defects have been noted in post-mortem samples from SMA patients (Kariya et al., 2008).

The establishment of the intermediate $Smn^{2B/-}$ mouse model has presented an excellent opportunity to observe neuromuscular pathology over an extended disease time course (Bowerman et al., 2012). The available mouse models of SMA have generally been concentrated at the severe end of the SMA spectrum, with the most widely used models, including $Smn^{-/-}$;*SMN2* and $Smn^{-/-}$;*SMN2*;*SMNΔ7*, displaying an early post-natal onset, with death occurring prior to 2 weeks of age (Le et al., 2005; Monani et al., 2000). The recent development of the $Smn^{2B/-}$ mouse model represents a significant advance within the field due to its extended life span, displaying a disease onset of around 10 days and mean life expectancy of 28 days thus allowing the study of SMA pathology over a more prolonged time course (Bowerman et al., 2012; Hammond et al., 2010). In contrast to the rapid and progressive breakdown of neuromuscular connectivity in more severe models, the $Smn^{2B/-}$ mouse model allows us to investigate the potential compensatory mechanisms, such as sprouting, occurring at the NMJ throughout the disease time course.

The reason for NMJ vulnerability in SMA is currently unclear. Defects in neurite outgrowth in cellular, xenopus and zebrafish models of SMA have been reported (McWhorter et al., 2003; Rossoll et al.,

Abbreviations: AAL, Adductor Auris Longus; AchR, Acetylcholine receptor; AS, Auricularis Superior; BotA, Botulinum Toxin type A; BTX, Bungarotoxin; LAL, Levator Auris Longus; NMJ, Neuromuscular Junction; SMA, Spinal Muscular Atrophy; Smn, Survival Motor Neuron; tSC, terminal Schwann cell; TVA, Transversus Abdominis.

* Corresponding author at: Ottawa Hospital Research Institute, 501 Smyth Road, Ottawa, Canada K1H 8L6.
E-mail address: rkothary@ohri.ca (R. Kothary).
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.nbd.2012.08.019

2003; Ymlahi-Ouazzani et al., 2010). However, such defects did not translate into overt developmental abnormalities in mouse models (McGovern et al., 2008; Murray et al., 2010a). Given the convergence of the mechanisms responsible for neurite outgrowth and synaptic maintenance, the observed defects in outgrowth observed may allude to synaptic maintenance abnormalities. The hypothesis that defects in synaptic maintenance/remodelling contribute to and/or underlie SMA is supported by the observation that selectively vulnerable populations of motor units in severe SMA mouse models display a marked decrease in paralysis-induced plasticity compared to more resistant motor units (Murray et al., 2008a).

Both synaptic maintenance and remodelling are a product of a network of pathways and interactions between muscle, nerve and terminal Schwann cell. Therefore, investigation of remodelling potential can reveal physiological deficits in NMJ functionality, which would have otherwise been masked. Dramatic NMJ remodelling occurs during development, disease, and following traumatic or chemical insult (Sanes and Lichtman, 1999). It is often typified by neuronal sprouting whereby neuronal processes extend from the synaptic terminal (terminal sprouting) or internode (nodal sprouting) which commonly innervate novel receptor clusters or denervated endplates (Santos and Caroni, 2003).

To investigate the parameters involved in synaptic maintenance and remodelling, we have undertaken an in-depth characterisation of NMJ remodelling in the $Smn^{2B/-}$ mouse model of SMA. We have investigated the incidence and prevalence of terminal and nodal sprouting in a range of differentially vulnerable muscles in $Smn^{2B/-}$ mice and report that there is a modest degree of terminal sprouting which is correlative with NMJ loss. We also report a significant level of highly elaborate and novel type of nodal sprouting which appears correlative with a decrease in NMJ loss. We have used botulinum-induced paralysis to assess whether the capacity for paralysis induce remodelling is changed in $Smn^{2B/-}$ mice and report that NMJs from SMA mice display a significant reduction in remodelling as well as defects in neurally-directed clustering of acetylcholine receptors (AChRs). We have used a denervation–reinnervation protocol to assess the rate of axon growth and demonstrate that the reduction in sprouting cannot be attributed to a reduced rate of axonogenesis. Finally, quantification of terminal Schwann cell numbers has revealed a progressive loss of terminal Schwann cells which may contribute to the defects described. This work reveals a disruption in NMJ remodelling in the $Smn^{2B/-}$ mouse model which could contribute to or underlie pathogenesis in SMA.

Materials and methods

Mouse maintenance and surgery

The $Smn^{2B/-}$ mice were established in our laboratory and maintained in the University of Ottawa vivarium on a C57BL/6×CD1 hybrid background. Mice were sacrificed by intra-peritoneal injection of Avertin or by cervical dislocation. All animal procedures were performed in accordance with institutional guidelines (Animal Care and Veterinary Services, University of Ottawa).

Tibial nerve crush/cut experiments were performed on P14 mice. Mothers were pre-dosed with buprenorphine prior to surgery. Mice were anesthetised by inhalation of isofluorane (2% in 1:1 N_2O/O_2). A 2 mm incision in the skin was made just above the heel to expose the tibial nerve. Blunt dissection was used to tease away overlying muscle, taking care not to damage neighbouring blood vessels. For nerve crush experiments, the tibial nerve was crushed using number 5 fine forceps for 15 s. For tibial nerve cut experiments, a 1–2 mm section of the tibial nerve was removed. The incision was closed with surgical glue. Mice were allowed to recover from anaesthetic before being returned to standard cages.

Administration of substances

Botulinum toxin type A (BOTOX, 0.5 μl/g of a 5 ng/ml solution; Allergan) was injected subcutaneously above the cranial muscle group at postnatal days 10 and 12. Mice were sacrificed and muscles analysed at P16.

Muscle dissection and immunohistochemical staining

Muscles were labelled by immunohistochemistry as described previously (Murray et al., 2008a). Briefly, muscles were immediately dissected from recently sacrificed mice and fixed in 4% paraformaldehyde (Electron Microscopy Science) in PBS for 15 min. Post-synaptic AChRs were labelled with αBTX for 30 min. Muscles were permeabilised in 2% Triton X-100 in PBS for 30 min, then blocked in 4% bovine serum albumin (BSA)/1% Triton X-100 in PBS for 30 min before incubation overnight in primary antibodies [Neurofilament (NF; 2H3)—Developmental Studies Hybridoma Bank; synaptic vesicle protein 2 (SV2)—Developmental Studies Hybridoma Bank; S100—Dako; all 1:250] and visualised with DyLight-conjugated secondary antibodies [DyLight goat anti-mouse; DyLight goat anti-rabbit; all 1:250, Jackson]. Muscles were then whole-mounted in Dako Fluorescent mounting media. Images were taken with a Zeiss LSM-510 meta confocal microscope.

Electron microscopy

Tibial nerve preparations were dissected and pinned out on wax before being immersion-fixed in 0.1 M cacodylate buffer containing 4% formaldehyde and 2.5% glutaraldehyde for 4 h before post-fixation in 1% osmium tetroxide for 45 min. Preparations were dehydrated in ascending alcohol series (50%, 70%, 90%, 100% ×2) and acetone. Preparations were embedded in Spur monomer. Ultra-thin sections (75–90 nm) were cut and collected on mesh grids, stained with uranyl acetate and lead citrate. Sections were viewed in a Hitachi H7100 transmission electron microscope.

Quantification and statistics

The percentage of fully occupied endplates was determined by classifying each endplate in a given field of view either fully occupied (pre-synaptic terminal (SV2 and NF) completely overlies endplate (BTX)), partially occupied (pre-synaptic terminal only partially covers endplate (BTX)), or vacant (no pre-synaptic label overlies endplate). At least 3 fields of view were analysed per muscle totalling >100 endplates per muscle. The number of tSCs per endplate was determined by taking confocal z-stacks of images labelled with S100, BTX and DAPI, and manually scanning through z series above and below the endplate to identify the number of DAPI positive nuclei corresponding to a S100 stain. At least 3 fields of view were analysed per muscle totalling >30 endplates per muscle. The number of sprouts per endplate was quantified by counting the number of neurofilament-positive projections from the pre-synaptic terminal or pre-terminal node on confocal z projections. Sprout length was measured using ImageJ software. Ectopic endplates were identified by an endplate<5 μm in diameter which were innervated by terminal or pre-terminal nodal sprouts. The number of axons per nerve was determined by counting the number of myelinated axon profiles present in a field of view of defined area on images obtained from the electron microscope at 10,000× magnification to give a value of average number of axons per square micrometer. The cross-sectional nerve area was then determined by tracing nerve area on images from toluidine blue stained nerve sections visualised through a light microscope. The number of axons per nerve was then extrapolated by multiplying the number of axons per micrometer squared by the nerve cross-sectional area. Measurements represent the mean from 3 nerve sections and the experimenter was blind