

TRANSGENIC MOUSE MODEL FOR SPINAL MUSCULAR ATROPHY: MORPHOLOGICAL CHARACTERIZATION AND GENE THERAPY APPROACH

PhD thesis of Paolo d'Errico



PhD Program in Translational and
Molecular Medicine (DIMET)- XXV cycle
Academic year 2011-2012

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*“La sapienza offre un riparo, come
l’offre il denaro; ma l’eccellenza della
scienza sta in questo, che la sapienza fa
vivere coloro che la posseggono.”
(Ecclesiaste 7:12)*

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CHAPTER 1

GENERAL INTRODUCTION

Spinal Muscular Atrophy (SMA) is a severe autosomal recessive disease characterized by degeneration of motor neurons. SMA is the leading genetic cause of infant death, with an incidence of 1:10000 live births and an estimated carrier frequency of 1:35 (*Pearn et al., 1980; Prior et al., 2009*). The symptoms of SMA reflect degeneration of spinal motor neurons and cranial motor nuclei, leading to a progressive muscle paralysis, respiratory failures and, in more severe cases, to death. The disease is caused by homozygous mutations of the Survival Motor Neuron 1 (*SMN1*) gene, located on chromosome 5q13, and encoding for an ubiquitous protein (full-length SMN or FL-SMN). In humans, centromeric copy of *SMN* gene (*SMN2*) generates mostly a protein lacking exon 7 ($\Delta 7$ -SMN) that is not functional and rapidly degraded (*Lorson et al., 1999; Monani et al., 1999, Gennarelli et al., 1995*). Despite the housekeeping function of SMN protein, reduced levels result in a predominantly motor neuron suffering; this aspect still remains the main issue to clarify. Currently SMA remains without efficacious and definitive cure.

Subtypes and Clinical aspects

Spinal muscular atrophy is clinically heterogeneous and its phenotype has been divided into three main types (I-III) based on the age of onset, severity of symptoms and development of motor milestones (*Munsat et al., 1992; Russman et al., 2007*).

SMA type I (Werdnig-Hoffmann disease) is the most severe form, with an onset before 6 months and death occurring within the first 2 years of life due to feeding and respiratory difficulties. Infants are hypotonic and typically have a symmetrical flaccid paralysis, a bed shaped chest and frog-leg posture.

SMA type II becomes apparent before 18 months and is a more slowly progressive disease with a variable prognosis. Children are able to sit independently, but are not able to stand or walk without artificial supports. Children will develop, if untreated, severe scoliosis and respiratory ventilation defect. The majority reaches the adult life, but life expectancy is highly variable.

SMA type III (Kugelberg-Welander disease) has symptomatic onset after 18 months of age and all patients are able to walk independently, but there is a marked heterogeneity in motor functions. Patients have a normal lifespan while the proximal muscle weakness is progressive.

Finally, in the adult form (**SMA type IV**) the symptomatic onset becomes apparent after age 5 years, and holds a full life expectancy.

The common clinical features of all SMA patients consist of symmetrical muscle weakness (more proximal than distal) associated with muscle atrophy. Muscle biopsy shows skeletal muscles

denervation with little or no sensory involvement and no marked decrease of motor nerve conduction velocities. Cognitive functions remain in a normal range.



Fig. 1. Children affected by different forms of SMA.

Genetic basis of SMA

SMN gene was identified and characterized by laboratory of J. Melki and A. Munnich in 1995 (*Lefebvre et al., 1995*). *SMN* gene is located on a 500 Kb duplicated region of chromosome 5q and is highly conserved in different species. However, only in humans, two forms of the *SMN* gene exist on each allele: a telomeric copy (*SMN1*) and a centromeric copy (*SMN2*) that originated as result of intra-chromosomal duplications during evolution (*Schmutz J., et al,2004*). The phenotype of SMA is due, in 96% of cases, to homozygous mutation of *SMN1* gene; the remaining 4% of cases are compound

heterozygotes, with a deletion on one allele and point mutations on the other (*Wirth B. et al., 2000*). *SMN2* differs from *SMN1* by only 5 nucleotides without any effect on the amino acids sequence. There is one nucleotide substitutions in intron 6, two in intron 7, one in exon 7 and one in the non-coding exon 8 (*Lorson et al., 1999*). *SMN* gene is highly ubiquitously expressed; its mRNA is about 1880 nucleotides long and is composed of 9 exons encoding a 38 kDa protein of 294 aminoacids (*Lefebvre et al., 1995*). The major product of *SMN1* gene is the functional “full-length” FL-SMN protein, while *SMN2* mostly produces a protein lacking exon 7 (SMN Δ 7), due to exon 7 skipping, that is unstable and rapidly degraded (*Lorson et al., 1998; Monani et al., 1999; Vitte et al., 2007*). Approximately, 10 % of SMN2 is translated into the FL-SMN protein through correct splicing and can modulate the severity of the phenotype as it can exist in multiple copies on a single chromosome and its number is inversely proportional to the SMA severity (*Vitali et al., 1999; Feldkotter et al., 2002*). *SMN2* copy number usually varies between one and four copies and the expression corresponding to five copies of *SMN2* gene may compensate the absence of the *SMN1* gene (*Prior et al., 2004*). The different splicing between these two genes is due to a Cytosine (C) to Thymidine (T) transition at position 6 of exon 7 in *SMN2* (Fig. 2). This nucleotide change disrupts an exon splicing enhancer (ESE) which is normally recognized by the splicing factor SF2/ASF, with the result that the splice site is not longer recognized and exon 7 is excluded (*Cartegni and Krainer, 2002*).

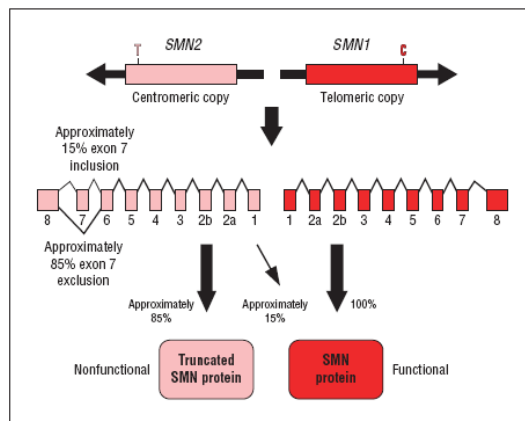


Fig. 2. Schematic of SMN gene. SMA patients have deletions in both copies of SMN1. However, the SMN2 is expressed and mostly produces a transcript lacking exon 7 because of a C to T transition at the beginning of exon 7. A small amount of FL-SMN protein is produced from SMN2 gene that may regulate the SMA gravity.

In addition, the same C to T nucleotide transition results in creation of an exon splicing silencer that binds the splicing repressor heterogeneous ribonuclear protein (hnRNP) A1, promoting the exon 7 exclusion in *SMN2* gene (Kashima *et al.*, 2007). Another *SMN2* base change, the A to G substitution at position 100 of intron 7, creates an intron splicing silencer (ISS) sequence that functions as an additional *SMN2* specific hnRNPA1 binding site that represses exon 7 inclusion *in vivo*. Mutations that disrupt, *in vitro*, the hnRNPA1 binding motif, prevent the binding of the protein increasing the frequency of exon 7 inclusion (Kashima & Manley, 2007). The truncated transcript lacking exon 7 encodes a putative shorter protein in which the last 16 amino acids of the FL- SMN protein are replaced by 4 amino acids encoded by exon 8.

Given to the relative homogeneity of the mutations in SMA patients, a rapid and sensitive molecular diagnosis is based on the homozygous deletion of *SMN1* gene. This involves a combined PCR, using specific primers for SMN exon 7, with restriction enzyme digestion assay of the SMN genes (*van der Stege et al., 1995; Rodrigues et al., 1995*). The number of *SMN1* copies may be determined with specific quantitative PCR assay, utilizing the single nucleotide difference in the exon 7 (*Feldkotter et al., 2002*).

SMN PROTEIN

Architecture of the SMN complex

SMN is an ubiquitous protein of 294 amino acids with a molecular weight of about 38 KDa. The SMN protein is found both in the cytoplasm and in the nucleus where it is concentrated in structures that are frequently found neighbouring or overlapping Cajal bodies (CBs), and hence called “Gemini of Cajal Bodies” or simply, gems (*Liu et al., 1996; Matera et al., 2003; Malatesta et al., 2004*). Cajal bodies, described for the first time in 1903, contain different molecules involved in transcription and in RNA processing. Double labeling experiments demonstrated that SMN and Cajal bodies co-localized depending on the cell type or tissue analyzed, but not in fetal tissues (*Liu et al., 1996; Young et al., 2000*), thus suggesting that gems and Cajal bodies are distinct nuclear structures, but with a functional relationship. Dreyfuss laboratory, in tandem with other groups,

demonstrated that SMN exist as an oligomer (*Pellizzoni et al., 1999; Lorson et al., 1998*) and it forms a large complex, of about 1 MDa, with a group of different proteins termed collectively as Gemin. The human SMN complex includes: Gemin2 (also called SMN-interacting protein-1 or SIP1) that links directly the N-terminal domain of SMN protein (*Liu et al., 1997; Ogawa et al., 2007*); Gemin3, with helicase function, links the SMN exon 7 (*Charroux et al., 1999*); Gemin4 (or Gip-1) interacts with SMN via Gemin3 (*Charroux et al., 2000*); Gemin5 (*Gubitz et al., 2001*), Gemin6 (*Pellizzoni et al., 2002*) linked to the complex via Gemin7 (*Baccon et al., 2002; Gubitz et al., 2004*); finally, Gemin8 interacts with Gemin4 and 7, whilst the latter recruits the Unr-interacting protein (UNRIP) and Gemin6 via direct interactions (*Carissimi et al., 2006; Otter et al., 2007*) (Fig. 3).

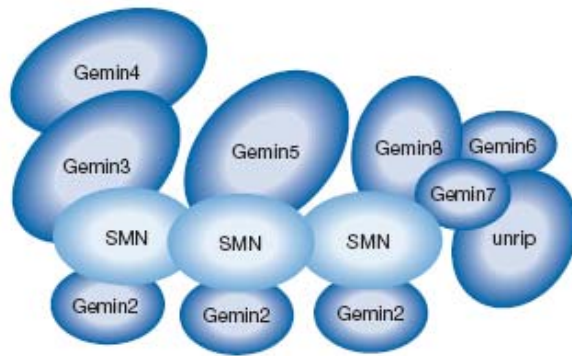


Fig. 3. Representation of SMN with other proteins with which it interacts in the SMN macromolecular complex.

It was observed that Gemin2 promotes the SMN oligomerization stabilizing the entire complex (*Young et al., 2000; Ogawa et al., 2007*). Gemin2-4 and 6-8 are core members of SMN complex, whilst Gemin5 and UNRIP are peripheral components and are rarely or detectable in gems, but both UNRIP and Gemin5 have been found to associate with the SMN complex only in the cytoplasm (*Grimmler et al., 2005; Hao et al., 2007*). Generation of SMN, Gemin2, 3 and 5 knock-outs results in lethality in a variety of organisms, thereby indicating that these components of SMN complex are essential for viability (*Briese et al., 2009; Shpargel et al., 2009; Kroiss et al., 2008*).

Role of SMN protein in the spliceosomal biogenesis

The SMN complex is implicated in the cytoplasmic assembly of the uridine-rich small nuclear ribonuclearproteins (UsnRNPs) into the spliceosome, a large RNA-protein complex that catalyzes the pre m-RNA splicing. Each snRNP is composed by a series of snRNAs molecules (U1, U2, U4, U5 and U6) associated with seven Sm proteins (termed B, D1-D3, E, F and G) and other Sm-like (Lsm) proteins. The Sm proteins bind the RNA and interact directly with SMN via arginine/glycine (RG) rich domains in the C-terminus (*Pellizzoni et al., 2001; Mourelatos et al., 2001*). Different experiments have shown that SMN complex is necessary to ensure a specific and stable interaction between the ‘ring’-shaped heptameric core made of Sm proteins on the short, single stranded, uridine-rich Sm site of Sm-class UsnRNAs (*Yong et al., 2002; Yong et al., 2004*)

(Fig.4). A methyltransferase complex consisting of the protein arginine-methyltransferase 5 (PRMT5), MEP50 and pICln recruit Sm proteins and enable their dimethylation on designated arginine residues by PRMT5, a modification that enhances the interaction between Sm proteins and SMN oligomers (*Chiari et al., 2009*).

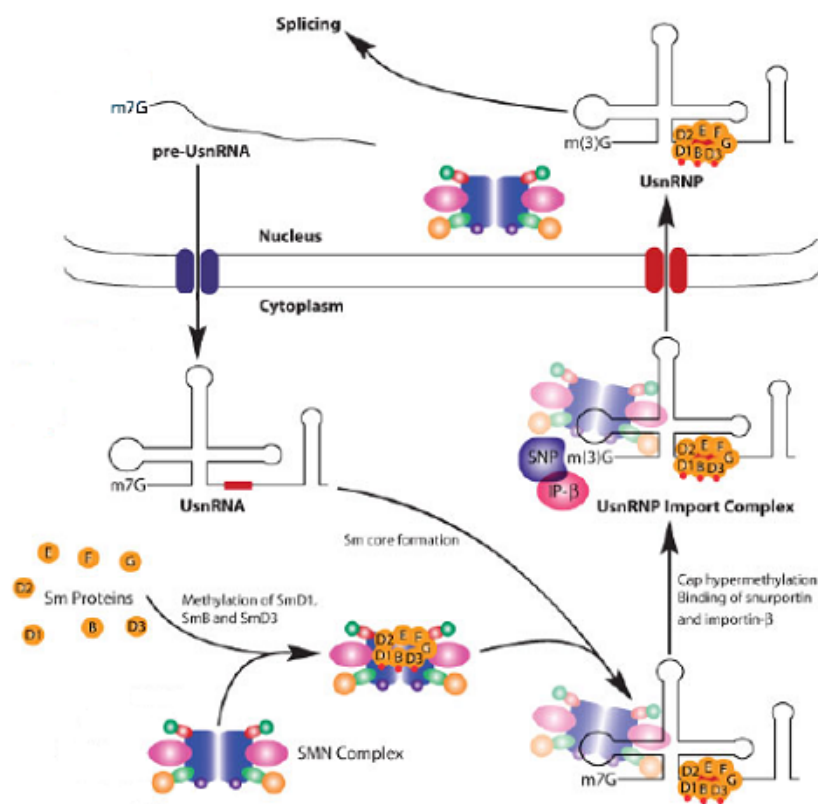


Fig. 4. Spliceosomal UsnRNP biogenesis pathway (*Briese et al., 2005*).

A correct Sm-core formation and the 5'-methylation (capping) of U snRNAs are necessary for the subsequent translocation of mature

snRNPs into the nucleus where they exert their splicing function (Fisher & Luchrmann, 1990; Will & Luhrmann, 2001).

The precise contribution of each member of the SMN complex is still a work in progress. Gemin5 was identified as the factor that allows the SMN complex to specifically recognize UsnRNAs *via* its WD repeats (Battle *et al.*, 2006; Lau *et al.*, 2009). UNRIP, another component with WD-repeats, might be important for the exchange of the Gemin6-Gemin7 heterodimer by the SmD3-SmB particle, which should ensure Sm ring closure within the SMN complex in preparation for the uploading on UsnRNAs (Ogawa *et al.*, 2009). The role of the other SMN component are still under analysis. SMN exon 3 contains a 53 amino acids sequence (aa 90-160) called Tudor domain (Ponting *et al.*, 1997) that plays an important role in assembling UsnRNPs (Buhler *et al.*, 1999). The first missense mutation identified in this domain is the E134K transition, found in SMA type I patients, that severely reduces the interaction between SMN and Sm proteins (Cuscò *et al.*, 2004). Another mutation was found, in two Japanese SMA I patients, in Tudor domain (W92S) that impairs the interaction between SMN and SmB (Kotani *et al.*, 2007). Different mutations accumulate in *SMN* gene, in particular the exons 3 and 6 represent ‘hotspots’ for mutations (Fig. 5).

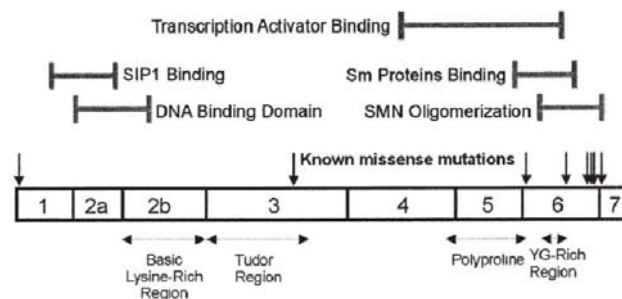


Fig. 5. Schematic representation of the SMN protein with its binding sites for different interacting proteins. The arrows indicate points of major mutations.

Role of SMN protein in the nucleus

In the nucleus, the SMN protein has other two important housekeeping functions: a direct involvement in pre-mRNAs splicing and a role in regulation of gene expression. The involvement of SMN protein in the splicing was first studied by Dreyfuss group, which has observed a splicing inhibition *in vitro* after co-incubation of nuclear extracts from HeLa cells with antibodies anti-SMN (Pellizzoni *et al.*, 1998). The exact mechanism by which SMN exerts this function is not clear, but an hypothesis is that SMN protein may modulate composition of the Sm and UsnRNPs proteins, previously assembled in the cytoplasm. Furthermore, SMN interacts, through a region of 16 amino acids in the C-terminal tail, with a family of heterogeneous nuclear ribonucleoproteins-Q (hnRNP Q) stably associated with pre-mRNAs (Mourelatos *et al.*, 2001). A possible role of SMN protein in gene regulation was hypothesized by observing its interaction with other proteins directly involved in this process such as RNA helicase A (RHA), RNA polymerase II (RNAPII) and some repressors of transcription (Pellizzoni *et al.*, 2001; Yan *et al.*, 2003).

Role of SMN protein in motor neuron axons

Motor neuron degeneration and muscle atrophy are widely accepted hallmarks of human SMA. However, the cellular site of action of SMN protein and how this contributes to SMA pathology is unclear.

Two hypotheses that could explain the particular vulnerability on motor neurons are postulated. In one of them, disturbed RNA processing specially relevant for motor neurons is proposed (*Pellizzoni et al., 2002; Gabanella et al., 2007; 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 snRNPs biogenesis. In the nervous system development, SMN changes its cellular localization and accumulates in axons *in vivo* (*Pagliardini et al., 2000*), and at branch points and growth cones in cultured motor neurons (*Jablonka et al., 2001*). SMN co-localizes with hnRNP-R, a protein involved in mRNAs transport in particular in motor neuron axons, thus suggesting a possible role of SMN in transport of specific mRNAs along the axon (*Rossol et al., 2002*). In particular, different *in vitro* experiments have shown that SMN interacts with actin-binding proteins and promotes the transport of mRNA for β -actin (*Rossol et al., 2003; 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 their localization at its site of action. Low levels of SMN may disturb local translation of β -actin resulting in low levels of this protein in the terminals. In addition, as F-actin levels have been shown to be important in axonogenesis, low levels of actin may explain the defective neurite length observed in

SMN deficient motor neurons cultures. Supporting this hypothesis it has been reported that the over-expression of plastin 3, a protein that binds actin filaments and increases F-actin levels, counteracts the axon growth defect in Smn-deficient mouse motor neurons in culture and in zebrafish (*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*). For this results, plastin 3 has been proposed as a genetic modifier for SMA. In addition, profilin IIa, another actin-binding protein, that negatively regulates neurite sprouting by increasing actin stability, is elevated in SMA models (*Bowerman et al., 2007*). Profilin IIa binds to the arginine- rich motifs in exon 5 of SMN protein (*Giesemann et al., 1999*) and promotes the F-actin polymerization sequestering free monomers. All these data suggest the possibility that SMN loss of function in axons contributes to the pathophysiology of SMA. According to this view, the SMN function specifically impaired in SMA is the association with a subset of axonal mRNAs and proteins pivotal for axon growth, transport, and maintenance in motor neurons.

Further evidence for a specific role of SMN in axons: the a-SMN protein

A new transcript of the *SMN* gene was recently identified and characterized by our laboratory (*Setola et al., 2007*). This new transcript originates from the retention of SMN intron 3 and codifies for a shorter protein termed axonal-SMN (a-SMN) because its

expression is largely restricted to motor neurons with further sub-cellular localization in axons. A premature stop-codon in retained intron 3 of a-SMN transcript leads to the synthesis of a shorter protein of about 19 KDa in mouse or human, and 22 KDa in rats that diverges from FL-SMN only for C-terminal tail. *In vivo*, a-SMN protein is highly expressed at embryonic stage and its expression is progressively reduced after birth, thus suggesting a possible involvement of a-SMN protein in motor neuron development during embryogenesis. Forced expression of human a-SMN in neuronal immortalized cell lines (NSC34) stimulates neurite extension in a time-dependent fashion (Fig. 6) and the over-expression in non-neuronal cell lines such as HeLa induces formation of filopodia containing actin filaments that colocalize with a-SMN protein (Fig. 7). This effects is not found in cells overexpressing FL-SMN.

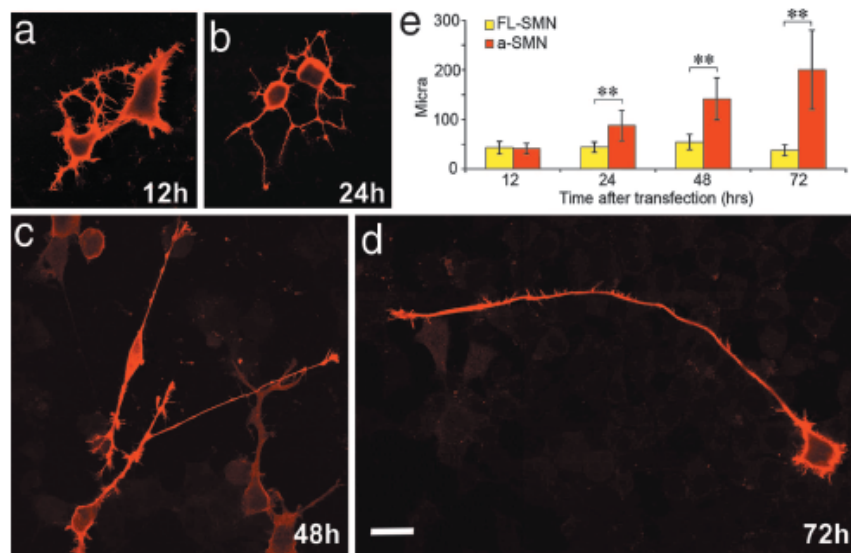


Fig. 6. Time course of human *a*-SMN overexpression. (a–d) Confocal images with an anti-tag antibody at 12 (a), 24 (b), 48 (c), and 72 h (d) after transfection. (e) Mean axon length at different time points in *a*-SMN and FL-SMN transfected motor neurons (Setola et al., 2007).

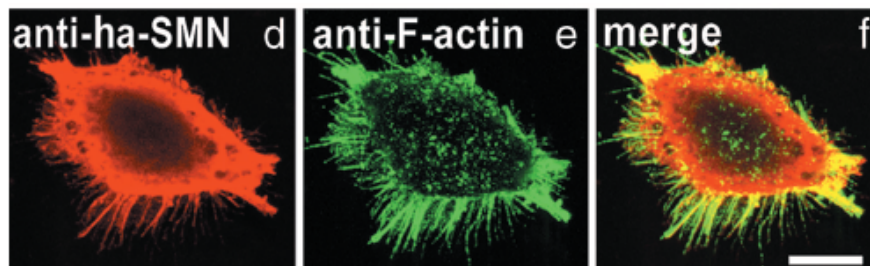


Fig. 7. Confocal images showing human *a*-SMN (d) (#910 anti-SMN) and F-actin (e) colocalization (f) in newly formed filopodia of HeLa cells transfected with ha-SMN (Setola et al., 2007).

Transfection experiments with different N-terminally tagged constructs revealed that exon 1/2a is important for axonal localization, and the remaining C-terminus is essential for axonogenesis. Retention of intron 3 provides the stop codon necessary to produce the an axonogenic polypeptide truncated at the exon 3/exon 4 junction. All this indicates that *a*-SMN plays an important role in motor neuron homeostasis and that its deficiency may be correlated with SMA pathogenesis.

SMA mouse models

Animal models are an important tool to clarify different aspects of pathogenesis and mechanisms characterizing a human disease. Since the *SMN* genes were identified, different animal models for SMA were generated, however mouse remains the most used animal model to study this disorder. Mice possess a single *Smn* gene, which has 82% amino acids identity with its human homolog and a similar expression pattern (Di Donato et al., 1997). Homozygous *Smn* knockouts provided the first direct evidence that the SMN protein is indispensable for cell survival because the complete *Smn* deletion (*Smn* $-/-$) results in massive embryonic cell death before implantation (Schrack et al., 1997) and therefore, not providing a useful model of SMA. In early 2000, two approaches were used to generate viable model mice. Frugier group selectively inactivated murine *Smn* in nerves using a conditional (*Smn*^{F7}) allele from which exon 7 is deleted (Frugier et al., 2000). The resulting neuronal mutants showed a decreased number of nuclear Gems and suffered motor neuron loss, muscle atrophy and died of neuromuscular disease at around postnatal day 25 (P25). Conditional mice with ablation of *Smn* only in muscle tissue displayed a prominent dystrophic phenotype, which could be modulated if SMN was knocked out in muscle satellite cells rather than in differentiated myofibers (Cifuentes-Diaz et al., 2001; Nicole et al., 2003). The complete absence of functional *Smn*, which never occurs in patients, suggests that these models provide only limited insights into the pathogenesis of SMA. To mimic the genetics of human SMA more accurately, two groups introduced the entire human

SMN2 gene into animals lacking murine *Smn* (Monani *et al.*, 2000; Hsieh-Li *et al.*, 2000). One to two copies of the *SMN2* transgene in *SMN2*; *Smn*^{-/-} mice rescue embryonic lethality. Newborn mutants are indistinguishable from control littermates, but by P2 begin to develop a clear phenotype characterized to inability to suckle, reduced size and progressive weakness. Approximately 40% of motor neurons are loss by 3 days of age, and their lifespan is about 4-6 days (Monani *et al.*, 2000). Eight copies of *SMN2* transgene were found not only to rescue embryonic lethality, but also to completely ameliorate the SMA phenotype, supplying the proof of concept study of the feasibility of modulating the *SMN2* gene for therapeutic purposes. Introduction of a second transgene, containing human *SMNΔ7* cDNA, into the severe model extends the lifespan from 6 to 13 days (Fig. 8) (Le *et al.*, 2005). Initially generated to assess potential *SMNΔ7* toxicity (Kerr *et al.*, 2007), this model (*SMN2*^{+/+}; *SMNΔ7*^{+/+}; *Smn*^{-/-}) is thought to be milder owing to the formation of heterologous complexes between FL-SMN and *SMNΔ7*, which might stabilize SMN and affect its turnover.

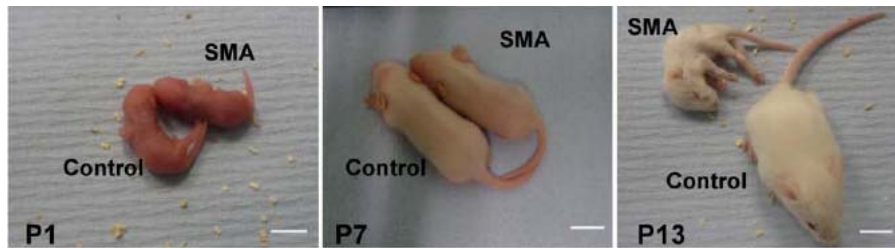


Fig. 8. Transgenic *SMAΔ7* (*SMN2*^{+/+}; *SMNΔ7*^{+/+}; *Smn*^{-/-}) mouse model; at birth (P1), the KO mice are indistinguishable from control littermates, but starting from P7 show the first signs of muscular weakness. At final stage of the disease (around P13), affected mice are visibly smaller compared to controls and are unable to right themselves.

A number of other transgenes containing point mutations in *SMN1* (A2G, A111G) have also been introduced into the severe model, resulting in increased lifespan (*Monani et al., 2003; Workman et al., 2009*). However, unlike *SMN2*, which generates a small amount of full-length protein, none of the other transgenes rescue the embryonic lethality caused by *Smn* deletion, indicating that FL-SMN is an absolute requirement for normal cellular function. Using a slightly different approach, a knock-in allele that disturbs endogenous *Smn* (*Smn2^{B/-}*), similar to human *SMN2*, was shown to result in more moderate severity, with a few animals living longer than 1 month (*Bowerman et al., 2009*).

SMA pathogenesis

The main pathological feature of SMA, present in all human patients, is a specific loss of lower motor neurons. Analysis of mouse models provided clear support for the notion that motor neurons are particularly vulnerable to low SMN levels. However, it is hypothesized that loss of motor neuron cell soma from the spinal cord occurred subsequent to the development of neuromuscular pathology, specifically defects at the neuromuscular junction (NMJ). NMJ pathology has been reported in human SMA patients (*Swodoba et al., 2005*) and across a variety of animal models. Prenatally, the neuromuscular system develops normally in SMA mice, prior to the

postnatal onset of disease. Early structural pathology at the NMJ occurs at both the pre-synaptic nerve terminal and in post-synaptic muscle fibers, and is characterized by significant nerve terminal loss, synaptic accumulation of neurofilament proteins, and decreased muscle fiber size. Although it is currently unclear how low levels of SMN cause these defects at the NMJ, the available data indicate that these pathologies may be a consequence of failed synaptic maintenance due to impaired axonogenesis.

Although SMA has traditionally been classified as a pure disease of lower motor neurons, it is becoming increasingly clear that additional cell and tissue types are vulnerable to reduced levels of SMN. The emerging evidence therefore challenges the idea that SMA is solely a disease of the neuromuscular system and suggests that it may be a multi-system disorder. One significant piece of evidence supporting this hypothesis came from a recent study by Park and colleagues (*Park et al., 2010*). These authors observed that the selective depletion of SMN in mouse motor neurons led to a dystrophic phenotype rather than full blown SMA, thereby demonstrating that SMN loss in cells and tissues other than motor neurons is required to generate SMA disease pathogenesis (*Park et al., 2010*).

Motor neuron survival and skeletal muscle development are closely linked and depend upon continued cellular contact between these two cell types; therefore, neuronal dysfunction can contribute significantly to muscle atrophy. However, it is likely that intrinsic abnormalities in SMA skeletal muscle cells also contribute directly to disease pathogenesis. Several different strategies using animal models of

SMA also support the notion that SMN is crucial to the normal function of muscle (*Rajendra et al., 2007; Gavrilina et al., 2008; Martinez et al., 2012*) and, in some cases, survival (*Chang et al., 2008*). Moreover, defects in muscle differentiation have been reported in a mouse model of severe SMA (*Hayhurst et al., 2012*). Temporal analysis of a mouse model of severe SMA demonstrated that pathological changes in muscle (including increased levels of cell death) occurred independently of nerve degeneration (*Murray et al., 2008*), confirming that SMN has cell-autonomous roles in muscle.

Despite clear evidence supporting a direct role for SMN in skeletal muscle, the extent to which intrinsic skeletal muscle pathology contributes to overall SMA pathogenesis remains unclear. Restoring SMN in skeletal muscle alone is insufficient to correct disease pathology in SMA mice (*Gavrilina et al., 2008; Martinez et al., 2012*).

Disrupted sensory pathways have been also described in severe SMA patients (*Rudnik-Schoneborn et al., 2003*) and in a number of mouse models (*Gogliotti et al., 2012; Martinez et al., 2012; Mentis et al., 2011*). Here, the disruption of functional connectivity between sensory and motor neurons in the spinal cord occurred prior to motor neuron cell loss. In addition, selective vulnerability was demonstrated, with synapses onto motor neurons innervating proximal muscles affected prior to those located more distally (*Mentis et al., 2011*). However, other studies have suggested that these defects may occur as a secondary consequence of intrinsic motor neuron pathology (*Gogliotti et al., 2012*), given that sensory synapse loss is prevented by

selectively increasing SMN levels in motor neurons (*Gogliotti et al., 2012*). Low levels of SMN have been shown to affect other neuronal populations in the brain, particularly those that normally have a high basal level of SMN expression (*Wishart et al., 2010*). For example, low levels of SMN disrupt cellular proliferation and impaired neurogenesis, leading to abnormal development of the hippocampus in a mouse model of severe SMA (*Wishart et al., 2010*). This indicates a potentially important role for SMN in regulating brain development, and evidence from several studies of human SMA patients suggests that pathological changes in the brain (particularly the thalamus) may be a relatively common event at the severe end of the disease spectrum (*Ito et al., 2004; Shishikura et al., 1983*). Treatments that target the entire nervous system may therefore be required to treat all symptoms of SMA.

Alongside evidence revealing that cells other than motor neurons contribute to SMA disease pathogenesis, improved clinical diagnostic tools for human patients and the development of new transgenic animal models has increased the reporting of pathology in cells and tissues beyond the neuromuscular system. Cardiac arrhythmias and abnormalities are one of the most common peripheral phenotypes reported in patients with severe SMA (*Rudnik-Schoneborn et al., 2008*) and severe SMA mouse models (*Gogliotti et al., 2012; Bevan et al., 2010; Heier et al., 2010*). In a model of severe SMA, mice developed bradycardia and dilated cardiomyopathy; however, in a significant finding, these cardiac defects improved following treatment with therapeutics that increased the levels of SMN (*Bevan et*

al., 2010; *Heier et al.*, 2010). Structural defects in the heart precede motor neuron dysfunction (*Shababi et al.*, 2010) and can exist independently of motor unit pathology and muscle paralysis (*Gogliotti et al.*, 2012). Overall, these observations indicate a vital role for SMN in cardiac development and function (*Shababi et al.*, 2012), suggesting that targeting the heart (either directly or indirectly) will be required to fully ameliorate SMA symptoms. Vascular defects, such as distal digital necrosis, are commonly reported in severe SMA patients, particularly when survival has been prolonged with the aid of invasive ventilation (*Araujo Ade et al.*, 2009; *Rudnik-Shonrborn et al.*, 2010). Necrosis has also been reported in the tail and ear pinnae of both mild (*Hsieh-Li et al.*, 2000) and severe SMA mice with prolonged survival due to drug treatment (*Foust et al.*, 2010; *Valori et al.*, 2010). The underlying cause of necrosis is likely to be downstream effects of SMN deficiency on the vasculature itself, as dramatic differences in muscle capillary phenotypes, independent of neuromuscular pathology, have been reported in severe SMA mice (*Schreml et al.*, 2012; *Somers et al.*, 2012). Thus, the cardiovascular system as a whole appears to be an important pathological target in SMA.

Metabolic abnormalities have been reported in SMA patients and mouse models, suggesting that the pancreas is vulnerable to low levels of SMN (*Bowerman et al.*, 2012). Severe SMA patients have been shown to be mildly hyperglycemic with the pancreas having a disorganized cellular structure, whereas an intermediate SMA mouse model has been shown to be glucose intolerant, hyperglucagonemic,

hyperglycemic, insulin sensitive, and to have pancreatic development defects (*Bowerman et al., 2012*).

Perhaps most strikingly, low levels of SMN have recently been shown to have significant effects on the liver, implicating this organ in disease pathogenesis. Liver-specific deletion of SMN leads to defects in embryonic liver development and iron overload (*Vitte et al., 2004*), indicating a crucial role for SMN in the liver. Indeed, results from a significant new study support this hypothesis (*Hua et al., 2011*). SMA mice are small in size, indicating that their growth may be retarded, and liver-derived insulin-like growth factor 1 (IGF-1) is known to have a role in supporting both normal postnatal growth and cardiac development and function. Indeed, significantly low levels of IGF-1 were reported in a severe mouse model of SMA (*Hua et al., 2011*), and further investigation revealed that the low levels of IGF-1 observed in these SMA mice were the consequence of decreased expression of IGF-binding protein acid labile subunit (IGFALS), a hepatic protein that stabilizes IGF-1. Reduced gene expression levels of *Igfals* were shown to precede the development of neuromuscular defects and, crucially, this phenotype could be reversed by increasing levels of full-length SMN (*Hua et al., 2011*).

There is now strong evidence that multisystem phenotypes result from low levels of SMN in SMA, with cells and tissues throughout the body demonstrating a broad spectrum of vulnerability.

Gene therapy approach

Currently there is no cure for SMA, but several approaches were developed in order to ameliorate the SMA phenotype and prolong the survival. All these approaches aim to increase levels of SMN protein in tissues stimulating the transcription of the *SMN2* gene, preventing the exon 7 skipping or stabilizing the SMN protein. One of the first drug therapies to be trialed was a class of histone deacetylase inhibitor (HDAC-Inhibitors) that increase SMN levels by driving endogenous *SMN2* promoter activity; however, they are not gene-specific and have likely detrimental off-target effects. Nonetheless, SMA mice treated with some of these drugs demonstrated increased survival and improved motor performance (Avila *et al.*, 2007). In mouse models, the best reported median survival following treatment is 38 days compared with 13 days for untreated mice, by using trichostatin A in combination with nutritional supplements (Narver *et al.*, 2008).

A more direct therapeutic approach to SMA therapy is to correct the fundamental cause of the disorder by replacing the missing *SMN1* gene. In the last three years important results were obtained by using adeno-associated viral vectors (AAV) to delivery *SMN1* gene in affected mice. First evidences of an efficacious gene therapy approach were obtained by Azzouz *et al.*, who restored SMN levels in motor neurons after multiple injections in muscle with by using lentiviral vectors (Azzouz *et al.*, 2004). These first gene therapy experiments have shown a reduction of motor neuron loss and an increased life

expectancy of about 30% in treated mice. Current approaches have used adeno-associated viruses to augment SMN levels in SMA models. Self-complementary AAV serotype 9 (scAAV9) vectors have been used most extensively as they show early gene expression, efficacious transduction of motor neurons and astrocytes, and efficiently cross the blood-brain barrier (BBB) after IV/ICV (intravenous; intra-cerebroventricular) injections (*Passini et al., 2010; Foust et al., 2010; Valori et al., 2010*). scAAV9-mediated SMN replacement successfully extended survival, improved weight, and ameliorated neuromuscular phenotype in SMA mouse models. In addition, following treatment there were improvements in early heart defects (*Bevan et al., 2010; Shababi et al., 2012*) and muscle fibers size (*Passini et al., 2010; Glascock et al., 2012a and b*). The most successful gene therapy trial has demonstrated a greater than 50% survival at 250 days following pre-symptomatic systemic delivery, compared with a survival of about 13 days (*Foust et al., 2010*).

Interestingly, survival rates and the extent of phenotypic rescue differ greatly between individual animals within a single study, as well as between different studies, using both similar and distinct approaches. An important caveat to this approach is that injections done in the mice have their maximal effect on postnatal day 1; the effect falls rapidly with advancing age so that injections on day 5 had only a partial effect, while injections on day 10 had no effect. This observation suggests that there may be a critical period in development during which sufficient SMN protein is essential for the health of motor neurons. Initial studies used IV administration, but more recent

reports have shown that ICV administration of the same amount of viral particles is more efficient in rescuing the phenotype of diverse SMA model mice than the IV route (*Glascocock et al, 2011; Glascocock et al, 2012*). Complementation of FL-SMN deficiency does not fully restore the clinical phenotype. In fact, in successfully treated mice was not observed the body weight restoring observed in healthy littermate and frequently present tail and ear necrosis, eyelid inflammation and distal necrosis of the limbs: these defects can be so severe as to require sacrifice. In addition, treated mice can die suddenly without evidence of motor impairment of a sufficient degree to explain death. For these reasons a more detailed comparisons of peripheral versus central delivery routes are therefore required, incorporating analysis of a wide range of cells and tissues, both within and beyond the neuromuscular system.

CHAPTER 2

SCOPE OF THE THESIS

Chapter 3

To provide on one side evidence that a-SMN is involved in the pathogenesis of SMA, and, on the other, to investigate the cellular and molecular mechanisms underlying the a-SMN-induced axon growth, we have verified the involvement of a-SMN in SMA by analyzing the effect of SMA-derived *SMN1* mutations on the axonogenic properties of a-SMN. A number of intragenic mutations are distributed in the 5' portion of the *SMN1* gene, thus altering the structure of the a-SMN transcript and protein. We have verified whether *SMN1* mutations (A2G; A111G; E134K) may alter the axonogenic properties of a-SMN in our over-expression system by confocal immunofluorescence experiments and morphometric analysis. Modifications of different cell parameters induced by the different a-SMN constructs were quantified and statistically analyzed, with particular attention to axonal arborization and growth.

Chapter 4

To define the a-SMN role in motor neuron homeostasis and to study the axonogenic properties of a-SMN, we generated a cell model with tetracycline-dependent conditional expression of the protein in motor neuronal NSC34 cells. In basal conditions, NSC34 express the FL-SMN protein, whereas it does not express detectable a-SMN at the western blot

level. The selected cell lines were used to evaluate the axonogenic phenotype by morphometric analysis at the confocal immunofluorescence level with antibodies recognizing a-SMN and established axonal markers. Moreover, these cell clones were used for whole-genome gene-expression-microarray experiments to establish whether a-SMN induction by TET causes significant and consistent changes in the activity of selected motoneuronal intracellular pathways.

Chapter 5

We characterized at morphological level, spinal cord and brain of transgenic SMA Δ 7 mouse model, mimicking SMA type II, in order to better define which neuron populations are more vulnerable to SMA progression during development. These analysis will allow to have a model to study the cellular effects after the over-expression of FL-SMN and a-SMN proteins by using adeno-associated viral vectors.

Chapter 6

We administered scAAV9/FL-SMN or scAAV9/a-SMN in SMA Δ 7 mice, with IV (intravascular) and ICV (intracerebroventricular) injections in order to study the biological effect of over-expressed- FL-SMN and a-SMN *in vivo*. We first analyzed the protein expression profile in different tissues and then the therapeutic effect on SMA phenotype. We will explore whether a-SMN, alone or synergistically with FL-SMN, has a therapeutic value in SMA mice or can modulate the motoneuronal fate.

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CHAPTER 3

SMA pathogenic mutations impair the axonogenic properties of axonal-SMN (a-SMN)

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Journal of Neurochemistry, 2012, vol 121, pag 465 - 474

ABSTRACT

The axonal SMN (a-SMN) protein is a truncated isoform of *SMN1*, the spinal muscular atrophy (SMA) disease gene. a-SMN is selectively localized in axons and endowed with remarkable axonogenic properties. At present, the role of a-SMN in SMA is unknown. As a first step to verify a link between a-SMN and SMA, we investigated by means of over-expression experiments in NSC34 motor neurons whether SMA pathogenic mutations located in the N-terminal part of the protein affected a-SMN function. We demonstrated here that either *SMN1* missense mutations or small intragenic re-arrangements located in the Tudor domain consistently altered the a-SMN capability of inducing axonal elongation *in vitro*. Mutated human a-SMN proteins determined in almost all NSC34 motor neurons the growth of short axons with prominent morphologic abnormalities. Our data indicate that the Tudor domain is critical in dictating a-SMN function possibly because it is an association domain for proteins involved in axon growth. They also indicate that Tudor domain mutations are functionally relevant not only for FL-SMN but also for a-SMN, raising the possibility that also a-SMN loss of function may contribute to the pathogenic steps leading to SMA.

INTRODUCTION

Autosomal recessive spinal muscular atrophy or SMA is the leading genetic cause of infant mortality, with an incidence of 1:10.000 live births and an estimated carrier frequency of 1:31 (Prior et al. 2010). It is due to motor neuron loss leading to progressive amyotrophic paralysis, respiratory failure and eventually death in the more severe cases. SMA is classified into three main clinical types (I-III), in relation to the age of onset and disease severity. All clinical types of SMA are mainly caused by homozygous deletion of the Survival of Motor Neuron gene *SMN1* (Hahnen et al. 1995; Lefebvre et al. 1995; Rodrigues et al. 1995). However, a minority of SMA patients (about 4%) are compound heterozygous with one deleted *SMN1* allele and the other *SMN1* allele carrying a subtle mutation (Lefebvre et al. 1998; Parsons et al. 1998).

The major product of the *SMN1* gene is the “full-length” FL-SMN protein, a well-established assembly factor for small nucleolar ribonucleoprotein particles (snoRNPs) and small nuclear RNPs (snRNPs) that plays a key role in spliceosomal biogenesis and mRNA splicing (Pellizzoni et al. 1998; Zhang et al. 2008). However, SMN is also specifically localized in motor neuron axons in vivo (Pagliardini et al. 2000), and recent papers have indicated that SMN plays a role in motor axon development, which may be independent from that played in snRNPs biogenesis (Rossol et al. 2002; Zhang et al. 2006; Shafey et al. 2008). In line with this hypothesis, a novel product of the *SMN* gene, the axonal SMN or a-SMN protein, has been recently identified

(Setola et al. 2007; Gunadi et al. 2008). a-SMN is a truncated, alternatively spliced isoform of the *SMN1* gene, originating from the retention of intron3. Like FL-SMN, a-SMN is preferentially encoded by the *SMN1* gene (Setola et al. 2007). At variance with FL-SMN, a-SMN does not localize in nuclear gems, but it is more selectively localized in axons and endowed with remarkable axonogenic properties (Setola et al. 2007).

SMN1 mutations have been already used in *in vitro* and *in vivo* assays to verify what functional alteration of the FL-SMN protein could be linked to SMA pathogenesis. Previous data have shown that SMA derived mutations impair functional properties of the FL-SMN protein both related to splicing, such as the formation of gems and the binding to the Sm class of snRNPs or to gemin proteins, and to axon growth, like the ability in rescuing motor axon defects in *Smn* deficient zebrafish motor neurons or in stimulating axon growth in cultured cells (Mohaghegh et al. 1999; Jones et al. 2001; Lefebvre et al. 2002; Sun et al. 2005; Carrel et al. 2006; Kotani et al. 2007; Nölle et al. 2011).

Some SMA pathogenic mutations are localized in the 5' portion of the *SMN1* gene (Sun et al. 2005) encoding the N-terminal part of the protein, particularly the Tudor domain, and they therefore alter the structure of the a-SMN transcript and protein. We have selected five mutations (W92S, A111G, E134K, 133-134 4bp del, and 147-148 5bp del) described in type I patients (Brahe et al. 1996; Clermont et al. 1997; Mohaghegh et al. 1999; Cuscò et al. 2003, Sun et al. 2005; Kotani et al. 2007). In the present paper, by means of over-expression

experiments in immortalized motor neurons, we have verified whether the selected pathogenic mutations of the *SMN1* gene could alter the functional properties of a-SMN in stimulating axon growth. Our data demonstrated that all mutated a-SMN proteins displayed altered axonogenic function, thus representing the first indication possibly linking a-SMN loss of function to SMA pathogenesis.

MATERIALS AND METHODS

Cultured NSC34 motor neurons. NSC34 are hybrid cell lines obtained by fusing a mouse neuroblastoma cell line with embryonic, motoneuron-enriched spinal cord cells (Cashman et al. 1992). NSC34 motor neurons were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Rockville, MD, USA) supplemented with 5% fetal bovine serum, 1mM glutamine and antibiotics (penicillin G K-salt, 100UI/ml and streptomycin sulphate, 100 µg/ml) and grown at 37°C in a humidified atmosphere (5% CO₂ - 95% air) in 25 cm² flasks (Corning, Cambridge, MA, USA). The culture medium was replaced every 2-3 days. Every week cells were detached from the plates by mechanical dissociation in culture medium, and then replated at a density of 5 x 10⁴ cells/flask.

Plasmid generation and cell transfection. In all experiments we used expression vectors containing the wt human a-SMN (ha-SMN) sequence encompassing exon1 to the entire length of intron 3 (Setola et al, 2007). To produce mutated ha-SMN constructs, mutations were

inserted by using the Quick Change Site directed mutagenesis kit (Stratagene, La Jolla, CA, USA) following manufacture's instruction. PCR amplified and gel purified fragments for wt and mutated ha-SMN were in frame cloned in the pcDNA4/HisMaxTOPO expression vector (Invitrogen, Carlsbad, CA, USA). All clones were fully sequenced. The pEGFP vector (Clontech, Mountain View, CA, USA) was used as a control. Transfection was performed with Lipofectamine 2000 (Invitrogen) by standard procedures, the efficiency of transfection was around 15% of total cells. For western blot analysis, NSC34 cells were washed twice in ice-cold phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} ions, detached with a cell scraper in 2 ml of the same buffer, and centrifuged at 1.000g for 5 min. For immunofluorescence analysis, cells were fixed after 24, or 48, or 72 h post-transfection with 4% paraformaldehyde in 4% sucrose in phosphate buffer pH. 7.2 (PB).

Antibodies. The anti-peptide polyclonal antibody directed against the C-terminal region of the human a-SMN (#910) was prepared in rabbits by NeoMPS (Strasbourg, France) and used at 1:1.000 dilution in all experiments. Mouse anti-SMN (clone 8) was purchased from BD Biosciences (Franklin Lakes, NJ, USA; diluted 1:25.000); mouse anti-tag (anti-Xpress) from Invitrogen (diluted 1:500); mouse anti-neurofilament 200 (NF-200) and mouse anti-Tubulin from Sigma-Aldrich (St. Louis, MO, USA; diluted 1:500); anti-peptide polyclonal paraplegin, diluted 1:500 (residues 89–304, kindly provided by Dr. Franco Taroni; Di Bella et al. 2010).

Western blot analysis. Transfected NSC34 cells were lysed in buffer containing 0.1M Na-phosphate (pH 7.4), 0.2% Triton X-100, 0.1mM EDTA, 0.2mM PMSF, 1mg/ml aprotinin and 1mg/ml leupeptin by three-repeated freezing and thawing cycles. The resulting cell lysates were centrifuged at 13.000 g. Proteins were separated by SDS-PAGE (12% acrylamide) and electro-blotted on nitrocellulose paper for 1h at 180mA. The nitrocellulose was blocked overnight with 10% no-fat milk in Tris buffered saline (TBS). The primary antibodies were diluted in 3% no-fat milk in TBS and incubated with the nitrocellulose for 1.5 h. The membranes were rinsed in TBS-tween 20, and incubated with the peroxidase-coniugated secondary antibodies (anti-mouse IgG 1:10.000, Kierkegard and Perry Lab, Gaithersburg, MD, USA) in 3% no-fat milk in TBS for 45 min. After washes in TBS/tween 20, the immunoreactive signal was revealed by enhanced chemiluminescence (ECL; Amersham International, Little Chalfont, UK). The mouse anti-actin (from Chemicon, Temecula, CA, USA; diluted 1:5.000) was used as loading control.

Immunofluorescence on NSC34 motor neurons. After fixation, to avoid crosslink with non-specific epitopes, NSC34 cells were rinsed three times in low concentration salt buffer (150mM NaCl and 10mM PB at pH 7,4) and three times in high concentration salt buffer (500mM NaCl and 20mM PB at pH 7.4), and then incubated in goat serum dilution buffer (3% normal goat serum, 0.1% Triton X-100, 500mM NaCl and 20mM PB at pH 7.4). The primary antibodies were incubated over-night at 4°C. The secondary Alexa Fluor 546 and 488 antibodies were incubated for 1 h at room temperature. In selected

experiments, transfected NSC34 cells were processed for sequential double-labeling immunofluorescence. Cells were incubated with monoclonal antibodies first (anti-Xpress or anti-tubulin) and polyclonal antibodies thereafter (anti-paraplegin and anti-NF200, or #910 anti-ha-SMN). To avoid interference between fluorophore signals, Alexa 488 and 647 far-red secondary antibodies were used. After all experiments, cells were repeatedly rinsed, coverslipped with Fluorsave (Calbiochem, Darmstadt, Germany) and examined on a confocal microscope (Radiance 2100, Bio-Rad, Hercules, CA; Leica SP5, Wetzlar, Germany; Nikon A1, Tokyo, Japan).

Cell measurements and statistical analysis. Confocal microscope images of cells of interest were selected. Axon length, axon abnormalities, and the density of transfected cells were quantified at different time points after transfection (24, 48, 72 h) by means of Image-ProPlus software. For statistical evaluation, at least fifty neurons transfected with a control empty vector expressing GFP, wt or mutated ha-SMN were analyzed at every time-points considered (n= 3 experiments). To quantify the density of anti-tag labeled neurons, three adjacent non-overlapping 0,36 mm² subfields were chosen for each construct. Density values (number of cells/area) from triplicate experiments were averaged per wild-type and mutated ha-SMN. Data were statistically analyzed by one-way or two-way ANOVA followed by Bonferroni as post hoc comparison test.

RESULTS

Preparation of α -SMN mutated constructs. *SMN1* pathogenic mutations from compound heterozygous SMA patients have been already used in *in vitro* and *in vivo* assays to verify FL-SMN functional alterations. The selected constructs (W92S, A111G, E134K, 133-134 4bp del, and 147-148 5bp del: Fig. 1A) were obtained by inserting point mutations or small intragenic rearrangements in the already available human α -SMN vector (pcDNA4/wt ha-SMN: Setola et al. 2007: Fig. 1A). As summarized in Figure 1A, the selected constructs produced either **i)** α -SMN proteins with single aminoacid substitutions (W92S, A111G, and E134K mutated ha-SMN proteins), or **ii)** SMN proteins identical to α -SMN and FL-SMN in the N-terminal part but with a different C-terminal tail, encoded in part by the normal sequence of exon 3, in part by the frameshifted sequence of exon 3 (133-134 4bp del and 147-148 5bp del: see Fig.1A). The sequence of all mutated constructs was verified by sequence analysis.

Expression levels of the different α -SMN polypeptides. We next transiently transfected the selected constructs in NSC34 motor neurons. As illustrated in Fig. 1, western blot analysis of cell lysates demonstrated that all constructs determined the production of ha-SMN polypeptides of the expected molecular size (Fig. 1B-C). The optical density quantification of WB immunoreactive bands (ha-SMN *vs* actin) revealed that wt and mutated ha-SMN proteins with single aminoacid substitutions produced at both 24 and 48 h after

transfection comparable protein amounts (Fig. 1 B-C). In contrast, 147-148 5bp del ha-SMN expression levels were clearly reduced at 48 h if compared to 24 h post-transfection (Fig. 1D). The expression levels of the 133-134 4bp del ha-SMN were already low at 24 h and almost absent at 48h after transfection (Fig. 1B-D). These findings could signify that the latter SMN proteins exert a negative effect not only on axon growth (see below) but also on cell survival and homeostasis. We checked this possibility by analyzing the density of cells transfected with the different ha-SMN constructs at 24, 48, and 72 h (Fig. 1E). As indicated in Fig. 1E, the number of transfected NSC34 motor neurons declined slowly over time for all constructs considered. This indicates that the frameshift mutated ha-SMN proteins are not toxic for motor neurons but rather less produced or less stable/more rapidly degraded.

Effect on axon growth. We have already shown that a-SMN is capable of inducing time-dependent axon growth upon forced expression in NSC34 motor neurons (Setola et al., 2007). We used the same experimental approach to compare the axonogenic properties of wt and mutated ha-SMN proteins. To this end, we analyzed a sizeable number of NSC34 motor neurons transfected with a control vector expressing GFP and the different ha-SMN constructs at three different time points (24, 48, 72 h after transfection) taken from triplicate experiments. As illustrated in Fig. 2A, the forced expression of the ha-SMN protein determined time dependent axon growth in NSC34 cells, in keeping with what reported previously by our group (Setola et al. 2007). No increase in axon length was observed after transfection with

the GFP-expressing vector. The ha-SMN polypeptides carrying single aminoacid substitutions were capable of progressively inducing axon growth in a way similar to that of wt ha-SMN (Fig. 2A-B). However, quantification of axonal length revealed that the axonogenic function of all missense mutated ha-SMN was impaired in comparison to that of wt ha-SMN and that reduction of axon growth was statistically significant (at 24 h for W92S/ha-SMN and E134K/ha-SMN, at 72 h for A111G/ha-SMN: Fig. 2A-B). The axonogenic function was completely abrogated for the two ha-SMN polypeptides with primary structure of the C-terminus more divergent from that of the ha-SMN protein. In NSC34 motor neurons transfected with 147-148 5bp del/ha-SMN, axon growth was not different from that of cells transfected with the GFP control vector. The axonogenic function of 133-134 4bp del/ha-SMN was completely abolished: the transfected motor neurons did not show any tendency to grow at all considered time-points (Fig. 2A-B).

Axonal abnormalities. We next verified the morphology of neurites whose growth was stimulated by the different human a-SMN constructs and the GFP vector as control. The NSC34 cells transfected with the GFP vector (Fig. 3A) and wt a-SMN (Fig. 3G) showed normal neurites at all time points considered. By contrast, cells transfected with mutated ha-SMN polypeptides were characterized by neurites not only shorter than those of cells over-expressing wt ha-SMN but also displaying clear morphologic abnormalities. In particular, we noticed the presence of evident swellings along the course of neurites. The swellings were present in cells transfected with

all mutated ha-SMN proteins, at all time points considered, along the neuritic shaft close to the cell body (Fig. 3B-C), at branching sites (Fig. 3B and D) and up to the growth cone (Fig. 3E). Irregularly shaped, enlarged growth cones were not infrequently found at the end of neurites with multiple swellings (Fig. 3B and E). In addition to the presence of the swellings, neurites also displayed an irregular course, as they were frequently angled (Fig. 3F and I) and reverberating toward the cell body (Fig. 3H and J). These abnormal features were present at all time points considered, but they tended to increase progressively at later time points (see below). In cells transfected with the frameshift mutated ha-SMN proteins, the over-expressed proteins tended to accumulate in granules within the cell body frequently in the region facing the growing abnormal neurite (Fig. 3K-L).

For a better estimate of the morphologic abnormalities induced by the different mutated ha-SMN proteins, we quantified the percentage of cells showing axonal defects at the different time points. As indicated in Figure 4, no abnormalities were observed in NSC34 cells transfected with wt ha-SMN at 24 h after transfection. At 48 and 72 h after transfection, only a minority of wt ha-SMN transfected cells (about 10%) were characterized by a few swellings along the neuritic shaft. By contrast, at all time points considered, the majority of cells transfected with the mutated ha-SMN displayed axonal abnormalities (Fig. 4). In particular, the negative effect of the missense mutations was progressive over time, whereas the frameshift mutations induced even after 24 h axonal abnormalities (and a higher number of axonal abnormalities per axon: data not shown) in almost all transfected

motor neurons (Fig. 4A). If compared to wt ha-SMN, all mutations eventually prove to be detrimental to the axonal morphology and growth in a highly significant fashion (Fig. 4B).

Cytoskeletal abnormalities and altered localization of subcellular organelles. To characterize further the morphologic abnormalities reported above, we performed double-labeling confocal experiments with cytoskeletal and mitochondrial markers in cells transfected with wt and mutated ha-SMN. Double-labeling staining revealed that cells transfected with wt ha-SMN showed neurites and growth cones characterized by regular organization of microtubules and no evidence of structural abnormalities or mitochondria accumulation (Fig.5A). By contrast, in cells transfected with mutated ha-SMN swellings were consistently associated with irregularities of the axonal cytoskeleton. As illustrated in Fig. 5, microtubules were consistently splayed apart in the axonal swellings (Fig. 5 B-D). In addition, in the neurites characterized by swellings, irregular disposition of microtubules was observed also at branching points (Fig. 5B, inset) and in the growth cone (Fig. 5C, inset). Swellings were also consistently characterized by accumulation of mitochondria (Fig. 5E). Interestingly, mitochondria tended to be concentrated mostly in the proximal part of the swellings (Fig. 5E). In addition, in cells transfected with the frameshift mutated ha-SMN, the protein accumulation in granules within the cell body was always associated with axonal swellings characterized by neurofilament irregularities (Fig. 5F). The quantification of neurofilament/tubulin accumulation in axon swellings (Fig. 5G) and growth cones (Fig.5H) confirmed the

significantly higher frequency of cytoskeletal abnormalities in cells transfected with mutated vs wt ha-SMN at all time points considered.

DISCUSSION

The role of the shorter SMN isoform a-SMN in SMA is presently unknown. As a first step to verify a possible link between a-SMN and the human disease, we have here investigated whether SMA pathogenic mutations coding for the SMN N-terminus possess biological relevance as to affect a-SMN functional properties. We demonstrated here that either missense mutations or small intragenic re-arrangements of the SMN transcript coding for the Tudor domain clearly and consistently altered the a-SMN capability of inducing axonal elongation *in vitro*. Point mutations reduced significantly whereas frameshift mutations abrogated completely the ha-SMN function in axon growth. In addition, mutated human a-SMN proteins determined in almost all NSC34 motor neurons the growth of axons with prominent morphologic abnormalities.

SMA is clearly a loss of function disease, whose pathogenesis is related to reduced levels of the SMN proteins, and no toxic effects of abnormal SMN proteins have been reported in compound heterozygous patients with one deleted and one mutated allele. However, the basal amount of endogenous a-SMN in NSC34 motor neurons is very low, as a-SMN is undetectable in these cells at the western blot level (data not shown). Therefore, NSC34 cells represent a convenient biologic cellular context to compare the differential

effect between over-expressed wt and mutated human a-SMN proteins. A similar strategy has been also used in different labs to verify the effect of these mutations on the FL-SMN function (Mohaghegh et al. 1999; Jones et al. 2001; Lefebvre et al 2002; Sun et al. 2005; Kotani et al. 2007; Fallini et al. 2011; Nölle et al. 2011).

The presence of axonal abnormalities associated with reduced axon growth may reflect the heavy over-expression of the constructs in our experimental conditions. However, similar to our observations in NSC34 cells expressing mutated ha-SMN proteins, axonal swellings, morphologic abnormalities of axonal shafts and growth cones, and abnormal accumulation of neurofilaments were also reported in both SMA patients and experimental SMA models. Neuropathology studies on SMA type I patients demonstrated dystrophic axons, neuronal swellings and accumulation of neurofilaments (Murayama et al. 1991; Devriendt et al. 1996; Korithenberg et al. 1997; Omran et al. 1998). In SMA mice prominent axonal swellings (McGovern et al. 2008) or neurofilament accumulation in neuromuscular junctions (Cifuentes-Diaz et al. 2002) were reported. Cultured motor neurons and sensory neurons from severe (SMN2 +/+; Smn^{-/-}) SMA mice showed decreased axonal length and reduced growth cones size (Rossoll et al. 2003; Jablonka et al. 2006; Ting et al. 2007). Smn knockdown in zebrafish to levels similar to those occurring in SMA patients determined the development of altered motor neurons with truncated or excessively branched axons that reached the target muscles with inappropriate trajectories (Mc Whorter et al. 2003).

Regarding the potential severity of the considered mutations in SMA pathogenesis, it should be noted that the W92S, A111G, E134K and 147-148 5bp del mutations were described in type I patients (Brahe et al. 1996; Clermont et al. 1997; Mohaghegh et al. 1999; Sun et al. 2005; Kotani et al. 2007), thus indicating that even subtle abnormalities of the Tudor domain are critical for SMN function. These mutations were previously reported to disrupt the SMN interaction with fundamental components of nuclear RNA-protein complexes, such as fibrillarin and the Sm proteins (Bühler et al. 1999; Jones et al. 2001; Sun et al. 2005), thus impairing snoRNP and snRNP biogenesis. However, it has been more recently demonstrated that FL-SMN also interacts through the Tudor domain with HuD, a protein of the neuronal ELAV family, in both the cytoplasm and axons of cultured motor neurons to form multiprotein complexes that are actively transported along the axon (Akten et al. 2011; Fallini et al. 2011; Hubers et al. 2011). The E134K mutation significantly impaired the interaction SMN-HuD in the axonal compartment (Fallini et al. 2011; Hubers et al. 2011). Given that the ha-SMN sequence contains the entire Tudor domain, it is possible that this specific region might subserve the anchoring of ha-SMN to other proteins or mRNA involved in neuronal development, growth and transport (such as HuD, neuritin, GAP43), and that abnormalities in this region may explain the a-SMN loss of function reported here. The relevance of the Tudor and the shorter protein domain encoded by intron3 is also suggested by the relationship between the severity of mutation and

axonogenic function, since the more the ha-SMN C-terminal sequence is altered, the more the a-SMN axonogenic properties are affected.

The 133-134 4 bp del mutation has been mostly reported in Spanish SMA families, associated with a wide variety of clinical manifestations, ranging from severe type I to asymptomatic patients (Cuscò et al. 2003). This mutation was the most severe in our *in vitro* experiments in affecting the a-SMN axonogenic functions. However, as clearly indicated by our data on the survival of transfected motor neurons, the mutated 133-134 4bp del ha-SMN did not possess toxic effects (as shown in Fig.1E) but it was likely more rapidly degraded. Therefore, other factors such as the number of copies of SMN2 and the ratio between FL and $\Delta 7$ proteins may underscore the important difference in severity reported in the Spanish families (Cuscò et al. 2003; Also-Rallo et al. 2011).

The double labeling experiments demonstrated that the axonal swellings induced by mutated human a-SMN proteins were structural abnormalities of the axonal shafts with altered cytoskeletal elements and altered distribution of subcellular organelles such as mitochondria. These findings may indicate that the a-SMN protein plays a relevant role in the establishment of the proper cytoskeletal structure of the axon, and that axonal transport may be impaired as a consequence of such cytoskeletal impairment. The relevance of a-SMN in the proper building up of the axon is also suggested by the accumulation of the mutated a-SMN polypeptides (particularly the 133-134 4 bp del ha-SMN, the mutated a-SMN with the C-terminus

most divergent from the wt ha-SMN) in the region of the cell body facing the newly growing neurite (see Fig. 3L).

In summary, we have demonstrated here that SMA pathogenic mutations strategically placed in the Tudor domain deeply alter the functional capabilities of a-SMN. The clear relationship between a-SMN structural alteration and functional impairment indicates that the Tudor and intron3-encoded domains are critical in dictating a-SMN function possibly because they work as an association domain for proteins involved in axonal growth or maintenance. Two limitations of our experimental approach should be underlined. First, these data do not provide a direct proof that a-SMN is pathogenetically linked with SMA. Rather, they more simply indicate that Tudor domain mutations are functionally relevant not only for FL-SMN function, raising the possibility of diverse, multiple pathogenic steps leading to the disease in humans. Second, for the limitations intrinsic to immortalized NSC34 cells, the potential pathogenic role of a-SMN for SMA would ultimately need to be studied in more physiologic biological systems, such as primary motoneuronal cultures or motor neurons *in vivo*.

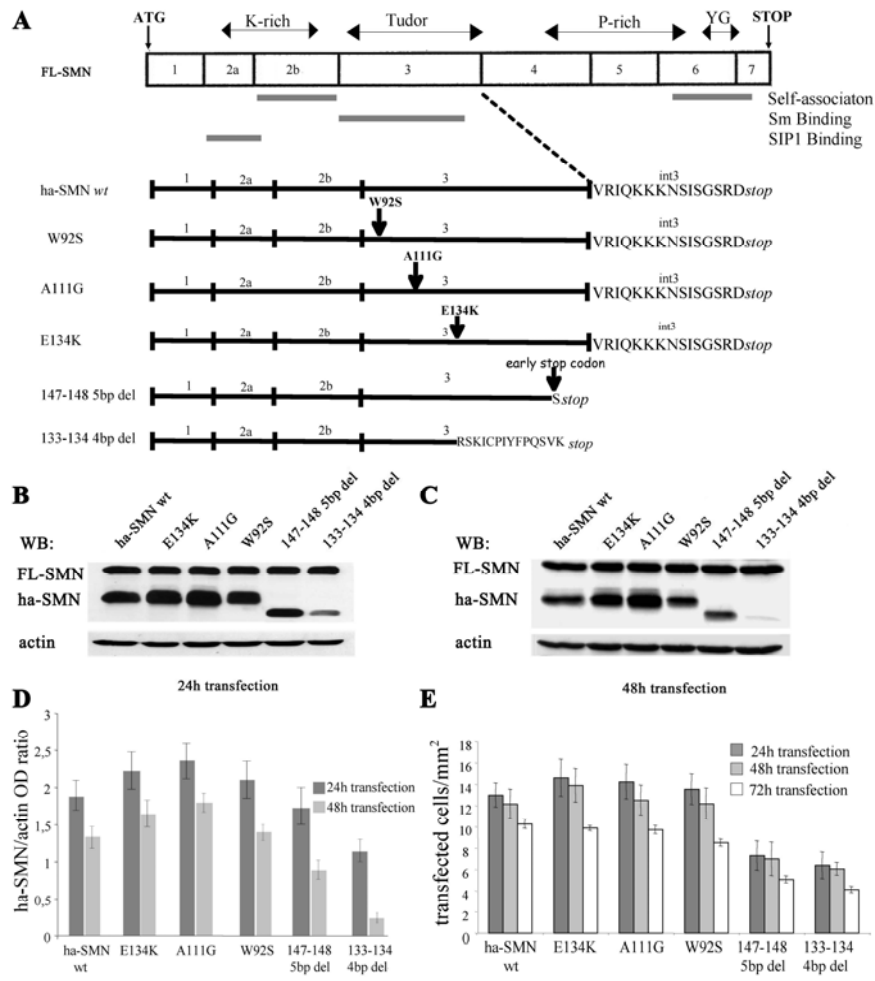


Fig. 1.A. Schematic representation of the full-length SMN protein, and the wt and mutated ha-SMN constructs employed in the present study. Note that the W92S, A111G, and E134K mutated ha-SMN all retain the wt ha-SMN C-terminus with the Tudor and the intron 3 encoded domains. By contrast, this domain is disrupted in the ha-SMN proteins with frameshift mutations, particularly in the 133-134 4bp del polypeptide. **B-C.** WB analysis showing the production of the different a-SMN polypeptides of the expected molecular size at 24 (B) and 48 h after transfection (C). Blots were probed with an antibody directed against the SMN N-terminus (clone 8, BD) recognizing both endogenous FL-SMN (upper bands) and transfected ha-SMN (lower bands). **D.** Bargraphs showing the expression levels of wt and mutated ha-SMN proteins (OD ratio vs actin) after 24-48 h transfection. **E.** Bargraphs showing the cell density of NSC34 motor neurons at different time points after transfection (transfected cells/mm²). The density of NSC34 cells similarly decreased over time for all constructs considered, even if was lower for the frameshift mutated ha-SMN at all time points considered.

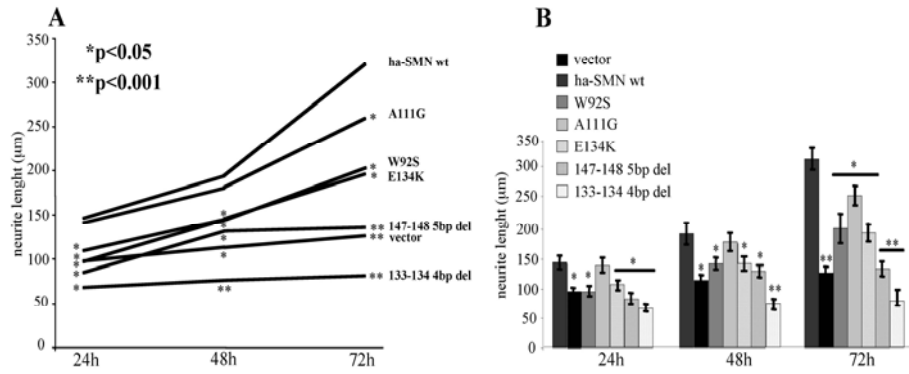


Fig. 2.A. Diagram illustrating the quantification of mean axonal elongation in NSC34 motor neurons transfected with the “empty” GFP vector, and the wt and mutated ha-SMN proteins at different time points. Data are indicated as mean $\pm$ s.e. of triplicate experiments. Note that cells transfected with 133-134 4bp del ha-SMN did not show any axonal growth at the different time-points considered. **B.** Bargraphs illustrating the statistical evaluation at the three time points considered. Axon length (for control vector vs wt vs mutated ha-SMN) was analyzed with two-way ANOVA followed by Bonferroni as post hoc comparison test.

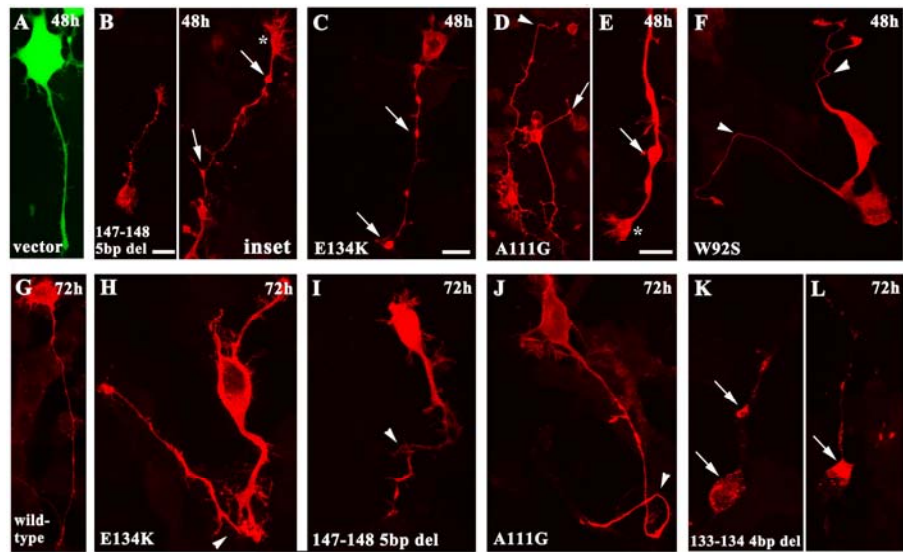


Fig. 3. Morphologic features of NSC34 motor neurons transfected with control GFP vector, wt and mutated ha-SMN proteins. Confocal images of transfected cells revealed by anti-tag antibody. Note the clear axonal abnormalities in all cells transfected with mutated ha-SMN, such as swellings along the axon shaft (arrows in B-C), at branching points (arrows in B and D) and close to the growth cones (arrows in E); irregular growth cones (asterisks in B and E); angled or reverberating axons (arrowheads in D, F, H-J). Also note that the frameshift mutated proteins tended to accumulate in the swellings and in the cell body facing the growing abnormal neurite (arrows in K-L). Scale bars: B, D and G: 35 μ m; A, inset in B, C-F and H-L: 25 μ m.

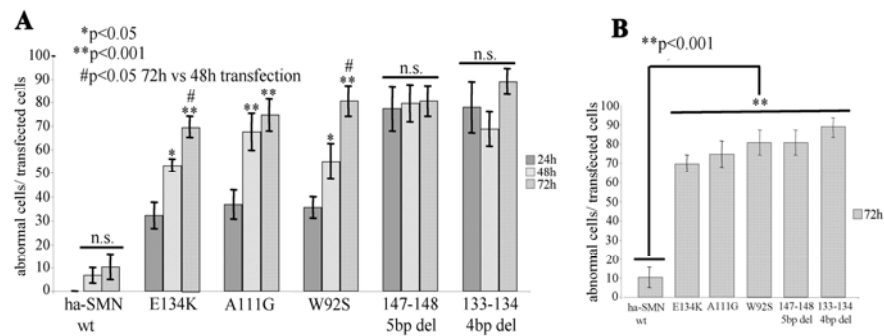


Fig. 4.A. Histogram showing the percentage of transfected cells with axonal abnormalities at the different time points considered. Note that the negative effect of the missense point mutations was significantly progressive over time, whereas the frameshift mutations induced axonal defects even after 24 h. **B.** By contrast, only 10% of cells transfected with wt ha-SMN displayed axonal abnormalities at 72 h. The difference was significant for all considered mutated ha-SMN proteins.

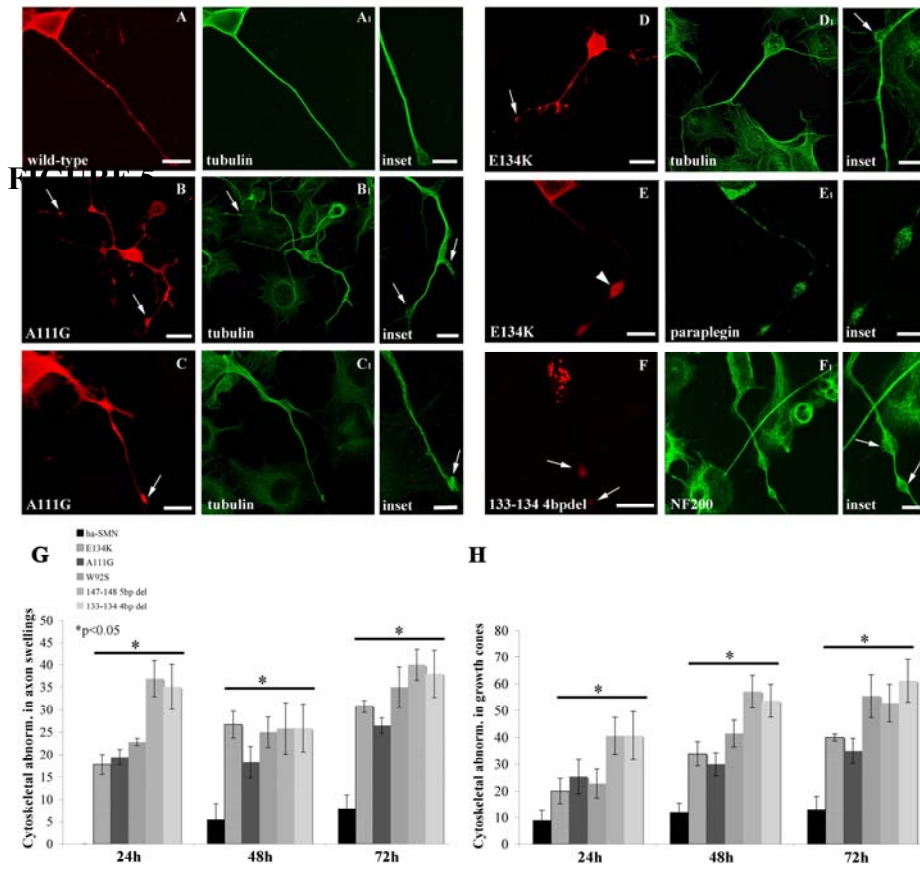


Fig. 5. Confocal images of NSC34 cells transfected with wt or mutated a-SMN constructs, double labeled for ha-SMN (red, pseudo-color from Alexa 647: #910 in A-D and anti-tag in E-F), and anti-tubulin (green, from Alexa 488: A₁-D₁), anti-paraplegin (green: E₁) and anti-neurofilaments NF-200 (green: F₁) antibodies. Note the irregular disposition of microtubules in axonal swellings, branch points and growth cones (arrows in B-D, insets) and the neurofilament irregularities in axonal swellings (arrows in F, inset). Also note the accumulation of mitochondria in axonal swellings (arrowhead in E and inset). Scale bars: A, B, D-F: 25µm; C: 20µm; inset in A-F: 10µm. **G-H** Bargraphs showing the presence of cytoskeletal abnormalities in swellings and growth cones in cells transfected with wt and mutated ha-SMN constructs at the different time points considered. The significant difference in cytoskeletal abnormalities between wt and mutants was obtained with one-way ANOVA followed by Bonferroni as post hoc comparison test.

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CHAPTER 4

Human axonal Survival of Motor Neuron (α -SMN) protein stimulates axon growth, cell motility, C-C motif ligand 2 (CCL2) and insulin-like growth factor-1 (IGF1) production

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The Journal of Biological Chemistry Vol. 287. N. 31, pag.26782-25794

SUMMARY

Spinal muscular atrophy (SMA) is a fatal genetic disease of motoneurons due to loss of full-length Survival of Motor Neuron (FLSMN) protein, the main product of the disease gene SMN1. Axonal SMN (a-SMN) is an alternatively spliced isoform of SMN1, generated by retention of intron 3. To study a-SMN function, we generated cellular clones for the expression of the protein in mouse motoneuron-like NSC34 cells. The model was instrumental in providing evidence that a-SMN decreases cell growth and plays an important role in the processes of axon growth and cellular motility. In our conditions, low levels of a-SMN expression were sufficient to trigger the observed biological effects, which were not modified by further increasing the amounts of the expressed protein. Differential transcriptome analysis led to the identification of novel a-SMN regulated factors, i.e. the transcripts coding for the two chemokines, CC motif ligands 2 and 7 (CCL2 and CCL7), as well as the neuronal and myotrophic factor, insulin-like growth factor-1 (IGF1). a-SMN dependent induction of CCL2 and IGF1 mRNAs resulted in increased intracellular levels and secretion of the respective protein products. Induction of CCL2 contributes to the a-SMN effects, mediating part of the action on axon growth and random cell motility, as indicated by chemokine knockdown and re-addition studies. Our results shed new light on a-SMN function and the underlying molecular mechanisms. The data provide a rational framework to understand the role of a-SMN deficiency in the etiopathogenesis of SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is a disease causing selective motoneuron death. SMA affects the pediatric population and it is the leading genetic cause of infant mortality (1). Loss of telomeric SMN1 (Survival of Motor Neuron 1), the pathogenetic gene, is at the basis of SMA, while SMN2, the duplicated centromeric gene, modulates the severity of this disease (2). SMN1 codes for a functional protein, full-length SMN (FL-SMN), while the primary product of SMN2 is A7-SMN, an unstable protein of uncertain significance (3). FL-SMN is an ubiquitous protein localizing to the nucleus and cytoplasm of many cell types (4). It is well established that FL-SMN acts as an assembly factor for small nuclear ribo-nucleoprotein particles or small nucleolar RNPs involved in mRNA splicing (2,5). However, it is unclear how reduced levels of an ubiquitous protein like FLSMN lead to the selective degeneration of motor neurons in SMA.

We demonstrated that FL-SMN is not the sole product of the SMN1 gene, which generates a second and much less abundant protein via an alternative splicing event resulting in retention of intron 3 (6). The alternative protein product is shorter than FL-SMN because of the presence of an in-frame stop codon located in intron 3. The same splicing variant is observed in mice and rats, indicating evolutionary conservation of the transcript and corresponding protein (7). Unlike FL-SMN, expression of this novel gene product is relatively tissue-specific and temporally restricted. In fact, the a-SMN mRNA and protein are detectable in spinal cord motor neurons and some

peripheral tissues, such as liver and heart, only during the late phases of embryogenesis and early post-natal life (6). In the motor neuron, the SMN splicing variant localizes to axons and is excluded from the nucleus. For this reason we named the protein axonal-SMN (a-SMN). It is conceivable that loss of function of human a-SMN contributes to the pathogenesis of this disease (8).

In the present report, we describe the development of a cellular model for the expression of a-SMN in NSC34 motoneuron-like cells. The model was used to support the functional significance of a-SMN in axonogenesis and to establish an important role for the protein in the control of cell motility. In addition, whole-genome gene expression studies permitted the identification of IGF1 (insulin-like growth factor-1), CCL2 and CCL7 (C-C motif ligands 2 and 7) as factors associated with a-SMN expression. Functional studies performed on CCL2 indicate that the chemokine contributes to the axonogenic and pro-motility action of a-SMN.

EXPERIMENTAL PROCEDURES

Cell cultures

The NCS34 cell line (9) was maintained in low-glucose (1 gr/l) DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 5% TET-System-approved fetal calf serum (Clontech, Mountain View, CA). To obtain stable deposition of neuronal axons, cells were grown in culture dishes pre-coated for one hour with Matrigel Matrix Basement Membrane (200 Ig/ml, BD, Bedford, MA) (10). The TR4 clone was grown in the presence of 10 Ig/ml Blasticidine S (Invitrogen), while a-SMN expressing clones were cultured in the presence of 10 Ig/ml of Blasticidine S and 50 Ig/ml Zeocin (Invitrogen). Induction of a-SMN was performed in medium containing 1 Ig/ml tetracycline (TET) without addition of other antibiotics.

Plasmid generation and transfections

The cDNA coding for human a-SMN (6) was amplified using the following oligonucleotides: forward primer, 5'-ctaagcttatggcgatgagcagcggcgga-3' (consisting of the underlined HindII site, placed upstream of the sequence corresponding to nt.164-185 of NM_000344) ; reverse primer, 5'-agtctagagcaggtttttgtataagggt-3' (consisting of an XbaI site placed upstream of the sequence complementary to nt.119-140 of SMN1 intron 3).

The resultant cDNA fragment encoding a-SMN was digested with HindIII/XbaI and inserted into the pcDNA4/TO plasmid (Invitrogen) digested with the appropriate enzymes. The human FLSMN cDNA has already been described (6).

Transient transfection of NSC34 cells with the human a-SMN and FL-SMN cDNAs was performed as described (6). Transfections were performed with the FuGENE HD Transfection Reagent (Roche, Mannheim, Germany), according to the manufacturer's instructions.

Generation of a-SMN expressing NSC34-derived cellular clones.

For the forced expression of human a-SMN, we used a tetracycline (TET) dependent system and an approach involving a two-step selection strategy. The NSC34 cell line was stably transfected with a TET-repressor plasmid construct (pcDNA6/TR, Invitrogen). We obtained a number of cell clones characterized by resistance to blasticidin S that were subsequently isolated by serial dilution. Four clones were evaluated for the expression of the TET-repressor by transient transfection of an appropriate TETdependent β -galactosidase reporter construct.

The cell clone TR4 was chosen for further studies, given the low level of reporter expression in basal conditions and significant induction upon challenge with TET. TR4 was transfected with a construct containing the entire coding region of the human a-SMN cDNA under the control of a promoter bearing TET-repressor binding sites.

After selection for zeocin resistance, numerous clones were isolated by serial dilution.

Antibodies

The anti-peptide polyclonal antibody raised against the C-terminal region of the human a-SMN (#910) was prepared in rabbits by NeoMPS (Strasbourg, France) and used at 1:500 dilution in all the experiments. The following antibodies were also used: mouse anti-SMN (clone 8) from BD Biosciences (Franklin Lakes, NJ, USA; diluted 1:25.000), directed against the N-terminal region of the SMN protein; mouse anti-actin from Chemicon (Temecula, CA, USA; diluted 1:5.000 for WB and 1:500 for IF experiments); mouse anti- α -tubulin (Sigma- Aldrich; diluted 1:500); mouse anti-JIP1 from Santa Cruz Biotechnology (Santa Cruz, CA, USA; diluted 1:200); mouse anti-CCL2 and mouse anti-IGF1 from R&D Systems (Minneapolis, MN, USA; diluted 1:20).

Morphological analysis and immunofluorescence (IF) experiments

For the evaluation of cellular morphology, cells were fixed with 4% paraformaldehyde/4% sucrose, or with 4% paraformaldehyde/2% glutaraldehyde for 30 min at room temperature. For IF, NSC34 cells were rinsed three times in low-salt (150 mM NaCl) 10mM phosphate buffer, pH 7.4 and three times in high-salt (500mM NaCl) 20 mM phosphate buffer, pH 7.4, to avoid crosslinking with nonspecific epitopes. Subsequently, fixed cells were incubated in goat serum dilution 20Mm phosphate buffer, pH 7.4, containing 3% goat serum, 0.1% Triton X-100 and 500 mM NaCl.

The primary antibodies were incubated overnight at 4°C. The secondary Alexa Fluor 546 and 488 antibodies were incubated for 1

hour at room temperature. After all experiments, cells were repeatedly rinsed with Fluorsave (Calbiochem, Darmstadt, Germany) and examined with a confocal microscope (Radiance 2100, Bio-Rad, Hercules, CA; Olympus Fluoview, Olympus, Segrate, Italy). For fluorescence intensity analysis of JIP1, fifteen confocal nonoverlapping images were chosen at 400x magnification from triplicate experiments.

Background fluorescence intensity was subtracted. Fluorescence was quantified using Image-ProPlus software and expressed as mean + SD. To quantify the surface area occupied by growth cones, NSC34 and a-SMN81 cells were labeled with DNase Alexa Fluor® 488 (Invitrogen), binding and highlighting G-actin (11), for 15 min at room temperature. Confocal images at 600X were taken from at least 30 growth cones in triplicate experiments and analyzed with the Image-Pro Plus software. Data were statistically analyzed by one-way ANOVA followed by the Bonferroni post hoc comparison test. For the atomic force microscopy (AFM) experiments, cells were grown on glass bottom dishes (WillCo-Wells, model GWSt 5040) for 72 hours, fixed in 2% glutaraldehyde phosphate buffer at pH 7.4 (for 30 min), gently washed in bi-distilled water and stored in 0.6% glutaraldehyde solution at 4°C. Cells were analyzed using a BioscopeII Atomic Force Microscope equipped with the Digital Instruments NanoScope III (Veeco, Santa Barbara, CA) and integrated with the inverted optical microscope Axio Observer A1 (Zeiss, Oberkoken, Germany). AFM images (512 x 512 pixels) were recorded at room temperature in contact mode (in both height and deflection modes) with non-

conductive silicon nitride tips Model MLCT (Veeco, Santa Barbara, CA), with typical nominal spring constants ranging from 0.01 N/m to 0.60 N/m and nominal resonance frequency ranging from 7 KHz to 125 KHz. The scan range was 65 μm and the scan rate was 0.5 $\mu\text{m}/\text{sec}$. Randomly selected cells were scanned and qualitatively analyzed to evaluate morphologic features of cell bodies, neurites and growth cones.

Gene expression analysis and validation studies

Total RNA was prepared using miRNeasy Mini Kit (Qiagen, Valencia, CA). Transcriptome analysis was performed with the use of Whole Mouse Genome Oligo Microarray Kit (Agilent, Palo Alto, CA) and the results were deposited in the GEO database (accession: E-MEXP-3402).

The levels of the a-SMN transcript were measured by SYBR Green-based Real Time PCR, using amplimers specific for the a-SMN mRNA; 5'-ttaccagctaccattgcttcaat-3' (nt. 487- 509 in NM_000344) and 5'- tagtctctgctccagaaattgaat-3' (complementary to nt. 23-47 of intron 3, numbering the 5'-end nucleotide of intron 3 as 1). Validation of the microarray results were performed with the following Taqman probes: Ccl2, Mm00441242_m1; Ccl7, Mm01308393_g1; Igfl, Mm00439560_m1; cMyc, Mm00487804_m1; Actb, Mm01205647_g1 (Applied Biosystems, Carlsbad, CA).

Measurement of CCL2 and IGF1 proteins

Cells were lysed and the protein extracts were used for Western blot analysis according to a routine procedure detailed elsewhere (6). The levels of CCL2 and IGF1 proteins in the cell lysates and the conditioned medium were measured by ELISA using mouse CCL2/JE/MCP-1 and mouse/rat IGF1 Immunoassay Kits (R&D Systems), respectively.

Silencing experiments

Synthetic anti-a-SMN (target sequence located in intron 3: 5'tgtcattcctgaaagtgg3') and non-targeting siRNAs were purchased from Dharmacon (ThermoFisher Scientific, Lafayette, Co). Transfection (11M siRNAs) was performed following the manufacturer's instructions.

Synthetic anti-CCL2 siRNAs [Ccl2MSS276974(3_RNAI) and Ccl2MSS276976(3_RNAI)] and the relative control siRNA (Cat. No. 12935-400, high GC Duplex) were purchased from Applied Biosystems and transfected with Lipo2000 (Invitrogen) according to the instructions of the manufacturer. For the experiments involving cell motility with anti-CCL2 siRNAs, cells were cotransfected with the green fluorescent protein (GFP) expressing plasmid pEGFP-N1 (Clontech, Mountain View, CA). After 72 hours, the efficiency of RNA interference was monitored by RT/real-time PCR using the Taqman probes described above.

Single cell motility and measurement of axon length

Single-cell-motility assays were performed on matrigel (200 Ig/ml) coated substrate (12), as described (13) using the Imaging Station Cell[^]R (Olympus, Segrate, Italy). Image sequences, converted to .tif format files, were analyzed with the image-analysis software ImageJ (W. Rasband, National Institute of Health, Bethesda, MD). In particular, each cell in the field was tracked individually using the “Manual Tracking” plug-in distributed with the ImageJ software. At 24h and 60h, the length of neurites was manually measured outlining each neurite with the free-hand line tool of ImageJ.

Statistical analysis

Unless otherwise indicated, statistical evaluation of the quantitative results was performed according to one-way ANOVA followed by post-hoc test.

RESULTS

Characterization of NSC34-derived cell lines expressing human α -SMN

NSC34 is an immortalized murine hybrid neuroblastoma/spinal cord cell line with a motoneuron-like phenotype (9). NSC34 cells express readily measurable amounts of endogenous full-length SMN (FL-SMN) upon Western blot analysis (6, 8). However, in the same experimental conditions, we were unable to determine detectable levels of the α -SMN counterpart (DL, unpublished observations). For these reasons, we used NSC34 cells to obtain regulated expression of human α -SMN and to study its biological function. We established a cellular model showing basal expression and tetracycline (TET)-dependent induction of the protein. First, we generated the NSC34-derived TR4 cell line, which is characterized by stable insertion of the TET-repressor. Similar to NSC34, TR4 cells showed a motoneuron-like morphology (data not shown). Subsequently, a human α -SMN expression plasmid was introduced in TR4 cells and several derived clones were isolated by serial dilution. We evaluated the expression of the α -SMN mRNA in basal conditions and after treatment with TET for 24 hours in numerous clones. Seven representative clones are shown in Fig. 1A.

Detectable amounts of human α -SMN mRNA were observed in all the clones analyzed even in the absence of TET. TET up-regulated the expression of the α -SMN transcript and protein in all the clones tested (Fig. 1B and 1C). The kinetics of α -SMN induction were very similar

in all clones, as illustrated for aSMN-81 cells (Fig. 1C). TET-dependent induction of a-SMN protein was evident after 12 hours, peaked at 48 hours and diminished by 72 hours.

Immunofluorescence (IF) studies performed in the a-SMN-81 cell line confirmed the Western blot data, revealing a-SMN labeling in basal conditions and TET-dependent induction of the a-SMN signal (Fig. 1D). The a-SMN protein showed the expected intra-cellular localization (6), which was limited to the soma/neuritis (Fig. 1D). This is at variance with what has been described for FL-SMN whose localization extends to the nucleus.

a-SMN causes growth inhibition, stimulates axonogenesis and increases cell motility

We evaluated the consequences of a-SMN expression on various aspects of cellular homeostasis. Initially, we compared the growth of a-SMN22 and a-SMN81 clones, two extremes in terms of basal a-SMN levels and TET-dependent induction, with that of the NSC34 and TR4 controls. We performed these experiments upon serum starvation (0.5%) and subsequent synchronization by serum re-addition (10%).

After a lag-time of approximately 24-36 hours, NSC34 and TR4 cells started to grow and reached confluency by 7 days (Fig. 2A). In contrast, the a-SMN22 and a-SMN81 cells were characterized by a slower growth rate and did not reach confluency even 10 days after re-addition of serum. The growth of the two clones and the parental cell lines was left largely unaffected by treatment with TET. This suggests that the amounts of a-SMN expressed in basal conditions are enough to cause maximal reduction of cell growth.

As growth inhibition is often accompanied by cell differentiation (13,14), we evaluated whether basal expression of a-SMN was associated with increased neuronal maturation. All the a-SMN expressing clones, including a-SMN81, presented a morphology characterized by signs of neuronal differentiation, with increases in axonal thickness, elongation and branching relative to the NSC34 or TR4 controls. As illustrated for a-SMN81 cells grown in basal conditions (Fig. 2B and Fig. 2C), these morphological changes were particularly evident upon immunofluorescence (IF). IF studies conducted with specific anti-tubulin and anti-actin antibodies demonstrated the presence of longer and more branched neurites in a-SMN81 than in NSC34 cells (Fig. 2B). Increased length and branching of axons was paralleled by increased complexity of growth cones, as indicated by the specific markers, JIP-1 (Fig. 2C, left) (15) and Gactin (Fig. 3A, left) (16,17). JIP-1 associated fluorescence was significantly higher in growth cones of a-SMN81 cells relative to the NSC34 controls (Fig. 2C, right). Induction of a-SMN by TET did not affect JIP-1 levels. After staining for G-actin, quantitative determination of growth cones' size revealed that the areas occupied by these structures were significantly larger in a-SMN81 relative to NSC34 cells (Fig. 3A, right).

Addition of TET to the medium of growing a-SMN81 cells did not alter this parameter significantly. Sustained axonogenesis of a-SMN expressing clones was confirmed by AFM analysis, comparing

a-SMN81 and TR4 cells (Fig. 3B). More numerous and thicker filopodia emerging from the cell body, as well as more complex neurites and growth cones were observed in the a-SMN81 clone.

We quantified the axonogenic effect of a-SMN expression by image analysis. The average axon length determined in a-SMN22, a-SMN81 and a-SMN6 cells grown in basal conditions was superior to that determined in the TR4 controls (Fig. 4A). As exemplified in the case a-SMN81 and a-SMN6 cells, TET caused no further increase in average axon length at the two time points considered (Fig. 4B).

Axon outgrowth and cell motility are often associated processes (18), both involving reorganization of tubulin and actin, two main components of the cytoskeleton (19,20). Given the changes induced by a-SMN on tubulin and actin organization (see Fig. 2B), we determined the effect of a-SMN expression on cell motility.

In basal conditions, the a-SMN22, a-SMN81 and a-SMN6 clones were all characterized by increased motility relative to the TR4 (Fig. 4C and 4D) and the NSC34 controls (Fig. 4D). a-SMN81 was the clone characterized by the highest level of motility, although it was not the clone expressing the largest a-SMN amounts, probably as a result of other and as yet to be defined concomitant factors. Addition of TET to the medium did not lead to any further enhancement of cell motility, as illustrated for a-SMN81 cells and TR4 controls. The increase in cell motility is the consequence of an effect on the random component, as indicated by the lack of directionality observed in cell movement (Fig. 4D).

Taken together our data demonstrate that a-SMN expression exerts effects on cell growth, axonogenesis and cell motility. These effects are already maximal in basal conditions and are not modified by TET addition. Within the range of the a-SMN expression levels determined in our clones, the results obtained are consistent with a threshold effect.

Human a-SMN expression perturbs the NSC34 transcriptome

To identify genes of functional significance for the growth inhibitory, proaxonogenic and pro-motility action of a-SMN, we defined the transcriptomic profiles of TR4, a-SMN81 and a-SMN22 cells grown for different amounts of time (24, 56 and 72 hours) in the absence/presence of TET, using whole-genome gene-expression microarrays (Fig. 5A).

Consistent with the functional data, we did not identify genes significantly regulated by TET in a-SMN81 and a-SMN22 cells. This is similar to what was observed in TR4 cells. Thus we refined our analysis, looking for genes whose expression was quantitatively and directly or inversely correlated to a-SMN protein levels in the various conditions considered, using a template-matching algorithm (Pavlidis Template Matching, PTM).

PTM resulted in the identification of genes whose expression was consistently higher (77 genes) or lower (53 genes) in a-SMN81 and a-SMN22 relative to the control TR4 cells, at all the time points analyzed (Fig. 5A; up-regulated genes, left and down-regulated genes, right).

However, we did not find transcripts whose levels were quantitatively and strictly correlated to the amounts of the a-SMN transgene product expressed (Table I lists the top 20 up- and downregulated genes). In fact, up-regulation or down-regulation of all these genes was already maximal in untreated a-SMN81/a-SMN22 cells, and expression was not influenced by TET or by time. The observations were confirmed by principal component analysis (PCA), which showed remarkable differences in the transcriptomic profiles of TR4 and a-SMN81 or a-SMN22 cells, regardless of TET treatment (Fig. 5A, bottom right).

This is similar to what was observed for the effects exerted by a-SMN on growth, axonogenesis and motility. Taken together, our gene expression data are consistent with the idea that amounts of a-SMN above a threshold, which is overcome by basal expression of the transgene, are enough to produce the observed perturbations in the transcriptome.

We focused our attention on four genes because of their potential significance for the a-SMN-dependent effects on cell growth, axonogenesis and motility (Ccl2, Ccl7 Igf1 and cMyc). The two chemokines, CCL2 and CCL7 possess chemoattractant properties (21), and control neuronal development as well as repair from injury (22,23). IGF1 regulates the growth and survival of neuronal cells (24,25). cMyc (vmyc myelocytomatosis viral related oncogene) is a well known protein involved in cell proliferation (26). To verify the association between the expression of a-SMN and these genes, we used RT/real-time PCR to compare the levels of the four transcripts in a larger panel of cell clones (Fig. 5B). Increased levels of CCL2,

CCL7 and IGF1 were present in all the a-SMN expressing clones considered, and reduced cMyc expression was found in six out of seven clones.

In all cases, mRNA levels were not influenced by treatment with TET, further confirming the a-SMN threshold effect hypothesized on the basis of the microarray results.

a-SMN-induced expression of CCL2, CCL7 and IGF1

To strengthen the cause/effect relationship between a-SMN expression and CCL2, CCL7 or IGF1 up-regulation and to evaluate the specificity of these effects, NSC34 cells were transiently transfected with expression plasmids containing either the human a-SMN or the human FL-SMN cDNA (6) (Fig. 6A).

Specific expression of the human a-SMN and human FL-SMN mRNAs was verified after transfection of the corresponding cDNAs, upon RT/real-time PCR analysis (data not shown).

Forced expression of human a-SMN resulted in increased levels of the mRNAs encoding CCL2, CCL7 and IGF1. In contrast, no significant change of the three transcripts was observed after transfection of the FL-SMN cDNA. This indicates that induction of CCL2, CCL7 and IGF1 is specifically associated with a-SMN expression. To support the results, we conducted mirror experiments in the representative a-SMN6 clone, knocking down a-SMN with a specific siRNA (Fig. 6B). Transient transfection of the siRNA caused a significant decrease in the levels of the a-SMN mRNA, which was accompanied by a

reduction in the amounts of the transcripts encoding CCL2, CCL7 and IGF1.

We determined whether induction of the IGF1 and CCL2 mRNAs resulted in increased intracellular accumulation and secretion of the corresponding proteins. In basal conditions, a-SMN22, a-SMN81 and a-SMN16 cells were characterized by intracellular levels of IGF1 protein well above those observed in the TR4control cell line (Fig. 6C, left). This was accompanied by augmented secretion of the growth factor (Fig. 6C, right). Addition of TET to the medium did not alter secretion of IGF1 (data not shown). IF studies demonstrated that a-SMN expression did not alter the intracellular distribution of IGF1, which localized predominantly to the cell body and axons (Fig. 6D).

Similar to IGF1, a-SMN22, a-SMN81 and a-SMN6 cells grown in basal conditions synthesized and secreted much more CCL2 than the two control NSC34 and TR4 cell lines (Fig. 7A). Significantly, the levels of CCL2 found in the medium are higher than the range of chemokine concentrations observed in human serum (30-150 pg/ml) (27). Even in this case, treatment of cells with TET had no significant effect on the amounts of CCL2 protein secreted by the various clones (data not shown). IF experiments confirmed augmented intracellular CCL2 in a-SMN22 and a-SMN6 cells. The majority of intra-cellular CCL2 was distributed in the form of granular structures in the perinuclear area of the cell body, consistent with localization in the secretory machinery (Fig. 7B).

CCL2 is involved in the control of cell motility and axonogenesis

We studied the functional links between a-SMN expression and induction of CCL2. We evaluated whether the protein exerted any effect on axon growth and cell motility, two processes controlled by a-SMN expression. To this purpose, parental NSC34 cells were challenged with CCL2 and subjected to time-lapse microscopy. CCL2 caused a measurable and significant augmentation in the average length of the axons which was evident after 24 hours and increased with time (Fig. 7C). In addition, the chemokine produced a statistically significant and reproducible increase in the motility of NSC34 and TR4 cells relative to vehicle-treated controls (Fig. 7D). Thus, our data are consistent with a role of CCL2 secretion in cell motility and axon deposition by a-SMN.

To evaluate the involvement of CCL2 in a-SMN induced motility and axon growth, we knocked down CCL2 in a-SMN6 cells with two siRNAs (Fig. 7E, left). Forty eight hours after transfection with the two siRNAs, cells were re-seeded for time-lapse analysis of axon growth and cell motility. Within 24 hours, we observed a significant decrease in the average length of axons in CCL2 silenced cells relative to cells transfected with a control siRNA (Fig. 7E, right). In the same experimental conditions, we observed a significant reduction in cell motility after treatment with the two CCL2 targeting siRNAs. We confirmed the results in the a-SMN22 clone (data not shown). These data further demonstrates that CCL2 contributes to the motility and axon growth responses triggered by a-SMN.

DISCUSSION

a-SMN is a recently identified and motor-neuron-specific product of SMN1, the disease-gene of SMA (6). Synthesis of a-SMN in the mouse CNS and spinal cord is limited to the last phases of fetal development and the first few days of neonatal life. This represents the time window characterized by maximal development of the CNS with establishment of the majority of synaptic circuits. To better define the function of a-SMN, we developed a cellular model for the expression of a-SMN. The model was instrumental in providing new insights into the biological activity of a-SMN.

The cellular paradigm described herein allowed regulated expression of human a-SMN in NSC34 cells, which is considered a proxy of the motoneuron (28-30). Our model was characterized by basal expression of a-SMN in untreated cells and by time-dependent induction of the protein upon challenge with TET. Basal expression of the protein was an intrinsic characteristic of the cellular system, and highlighted an interesting aspect of a-SMN biological activity, i.e. threshold effects.

Altogether the data were consistent with the idea that maximal a-SMN biological activity was already evident when the intra-cellular amounts of the protein exceeded a threshold level. This may account for the tight quantitative and timedependent regulation of a-SMN expression and the low levels of protein observed in the developing CNS and spinal cord.

In our cellular model, a-SMN was shown to play a role in biological processes of pivotal importance for motoneuron homeostasis. We documented that a-SMN controls cell motility and axon outgrowth, stimulating both processes.

Stimulation of axon outgrowth was accompanied by an a-SMN-dependent increase in the surface area of growth cones (see Fig. 3A). The observation is interesting, since it has been reported that the area occupied by growth cones is reduced in motoneurons from a SMA mouse model (31-33). Increase in cell motility and axon outgrowth effects were accompanied by a cytostatic action on the motoneuron. a-SMN-dependent growth inhibition and axon outgrowth may be coupled processes and the result of a differentiating action along the neuronal pathway. In fact, terminal cellular differentiation is invariably associated with growth arrest in various cell types (14), including neurons (34-37). In addition, some of the morphological features associated with a-SMN expression in NSC34 cells were reminiscent of those induced by treatment of motoneurons with the prototypic differentiating agent, all-trans retinoic acid (38-41). It is likely that a-SMN induced cellular motility and axonogenesis are also coupled processes. In fact, coupling of cell motility and axon growth has been demonstrated in other cellular models of neuronal differentiation (42-44). Furthermore, establishment of neuronal circuits is the result of axon and neuronal cell movement to their correct destinations by guidance cues in the developing brain (45).

Therefore, it is possible that the primary function of a-SMN in neurons, and particularly in motoneurons, is to control differentiation

and motility ultimately resulting in the modulation of directional axon growth. Loss of a-SMN and consequent defects in motoneuron motility may contribute to the pathogenesis of SMA. Indeed, the position of spinal motoneurons in type I SMA patients suggests abnormal cell migration, as these cells are aberrantly located along the ventral roots outside the ventral horn (46). A role in neuronal migration is also suggested by the specific SMN localization in the region of the germinative neuroepithelium where young postmitotic neurons start migration during human brain development (47).

Our transcriptome analysis provided important clues as to some of the molecules potentially involved in a-SMN induced cell motility and axonogenesis. We identified the growth factor, IGF1, as well as the chemokines, CCL2 and CCL7, as possible mediators of a-SMN biological activity. The transcripts encoding the three factors were up-regulated in all the a-SMN expressing clones analyzed and induction was subject to the same threshold effects observed in the case of cell motility and axon growth. Experiments involving transient transfection or silencing of a-SMN in NSC34 cells support a cause-effect relationship between a-SMN expression and induction of CCL2/CCL7 and IGF1. Studies involving addition of CCL2 to the growth medium indicated that the cytokine increased the motility of NSC34 and TR4 cells.

Complementary studies performed in the a-SMN expressing clone a-SMN6, demonstrated that selective silencing of the CCL2 gene was associated not only with a decrease in random motility but also with a reduction in axon growth.

Collectively, the evidence gathered suggests that the chemokine mediates part of the effects afforded by a-SMN on the two cellular processes.

It is possible that CCL2 contributes to the control of motility and axon growth via modulation of the cytoskeletal dynamics (48,49). In fact, a-SMN seems to affect tubulin and actin reorganization (see Fig. 2B). Regulation of CCL2 and CCL7 has farreaching implications as to the potential relevance of a-SMN functional inactivation for SMA etiopathogenesis. In fact, CCL2 and CCL7 recognize the same CCR2 receptor, which, along with CCR5, is considered to be an important player in myoblast proliferation after skeletal muscle injury (50). Thus, loss of a-SMN activity and degeneration of the motoneuron axons in SMA may result in CCL2 and CCL7 deficits with consequent effects on muscular trophism. With respect to this, there is recent evidence indicating that CCL2 is involved not only in peripheral and CNS inflammatory responses but also in controlling the normal activity of the neuron.

CCL2 and its cognate receptor are constitutively expressed in several brain regions (50) and enhance neuronal excitability as well as synaptic transmission via pre-synaptic mechanisms (51).

Our results provide the first demonstration that synthesis and secretion of CCL2 (52,53) contribute to neuronal axonogenesis and motility in a cell autonomous manner. In our model, the effect of CCL2 on cell motility and axon growth is neither directional nor gradient-dependent, two characteristics that are typical of the chemoattractive action exerted by the chemokine on other cell types. The

demonstration that CCL2 exerts functions other than chemotactic activity on neuronal cells adds to the emerging evidence that chemokines are involved in neuronal plasticity (54-56).

Up-regulation of IGF1 has relevance for the physiological activity of a-SMN and its involvement in SMA. In fact, a-SMN stimulates synthesis, secretion, and axonal transport of IGF1. The protein possesses neurotrophic properties (57) mediated by activation of the PI3K/AKT pathways (58,59) and it is involved in myoblast proliferation as well as induction of myogenic differentiation. Through IGF1, a-SMN could exert an action in modulating the maturation of the neuromuscular unit during development. In addition, a recent report demonstrated that the levels of IGF1 were very low in the serum of a SMA mouse model (60) and the amounts of the circulating growth factor were restored to normal levels upon reexpression of the full length form of SMN2.

Finally, increased expression of IGF1 extended the survival of animals with spinal and bulbar muscular atrophy (SBMA) and increased median survival in a SMA (A7) mouse model (61-63).

Further studies are needed to establish whether a-SMN deficiency and consequent impairment of the cellular processes controlled by the protein play a role in the etiopathogenesis of SMA.

Table I Top over- and under-expressed genes in α -SMN expressing clones

Symbol	Name	TR4		α -SMN 81		α -SMN 22		fold vs TR4	fold vs TR4
		mean	sd	mean	sd	mean	sd		
Scg2	Secretogranin II	5.7	0.4	8.9	0.7	12.2	0.1	9.2	94.7
Ccl7	Chemokine (C-C motif) ligand 7	6.0	0.8	9.4	0.5	10.7	0.5	10.7	26.0
Kif21a	Kinesin family member 21A	6.4	1.0	9.6	0.6	10.6	0.2	9.0	18.4
Ccl2	Chemokine (C-C motif) ligand 2	10.8	1.5	14.1	0.4	14.5	0.4	10.2	12.7
Serpinb8	Serine peptidase inhib, clade B- 8	5.7	0.8	8.7	0.3	9.0	0.2	7.6	9.6
Sulf1	Sulfatase 1	5.1	0.5	7.6	0.3	7.9	0.3	5.5	7.0
Rnf152	Ring finger protein 152	5.1	0.4	7.3	0.4	7.9	0.2	4.5	7.1
Ly6c1	Lymphocyte antigen 6 complex, C1	4.4	0.2	6.2	0.4	7.4	0.2	3.5	7.9
Igf1	Insulin-like growth factor 1	4.3	0.2	5.5	0.3	6.9	0.2	2.3	6.0
Ppp1r14a	Protein phosphatase 1, reg sub 14A	6.8	0.6	8.2	0.2	8.9	0.4	2.5	4.3
Ly6a	Lymphocyte antigen 6 complex, A	4.3	0.3	5.7	0.4	6.3	0.3	2.7	4.1
Tmem37	Transmembrane protein 37	8.4	0.4	10.1	0.4	10.2	0.2	3.2	3.4
Fnl1	Fibronectin 1	7.1	0.5	8.9	0.5	8.8	0.3	3.3	3.2
Tmem159	Transmembrane protein 159	9.0	0.4	10.9	0.3	10.4	0.1	3.7	2.7
Frrs1	Ferric-chelate reductase 1	4.9	0.5	6.1	0.4	6.8	0.4	2.5	3.9
Cflar	CASP8 and FADD-like apopt reg	6.2	0.6	7.9	0.3	7.5	0.1	3.2	2.5
Hoxd3	Homeo box D3	4.0	0.3	5.3	0.3	5.6	0.3	2.5	3.0
Cyb5	Cytochrome b-5	9.1	0.5	10.7	0.2	10.3	0.1	3.0	2.2
LOC433944	hypothetical gene (AK083542)	4.5	0.4	5.9	0.2	5.8	0.3	2.7	2.5
Col1a1	Collagen, type I, alpha 1	6.5	0.5	8.0	0.2	7.7	0.2	2.9	2.3
Cabin1	Calcineurin binding protein 1	9.1	0.2	8.3	0.4	8.1	0.2	0.6	0.5
NA	NA	11.4	0.2	10.4	0.2	10.5	0.1	0.5	0.5
Myc	Myelocytomatosis oncogene	13.5	0.2	12.8	0.3	12.4	0.2	0.6	0.5
Alx1	ALX homeobox 1	7.8	0.1	6.7	0.2	7.0	0.2	0.5	0.6
Dnajc13	DnaJ (Hsp40) homolog, C-13	10.9	0.4	10.0	0.3	9.9	0.2	0.5	0.5
Pdcd6ip	Programmed cell death 6 int prot	10.2	0.3	9.3	0.3	9.1	0.3	0.6	0.5
Stat3	Signal transducer and act transcr 3	11.6	0.4	10.5	0.2	10.7	0.2	0.5	0.5
Eya4	Eyes absent 4 homolog (Dm)	10.2	0.4	9.2	0.2	9.1	0.2	0.5	0.5
Nhecl1	Na+/H+ exchanger domain cont 1	5.3	0.3	4.4	0.2	4.2	0.3	0.5	0.5
NA	NA	11.0	0.2	10.2	0.3	9.7	0.1	0.6	0.4
Lss	Lanosterol synthase	10.7	0.1	9.7	0.5	9.7	0.2	0.5	0.5
Ulk4	Unc-51-like kinase 4 (C. elegans)	6.7	0.4	5.6	0.2	5.5	0.3	0.5	0.4
Man1a	Mannosidase 1, alpha	8.7	0.3	7.5	0.2	7.6	0.3	0.4	0.5
NA	NA	10.0	0.2	8.9	0.2	8.8	0.1	0.5	0.4
Iqgap2	IQ motif cont GTPase act prot 2	10.2	0.3	9.0	0.4	9.0	0.2	0.4	0.4
Mtap7	Microtubule-associated protein 7	8.1	0.5	6.8	0.3	6.8	0.1	0.4	0.4
Dalrd3	DALR anticodon bind domain cont	8.5	0.2	7.1	0.3	7.3	0.2	0.4	0.4
1700080O16Rik	RIKEN cDNA 1700080O16 gene	5.6	0.4	4.3	0.3	4.1	0.3	0.4	0.4
Slc38a3	Solute carrier family 38, member 3	7.3	0.3	6.0	0.4	5.7	0.2	0.4	0.3
NA	NA	7.8	0.7	4.6	0.3	4.1	0.4	0.1	0.1

The table contains the quantitative results obtained after microarray gene expression profiling of a-SMN81, a-SMN22 and TR4 cells. Data are expressed in log2 of the intensities and represent 8- 16 samples for each cell line taken at different times in the absence and presence of TET (columns 3-8). The last two columns show the fold change in the two a-SMN expressing clones relative to the control TR4 clone. The top 20 genes concordantly over- and under-expressed in a- SMN81 and a-SMN22 clones are shown.

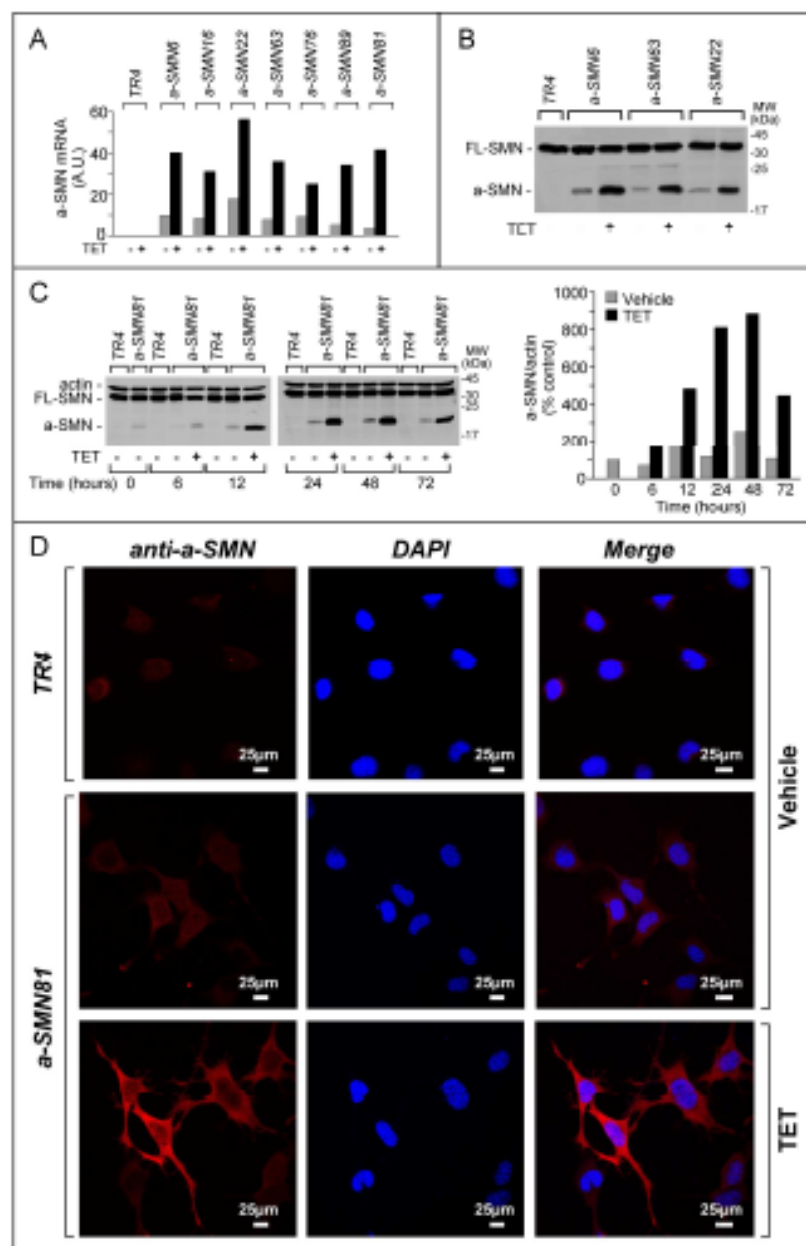


FIGURE 1

Fig. 1 TET-dependent induction of the a-SMN transcript and protein

(A) The indicated a-SMN expressing clones and the parental cell line, TR4, were grown to confluency. Cells were subsequently treated with medium in the presence/absence of TET for 24 hours. Total RNA was isolated and used for RT/Real Time PCR analysis. The relative quantity of a-SMN transcript is expressed in arbitrary units (A.U.) following normalization for the actin transcript. **(B)** The indicated a-SMN expressing clones and TR4 cells were treated with or without TET for 24 hours. Protein extracts were separated on 12 % denatured polyacrylamide gel electrophoresis and subjected to Western blot analysis using anti-SMN antibodies (clone 8). MW = molecular weight. **(C)** The a-SMN81 clone and the TR4 counterpart were treated with TET for the indicated amount of time and protein extracts were analyzed by Western blot as in (B). The densitometric quantification of the a-SMN bands following normalization for actin is illustrated by the bar graph on the right. MW = molecular weight. **(D)** The a-SMN81 and TR4 clones were treated with or without TET for 24 hours and were analyzed by means of confocal IF after staining with anti-human a-SMN antibodies (No. 910) (left panels) and counterstaining with the nuclei-specific dye, DAPI (middle panels). Merged images are shown on the right.

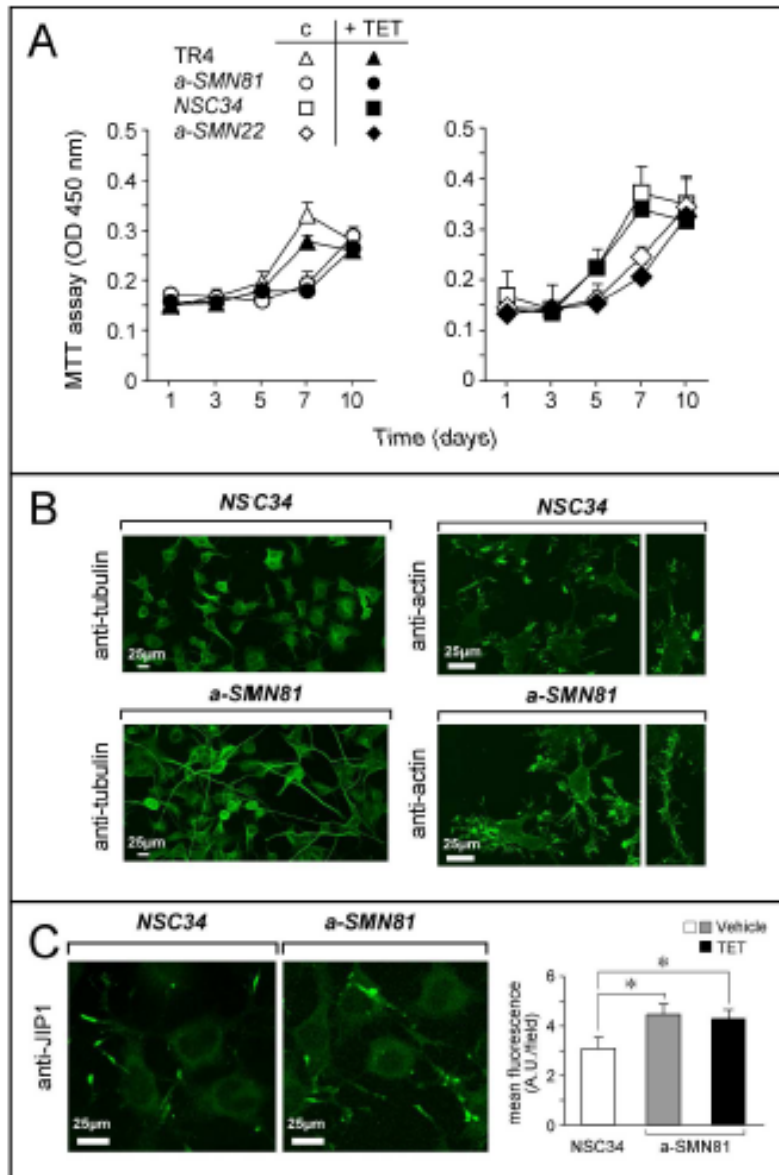


FIGURE 2

Fig. 2 Growth and morphology of the a-SMN expressing clones

(A) NSC34, TR4, a-SMN81 and a-SMN22 cells were grown in medium containing 0.5% serum for 3 days. Subsequently, cells were trypsinized and seeded at 20,000 cells/ml in medium containing 5% serum. Cell growth, in the presence/absence of TET, was followed using the MTT assay. Each value is the Mean+SD of three separate cultures. **(B)** and **(C)** NSC34 and a-SMN81 cells were grown in basal conditions. The morphological features of the cells were analyzed by means of confocal IF with anti- α - tubulin (B, left), anti-actin (B, right) and anti-JIP1 (C) antibodies. The bar-graph on the right shows quantification of the JIP-1 fluorescence results.

* Significantly different ($p < 0.05$, one-way ANOVA followed by the Bonferroni post hoc comparison test).

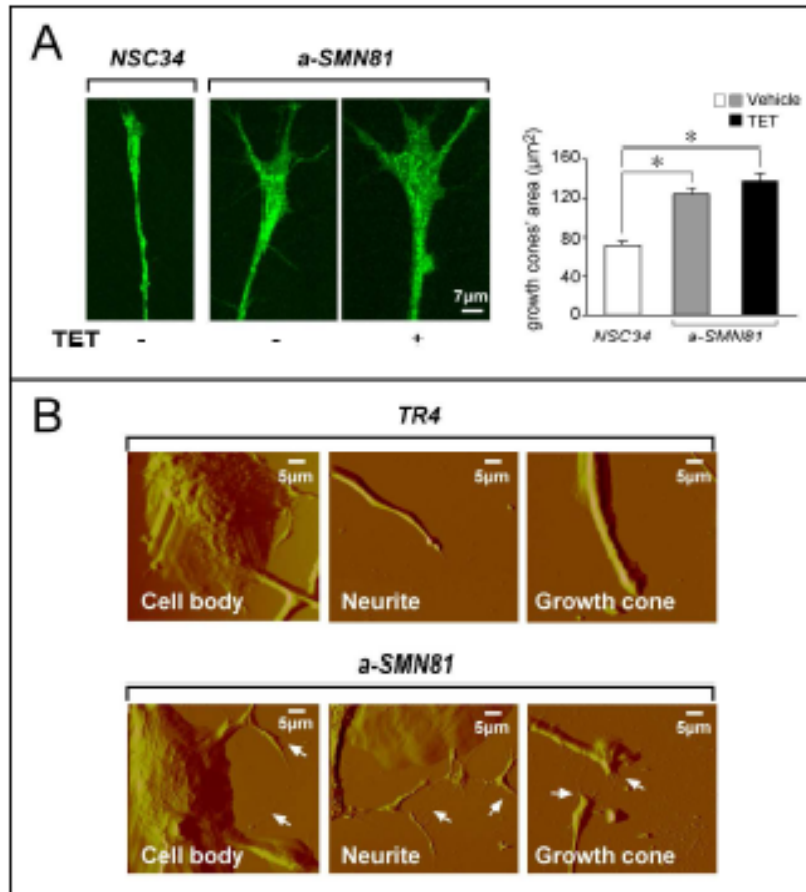


FIGURE 3

Fig. 3 Morphology of axons and growth cones in NSC34, TR4 and a-SMN81 cells
(A) To determine the surface area occupied by growth cones, NSC34 and a-SMN81 cells grown in the absence or presence of TET, as indicated, were stained for G-actin, for 15 min at room temperature. Confocal images were taken from at least 30 growth cones in triplicate experiments and analyzed with the Image-Pro Plus software. Left: Representative images. Right: Quantification of the growth cones' surface area. Data were analyzed by one-way ANOVA followed by the Bonferroni post hoc comparison test. * $p < 0.05$. **(B)** Representative atomic force micrographs of TR4 and a-SMN81 cells illustrate the morphological features of cell bodies, neurites and growth cones (arrows).

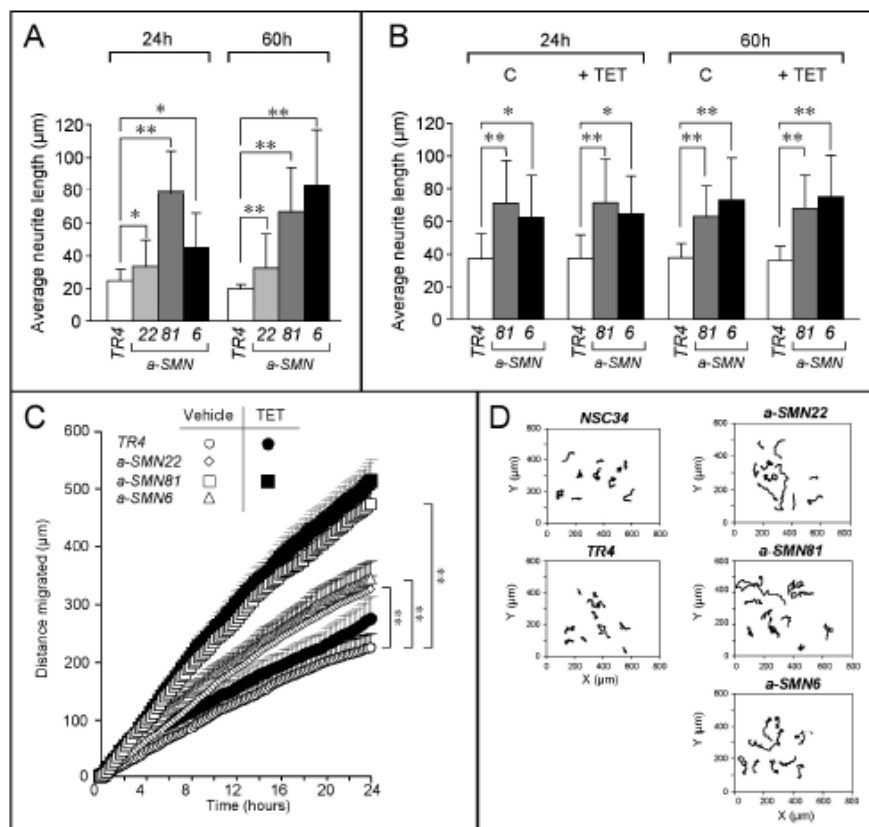


FIGURE 4

Fig. 4 *Axonogenesis and cell motility of the a-SMN expressing clones*

TR4, a-SMN6 and a-SMN81 clones were seeded at 3,000 cells/ml in the presence/absence of TET. Neurite length (after 24 and 60 hours of treatment) and random cell motility were determined by time-lapse microscopy. **(A)** and **(B)** Average neurite length were calculated on at least 40 individual cells per each experimental point. **(C)** Random cell motility was also calculated on at least 40 individual cells per each experimental point. **(D)** The migration pathway of ten representative cells/each group is illustrated.

** Statistically significant according to two-way ANOVA ($p < 0.01$); * Statistically significant according to two-way ANOVA ($p < 0.05$).

Fig. 5 *Transcriptome profiles of TR4, a-SMN22 and a-SMN81 cells and validation of the results* TR4, a-SMN22 and a-SMN81 cells were treated for 24, 56 and 72 hours with or without TET. Total RNA was extracted, labeled and used to hybridize whole-genome gene expression microarrays. **(A)** Upper: The panels (left = dose-dependent up-regulation; right = dose-dependent down-regulation) show the templates used for Pavlidis Template Matching (PTM) analysis which correspond to the a-SMN protein levels determined by Western blot (see Fig. 1C). Middle: The expression profiles of the genes significantly matching the templates ($p < 0.01$) are shown. Lower: The heat-maps of the same genes up or down-regulated in a-SMN81 and a-SMN22 clones are illustrated. Bottom right: A Principal Component Analysis (PCA) of the transcriptomes determined in TR4, a-SMN22 and a-SMN81 cells is presented (magenta = without TET; blue = with TET). **(B)** The relative mRNA levels of CCL2, CCL7, IGF1 and cMyc determined in the indicated a-SMN expressing clones, as well as NSC34 and TR4 control cells were validated by RT/Real Time PCR. The data are expressed in fold change relative to the NSC34 control taken as 1. The results were obtained from triplicate cultures and are expressed as the Mean+SD.

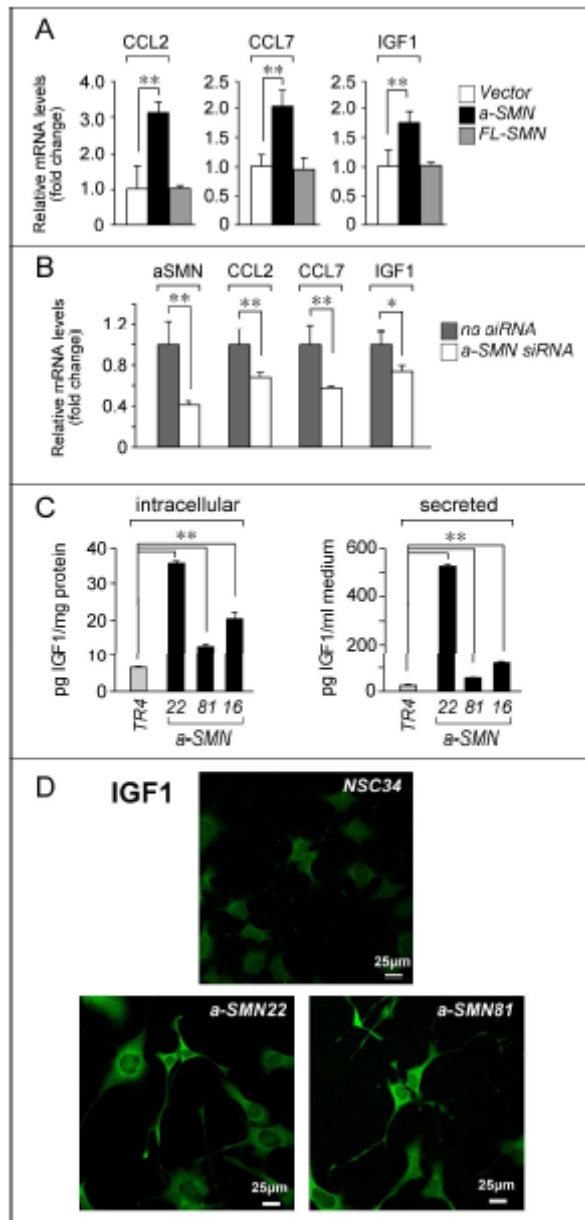


FIGURE 6

Fig. 6 *Regulation of target genes by a-SMN: a-SMN-dependent induction and sub-cellular localization of the IGF1 protein* **(A)** NSC34 cells were transiently transfected with a-SMN or FL-SMN cDNAs or with the void vector. After 48 hours cells were harvested and the total RNA was extracted. The levels of CCL2, CCL7 and IGF1 transcripts were measured by RT/Real Time PCR. The data are expressed in fold change relative to the control (vector) taken as 1. The results represent triplicate cultures and are expressed as the Mean+SD. **(B)** a-SMN6 cells were transfected with siRNAs specific for human aSMN or with scrambled control siRNAs (ns siRNA). After 72 hours, total RNAs were extracted and the levels of aSMN, CCL2, CCL7 and cMyc transcripts were measured. The data are expressed in fold change relative to the control (ns siRNA) taken as 1. The results represent triplicate cultures and are expressed as the Mean+SD. **(C)** The indicated a-SMN expressing clones as well as the parental cell line, TR4, were grown in DMEM supplemented with 5% serum to confluency. Medium was changed and, 24 hours later, the levels of the IGF1 protein in cell extracts and conditioned medium were measured with specific ELISA assays. **(D)** NSC34, a-SMN22 and a-SMN81 cells were grown and subjected to immuno-fluorescence staining with an anti-IGF1 antibody.

** Statistically significant according to Student's t-test ($p < 0.01$);

* Statistically significant according to Student's t-test ($p < 0.05$).

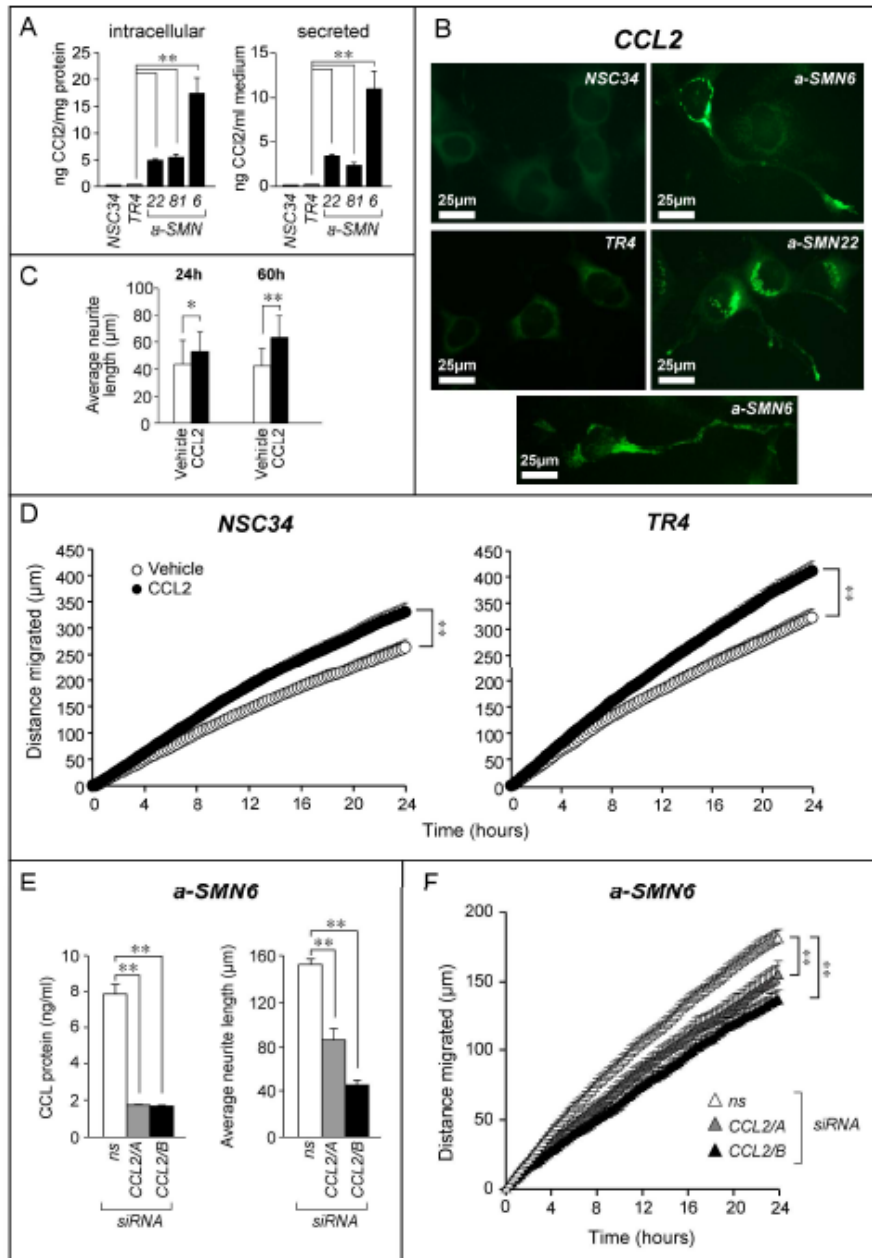


FIGURE 7

Fig. 7 *a*-SMN-dependent induction and sub-cellular localization of the CCL2 protein: control of axon growth and cell motility by CCL2. **(A)** The indicated a-SMN expressing clones as well as the parental cell lines, NSC34 and TR4, were grown to confluency in DMEM supplemented with 5% serum. Medium was changed and, 24 hours later, the levels of the CCL2 protein in cell extracts and conditioned medium were measured with specific ELISA assays. **(B)** NSC34, TR4, a-SMN6 and a-SMN22 cells were grown and subjected to immunofluorescence staining with an anti-CCL2 antibody. **(C)** NSC34 cells were treated in the presence/absence of CCL2 for the indicated amount of time. The average neurite length was determined. **(D)** NSC34 and TR4 cells were seeded at the density of 3,000 cells /ml in the presence/absence of CCL2. Random cell motility was measured after the indicated amount of time. **(E)** Cells (a-SMN6) were transfected with two different CCL2 targeting siRNAs (CCL2/A and CCL2/B) and a relative control siRNA (ns, 80 nM) in the presence of a GFP expressing plasmid (pEGFPN1, 10 nM). Left: CCL2 knock-down. Six hours after transfection, the culture medium was changed. Twenty four hours later, the amount of secreted CCL2 was measured in the conditioned medium of transfected cells. Each value is the mean+SD of three replicate cultures. Right: Axon growth. Forty eight hours after transfection with the siRNAs, cells were re-seeded for time-lapse analysis of axon growth. After 24 hours, the average neurite length of GFP-positive cells was measured. Each value represents the mean+SD of at least 20 cells. **(F)** Cell motility. The random cell motility of GFP-positive transfected cells was measured starting twenty four hours after re-seeding of transfected cells.

** Statistically significant according to two-way ANOVA ($p < 0.01$);

* Statistically significant according to two-way ANOVA ($p < 0.05$).

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CHAPTER 5

Region specific neuronal degeneration in a transgenic mouse model for SMA type II

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Manuscript in preparation

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease that represents the most common inherited cause of infant mortality with an incidence of around 1 in 10000 live births and a carrier frequency of 1:31 (*Prior et al., 2010*). The major pathological characteristic of SMA is degeneration of spinal motor neurons associated with a progressive atrophy of the limb and trunk muscles and, in severe cases, death. Based on the age of onset and disease severity, SMA has been classified into three main clinical types (I-III) that are all caused by homozygous loss or mutation of the Survival Motor Neuron 1 (*SMN1*) gene (*Lefebvre et al., 1995*). In humans, there are two *SMN* genes, the telomeric *SMN1* coding for an ubiquitous protein (full-length SMN or FL-SMN), and its centromeric homolog *SMN2* that generates mostly a protein lacking exon 7 ($\Delta 7$ -

SMN) that is thought to be not functional and rapidly degraded (Lorson *et al.*, 1999; Monani *et al.*, 1999; Gennarelli *et al.*, 1995). The *SMN2* gene does, however, produce a 10% of full-length protein, therefore, copy number of *SMN2* can modulate the severity of SMA (Vitali *et al.*, 1999; Wirth *et al.*, 2006). The 38 KDa FL-SMN protein is expressed in cytoplasm and nucleus and plays a critical role in snRNPs assembly and pre-mRNAs maturation (Pellizzoni *et al.*, 1998; Zhang *et al.*, 2008). However, SMN also localizes in motor neuron axons *in vivo* (Pagliardini *et al.*, 2000) and a more specific role of the protein in axons development was documented (Rossol *et al.*, 2003; Shafey *et al.*, 2008). Currently, the molecular and cellular mechanisms by which mutations of the *SMN1* gene are responsible for a selective neuromuscular system involvement remain unclear.

To gain insight into the pathogenesis of SMA, different mouse models of SMA were generated (Monani *et al.*, 2000; Le *et al.*, 2005; Li *et al.*, 2000); the most commonly used is the SMA Δ 7 mouse (Le *et al.*, 2005) that recapitulates human disease. These mice manifest early impairments of motor behavior correlated with a motor neuron suffering, and have a lifespan of about 2 weeks. Although understanding of the molecular biology of disease in the SMA Δ 7 mouse model has evolved, there are still discrepancies on the number and subgroups of spinal motor neurons selectively involved in SMA progression. Furthermore, morphological analysis of the brain in SMA Δ 7 mouse model and evaluations of a possible vulnerability of upper motor system have never been carried out.

In the present study we used this knockout transgenic mouse (SMN2^{+/+}; SMN Δ 7^{+/+}; Smn^{-/-}) to characterize the spinal cord and brain pathology during development. We report a progressive motor neuron loss in affected mice; this degeneration begins early in disease progression, but it's not evident at late embryonic stage, suggesting that gross prenatal development is largely unaffected by Smn depletion. We observe also that motor neuron loss is not generalized since only larger motor neurons in specific pools innervating proximal muscles result to be vulnerable to SMA. Finally, quantification of cell density in the motor cortex revealed a loss of pyramidal neuron specifically in layer V while the other cortical layers result to be spared, indicating that SMA related neuronal degeneration is not restricted to lower motor-system.

MATERIALS AND METHODS

Experimental animals

All the procedures involving animals were performed in accordance with the European Community Council guidelines (1986/609/EEC), and University of Torino's institutional guidelines on animal welfare (DL 116/92); mice had free access to food and water; efforts were made to minimize the number of animals used and their suffering.

The original breeding pairs of SMN Δ 7 mice (Smn^{+/-}; SMN2^{+/+}; SMN Δ 7^{+/+}) were purchased from Jackson Laboratory (stock number 005025; Jackson Lab, Maine, USA). The colony was maintained by

interbreeding carrier mice, and the offspring were genotyped by PCR assays on tail DNA according to the protocols provided by Jackson Laboratory.

Data were obtained from tissues harvested from SMA ($\text{Smn}^{-/-}$; $\text{SMN2}^{+/+}$; $\text{SMN}\Delta 7^{+/+}$) and WT ($\text{Smn}^{+/+}$; $\text{SMN2}^{+/+}$; $\text{SMN}\Delta 7^{+/+}$) mice sacrificed at embryonic stage (E19.5) and between postnatal day 4 (P4, pre-symptomatic stage) and P13 (final stage), considering P0 as the day of birth.

Histological examination

WT and SMA pups were deeply anaesthetized by intraperitoneal injection of 4% chloral hydrate (10 ml/Kg) and were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffer (0.1 M PB; pH 7.2). Brains were removed, weighed and immersed in fixative for 2 h at 4°C. Samples were transferred overnight into 30% sucrose in 0.1 M PB at 4°C for cryoprotection, embedded in cryostat medium (Killik; Bio-Optica, Milan, Italy) and cut on the cryostat (Microm HM 550). P4 and P9 brains were cut in serial coronal 20 μm -thick sections and mounted onto gelatin-coated slides to be processed for immunostaining.

P4, P13 and E19.5 cervical spinal cords were dissected and embedded in warm 6% Agar (Fluka BioChemika), the samples were cut on the vibratome in serial transverse 28 μm -thick (for the embryos) and 30 μm -thick sections (for the pups) respectively.

Histology, immunohistochemistry and confocal imaging

For light microscopy, spinal cord sections were blocked with 5% BSA, 1% Triton X-100 for 2 h at room temperature (RT), treated with 3% H₂O₂ to remove the endogenous peroxidase activity, rinsed three times in PBS and then incubated with goat polyclonal anti-Choline Acetyl Transferase (ChAT) antibody (Millipore; diluted 1:150 in 0.01M PBS, 3% BSA, 0.5% Triton X-100) over night at RT in a humidified chamber. After rinsing for 30 min in PBS, the sections were incubated with biotinylated donkey anti-goat IgG (Santa Cruz Biotechnology; diluted 1:200 in PBS, 3% BSA, 0.5% Triton X-100) for 2 h, rinsed for 15 min in PBS and then incubated with ExtrAvidin-peroxidase (Sigma; diluted 1:4000 in PBS, 3% BSA) for 1 h. Peroxidase staining was obtained by incubating the sections in 0.075% DAB and 0.002% H₂O₂ in 50 mM Tris-HCl pH 7.5. Section were air dried, dehydrated and coverslipped with DPX. The sections adjacent to the immunoreacted ones were counterstained with 0.1% thionin.

For the confocal images, the free-floating sections were pretreated with 4% sucrose in PBS, permeabilized 30 min in 100% cold methanol, blocked with 10% normal goat serum (NGS) in PBS with 0.2% Triton X-100 for 1 h at RT and then incubated overnight at 4°C with monoclonal mouse anti- Glial Fibrillary Acid Protein (GFAP) antibody (Millipore, diluted 1:500 in PBS, 1% NGS and 0.2% Triton X-100). After rinsing in PBS, the sections were incubated with biotinylated anti-mouse IgG (diluted 1:200 in PBS and 1% NGS) for 1 h, and then incubated with Cy2-conjugated Streptavidin (Jackson ImmunoResearch; diluted 1:200 in PBS, 1% NGS) for 1 h. After

repeated rinsing, the section were incubated in Neurotrace Fluorescent Nissl Stains (Molecular Probes; 1:200 in PBS), rinsed again and finally mounted on slides in water, coverslipped with Fluorsave (Calbiochem, Darmstadt, Germany) and examined on a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA).

Nissl Staining

Brain and spinal cord sections were stained for Nissl substance to perform cell counting. Sections were mounted on 2% gelatin-coated slides and air-dried overnight; slides were hydrated in distilled water for one minute before placing in 0.1% Cresyl violet acetate (Sigma Aldrich, Milano, Italy) for six minutes, dehydrated in an ascending series of ethanol, cleared in xylene and cover-slipped with Eukitt (Bioptica, Milan, Italy).

Spinal cord stereological counts

We have performed stereological counts at the cervical C3-C5 segment for pups and entire cervical tract for embryos, stained with cresyl violet (for each genotype: E19.5, n=3; P4, n=3; and P13, n=4) and immunoreacted for ChAT (for each genotype: P4, n=3; P13, n=4). For stereological counts on Nissl-stained sections, the nucleoli of the motor neurons in the ventral horns of the spinal cord were counted at 40X: only neurons with an area $\geq 80 \mu\text{m}^2$ (classified as alpha motoneurons) and located in a congruent position were counted (Vercelli et al., 2008). On the other hand, as concerns ChAT-immunoreacted sections, we counted all ChAT-positive neurons

located in the ventral horns. A total estimated number of alpha motor neurons was obtained with a stereological technique, the Optical Fractionator (*West et al., 1991*), by using a computer-assisted microscope and the StereoInvestigator software (MicroBrightField, Williston, VT, USA). Cells were counted on the computer screen using an Optronics MicroFire digital camera mounted on a Nikon Eclipse E600 microscope. From the reconstructed segment we have also obtained data relative to the volume of the ventral gray matter. In details at P4 we reconstructed one 30 μm -thick ChAT-positive section every five, and one 30 μm -thick Nissl-stained section every fifteen; on the other hand, at P13 we reconstructed one 30 μm -thick (both ChAT-positive and Nissl-stained) section every eighteen; moreover at E19.5 we reconstructed one 28 μm -thick (only Nissl-stained) section every eighteen. Additionally, in P4 and P13 mice, we have also analyzed the body size dimension of the ChAT-positive motor neurons present at C3-C5 level.

Spinal motor neuron pool analysis

Three cervical motor neuron pools (phrenic nucleus in C4, deltoid and biceps in C5-C6, forearm in C8-T1) were identified in sections immunoreacted with ChAT according to the Paxinos Spinal cord Mouse Atlas (*The Spinal Cord, Watson C.- Paxinos G., 2009*). Number of considered motor neuron pools varied according to the age and genetic background of mice. Sections were photographed with the Axioplan 2 Imaging microscope (Zeiss) and cell body areas measured on 40X magnification fields using Axiovision Rel. 4.8 (Zeiss). Mean

motor neuron area was calculated for each pool, and size distribution histograms (expressed in percentage) were constructed by grouping cell body cross-sectional areas in 100 μm^2 bins. This analysis was performed on P4 and P13 mice (n=3, n=4 respectively, for each genotype). Only cell bodies with clear nucleolus were measured in order to avoid double counting of the same cell.

Motor cortex analysis

Cortical sections at P4 (n=3 for each genotype) and P9 (WT n=4, SMA n=3) stained with Cresyl violet (see above Nissl staining protocol), were analyzed using the Neurolucida software program (Microbrightfield Inc., VT, USA).

Briefly, motor cortex (relative to one hemisphere) was identified and stereological cell counts were performed in two layers (layer II/III and layer V). The nucleoli were counted at 40X magnification: one 20 μm -thick section every five was reconstructed in a cerebral segment corresponding to plates 29, 36 and 39 of the Paxinos and Watson atlas (*Franklin and Paxinos 1997*). The density of neurons was obtained using Neurolucida Explorer (MicroBrightField, Inc.) for computer-aided microscopy (Microbrightfield Inc., VT, USA).

Moreover, we measured the neuronal soma size both in layer II/III and in layer V of motor cortex in random sampling (WT mice N=3; SMA mice N=3): we delineated the contours of 100 cells (showing the nucleolus and avoiding the smaller glial cells) with Neurolucida software and we analyzed the data with Neurolucida Explorer.

Astrogliosis quantification

To evaluate astroglial activation, GFAP immunoreactivity was analyzed. The density of immunopositive profiles was quantified by 2 blind observers. For quantitative analysis, we considered two areas, in the motor cortex and in ventral horns of C5 spinal cord, for each animal (WT mice N=3; SMA mice N=4) at P13. These areas were photographed at 40X magnification using Zeiss AxioCam MRc5 connected to the Axioplan2 Imaging microscope (Zeiss) and the fluorescence intensity, expressed in Arbitrary Units (AU), of the considered area was quantified using the ImageJ software.

Western Blot Analysis

Spinal cords were removed from P4 and P13 mouse after rapid decapitation and were immediately frozen in dry ice and preserved at -80° C until use. Samples were homogenized in buffer containing 20 mM Hepes (pH 7.4), 1 mM DTT, 0.1 mM PMSF and Complete (Roche, Indianapolis, IN). Protein extracts were separated by 12 % SDS-PAGE, electroblotted onto nitrocellulose membranes, probed with goat polyclonal anti- ChAT antibody (Millipore; diluted 1:150 in 3% non-fat milk) and analyzed with ECL. A monoclonal antibody against F-actin was used as loading control (Chemicon; 1:4000).

Statistics

Data are shown as mean $\pm$ SEM (standard error of the mean) and inter-group differences were statistically compared with unpaired

Student's t-test, two tails. Differences were considered significant when $p \leq 0.05$.

RESULTS

Time course of spinal motor neuron loss in SMA

To study the onset and progression of motor neuron loss in SMA Δ 7 spinal cord, we initially performed stereological counts on ChAT immunoreacted sections corresponding to the cervical C3-C5 region. We began our analysis on spinal cord at late and pre-symptomatic time points (P13 and P4 respectively). Even when observed at low magnification, the area occupied by ChAT-positive (ChAT+) motor neurons in the ventral horns of affected mice, results visibly smaller compared to WT mice, mostly at P13 (Fig. 1A). In detail, motor neuron counts, already at P4, showed slight inter-group differences; the total number of ChAT+ cells was reduced, but not significantly, in SMA mice by 30% (WT: 3024.50 ± 448.83 cells; SMA: 2117.63 ± 104.05 cells, $p=0.073$). Such differences significantly increased to 41% at P13 (WT: 4705.34 ± 630.67 cells; SMA: 2863.68 ± 280.27 cells, $*p < 0.05$, unpaired t-test) (Fig. 1B).

By western-blot analysis, we observed an increase of the ChAT expression between P4 and P13 (Fig. 1C) that could explain the unlikely increase of the motor neuron number during the days. Therefore, in addition to the ones previously described, we performed other stereological counts. In this way, at P4, we counted in WT

7703.20 $\pm$ 2173.38 motor neurons, and in SMA 6103.17 $\pm$ 323.51 motor neurons. Similarly to the previous results, at P13 differences result highly increased, showing a number of 7594 $\pm$ 1024.37 motor neurons in WT and 5293.83 $\pm$ 966.25 motor neurons in SMA mice (Fig. 2B). By using Nissl staining we did not observe any variance in motor neuron number between WT mice, confirming the need to use both staining methods to perform a more proper cell quantification. Additionally, as a consequence to the emerging differences already observed at P4, we decided to extend our analysis at late embryonic time point (E19.5) in order to investigate whether motor neuron degeneration is already present at pre-natal stage. The motor neuron counts performed in the entire Nissl-stained cervical segment did not reveal a decreased cell number or significant differences in ventral horns of SMA compared to WT mice (WT: 9097.08 $\pm$ 1439.59 motor neurons, SMA: 10404.12 $\pm$ 1018.96 motor neurons, $p > 0.05$, unpaired t-test) (Fig. 2B). Together, these data shown that in the cervical region, as well as already documented for the lumbar tract (*Le et al., 2005*), the motor neuron loss begins earlier than expected in SMA progression (before that muscular deficit becomes evident), even if the embryonic quantification excluded a possible aberrant motor neuron developmental process.

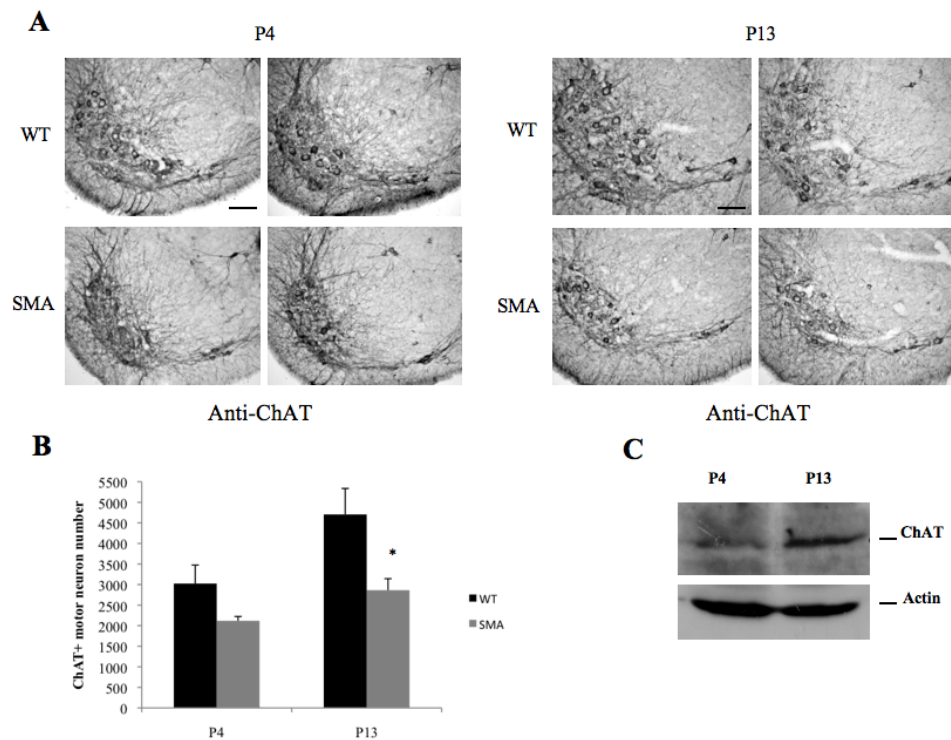


Fig. 1 (A) Coronal sections of ChAT positive motor neurons in the same cervical region of the spinal cord from WT and SMA mice at pre-symptomatic (P4) and final stage of the disease (P13). The area occupied by motor neurons in the ventral horns of SMA mice was clearly reduced if compared to WT. This difference is already evident at P4, but very pronounced at P13; (B) stereological counts of ChAT + motor neurons in C3-C5 tract of the spinal cord revealed that the total number was reduced by 41% ($p < 0.05$, $n = 4$ per genotype) in affected compared to WT mice at P13; at pre-symptomatic stage (P4) there is already 30% ($p > 0.05$, $n = 3$ per genotype) of reduction of ChAT + motor neurons in SMA mice; (C) Western-blot revealed the different expression profile of ChAT during development. Scale bar=100 μ m.

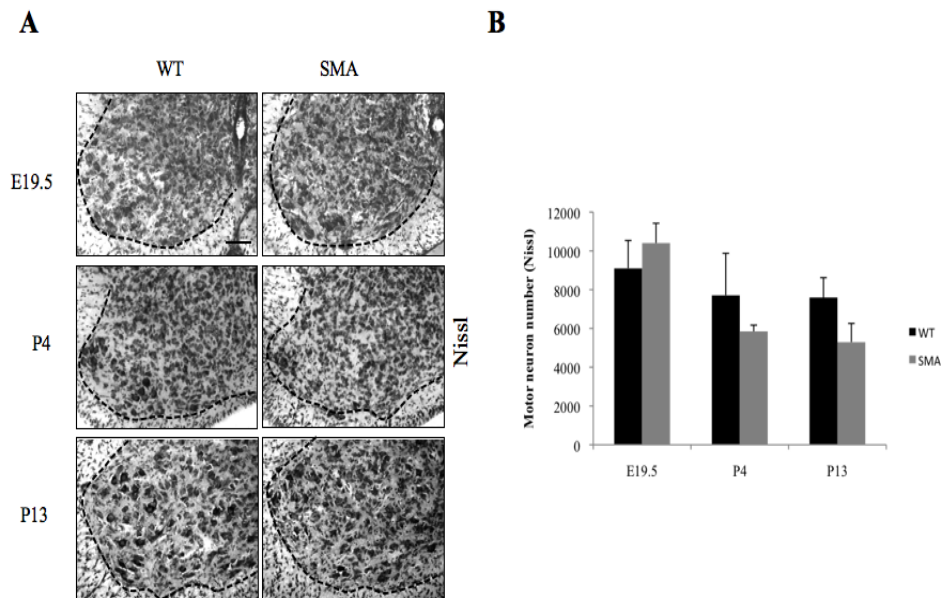


Fig. 2 (A) Nissl-stained sections of cervical spinal cord at different developmental stage (E19.5, P4 and P13); (B) stereological count of cervical motor neurons confirmed the cell loss observed with ChAT immunolabeling experiments at P4 and P13 time points, and did not revealed a decreased motor neuron number at pre-natal period. Scale bar= 100 μ m.

Loss of larger motor neurons in SMA

In the cervical C3-C5 segment, we have measured the ChAT+ motor neuron body size. At P4 and P13, motor neuron area was significantly reduced by 20% and 28%, respectively, in SMA mice as compared to WT (Fig. 3A). The average transverse area at P4 was: $394.70 \pm 4.73 \mu\text{m}^2$ in WT and $323.98 \pm 5.27 \mu\text{m}^2$ in SMA ($^{***}p < 0.001$, unpaired t-test), whereas at P13 it was: $437.92 \pm 13.13 \mu\text{m}^2$ in WT and $316.38 \pm 17.68 \mu\text{m}^2$ in SMA ($^{***}p < 0.001$, unpaired t-test). In order to understand whether reduced average area is due to a shrinkage of spared motor neurons or to a loss of larger ones, we calculated the percentage of soma size distribution. Both at P4 and P13, the number of larger motor neurons (cross-area $> 501 \mu\text{m}^2$) in SMA mice resulted significantly reduced compared to WT mice (Fig. 3D, 3E). In detail at P4, larger motor neurons were $20.54 \pm 1.54 \%$ in WT compared to $9.7 \pm 0.99\%$ in SMA ($^{**}p < 0.01$, unpaired t-test); at P13, WT larger motor neurons were $37.14 \pm 4.14\%$ compared to SMA: $8.73 \pm 2.33\%$ ($^{***}p < 0.0001$, unpaired t-test). These data, if considered together with the reduction of total motor neuron number, suggest that in the considered cervical tract, the difference in the average area between WT and SMA mice is correlated to a more selective degeneration of larger groups of motor neurons.

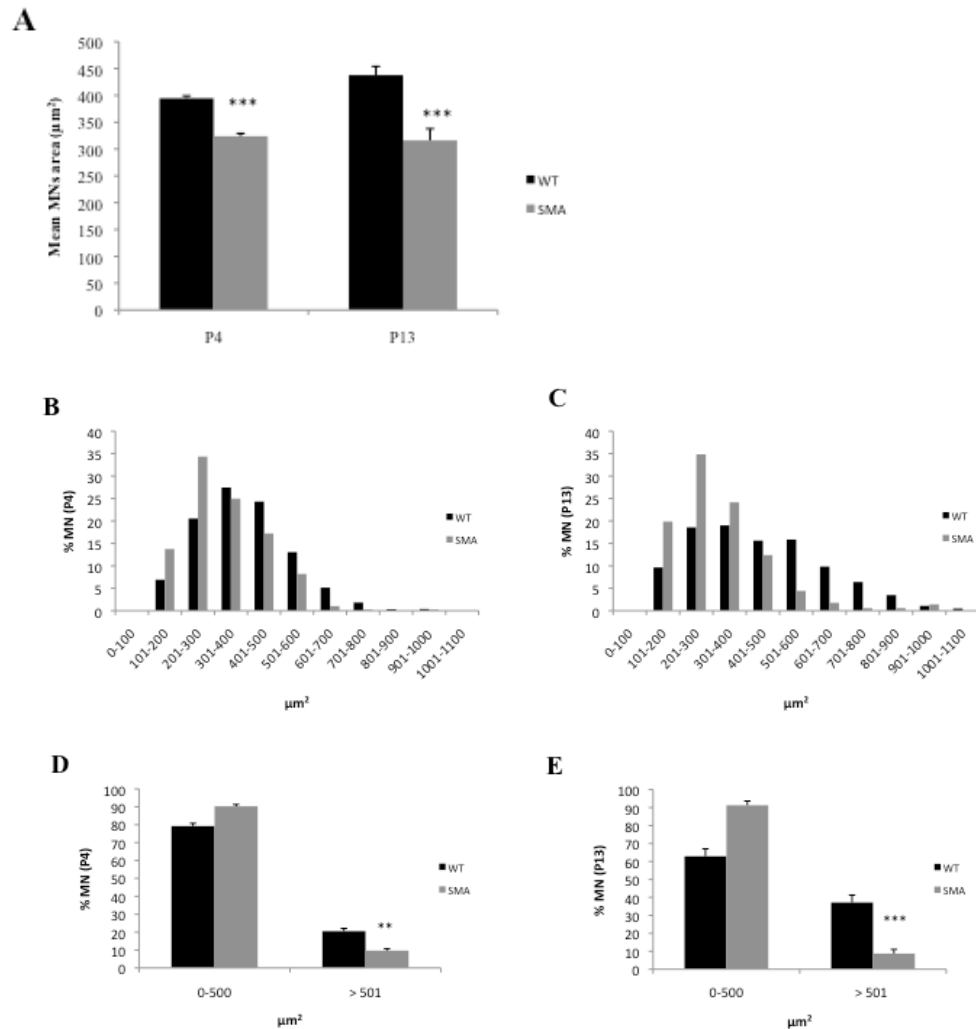


Fig. 3 (A) Quantitative analysis of motor neuron body size in C3-C5 spinal tract revealed an overall reduction of somatic area in both P4 and P13 affected mice (20% and 28% respectively, $p < 0.001$, $n = 3$ and $n = 4$ per genotype). The area reduction is probably correlated to degeneration of larger motor neurons; (B, C) Soma size distribution (bins= $100\mu\text{m}^2$) of affected mice showed a decreased number of the larger motor neurons; (D, E) the difference between WT and SMA mice become particularly significant for cells with area $> 501\mu\text{m}^2$.

Vulnerability of motor neuron sub-populations innervating proximal muscles

SMA is known to affect in particular proximal, axial and respiratory muscles. For this reason, we examined three specific motor neuron populations of the cervical region (Fig. 4A). By their location, we selected: (i) phrenic nucleus innervating diaphragm, (ii) C5-C6 lateral pools innervating forelimbs proximal muscles and (iii) C8 lateral pools innervating forelimbs distal muscles, in order to better understand, during development, which resulted more affected in SMA mice. In detail, to test whether the loss of the largest motor neurons, described above, took place in specific motor neuron sub-populations, we analyzed the mean area and frequency of body size distribution in each of these three pools at P4 and P13. We found that only in motor neuron population innervating proximal forelimb muscles there was a significant somatic area reduction in SMA mice compared to WT. This body size reduction was about 27% at P4 (WT: $526.51 \pm 18.36 \mu\text{m}^2$, SMA: $386.34 \pm 27.54 \mu\text{m}^2$, ** $p < 0.01$, unpaired t-test) and it reached 37% at P13 (WT: $599.68 \pm 23.90 \mu\text{m}^2$, SMA: $377.93 \pm 14.89 \mu\text{m}^2$, *** $p < 0.001$, unpaired t-test) (Fig. 4B). Furthermore, in this pool, the analysis of cell distribution revealed that, in SMA mice, the percentage of larger motor neurons (area $> 501 \mu\text{m}^2$) was significantly reduced compared to WT mice (Fig. 4C). This difference was already clear at P4, but much more evident at late symptomatic disease time point (39% and 55% loss at P4 and P13 respectively, * $p < 0.05$ and *** $p < 0.001$, unpaired t-test). In the other two motor neuron populations we found a slight reduction of larger

motor neurons that was not significant and there was no decrease of mean somatic area in SMA mice, both at P4 and P13 (Fig. 4B). This suggests that, in SMA progression, there is not a widespread degeneration of spinal motor neurons, but that specific sub-populations result more selectively involved.

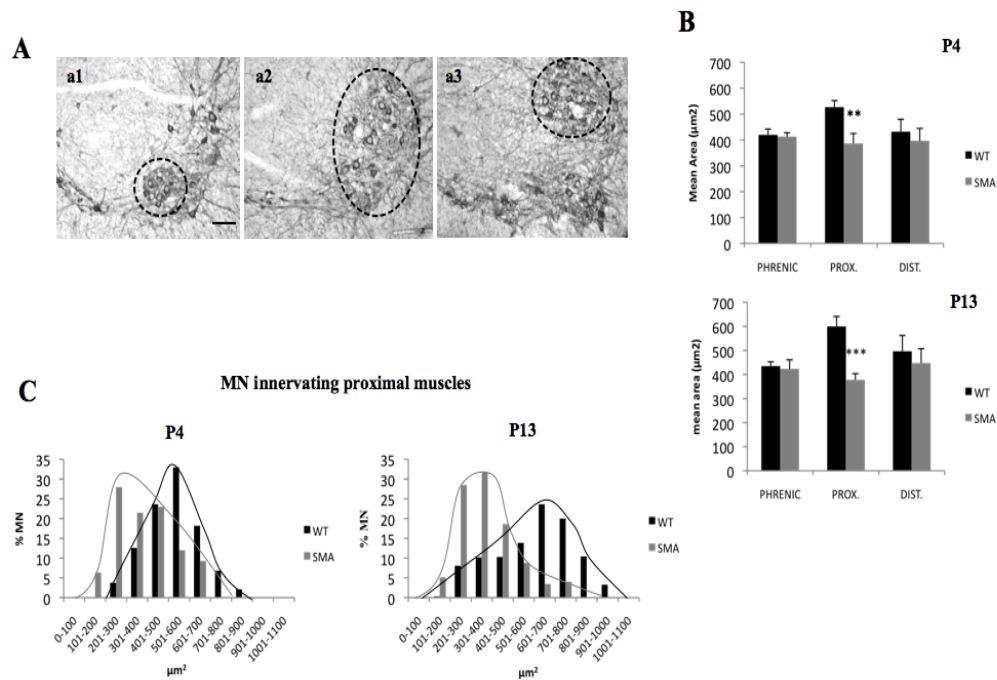


Fig. 4 (A) Chat immunoreactivity of serial cervical sections allowed to distinguish 3 specific motor neuron pools by their locations: the phrenic nucleus (a1) innervating diaphragm, C5-C6 lateral MN innervating proximal forelimb muscles (a2) and C8 lateral MN innervating distal forelimb muscles (a3); (B) measurement of mean area of the 3 different populations revealed that only in lateral pool innervating proximal forelimb muscles there was a significant reduction of mean somatic area both at P4 (upper) (reduction of 27%, $p < 0.01$, $n = 3$) and P13 (lower) (reduction of 37%, $p < 0.001$, $n = 4$). (C) body size distribution of motor neuron pools innervating proximal muscles revealed a clear loss of larger cells in SMA mice compared to WT.

Scale bar = 100 μm .

Pyramidal neuron loss in layer V of motor cortex

The effect of reduced levels of SMN protein was not yet studied in brains of SMA Δ 7 mice and a possible involvement of the upper motor system remains still unclear.

Therefore, we investigated at morphological level, the postnatal brain at pre- and late-symptomatic time points, focusing our assessment at the motor cortex level, specifically in the layers II/III and V. A first comparison of whole brains from WT and SMA mice, revealed a clear reduction in brain size and weight of affected mice at P13, whereas such difference was not evident at P4 (Fig. 5A and 5B). Stereological counts were performed on Nissl-stained coronal brain sections to quantify the cell density in motor cortex layers V (Fig. 5D) that projects efferent fibers directly to the spinal cord. At P4, the measured cell density in layer V was slightly reduced in SMA mice compared to WT, but not significantly (2896685.87 ± 533543.63 cells/mm³ in WT, 2278320.80 ± 220423.53 cells/mm³ in KO; $p > 0.05$, unpaired t-test); on the contrary, by P9 we observed a significant cell density reduction of 52% in affected mice (2219833.38 ± 252334.76 cells/mm³ in WT, 1056858.61 ± 216239.86 cells/mm³ in KO; $**p < 0.01$, unpaired t-test). However, at this age, the layers II/III did not present differences among the two groups and it seems to remain unaffected in SMA mice (data not shown), suggesting that distinct cortical layers were differentially susceptible in SMA progression. We also measured, at P9, the mean area of pyramidal neurons in layer V and II/III, and we observed a reduction in the soma dimension in both layers, but not significant as in the spinal cord (data not shown); in particular we

noted the lack of neurons with an area bigger than $100\text{-}110\text{ }\mu\text{m}^2$ (in layer V and $60\text{-}70\text{ }\mu\text{m}^2$ in layer II-III respectively, this probably reflecting the overall reduction of the brain size. These data suggest that low levels of SMN protein are not only associated with an impairment of lower motor neurons, but also that the upper motor system is involved in SMA progression, even though at a subclinical level.

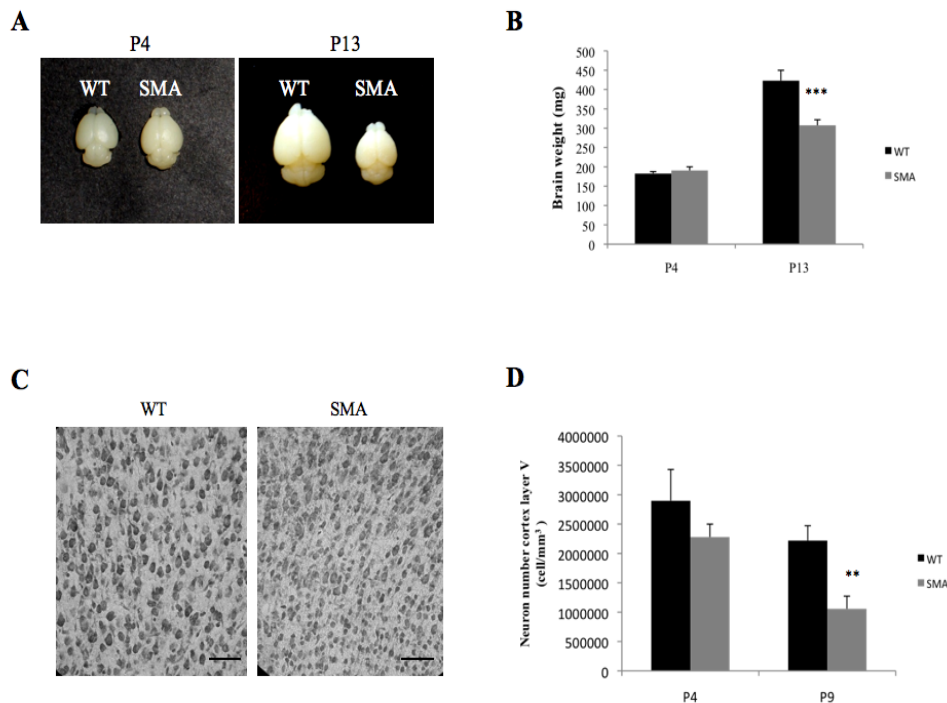


Fig. 5 Modified brain size and weight in mice with SMA. (A) Photograph of brains from P4 and P13 mice showed a clear size reduction of SMA brain at P13, while differences at P4 were not evident; (B) Bar chart showing brain weight from SMA and WT littermate at P4 (WT, n=5; SMA, n=4) and P13 (n=7 for each genotype). There was no difference between the two genotypes at pre-symptomatic stage, while we observed a weight reduction of about 30% at a late-symptomatic time point ($p<0.001$);

(C) Nissl-stained brain sections showing motor cortex layer V of WT versus SMA mice. Scale bar=50 μm ; (D) Stereological counts in Nissl-stained brain sections at P9 showed a significant decrease by 52% ($p < 0.01$, $n=3$ per genotype) of neuron density in layer V of P9 SMA mice. At P4 there was a cell density reduction but not statistically significant.

Astroglial activation in SMA mice

Finally, we evaluated the astroglial activation in the spinal cord and brain of affected mice in order to observe whether SMA disease is associated to a glial over-expression, a common feature in many degenerative disorders. We analyzed the astroglial activation on sections immunoreacted with GFAP, from P4 and P13 mice and the fluorescent intensity was quantified using ImageJ software (Fig. 6). In the brain and spinal cord of affected mice we did not observe astrogliosis at P4, but a slight astroglial activation started from P7 and gradually increased until P13, in which was massive (Fig. 6B and 6D, *** $p < 0.001$, unpaired t-test). Interestingly, the astrogliosis in the brain cortex resulted more intense along the layer V (Fig. 6C) of affected mice, supporting the evidences (on brain cortex) described above. On the other hand, in the spinal cord, the astrogliosis did not seem more intense in lamina IX, but it was extended both in dorsal and ventral horns. Astrocytes exert, in the central nervous system, trophic and support functions, and so their activation in SMA disease may represent a compensatory mechanism activated by neuronal suffering.

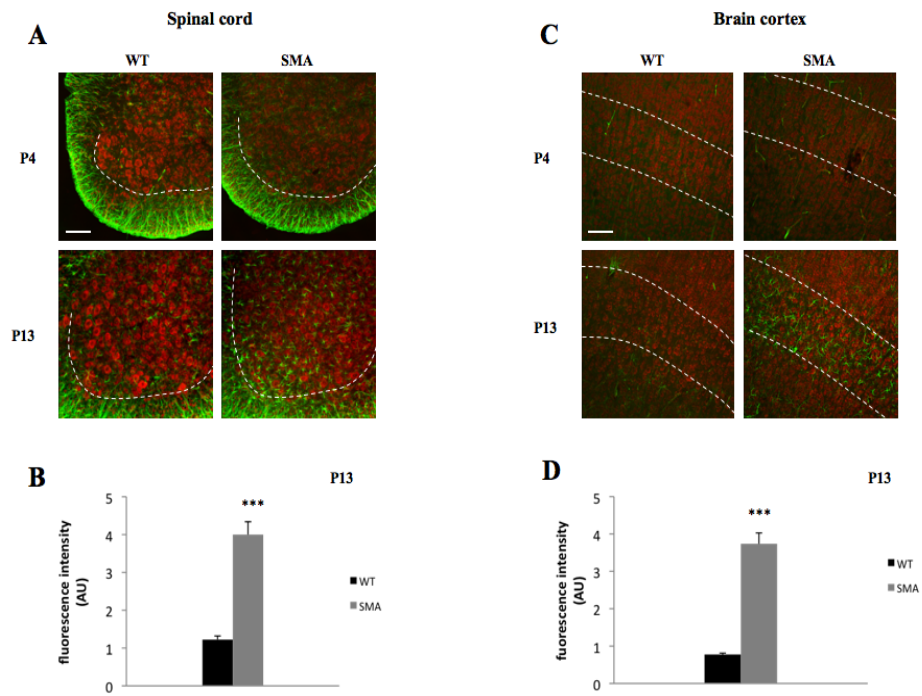


Fig. 6 Cervical spinal cord (A) and brain (C) from WT and SMA mice stained with Neurotrace (red) and reacted with anti-GFAP for glia (green): evidence of astroglial activation in SMA at P13 in both tissues. At P4 there were not evident signs of gliosis, thus suggesting a correlation with SMA progression; (B, D) quantification of fluorescence intensity (expressed in arbitrary units, AU) of immunolabeled spinal cord and brain sections at P13. Scale bar= 100 μ m.

DISCUSSION

In the present study we used the transgenic SMA Δ 7 mouse model (SMN2^{+/+}; SMN Δ 7^{+/+}; Smn^{-/-}) (Le et al., 2005) to document, at morphological level, the neuro-pathologic effects caused by reduced levels of SMN protein. First, we analyzed spinal cord from affected mice in order to accurately quantify the onset and progression of

motor neuron loss, and then to establish whether specific motor neuron groups were more vulnerable to SMA. Next, we extended our analysis to the brain, focusing our attention on motor cortex, to explore whether the upper motor system was also involved in SMA pathogenesis. In summary, at spinal level, motor neurons of affected mice showed three main pathological features: (i) severe motor neuron degeneration already present at pre-symptomatic stage (P4), but not detectable at pre-natal period (E19.5); (ii) vulnerability of specific motoneuronal sub-populations and (iii) cell death restricted to the larger cells. At brain level, the cell density quantification in different layers of motor cortex revealed a significant reduction of pyramidal neurons specifically restricted to the layer V in SMA mice at late-symptomatic stage, thus suggesting that (i) lower motor system is not the only neuronal compartment affected in SMA progression and that (ii) there is a differential susceptibility between cortical layers.

Spinal cord

In the spinal cord, we first counted motor neurons in the C3-C5 cervical tract at pre- (P4) and late-(P13) symptomatic stage extending then our analysis at pre-natal stage (E19.5). Compared to controls, SMA mice did not display any difference of motor neuron number at E19.5, thus suggesting lack of aberrant developmental defects. At P4, an approximately 30% of reduction was already present, while, between P4 and P13 we observed a further loss of motor neurons, but not so dramatic (10% more cell reduction). Taken together, these data suggest that in early perinatal days there was a rapid and more severe

cell death, followed by a period of stability characterized by a minor motoneuronal loss and, probably, by further systemic complications that develop later and could worsen the clinical phenotype until death (Heier *et al.*, 2010; Vitte *et al.*, 2004; Hua *et al.*, 2011). In our analysis stereological counts were performed by using two different approaches: immunohistochemistry experiments by using the specific motor neuron marker anti-ChAT, and Nissl staining. Together, these two methods allow a better quantification; because anti-ChAT selectively distinguishes motor neurons, but it is differentially expressed during development; while Nissl staining allows to compare neuron number at different time points, but it forces to establish a cut-off for motoneuronal counting, thus risking to exclude from the count some motor neurons or, *viceversa*, include wrong cells.

In SMA patients, proximal and axial muscles are the most severely affected. Analyzing specific motor neuron pools, we demonstrated that this muscular vulnerability reflected a differential susceptibility at the motoneuronal level. In SMA mice, lateral motor neuron populations innervating proximal fore-limbs muscles, resulted the most affected. In more detail, after quantification of cell body distribution, we observed that in these more vulnerable motor neuron pools there was a loss of the larger cells, while those smaller seem to be strikingly preserved. These results suggested that, in the spinal cord, there is not a widespread motor neuron degeneration; conversely specific pools are more affected and, interestingly, not all motor neurons in the same location, respond to reduced levels of SMN in the same way. Our results support and extend similar analysis performed

on lumbar spinal motor neurons and their neuromuscular junction (NMJ) (*Murray et al., 2008; Mentis et al., 2011; Ling et al., 2012*). In particular, *Murray et al.* described differential vulnerability between motor units innervating neighboring skeletal muscles and, based on NMJ defects, suggested that certain sub-populations innervating the same muscle may be preferentially affected in SMA mice. This hypothesis is now well supported by our results that show a more accurately distinction on which motor neurons are resistant and which more susceptible to SMA degeneration. Moreover, our study explains two main issues: the modest motor neuron reduction observed (30-40%) contrasting the severe motor phenotype of SMA Δ 7 mice, and discrepancies of motor neuron quantification between different studies, although performed on the same mouse model (*Le et al., 2005; Kariya et al., 2008; Passini et al., 2010*). However, additional studies are required to better identify the pattern of neuronal loss and to identify which classes of skeletal muscles (fast-synapsing or delayed-synapsing; *Sun et al., 2002*) are innervated by the vulnerable large cells. Mechanisms leading to a differential response of motor neurons to reduced SMN levels, remain elusive and controversial. Some studies, in fact, suggest that loss of cell soma from the spinal cord occurred subsequently to defects at the NMJs (*Chan et al., 2003; Boon et al., 2009; Murray et al., 2008*), that are characterized by nerve terminal loss and accumulation of neurofilaments at the motor endplate (*Cifuentes-Diaz et al., 2002; Lee et al., 2011*); on the other hand, a recent study indicates that the disruption of functional connectivity of sensory synaptic inputs on motor neurons occurred

prior to their loss (*Mentis et al., 2011*) and a selective vulnerability was demonstrated, with a primary deafferentation onto motor neurons innervating proximal muscles compared to those located more distally.

The reduction of mean motor neuron body size that we documented in SMA Δ 7 mice related to a loss of larger cells, indicates that in this severe model there is not an enlargement of spared motor neurons, observed instead in milder mouse models (*Fulceri et al., 2012*) and in some SMAIII patients (*Crawford and Pardo 1996; Vogt et al., 1997*). The pathological increase of motor neuron cell body in these mice is probably correlated to the delayed progression of the disease that may also explain the heterotopy of motor neurons, which are migratory and misplaced, in mild SMA mice and patients (*Fulceri et al., 2012; Simic et al., 2008*), never described in more severe models.

Brain

SMN low levels affect non-motor regions in the brain, such as the hippocampus, in a severe SMA mouse model (SMN2^{+/+};Smn^{-/-}) with a life-span of 6 days (*Wishart et al., 2010*). Here, we have quantified the cell density of upper motor neurons.

SMA shares several aspects with another motor neuron disease, the amyotrophic lateral sclerosis (ALS): indeed ALS is a neurodegenerative disease characterized by loss of motor neurons, denervation of target muscles, muscle atrophy and paralysis (*Bruijn et al., 2004*): in ALS both upper and lower motor neurons are affected (*Chou and Norris, 1993*). Even though, as generally reported in

literature, SMA patients “only showed decreased numbers of anterior horn cells in the spinal cord, with no change in the cortical pyramidal cells, including the Betz cells, and no degeneration of the pyramidal tracts” (*Huang and Luo, 1983*), by analyzing SMA Δ 7 murine brain, we have surprisingly detected that also upper motor neurons degenerate: in fact we observed a selective degeneration of pyramidal neurons of layer V of the motor cortex. Indeed in the II-III layer of the motor cortex of SMA mice we did not observed a reduction in the cell number, but only in the cell body dimensions.

Finally, we observed a strong glial activation both in the spinal cord and brain of affected mice at P13. In line with our findings, the astrocytosis in the motor cortex was more marked along the layer V in which there are pyramidal cells (upper motor neurons) that project their afferents directly to the spinal cord, and that resulted mostly affected. The astrocytic network plays an important role in synaptic function and plasticity by providing metabolic support to neurons and participating to the reuptake and release of some neuro-transmitters (*Henneberger et al., 2010; Rouach et al., 2008*). Astrocytes become reactive in response to several insults and their role in the pathogenesis of neurodegenerative disease is largely recognized. Therefore, astrocyte over-expression observed in SMA mice may indicate which regions (particularly in the brain) are more suffering and therefore require a greater trophic support.

We are aware that our observations are inconsistent with the human pathology features, as previously reported, but probably we have detected specific characteristic of the murine model. On the other

hand also human samples can show some specific features, such as the presence of heterotopic neurons in the spinal cord (*Simic et al., 2008*), that we did not observe in our samples.

Some authors tried to unravel the mechanism leading to motor neuron death: one hypothesis is that apoptosis occurs predominately during earlier stages of SMA, whereas in the later stages of disease prevails necrosis (*Simic et al., 2008*). However such mechanisms remain still elusive. Therefore in this context we can only speculate about the upper motor neuron fate. One hypothesis could be that the reduction of cortical motor neurons is a consequence of lower motor neuron death: if spinal motor neurons die first, upper motor neurons could consequently degenerate, losing their target. However at P9 in the motor cortex of affected mice we observed a cell density reduction of 52%, whereas not even at P13 spinal motor neurons result halved in SMA mice compared to WT. Therefore we cannot exclude that upper motor neuron death triggers spinal degeneration. For this reason in the future we intend to better examine these aspects, in order to clarify some pathological aspects and some molecular mechanisms of the disease.

Conclusion

In summary, the present study explored, in more detail, the effect of reduced levels of SMN protein in lower and upper motor neuron compartment, extending current understanding of SMA pathogenesis. Our findings complicate the SMA clinical picture, but provide also more details of regions and neuronal cells selectively involved in

SMA progression. Different promising therapeutic approaches are developing for SMA, and the ability to target specifically all cell types involved in SMA pathogenesis, may be fundamental to obtain a more efficacious therapeutic success.

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CHAPTER 6

Gene therapy approach by using AAV9 on SMA Δ 7 mouse model

INTRODUCTION

Spinal Muscular Atrophy (SMA) is the leading genetic cause of childhood mortality, characterized by progressive loss of spinal motor neurons leading to muscle weakness and ultimately to respiratory insufficiency causing death. The transmission of SMA is autosomal recessive with an incidence of 1:10000 and a very high carrier frequency of 1:31 (*Prior et al., 2010*). The biological cause of this devastating disease lies behind the lack of the Survival Motor Neuron gene 1 (SMN1) located in chromosome 5q13 (*Lefebvre et al., 1995*). Humans are the only species endowed with two copies of the *SMN* gene, *SMN1* and *SMN2*. The SMA severity is regulated depending on the number of *SMN2* copies present on their genome (up to 4 copies in total) (*Lefebvre et al., 1997*). The two *SMN* genes differ for a single base pair transition from C to T at the level of exon 7, which is responsible for a different ratio of alternative splicing between the full-length-SMN (FL-SMN) protein and the un-functional delta 7 protein (*Lorson et al., 1999; Cartegni & Krainer 2002*). The FL-SMN protein is expressed ubiquitously and constitutes the core of a ribonucleoprotein complex which exerts housekeeping functions such as the recycling of pre-mRNAs and spliceosome assembly (*Meister et*

al., 2001; Pellizzoni *et al.*, 2002; Gabanella *et al.*, 2007). A specific function of FL-SMN in axons may explain the specific motor neuron loss typical of SMA (Rossoll *et al.*, 2003; Oprea *et al.*, 2008). The role of SMN in axons has recently received further support with the identification, by our group, of an axonal form of SMN (a-SMN: Setola *et al.*, 2007). In comparison to the FL-SMN protein, a-SMN expression is largely restricted to motor neurons, with further sub-cellular localization in axons. In contrast to FL-SMN, forced expression of a-SMN stimulates motor neuron axonogenesis (Setola *et al.*, 2007). The a-SMN protein is mainly produced by the *SMN1* gene. All this indicates that a-SMN deficiency may play an important role in the pathogenesis of SMA. The role of a-SMN and its relevance to SMA are difficult to clarify due to the full sequence homology with FL-SMN (spanning from exon1 to exon3) and the limited expression during embryogenesis that restricts the possibility of *in vivo* studies.

In the past decade, recombinant adeno-associated viruses (AAV) have increasingly been recognized as potent vectors for gene therapy in the central nervous system, due to their neuronal tropism, stable transgene expression in quiescent cells, low pathogenicity and low rate of integration in the host genome. Successful phenotypic rescue of SMA transgenic mice by IV/ICV (intravenous; intra-cerebroventricular) delivery of FL-SMN expressing AAV viral vectors (Passini *et al.*, 2010; Foust *et al.*, 2010; Valori *et al.*, 2010; Dominguez *et al.*, 2011; Bevan *et al.*, 2010; Glascock *et al.*, 2011, 2012; Shababi *et al.*, 2012) has recently provided proof of principle for an effective gene-therapy approach to SMA. However, relevant issues still need to be solved.

First, complementation of FL-SMN deficiency does not fully restore the clinical phenotype. Second, successfully treated mice frequently present ear inflammation and necrosis, ocular swelling and inflammation, distal necrosis of the limbs: these defects can be so severe as to require sacrifice. Third, the most effective way of administration is still debated. Fourth, it is not entirely clear what cell types need to be targeted in order to obtain rescue of the phenotype. Motor neurons are obviously the most relevant cellular targets. However, AAV9-FL-SMN treated mice can die suddenly without evidence of motor impairment of a sufficient degree to explain death (*Glascocock et al 2011; see also Gogliotti et al 2012*). In the present study, we examined the ability of double-stranded (ds; or self-complementary, sc) AAV9-mediated gene therapy approach to deliver the FL-SMN or a-SMN proteins to spinal cord motor neurons and tested their ability to rescue the phenotype of the SMA Δ 7 mice, the most commonly used SMA mouse line, mimicking human SMA type-II phenotype (*Le et al., 2005*). We explored two routes of delivery in order to obtain the most precise targeting to neurons, intravascular (IV) and intracerebroventricular (ICV). Only the ICV injections resulted in a high level transduction of spinal cord and brain leading to a substantial increase of the lifespan of the SMA Δ 7 mice, thus shedding light on the relevance of the choice of the delivery route when considering therapeutical approaches in humans patients.

RESULTS and DISCUSSION

Intravascular delivery of FL-SMN and a-SMN proteins does not alter disease course of SMA $\Delta 7$ mice

In a first attempt to deliver FL-SMN and a-SMN proteins to motor neurons of SMA $\Delta 7$ mice (SMN2^{+/+}, Smn $\Delta 7$ ^{+/+}, Smn^{-/-}), we chose the intravenous (IV) route. Newborn SMA mice (P1) were genotyped and then IV injected at P2 with double-stranded AAV serotype 9 for CMV driven expression of human FL-SMN or human a-SMN (dsAAV9/FL-SMN, n=5; dsAAV/a-SMN, n= 5) and compared to control (n=5) and SMA uninjected (n=6) groups. SMA neonates were positioned over a trans-illuminator to reveal the temporal/facial vein, and injected with a sterilized fine glass micropipette connected to a 5 ml syringe by a tubing system. Approximately $5.5-8 \times 10^{10}$ vps (viral particles), for both FL-SMN or a-SMN, in a final volume of 30 μ l were administered in a single injection. This strategy of administration was able to induce the expression of FL-SMN or a-SMN proteins in peripheral tissues (liver, heart), but not in the CNS (brain and spinal cord), as demonstrated by our western-blot data (Fig. 1). However, neither scAAV9/FL-SMN nor scAAV9/a-SMN were able to extend survival (Fig. 2A) of SMA mice, or ameliorate their motor phenotype, as shown by performing the “righting-reflex test” (Fig. 2B). SMA treated mice remained significantly smaller than controls and showed weight loss starting from P9-P10, as in untreated SMA mice (Fig. 2C). We also checked expression of FL-SMN and a-SMN proteins at the morphology level by means of IF, but this strategy was insufficient for

targeting motor neurons (or other CNS cell types as well). It is possible that failure in rescuing (particularly for FL-SMN) is related to the insufficient titer of our viral preparation, given the different results obtained by different groups using the same strategy of administration.

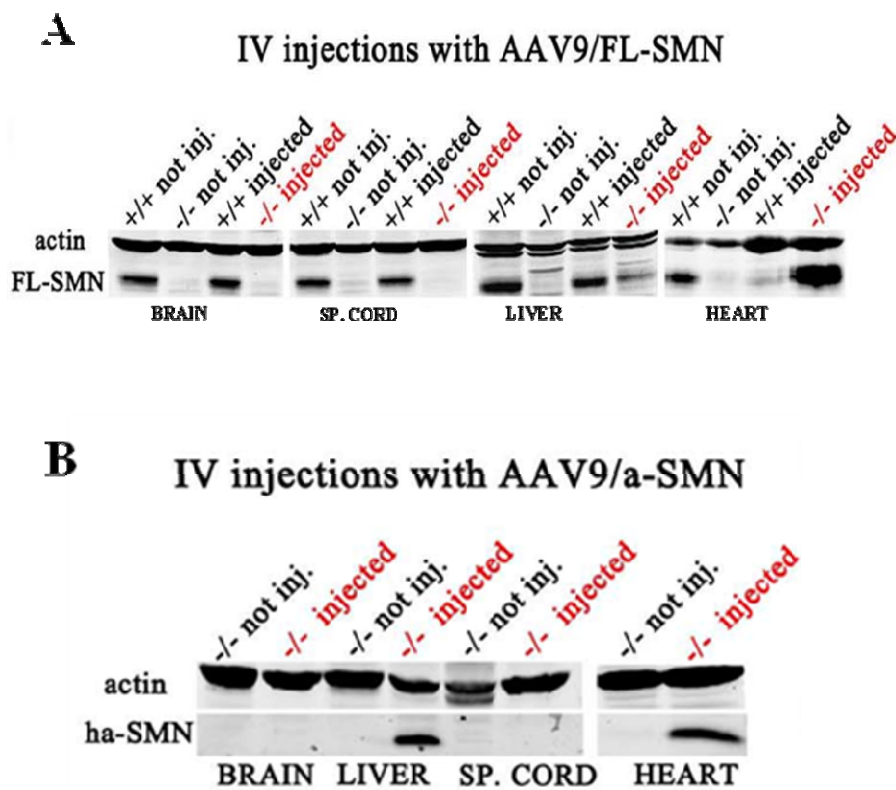


Fig.1 Western blot experiments revealed expression of FL-SMN (A) or a-SMN (B) proteins in peripheral tissues (liver and heart), but not in the CNS (brain and spinal cord) in IV treated SMA Δ 7 mice at P13-P15.

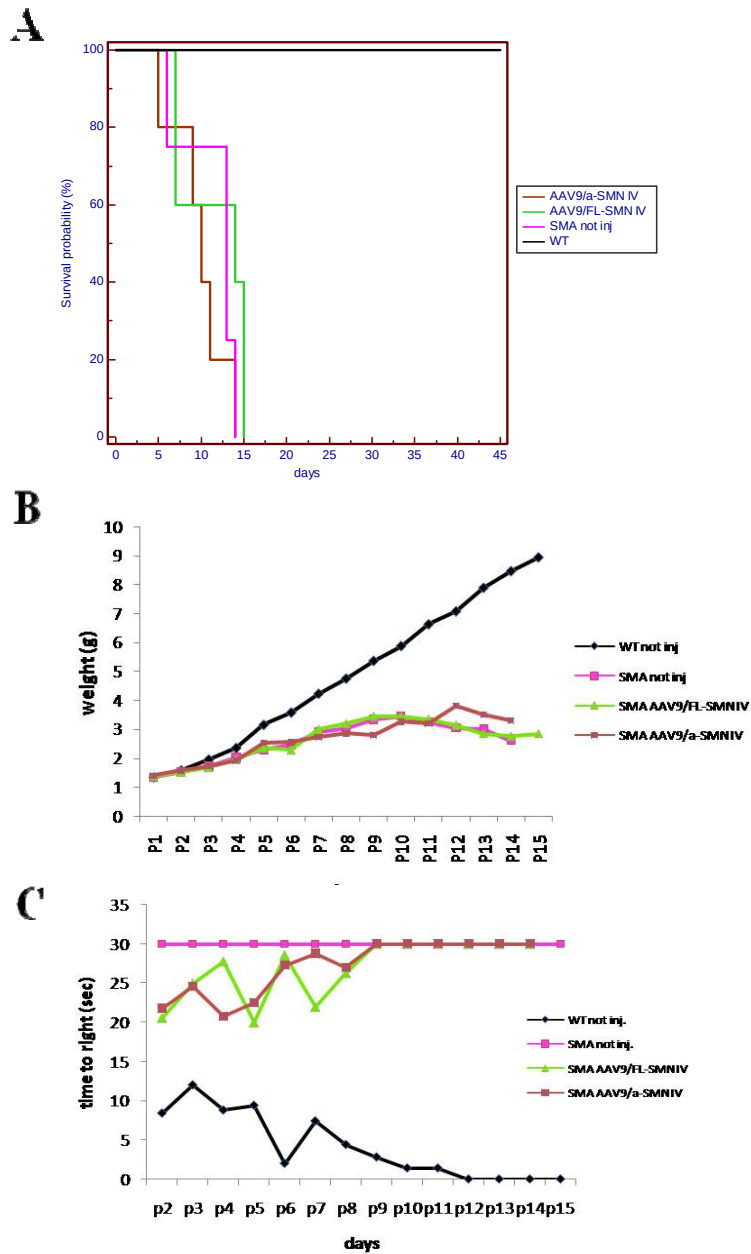


Fig. 2 (A) Kaplan-Meier survival curve of WT (n=5), untreated (n=6) and IV treated (n=5) SMA animals with either FL-SMN or a-SMN isoforms; neither scAAV9/FL-SMN nor scAAV9/a-SMN were capable of extending survival of affected mice. (B)

Injected mice remained significantly smaller starting from P4 and did not gain weight if compared to SMA untreated mice. (C) Righting-reflex test showed an impairment of motor functions in IV treated SMA mice comparable to untreated SMA mice, with both viruses.

ICV delivery resulted in high CNS levels of SMN transduction and prolonged lifespan of scAAV9/FL-SMN injected SMA mice in a dose-dependent manner

In order to transduce neuronal compartment, we decided to physically overcome the blood-brain barrier focusing on the ICV administration. In all injections, SMA pups were placed under cryo-anesthesia, positioned over a trans-illuminator to reveal the anatomical structure for administration into the lateral ventricle, and injected with a sterilized fine glass micropipette. Considering the failure in rescuing the SMA phenotype of our IV experiments and the volume restrictions of the ICV technique, a series of four injections per animal were performed. Two different doses of scAAV9/FL-SMN (total viral load of 5×10^{10} and 2×10^{11} vps) and one dose of scAAV9/a-SMN (total viral load 4×10^{10} vps) were injected into the lateral ventricles of SMA affected mice (scAAV9/FL-SMN: n=4 low dose, n=4 high dose; scAAV9/a-SMN: n=4). Final volume of 24 μ l were administered in 4 injections, 2 at P2 and 2 at P3.

First, SMN protein levels were examined at P13-P15 in disease relevant tissues. Following the ICV injection directly into the brain, both SMN proteins were still highly expressed in peripheral tissues (liver and heart), but also dramatically increased in the brain and spinal cord if compared to IV injections (Fig. 3). In more detail, we

analyzed, at morphological level, the expression of both SMN proteins in the spinal cord and brain in order to examine which neuronal regions and cell types were specifically targeted by the virus. In the spinal cord, immunofluorescence experiments clearly indicated that motor neurons were preferentially target (Fig. 4). We found motor neuron labeling throughout the spinal cord (i.e., at cervical, thoracic, and lumbo-sacral levels), although the number of labeled motor neurons varied at the different spinal levels in the different mice considered. We evaluated, through double-labeling with SMN and ChAT (specific motor neuron marker) antibodies, that the percentage of transduced motor neurons in spinal cords of WT mice was up to 70% for AAV9/FL-SMN and 40% for AAV9/a-SMN. In brain, different regions were targeted by the virus (Fig. 5). In particular, we noted an high SMN expression in cortex (cingulated, piriform and entorhinal cortex, Fig. 5A-D) and in the hippocampus CA2. Furthermore, some nuclei in brainstem resulted transduced by the virus such as facial (Fig. 5E) and hypoglossal nuclei. Interestingly, a diffuse a-SMN immunostaining pattern consistent with glial cell morphology was observed in brain of injected SMA mice at P12 and double labeling of ha-SMN and GFAP confirmed that the majority of the AAV9/a-SMN-transduced cells were astrocytes (Fig. 5G). In contrast FL-SMN expression, after scAAV9/FL-SMN injections resulted in distinct cell bodies with neuronal morphology (Fig. 5A, B), while a restricted number of transduced cells resulted to be astrocytes. This different expression pattern may be correlated to a different tropism of the virus, but every possible explanation remains elusive

yet. In neurons, FL-SMN accumulates in granules mostly inside the cell body, while a-SMN has a diffuse pattern and accumulates along the dendritic and axonal extensions, confirming our *in vitro* observations.

As shown in the Kaplan-Meier curve (Fig. 6A), only the ICV injection of scAAV9/FL-SMN was able to extend survival (at present, up to 70 days) and ameliorate the motor phenotype, while the disease course of scAAV9/a-SMN injected mice was comparable to uninjected SMA mice (Fig. 6C). However, further studies are necessary before confirming the ineffectiveness of a-SMN; in fact the lack of a phenotypic improvement may be due to an insufficient titer of the virus or to a synergistic role between the two SMN proteins that require a co-infection of AAV9/a-SMN with AAV/FL-SMN.

All mice treated with scAAV9/FL-SMN gained more weight than the SMA controls, but they remained significantly smaller than the WT animals (Fig. 6D); some treated mice started losing weight progressively at a certain time point after injections. Animals were sacrificed for morphologic analysis when the weight curve was predictive of forthcoming death.

In the righting reflex test, that measures overall muscle strength and coordination, AAV9/FL-SMN -treated mice showed a significant improvement that was normalized by days 12 (Fig. 6C) and a visible improvement in their spatial-locomotive function. Two doses of scAAV6/FL-SMN (see above) were injected into the brain of SMA affected mice resulting in amelioration of the disease course in terms of lifespan (Fig. 6A) and body weight (Fig. 6D). In fact, mice injected

with low doses of virus had a mean lifespan of 30 days while the high concentration of virus extended survival beyond 70 days, thus suggesting the importance in delivering the sufficient amount of virus in order to obtain beneficial effects.

Interestingly, even at the time of sacrifice treated mice showed relatively preserved motor function: they were still easily ambulant in their cages, with some evidence of clasping when suspended through their tails. Therefore, they seemed to die not simply as a consequence of motor impairment. The surviving treated mice were weaned at 35-40 days (weaning of the littermates was regularly performed at 3 weeks), and food-assisted with water-wet pellets to facilitate mastication, throughout the rest of their life. In our study, all treated mice developed initial signs of tail necrosis between P25-P30, then a rapid process of necrosis occurred also in ear pinna and hind paws (Fig. 7). Moreover treated mice showed inflammation of the eyelids (uni- or bi-lateral) and sometimes dental defects that did not allow to chew properly (Fig. 7D). Death of SMA-scAAV6/FL-SMN mice in the first part of the survival curve occurred unexpectedly and was often associated with a decrease in body mass. For the long-surviving mice, respiratory distress symptoms (labored breathing and dyspnea) and severe necrosis of distal tissues were interpreted as a clear aggravation of the clinical course and therefore animal euthanasia was required for ethical reasons.

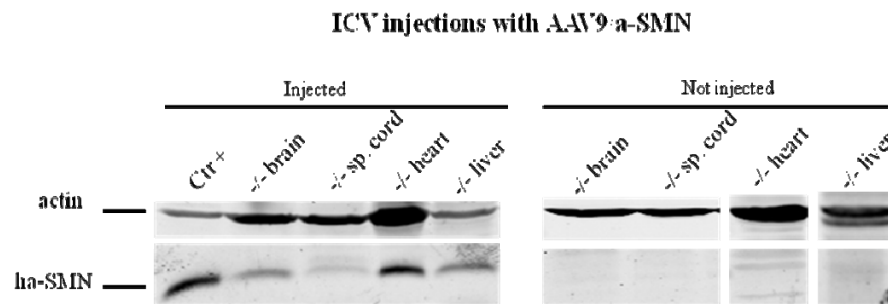


Fig. 3 Western-blot analysis revealed that protein expression (showed above only a-SMN protein) was increased also in brain and spinal cord tissues following ICV treatment with scAAV9, compared to the complete absence of a-SMN protein in untreated SMA mice. High protein expression levels were found in extra-neural tissues (liver and heart).

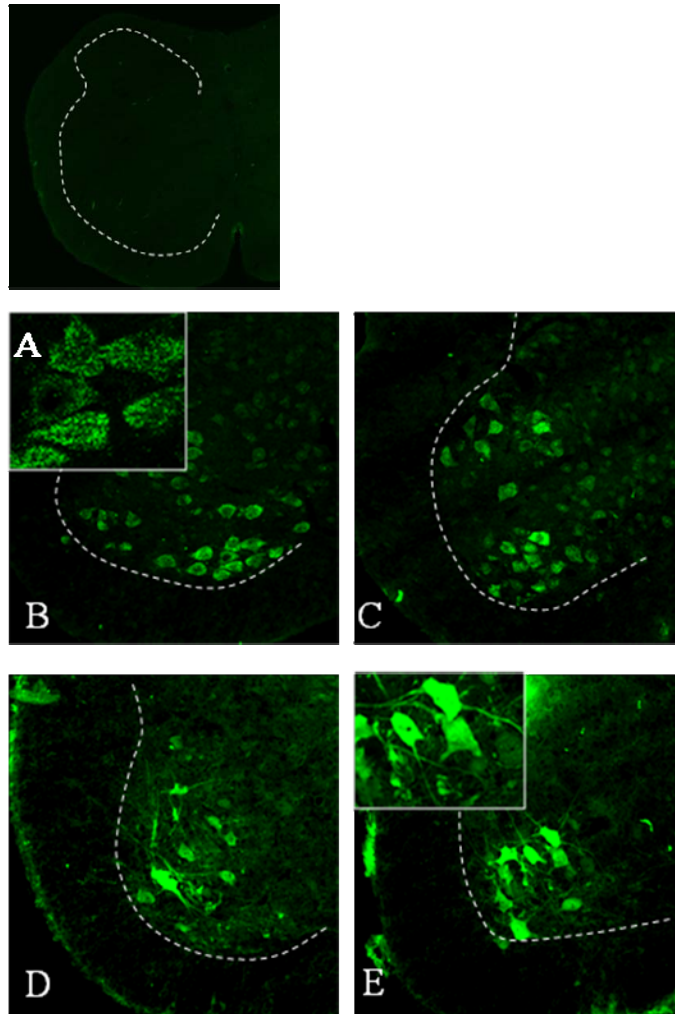


Fig. 4 Immunolabeling of cervical (B, D) and lumbar (C, E) tract of spinal cord with FL-SMN (B, C) or a-SMN (D, E) antibodies showed that a subset of motor neurons were transduced by scAAV9/SMN after ICV treatment. High magnification shows the punctuate pattern of FL-SMN protein inside the cytoplasm and nucleus of motor neurons (inset in B), while a-SMN localizes, with a diffuse staining, in cell bodies and in dendrite extensions (inset in E).

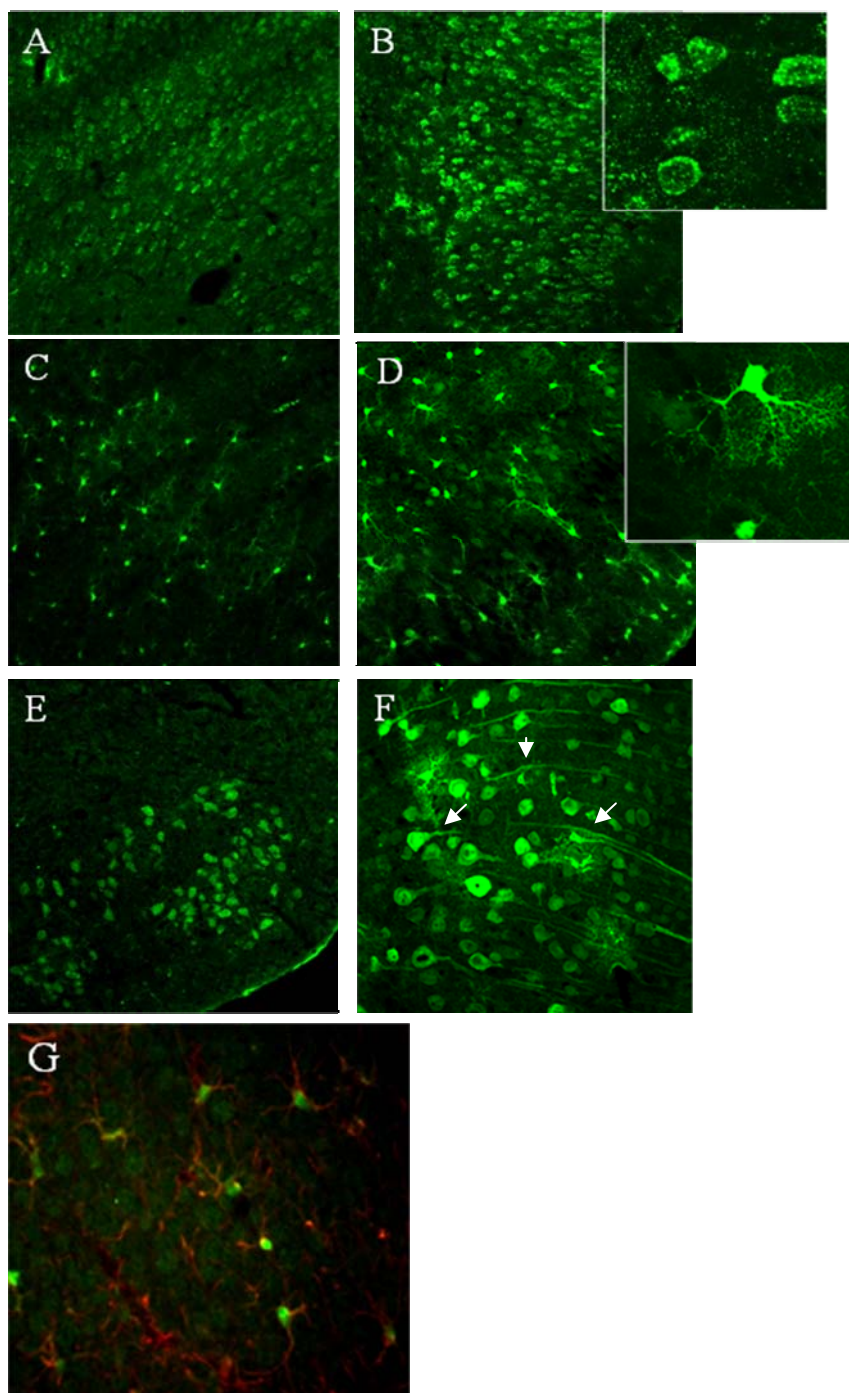


Fig. 5 Immunolabeling of different brain regions of ICV injected mice with AAV9/FL-SMN (A, B) or AAV9/a-SMN (C-F) showed a virus tropism for different regions of the brain. In particular, brain cortex (A, C= cingulated cortex; B, D= piriform cortex) resulted highly transduced by the virus and neurons of the facial nucleus (E). In brain cortex, a diffuse a-SMN immunostaining pattern consistent with glial cell morphology was observed (G), but in transduced cortical neurons, a-SMN was particularly expressed along the axons (F, arrows). By contrast, scAAV9/FL-SMN mainly targeted neurons.

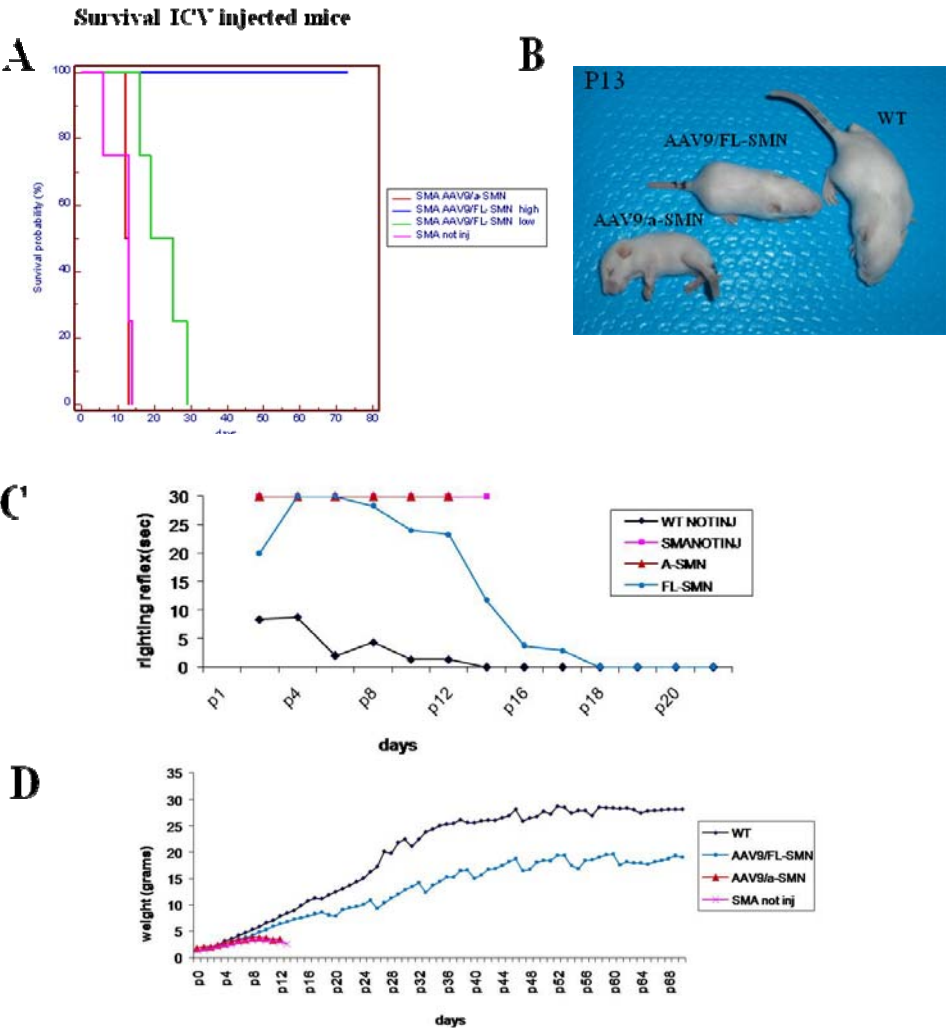


Fig. 6 (A) Survival curve of untreated (n=4) and ICV treated SMA mice (n=4) showed a significant extension of survival in mice injected with AAV9/FL-SMN in a dose-dependent manner. (B) P13 mice showed rescue of phenotype after AAV9/FL-SMN injections compared to AAV9/a-SMN injected and WT untreated mice. AAV9/FL-SMN treated mice performed significantly better than untreated SMA mice on the righting reflex test (C), and were heavier than untreated SMA controls from day P11 and onwards (D).

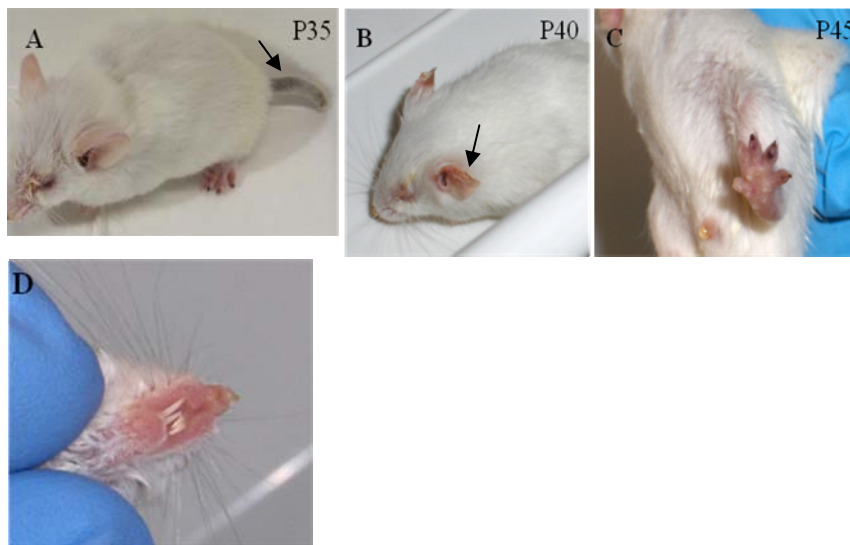


Fig. 7 From P30, injected mice developed necrotic peripheral tissues; in particular tail (A), ear pinna (B) and hind-paws (C) were particularly affected. (D) Sometimes treated mice developed dental defects.

METHODS

Recombinant AAV vectors production and titration.

The FL-SMN and a-SMN coding sequences have been cloned in a AAV vector carrying a CMV enhancer, a modified chicken b-actin promoter and bovine growth hormone polyadenylation signal. The AAV viruses with serotype 9 capsid have been produced by transfecting in HEK293 cells the AAV specific vector, the rep/cap-plasmids p5E18-VD2/9 and the pAdDF6, a plasmid construct carrying all essential Ad helper genes, in a molar ratio of 1:1:2. At 48 h after transfection, cells were collected by scraping, and lysed by freeze-and-thaw cycling. Virus particles were purified through isopycnic CsCl gradient centrifugation. We obtained a final titer of 2.56×10^{12} /ml and 7.2×10^{13} virus particles (*scAAV9/FL-SMN*) and 1.80×10^{12} /ml virus particles (*scAAV9/a-SMN*) determined by quantitative polymerase chain reaction of DNase I-treated stocks.

Animal handling and genotyping

All the procedures involving animals were performed in accordance with the European Community Council guidelines (1986/609/EEC); mice had free access to food and water; efforts were made to minimize the number of animals used and their suffering. The original breeding pairs of SMN Δ 7 mice (*Smn*^{+/-}; *SMN2*^{+/+}; *SMN Δ 7*^{+/+}) were purchased from Jackson Laboratory (stock number 005025; Jackson Lab, Maine, USA) and the colony was maintained by interbreeding carrier mice. The day of birth was counted as P1 and the neonates were genotyped

within 24 h by PCR assays on tail DNA according to the protocols provided by Jackson Laboratory.

In vivo injections

For intravascular (IV) injections, 30 µl of virus were injected at P2 in the facial/temporal vein by using a sterilized fine glass micropipette connected to a 5 ml syringe by a tubing system. Approximately $5.5\text{--}8 \times 10^{10}$ vps, for both FL-SMN or a-SMN were administered in a single injection. For intracerebroventricular (ICV) administration, SMA pups were placed under cryo-anesthesia, positioned over a trans-illuminator to reveal the anatomical structure and injected with a sterilized fine glass micropipette. A series of four injections were given to obtain a final titer of 5×10^{10} , 2×10^{11} vps (AAV9/FL-SMN, low and high titer respectively) or 4×10^{10} vps (AAV9/a-SMN) in a final volume of 24 µl administered with two injections at P2 (AM and PM) and two at P3 (AM and PM).

Animal behavior and motor function analysis

Mice were daily monitored for body weight and their general conditions (hind-limb clasping, necrosis) . Righting reflex was used as a pre-weaning test and performed every day from P2 to P20. Mice were placed on their back and the ability to turn themselves and stand on the four paws was measured over 30 seconds of time.

Histological analysis

Mice were deeply anesthetized with intra-peritoneal injection of 4% chloral hydrate (10 ml/Kg) and transcardiacally perfused with saline

followed by 10 ml of 4% paraformaldehyde (PFA 4%). Spinal cord and brain were post-fixed in 4% paraformaldehyde for three hours (hrs) at room temperature (RT), then moved into a 30% Sucrose solution and left overnight at 4°C. The next day tissues were mounted on dry ice in cryoembedding fluid. Sections of 20 µm thickness were cut at the cryostat and collected in PBS. Immunofluorescence was performed under standard condition. Briefly, tissue sections were incubated with a blocking solution containing normal goat serum (NGS) 10%, Triton-X 100 0.2% for 1 hr at RT. Following this step, sections were incubated overnight at 4°C with the primary antibody: mouse anti-SMN (1:350, Becton Dickinson), mouse anti-glial fibrillary acidic protein (GFAP, 1:500, Millipore), rabbit anti-ha-SMN (#910, NeoMPS, 1:100), or goat anti- Choline Acetyl Transferase (ChAT, 1:200, Millipore). The following day, sections were rinsed in PBS for 30 min and incubated with the corresponding secondary biotinylated antibody (diluted 1:200 in PBS and 1% NGS) for 1 h, and then incubated with Cy2-conjugated Streptavidin (Jackson ImmunoResearch; diluted 1:200 in PBS, 1% NGS) for 1 h. After rinsing, sections were washed in water and coverslipped with Fluorsave (Calbiochem, Darmstadt, Germany). Image acquisition was performed with a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA) and analyzed with Photoshop[®] softwares. For motor neuron counts, one section every 13 was mounted on slide and stained with anti-SMN and anti-ChAT antibodies.

Western Blot Analysis

Spinal cord, brain, liver and heart were removed from mouse after rapid decapitation and were immediately frozen in dry ice and preserved at -80° C until use. Samples were homogenized in buffer containing 20 mM Hepes (pH 7.4), 1 mM DTT, 0.1 mM PMSF and Complete (Roche, Indianapolis, IN). Protein extracts were separated by 12 % SDS-PAGE, electroblotted onto nitrocellulose membranes, probed with mouse anti-SMN (Becton Dickinson, diluted 1:10000 in 5% non-fat milk) or rabbit anti-ha-SMN (#910, NeoMPS, diluted 1:100 in 5% non-fat milk). Odyssey secondary antibodies were used (goat anti-mouse IRDye 680 and goat anti-rabbit IRDye 680) diluted 1:15000 in non-fat milk. A monoclonal antibody against F-actin was used as loading control (Chemicon, 1:4000).

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CHAPTER 7

SUMMARY AND CONCLUSIONS

Spinal Muscular Atrophy or SMA is a severe autosomal recessive disease characterized by motor neuron degeneration. SMA is the leading genetic cause of infant mortality and, in affected children, motor neuron loss leads to progressive amyotrophic paralysis, respiratory failure, and, in more severe cases, death. SMA is classified into three main clinical types (I-III), in relation to the age of onset and disease severity. SMA is a monogenic disease caused by disruptions of the Survival of Motor Neuron 1 (*SMN1*) gene. The severity of the disease is related to the presence, peculiar to the human species, of the almost identical *SMN2* copy gene. The *SMN2* gene mainly expresses an exon 7 truncated SMN form ($\Delta 7$ -SMN) and only low amounts of the functional “full-length” FL-SMN protein. Thus, the presence of more *SMN2* copies may ameliorate the clinical course of SMA. In addition, a truncated form of SMN is expressed in neurons. This protein, termed axonal SMN (a-SMN), is translated from an *SMN1*-derived mRNA, which retains intron 3, containing a premature stop codon, and thereby producing a truncated shorter protein (*Setola et al., 2007*) expressed during embryogenesis and early postnatal life. In comparison to the FL-SMN protein, a-SMN expression is largely restricted to motor neurons with further sub-cellular localization in axons and dendrites. In contrast to FL-SMN, forced expression of a-SMN stimulates motor neuron axonogenesis (*Setola et al, 2007*). All

this indicates that a-SMN deficiency may play an important role in the pathogenesis of SMA. However, a clear understanding of the disease pathogenesis and the overall functions of SMN protein is still to be determined and a definitive cure for patients is not still available.

The final goal of my PhD project was to obtain further insights into the role exerted by a-SMN in SMA pathogenesis. Achievement of this major aim requires a better characterization of the a-SMN biological function in motor neuron homeostasis as well as a definition of the underlying molecular mechanisms and determinants. Our studies involved both *in vitro* and *in vivo* approaches. In particular, my project focused on the use of a transgenic SMA mouse model, in order to obtain first, a fine morphological characterization of the model and then a read out for development of a possible gene therapy approach by using SMN proteins.

In **chapter 3**, to support the hypothesis that a-SMN loss of function plays a role in the pathogenesis of SMA, we investigated by means of over-expression experiments in NSC34 motor neurons, whether SMA pathogenic mutations located in the N-terminal part of the protein affected a-SMN function. *SMN1* missense mutations (W92S; A111G; E134K) or small intragenic rearrangements (133-134 4bp delAGAG; 147-148 5bp del GAAGT) were inserted in expression vectors containing the wt human a-SMN (ha-SMN) sequence to produce mutated ha-SMN constructs. All mutations were reported in SMA type I patients and they were located in the region of the *SMN1* gene

coding for the Tudor domain. After transient transfection in NSC34 motor neurons, we compared the rate of axon growth induced by control GFP vector, and wild-type and mutated human a-SMN proteins. Quantification of axonal elongation in a large number of NSC34 motor neurons demonstrated that missense point mutations reduced significantly whereas frame-shift mutations abrogated completely the ha-SMN function in axon growth. We also verified the morphology of neurites whose growth was stimulated by the different human a-SMN constructs and the GFP control vector. Our data demonstrated that cells transfected with mutated ha-SMN polypeptides were characterized by neurites not only shorter but also displaying clear morphologic abnormalities. Evident swellings along the neuritic shaft close to the cell body, at branching sites and up to the growth cone were present in cells transfected with all mutated ha-SMN proteins. Double-labeling confocal staining with cytoskeletal and mitochondrial markers revealed that swellings and abnormal growth cones were consistently associated with irregularities of the axonal cytoskeleton. Axonal swellings were also consistently characterized by accumulation of mitochondria. Similar morphologic abnormalities of axonal shafts and growth cones, and abnormal accumulation of neurofilaments were also reported in SMA patients and experimental SMA models. Taken together, our findings indicate that **i)** the a-SMN protein plays a relevant role in the establishment of the proper cytoskeletal structure of the axon, and that axonal transport may be impaired as a consequence of such cytoskeletal abnormalities; **ii)** the Tudor domain is critical in dictating a-SMN function possibly

because it is an association domain for proteins involved in axon growth; **iii**) Tudor domain mutations are functionally relevant not only for FL-SMN but also for a-SMN, raising the possibility that also a-SMN loss of function may contribute to the pathogenic steps leading to SMA.

In **chapter 4**, to get further insight into the biological function of a-SMN in the motor neuron cell context, we described the production of NSC34 cell clones with conditional, tetracycline (TET)-dependent expression of a-SMN. The analysis of our cellular model provided two main results. First, it demonstrates that a-SMN stimulates random cell motility and axon growth. Growth inhibition and axon growth may be coupled and the result of the differentiating action set in motion by a-SMN in physiologic condition *in vivo*. In fact, terminal cellular differentiation is invariably associated with growth arrest in various cell types, including neurons, and some morphology features associated with a-SMN over-expression are reminiscent of those induced in motor neurons by the differentiating agent retinoic acid. By the same token, it is likely that cellular motility and axonogenesis are also coupled. In developing brain, establishment of neuronal circuits is the result of axon and neuronal cell movement to their correct destinations by guidance cues, and coupling of cell motility and axon outgrowth has also been demonstrated in cell models. It is thus possible that the primary function of a-SMN in the developing brain and spinal cord is to modulate some aspect of neuronal differentiation and motility that ultimately result in the control of directional axon

growth. Second, we identified the chemokines CCL2 and CCL7 and the growth factor IGF1 as potential mediators of a-SMN biological activity in axon growth and cell motility of motor neurons. Indeed, the transcripts encoding the three factors were up-regulated in all the a-SMN over-expressing clones analyzed, and the cause-effect relationship between a-SMN expression and induction of CCL2, CCL7 or IGF1 proteins was confirmed by experiments of a-SMN transient transfection in NSC34 cells. Recent evidence indicates that CCL2 is involved not only in peripheral and CNS inflammatory responses but also in controlling normal neuronal activity. CCL2 and its receptor CCR2 are synthesized by neurons and constitutively expressed in several brain regions, and CCL2 enhances neuronal excitability and synaptic transmission *via* pre-synaptic mechanisms. These results support the emerging concept that chemokines function as neuromodulators in the CNS. Our results are relevant not only because they identify CCL2 as a mediator of a-SMN activity, but also because they represent the first demonstration that CCL2 synthesis and secretion contribute to neuronal axonogenesis and motility in a cell autonomous manner. We propose that CCL2 acts as a trophic factor for the growth of motor neuron axons and this may represent a novel biological activity of the cytokine. Our data indicate the secreted form of CCL2 mediates the pro-motility and axogenic activities of a-SMN, and morphologic data demonstrate that a fraction of the protein localizes to the growth cones and developing axons. Co-localization of a-SMN and CCL2 suggests that the two proteins may physically interact. Up-regulation of IGF1 has also far-reaching

implications for the physiological activity of a-SMN, as IGF1 is endowed with neurotrophic properties mediated by activation of the PI3K/AKT pathways. IGF1 treatment delays disease onset and progression in mouse models of amyotrophic lateral sclerosis (*Kaspar et al., 2003; Dobrowolny et al., 2005*) and skeletal muscular dystrophy (*Musaro et al., 2001*). In addition, increasing IGF1 in muscles extends the survival of D7 SMA mice (*Bosch-Marce et al., 2011*). Finally, reduced levels of circulating IGF1 are present in a severe SMA mouse model and may be restored by effective antisense oligonucleotide treatment (*Hua et al., 2011*). Thus, the a-SMN/IGF1 link may play a relevant role also in SMA diseased conditions.

In **chapter 5**, we have characterized, at the morphological level, spinal cord and brain of the transgenic SMA Δ 7 mouse model (SMN2^{+/+}; SMN Δ 7^{+/+}; Smn^{-/-}, *Le et al., 2005*) during development, in order to better understand the impact of reduced levels of SMN protein in specific motor neuron sub-populations and in the motor cortex. After stereological counts, in the cervical spinal tract at different developmental stages (E19.5, P4 and P13), we found in affected mice, a reduced number of motor neurons already at pre-symptomatic time point and no difference at pre-natal stage, thus suggesting a severe and rapid perinatal cell loss, but no evident signs of developmental defects. The analysis of mean motor neuron area, together with a body size distribution, revealed a clear loss of motor neurons with larger soma in affected compared to WT mice. Then, we focused our attention on specific motor neuron pools innervating

proximal, distal or respiratory muscles, in order to see whether some groups are differentially vulnerable to SMA. By analyzing the mean area and the distribution, we found that only larger motor neurons within the pool innervating proximal muscles result more vulnerable to degeneration, while other sub populations were spared, thus excluding a widespread motor neuron loss. Finally, cell density quantification of the motor cortex revealed that, in SMA mice, there was a strong and selective reduction of the upper motor neurons in layer V, while in the layer II-III of SMA mice we did not observe a reduction in the cell number, but only in the cell body size. Therefore, the upper motor system involvement exclude, at least in this model, that SMA is specifically a lower motor neuron disease, supporting other studies that described, in a more severe mouse model, defects in non-motor regions of the brain such as the hippocampus (*Wishart et al., 2010*). Our observations give insights on some clinical aspects never documented before, allowing to study, in more detail the cellular effect of FL-SMN and better define the role of a-SMN *in vivo*, after restoring of protein levels by gene therapy approaches.

In **chapter 6**, are reported preliminary results of a viral gene transfer approach to deliver human FL-SMN or a-SMN in mice by using AAV9 vectors. This approach allowed to study, in particular, the potential role of a-SMN *in vivo* and will establish whether increasing a-SMN levels can ameliorate the clinical phenotype of SMA.

We first focused our attention on the IV (intravascular) route of administration via the facial/temporal vein (post-natal day 2, P2)

because it allows delivery of larger volumes of viral preparations. This strategy of administration was able to induce the expression of FL-SMN or a-SMN proteins in peripheral tissues (liver, heart, and liver), but not in the CNS (brain and spinal cord). However, neither *scAAV9*/FL-SMN nor *scAAV9*/a-SMN was able to extend survival. We then focused on the ICV (intracerebroventricular) administration. Multiple injections per animal were performed in newborn mice in order to by-pass the blood-brain barrier. After ICV injections both brain and spinal cord were infected by the virus. A more detailed analysis, performed with immunohistochemistry experiments, revealed that in the entire spinal cord, motor neurons were preferentially transduced, and in the brain different neuron populations were targeted. Interestingly, after a-SMN injections, most of the cells transduced in the brain resulted to be astrocytes. The injected mice were daily monitored for survival, weight gain, motor behavior and for development of pathologic effects such as peripheral necrosis and eye inflammation. Injection of *scAAV9*/FL-SMN was able to extend survival in a dose-dependent manner and ameliorate their motor function; conversely *scAAV9*/a-SMN delivery was not effective in increasing SMA mice survival or ameliorating their phenotype. However, before excluding an effective role of a-SMN in SMA, other experiments and observations are necessary.

FUTURE PROSPECTS

The role of a-SMN protein and, in particular, its involvement in axonal growth and cell motility were well documented *in vitro*. However, the contribution of a-SMN loss to the SMA phenotype is still unclear. To elucidate the possible role of a-SMN *in vivo* and to study in more detail the biological effect of restored levels of FL-SMN protein after gene transfer approach, we will analyze, in spinal cord and brain, those regions and cell populations that we found most affected in SMA Δ 7 mice. The following experimental groups will be soon considered: 1) mice ICV treated with higher-titer scAAV9/FL-SMN or scAAV9/a-SMN and 2) mice ICV treated with simultaneous administration of AAV9/FL-SMN and AAV9/a-SMN in order to evaluate whether the co-administration could represent a better therapeutic option than restoring FL-SMN levels alone. In a next future we also plan to generate an scAAV9/a-SMN placed under control of neuron specific promoters to obtain a more efficient neuron transduction, particularly into the brain. First, treated and control mice will be daily followed-up for survival and motor behavior. Weight gain, Kaplan-Meier survival curves, and hind-limb posture after tail suspension will be monitored in the different treatment groups. Injected mice of the different experimental groups will be followed-up throughout their life for the development of pathologic effects. Behavioral tests will be performed to check motor function: at early time points we will perform the righting reflex test (time to right after placing mice on their backs) to monitor gross mobility and the hind-limb suspension test for proximal hind-limb muscle strength; at later

time points we will evaluate the Rotarod test for balance, the Treadmill for speed and resistance on a moving platform, the Paw grip endurance test for the ability in remaining suspended to an inverted grid, the footprint analysis for primary locomotor gait, and the activity cage to monitor movements in a cage environment. Then, we will carefully analyze the cell populations particularly vulnerable to SMA in spinal cord and brain via Nissl staining and confocal IF experiments; subsequently we will study synaptic inputs to motor neurons (in treated vs untreated mice) using VGluT1 or synaptophysin as pre-synaptic markers (*Ling et al., 2010; Mentis et al., 2011*). In the periphery, we will analyze: **i)** the morphology and innervation of neuromuscular junctions (through double fluorescence staining with α -bungarotoxin and neurofilament/synaptic vesicle2 antibodies; **ii)** the cardiac function (with ecocardiography), and morphology (gross anatomy and whole-mount tyrosine hydroxylase immunostaining of the sympathetic innervations: *Heier et al., 2010*). Finally, the expression and cellular localization of CCL2 and IGF1 (analyzed by RT-PCR, WB, and confocal IF) will be evaluated to confirm the link between these two proteins and a-SMN (and possibly FL-SMN) in SMA conditions *in vivo*. We will also verify the impact of the different treatments, in view of developing novel and effective therapeutic strategies.

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PERSONAL PUBLICATIONS

SMA pathogenic mutations impair the axonogenic properties of axonal-SMN (a-SMN)

Denise Locatelli, **Paolo d'Errico**, Silvia Capra, Adele Finardi, Francesca Colciaghi, Veronica Setola, Mineko Terao, Enrico Garattini, and Giorgio Battaglia.
Journal of Neurochemistry, 2012, vol 121, pag 465 - 474

Human Axonal Survival of Motor Neuron (a- SMN) protein stimulates axon growth, cell motility, C-C motif ligand 2 (CCL2), and insulin- like growth factor-1 (IGF1) production

Locatelli D., Terao M., Fraelli M., Zanetti A., Kurosaki M., Lupi M., Barzago MM., Uggetti A., Capra S., **d'Errico P.**, Battaglia G., Garattini E.
The Journal of Biological Chemistry Vol. 287. N. 31, pag.26782-25794

RINGRAZIAMENTI

Ringrazio tutte le persone che mi hanno aiutato nella realizzazione di questo lavoro; chi, direttamente o indirettamente, ha contribuito agli studi descritti nel mio progetto scientifico.

In particolare ringrazio Marina Boido, per aver collaborato con me al lavoro sulla caratterizzazione animale, Denny e Paola per avermi aiutato nella stesura della tesi e per il loro supporto morale e tecnico.

Un ringraziamento speciale a mia moglie Simona per aver sopportato pazientemente la mia “assenza” e per il suo aiuto nella stesura della bibliografia.