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SYMPOSIUM: Recent Advances in Hereditary Neuromuscular Diseases of Childhood

Spinal Muscular Atrophy: Present State

Henning Schmalbruch[1] and Georg Haase[2]

[1] Department of Medical Physiology, Division of Neurophysiology, University of Copenhagen, Denmark
[2] INSERM U.382, Institut de Biologie du Développement de Marseille, Campus de Luminy, Marseille, France

Spinal muscular atrophy (SMA) is a hereditary neurodegenerative disease caused by homozygous deletions or mutations in the *SMN1* gene on Chr.5q13. SMA spans from severe Werdnig-Hoffmann disease (SMA 1) to relatively benign Kugelberg-Welander disease (SMA 3). Onset before birth possibly aggravates the clinical course, because immature motoneurons do not show compensatory sprouting and collateral reinnervation, and motor units in SMA 1, in contrast to those in SMA 3, are not enlarged. Genetic evidence indicates that *SMN2*, a gene 99% identical to *SMN1*, can attenuate SMA severity: in patients, more *SMN2* copies and higher SMN protein levels are correlated with milder SMA. There is evidence that SMN plays a role in motoneuron RNA metabolism, but it has also been linked to apoptosis.

Several mouse models with motoneuron disease have been successfully treated with neurotrophic factors. None of these models is, however, homologous to SMA. Recently, genetic mouse models of SMA have been created by introducing human *SMN2* transgenes into *Smn* knockout mice or by targeting the *Smn* gene knockout to neurons. These mice not only provide important insights into the pathogenesis of SMA but are also crucial for testing new therapeutic strategies. These include *SMN* gene transfer, molecules capable to up-regulate *SMN* expression and trophic or antiapoptotic factors.

Introduction

Spinal muscular atrophy (SMA) is a hereditary motoneuron disease often causing death in early childhood. The identification of mutations in the survival motor neuron (*SMN1*) gene in patients with SMA has helped to ascertain the diagnosis in individual patients and brought important insights into the pathogenesis of SMA. These discoveries and the development of genetic animal models of SMA might open new avenues to future therapies for this devastating disease.

SMA is traditionally classified according to clinical criteria: children with infantile Werdnig-Hoffmann's disease (SMA 1) never learn to sit unaided; prognosis is poor when symptoms are present at birth or before the age of two months (140). Eighty per cent of children with SMA 1 die within the first year, and survival beyond the age of 2 years is exceptional (37). Some cases present with diminished fetal movements and postnatal asphyxia and resemble congenital axonal neuropathy (37, 74, 85). Children with intermediate SMA 2 learn to sit but not to walk, whereas those with the chronic juvenile Kugelberg-Welander form (SMA 3) eventually walk. In 569 patients with SMA 2 and 3, life expectancy was statistically normal for those with SMA 3, while survival rates for patients of type 2 was 99% and 69% after 5 and 25 years, respectively. The probability of still being able to walk 10 and 40 years after onset was 70% and 22% for SMA 3 patients when the disease had started before the age of 3 years, and 97% and 59% when it had started after the age of 3 years (154, 155). In 504 patients, a moderate elevation of the serum creatine kinase was found in patients with SMA 3 but not in those with SMA 1 or 2 (119). This suggests that secondary myopathy only occurs in SMA 3.

Molecular Genetics of SMA

In 1990, genetic linkage analysis showed that SMA types 1 to 3 map to chromosome 5q11.2-13.3 (13, 50, 90, 92). Further refinement of the genetic map allowed prenatal diagnosis in SMA families (36, 91). The physical map revealed that the SMA region contained an inverted duplication of >500 kB with specific low copy repeats, retrotransposons and pseudogenes which make this region prone to large-scale deletions (44, 93, 130). Four genes have been identified in this region: the survival motor neuron (*SMN1*) gene (77), the neuronal apoptosis inhibitory protein (*NAIP*) gene (118, 141), the *p44* gene, which encodes a subunit of the basal transcription factor TFIIH (17, 22) and *H4F5*, a gene with unknown function (121). The four genes are duplicated with a telomeric and a centromeric copy (Figure 1A).

Corresponding author:
Henning Schmalbruch, M.D., Dept. of Medical Physiology, Division of Neurophysiology, The Panum Institute, Blegdamsvej 3 c, DK2200 Copenhagen, Denmark; Tel.: +45 3532 7464; Fax: +45 3532 7499; E-mail: H.Schmalbruch@mfi.ku.dk

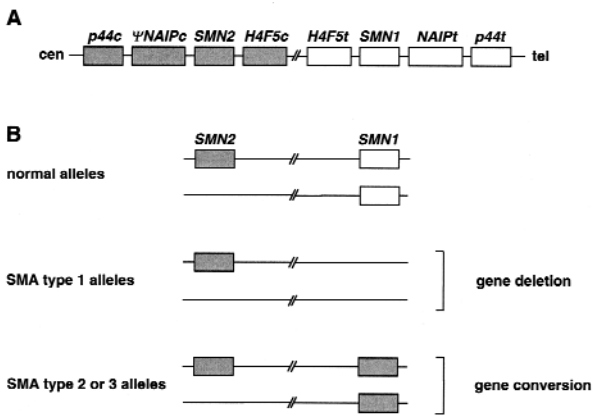

Figure 1. **A**. Diagrammatic representation of the duplicated region on chromosome 5q13, showing positions of the *p44*, *NAIP*, *SMN* and *H4F5* genes. *ΨNAIP* is a *NAIPc* pseudogene. **B**. Model of SMN gene deletion and conversion events proposed by Campbell *et al* (21). Normal alleles contain one functional *SMN1* gene (open box) and one, or in rare cases zero, *SMN2* genes (shaded boxes). Deletion of *SMN1* does not alter the number of *SMN2* copies and causes severe SMA. Gene conversion of *SMN1* into *SMN2* increases the number of *SMN2* copies and most often underlies milder SMA.

While deletions in either of the four genes could be detected in SMA patients, it is now clear that the telomeric *SMN* gene, termed *SMN1* (*SMNt*), is the only SMA-causative gene.

The *SMN1* gene covers about 20 kb, contains nine exons and is to 99% identical with its centromeric copy *SMN2* (*SMNc*). *SMN1* differs from *SMN2* by the length of a CA dinucleotide repeat in the 5′ upstream region (39, 100), by three nucleotide substitutions in introns and by two nucleotide substitutions in exons 7 and 8 (16, 77, 99). For routine diagnosis, the *SMN1* and *SMN2* genes are distinguished at the level of exons 7 and 8 by SSCP (single-strand conformation polymorphism) (77) or by enzymatic digestion of PCR products (146).

To date, *SMN1* gene analysis has been performed in more than 700 SMA 1 to 3 patients from different geographic origin. Homozygous *SMN1* gene deletions were found in 90% to 100% of cases (20, 24, 54, 77, 117, 146). Subtle mutations accounted for 3.4% of cases in a study on 525 typical SMA patients (150). These subtle mutations comprise point mutations (55, 113, 137), micro-deletions or insertions (8, 20, 111, 150) or small duplications (112) which are scattered through exons 1 to 7. In control individuals, the *SMN1* gene was always present whereas the *SMN2* gene was absent in about 5% without any pathological consequence (77).

Several observations pointed to the existence of SMA modifier genes: *1)* The *SMN1* deletion alone could not account for the clinical SMA severity because over 90% of SMA patients showed *SMN1* exon 7 deletions irrespective of the type of SMA. *2)* Rare families have been reported with asymptomatic individuals displaying the same *SMN1* haplotype as affected relatives (30, 54, 149, 150). *3)* The analysis of several genetic markers in the SMA region has revealed a tight correlation between the extent of the deletion and disease severity. Indeed, 68% of SMA type 1 patients displayed large scale deletions encompassing the *SMN1*, *NAIPt* and *p44t* gene, in contrast to less than 5% of SMA type 2 and SMA type 3 patients (17, 18, 147). *NAIP* was an interesting candidate as SMA modifier gene because of its sequence homology to two baculovirus IAPs (inhibitor of apoptosis proteins) (29). Several studies demonstrated that *NAIP* overexpression can attenuate neuronal apoptosis *in vitro* (94) or *in vivo* when induced by ischemia (151) or axonal injury (115). Further studies will be required to affirm — or to disprove — a role of *NAIP* as a physiologically relevant SMA modifier gene. It has also been proposed that the *SMN2* gene can act as a modifier gene in SMA (94). Genetic evidence supports the idea that *SMN2* can influence the SMA phenotype. To determine the number of *SMN2* copies in SMA patients or carriers, McAndrew *et al* (89) have used competitive PCR and Campbell *et al* (21) pulsed-field gel electrophoresis. Interestingly, the number of *SMN2* copies in SMA 2 or 3 chromosomes was found to be greater than in SMA 1 chromosomes. These results not only provided the much awaited genotype/phenotype correlation in SMA, but also suggested the existence of different genetic mechanisms responsible for severe and mild forms. Gene deletion is supposed to predominate in SMA 1 whereas gene conversion events, *i.e.* conversion of *SMN1* into *SMN2*, would prevail in SMA 2 or 3 (Figure 1B).

SMN gene analysis also allowed the separation of classical SMA from forms of spinal muscular atrophy that are associated with additional defects (SMA plus) and from other neuromuscular disorders. Homozygous *SMN1* deletions were found in patients with congenital cytoplasmic body myopathy (145), arthrogryposis multiplex congenita (15) and arthrogryposis multiplex congenita with cardiac defects that clinically would not have been suspected to have SMA (69). No SMN deletions were detected in the spinal form of Charcot-Marie-Tooth disease (56) or in amyotrophic lateral sclerosis (102, 108, 109).

Function of SMN

The *SMN* genes are transcribed into an mRNA of 1.5

kb which encodes a 294 amino acid protein of 38 kD predicted molecular weight. The expression of SMN mRNA or SMN protein is ubiquitous and has been detected in spinal cord, skeletal muscle, lung, liver, heart, kidney and liver (19, 31, 77, 78, 106) Expression of the *SMN* genes not only gives rise to full length transcripts but also to transcripts without exon 7 (SMNΔ7) or without exon 5 (SMNΔ5) (49, 77). Interestingly, *SMN1* produces predominantly full length SMN (90%), only 10% of SMNΔ5 and no detectable SMNΔ7. In contrast, *SMN2* expression leads to only 20 to 30% of full length SMN and predominantly produced SMNΔ7 (60%). The splicing of SMN2 pre mRNA into SMNΔ7 is due to a single nucleotide difference in exon 7 (codon 280: TTT in *SMN2* versus TTC in *SMN1*) (83, 99) and seems to yield a protein with decreased *in vitro* stability (82).

Several studies have investigated the expression of SMN protein in SMA patients. Using western blot or immunohistochemical analyses in several tissues including spinal cord, Lefebvre *et al* (78) and Coovert *et al* (31) found a marked decrease of SMN protein in fetal spinal cord in SMA patients as compared to normal controls. In fetal muscle of type 1 SMA, SMN immunoreactivity was also reported to be lost (19). Lefebvre *et al* (78) demonstrated a negative correlation between clinical SMA severity and SMN protein levels in lymphoblastoid cells. This study strongly suggested that, in milder forms of SMA, SMN protein derived from the *SMN2* gene could partially compensate for the absence of *SMN1*.

Yeast two hybrid screens and biochemical interaction studies were used to identify cellular partners of the SMN protein and implicated SMN in cellular functions as diverse as RNA metabolism and apoptosis. In yeast, Dreyfuss and colleagues detected SMN as binding partner of ribonucleoprotein U (80), and, when using SMN as a bait, demonstrated that SMN also interacted with itself, fibrillarin and with a novel protein called SIP-1 (SMN interacting protein-1 [81]). Further interactions of SMN with the proteins Gemin-3 and Gemin-4 were found by co-immunoprecipitation (25, 26). In HeLa cells, SMN, SIP-1 and Gemin-3 were localized within the cytoplasm and in novel nuclear structures which were adjacent to coiled bodies and therefore termed gems ("gemini of coiled bodies"). Gems appeared as 2 to 8 dot-like structures per nucleus but lacked expression of coilin, a marker of coiled bodies; for more details, see (87). The subcellular localization of SMN however is currently debated because the majority of

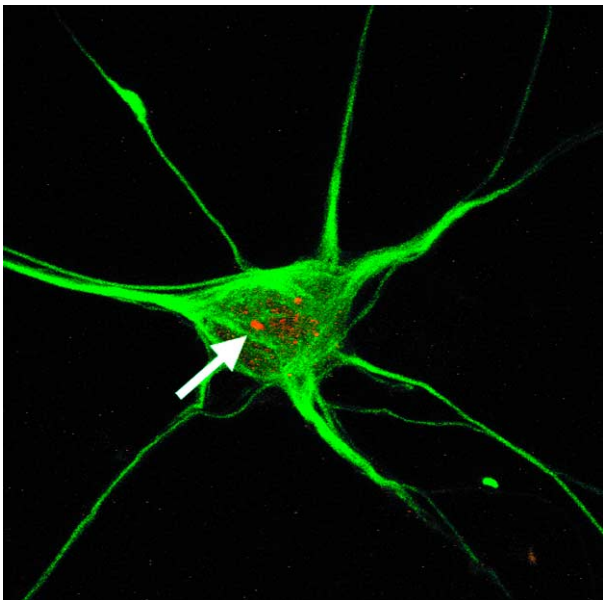

Figure 2. SMN is localized in distinct nuclear organelles of cultured motoneurons. Superimposed confocal images of immunofluorescence labeling with antibodies directed against SMN (in red) and neurofilament (NF 160 kD, in green). SMN is detected in nuclear organelles (arrow) which have the typical size and ovoid-like shape of coiled bodies and which are also immunoreactive for coilin (76). Figure kindly provided by Dr. B. Pettmann.

cells, including primary neurons, express SMN precisely in coiled bodies (4, 23, 76, 86) (Figure 2).

The interaction of SMN with an RNA binding protein and its localization in specific nuclear structures raised the hypothesis that SMN was involved in RNA metabolism. In eucaryotes, premessenger RNAs are spliced in spliceosomal complexes. These are assembled in a highly ordered manner: small uridine-rich RNAs are transcribed in the nucleus, exported into the cytoplasm, associate with specific Sm proteins to snRNPs (small ribonuclear ribonucleoproteins) and the complexes are reimported. Although the precise role of SMN in RNA processing is not understood, SMN seems to be important in the assembly and function of the spliceosomal complexes (42, 114): SMN/SIP-1 can bind to specific Sm proteins and SMN can stimulate splicing of heterologous pre mRNAs. On the other hand, some SMN mutations cause diminished interaction with Sm proteins or reduced pre mRNA splicing. Further studies are required to establish whether pre mRNA splicing is indeed impaired in motoneurons of SMA patients.

Programmed cell death has for a long time been viewed as an important feature in SMA. The study of Iwahashi *et al* (66) showing that SMN binds to and colocalizes with the anti-apoptotic molecule Bcl-2 has there-

fore gained considerable attention. Interestingly, when SMN and Bcl-2 were co-expressed in HeLa cells, they also synergized in counteracting the pro-apoptotic effects exerted by Bax or Fas. In cultured motoneurons however, SMN does not colocalize with Bcl-2 (76) which makes the role of SMN in preventing neuronal apoptosis questionable.

Histopathology and histogenesis

SMA is traditionally assumed to primarily affect motoneurons with subsequent axonal degeneration and denervation of muscle fibres. Autopsy studies (almost exclusively of SMA 1 patients; for review, see [35]) have thus focussed on anterior horn cells and have demonstrated neuronal loss. Autopsy studies are hampered not only by unavoidable artifacts but also by the fact that they usually demonstrate the end-stage of a disease. Degenerating motoneurons commonly described as ballooned contain accumulations of phosphorylated neurofilaments. Such ballooned neurons occur not only in the anterior horn but also in the thalamus and in dorsal root ganglia (103, 143) and even in motor nuclei of extraocular muscles (71) which tend to be clinically unaffected. Motoneurons of SMA 1 patients are said to lack the typical ubiquitin-positive skein-like inclusion of sporadic amyotrophic lateral sclerosis but contain granular ubiquitin-positive inclusions (88). Chou and Wang (27) found evidence for a defect of neurofilament assembly in chromatolytic but not in surrounding "normal" neurons and speculate that this would affect synapse formation on the motoneurons and also disturb the neuron-glia relationship. Reduced synaptophysin-staining of affected motoneurons which indicates loss of synapses has indeed been reported (64, 153). Nevertheless, "synaptic stripping" is also seen after experimental axotomy (9) and hence may be a non-specific result of the loss of target contact. Hayashi *et al* (59) found apoptotic (TUNEL-positive) neurons in the thalamus of 3 of 4 Werdnig-Hoffmann patients but no apoptotic cells in the spinal cord. While apoptosis as mechanism for neuronal death in SMA is discussed in analogy to neuronal death during normal development in experimental animals (14), evidence for apoptosis during motoneuron death in patients is lacking.

Hausmanowa-Petrusewicz (57) and her school propose that the surviving motoneurons in SMA 1, as well as the mucle fibres they supply, are immature (58) and that defective or delayed maturation of the motor units makes motoneurons and muscle fibers prone to die. Along these lines, it has been maintained that myonuclei in muscles of SMA 1 patients undergo apoptosis (41, 138). While Fitzsimmons and Hoh (43) and Biral *et al* (6) found fetal myosin in muscles from patients with SMA 1, this could not be confirmed by others (120, 136). Hence, the matter is still open for discussion.

Korinthenberg *et al* (74) observed three siblings with genetically confirmed SMA with a very severe phenotype; nerve biopsies and postmortem examination showed axonal damage in mixed and sensory nerves but a normal number of motoneurons which were, however, often chromatolytic. Chromatolysis indicates that the neuron has lost contact with its target. These findings might support the notion that the degenerative process starts in the periphery of the neuron and that muscles become denervated before the somata are lost. In defense of the classical interpretation, Imai *et al* (65) claim that a dying-back process can be excluded because in "less severe affected" SMA patients the conduction velocity of the motor nerves is equally reduced in proximal and distal nerves. The interpretation of this observation is complicated, because it was obtained in SMA 2 or 3 and because distal axonal degeneration even in normal individuals causes retrograde axonal atrophy with slowing of proximal nerve conduction; loss of axons does not necessarily reduce the conduction velocity of the nerve.

While there is no doubt that SMA is a denervating disease, the possibly ubiquitous role of SMN does not exclude that muscle plays a pathogenetic role. Muscle extract from SMA patients inhibits the neurite-growth promoting effect of neonatal chick muscle (61). Cloned satellite cells of human muscle when co-cultured with embryonic rat spinal cord form myotubes that eventually become innervated. Myotubes from SMA 1 and 2 patients but not from normal donors undergo degeneration 1-3 weeks after innervation. Degeneration is prevented when 50% of normal donor cells are added to the SMA satellite cells (10, 51).

A muscle biopsy from a child with SMA 1 profoundly differs from that from adult patients with SMA 3 or other adult-onset motor neuron diseases. The latter usually show typical neurogenic changes with atrophic type 1 and type 2 fibres with band-like or triangular cross sections, and large groups of type 1 and type 2 fibres indicating collateral reinnervation. Secondary myopathic changes may also be present. This is never seen in SMA 1 patients: the vast majority of fibres is almost circular in cross section and measure between 8 and 15 μm in diameter, while much fewer fibres are of normal size or even hypertrophic. The large fibres are almost exclusively of type 1 while the small fibres are of type 1 or 2, fibre type grouping does not occur. Large fibres may be

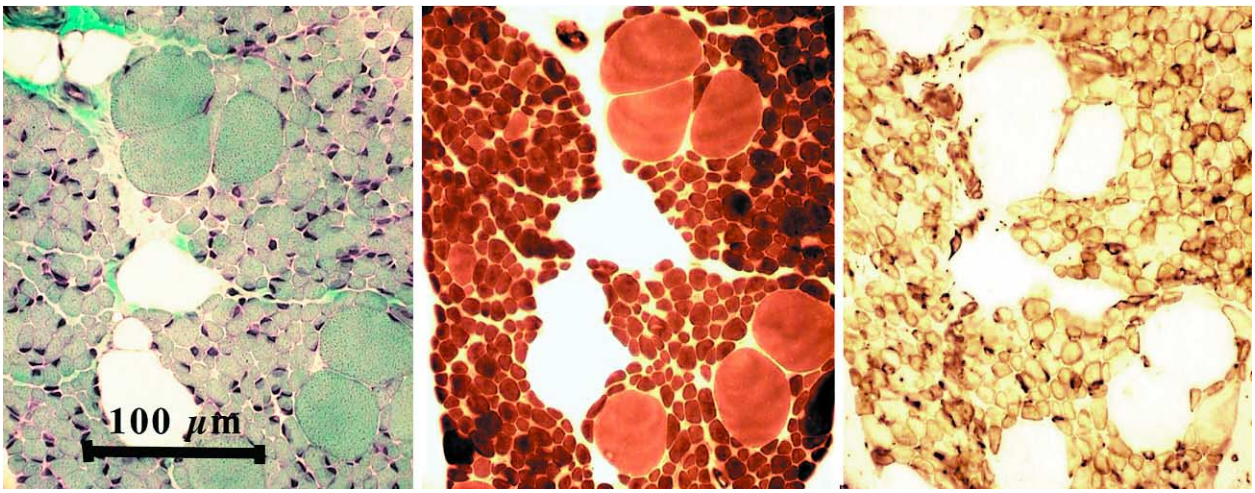

Figure 3. Typical muscle biopsy of a one-year old girl with SMA 1. The Gomori trichrome stain (left) shows few grossly hypertrophic and numerous small round fibres, mostly with peripheral nuclei. In addition, fat cells (unstained) are present. Consecutive sections stained for ATPase at pH 10.3 (middle) and with anti-vimentin (left) demonstrate that the hypertrophic fibres are of type 1 while most small fibres stain like type 2 fibres. The small but not the large fibres react with anti-vimentin.

of type 1, either because type 1 motor units are spared or because overload has induced type 2 to type 1 transformation. A characteristic and diagnostically useful feature of the small fibres is their intense reactivity with anti-vimentin (Figure 3). Lack of reinnervation and small motor units in SMA 1, and reinnervation and large motor units in milder cases have also been shown electrophysiologically (34). Replacement of muscle fibres by fat cells is an early event in SMA 1. A biopsy from a less severely affected young patient may show both features in different regions: type grouping in some fascicles and the typical SMA 1 appearance in others (Figure 4). These general histological differences are therefore unfit to predict the clinical course in the individual patient, possibly because the biopsy only comprises a minuscule part of the patient's muscular system. These differences might nonetheless help to understand why the clinical course differs between patients.

Animal models of spinal muscular atrophy

Histopathological and experimental research in motoneuron diseases in patients are hampered by the fact that motoneurons can only be investigated at autopsy when the disease has run its course; only skeletal muscle tissue is accessible before death. Therefore, understanding the development of the histopathological changes in SMA requires studies in animal models which also allow testing therapeutic approaches.

Models not homologous to SMA. The fact that symptoms of SMA 1 are often present at birth suggests that denervation of skeletal muscles starts *in utero*, possibly when the neuromuscular system is still immature. This situation is easily mimicked experimentally, because the neonatal neuromuscular system is less mature in rat than in man. Almost all axotomized motoneurons die in a newborn rat when the still unmyelinated sciatic nerve is cut. This target dependence of motoneurons decreases with age, and no motoneurons are lost when the nerve is cut at age 4 weeks (122). The loss is incomplete after neonatal nerve crush, because rapid axonal regeneration saves the motoneurons (70). It is not known why mature motoneurons survive axotomy while immature ones do not. Ciliary neurotrophic factor (CNTF) which is produced by Schwann cells, possibly acts as lesion factor of peripheral nerves (131, 134); nerves of newborn rats do not yet contain CNTF, and the onset of CNTF production roughly coincides with the loss of target dependence of motoneurons. CNTF was in fact the first factor able to delay motoneuron death after neonatal axotomy (132) and in a mouse mutant with hereditary motoneuron disease (133).

Partial denervation of immature rat muscle is not followed by collateral reinnervation as in adult rats, and the size of the motor units does not increase (5, 12, 84, 142). The denervated muscle fibres remain small and soon vanish to be replaced by fat cells. Degenerating small fibres are attacked by natural killer cells and are eventually phagocytosed by macrophages (125). Myonuclei often show ultrastructural signs of apoptosis, but histochemistry reveals only few nuclei with DNA breaks.It is

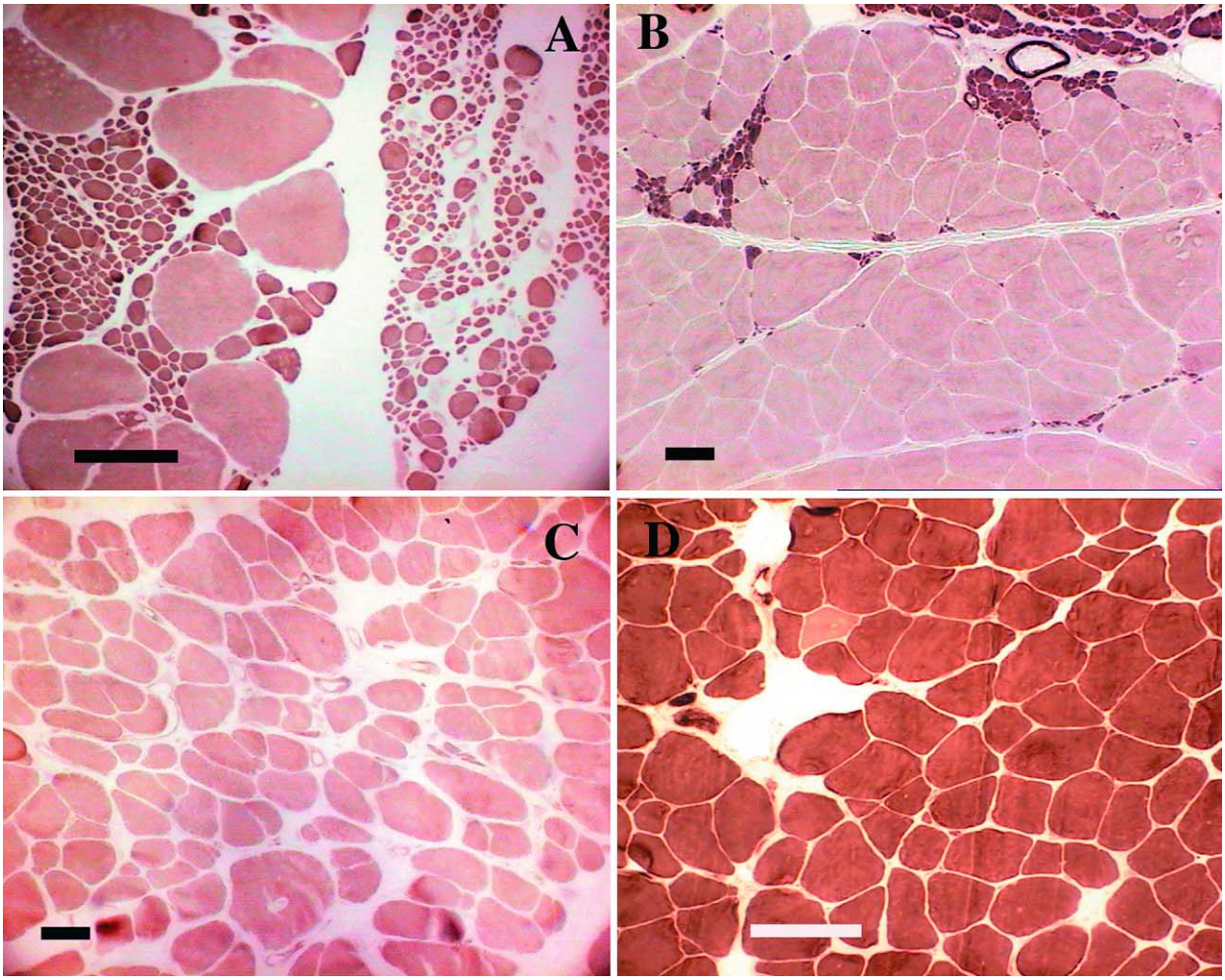

Figure 4. Examples of muscle biopsies that illustrate the histological variability of SMA 3. All sections have been stained for ATPase at pH 10.3. Bars: 100 μm.
The micrographs of this panel may suggest that that the small fibres increasingly vanish and that the number of innervated fibres determines the eventual fate of the muscle.
a: Modestly affected 5-year-old boy who is still able to play football (soccer) in kindergarden. The picture resembles that of the SMA 1 biopsy shown in Figure 3. Few hypertrophic fibres are surrounded by numerous small fibres without evidence of reinnervation. **b:** This 6-year-old girl has difficulties in getting up from a sitting position and uses both hands when climbing staircases. The muscles consist of normal-sized type 1 fibres and interspersed small type 2 fibres. **c** and **d**. This 29-year old man had been suspected to have Becker dystrophy. His main complaint is that he has difficulties in getting up from a sitting position; otherwise he is mildly affected. Biopsies from the anterior tibial and medial vastus muscles show almost complete fibre type homogeneity: the biopsy from the anterior tibial muscle consists of type 1 fibres (**c**) and that from the vastus (**d**) consists of type 2 fibres; only one type 1 fibre is seen. The histological picture in c has myopathic features.

not known whether the immature motoneurons are unable to sprout or whether the denervated muscle fibres do not induce sprouting. There is strong evidence that Schwann cells play an essential role during sprouting, and that they die after neonatal but not adult denervation (144). Four weeks later however, the previously denervated muscle fibres reject innervation by mature motor axons (124). The general appearance of a rat muscle which has been incompletely denervated at birth after few weeks strongly resembles that of a muscle biopsy from a patient with SMA 1 with fatty infiltration, few hypertrophic fibres and numerous small fibres with circular cross-sectional shape, and the number of motoneurons is also reduced (123).

The lack of collateral reinnervation in SMA 1 relates to the inability of immature motor units to expand, and

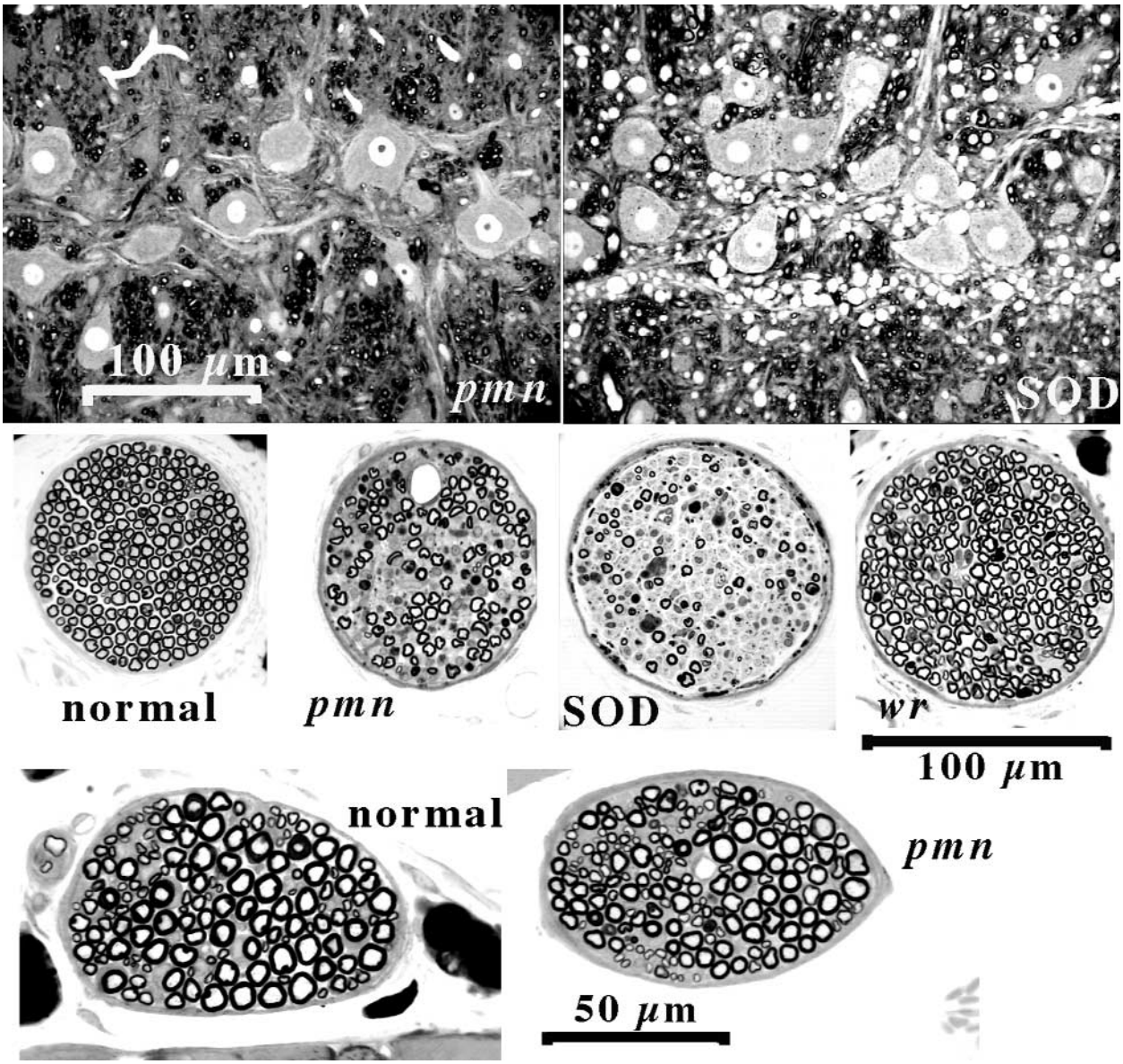

Figure 5. Essential histological findings in murine models of motoneuron disease. Epoxy sections 3 μm thick have been stained with p-phenylenediamine. *pmn*: progressive motor neuronopathy; SOD: transgenic SOD1(G93A) mice; *wr*: *wobbler* mice. **Top**: Longitudinal sections through the spinal cord at C4 showing somata of the motor nucleus of the phrenic nerve. The somata are normal and not reduced in number in severely affected *pmn* mice at day 30, while they show dramatic dendrictic swellings already in modestly affected SOD1 mice at day 115. **Middle**: Phrenic nerves show pronounced axonal degeneration in *pmn* and SOD1 mice and loss of axons as compared to normal. The axonal loss in SOD1 mice is much greater than the loss of somata of motoneurons. The phrenic nerves of *wobbler* mice (day 60) are normal despite the fact that neighbouring motor nuclei of the brachial plexus are partially depleted. **Bottom**: In contrast to severe axonal degeneration in the phrenic nerve of *pmn*, intercostal nerves appear well preserved. The micrographs were taken from the same distal branch of the IX. intercostal nerve.

this may contribute to the rapid clinical progression of SMA 1. The onset of SMA 2 and SMA 3 is later when motor units can expand, and collateral reinnervation helps to maintain muscle function and to ensure a protracted course of the disease. Whether the loss of motoneurons in SMA 1 is due to the inability of the muscle to keep them alive remains, however, speculative.

Hereditary motorneuron diseases have been observed in many animals (127, 135), but the phenotypes have

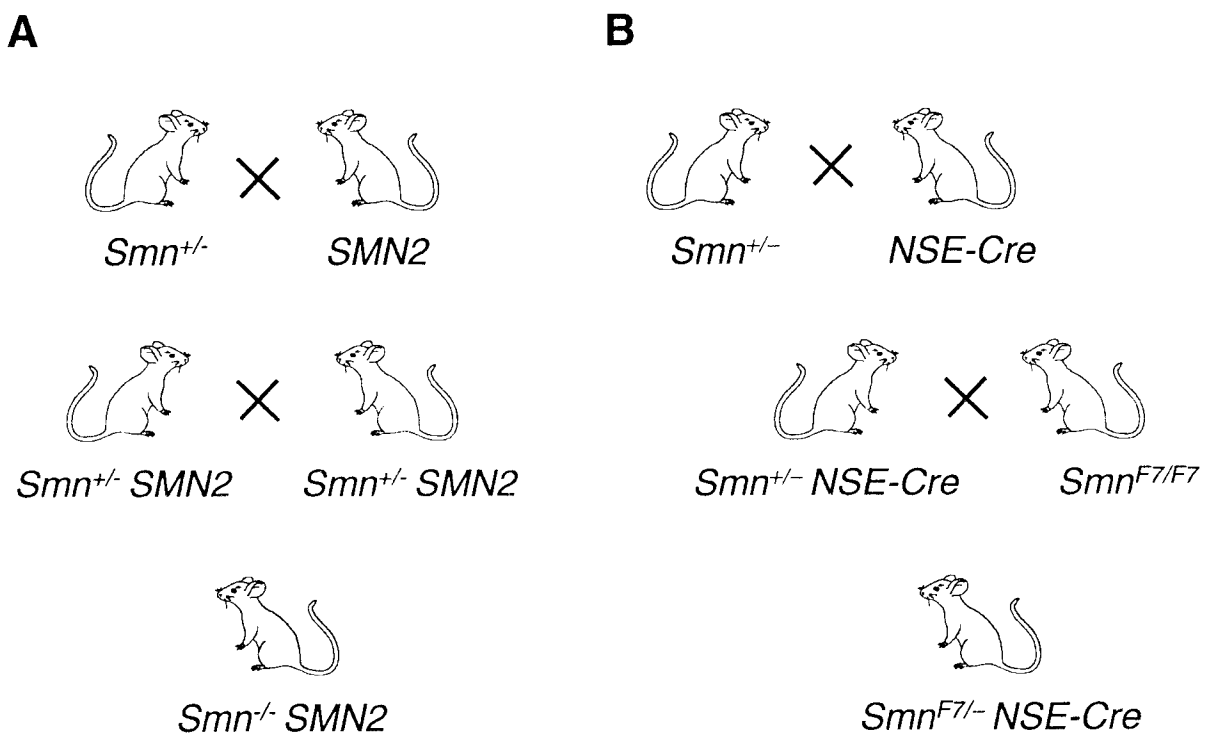

Figure 6. Genetic mouse models of SMA. **A**. Hsieh-Lo *et al* (63) and Monani *et al* (101) rescued the embryonically lethal phenotype of *Smn*$^{-/-}$ mice by introducing human *SMN2* transgenes. The *Smn*$^{-/-}$ *SMN2* mice (lower panel) were generated by first crossing heterozygous *Smn*$^{+/-}$ mice (upper panel) with transgenic mice for human *SMN2* and then by intercrossing *Smn*$^{+/-}$ *SMN2* mice (middle panel). **B**. The aim of Frugier *et al* (46) was to achieve a neuron-specific deletion of the *Smn* gene. Three types of mice were generated: transgenic mice expressing the Cre recombinase gene under the control of the neuron specific enolase promoter (*NSE-Cre*, upper panel), mice with a heterozygous *Smn* exon 7 deletion (here termed *Smn*$^{+/-}$, upper panel) and mice in which the *Smn* exon 7 was flanked by loxP recombination sites (*Smn*$^{F7/F7}$, *middle panel*). The Cre-mediated excision of *Smn* exon 7 in *Smn*$^{F7/-}$ NSE-Cre mice (lower panel) induces pathology.

only been properly characterized in a few mutations in mice and one in dogs.

Motoneurons innervating forelimb muscles show vacuolar degeneration in *wobbler* mice (38). Neuronal degeneration eventually comes to a halt, and most mice survive for more than a year, perhaps because the phrenic nerve is not affected. The deficit of myelinated fibres in the median nerve by far exceeds the deficit of motoneurons indicating that the disease starts distally (116; own unpublished observations). Also mice with progressive motor neuronopathy *pmn* present with a dying-back process of motor axons which starts at age 2 weeks and primarily affects hindlimbs and diaphragm. Death occurs already 5-7 weeks after birth when the loss of somata of spinal motoneurons if any is negligible (128) (Figure 5). It has been argued that *pmn* mice have an axonopathy rather than motoneuron disease (148). The same point might be raised against hereditary canine spinal muscular atrophy (HCSMA), a motoneuron disease in Britanny spaniels in which the number of anterior horn cells also remains normal (32, 33) despite widespread denervation of the muscles.

Transgenic mice with mutated genes for superoxide dismutase 1 from patients with familial ALS ("SOD1 mice") (52) show dendritic swellings and accumulation of neurofilaments in the proximal axon. Phrenic and intercostal nerves become affected and the animals die after several months (Figure 5). Distal axonal degeneration precedes neuronal death also in these mice (73). SOD1 mice eventually lose half of their spinal motoneurons (97); these authors were unable immunohistochemically to demonstrate apoptosis in sections of the cervical and lumbar spinal cord.

To summarize, distal motor axons degenerate before proximal axons and neuronal bodies in all hereditary motoneuron diseases investigated so far. Signs of degen-

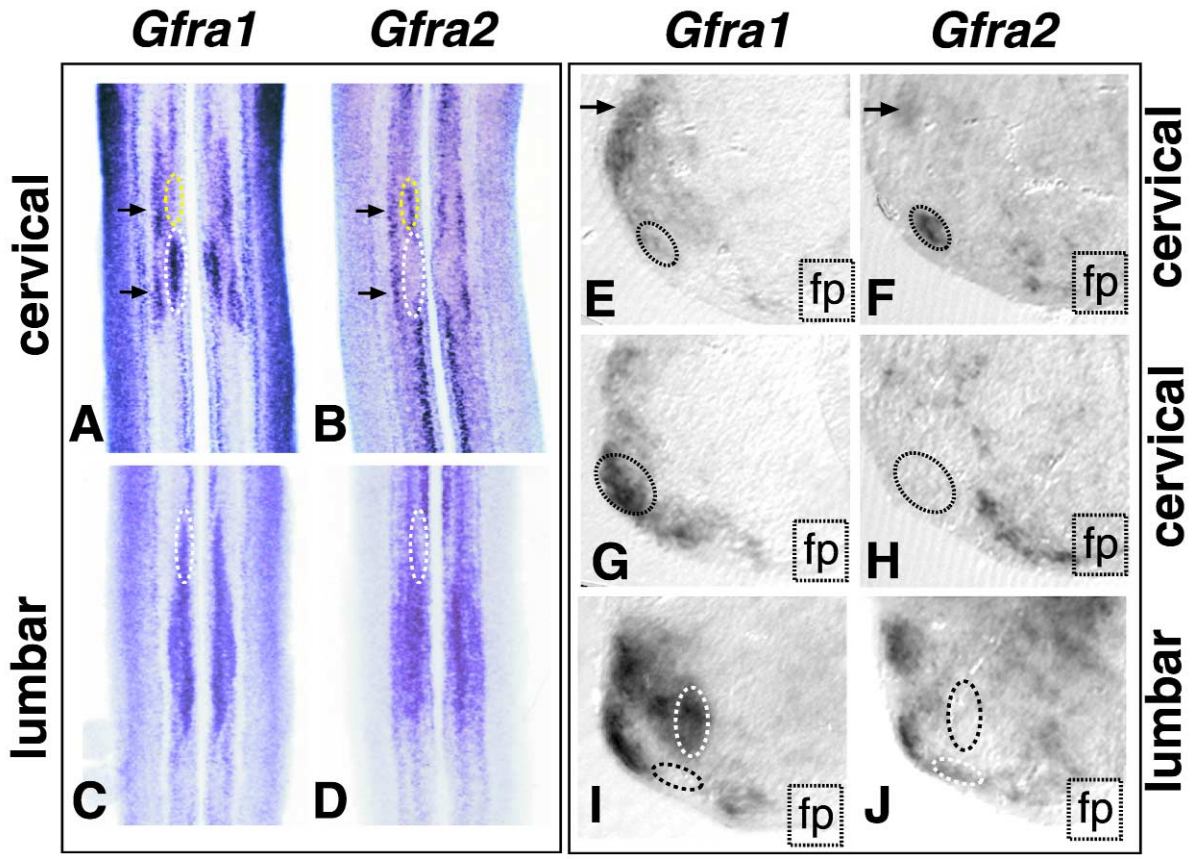

Figure 7. *Developing motoneurons (mouse E13.5) show distinct expression patterns of the genes Gfra1 and Gfra2 which encode the receptors for the neurotrophic factors GDNF and Neurturin.* **A-D**. Whole-mount *in situ* hybridisation was performed using probes to *Gfra1* (**A**, **C**) or *Gfra2* (**B**, **D**) on cervical (**A**, **B**) and lumbar (**C**, **D**) spinal cords. Circled in white are two subpopulations of motoneurons which strongly express *Gfra1*, and in yellow one which expresses *Gfra2*. Arrows point to motoneurons that express both *Gfra1* and *Gfra2*. The circled motoneuron subpopulations are located in the lateral motor column (LMC) but appear to be close to the midline because the spinal cord preparation has been flattened.
E-J. Transverse sections of these preparations were cut at cervical (**E-H**) and lumbar levels (**I**, **J**). The panels show the half-ventral horn of each section; fp indicates the floor-plate. Hybridisation using the *Gfra1* probe (**E**, **G**, **I**) stained more motoneurons than hybridisation using a *Gfra2* probe (**F**, **H**, **J**). Arrows point to the same *Gfra1*- and *Gfra2*-positive motoneuron column as in A and B; dashed lines delineate motoneuron groups that are positive for only one α-receptor. See also Garcès *et al* (47).

eration have been found in neuromuscular synapses even before the onset of overt clinical deficits. Degeneration was most pronounced in those parts of the muscles which were less apt to sprout in response to botulinum toxin A. Botulinum toxin A induced sprouting preferentially on slow-twitch muscle fibres of normal mice, and synapses of such fibres were in diseased mice spared up to the terminal phase (45).

Two additional mouse mutants, motor neuron disease *mnd* (95, 96) and motor neuron disease-2 *mnd-2* (68), have been described. Nevertheless, *mnd* eventually turned out to have ceroid lipofuscinosis rather than motoneuron disease (11, 110), and *mnd-2* does not have neuromuscular disease at all (126).

Genetic animal models of SMA. In contrast to humans, mice and rats possess only one *Smn* gene (3), and to date, no *Smn* mutations have been detected in the various spontaneous mouse mutants with motoneuron degeneration. This might be related to the essential role of *Smn* in early development. As shown by Schrank *et al* (129), embryos of *Smn* knockout ($Smn^{-/-}$) mice display a massive apoptosis at the early blastocyst stage and die before implantation. Earlier on, the presence of maternal SMN transcripts and protein probably compensates for the missing embryonic *Smn* expression.

In order to generate animal models for SMA, SMN levels thus had to be reduced in a less drastic way or in a more tissue- or time-restricted manner. Heterozygous

Smn (*Smn*$^{+/-}$) mice for instance, which have a roughly 50% reduction of SMN protein levels in the spinal cord, developed mild spinal muscular atrophy. At birth, the number of spinal motoneurons was normal, but about half of them were lost during the first 6 months of life with no further loss between 6 and 12 months (67).

Another strategy was to generate viable *Smn* KO mice by introducing *SMN2* transgenes. Such mice were obtained by crossing heterozygous *Smn*$^{+/-}$ mice with transgenic mice for human *SMN2* along with a part of NAIP and p44 (63), or with transgenic mice for human *SMN2* only (101) (Figure 6A).

These mice were affected but the clinical picture varied, even between littermates. Some died immediately after birth, others lived for a few weeks and a third group survived and bred normally. The phenotype of the last group of mice was only characterized by short and enlarged tails (63). Interestingly, the mice with the most severe phenotypes carried low copy numbers of *SMN2* transgenes (101) or produced low levels of SMN protein (63), whereas milder phenotypes were associated with higher copy numbers.

Melki and coworkers have chosen a different approach (46) (Figure 6). They produced a conditional deletion of mouse *Smn* exon 7 by using the Cre/loxP recombination system. When the Cre recombinase gene was expressed ubiquitously, mice with the homozygous deletion died during early fetal life. Next, the Cre recombinase gene was expressed under the control of the NSE (neuron specific enolase) promoter in order to specifically induce the *Smn* deletion in neurons. These mice survived, were normal at birth but two weeks later developed motor deficits and tremor; they died 17-36 days after birth. Skeletal muscles showed pronounced histological signs of denervation, but somata of motoneurons were apparently normal in number (Melki, personal communication). This elegant study suggests that *Smn* deletion in neurons is the *primum movens* towards an SMA phenotype. Mice in which Cre-mediated *Smn* gene deletion is induced specifically in skeletal muscle have also been constructed and the publication of their phenotype is eagerly awaited.

The generation of these four SMA animal models has provided important insights into the pathogenesis of SMA. Nevertheless, the mouse phenotypes have only been incompletely characterized. Frugier *et al* (46) were the only ones histologically to demonstrate denervation of skeletal muscles, while Monani *et al* (101) failed to find neurogenic changes in quadriceps and gastrocnemius muscles. Frugier *et al* (46) in semithin plastic sections for light microscopic describe "indentations" of the nuclei of motoneurons as characteristic for their SMA model. Similar nuclear structures are seen in grazing sections in normal mice as well, and the authors do not report that the "indentations" allowed distinguishing unmarked spinal cord sections from wild-type and KO mice. Jablonka *et al* (67) and Monani *et al* (101) document a reduction of the number of spinal and facial motoneurons. Motoneurons are described as chromatolytic (63, 67) although it has been established that chromatolysis of motoneurons in mice is inconspicuous (79). Convincing histological and electrophysiological assessment of muscle and peripheral nerves comparable to the diagnostic evaluation of SMA patients and the demonstration that the disease is restricted to the motor system are thus lacking. In view of the previous incorrect classifications of the spontaneous mutants *mnd* and *mnd2* (see above) such studies seem essential.

Therapeutic horizons

Mouse models of SMA are not only required for a better understanding of the molecular pathogenesis of SMA but also for testing future therapeutic strategies. These might be directed at the *SMN* genes or to cellular pathways known to be involved in motoneuron degeneration.

A straightforward approach in SMA would be to replace the lost *SMN1* gene by means of gene transfer. When considering *SMN1* gene transfer into spinal cord, several types of viral vectors derived from lentiviruses (28, 62), adenoviruses (1), adeno associated viruses (2), herpesviruses (72) or polioviruses (7) show promise because they can efficiently transduce motoneurons *in vivo*. Each of these vector types has specific limitations with respect to cytotoxicity, immunogenicity, biosafety, stability of gene expression, production yield, etc. Even more importantly, all vectors face a common problem: when injected into the CNS parenchyme, their diffusion is restricted to a few millimeters; when injected into a peripheral target muscle, the retrograde infection of motoneurons — if operating for the given vector — is limited to a single motor pool. A prerequisite to viral SMN gene transfer seems therefore to identify those motoneuron subpopulations that are most sensitive to the degenerative process and that are critical for the animal's survival. Indeed, not all motoneuron groups are equally affected in human SMA. Clinical observations suggest that progression tends to come to a halt after some time (35). Muscle histology of children with SMA 3 might be interpreted to mean that the small fibres vanish and that the fate of the muscle and thus also of the patient depends on the number of surviving innervated fibres (Figure 4). If this were correct, primary pathology

of SMA would eventually become arrested although loss of function due to secondary complications might be progressive. The relative preservation of the diaphragm and hence of phrenic motoneurons in SMA patients has been histologically confirmed (75). Comparable observations have been made in mice. The phrenic nerve is completely spared in *wobbler* mice despite degeneration of brachial motoneurons, while it degenerates in SOD1 and *pmn* mutant mice in which lumbar neurons are the most affected. In *pmn*, phrenic motor axons are almost completely lost after 3 weeks although the mice live for 2 to 4 additional weeks; this may be due to the relative preservation of intercostal nerves (Figure 5).

Analyses in SMA patients indicate that disease severity is tightly correlated with the level of *SMN2* expression as evidenced by *SMN2* gene copy number, SMN protein levels or number of gems. The mouse models have further corroborated that the *SMN2* gene can partially compensate for the missing *SMN1* gene and attenuate disease severity. It might therefore be of therapeutic interest to pharmacologically activate *SMN2* gene expression. Several academic and industrial groups have established high throughput screens for molecules that activate the *SMN2* promoter. The *SMN2* promoter indeed contains numerous putative binding sites for transcription factors implicated in neuronal differentiation or survival such as AP-2, E2F-1, GATA-2, HNF-3, N-Oct-3 or YY1 (39, 100). Screens are performed on primary motoneurons, neural cell lines or heterologous cells and monitor the *SMN2* promoter activity by the expression of reporter genes or by the induction of phenotypic effects such as an increase in the number of gems. In addition, screens have been devised to identify molecules capable to revert the abnormal splicing pattern of the *SMN2* gene (156).

Neurotrophic factors and, more recently, anti-apoptotic peptides have also gained considerable interest as therapeutic candidates in neurodegenerative diseases; for review, see (60, 104, 139). Clinical studies on patients with amyotrophic lateral sclerosis treated with repeated subcutaneous injections of CNTF, BDNF and IGF-1 were unsuccessful. This failure is widely attributed to the limited bioavailability or the toxic side effects of the recombinant proteins and might be overcome by improved modes of delivery such as intrathecal infusion or gene transfer. Combinations of several neurotrophic factors might also be necessary to reduce motoneuron death more efficiently. Mitsumoto *et al* (98) have shown additive effects of CNTF and BDNF in the *wobbler* mouse model, and we have demonstrated additive effects of CNTF and NT-3 in *pmn* mice (53). Recent studies in several animal species provided direct evidence for the idea that the trophic requirements may differ between distinct subpopulations of motoneurons (see review 40). Motoneurons from different parts of the spinal cord (Figure 7) and from different motor nuclei express different molecular markers and receptors for neurotrophic factors (See review 40). Hence, the survival promoting effect of neurotrophic factors during development, after axotomy or *in vitro* differs for different subpopulations of motoneurons (hepatocyte growth factor/scatter factor HGF/SF: (105, 152); glial cell derived growth factor GDNF: (47, 107); fibroblast growth factor FGF: [48]). It may be hoped that this research broadens our knowledge of the target-neuron relationship and eventually helps to identify better combinations of neurotrophic factors capable to rescue degenerating motoneurons in SMA.

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