Defective Neuromuscular Junction Organization and Postnatal Myogenesis in Mice With Severe Spinal Muscular Atrophy

Elisabet Dachs, BS, Marta Hereu, BS, Lídia Piedrafita, BS, Anna Casanovas, MD, PhD, Jordi Calderó, MD, PhD, and Josep E. Esquerda, MD, PhD

Abstract
A detailed pathologic analysis was performed on Smn−/−;SMN2+/+ mice as a mouse model for human type I spinal muscular atrophy (SMA). We provide new data concerning changes in the spinal cord, neuromuscular junctions and muscle cells, and in the organs of the immune system. The expression of 10 synaptic proteins was analyzed in 3-dimensionally reconstructed neuromuscular junctions by confocal microscopy. In addition to defects in postsynaptic occupancy, there was a marked reduction in calcitonin gene–related peptide and Rab3A in the presynaptic motor terminals of some, but not all, of the skeletal muscles analyzed. Defects in the organization of presynaptic nerve terminals were also detected by electron microscopy. Moreover, degenerative changes in muscle cells, defective postnatal muscle growth, and prominent muscle satellite cell apoptosis were also observed. All of these changes occurred in the absence of massive loss of spinal cord motor neurons. On the other hand, astrogia, but not microglia, increased in the ventral horn of newborn SMA mice. In skeletal muscles, the density of interstitial macrophages was significantly reduced, and monocyte chemotactic protein-1 was downregulated. These findings raise questions regarding the primary contribution of a muscle cell defect to the SMA phenotype.

Key Words: Apoptosis, Macrophage, Mouse model, Motoneuron, Neuromuscular junction, Skeletal muscle, Spinal muscular atrophy.

INTRODUCTION
Spinal muscular atrophy (SMA) is a motoneuron (MN) disease caused by deletions or inactivating mutations of the Survival Motor Neuron 1 (SMN1) gene, which results in reduced dosage of full-length SMN protein (1). Spinal muscular atrophy is the second most common fatal autosomal recessive childhood disorder with an incidence of approximately 1 per 6,000 births (2, 3). The deletion of SMN1 homologs in other animals is lethal at early embryonic ages (4). Because the human genome contains a variable number of copies of a second paralogous gene, SMN2, the absence of SMN1 is partially compensated by SMN2. This is because the SMN2 gene mostly generates transcripts lacking exon 7 and produces a truncated and rapidly degraded nonfunctional SMNΔ7 protein. However, approximately 10% to 20% of SMN2 transcripts are correctly spliced and produce full-length SMN. Because there is polymorphism with respect to the number of copies of SMN2, the levels of full-length SMN in SMA patients may vary and inversely correlate with the severity of the disease (5, 6). Spinal muscular atrophy is a ubiquitously expressed protein that plays a prominent role in the assembly of small nuclear ribonucleoproteins in the nuclear Cajal body and in pre-mRNA splicing (7). How reduced levels of SMN cause a MN disease in humans is not currently understood. Two alternatives have been proposed: 1) the SMA phenotype is a consequence of cell-specific defective small nuclear ribonucleoprotein biogenesis and pre-mRNA splicing; or 2) the disease is due to the loss of the MN-specific function of the SMN protein (3, 8). SMN seems to be crucial for the transport of mRNA in neurons; therefore, disruption of this function may be pivotal in the pathogenesis of SMA (9).

On the basis of conventional postmortem neuropathologic studies of the end-stages of disease, it has been historically assumed that the loss of ventral horn MNs and subsequent skeletal muscle atrophy are the most characteristic features of human SMA (10). To gain further insight into the pathogenesis of SMA, mouse models of this disease have been generated (11–16). Detailed studies charting the progression of the disease in SMA mice have concluded that defects in axons and their synaptic terminals at the neuromuscular junctions (NMJs) are associated with the most prominent and earliest changes (17–21). Compromised NMJs have also been described in drosophila and zebrafish Smn mutants (22, 23). This concept may be relevant to other MN diseases such as amyotrophic lateral sclerosis (ALS). Indeed, in ALS, it has been reported that synaptic disconnection at NMJs is a key aspect of the pathology and occurs earlier than...
MN death (24–27). If clinical manifestations of MN diseases result from damage in the distal motor axon but not from activation of the death pathway, new therapeutic strategies should be directed to protecting distal axons and synaptic motor terminals. Thus, a more precise knowledge of the nature of NMJ pathology may contribute to the definition of new targets for therapy.

Here, we report a detailed pathologic analysis of the Smn\(^{--}\);SMN2 mouse model of type I SMA and provide new data concerning changes detected in the spinal cord, skeletal muscle (including NMJs), and immune system organs. We analyzed the expression of 10 synaptic proteins using multiple fluorescent labeling and confocal microscopy in NMJs and identified (in addition to defects in postsynaptic occupancy) marked and early downregulation of calcitonin gene–related peptide (CGRP) and Rab3A in the presynaptic motor terminals of some, but not all, of the SMA muscles analyzed. Defective postnatal muscle growth, degenerative changes in skeletal muscle cells, and prominent muscle satellite cell apoptosis were also detected. All of these changes occur in the absence of the massive loss of spinal cord MNs. These findings suggest a primary contribution of muscle defects to the SMA phenotype. Preliminary results of this work were presented in an abstract form (28).

**MATERIALS AND METHODS**

**Animals**

SMA type I mice FVB.Cg-Tg(SMN2)\(^{89Ahmb}\)Smn\(^{tmi1Msd}\)/J were purchased from The Jackson Laboratory (Sacramento, CA). Heterozygous animals were crossed to obtain homozygous Smn\(^{--}\);SMN2\(^{+/+}\). Littermates homozygous for the mutation and their Smn\(^{+/+}\);SMN2\(^{+/+}\) (referred as wild-type [WT]) were used for the experiments. Animals were identified by marking their feet with green tattoo paste (Fine Science Tools GmbH, Heidelberg, Germany) at postnatal day (P) 0; and a piece of their tail was snipped for genotyping. The REDExtract-N-Amp Tissue PCR Kit (Sigma, St Louis, MO).

---

**FIGURE 1.** Spinal cord changes in severe spinal muscular atrophy (SMA) mice. (A) Number of motoneurons (MN) in the ventral horn of the lumbar spinal cord of severe SMA mice. Note that a moderate, but significant, MN loss is only observed at terminal ages (P5–6). (B) Quantification of Iba-1 positive microglial cells in the ventral horn shows no differences between wild-type (WT) and SMA. (C) Glial fibrillary acidic acid protein (GFAP)–positive astroglia are increased around MNs in postnatal SMA mice. (D, E) Representative images of GFAP immunostaining (red) adjacent to MN cell bodies visualized after fluorescent Nissl staining (green) in samples from WT and SMA mice. Numbers in parenthesis in the graphs indicate numbers of animals analyzed. *, p < 0.05; and **, p < 0.01 versus WT (Student t-test). Scale bars: E = 25 μm (valid for [D]).
was used for genomic DNA extraction and polymerase chain reaction setup. The primers used were as follows: WT forward 5′ CTCCGGGATATTGGGATTG 3′, SMA reverse 5′ GGTAACGCCAGGGTTTTC 3′ and WT reverse 5′ TTTCTTCTGGCTGTGCCTTT 3′. Electrophoresis in 1% agarose gel and SYBR safe DNA stain (Molecular Probes, Eugene, OR) was run at 100 mV. In some experiments, newborn (P0–1) Smn+/−, SMN2−/− mice were subjected to sciatic nerve axotomy: the right sciatic nerve was transected and ligated at the midtigh level under anesthesia by hypothermia. All experimental procedures used had been approved by the Committee for Animal Care and Use of the University of Lleida, in line with Generalitat de Catalunya norms (DOGC 2073, 1995).

**Histology**

Samples were fixed in Carnoy solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) to count healthy MNs. Serial paraffin sections (8 μm for E18; 12 μm for P0–1 and P5–6) obtained through the entire lumbar segment of spinal cord were stained with Cresyl violet, and every 10th section was counted according to established procedures (29).

**Immunocytochemistry and TUNEL Staining**

The SMA and WT animals analyzed were pooled in 3 different groups: E18, P0–1, and P5–6. Spinal cords and the diaphragm (Diaph), intercostalis (IC), sternomastoid (SItM), and lingual (Ling) muscles were separately dissected and fixed by immersion in 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (PB) at pH 7.4 for 1 to 24 hours. Skin-removed whole limbs were equally fixed, and tibialis anterior (TA), gastrocnemius (GN), soleus (SOL), and extensor digitorum longus (EDL) muscles were then separately dissected. Samples were cryoprotected with 30% sucrose in PB, embedded in Tissue Freezing Medium (TFM Triangle Biomedical Sciences, Durham, NC), frozen, and stored at −80°C for cryostat sectioning. For spinal cords, transverse serial cryostat sections (16 μm thick) from thoracic and lumbar segments were obtained.

For immunocytochemistry (ICC), sections were sequentially rinsed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100, blocked in 10% normal goat serum, and incubated with the primary antibody overnight at 4°C. The primary polyclonal rabbit antibodies used were anti-CGRP (Sigma; 1:1000), anti-Synaptophysin (SyPhys; Dako, Glostrup, Denmark; 1:100), anti–vesicular acetylcholine transporter (VACHT; Synaptic Systems, Göttingen, Germany; 1:1000), anti-Rab3A (Synaptic Systems; 1:200), anti–synaptobrevin-1 (VAMP-1; Synaptic Systems; 1:1000), anti–synaptotagmin 1 (SyTg; Synaptic Systems; 1:100), anti–cytostatin c (CSP; Chemicon; 1:100), anti-bassoon (Synaptic Systems; 1:200), anti–ionized calcium-binding adaptor molecule 1 (Iba-1; Wako Pure Chemical Industries, Osaka, Japan; 1:500), anti–neurofilament 160 kDa (NF) (Chemicon, Temecula, CA; 1:500), and anti–calcium channel (α1B subunit) (Ca2.2; Sigma; 1:100). Other antibodies used included chicken polyclonal anti-NF 68 kDa (Abcam, Cambridge, UK; 1:1000), chicken polyclonal anti–glial fibrillary acidic protein (GFAP; Abcam; 1:1000), mouse monoclonal anti–synaptic vesicle protein 2 (SV2; Hybridoma Bank, Iowa City, IA; 1:1000), mouse monoclonal anti–Pax7 (Hybridoma Bank; 1:100), and rat monoclonal anti–laminin-2 (Sigma; 20 μg/mL). In some cases, the chicken polyclonal anti-NF 68 was used instead of the rabbit anti–NF 160 to perform multiple immunolabeling with other rabbit antibodies. The anti-NF antibodies gave similar results and will be referred to as “NF.” Washed sections were incubated at room temperature (RT) for 1 hour with appropriate Cy3- and Cy5-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and with either AlexaFluor 488– or AlexaFluor 647–labeled α-Bungarotoxin (Molecular Probes; 1:500) to identify postsynaptic acetylcholine receptors. Sections were also labeled with 4′, 6-diamidino-2-phenylindole dihydrochloride (50 ng/mL; Molecular Probes) for DNA. Spinal cord sections were also labeled with fluorescent NeuroTrace Nissl staining (Molecular Probes).

Neuromuscular junctions were scored in a blinded manner and assigned to 1 of 3 groups according to the intensity of their immunoreaction: positive, mild (a very low signal but still distinguishable from the background), and negative.

To assess macrophage numbers, the sections were processed for immunoperoxidase staining with Iba-1 antibody, according to standard ABC (Vector Laboratories, Burlingame, CA) procedure.

Apoptosis was evaluated in muscle sections using TUNEL staining (In Situ Cell Death Detection Kit; POD, Roche, Penzberg, Germany) or active caspase-3 immunolabeling (Cell Signaling Technology, Danvers, MA; 1:200).

For Pax7 ICC, unfixed legs frozen in liquid N2-cooled isopentane were used. Cryostat sections (7 μm) were then

**FIGURE 2.** Expression of some synaptic proteins in neuromuscular junctions (NMJs) of intercostalis (IC) muscles from wild-type (WT) and spinal muscular atrophy (SMA) littermates. (A) The percentage of labeled NMJs of WT (○) and SMA(−) was evaluated in newborn (P0–1) and end-stage (P5–6) animals. Samples from 4 to 12 animals were analyzed for each immunostaining. To identify synaptic sites, specific immunostaining was combined with α-Bungarotoxin (α-Bgtx) labeling. Although the expression of most synaptic proteins is reduced in SMA, most of their NMJs remain innervated at end-terminal stages. The calcitonin gene–related peptide (CGRP) and Rab3A are markedly depleted in SMA NMJs. (B–I) To illustrate the compartmentalization of CGRP in a particular pool of synaptic vesicles, triple-labeled NMJs are shown after confocal microscope imaging. (B–E) CGRP (red) and the synaptic vesicle marker SV2 (blue) do not colocalize at NMJ delimited by α-Bgtx labeling. (F–I) In contrast, synaptophysin (SyPhys) (red) and SV2 (blue) show almost complete overlap. (J–M) Representative images of SyPhys (red in [J, K]) and CGRP (red in [L, M]) immunostaining of presynaptic protein expression in WT (J, L) and SMA (K, M) NMJs. Postsynaptic membranes are labeled with α-Bgtx (green), *p < 0.05; **p < 0.01; and ***p < 0.001 versus WT (one-way ANOVA followed by a post hoc Bonferroni test). Scale bars: I = 10 μm (valid for [B–H]); M = 10 μm (valid for [J–L]).
fixed for 10 to 30 minutes in 4% PFA in 0.1 mol/L PB and processed for Pax7 immunofluorescence in combination with laminin or TUNEL labeling.

For CGRP studies in the spinal cord, cryostat sections serially obtained through the entire thoracic and lumbar segments and labeled by immunoperoxidase were used. Numbers of CGRP-positive MNs were counted in every 30th section of thoracic and lumbar segments. Alternative sections were stained with Cresyl violet for evaluation of MN numbers.

Imaging was performed with an Olympus BX51 epifluorescence microscope (Olympus, Hamburg, Germany) equipped with a DP30BW camera or with a FluoView 500 Olympus laser scanning confocal microscope. Digital images were analyzed with Visilog 6.3 software (Noesis, Orsay, France).

Whole Mount Preparations
Flat muscles such as the Diaphragm, levator auris longus, and transversus abdominis (THA) were dissected and briefly fixed in 4% PFA (1 hour), washed in PBS, and frozen at −20°C. The samples were treated with 20% normal horse serum containing 0.4% Triton X-100 (6-8 hours) and incubated with primary antibodies on a shaking plate for 48 hours at 4°C. The primary antibodies used were anti-CGRP, anti-NF 160 kDa, anti-SyPhy, anti-Rab3A, and anti-SV2. Muscles were extensively washed with PBS containing 0.02%–TWEEN 20 and incubated with the appropriate secondary antibody overnight at 4°C. Slides were washed with PBS, mounted in 30% glycerol, and 3-dimensionally (3D) imaged by confocal microscope. For 3D reconstructions, NMs were optically sectioned into 0.5-μm thickness using a 60× N/A 1.4 objective. In some cases, NMs were 3D-reconstructed using Imaris Software (Bitplane, Zurich, Switzerland).

Muscle Fiber Morphometry
The SMA and WT mice legs were dissected, embedded in tragacanth gum, and snap-frozen in liquid N2-cooled isopentane. Cryostat sections (16 μm) were obtained and labeled with AlexaFluor 488–labeled wheat germ agglutinin (Molecular Probes; 25 μg/mL) to delimitate individual muscle fibers for morphometry. Images were taken with the confocal microscope and analyzed with Visilog 6.3 (Noesis).

Electron Microscopy
Muscles were microdissected in Hanks solution, pinned onto Sylgard (Down Corning, Wiesbaden, Germany) plates, and fixed in a solution containing 1% glutaraldehyde and 1% PFA in 0.1 mol/L PB, pH 7.4, for 1 hour at 4°C. The samples were trimmed in PB, postfixed in 1% OsO4, and embedded in EMbed 812 (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were taken from selected areas containing NMJs (identified previously in toluidine blue-stained 1-μm-thick semithin sections), counterstained with uranyl acetate and lead citrate, and then examined in a Zeiss EM 910 (Zeiss, Oberkochen, Germany) electron microscope.

Western Blot
Animals were decapitated for subsequent dissection of their distal limb muscles, which were then rapidly frozen in N2, and stored at −80°C until use. Limb muscles were fragmented with ultra Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany) and homogenized with 200 μL of warmed blending buffer 1 × SR (2% SDS and 125 mmol/L Tris-HCl, pH 6.8) and protease inhibitor (Sigma; ref. P8340) for 3 × 10 seconds. The homogenized muscle was heated to 100°C for 5 minutes and centrifuged for 5 minutes at 12,000 rpm, and the supernatant was collected. The protein concentration was determined by BIO-RAD Micro DC protein assay (BIO-RAD, Madrid, Spain). The loading buffer 4 × SS (20% sucrose and 0.05% bromophenol blue, 0.1% sodium azide) containing 5% to 10% β-mercaptoethanol (Sigma) and 20 to 30 μg of protein was loaded in a 15% polyacrylamide electrophoresis gel. Proteins were electrotransferred to a PVDF (Immobilon-P; Millipore, Bedford, MA) membrane in Tris-glycine-methanol buffer. The membrane was blocked with 5% fat-free dried milk dissolved in 0.1% Tween 20 in Tris-buffered saline pH 8 (TBST), for 1 hour at RT, and then extensively washed in TBS. The membrane was incubated overnight with the primary rabbit antibody anti–monocyte chemotactic protein-1 (MCP-1; Cell Signaling Technology; 1:1000) at 4°C with gentle agitation. The membrane was washed in TBST, incubated with a peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5000; Amersham Biosciences, Buckinghamshire, UK) in TBST I for 30 minutes at RT, washed in TBST, and then visualized using a SuperSignal Chemiluminescent detection kit (Pierce, Rockford, IL), as described by the manufacturer.

Statistical Analysis
Data are expressed as mean ± SEM. The statistical analysis was assessed by either one-way ANOVA (followed by post hoc Bonferroni test) or Student t-test. In all cases, differences were considered to be statistically significant if p < 0.05.

RESULTS
Phenotype, Survival, and Spinal Cord Changes in Severe SMA Mice
Nearly half of newborn Smmn−/−;SMN2+/+ mice died on the first postnatal day (46.5% n = 39). Surviving animals showed a deficiency in weight gain and did not live beyond P7 (not shown). It seems that food intake deficiency, affecting the nutritional status of ill pups, is not a major contributor of SMA phenotype, that is, although all the animals had reduced body weights and muscular atrophy, only approximately 50% of end-terminal mice had an empty stomach at the time of sampling. Overall, these findings are consistent with previously published results relating to the same model (13, 30).

In animals at advanced stages of the disease, moderate (approximately 20%) but significant loss of ventral horn MNs in the lumbar spinal cord was detected (Fig. 1A). In newborn animals (P0–1), some MN loss was observed, but because of individual variability, the change was not significant. No differences in MN numbers were observed at the lumbar spinal cord for embryonic ages (E18), indicating that naturally occurring MN programmed cell death, which takes place between E13 and E15 in the mouse spinal cord (31), was not altered as a consequence of SMN deficiency. To detect other possible
changes to the spinal cord of SMA mice, immunostaining was performed for GFAP and Iba-1 to visualize astroglia and microglia, respectively. Whereas no changes were found in microglial cells (Fig. 1B), astroglial processes around MNs increased in SMA. Quantification of the relative area of GFAP-positive structures within the ventral horn demonstrated that astroglia increased in SMA (3-fold in newborns and 5-fold at terminal stages of the disease). No changes were found in late embryonic (E18) stages (Figs. 1C–E).

Defective Expression of Synaptic Proteins in Smn<sup>j/j</sup>;SMN2<sup>+/+</sup> NMJs

Because severely paralyzed animals showed only a moderate MN loss in the terminal stage of the disease, we searched for changes in muscle and NMJs that could account for the motor deficit. Impairment of axonal transport proteins, including actin, and ribonucleoproteins throughout the motor nerves may be crucial in the pathogenesis of SMA (32, 33). To detect early changes in NMJ proteins in severe SMA mice, we analyzed the expression of different presynaptic markers in the IC muscle using ICC.

### TABLE. Immunohistochemical Analysis of Various Different Muscles From Wild-Type and Spinal Muscular Atrophy Mice at P5

<table>
<thead>
<tr>
<th>Muscle</th>
<th>% of Neuromuscular Junctions With Positive Immunoreactivity (WT)</th>
<th>% of Neuromuscular Junctions With Positive Immunoreactivity (SMA)</th>
<th>% of Neuromuscular Junctions With Positive Immunoreactivity (SMN2&lt;sup&gt;+&lt;/sup&gt;/+)</th>
<th>% of Neuromuscular Junctions With Positive Immunoreactivity (SMN2&lt;sup&gt;+&lt;/sup&gt;/+)</th>
<th>% of Neuromuscular Junctions With Positive Immunoreactivity (SMN2&lt;sup&gt;+&lt;/sup&gt;/+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus (3–9)</td>
<td>92.5 ± 2.26</td>
<td>71 ± 3.3</td>
<td>11.9 ± 3.9</td>
<td>6.7 ± 1.4</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>Gastrocnemius (4–5)</td>
<td>94.9 ± 3.09</td>
<td>73 ± 5.2</td>
<td>8.2 ± 4.2</td>
<td>6.7 ± 5.2</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>Extensor digitorum longus (4–6)</td>
<td>94.9 ± 3.09</td>
<td>73 ± 5.2</td>
<td>8.2 ± 4.2</td>
<td>6.7 ± 5.2</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>Tibialis anterior (4–5)</td>
<td>94.9 ± 3.09</td>
<td>73 ± 5.2</td>
<td>8.2 ± 4.2</td>
<td>6.7 ± 5.2</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>Dorsal diaphragmaticus (4–6)</td>
<td>94.9 ± 3.09</td>
<td>73 ± 5.2</td>
<td>8.2 ± 4.2</td>
<td>6.7 ± 5.2</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>Interosseus (4–6)</td>
<td>94.9 ± 3.09</td>
<td>73 ± 5.2</td>
<td>8.2 ± 4.2</td>
<td>6.7 ± 5.2</td>
<td>4.2 ± 0.9</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of endplates with positive immunoreactivity (% SEM). Numbers in parenthesis indicate the number of animals examined.

*<i>p</i> < 0.01, versus WT (Student's t-test).
†<i>p</i> < 0.001, versus WT (Student's t-test).
‡<i>p</i> < 0.05, versus WT (Student's t-test).
§<i>p</i> < 0.01, versus WT (Student's t-test).

CGRP, calcitonin gene-related peptide; NF, neurofilament; SMA, spinal muscular atrophy; SyPhys, synaptophysin; WT, wild-type.

FIGURE 3. Calcitonin gene-related peptide (CGRP) expression in lumbar and thoracic ventral horn MNs in wild-type (WT) and spinal muscular atrophy (SMA) mice. (A) Thoracic MNs of newborn and terminal SMA mice show a significant reduction in CGRP-positive MNs, which does not occur in the lumbar spinal cord. Numbers in parenthesis indicate number of animals analyzed. **, <i>p</i> < 0.01 versus WT (Student's t-test). (B, C) CGRP immunoreactivity in a P5 WT ventral horn. Calcitonin gene–related peptide expression is confined to MN somata; some neurites show a granular pattern. Scale bars: B = 100 μm; C = 25 μm.
FIGURE 4. Reduced Ca_{2.2} Ca^{2+} channel subunit immunoreactivity in spinal muscular atrophy (SMA). (A-D) Sections of intercostalis (IC) muscle from P0 wild-type (WT) (A, B) and SMA (C, D) mice immunolabeled for Ca_{2.2} Ca^{2+} channel subunit (red) showing its clustering at the presynaptic membrane of neuromuscular junctions (NMJs) identified by α-Bungarotoxin (α-Bgtx) binding (green); the antibody used also displays some nonspecific nuclear staining (arrows), as evidenced by its colocalization with 4’, 6-diamidino-2-phenylindole dihydrochloride (blue). Presynaptic Ca_{2.2} immunoreactivity is reduced in SMA. (E) Pixel intensity of Ca_{2.2} signal evaluated in IC muscle NMJs from 3 WT and 4 SMA P0–1 mice; the number of measured synapses is indicated in the bars. (F, G) High-magnification micrographs of Ca_{2.2} taken with a confocal microscope showing immunoreactivity in the presynaptic membrane of newborn WT NMJs ([F], red), which is absent or reduced in NMJs from newborn SMA ([G]); postsynaptic membrane was labeled with α-Bgtx (green). (H, I) Densitometric analysis along a line crossing presynaptic and postsynaptic membrane (represented in [F] and [G]) demonstrating the pixel intensity values for Ca_{2.2} (red) and α-Bgtx (green). Scale bars: D = 10 μm (valid for [A–C]); G = 1 μm (valid for [F]).
vesicle antibodies was used in combination with postsynaptic membrane labeling with α-Bungarotoxin (Fig. 2A). The results shown in Figure 2A (in which mild and positive NMJ immunoreactivity results were pooled) indicate that most NMJs in terminally affected SMA animals were still innervated. Only ~10% of synapses were devoid of the widely expressed synaptic vesicle proteins SyPhys or SV2; these 2 markers colocalized within the presynaptic compartment (Figs. 2B–E). Presynaptic cytoskeletal NF followed a similar profile. The proteins involved in the acquisition of cholinergic phenotype and synaptic vesicle functionality (e.g. VACHT, VAMP-1 [involved in the exocytosis machinery], the calcium sensor SyTg, or Rab3A) were downregulated early (P0) in SMA. This was particularly prominent in the case of Rab3A because the number of Rab3A-depleted NMJs was ~25% higher in P0–1 SMA than in WT. A marked decline in Rab3A was also observed at P5–6 SMA. Rab3A is involved in the activity-dependent transport of synaptic vesicles to, and their docking at, the active zone (34). Calcitonin gene–related peptide was also markedly downregulated in P0–1 SMA NMJs. This neuropeptide coexists with acetylcholine in separate vesicles in vertebrate NMJs (Figs. 2F–I). It is stored in large dense core vesicles and shows remarkable developmental and plasticity regulation. For example, CGRP is transiently high in developing and regenerating NMJs (35–39). In contrast to newborn WT IC, in which ~22% of the NMJs were devoid of CGRP, this neuropeptide was absent in more than 60% of innervated SMA NMJs. It is interesting to note that sensory/visceral CGRP immunoreactivity was well preserved in SMA affected muscles (not shown), suggesting selective depletion of CGRP in the motor nerves and NMJs.

The expression of some synaptic proteins was also determined in embryonic (E18) SMA IC NMJs. No changes in early motor innervation were detected after Rab3A, NF 160 kDa, or SV2 or SyPhys immunolabeling. However, CGRP was significantly depleted in SMA NMJs (not shown). The development of postsynaptic apparatus was also found to be impaired in SMA, showing a reduction in the area of clustered acetylcholine receptors adjacent to the nerve terminal (~23% on E18 and 65% on P5–6).

We next explored the expression of the representative NMJ proteins CGRP, SyPhys, and Rab3A to follow synaptic alterations in different SMA muscles (Table). SOL, TA, GN, EDL, Ling, Stm, and Diaph were all examined in cryostat sections. In contrast with the data obtained in the IC, CGRP downregulation was not found in TA, GN, Ling or EDL muscles in SMA. However, Diaph and, to a less extent, Ling both showed significantly decreased CGRP in SMA mice. Although not significant, data from Stm also indicated that CGRP might be downregulated in this muscle. Changes in CGRP immunoreactivity could not be assessed in SOL because the peptide was absent in motor nerves of both WT and SMA mice in this muscle. Muscles showing CGRP downregulation usually displayed reduced Rab3A, as was seen in IC. However, denervated synapses, identified by their lack of SyPhys, were rarely found in all the muscles examined at the terminal stage of the disease.

Immunoreactivity of CGRP was also determined in ventral horn MNs (Figs. 3A–C). As in the different muscle groups, CGRP was significantly depleted in thoracic but not in lumbar MNs in newborn SMA mice. Calcitonin gene–related peptide downregulation in thoracic MNs was not a consequence of the loss of any particular MN subtype because no significant loss of thoracic MNs was detected at this age (data not shown).

By ICC, Smn-deficient cultured MNs exhibited a reduced accumulation of the pore-forming subunit (Ca_{v2.2}) of the N-type voltage-gated Ca^{2+} channels (VGCCs) in growth cones, which correlated with a reduced frequency of local Ca^{2+} transients (40). We applied the same antibody to tissue sections of SMA IC muscles. Although the immunoreaction showed some background and notable nonspecific nuclear staining, examination at high power under a confocal microscope revealed that the presynaptic clustering of Ca_{v2.2} immunoreactivity was lost or reduced in samples from newborn SMA animals (Figs. 4A–I).

The entire neuromuscular innervation was also studied in whole-mount preparations of flat muscles immunostained for NF, SyPhys, SV2, Rab3A, and CGRP. Although denervated NMJs were scarce, CGRP expression was markedly downregulated in the TVA of newborn SMA mice, but no clear evidence of Rab3A downregulation was seen until P5–6 in this muscle (Table). Neuromuscular junctions were imaged at high resolution with the confocal microscope, and a 3D reconstruction was made (Figs. 5A–H; Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A242). The complexity of the presynaptic apparatus and how and in which extent this fits with the postsynaptic membrane were evaluated in newborn TVA after SV2 plus NF immunostaining (Figs. 5I–N). The results indicated that presynaptic organization was simplified in SMA; endplates had values of terminal axon arborization that were half those of the controls. In addition, occupation of the postsynaptic membrane area decreased more than 3-fold in SMA (Fig. 5O). We detected other changes in SMA muscles that were in line with other published descriptions (17, 18), including NF accumulation in the presynaptic terminals (Figs. 5P, Q) and axonal swellings in the intramuscular nerves (Fig. 5R). Neuromuscular junctions containing NF aggregates in presynaptic terminals were quantified in whole mounts of TVA muscle at P5. In SMA mice, the percentage was 48.7% ± 5.4%, whereas no NF aggregates were found in WT mice (number of NMJs analyzed = 97 from 4 WT mice and 235 from 6 SMA mice).

At P0–1, many of the motor axon terminals in both levator auris longus and Diaph not only were restricted to postsynaptic territories but also overgrew and overshot the NMJs (not shown), but this was also observed in the Smn^{+/−};SMN2^{+/−} that we used as a control. A similar observation was recently reported in this model (41, 42).

**Ultrastructural Changes in Muscles of SMA Mice**

Intercostalis, TVA, Diaph, and TA muscles were analyzed by electron microscopy in newborn and terminal SMA mice and their WT littermates (Figs. 6A–J). From the qualitative point of view, comparable alterations were seen in all these muscles. These changes involved NMJs, muscle cells, and intramuscular nerves. At the NMJs, a large variety was
observed in the size of presynaptic buttons, which is a normal feature of immature (P5–6) remodeling synapses. However, in SMA mice, there were tiny presynaptic terminals. In addition, presynaptic nerve terminals were often detached from the postsynaptic membrane, and there were large areas of postsynaptic membrane unoccupied by synaptic terminals (Figs. 6A, B). In some cases, detached nerve endings appeared to be surrounded by terminal Schwann cell processes, suggesting the active participation of these cells in the removal of nerve terminals from the postsynaptic sites (Fig. 6H). Synaptic vesicles were abundant in large synaptic terminals and their sizes were similar to those in WT (WT = 51.7 ± 0.3 nm, SMA = 53.4 ± 0.3 nm). However, the number of active zones and their associated vesicles was reduced in SMA, suggesting that a deficiency in neurotransmitter release could have arisen from this alteration (Figs. 6C, D, J). The number of autophagosome/lysosome-like structures was also increased inside presynaptic terminals in SMA (Fig. 6E). Presynaptic NF accumulation was also seen in a form similar to that previously described (17).

Most of the intramuscular nerves contained normally appearing axons, but occasional focal accumulation of autophagosome-like structures, indicative of axonal degeneration, was seen (Fig. 6G). Spinal muscular atrophy muscles showed focally localized areas of Z band disruption and myofibrillar disorganization. This was particularly evident in the TA and TVA of end-terminal mice (Fig. 6F).

Intercostalis and TA SMA muscles frequently exhibited mononucleated apoptotic cells within the muscle basal lamina (Figs. 7A–B). We deduced that these corresponded to muscle satellite cells because this morphology fits in with the definition of these cells (43). The apoptotic satellite cells often appeared to be engulfed by macrophage-like cells lying inside the muscle basal lamina. These macrophage-like cells sometimes contained several apoptotic bodies (not shown).

**Apoptotic Cell Death Is Increased in SMA Muscles**

Muscle apoptosis was further evaluated after performing either active caspase-3 immunostaining or TUNEL reaction on IC and TA muscle sections. Both techniques gave similar results. As shown in Figures 7E–H, the density of active caspase-3 cells within muscle in SMA was similar to that in WT at the time of birth, but muscle apoptosis in SMA was approximately 5 to 20 times greater than in the controls in both of the muscles examined at P5–6. By light microscopy, the apoptotic cells were located adjacent to muscle fibers (Fig. 7G). Electron microscopy demonstrated that muscle cell apoptosis was frequently observed in satellite cells (Figs. 7A–B'). When TUNEL staining was combined with laminin ICC to delineate muscle fiber basal lamina, it was found that 46.3% (±5.5%, n = 3 TA muscle from different P5–6 SMA mice) of TUNEL-positive nuclei and muscle fibers shared the same basal lamina (Figs. 7C, C'), as expected for muscle satellite cells (44). TUNEL staining was also combined with the satellite cell marker Pax7. In that case, TUNEL-positive nuclei did not show Pax7 immunoreactivity (Figs. 7D, D'). Because the apoptotic process involves abrupt changes in chromatin structure and proteolytic activity, it is possible that a transcription factor such as Pax7, which localizes within the nucleus, could be destroyed concomitantly with apoptosis; alternatively, satellite cells might die when Pax7 is down-regulated (45). Active caspase-3 immunostaining on P0–1, both in WT and SMA, revealed the presence of large and elongated apoptotic muscle fibers together with apoptotic mononucleated cells, indicating that the process of transient and normally occurring muscle cell death appears at this age (Fig. 7F). A considerable number (53.7%) of muscle apoptosis in SMA corresponded to interstitial cells. Although exploration of the nature of these cells is out of the scope of the present study, we suggest that they may correspond to interstitial macrophages clearing apoptotic satellite cells or/muscle resident (interstitial myogenic nonsatellite) stem cells (46).

To explore whether muscle apoptosis is a process linked to Smn<sup>−/−</sup>:SMN2<sup>+</sup> phenotype or, alternatively, a secondary response to denervation, sciatic nerve axotomy was performed on newborn (P0–1) Smn<sup>−/−</sup>:SMN2<sup>+</sup> mice. Cleaved caspase-3 immunoreactivity was evaluated in denervated TA muscles 48 and 72 hours after axotomy. Contralateral (normally innervated) TA muscles were used as controls. No significant differences in the number of apoptotic profiles were found between ipsilateral (denervated) and contralateral TA muscles (48 hours after axotomy: ipsilateral = 5.1 ± 1.7, contralateral = 8.3 ± 4.4, n = 3; 72 hours after axotomy: ipsilateral = 23.8 ± 12.6, contralateral = 24.8 ± 12.7, n = 2; expressed in apoptosis/mm<sup>2</sup>). By contrast, it should be noted that, in newborn rats, virtually all spinal cord MNs have already died by apoptosis 48 hours after sciatic nerve transection (47).

Analysis of the postnatal growth of TA muscle after measuring individual muscle fiber size was concordant with a
defect in postnatal myogenesis. The distribution frequency of muscle fiber size in newborn animals was similar for both the SMA and WT TA muscles (Figs. 8A–D). However, it seems that muscle growth was further arrested, that is, the fiber size histograms on P5–6 were similar to P0–1 in SMA mice. This contrasted with WT mice, in which the P5–6 histogram was shifted to the right because of normal muscle growth. The characteristic, large hypertrophic fibers usually present in human SMA muscles were not seen in severe SMA mice.

Depletion of Interstitial Macrophages in Muscles and Atrophy of Immune Organs in SMA Mice

Because apoptosis with subsequent macrophage recruitment was apparently stimulated in SMA muscles, we analyzed the status of interstitial macrophages in these muscles. Sections of TA, IC, Ling, and StM muscles were immunostained with anti–Iba-1 antibody to detect the macrophage population; positive cell densities were also assessed (Figs. 9A–D). Surprisingly, in P5–6 SMA, all the muscles examined displayed a notable reduction of macrophage density. When these measurements were made in newborns, no differences were observed between WT and SMA (Fig. 9E). Because MCP-1 is a factor involved in the recruitment of monocytes in connective tissues, the expression of this protein was analyzed in limb WT and SMA muscle extracts. As shown in Figures 9F and G, MCP-1 was significantly downregulated in P5–6 SMA.

To determine whether macrophage depletion was tissue-specific, the density of Iba-1–positive cells was also measured in the spleen and liver, but no differences between SMA and WT were observed in these organs. However, the weights of the spleen and thymus were reduced by approximately 4- to 6-fold in P5–6 SMA versus WT. Weights of other organs, such as the kidney and liver, were only reduced 2-fold in SMA, following the same profile observed for the whole body (Figs. 9H–J).

In most SMA mice, histologic studies showed that there was massive cortical lymphocyte apoptosis in the thymus at terminal disease stages (8’11 of the animals examined). Apoptosis was demonstrated by several techniques: hematoxylin and eosin staining of paraffin sections, 4’,6-diamidino-2-phenylindole dihydrochloride staining on cryostat sections, toluidine blue–stained plastic sections, and electron microscopy (Figs. 9K–N). No relationship was found between thymus apoptosis and gastric milk depletion.

DISCUSSION

Our phenotypic analysis of Smn−/−, SMN2+/+ mice shows that instead of MN death, early NMJ defects are the pathologic hallmark of SMA. For example, the number of lumbar MNs was not significantly reduced in newborn SMA mice, and only a moderate loss (~20%) was observed at the end stage of the disease. However, some early alteration may occur at the spinal cord because detectable ventral horn astrocytosis was found before MN death. Astroglial activation is frequently associated with MN pathology in ALS and after axotomy; it may have complex and opposite, that is, either neuroprotective or neurotoxic, effects (48, 49). How reactive astrocytes affect MN function and synaptic connectivity in SMA should be investigated further. It is interesting to note that the number of glutamatergic terminals on MN cell bodies is reduced in SMA mice (50, 51). The absence of noticeable spinal cord microgliosis suggests that, in contrast to the SMA Δ7 model, neuroinflammation is not a relevant mechanism in the most severe SMA mice (50). Nevertheless, several alterations in NMJs, which correlate with the disease progression, were seen. Some of these data are in line with other reports on the SMA mouse model in which NF accumulation and the simplification of presynaptic terminals are described as early detectable pathologic features (17–21). In addition, we provide evidence that some molecular components of presynaptic terminals were downregulated in normally innervated NMJs of the most severely affected proximal muscles (i.e., IC, TVA, and StM). In these muscles, there was a remarkable fall in Rab3A and CGRP, whereas other synaptic vesicle markers such as SyPhys and SV2 were normal or slightly decreased. Rab3A is a GTP-binding protein involved in targeting synaptic vesicles to the active zones and in neurotransmitter release in nerve terminals (52, 53). In Rab3A null mutants, the activity-dependent recruitment of synaptic vesicles to active zones and the recovery of synaptic transmission after exhaustive stimulation were both impaired (34). Therefore, it is conceivable that Rab3A depletion in SMA results in comparable defects in transmitter release in neuromuscular junctions (NMJs) and muscle cells in spinal muscular atrophy (SMA) mice. (A) NMJ from P5 WT intercostalis (IC) muscle shows normal organization of nerve terminals (nt, red) establishing synaptic contacts with a skeletal muscle cell (musc, green). The synaptic terminals are covered by terminal Schwann cells (SC, yellow). (B) A NMJ taken from P5 SMA IC muscle shows a reduced number and size of nerve terminals leaving unoccupied a large portion of the postsynaptic membrane (arrows). (C, D) Clustered synaptic vesicles close to presynaptic membrane, which form active zones, in wild-type (WT) muscle (arrows in [C]); in SMA, synaptic vesicles are not organized in active zone clusters (D). Images are from IC synaptic terminals. (E) Autophagosome-like vacuoles and lysosomal inclusions (*) are frequently seen within presynaptic axon terminals in a P5 SMA tibialis anterior (TA) muscle. (F) Focal disruption of myofilament organization with dissolution of Z band structure in P5 SMA TA muscle (*) compared with the normal appearance of an adjacent muscle cell displaying clearly visible Z bands (arrow). (G) Degenerating myelinated (my) axon within an intramuscular nerve from P5 SMA TA containing an abnormal accumulation of multilamellar organelles and membrane vesicles. (H) An axon terminal (nt in red) from P5 SMA tibialis anterior (TA) appears engulfed by Schwann cell (SC) processes (yellow) and detached from the postsynaptic membrane; postsynaptic muscular cell is labeled in green. Some atrophic nerve terminals (red) remain in close contact with postsynaptic membrane. (I) NMJ from P5 SMA diaphragm (Diaph) displaying normal ultrastructure of nerve terminals (nt) with well-organized active zones (arrows) and folded postsynaptic membrane. (J) Quantification of the number of synaptic vesicles docked at the active zones; the number of vesicles was referred to in relation to the length of analyzed presynaptic membrane; the data are from 13 scored endplates. *, p < 0.05 versus WT (Student t-test). Scale bars: A = 3.6 μm; B = 2.3 μm; C = 0.1 μm (valid for [D]); E = 1.16 μm; F = 2.3 μm; G = 1.8 μm; H = 0.9 μm; I = 0.5 μm. Sometimes, structures were manually dashed with false color.
neuromuscular synapses. In fact, the evoked neurotransmitter release has been found decreased in severe SMA mice before the appearance of extensive denervation (21, 54). Our data on active zone reduction in SMA presynaptic terminals is consistent with the observed molecular and functional alterations and in accordance with data published in the SMA Δ7 model (21). The organization of presynaptic active zones is tightly linked to VGCCs, which play an important role as scaffolding proteins through their interaction with laminin β2 (55). Therefore, the downregulation of VGCCs we found in SMA is in concordance with the reduction in active zones.

We observed early depletion of CGRP in presynaptic terminals in apparently normal synapses. As CGRP was not equally expressed in different muscles and ages (56), its implication in SMA can only be assessed when compared with appropriate matched controls. For example, CGRP was found to be downregulated in proximal SMA-affected muscles such as IC and TVA, whereas it was normally expressed in less affected distal muscles. However, in slow-twitch SOL muscle, CGRP was absent in both SMA and controls. Calcitonin gene–related peptide is normally present in the CNS and, particularly, in cell bodies of large subpopulations of cranial and spinal MNs (57–60). This peptide is regulated during the development, plasticity, and regeneration of nerves and NMJs (36–39, 61, 62). It has been reported that CGRP enhances the postsynaptic response at developing NMJs through CGRP-receptor binding and cAMP-dependent signaling (63). It has also been proposed that CGRP is a nerve-derived activator of the transcription of postsynaptic acetylcholine receptors (64). Whether CGRP reduction in SMA contributes to depressed neuromuscular transmitter transmission should be elucidated. It seems that the depletion of CGRP-containing vesicles in SMA NMJs may not be a consequence of reduced axonal transport because MN cell bodies in the thoracic spinal cord, which innervate proximal muscles, also showed reduced CGRP levels. The possibility that an axonal deficiency in mRNA axonal transport accounts for CGRP depletion in SMA should also be investigated because an intra-axonal synthesis of CGRP occurs during peripheral nerve regeneration (62). The specificity of CGRP downregulation in SMA motor system should be noted because the expression of this peptide in the sensory and autonomic nervous system remained.

FIGURE 7. Muscle satellite cell apoptosis in spinal muscular atrophy (SMA) mice. (A–B) Electron micrographs of a skeletal muscle fiber taken from P5 SMA intercostalis (IC) showing 2 examples of apoptotic mononucleated satellite cells (pseudocolored in red) with appropriate matched controls. For example, CGRP was found to be downregulated in proximal SMA-affected muscles such as IC and TVA, whereas it was normally expressed in less affected distal muscles. However, in slow-twitch SOL muscle, CGRP was absent in both SMA and controls. Calcitonin gene–related peptide is normally present in the CNS and, particularly, in cell bodies of large subpopulations of cranial and spinal MNs (57–60). This peptide is regulated during the development, plasticity, and regeneration of nerves and NMJs (36–39, 61, 62). It has been reported that CGRP enhances the postsynaptic response at developing NMJs through CGRP-receptor binding and cAMP-dependent signaling (63). It has also been proposed that CGRP is a nerve-derived activator of the transcription of postsynaptic acetylcholine receptors (64). Whether CGRP reduction in SMA contributes to depressed neuromuscular transmitter transmission should be elucidated. It seems that the depletion of CGRP-containing vesicles in SMA NMJs may not be a consequence of reduced axonal transport because MN cell bodies in the thoracic spinal cord, which innervate proximal muscles, also showed reduced CGRP levels. The possibility that an axonal deficiency in mRNA axonal transport accounts for CGRP depletion in SMA should also be investigated because an intra-axonal synthesis of CGRP occurs during peripheral nerve regeneration (62). The specificity of CGRP downregulation in SMA motor system should be noted because the expression of this peptide in the sensory and autonomic nervous system remained.

FIGURE 8. Relative frequency histogram of muscle fiber areas in tibialis anterior (TA) from newborn and end-stage spinal muscular atrophy (SMA) mice and age-matched wild-type (WT) mice. (A, B) Representative images of wheat germ agglutinin (WGA)–lectin labeled limb muscle cryostat sections from P5 WT (A) and SMA (B) used for histometry. (C, D) Histograms of muscle fiber size (±SEM) from newborn (P0–1) and P5–6 SMA tibialis anterior (TA) (n = 3–6). Note the histogram left shifting in P5–6 SMA. Scale bar: A = 60 μm (valid for [B]).

© 2011 American Association of Neuropathologists, Inc.
cell death in mammalian muscle, there is evidence that muscle
regenerative and reparative events.

Increased muscle satellite cell apoptosis in muscles of
P5–6 SMA animals correlated with disease progression. Apoptosis in other muscle cell types was virtually absent after
extensive ultrastructural screening. Therefore, the mononucleated active caspase-3- and TUNEL-positive cells observed
by light microscopy were likely mainly satellite cells. Whether satellite cell apoptosis is a consequence of SMN deficiency
or a secondary response to muscle denervation, or both, can-
not be yet stated. It is believed that satellite cells are the
source of myogenic precursors for postnatal muscle growth
and that this is largely due to the formation of secondary
myotubes (66). During the embryonic development of rats,
primary myotubes develop autonomously even in the absence
of innervation, but the generation of secondary myotubes is
regulated by nerves (67). Our histograms showing the arrest
of muscle fiber growth in SMA suggest that the generation
of secondary myotubes was impaired and that satellite cell
apoptosis might account for this. On the other hand, deletion
of Smn exon 7 directed to muscle satellite cells is sufficient
to induce severe myopathy, indicating that muscle in itself
is an important contributor to SMA pathology (68). Likewise,
SMN gene mutation restricted to skeletal muscle results in
myopathy induced by SMN deficiency (68). Our data showing apoptosis in SMA satellite cells are parallel
observations of a large number of apoptotic nuclei in muscle
biopsies from children with SMA (69, 70).

In contrast to P5–6 mice, we observed that both WT and
SMA newborn (P0–1) muscles had apoptosis. This probably
reflects processes of normal developmental muscle remodel-
ing. Although, as far as we know, there are no available sys-
tematic studies on developmentally regulated programmed
cell death in mammalian muscle, there is evidence that muscle
cell death occurs during normal development in the chick
embryo (71).

With regard to the presumed specific role of SMN protein in muscle cells, it has been reported that SMN par-
ticipates in the structural organization of the Z band (72). Our
frequent observation that areas with focal myofibrillar dis-
organization in the Z band were preferentially disrupted
whereas adjacent myosin and actin filaments remained unaf-
ected in SMA muscle is consistent with this.

On the basis of the finding of stimulated apoptosis within SMA muscle, we expected that the recruitment of macrophages destined to eliminate dying cells would lead to an increased density of interstitial macrophages in the muscle connective tissue. However, we observed just the opposite: SMA muscles exhibited between 25% and 50% lower macro-
phage density versus controls. It is known that macrophages
exert an antiapoptotic action on muscle satellite cells, as has
been shown in vitro; it has also been also demonstrated that
MCP-1 is one of the major factors influencing monocyte
chemotaxis by muscle precursor cells (74). Although we
found that MCP-1 was decreased in SMA muscle extracts,
whether macrophage depletion is related to increased muscle
precursor apoptosis and a deficiency in postnatal myogenesis
should be investigated further. In any case, macrophage deple-
tion was tissue-specific because it was not observed in either
the liver or the spleen.

There was massive apoptosis in the thymus cortex of
SMA mice. A similar phenotype of massive death of the cor-
tical lymphocytes was reported in TrkB-deficient mice and a
role for TrkB signaling in the regulation of thymus apoptosis
was suggested (75). Similarly, SMN may be involved in the
mechanisms that determine cortical thymus lymphocyte apo-
ptosis, but we do not have evidence to support this hypothesis.
Alternatively, enhancement of thymocyte apoptosis could
be a secondary response in terminally stressed animals. In
fact, glucocorticoids and restraint stress can induce apoptotic
thymus involution (76, 77). However, the fact that we ob-
served a selective atrophy of immune organs such as the
thymus and spleen suggests a possible primary defect in the
immune system in severe SMA mice. It has recently been

FIGURE 9. Muscle macrophage depletion and immune organ atrophy in terminal spinal muscular atrophy (SMA) mice. (A–D) Tibialis anterior (TA) muscle cryostat sections of P6 wild-type (WT) (A, B) and SMA (C, D) mice immunostained for Iba-1 (green) to
assess macrophage density. Higher power images of interstitial macrophages are shown in (B) and (D) after counterstaining with phallloidin (red) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue) to detect actin and DNA, respectively. (E) Quantification of muscle interstitial macrophage density showing a significant decrease in SMA at the end stage. Numbers in parenthesis indicate the number of animals analyzed. (F) Western blot from extracts of whole limb muscles showing monocyte
chemotactic protein-1 (MCP-1) downregulation in end stage SMA mice. (G) Quantification of MCP-1 Western blot data. (H) A representative example of spleen macrophomorphology showing conspicuous atrophy in the P5 SMA versus WT. (I) Plots of relative
organ weight indicate the selective spleen atrophy in SMA mice. (J) Plots of organ weight ratio P5–6 WT/SMA demonstrate the
selective atrophy of immune system organs. Data from (I) represent the mean ± SEM of 4 to 11 animals. (K–N) Representative images of thymus histology from P6 WT and SMA mice. Cortical lymphocytes in SMA display massive apoptosis demonstrable after
DAPI staining (M) and electron microscopy (* in [N]). **, p < 0.01; and ***, p < 0.001 versus WT (Student t-test). Scale bars: A =
100 μm (valid for [C]); B = 10 μm (valid for [D]); M = 20 μm (valid for [K]); N = 5 μm (valid for [L]). Small divisions in (H) are in
millimeters.

© 2011 American Association of Neuropathologists, Inc.
demonstrated that SMN depletion in cell types other than MNs may significantly contribute to SMA pathology (50). In addition, systemic immune aberrations and spleen atrophy have been reported in a mouse ALS model (78).

In summary, our results sustain the idea that the maintenance of NMJ innervation is severely affected in SMA and that defective skeletal muscle development may be a crucial pathogenetic element in this disease. In addition, the conspicuous pathologic alteration detected in the immune system organs of SMA animals deserves further attention.

ACKNOWLEDGMENTS

The authors thank Drs Lucia Tabares, Rocío Ruiz, Neus Garcia, María Ángel Lanuza, and Dolors Ciutat for helping with muscle microdissection and Montse Ortega for technical assistance.

REFERENCES

16. Le TT, Pham LT, Butchbach ME, et al. SMNDelta7, the major product of the centromeric survival motoneuron (Smn2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. Hum Mol Genet 2005;14:845–57
28. Dachs E, Hereu M, Esquerda JE. Defective postnatal development of neuromuscular junctions and conspicuous muscle pathology is observed in mice with severe spinal muscular atrophy [abstract]. Presented at the 13th Annual International Spinal Muscular Atrophy Conference. Research Group Meeting; Cincinnati, OH; June 18–20, 2009
47. Colemen WL, Bliil CA, Bykhowskaia M. Rab3a deletion reduces vesicle docking and transmitter release at the mouse diaphragm synapse. Neuroscience 2007;48:1–6
49. Chen J, Billings SE, Nishimune H. Calcium channels link the muscle-derived synapse organizer laminin β2 to Bassoon and CAST/Erc2 to organize presynaptic active zones. J Neurosci 2011;31:512–25
71. Coleman WL, Bill CA, Bykhowskaia M. Rab3a deletion reduces vesicle docking and transmitter release at the mouse diaphragm synapse. Neuroscience 2007;48:1–6
73. Chen J, Billings SE, Nishimune H. Calcium channels link the muscle-derived synapse organizer laminin β2 to Bassoon and CAST/Erc2 to organize presynaptic active zones. J Neurosci 2011;31:512–25