Developmental Biology 330 (2009) 153–166

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Abnormal development of the neuromuscular junction in *Nedd4*-deficient mice

Yun Liu [a], Ronald W. Oppenheim [b], Yoshie Sugiura [a], Weichun Lin [a,*]

[a] *Department of Neuroscience, UT Southwestern Medical Center, 6000 Harry Hines Blvd, Dallas, TX 75390-9111, USA*
[b] *Department of Neurobiology and Anatomy, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA*

ARTICLE INFO

Article history:
Received for publication 4 December 2008
Revised 23 March 2009
Accepted 25 March 2009
Available online 1 April 2009

Keywords:
Neuromuscular junction
Mouse genetics
Motoneuron
Synaptogenesis
Mammalian development

ABSTRACT

Nedd4 (*n*eural precursor cell *e*xpressed *d*evelopmentally *d*own-regulated gene *4*) is an E3 ubiquitin ligase highly conserved from yeast to humans. The expression of Nedd4 is developmentally down-regulated in the mammalian nervous system, but the role of Nedd4 in mammalian neural development remains poorly understood. Here we show that a null mutation of *Nedd4* in mice leads to perinatal lethality: mutant mice were stillborn and many of them died in utero before birth (between E15.5–E18.5). In *Nedd4* mutant embryos, skeletal muscle fiber sizes and motoneuron numbers are significantly reduced. Surviving motoneurons project axons to their target muscles on schedule, but motor nerves defasciculate upon reaching the muscle surface, suggesting that Nedd4 plays a critical role in fine-tuning the interaction between the nerve and the muscle. Electrophysiological analyses of the neuromuscular junction (NMJ) demonstrate an increased spontaneous miniature endplate potential (mEPP) frequency in *Nedd4* mutants. However, the mutant neuromuscular synapses are less responsive to membrane depolarization, compared to the wildtypes. Ultrastructural analyses further reveal that the pre-synaptic nerve terminal branches at the NMJs of *Nedd4* mutants are increased in number, but decreased in diameter compared to the wildtypes. These ultrastructural changes are consistent with functional alternation of the NMJs in *Nedd4* mutants. Unexpectedly, Nedd4 is not expressed in motoneurons, but is highly expressed in skeletal muscles and Schwann cells. Together, these results demonstrate that Nedd4 is involved in regulating the formation and function of the NMJs through non-cell autonomous mechanisms.

Published by Elsevier Inc.

Introduction

Protein ubiquitination and ubiquitin–proteasome mediated degradation have emerged as important mechanisms regulating synapse formation and function (Bingol and Schuman, 2006; Ding et al., 2007; Ehlers, 2003; Speese et al., 2003; Watts et al., 2003). The ubiquitination process is a cascade of reactions catalyzed, sequentially, by the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin ligase (Scheffner et al., 1995), each of which are critically involved in synaptogenesis. For example, bendless, an E2 enzyme in *Drosophila*, is required for the giant fiber axon to synapse with its motoneuron target (Thomas and Wyman, 1984); UBC-25, an E2 enzyme in *C. elegans*, is required for the maintenance of neuromuscular function (Schulze et al., 2003). Furthermore, mutations of rpm-1 (*r*egulator of *p*re-synaptic *m*orphology), a RING (*r*eally *i*nteresting *n*ovel *g*ene) finger-domain E3 ligase in *C. elegans* (Schaefer et al., 2000; Zhen et al., 2000), or *highwire*, the homologue of *rpm-1* in *Drosophila* (DiAntonio et al., 2001; Wan et al., 2000; Wu et al., 2005) lead to neuromuscular synaptic overgrowth and transmission defects. Similarly, axonal projection and neuromuscular defects have been reported in the mouse mutant *Phr1* (*pam, highwire, rpm-1*), the murine orthologue of *highwire*/*rpm-1* (Bloom et al., 2007; Burgess et al., 2004; Lewcock et al., 2007), and in zebrafish mutant *Esrom*, the fish orthologue of *highwire*/*rpm-1* (D'Souza et al., 2005).

Like *rpm-1*/*highwire*/*Esrom*/*Phr1*, Nedd4 is an E3 ligase, but it is a member of a distinct family of E3s, the HECT (*h*omologous to *E*6-AP *c*arboxyl *t*erminus) domain E3 family (Ingham et al., 2004; Rotin et al., 2000)). Nedd4 contains one C2 domain, three (or four) WW domains and one HECT domain; such domain architecture is highly conserved from yeast to humans (Ingham et al., 2004; Kumar et al., 1997; Staub and Rotin, 2006). In mammals, Nedd4 is known to down-regulate the epithelial sodium channel (ENaC) (Abriel et al., 1999; Dinudom et al., 1998; Goulet et al., 1998; Harvey et al., 1999; Staub et al., 1996) and other voltage-gated sodium channels including neuronal specific (Fotia et al., 2004) and cardiac specific (van Bemmelen et al., 2004) voltage-gated sodium channels. Studies in *Drosophila* have shown that DNedd4 (the *Drosophila* orthologue of Nedd4) regulates cell surface expression of the roundabout (Robo) receptor (Myat et al., 2002) and the transmembrane protein Commissureless (Comm) (Ing et al., 2007), both of which are crucial for target recognition and stabilization of the fly NMJ. It is thought that DNedd4 promotes neuromuscular synaptogenesis in *Drosophila* by facilitating the endocytosis of Comm (Ing et al., 2007; Wolf et al., 1998).

* Corresponding author. Fax: +1 214 648 1801.
E-mail address: weichun.lin@utsouthwestern.edu (W. Lin).

0012-1606/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.ydbio.2009.03.023

A question arises what role(s) Nedd4 plays in the formation and function of the mammalian NMJs. In fact, Nedd4 was initially identified as a developmentally down-regulated gene from the mouse brain; Nedd4 mRNA is detected in the neural tube (E8.5), the head (E10–E11) and mouse brain (E13-neonatal stage) and its level regresses as development proceeds (Kumar et al., 1997; Kumar et al., 1992). However, the role of Nedd4 in the development of mammalian nervous system remains largely unknown. To address this issue, we have examined mutant mice deficient in Nedd4. Our analyses show that *Nedd4* mutants are perinatal lethal and display profound defects in neuromuscular formation and function. In *Nedd4* mutants, the skeletal muscle fiber sizes and motoneuron numbers are significantly reduced. Surviving motoneurons project axons to their muscles on schedule, but motor nerves defasciculate upon reaching the muscle surface, suggesting Nedd4 is not required for muscle target recognition, but is important for proper interaction between the nerve and the muscle. Interestingly, neuromuscular synapses in *Nedd4* mutants are established in the central region of the muscle, in a pattern similar to the wildtypes. However, an individual synapse in the *Nedd4* mutant is composed of more numerous, yet smaller nerve terminal profiles, compared to the wildtypes. Consistent with these structural defects, electrophysiological analyses revealed functional impairments of neuromuscular synaptic activities in *Nedd4* mutant embryos. Interestingly, Nedd4 was not detected in motoneurons, but was highly expressed in skeletal muscles and Schwann cells in the periphery. These results suggest that Nedd4 functions through non-cell autonomous mechanisms to regulate nerve terminal differentiation and function.

Materials and methods

Animals

Nedd4 mutant mice (*Nedd4*$^{Gt(IRESBetageo)249Lex}$) were generated by Lexicon Genetics Incorporated, via retroviral insertion strategy (Raymond and Soriano, 2006). The *Nedd4* gene was disrupted between exons 17 and 18, and this strategy also introduced a reporter gene, β-*galactosidase* (*lacZ*), into the *Nedd4* locus, thus the expression of *lacZ* could be used to identify cells expressing endogenous *Nedd4* (http://www.mmrrc.org/strains/11742/011742.html). We have obtained the heterozygote *Nedd4*$^{Gt(IRESBetageo)249Lex}$ mice, which are viable, fertile and devoid of gross phenotypic defects (http://www.mmrrc.org/strains/11742/011742.html), from the Mutant Mouse Regional Resource Centers (MMRRC). To generate homozygous mutants (*Nedd4*$^{Gt(IRESBetageo)249Lex/Gt(IRESBetageo)249Lex}$, hereafter as *Nedd4*$^{-/-}$, or *Nedd4* mutant), heterozygotes were time-mated, and the day when a vaginal plug first appeared was designated as embryonic (E) day 0.5. After selected intervals of development, embryos (E10.5–E18.5) were collected by Cesarean section of anesthetized pregnant female mice. *Nedd4* mice were genotyped by PCR, using the following primer set: *Nedd4* wild type allele-forward GGA GTC TTT GGA TAT TGT AAG AGC, reverse GAG CGT GCG CCT CAC AAG TAT GA; *Nedd4* mutant allele-forward AAA TGG CGT TAC TTA AGC TAG CTT GC; reverse GAG CGT GCG CCT CAC AAG TAT GA. All experimental protocols followed NIH Guidelines and are approved by the UT Southwestern Institutional Animal Care and Use Committee.

Immunofluorescence

Immunofluorescence staining was carried out as previously described (Liu et al., 2008). Briefly, muscle samples were fixed in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.3) at 4 °C overnight, blocked in dilution buffer (500 mM NaCl, 0.01 M phosphate buffer, 3% BSA and 0.01% thimerosal), and then incubated with primary antibodies. The sources of primary antibodies were as follows: S100β (1:500, Swant Swiss Antibodies, Switzerland), neurofilament (NF150) (1:1000, Chemicon, Temecula, CA), synaptophysin (1:100, Dako, Carpinteria, CA), SV2 (1:1000, Developmental Studies Hybridoma Bank, the University of Iowa, Iowa City, IA), HB9 (1:5000, gifts from Dr. Samuel Pfaff, Salk Institute, La Jolla, CA) (Thaler et al., 1999), MuSK (1:500, gift from Dr. Steve Burden, Skirball Institute, NYU Medical Center, NY) and synaptotagmin-2 (1:1000, gift from Dr. Thomas Südhof, Stanford University School of Medicine, Palo Alto, CA) (Pang et al., 2006). Samples were then incubated with fluorescein isothiocyanate-conjugated secondary antibodies and Texas-Red conjugated α-bungarotoxin (α-bgt) (2 nM, Molecular Probes), washed with PBS and mounted in 90% glycerol, 10% Tris Buffer (pH 8.5, 10 mM) containing *n*-propyl gallate (20 mM) to reduce photobleaching (Giloh and Sedat, 1982). Quantification of fluorescence intensity and sizes of AChR clusters was generated from confocal images acquired with identical, sub-saturating gains. The mean gray value (integrated density/total pixels), area, perimeter and Feret's diameter (the length of the greatest axis) were measured using NIH ImageJ.

X-gal staining for LacZ (β-galactosidase)

Detection of β-*galactosidase* (*LacZ*) activity was carried out based upon previously described procedures (MacGregor et al., 1995; Sanes et al., 1986). Briefly, embryonic (E11.5–E18.5) tissue sections (12 μm) or whole diaphragm muscles (E13.5–E18.5) of heterozygote or wildtype (as negative control) mice were fixed in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer for 1 h, washed three times for 10 min each time in PBS and then incubated in an incubation buffer containing sodium phosphate (150 mM), $MgCl_2$ (2 mM), sodium deoxycholate (0.01%), NP-40 (0.02%), potassium ferricyanide (5 mM), potassium ferrocyanide (5 mM), and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (1 mg/ml, X-Gal), at 37 °C. Images were acquired on an Olympus BX51 upright microscope with Nomarski optics (for sections) or a Zeiss stereo microscope (SteREO Discovery) (for wholemount diaphragm muscles).

Western blot

Embryonic tissues were collected at E18.5 and homogenized in Tris buffer containing 50 mM Tris–NaOH, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF and protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA). Tissue homogenates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked in 5% milk in Tris-buffered saline. The membrane was incubated with anti-β-galactosidase (Cappel, 1:1000), or anti-Nedd4 (1:10,000, Upstate), or mouse monoclonal anti-alpha-tubulin (1:1000, Sigma-Aldrich) antibodies, followed by peroxidase-conjugated secondary antibodies (1:10,000, Biomol), and visualized with Enhanced Chemiluminescence (ECL, Amersham Biosciences) reagents.

Acetylcholinesterase (AChE) assay

Detection of AChE was based on the methods previously described (Enomoto et al., 1998). Briefly, diaphragm muscles were fixed with 2% PFA, rinsed in PBS and incubated in 0.2 mM ethopropazine, 4 mM acetylthiocholine iodine, 10 mM glycine, 2 mM cupric sulfate, and 65 mM sodium acetate solution at pH 5.5, for 2–4 h at 37 °C. Staining for AChE was developed by incubating the wholemount diaphragm for 2–5 min in sodium sulfide (1.25%, pH 6.0), followed by extensive wash. The diaphragms were then cleared with 50% glycerol in PBS and flat mounted and images were acquired on a Zeiss stereomicroscope.

Morphometric analysis

Wildtype or heterozygote ($N = 5$) and *Nedd4* mutant ($N = 3$) embryos (E18.5) were immersion fixed in Bouins solution following decapitation and evisceration. The trunk was trimmed resulting in the

Download English Version:

https://daneshyari.com/en/article/10933311

Download Persian Version:

https://daneshyari.com/article/10933311

Daneshyari.com