Synaptic Defects in Spinal Muscular Atrophy Animal Models

Laura Torres-Benito, Rocío Ruiz, Lucía Tabares

Department of Medical Physiology and Biophysics, School of Medicine, University of Seville, 41009 Seville, Spain

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ABSTRACT: Proximal spinal muscular atrophy, the most frequent genetic cause of childhood lethality, is caused by homozygous loss or mutation of the *SMN1* gene on human chromosome 5, which codes for the survival motor neuron (SMN) protein. SMN plays a role in the assembly of small nuclear ribonucleoproteins and, additionally, in synaptic function. SMN deficiency produces defects in motor neuron β-actin mRNA axonal transport, neurofilament dynamics, neurotransmitter release, and synapse maturation. The underlying molecular mechanisms and, in particular, the role of the cytoskeleton on the pathogenesis of this disease are starting to be revealed. © 2011 Wiley Periodicals, Inc. Develop Neurobiol 72: 126–133, 2012

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SMN: AN ESSENTIAL PROTEIN

Low levels of survival motor neuron (SMN) protein result in spinal muscular atrophy (SMA), the most frequent genetic cause of early childhood lethality (Crawford and Pardo, 1996). SMA is an autosomal recessive degenerative disease characterized by symmetrical muscular weakness and atrophy of limb and trunk muscles. Currently, there is no effective treatment for SMA. SMN is encoded by two genes that lie on a duplicated region of human chromosome 5 [5q13; (Lefebvre et al., 1995)]. *SMN1* produces full-length SMN (SMN-FL), and *SMN2* produces an unstable truncated form of SMN (SMNΔ7) and a small amount (∼10%–20%) of SMN-FL, which results from alternative splicing (Gennarelli et al., 1995; Lorson et al., 1999; Monani et al., 1999). SMA is caused by mutations or loss of the *SMN1* gene and retention of the *SMN2* gene (Lefebvre et al., 1995). In the absence of a functional *SMN1* gene, the severity of the disease depends on the amount of SMN-FL produced by *SMN2* (the *SMN2* copy number varies in the population). Low number of SMN2 copies results in severe childhood forms of the disease that lead to rapid paralysis and early death (types I and II), whereas higher number of copies lead to milder forms (types III and IV) in which patients reach normal age despite impairment by muscle paralysis.

SMN is a 38 kDa protein ubiquitously expressed in all tissues that localize to both the cytoplasm and nucleus of cells. The best-characterized SMN function is its participation in the assembly of small nuclear ribonucleoproteins as part of a multiprotein complex in the spliceosome (Fischer et al., 1997; Liu et al., 1997; Meister et al., 2001; Pellizzoni et al., 2002). Although the absence of SMN is embryonically lethal, low levels of SMN produce loss of α-motor neurons in the spinal cord, which causes muscle weakness and muscle atrophy in particular in proximal muscle groups of the body axis. It is still not understood, however, why reduced levels of the ubiquitously expressed SMN

Correspondence to: L. Tabares (ltabares@us.es).

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protein specifically cause motor neuron disease. Currently, two hypotheses are postulated (Monani, 2005; Burghes and Beattie, 2009; Chari et al., 2009). In one of them, disturbed RNA processing specially relevant for motor neurons is proposed (Pellizzoni et al., 2002; Wan et al., 2005; Winkler et al., 2005; Eggert et al., 2006; Gabanella et al., 2007; Pellizzoni, 2007; Zhang et al., 2008; Burghes and Beattie, 2009). In the other, it is postulated that SMN has additional functions in axons and synapses, which are independent of its role in small nuclear ribonucleoprotein biogenesis (Fan and Simard, 2002; McWhorter et al., 2003; Rossoll et al., 2003; Carrel et al., 2006; Eggert et al., 2006; Gabanella et al., 2007; Pellizzoni, 2007; Burghes and Beattie, 2009). Moreover, SMN is found not only in the cell soma but also in axons *in vivo* (Pagliardini et al., 2000), and at branch points and growth cones in cultured motor neurons (Jablonka et al., 2001). Within axons and growth cones, SMN localizes in granules and is rapidly transported bi-directionally (Zhang et al., 2003) together with Gemin2 and Gemin3 (Zhang et al., 2006).

SMN-Deficient Animal Models: Development, Axonal, and Synaptic Dysfunctions

In various invertebrates and vertebrates animal models of SMA, morphological and functional evidence shows that the loss of function of SMN leads to important defects, ranging from embryonic lethality to impairment of the motor system. The severity of the phenotype is inversely proportional to the level of SMN expression. The study of model organisms with motor defects is of major importance for the understanding of the molecular mechanisms implicated in the pathophysiology of SMA in humans, as the dysfunction of the motor system is its most striking alteration. Interestingly, the synaptic and axonal defects observed in these models do not seem to be caused by motor neuron cell death, which is generally a relatively late manifestation of the disease (Kariya et al., 2008; Kong et al., 2009).

In *Caenorhabditis elegans* the knockdown of smn-1 (the worm orthologue of human SMN) by RNA interference, results in a severe embryonic lethal phenotype (Miguel-Aliaga et al., 1999; Fraser et al., 2000; Sonnichsen et al., 2005). A less complete removal of smn-1 produces an arrest in late larval development [Fig. 1(A)], reduced lifespan, sterility, and locomotor dysfunction (Briese et al., 2009).

In Drosophila, Smn deficit also produces high larval lethality, alterations in late stages of development and motor behavior defects. Drosophila endplate electrophysiological recordings show that postsynaptic currents are reduced at the neuromuscular junction (NMJ) of SMA mutants [Fig. 1(B, a)]. The coexistence of this functional defect with synaptic motor neuron bouton disorganization [Fig. 1(B, b)] led to the suggestion, for the first time, of a primary defect at the SMA end-plates (Chan et al., 2003). Additionally, fly motor neurons display pronounced axon routing and arborization deficits (Rajendra et al., 2007) that suggest, in addition, a possible defect in axonogenesis.

In Xenopus, Smn deficit also induces alterations ranging from developmental arrest at gastrulation (Winkler et al., 2005) to motor neuron defects characterized by reduced axonal outgrowth and abnormal formation of branching extensions (Ymlahi-Ouazzani et al., 2010).

Similarly, in a zebrafish embryo Smn-deficient model, motor axons show abnormal growth along the body muscles. Frequently, mutant axons are short, display truncations, and excessive branching [Fig. 1(C)], and do not succeed in finding their most distal targets (McWhorter et al., 2003; Carrel et al., 2006; Oprea et al., 2008; Hao et al., 2011).

In mice, both *in vitro* and *in vivo* SMA models have been used for studying the pathophysiology of the disease. Embryonic SMN-deficient spinal motor neurons in culture exhibit reduced axon elongation and growth cone size (Rossoll et al., 2003; Zhang et al., 2003; Garcera et al., 2011), similar to what has been reported in fly, frog, and zebrafish SMA models. In addition, Smn-deficient motoneurons exhibit severe defects in clustering Cav2.2 channels in axonal growth cones and reduced frequency of local Ca^{2+} transients (Jablonka et al., 2007).

Despite the axonogenesis defect observed in SMN-deficient motor neurons *in vitro*, in acute neuromuscular preparations from the same SMA mouse model, this defect is not observed. Furthermore, in the SMA mouse models, no defect in axonal formation at any stage of development is found (McGovern et al., 2008). In *ex vivo* preparations, the number of axonal branches innervating single muscle fibers is not different from wild-type littermates (Kariya et al., 2008). Moreover, during the early postnatal age of SMNΔ7 SMA mice (a widely used SMA mouse model) muscle fibers are multi-innervated (Murray et al., 2008), and the multiple motor nerve inputs contacting a single muscle fiber are functional (Ruiz et al., 2010). Therefore, the reduction of SMN levels in mice does not interfere with the correct formation of synapses *in vivo*. This suggests the existence of compensatory mechanisms during axonogenesis,

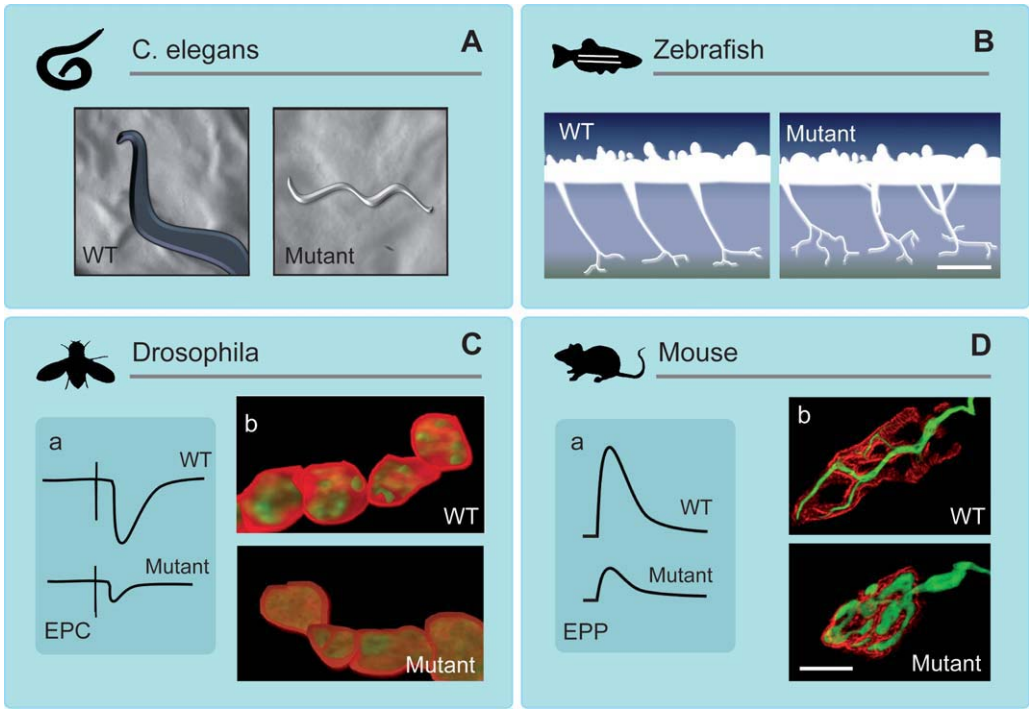

Figure 1 Schematic representation of neuromuscular defects in different animal models. **A**. *C. elegans* knockdown *smn-1* shows growth arrest and locomotor dysfunction in 4 day larvae. **B**. Zebrafish 48 h embryos with reduced levels of *smn* display truncated and excessively branched motor nerves. Scale bar: 50 mm. **C**. NMJ features in *smn Drosophila* model. (a) End-plate postsynaptic currents (EPCs) are reduced in third instar *smn* mutant larvae. (b) NMJ representing postynaptic receptors (red) and synaptic vesicles (green). The mutant shows disorganized boutons compared with the WT. **D.** NMJ features in SMND7 mouse model. (a) Representative end-plate potential (EPP) traces from TVA at postnatal day 7 (P7). The mean size of the EPP in the mutant is significantly decreased. (b) Immunostaining of postsynaptic receptors (red) and neurofilaments (green). Mutant NMJs exhibit reduced postsynaptic size and massive NF accumulation in presynaptic terminals. Scale bar: 10 micrometers.

which could be related to the complex environment in which mammalian motor axons normally develop.

Electrophysiological recordings of the synaptic activity at mouse NMJs from different SMA models have shown a number of abnormalities (Table 1). The most relevant finding is the decrease in the amount of neurotransmitter released per action potential (quantum content, QC). In SMNΔ7 mice, the most functionally affected muscle is transversus abdominis (TVA; a proximal slow-twitch muscle) with ~50% reduction in QC [Fig. 1(D, a)], followed by distal and faster muscles such as tibialis anterior (TA; ~40% reduction) and extensor digitorum longus (EDL; ~25% reduction) (Table 1). In addition, levator auris longus (LAL), a uniformly fast muscle shows a special gradient of alteration, with the caudal division degenerating before rostral division (Murray et al., 2008; Ruiz et al., 2010).

The amount of neurotransmitter released per fused synaptic vesicle (quantal size) seems not to be altered in motor SMA terminals, as shown by the similar size of the miniature end-plate currents in control and mutant mouse terminals (Table 1). Therefore, the reduction in QC (Kong et al., 2009; Ling et al., 2010; Ruiz et al., 2010) could be due to a decrease in release probability and/or in the number of docked vesicles. The vesicle release probability has been shown to be decreased in several SMA muscles (Table 1). In addition, a decrease in total and docked vesicles has been found using electron microscopy (Kong et al., 2009). The diameter and morphology of the vesicles seem to be not altered (Kong et al., 2009), whereas the vesicle distribution is not normal in comparison with WT because the vesicles, in SMA mouse model, are preferentially located in the periphery of the presynaptic terminal (Kariya et al., 2008). These data are indicative of synaptic pathology and suggest that the presynaptic motor compartment is especially vulnerable to the decrease of SMN levels.

Table 1 Morphological and functional synaptic alterations described in SMA mouse models in comparison to littermates controls

	Transversus Abdominis	Tibialis Anterior	Extensor Digitorum Longus	Gastrocnemius	Levator Auris Longus	Semitendinosus	Diaphragm
Quantal content	∼↓ 50% (6)	∼↓ 40% (3)	∼↓ 25% (5)		Not altered (rostral) (6) ∼↓ 50% (caudal) (6)	∼↑ 20% (#) (2)	
mEPP	↑Frequency (P7) ↑Amplitude (P14) (6)	Not altered (mEPC) (3)	↓ Frequency ↑ Amplitude (5)		Not altered (P7) (6)	Not altered (#) (2)	
Probability of release		↓ (3)	↓ (5)		↓ (6)		
RRP size			Not altered (5)				
Synaptic vesicles		∼↓ 56 % (total) (3) ∼ ↓ 32 % (docked) (3) ↓ (+) (1)		↓ (+) (1)			Not altered (2) ↓ (+) (1)
Presynaptic mitocondria		↓ density, but normal morphology (3)					↓ in size but not altered number (2)
Neurofilaments	Abnormal (P15) (6)	Abnormal (P14) (3) Abnormal (+) (1)	Abnormal (P14) (5) Abnormal (+) (1)	Abnormal (P5-14) (2) Abnormal (+) (1)	Abnormal (6)		
Postsynaptic Area	↓ (P6) (*) (4)	↓ (P5-13) (3) ↓ (+) (P30) (1)	↓ (+) (P30) (1)	↓ (P14) (2) ↓ (+) (P30) (1)	↓ (C2) (P14) (7)		

All data refer to $SMN^{\Delta 7}$ mouse model, except: (+) $Smn^{f7}/Smn^{\Delta 7}$, NSE-Cre^{+} mice; (*) $Smn^{-/-}$; SMN2 mice; (#) $SMN2^{+/+}$; $SMN1A2G^{+/-}$ mice; $Smn^{-/-}$ mice.
(1) Cifuentes-Diaz et al., 2002; (2) Kariya et al., 2008; (3) Kong et al., 2009; (4) Murray et al., 2008; (5) Ling et al., 2010; (6) Ruiz et al., 2010; (7) L. Tabares' lab, unpublished data.
mEPP: miniature endplate potential; mEPC: miniature endplate current; RRP: readily releasable pool; P: postnatal age (in days).

In the SMNΔ7 SMA mouse model, it has been reported that presynaptic mitochondria in the diaphragm are smaller than in wild-type littermates (Table 1), whereas no differences were found at the postsynaptic sites (Kariya et al., 2008). However, in the TA muscle from the same mouse model, a decrease in the number of mitochondria in the presynaptic terminals has been reported (Kong et al., 2009). In a neuronal cell model of SMA, decreased ATP levels and increased free radicals have been reported and attributed to mitochondrial dysfunction (Acsadi et al., 2009). Therefore, it is predictable that an impairment of mitochondrial function would increase the cytosolic Ca^{2+} load during intense stimulation in nerve terminals (Friel and Tsien, 1994). Although there is not direct proof of this in SMN-deficient terminals, in a particularly affected proximal muscle TVA an abnormal increase in the amount of Ca^{2+}-dependent asynchronous release during prolonged stimulation has been found, supporting the hypothesis of an altered intraterminal bulk Ca^{2+} concentration in highly altered synapses. Mitochondrial dysfunction has been proven in distinct motor neuron diseases, including ALS (Hervias et al., 2006; Nguyen et al., 2006), and Spinal and Bulbar Muscular Atrophy (Ranganathan et al., 2009). However, no studies of mitochondrial function in presynaptic terminals of SMA patients have been reported.

At the level of the postsynaptic site different morphological defects in the SMA mouse model have been demonstrated (Table 1). Although in wild-type mice the structure of neuromuscular synapses becomes more complex during postnatal development—ovoid AChR plaques are transformed into complex, pretzel-like shapes, characteristic of adult synapses (Sanes and Lichtman, 1999)—in SMA mutant mice the postsynaptic plaques do not fully progress to the mature form but remain small and oval-shaped with almost no gutters or perforations [Fig. 1(D, b)] (Kariya et al., 2008; Murray et al., 2008; Kong et al., 2009; Ruiz et al., 2010). Postsynaptic receptors remain in patches and postsynaptic membrane folds fail to form. In addition, the kinetics of postsynaptic responses remains slow in SMA mutant mice (Kong et al., 2009; Ruiz et al., 2010), while in the wild-type littermates it becomes faster probably following reorganization of pre- and postsynaptic elements towards a more efficient coupling. The molecular mechanisms underlying the maturation defects of the NMJ in SMA are, however, unknown.

Although in SMA mouse models no defect in axonal formation has been described, neurofilament accumulation in a high percent of terminal axons [Fig. 1(D, b)] has been found in almost all muscles affected by the disease (Table 1) (Cifuentes-Diaz et al., 2002; Kariya et al., 2008; Murray et al., 2008; Kong et al., 2009; Ruiz et al., 2010). It can be inferred that large neurofilament accumulation may participate in the motor neuron dysfunction and cause axonal degeneration by slowing down the transport of components required for axonal and synapse maturation and maintenance.

Molecular Mechanisms

SMN has been shown to form a complex with other proteins and transport mRNA for β-actin and different protein cargos along the axon (Rossoll et al., 2003; Glinka et al., 2010; Peter et al., 2011). Filamentous actin (F-actin) is an important cytoskeletal element in nerve terminals, which interact with different organelles and is crucial for localization of those at its site of action. For example, F-actin plays important roles in SV recycling (Shupliakov et al., 2002; Bloom et al., 2003). In addition, mitochondrial subcellular localization is achieved by stabilization of actin filaments through the RhoA/formins pathway (Minin et al., 2006).

Low levels of SMN may, consequently, disturb local translation of beta actin resulting in low levels of this protein in the terminal. In addition, as F-actin levels have been shown to be important in axonogenesis (growth, pathfinding, and branching), low levels of actin may explain the defective neurite length observed in SMN deficient motor neurons in culture.

Supporting the earlier hypothesis, recently, it has been reported that the overexpression of plastin 3, a protein that binds and bundles actin filaments and increases F-actin levels, counteracts the axonal growth defect in Smn-deficient mouse motor neurons in culture and in zebrafish (Oprea et al., 2008). Furthermore, in humans, unaffected *SMN1*-deleted females exhibit significantly higher expression of plastin 3 (Oprea et al., 2008). On the other hand, low levels of SMN in SMA mutant mice are accompanied by a decrease in plastin 3 (Bowerman et al., 2009). Based on all these results, it has been proposed that the gene that encodes plastin 3, *PLS3*, is a protective modifier for SMN deficiency. In addition, profilin IIa, another actin binding protein—but one that negatively regulates neurite sprouting by increasing actin stability (Da Silva et al., 2003)—is elevated in SMA models (Bowerman et al., 2007). The effect of profilin, together with other proteins such as cofilins, on actin is mediated by RhoA, a small GTPase, and by its kinase (ROCK), which phosphorylates actin (Da Silva et al., 2003). In SMA models, the RhoA/ROCK pathway is over-activated.

Summary and Future Questions

SMA is a severe neurodegenerative disease and the most common genetic cause of infant mortality. The disease is caused by the mutation of the *SMN1* gene in humans. Despite the need of SMN in all tissues, SMN is predominantly important for the neurodevelopment of the motor system, particularly in invertebrates and lower vertebrates SMA model organisms and for the maturation and maintenance of motor neuron terminals in higher vertebrates. Low levels of SMN in SMA mouse models cause abnormal synaptic organization and defective synaptic neurotransmission that lead to early neurodegeneration.

Although much has been discovered about SMA recently, many aspects regarding the regulation and function of SMN remain unknown. For example, at the molecular level, it would be enormously useful to identify all the modifier genes of *SMN*, to learn how the splicing of *SMN1* and *SMN2* can be regulated by different molecules, and to discern the precise interactions of SMN with other molecules in the spliceosome. Regarding the pathogenesis of the disease, still open questions are the special vulnerability of proximal lower motor neurons, the functional importance of SMN in axonal transport of different mRNAs and proteins implicated in synaptic organization and function, the role of SMN in synaptic maturation and maintenance, and the function of SMN in neurons other than motor neurons. Further biochemical, genetic, and functional studies are crucial to answer these basic questions, and will be of enormous value for designing new therapeutic approaches.

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