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ORIGINAL ARTICLE

Neuronal-Specific Roles of the Survival Motor Neuron Protein: Evidence From Survival Motor Neuron Expression Patterns in the Developing Human Central Nervous System

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Abstract

Despite recent data on the cellular function of the survival motor neuron (SMN) gene, the spinal muscular atrophy (SMA) disease gene, the role of the SMN protein in motor neurons and hence in the pathogenesis of SMA is still unclear. The spatial and temporal expression of SMN in neurons, particularly during development, could help in verifying the hypotheses on the SMN protein functions so far proposed. We have therefore investigated the expression and subcellular localization of the SMN protein in the human central nervous system (CNS) during ontogenesis with immunocytochemical, confocal immunofluorescence, and Western blot experiments using a panel of anti-SMN antibodies recognizing the full-length SMN protein. The experiments not only revealed the early SMN expression in all neurons, but also demonstrated the progressive shift in SMN subcellular localization from mainly nuclear to cytoplasmic and then to axons during CNS maturation. This finding was present in selected neuronal cell populations and it was particularly conspicuous in motor neurons. Our data support the idea of a specific role for SMN in axons, which becomes predominant in the ontogenetic period encompassing axonogenesis and axonal sprouting. In addition, the asymmetric SMN staining demonstrated in the germinative neuroepithelium suggests a possible role for SMN in neuronal migration and/or differentiation.

Key Words: Axonal sprouting, Axonal transport, CNS development, Neuronal differentiation, Neuronal migration, SMA, Spinal muscular atrophy.

INTRODUCTION

The survival motor neuron (SMN) gene, originally reported in 1995 (1), is the disease gene for spinal muscular atrophy (SMA), a severe and common childhood autosomal-recessive disease characterized by selective degeneration of spinal and bulbar motor neurons and by progressive amyotrophic paralysis (2). In humans, the SMN gene is contained in a 500-kb inverted repeat on chromosome 5q13. Therefore, the human SMN gene, in contrast to the *SMN* homologue in mice, rats, and other mammals (3–7), is present in two copies, telomeric (or SMN1) and centromeric (or SMN2). The SMN1 gene is deleted or mutated in more than 98% patients with SMA (8), and it is the essential gene responsible for the SMA phenotype. The SMN2 gene may exist in several copies in single human chromosomes, and the copy number of SMN2 may modulate the disease severity of SMA (9, 10). The two genes possess promoter regions with identical activities (11), which are downregulated during cellular differentiation in vitro (12). Both SMN genes produce a full-length mRNA and different transcripts formed by alternative splicing involving exons 7, 5, and 3 (13, 14). However, the predominant isoform from SMN1 is the full-length mRNA, whereas SMN2 produces mostly the mRNA lacking exon 7 and a C-terminus truncated protein isoform, which is thought to be unstable and rapidly degraded (1, 15).

The SMN protein is ubiquitously expressed in large multimeric complexes within the nucleus (in coiled bodies and "gems") and the cytoplasm of all cellular types, including motor neurons (3, 16). It is a multifunctional protein with many housekeeping functions, involved in spliceosomal snRNPs assembly and pre-mRNA splicing (17–19), in gene regulation at the transcriptional level (20–22), and, possibly, in regulating apoptosis (23–28).

Despite the data mentioned, the issue of why reduced levels of a housekeeping protein like SMN may eventually lead to motor neuron degeneration in SMA remains to be solved. Data from transgenic mouse models for SMA have suggested that the pathogenic mechanisms resulting in SMA could be determined by a still unknown role of SMN in motor neurons, particularly relevant during the postnatal development of the spinal cord (29–31). Recently, growing evidence has pointed out that the association between SMN and the cytoskeleton in axons and the axonal transport of SMN may be of relevance in the pathogenesis of SMA. The SMN protein is associated with cytoskeletal elements in motor neuron axons and axon terminals, particularly during

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early postnatal development (32), and it has been localized in the growth cones of neuritic extensions of cultured neuronal cells (33). In addition, the axonal transport of SMN has been very recently suggested in vitro in cultured neurons (34).

Understanding the pathogenesis of human SMA would be aided by a detailed analysis of the expression of the SMN protein in the human central nervous system (CNS). The specific hypothesis of the SMN protein function may be verified by clarifying where and when the SMN protein is actually expressed in different cellular compartments during CNS ontogenesis. Until now, the spatial and temporal profile of SMN protein expression has been investigated in the spinal cord of experimental animals (3, 32), but only in situ hybridization data are available on the SMN gene expression in human CNS during development (35). In this study, we investigated the expression and regional localization of the SMN protein during prenatal and postnatal development in the germinative neuroepithelium, spinal cord, brainstem, cerebellum, and cerebral cortex by immunocytochemistry and confocal immunofluorescence. The main finding of the study is that SMN subcellular localization in neurons progressively changes during CNS maturation, shifting from mainly nuclear to cytoplasmic and then becoming particularly evident in axons. In addition, our data suggest a possible role for SMN in young migrating neurons.

MATERIAL AND METHODS

Human Samples

Human samples were obtained from three sources: 1) spinal cords from five fetuses obtained from induced abortions at 6 weeks 3 days, 7 weeks, 7 weeks 3 days, and 8 weeks of gestation (cases 1–5), were made available to us through an experimental protocol on Parkinson and Huntington diseases approved by the French National Ethical Committee. These specimens were collected and stored in a sterile surviving medium, fixed by immersion in 4% paraformaldehyde and 0.4–0.5% glutaraldehyde in phosphate buffer (PB) at pH 7.4, and then embedded in paraplast. 2) Paraplast-embedded specimens of prenatal and postnatal cases were also obtained from the Department of Neurological Sciences of the University of Verona (seven fetuses: cases 6–12; and five infants; cases 13–15, 17, and 18) and from the Neuropathology Unit of the Neurological Institute in Milano (one infant, case 16; and one adult, case 19). Fetal spinal cord, brainstem, cerebellum, and cerebral cortex were obtained from miscarriages at 10, 14, 16, 20 (two cases), 21, and 22 weeks (Table 1). 3) Finally, four cortical samples (cases 20–23) were obtained as a byproduct of surgical resections from adult patients operated on for brain tumor removal in the Neurosurgery Department of the Neurological Institute (Table).

TABLE 1. Summary of Human Cases Analyzed in this Study

	n	Case	Age	Cause of Death	Spinal Cord	Brainstem	Cerebellum	Cerebral Cortex	Western Blot
Prenatal	1	H.E. 5.1	6 weeks, 3 days	Induced abortion	x				
	2	H.E. 10.2	6 weeks, 3 days	Induced abortion	x			x	x
	3	H.E. 5.2	7 weeks	Induced abortion	x				
	4	H.E. 10.3	7 weeks, 3 days	Induced abortion	x				x
	5	H.E. 5.3	8 weeks	Induced abortion	x				
	6	65 cs	10 weeks	Miscarriage	x				
	7	92/06	14 weeks	Miscarriage	x	x			
	8	96/02	16 weeks	Miscarriage		x	x	x	
	9	86 cs	20 weeks	Miscarriage		x		x	
	10	98/36	20 weeks	Miscarriage			x	x	
	11	11/00	21 weeks	Miscarriage	x				
	12	97/22	22 weeks	Miscarriage	x	x		x	
Postnatal	13	80/10	2 days	Respiratory failure		x	x	x	
	14	96/39	37 weeks, 46 days	Anoxia	x				
	15	90/23	2 months	CMV pneumonia			x	x	
	16	1144	6 months	Respiratory failure	x				
	17	92/23	8 months	Fallot tetralogy		x			
	18	91/21	13 months	CMV pneumonia	x				
	19	942	38 years	Medulloblastoma	x				
	20	B 1	70 years	Surgical sample				x	
	21	B 5	35 years	Surgical sample				x	
	22	B 132	24 years	Surgical sample				x	
	23	B 136	42 years	Surgical sample				x	x

CMV, cytomegalovirus.

Postnatal tissues were obtained from the following: 1) two newborns, the first born after 35 weeks of gestation and died at age 2 days from respiratory failure; the second born after 36 weeks of gestation and died at age 46 days from anoxia; 2) four infants, the first aged 2 months and died after cytomegalovirus pneumonia without CNS involvement, the second aged 6 months died from respiratory failure, the third aged 8 months died from cardiac failure as a consequence of Fallot tetralogy, and the fourth aged 13 months died after cytomegalovirus pneumonia without CNS involvement; 3) one adult, aged 38 years died after medulloblastoma of the fourth ventricle. None of the examined cerebral and spinal samples was characterized by histologic abnormalities (Table).

Antibodies

Three different anti-SMN-specific antibodies were used in immunocytochemical, confocal immunofluorescence, and Western blot experiments. The polyclonal antibody 1137 was raised in rabbit and directed against amino acid residues 1–15 (i.e. exon 1) of the N-terminal region of the human SMN protein sequence (3). The polyclonal antibody 854 was raised in rabbit and directed against amino-acid residues 4–21 (i.e. exon 1) of the N-terminal region of the rat SMN protein sequence (32). The commercially available monoclonal antibody, called TL, was directed against residues 14–174 (spanning exons 2a, 2b, 3, and part of exons 1 and 4) of a human SMN fusion protein (clone 8; Becton-Dickinson, Franklin Lakes, NJ). All anti-SMN antibodies recognized a major band migrating at approximately 38 kDa (3–32), likely corresponding to the full-length SMN protein (36). In the double-labeling confocal experiments, we used the monoclonal antibody against microtubule-associated protein 2 (MAP2; Sternberger Monoclonals Inc., Lutherville, MA) as specific marker for early generated and mature neurons.

Immunocytochemistry and Immunofluorescence

Paraplast-embedded specimens were cut in 5- to 10-μm coronal sections with a rotary microtome. The dewaxed and hydrated paraplast sections were boiled in 10 mM sodium citrate buffer, pH 6, for 5 minutes using a microwave oven set at 650 to 750 W to retrieve the antigen immunoreactivity, rinsed in phosphate-buffered saline (PBS), and incubated with 10% normal goat serum (NGS) and 0.2% Triton-X100 for 60 minutes to mask nonspecific adsorption sites. For single-labeling immunocytochemistry, sections were incubated overnight at 4°C with the anti-SMN antibodies (diluted 1:1000 in PBS-1% NGS and 0.2% Triton X-100). After rinsing for 30 minutes in PBS, the sections were incubated with a 1:200 dilution of biotinylated goat antirabbit IgG or goat antimouse IgG (Vector Laboratories, Burlingame, CA) for 75 minutes, rinsed for 30 minutes in PBS, and then incubated with the ABC elite complex (Vector) or Extravidine (Sigma-Aldrich, St. Louis, MO) for 75 minutes. Peroxidase staining was obtained by incubating the sections in 0.075% DAB and 0.002% H_2O_2 in 50 mM Tris buffer at pH 7.6. Sections were dried, dehydrated, and coverslipped with DPX. The sections adjacent to the

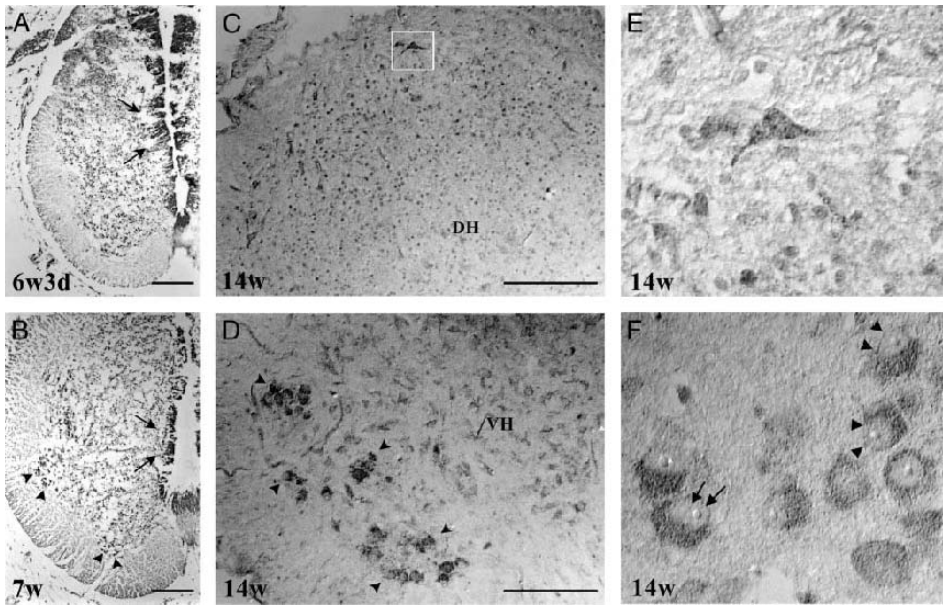

FIGURE 1. Survival motor neuron (SMN) staining in the developing spinal cord. **(A, B)** SMN staining at ages 6 weeks 3 days **(A)** and 7 weeks **(B)**; TL antibody. Note the even staining of the germinative neuroepithelium (arrows) and the more intense SMN immunoreactivity of motor neurons at 7 weeks (arrowheads in **[B]**). **(C–E)** SMN staining at age 14 weeks; 1137 antibody. Note the preferential nuclear labeling of dorsal horn neurons **(C)** and the presence of rare marginal lamina I neurons (**[C]**, arrows; the neuron within the boxed area is represented at higher magnification in **([E])**. Ventral horn motor neurons (**[D]**, arrowheads) display mostly cytoplasmic staining: their nuclei are located at the periphery of the perikarya (**[F]**, arrowheads) and contain discrete SMN-positive dots (**[F]**, arrows). Calibration bars = 100 μm.

immunoreacted ones were counterstained with 0.1% thionin. The specificity of the staining was tested by using the preimmune sera, omitting the primary antibodies, or preincubating the primary antibodies with an excess of antigenic peptide.

For sequential double-labeling immunofluorescence experiments, sections were incubated with the anti-SMN primary antibodies at 1:50 dilution and then for 1 hour with biotinylated goat antirabbit or goat antimouse IgG (Jackson ImmunoResearch Labs, West Grove, PA; diluted 1:250) followed by Cy2-conjugated streptavidin or rhodamine avidin D (Jackson; diluted 1:250). SMN-immunoreacted sections were then first incubated with anti-MAP2 (diluted 1:100) and thereafter incubated for 1 hour with Alexa Fluor 546 or Alexa Fluor 488 secondary antibodies (Molecular Probes, Eugene, OR; diluted 1:2000). All incubation with primary antibodies was performed overnight at 4°C followed by three rinses in PBS. After all experiments, sections were repeatedly rinsed, coverslipped with Fluorsave (Calbiochem, Darmstadt, Germany), and examined on a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA). Confocal images were saved in TIFF and then elaborated by means of Adobe Photoshop software.

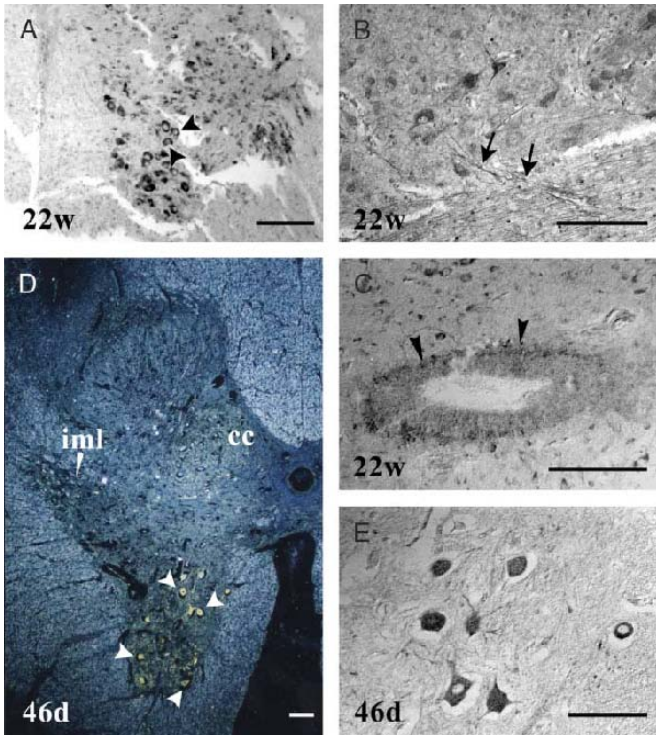

FIGURE 2. Survival motor neuron (SMN) staining in prenatal and postnatal spinal cord. **(A–C)** At 22 gestational weeks, motor neuron cell bodies (**[A]**, arrowheads; TL antibody) and axons entering the white matter (**[B]**, arrows; 854 antibody) are intensely SMN-immunoreactive; young neurons at the more external border of the spinal germinative neuroepithelium also display intense SMN staining (**[C]**, arrowheads; TL antibody). **(D)** Dark-field color photomicrograph of thoracic spinal cord at postnatal day 46; 1137 antibody. Note the intense SMN staining of motor neurons (arrowheads) and the SMN staining of neurons within the column of Clarke (cc) and the intermediolateral nucleus (iml). **(E)** Higher-power photomicrograph showing the intense SMN labeling of cell bodies and dendrites of postnatal motor neurons; 854 antibody. Calibration bars = 100 μm.

Neuronal Cell Counts

To quantify the SMN labeling in motor neurons at different developmental stages (22 months prenatally, 6 months and 38 years postnatally), we compared the number of thionine-stained and SMN-immunoreactive motor neurons in adjacent consecutive sections. Thionine-stained spinal cord sections were reconstructed by means of camera lucida and then matched with the adjacent sections immunoreacted with an anti-SMN antibody. Blood vessel and white matter–gray matter boundaries were used as reference marks. Individual motor neurons were easily identified on adjacent sections using a Nikon Microphot FXA microscope with a differential interference contrast illumination (Nomarski) at 200×. Only thionine-stained motor neurons in lamina IX displaying the nucleolus on the plane of the section were considered. For statistical evaluation, six different sections from the three different cases were taken into account. Data were analyzed by one-way test of variance (ANOVA) by comparing percentages at the different ages examined.

Western Blot Analysis

Specimens from two prenatal and one postnatal cases (Table) were snap-frozen on dry ice or by immersion in liquid nitrogen and stored at −80°C until processing. For the preparation of cellular membrane and cytosolic fractions, tissue samples were homogenized (Teflon-glass potter, 10 strokes, 700 rpm) in four volumes of buffer containing 20 mM HEPES, 1 mM DTT, and 1 mM EGTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF) at pH 7.4 in the presence of protease inhibitors (Complete; Boehringer Mannheim GmbH, Germany). The homogenized tissue was centrifuged at 1,000 g for 10 minutes. The resulting supernatant was centrifuged at 100,000 g for 45 minutes and the resulting pellet and supernatant were regarded as cellular membrane (M) and soluble (S) fractions, respectively. For nuclear fractionation, the homogenized tissue was processed in 10% NP-40 buffer, strongly mixed, and centrifuged at 1,000 g for 2 minutes. The obtained pellet was resuspended in buffer containing 0.4 M NaCl, 20 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 25% glycerol in the presence of protease inhibitors as specified previously, mixed for 30 minutes at 4°C, and centrifuged at 14,000 g for 20 minutes. The resulting pellet and supernatant were regarded as nuclear membrane and nuclear extract fractions, respectively.

Protein concentrations were determined using the Bio-Rad protein assay reagent based on the Bradford dye-binding procedure. Proteins were separated by SDS-PAGE (12% acrylamide) and electroblotted on nitrocellulose paper for 60 minutes at 180 mA. The nitrocellulose papers were blocked with 10% nonfat milk, and proteins were recognized

with the anti-SMN primary antibodies (TL, diluted 1:25.000; 1137 and 854, diluted 1:1.000). The nitrocellulose was then incubated with antirabbit (1:5.000; Sigma, St. Louis, MO) or antimouse (1:10.000; Kierkegaard and Perry Lab, Gaithersburg, MD) HRP-conjugated secondary IgGs, and the antigen–antibody complex was revealed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Freiburg, Germany). To verify the amount of proteins loaded in each lane, blots were subsequently probed with an antiactin antibody (Chemicon, Temecula, CA; diluted 1:5000). We also verified the subcellular purity of nuclear fractions using the nuclear-specific marker antihistones antibody (MAB052; Chemicon; diluted 1:2000).

RESULTS

In the present study, we investigated the expression and regional localization of the SMN protein within the human CNS during prenatal and postnatal development. We focused our attention on the SMN expression in the spinal cord and lower brainstem, given the selective involvement in SMA of spinal and bulbar motor neurons and in the cerebral and cerebellar cortices. In addition, we analyzed the early expression of the SMN protein in the germinative neuroepithelium. Finally, we verified the subcellular localization of the SMN protein at different developmental stages with confocal immunofluorescence and Western blot analysis.

Spinal Cord

At age 6 weeks and 3 days (Fig. 1A), all cells within the developing spinal cord were SMN-immunoreactive and the SMN labeling was particularly evident within the nucleus. Developing neuroblasts in the germinative neuroepithelium were also intensely and evenly SMN-positive (Fig. 1A, arrows). The same pattern of staining was observed at ages 7 and 10 weeks, but SMN immunoreactivity was slightly more intense in ventral horn motor neurons than in the other cellular types within the spinal cord (Fig. 1B, arrowheads). At 14 weeks gestational age (Fig. 1C–F), SMN labeling was particularly intense in the cytoplasm of motor neuron cell bodies (Fig. 1D, arrowheads); discrete dots of SMN immunoreactivity were evident within the nuclei (Fig. 1F, arrows), which were frequently located at the periphery of the perikarya (Fig. 1F, arrowheads). In the dorsal horn, most cells displayed SMN staining in the nucleus with the exception of a few elongated neurons marginally placed in lamina I at the border between the dorsal horn and the white matter, which were also characterized by intense SMN cytoplasmic staining (Fig. 1C, E). At age 22 weeks (Fig. 2A–C), SMN immunoreactivity was diffuse in the dorsal and ventral horns, but motor neurons were clearly more intensely labeled (Fig. 2A, arrowheads). SMN was clearly evident in the cytoplasm not only of cell bodies, but also of proximal dendrites. In addition, axons from motor

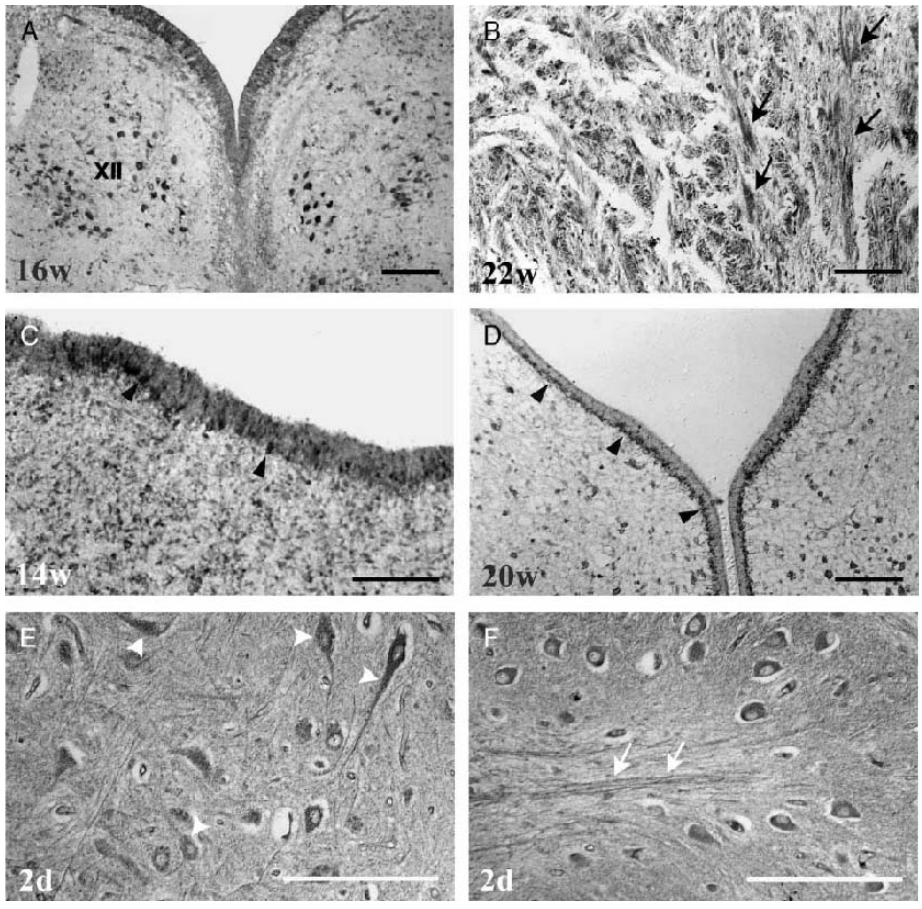

FIGURE 3. Survival motor neuron (SMN) staining in prenatal and postnatal brainstem. **(A, B)** Motor neurons of the hypoglossal nucleus (**[A]**, 16th gestational week), and corticospinal axons within the bulbar pyramids (**[B]**, arrows, 22nd gestational week) are intensely SMN-immunoreactive; TL antibody. **(C, D)** Young neurons located at the external portion of the germinative neuroepithelium display intense SMN staining at the 14th gestational week (**[C]**, arrowheads), and more conspicuously at the 20th gestational week (arrowheads in D); TL antibody. (**E, F**) In the postnatal brainstem, SMN staining is evident in cell bodies and dendrites (**[E]**, arrowheads, dorsal motor nucleus of the vagus) as well as in axons within the bulbar neuropil (**[F]**, arrows, inferior olivary nucleus); 1137 antibody. Calibration bars = 100 μm.

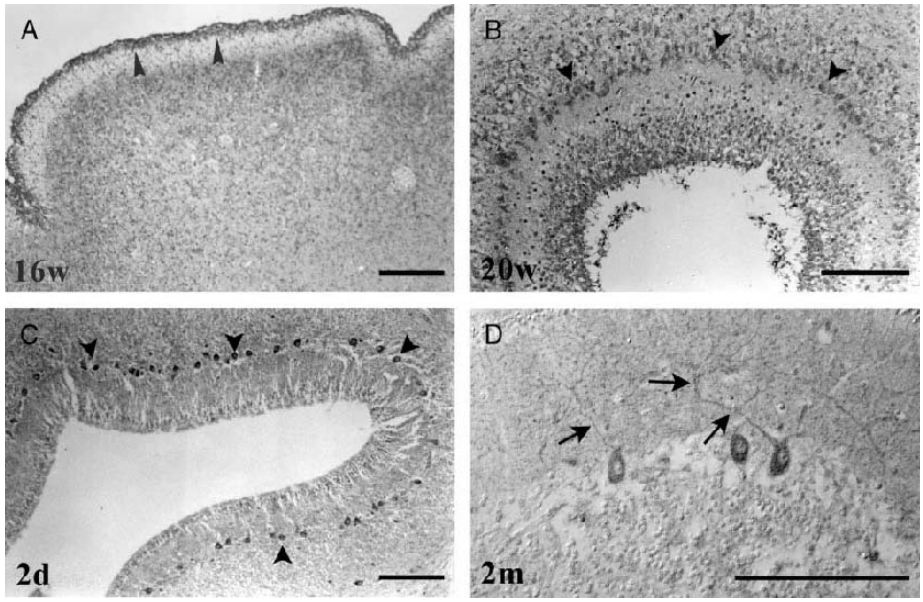

FIGURE 4. Survival motor neuron (SMN) staining in prenatal and postnatal cerebellum. **(A)** At the 16th gestational week, the external granular layer is intensely and evenly SMN immunoreactive (arrowheads); 1137 antibody. **(B)** At the 20th gestational week, Purkinje neurons (arrowheads) display both nuclear and cytoplasmic staining; TL antibody. **(C, D)** Intense cytoplasmic (**[C]**, arrowheads; TL antibody) and dendritic (**[D]**, arrows; 1137 antibody) SMN staining of postnatal Purkinje neurons. Note that SMN staining is more intense in some but not all Purkinje neurons. Calibration bars = 100 μm.

neurons entering the ventral roots were clearly and intensely SMN-immunoreactive (Fig. 2B, arrows). The cells in the germinative neuroepithelium were characterized by light SMN labeling, with the exception of intensely SMN-immunoreactive neurons located at the more external border of the germinal zone (Fig. 2C, arrowheads). In the neonatal spinal cord (Fig. 2D, E), the selective SMN labeling of motor neurons was more conspicuous (Fig. 2D, arrowheads). The SMN pattern of staining was very similar to that observed in the adult spinal cord (3). Neurons in the columns of Clarke (cc) and intermediolateral nuclei im were also SMN-immunoreactive, although less intensely than motor neurons (Fig. 2D). Within motor neurons, SMN staining was particularly evident in the cytoplasm of cell bodies and proximal dendrites (Fig. 2E), whereas immunoreactive puncta were rarely observed within the nuclei.

At all examined ages, the comparison between adjacent thionine-stained and immunoreacted sections revealed that the vast majority of motor neurons were SMN-positive (94.97% ± 4.73% at 22 prenatal weeks; 89.45% ± 5.22% at 6 months postnatal; 82.33% ± 12.47% in adulthood, age 38, means ± standard deviation, n = 6). Even if the percentage of SMN-immunolabeled motor neurons was slightly less in the adult if compared with the embryo, no significant differences in SMN labeling were evident at the three examined ages ($p = 0.13$; $F = 2.34$). The number of SMN-positive cell bodies decreased from prenatal to postnatal ages both in the cervical (24.0 SMN-positive motor neurons per section at 14 prenatal weeks; 22.6 per section at 22 prenatal weeks; 16.0 per section at 13 months postnatal) and thoracic (11.33 per section at one month postnatal; 7.0 per section in adults) spinal cords.

Brainstem

We examined the lower part of the brainstem at the level of the hypoglossal nucleus, inferior olivary nuclei, and bulbar pyramids at 16 and 22 weeks of gestation, at birth, and in an infant at 8 postnatal months (Table). At the 16th gestational week (Fig. 3A), the motor neurons of the hypoglossal nucleus were intensely SMN-immunoreactive both in the nuclei and cell bodies and proximal dendrites. Parasympathetic and sensory nuclei were also SMN-immunoreactive; the large neurons of the nucleus ambiguus and the neurons of the dorsal motor nucleus of the vagus and the sensory neurons of the cuneate and external cuneate nuclei displayed SMN labeling in both nucleus and cytoplasm (not shown). In addition, neurons in the inferior olivary nuclei

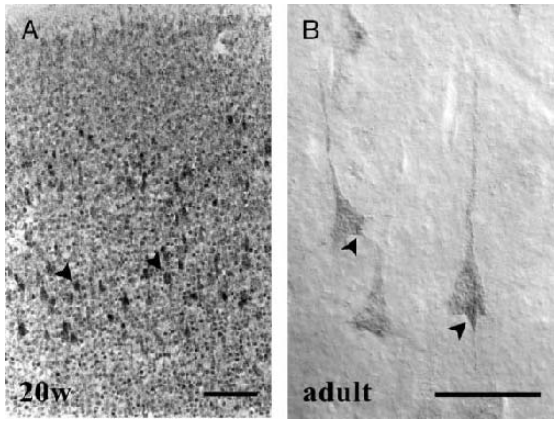

FIGURE 5. Survival motor neuron (SMN) staining of developing (**[A]**, arrowheads; TL antibody) and mature (**[B]**; 1137 antibody) pyramidal neurons. Note the preferential SMN nuclear staining of the developing cortical plate **(A)** and the SMN immunoreactivity at the axon hillock of mature layer V pyramidal neurons (**[B]**, arrowheads). Calibration bars = **(A)** 100 μm; **(B)** 75 μm.

displayed SMN labeling (not shown). The same pattern of staining was also observed at the 22nd gestational week. However, an additional feature was observed: strong SMN immunoreactivity in the axons of the descending corticospinal tract within the bulbar pyramids (Fig. 3B, arrows). We also examined the SMN staining of the germinative neuroepithelium at bulbar and pontine levels and at the mesodiencephalic junction at 14 and 20 weeks of gestation (Fig. 3C, D). At the 14th gestational week, some intensely SMN-immunoreactive young neurons were present in the more external portion of the germinative neuroepithelium, i.e. away from the ventricle (Fig. 3C, arrowheads). The asymmetric labeling of the germinative neuroepithelium was particularly striking at midpontine levels at the 20th week of gestation (Fig. 3D, arrowheads). At birth and at postnatal age 8 months, the regional distribution of the bulbar SMN staining was similar to that observed prenatally, but the pattern of staining was different. As observed in the spinal cord, the SMN immunoreactivity was mainly cytoplasmic, nicely outlining the cell bodies and proximal dendrites (Fig. 3E, arrowheads), whereas only rare, dot-like immunoreactivity was present in the nucleus. In addition, the SMN staining in corticospinal axons was clearly reduced at birth and barely appreciable at 8 months of age; however, thread-like SMN-immunoreactive structures, likely representing axonal fibers, were still present postnatally in the bulbar neuropil (Fig. 3F, arrows).

Cerebellum

We examined the developing cerebellum prenatally at 16 and 20 gestational weeks and postnatally at birth and age 2 months (Table). At 16 and 20 weeks gestation, no selective pattern of SMN staining was present within the cerebellum. The SMN immunoreactivity was present in the nucleus of young, nondifferentiated neurons in both the deep nuclei and the cerebellar cortex and in the densely packed small neurons of the external granular layer, i.e. the germinal layer for the cerebellar granule, stellate, and basket cells (Fig. 4A, arrowheads). At the 20th gestational week, Purkinje neurons displayed cytoplasmic labeling in addition to nuclear SMN staining (Fig. 4B, arrowheads). At birth, and more evidently at 2 months of age, the pattern of cerebellar SMN staining was markedly different and only Purkinje cells (Fig. 4C) and neurons in the deep cerebellar nuclei were SMN-immunoreactive. In particular, SMN was evident in the cytoplasm and developing apical dendritic trees of the Purkinje neurons (Fig. 4D). Notably, the SMN staining was more evident in some Purkinje neurons and less evident in others (Fig. 4C, arrowheads).

Cerebral Cortex

We examined the developing cerebral cortex prenatally at 16, 20, and 22 gestational weeks, and postnatally at birth, at age 2 months, and in surgical samples obtained from four adult patients. In the developing cerebral cortex at the 16th gestational week, the germinative neuroepithelium was thick, whereas the cortical plate was still relatively thin with many undifferentiated neurons with large nuclei surrounded by a thin rim of cytoplasm. At this developmental stage, SMN immunoreactivity was mainly nuclear and evident in most cells of the developing white matter and cortical plate. At the 20th and 22nd gestational weeks, the ventricular zone was much thinner and, conversely, the cortical plate much thicker. SMN immunoreactivity was still mainly nuclear; it was clearly reduced in the ventricular zone and more intense in the cortical plate. Within the cortical plate, small clusters of neurons with pyramidal shape displayed more intense SMN immunoreactivity (Fig. 5A, arrowheads). Postnatally, at birth, and in adulthood, SMN immunoreactivity was mainly confined in the cytoplasm of cell bodies and basal and apical dendrites of pyramidal neurons, particularly in layer V (Fig. 5B). Notably, in some layer V pyramidal neurons, SMN immunoreactivity was particularly intense at the axon

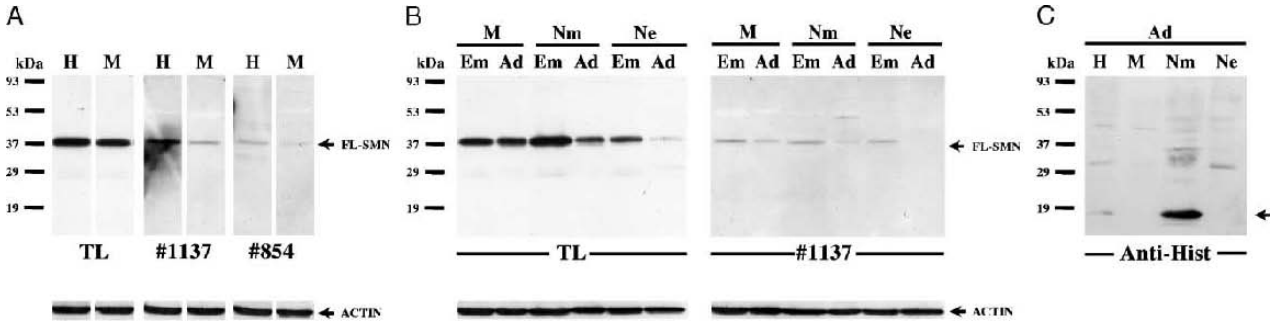

FIGURE 6. Representative Western blot analysis of survival motor neuron (SMN) protein expression in spinal and cerebral protein extracts from embryonic (Em) and adult (Ad) cases. **(A)** All the anti-SMN antibodies recognize a single immunoreactive band at approximately 38 kDa, corresponding to the full-length SMN protein (FL-SMN, arrow) in both the homogenate (H) and cellular membrane (M) fractions from embryonic spinal cord. **(B)** The SMN protein is downregulated during central nervous system maturation but more clearly decreased in nuclear membrane (Nm) and nuclear extract (Ne) than in cellular membrane (M) fractions. No SMN signal was obtained in the soluble cytosolic fraction (not shown). **(C)** The antihistone antibody reveals the H1 histone band at approximately 20 kDa in the nuclear membrane fraction only (arrows), thus confirming the subcellular purity of our preparation. The correct amount of loaded proteins in each lane was tested with an antiactin antibody (lower lanes, arrows).

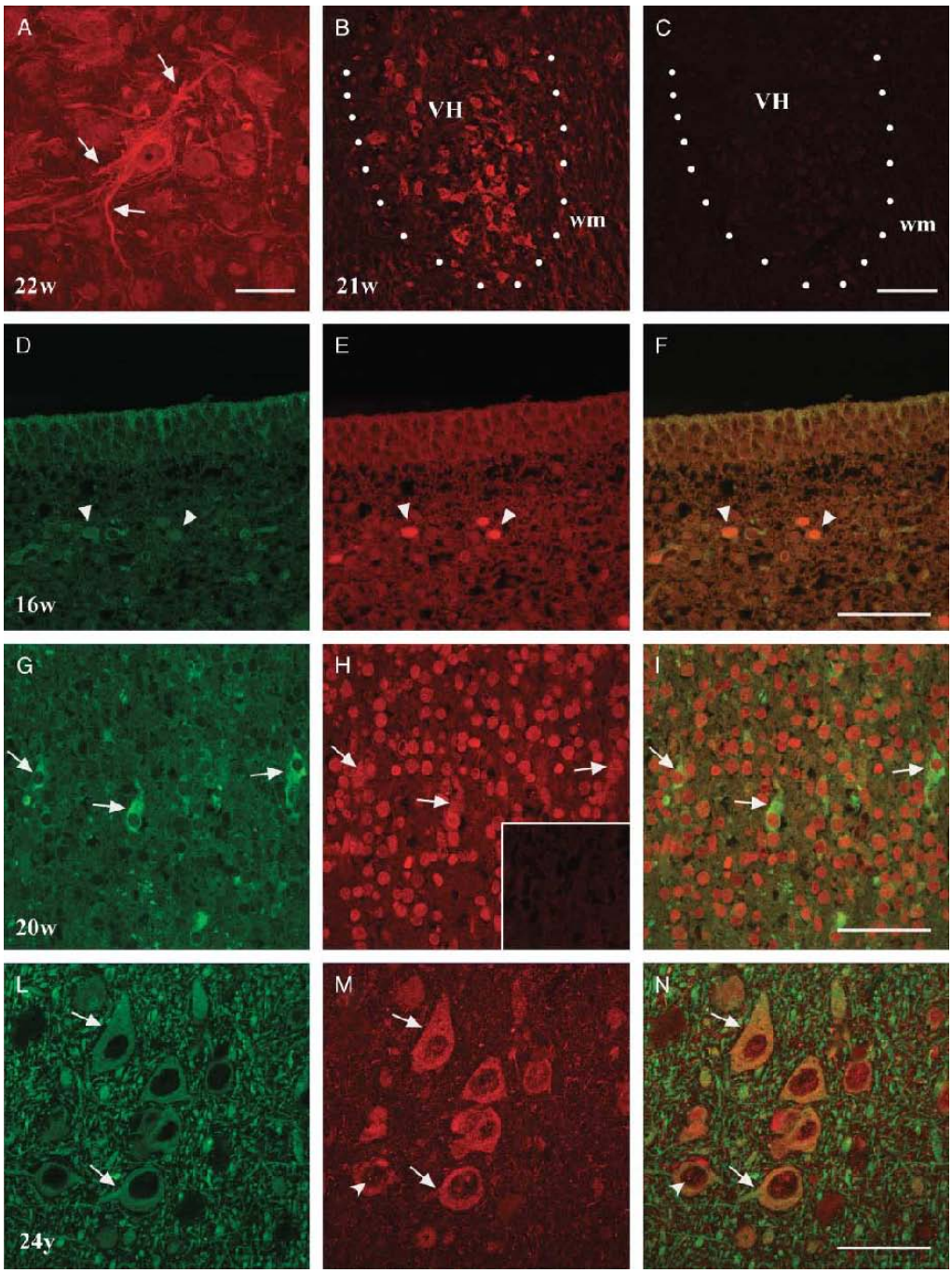

FIGURE 7. (A–C) Confocal immunofluorescence survival motor neuron (SMN) staining of fetal spinal motor neurons. Note the intense cytoplasmic and dendritic labeling **[A]**, arrows; 854 antibody) and the absence of motor neuron staining when the 1137 antibody **(B)** is preincubated with an excess of antigenic peptide **(C)**. **(B)** and **(C)** are adjacent consecutive paraffin sections. VH, ventral horn; wm, white matter. **(D–N)** The SMN staining progressively shifts from the nucleus to the cytoplasm during neuronal differentiation. MAP2 staining in green **(D, G, L)**, SMN staining in red **(E, H, M)**, merged images in **(F, I, N)**. Note that the SMN staining of young neurons migrating in the subventricular zone at the 16th gestational week is mainly nuclear **[E-F]**, arrowheads) surrounded by a thin rim of MAP2 immunoreactive cytoplasm **(D)**. In neurons of the developing cortical plate at the 20th gestational week, SMN staining is still mainly nuclear even if pale perikaryal SMN staining is present in MAP2-positive pyramidal neurons (**[G–I]**, arrows). By contrast, in postnatal cortical neurons, SMN staining is present in cytoplasm and proximal dendrites and colocalizes with MAP2 labeling (**[L, M]**, arrows). Note the dot-like labeling of nuclear gems (**[M, N]** arrowheads; 1137 antibody). Insert in **(H)** demonstrates the absence of nuclear labeling when omitting the anti-SMN antibody. Calibration bars = **(A–I)** 100 μm; **(L, N)** 50 μm.

hillock (Fig. 5B, arrowheads). Pyramidal and multipolar neurons were also SMN-immunoreactive in layer VI.

Subcellular Localization of Survival Motor Neuron During Development

Because the pattern of SMN immunoreactivity was mainly nuclear at early developmental stages in all spinal and cerebral areas considered and became predominantly cytoplasmic during CNS maturation, we verified the subcellular localization patterns of SMN with Western blot analysis and double-labeling confocal immunofluorescence. Western blot experiments first demonstrated that all three antibodies recognized a major SMN band at approximately 38 kDa, likely corresponding to the FL-SMN protein (36) in both the homogenate and cellular membrane fractions from spinal cord protein extracts of 6- to 7-week-old fetuses (Fig. 6A; Table). The specificity of the antibodies was further supported by the complete loss of the 38 kDa SMN staining after preincubation of the 854 and 1137 antibodies with corresponding antigenic peptides (3–32). In addition, the SMN protein expression was developmentally regulated, being more abundant in the embryonic than in the adult CNS in all the cellular fractions considered (Fig. 6B). Interestingly, the developmental decrease of the SMN signal was more evident in the nuclear membrane and nuclear extract fractions than in the cellular membrane fraction in keeping with the data from the immunocytochemical experiments.

Double-labeling confocal immunofluorescence experiments were carried out using an anti-MAP2 antibody, a cytoskeletal marker of early-generated and mature neurons (37). At the 16th gestational week, the SMN staining in the subventricular zone was mainly segregated from that obtained with the anti-MAP2 antibody. The SMN staining was particularly evident in the nuclei of young neurons migrating in the subventricular zone and it was surrounded by the MAP2 staining of the cytoplasm (Fig. 7D–F, arrowheads). In neurons of the developing cortical plate at the 20th gestational week, SMN was mainly localized in the nucleus; however, pale SMN staining was also present in the perikarya and proximal dendrites of some young pyramidal neurons clearly labeled by the anti-MAP2 antibody (Fig. 7G–I, arrows). By contrast, in postnatal cortical neurons, SMN staining was mainly located in the cytoplasm and proximal dendrites, where a clear pattern of colocalization with MAP2 was evident (Fig. 7L–N, arrows). Dot-like labeling of nuclear gems was also present (Fig. 7M, N, arrowheads). Taken together, the Western blot and double-labeling confocal experiments confirm the finding that SMN staining tends to shift progressively from the nucleus to the cytoplasm during neuronal differentiation.

DISCUSSION

In the present study, we have addressed the issue of where and when the SMN protein is expressed in neurons of the human CNS from early ontogenesis to postnatal ages. We have accomplished this investigation by using three anti-SMN antibodies (3, 32), all recognizing in Western blot experiments a protein band migrating at approximately 38 kDa, corresponding to the full-length SMN protein (Fig. 6A). The idea behind the present study is that the pattern of neuronal expression of SMN could help verifying the hypotheses on the SMN protein function so far proposed.

The Survival Motor Neuron Protein Is Expressed Early in Central Nervous System Neurons

Our data clearly demonstrate the early presence of SMN in the developing CNS. At the earliest examined embryonic age (6 weeks, 3 days), all cells within the spinal cord were immunoreactive for SMN, and there were no regional differences in SMN expression and no predominance in SMN expression by a single cellular type. This expression pattern of SMN was not only evident in the spinal cord of 6-week-old embryos, but also in the ventricular and subventricular zones of the developing neocortex at the gestational age of 14 weeks. At these early developmental stages, the SMN expression was mainly nuclear, and no staining differences were apparent between the nucleus and cytoplasm. Our confocal immunofluorescence analysis confirmed that the SMN staining pattern in young developing neurons was mainly nuclear by demonstrating that in migrating neurons in the subventricular zone of the developing neocortex at the 14th gestational week, the SMN-positive nuclei were surrounded by an MAP2-labeled but SMN-negative thin cytoplasmic ring (Fig. 7D–F).

The presence of SMN in the nuclei of all cells during early development is consistent with the housekeeping functions so far proposed for SMN such as the role in spliceosomal assembly and pre-mRNA maturation, which has been suggested as the basic cellular mechanisms leading to SMA (19). SMN is necessary for all cellular types during early CNS ontogenesis, as also clearly indicated by the massive cell death during early embryonic stages when *Smn* is homozygously disrupted in $Smn^{-/-}$ knockout mice (29).

The Survival Motor Neuron Staining Pattern Shifts From Nucleus to Cytoplasm and Axons During Neuronal Development

Early during CNS ontogenesis, the subcellular localization of SMN in neurons started to change and became progressively more clearly evident in the cytoplasm. This shift in staining pattern was evident not only in spinal motor neurons (Fig. 7A), in which the cytoplasmic expression was clearly predominant from age 14 weeks onward, but also in pyramidal neurons of the cerebral cortex (Fig. 7L–N) and cerebellar Purkinje neurons (Fig. 4C, D) at later developmental stages. The SMN expression shift from nucleus to cytoplasm was further supported by Western blot analysis, demonstrating that the SMN downregulation was clearly more evident in the nuclear subcellular fractions than in the cellular membrane fraction (Fig. 6B).

In addition, the SMN expression became clearly evident in axons at later developmental stages. At 22 weeks gestation, axons from motor neurons leaving the spinal cord and entering the ventral roots and axons in the descending corticospinal tract at the level of the bulbar pyramids were intensely SMN-immunoreactive. The axonal expression of SMN was

still clearly evident at early postnatal stages, like in the bulbar neuropil at birth (Fig. 3), and it tended later to decrease as demonstrated by the reduction and disappearance of SMN staining in the pyramidal tract.

The SMN staining of axons cannot be easily related to the housekeeping functions of SMN mentioned here, but rather suggests a novel role for SMN that becomes predominant in neurons at a certain phase of their development. Recently, many studies have suggested that SMN may play a role in axons. The SMN axonal localization has been clearly demonstrated in vivo in the early postnatal rat spinal cord (32) and in sciatic nerves (Battaglia, unpublished observations) and in vitro in growth cone-like structures of cultured neurons (33). In cultured neurons, live imaging has revealed the existence of bidirectional, cytoskeleton-dependent movements of the SMN protein, thus strongly indicating a role for SMN in axonal transport (34). In addition, a role for SMN in the process of axon growth has been suggested by different lines of evidence: 1) the impairment of motor axon outgrowth and branching determined by the antisense oligonucleotide-mediated knockdown of SMN in zebra fish in vivo (38); and 2) the colocalization in motor neuron axons between SMN and the heterogenous nuclear ribonucleoprotein hnRNP-R (39) and the SMN/hnRNP-R-mediated translocation of β-actin mRNA in distal parts of axons and growth cones (40). Taken together, these data point out the relevance of SMN in axons and suggest that impairment in the role of SMN in axonal transport or growth could be the key to understanding the pathogenesis of SMA.

Why Are Motor Neurons Selectively Impaired in Spinal Muscular Atrophy?

Another interesting feature of the SMN expression during CNS development was the progressive selectivity of the SMN staining. This feature was again particularly evident in spinal cord motor neurons. In the embryonic spinal cord at 6 weeks gestational age, the SMN staining is evenly diffuse, whereas at birth, motor neurons are by far the cellular type more strongly SMN-labeled (Fig. 2D). SMN staining in motor neurons is still present during adulthood, although not as intense as at birth. This pattern of staining may reflect the fact that motor neurons are strictly dependent on SMN expression at all developmental stages and in adulthood for their function and survival, and may account at least in part for their selective death when the SMN protein expression is dramatically reduced in SMA. However, other neurons did express high levels of SMN, as indicated by our data, such as the pyramidal neurons in the neocortex, the Purkinje neurons in the cerebellum, and neurons of somatosensory or olivary nuclei in the brainstem. In all of these neurons, SMN staining was intense and specific, likely reflecting the expression of high levels of the protein. Similar expression patterns were reported by in situ hybridization data in the sensory system, cerebral cortex, and cerebellum during human CNS development (35).

However, even if more widespread neuronal degeneration, extending beyond the motor neuron compartment, has been reported in severely affected patients with SMA (41, 42), the degeneration of motor neurons is by far the more relevant biologic condition in SMA from both the clinical and neuropathologic standpoint. It is possible that the peculiar structure of motor neurons affected in SMA, which innervate proximal muscles and are therefore characterized by extensive sprouting, may contribute to the selectivity of their degeneration: it has been indeed reported that surviving motor neurons in patients affected by severe SMA type I possess decreased ability to increase their motor unit territory (43).

Other Function for Survival Motor Neuron?

Finally, another interesting feature has been revealed by our analysis. The SMN staining was particularly intense in the more outer parts of the germinative neuroepithelium, i.e. in positions away from the ventricle. This asymmetric pattern of SMN labeling was striking in that it was present in different analyzed cases and in different parts of the germinative neuroepithelium, which give rise to different population of neurons. Because neuroepithelial germinal cells move toward the ventricular lumen for mitotic division and away from it after mitosis is completed, the "polarized" SMN staining likely reflects a particularly high expression of SMN by postmitotic young neurons starting their migration or final differentiation. A role for SMN in neuronal migration or differentiation has not been hypothesized so far. However, the recent data on SMN function have clearly shown that SMN is a multifunctional protein and that it has a close relationship with the neuronal cytoskeleton. A role for SMN at a certain stage of migration, possibly through a specific interaction with the neuronal cytoskeleton, is a possibility that deserves further attention in future studies.

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REFERENCES

1. Lefebvre S, Burglen L, Reboullet S, et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell 1995;80: 155–65
2. Pearn J. Classification of spinal muscular atrophies. Lancet 1980;1: 919–22
3. Battaglia G, Princivalle A, Forti F, et al. Expression of the SMN gene, the spinal muscular atrophy determining gene, in the mammalian central nervous system. Hum Mol Genet 1997;6:1961–71
4. DiDonato CJ, Chen XN, Noya D, et al. Cloning, characterization, and copy number of the murine survival motor neuron gene: Homolog of the spinal muscular atrophy-determining gene. Genome Res 1997;7: 339–52

5. Blazej RG, Mellersh CS, Cork LC, et al. Hereditary canine spinal muscular atrophy is phenotypically similar but molecularly distinct from human spinal muscular atrophy. J Hered 1998;89:531–37
6. Pietrowski D, Goldammer T, Meinert S, et al. Description and physical localization of the bovine survival of motor neuron gene (SMN). Cytogenet Cell Genet 1998;83:39–42
7. Rochette CF, Gilbert N, Simard LR. SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to Homo sapiens. Hum Genet 2001;108:255–66
8. Lefebvre S, Burglen L, Frezal J, et al. The role of the SMN gene in proximal spinal muscular atrophy. Hum Mol Genet 1998;7:1531–36
9. Campbell L, Potter A, Ignatius J, et al. Genomic variation and gene conversion in spinal muscular atrophy: Implications for disease process and clinical phenotype. Am J Hum Genet 1997;61:40–50
10. Vitali T, Sossi V, Tiziano F, et al. Detection of the survival motor neuron (SMN) genes by FISH: Further evidence for a role for SMN2 in the modulation of disease severity in SMA patients. Hum Mol Genet 1999;8:2525–32
11. Monani UR, McPherson JD, Burghes AH. Promoter analysis of the human centromeric and telomeric survival motor neuron genes (SMNC and SMNT). Biochim Biophys Acta 1999;1445:330–36
12. Germain-Desprez D, Brun T, Rochette C, et al. The SMN genes are subject to transcriptional regulation during cellular differentiation. Gene 2001;279:109–17
13. Gennarelli M, Lucarelli M, Capon F, et al. Survival motor neuron gene transcript analysis in muscles from spinal muscular atrophy patients. Biochem Biophys Res Commun 1995;213:342–48
14. Jong YJ, Chang JG, Lin SP, et al. Analysis of the mRNA transcripts of the survival motor neuron (SMN) gene in the tissue of an SMA fetus and the peripheral blood mononuclear cells of normals, carriers and SMA patients. J Neurol Sci 2000;173:147–53
15. Lorson CL, Androphy EJ. An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. Hum Mol Genet 2000;9:259–65
16. Liu Q, Dreyfuss G. A novel nuclear structure containing the survival of motor neurons protein. EMBO J 1996;15:3555–65
17. Liu Q, Fischer U, Wang F, et al. The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. Cell 1997;90:1013–21
18. Fischer U, Liu Q, Dreyfuss G. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. Cell 1997;90:1023–29
19. Pellizzoni L, Kataoka N, Charroux B, et al. A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. Cell 1998;95:615–24
20. Strasswimmer J, Lorson CL, Breiding DE, et al. Identification of survival motor neuron as a transcriptional activator-binding protein. Hum Mol Genet 1999;8:1219–26
21. Williams BY, Hamilton SL, Sarkar HK. The survival motor neuron protein interacts with the transactivator FUSE binding protein from human fetal brain. FEBS Lett 2000;470:207–10
22. Pellizzoni L, Charroux B, Rappsilber J, et al. A functional interaction between the survival motor neuron complex and RNA polymerase II. J Cell Biol 2001;152:75–85
23. Crawford TO, Pardo CA. The neurobiology of childhood spinal muscular atrophy. Neurobiol Dis 1996;3:97–110
24. Iwahashi H, Eguchi Y, Yasuhara N, et al. Synergistic anti-apoptotic activity between Bcl-2 and SMN implicated in spinal muscular atrophy. Nature 1997;390:413–17
25. Sato K, Eguchi Y, Kodama TS, et al. Regions essential for the interaction between Bcl-2 and SMN, the spinal muscular atrophy disease gene product. Cell Death Differ 2000;7:374–83
26. Simic G, Seso-Simic D, Lucassen PJ, et al. Ultrastructural analysis and TUNEL demonstrate motor neuron apoptosis in Werdnig-Hoffmann disease. J Neuropathol Exp Neurol 2000;59:398–407
27. Coovert DD, Le TT, Morris GE, et al. Does the survival motor neuron protein (SMN) interact with Bcl-2? J Med Genet 2000;37:536–39
28. Young PJ, Day PM, Zhou J, et al. A direct interaction between the survival motor neuron protein and p53 and its relationship to spinal muscular atrophy. J Biol Chem 2002;277:2852–59
29. Schrank B, Götz R, Gunnersen JM, et al. Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. Proc Natl Acad Sci U S A 1997;94:9920–25
30. Hsieh-Li HM, Chang JG, Jong YJ, et al. A mouse model for spinal muscular atrophy. Nat Genet 2000;24:66–70
31. Monani UR, Sendtner M, Coovert DD, et al. The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in SMN(−/−) mice and results in a mouse with spinal muscular atrophy. Hum Mol Genet 2000;9:333–39
32. Pagliardini S, Giavazzi A, Setola V, et al. Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. Hum Mol Genet 2000;9:47–56
33. Fan L, Simard LR. Survival motor neuron (SMN) protein: Role in neurite outgrowth and neuromuscular maturation during neuronal differentiation and development. Hum Mol Genet 2002;11:1605–14
34. Zhang HL, Pan F, Hong D, et al. Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. J Neurosci 2003;23:6627–37
35. Tizzano EF, Cabot C, Baiget M. Cell-specific survival motor neuron gene expression during human development of the central nervous system: Implications for the pathogenesis of spinal muscular atrophy. Am J Pathol 1998;153:355–61
36. Jablonka S, Schrank B, Kralewski M, et al. Reduced survival motor neuron (SMN) gene dose in mice leads to motor neuron degeneration: An animal model for spinal muscular atrophy type III. Hum Mol Genet 2000;9:341–46
37. Del Rio JA, Martinez A, Auladell C, et al. Developmental history of the subplate and developing white matter in the murine neocortex. Neuronal organization and relationship with the main afferent systems at embryonic and perinatal stages. Cereb Cortex 2000;10:784–801
38. McWhorter ML, Monani UR, Burghes AH, et al. Knockdown of the survival motor neuron (SMN) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. J Cell Biol 2003;162:919–31
39. Rossoll W, Kroning AK, Ohndorf UA, et al. Specific interaction of SMN, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: A role of SMN in RNA processing in motor axons? Hum Mol Genet 2002;11:93–105
40. Rossoll W, Jablonka S, Andreassi C, et al. SMN, the spinal muscular atrophy determining gene product, modulates axon growth and localization of β-actin mRNA in growth cones of motoneurons. J Cell Biol 2003;163:801–12
41. Devriendt K, Lammens M, Schollen E, et al. Clinical and molecular genetic features of congenital spinal muscular atrophy. Ann Neurol 1996;40:731–38
42. Korinthenberg R, Sauer M, Ketelsen UP, et al. Congenital axonal neuropathy caused by deletions in the spinal muscular atrophy region. Ann Neurol 1997;42:364–68
43. Hausmanowa-Petrusewicz I. Electrophysiological findings in childhood spinal muscular atrophies. Rev Neurol (Paris) 1988;144:716–20

