

REVIEW

The Neurobiology of Childhood Spinal Muscular Atrophy

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INTRODUCTION

Almost to the century after the initial clinical description of childhood spinal muscular atrophy (SMA) (Werdnig, 1891) a new era of investigation into the nature of SMA was inaugurated when Conrad Gilliam and his group at Columbia University established genetic linkage for this autosomal recessive disorder to chromosome 5q (Brzustowicz *et al.*, 1990). In 6 years the pace of research has accelerated markedly: an extensive international effort to identify the gene is documented in over 50 papers by 240 authors at a dozen centers. One early by-product of this search was the discovery that the SMA-critical region of chromosome 5q is unusually unstable and that this genetic instability is likely responsible for the high incidence and worldwide distribution of SMA (Carpten *et al.*, 1994; Theodosiou *et al.*, 1994; Thompson *et al.*, 1995; Crawford, 1996). By early 1995, the search had narrowed sufficiently for two neighboring but unrelated candidate genes to be proposed for the disorder (Lefebvre *et al.*, 1995; Roy *et al.*, 1995a). The arguments buttressing the candidacy of each of these two genes contrast strong biologic plausibility against high genetic probability. The *plausible* candidate gene, neuronal apoptosis inhibitory protein (*NAIP*), has displayed anti-apoptotic function—an activity that matches closely a standing hypothesis that SMA is a disorder of development. The *probable* candidate gene, survival motor neuron (*SMN*), is deleted in over 95% of patients with SMA and disabling point mutations have been found in a number of the remaining patients where they were pursued.

Investigations into the pathogenesis of SMA are now turning to the cellular biology of *NAIP* and *SMN* and

to the generation of gene-targeted animal models. Meanwhile, available animal models of motor neuron degeneration raise new questions about the multiple pathways of selective motor neuron vulnerability. New clinical insights and findings also raise some unexpected questions. The current vigor of SMA research stands as an outstanding illustration of how insight from multiple levels—from epidemiology, clinical trials, and individual patient investigations to pathology, biochemistry, classic genetics, and now molecular genetics and cell biology—contribute to and draw strength from one another.

EPIDEMIOLOGY AND NOSOLOGY

SMA, an autosomal recessive disease, is the most frequent monogenic cause of death in infancy and a major cause of childhood morbidity due to weakness. It is common in all areas of the world where incidence has been studied (Emery, 1991). In Europe and North America, the best estimates are that 1 in 16,000 to 25,000 infants dies of the severe infantile form, while an approximately equal number have a milder form. The disease exists as a spectrum, usually arbitrarily divided by clinical criteria into several groups. Infants with the severe form (known variously as Werdnig-Hoffmann disease, severe infantile SMA, or SMA type 1) generally manifest weakness before 3 months and certainly before 6 months and are so profoundly weak that they are never able to maintain a sitting posture. In this group, average life expectancy is 8 months, with 75 and 95% mortality by the first and second birthdays (Ignatius, 1994; Thomas & Dubowitz, 1994). Children with milder forms of SMA (also known as SMA type 2,

or chronic childhood SMA, and SMA type 3, or Kugelberg-Welander disease) generally manifest weakness after 6 months and at their best can either maintain a sitting posture (type 2) or are able to stand and walk (type 3) (Munsat, 1991). Both the length of survival of children with the milder forms of SMA and the maintenance of their function are greatly influenced by the quality of clinical care.

For much of the first 100 years of SMA research, clinicians tried to determine whether the severe SMA 1 and milder SMA 2 and 3 were individually distinct entities or were instead a single disease of varying severity. Beginning in the 1970s Pearn and then others have demonstrated that affected sibling pairs have a similar age of onset and severity of weakness (Pearn *et al.*, 1973; Feingold *et al.*, 1977; Pearn, 1980; Rudnik-Schoneborn *et al.*, 1994). Siblings with SMA 1 die at a comparable age in infancy, whereas siblings with SMA 2 or 3 achieve parallel functional milestones. Although the initial interpretation of this finding of similar disease severity within nuclear families favored the distinctiveness of the various SMA types, the recognition shortly thereafter of a wider range of disease severity within a number of extended families (Bouwsma & Leschot, 1986; Zerres *et al.*, 1987) argued that the range of SMA types was caused by a single gene with multiple pathogenic alleles. This issue was essentially settled in 1990 when both the severe and milder affected groups were linked to the chromosomal 5q11.3–13.1 region (Gilliam *et al.*, 1990; Melki *et al.*, 1990). These data strongly support either the model of a single gene with multiple alleles or a model of multiple tightly linked pathogenic genes.

CLINICAL SYNDROME AND DIAGNOSIS

The linkage of SMA to chromosome 5q provided, for the first time, a rational basis for diagnostic criteria for SMA. To aid geneticists in furthering the gene search, research criteria were initially drawn narrowly using 5q linkage in siblings to establish a cohort of patients with this “gold standard” diagnosis (Munsat *et al.*, 1990; Munsat, 1991; Munsat & Davies, 1992). Characteristics that might be ambiguous or overlap with another disorder, and that had been shown to be not linked to 5q in at least one family, were used to exclude the diagnosis. In fact, to the credit of a generation of superb clinicians, these criteria mirrored closely the diagnostic criteria established over several decades derived from semiology alone (Dubowitz, 1995). Af-

ected children have diffuse symmetric weakness of more proximal than distal muscles and greater weakness of the legs than the arms. The weakness is due to denervation demonstrable by electrophysiologic and biopsy criteria. Other organ involvement, congenital arthrogryposis, or evidence of more diffuse neurologic impairment undermine the diagnosis according to these strict criteria.

More recently, homozygous deletion of *SMN* has been demonstrated to be a sensitive genetic test for the disorder applicable to single cases (see below). Homozygous deletion of *SMN* will likely become a new gold standard sufficient in the proper clinical setting to certify the diagnosis. With this test, the range of the phenotype can be expanded without fear of erroneous diagnosis of marginal cases. Already this test has shown that about half of the children with classic clinical signs and symptoms of SMA, but who in addition have other unusual features either in the brain or outside the nervous system, appear to have SMA (Bürglen *et al.*, 1995; Rudnik-Schöneborn *et al.*, 1996). On the milder end of the spectrum are patients with substantially later onset and milder degrees of weakness who had previously been excluded because of overlap with other motor neuron diseases. Because this group of adults with SMA has homozygous deletion of *SMN*, the suggestion has been made that a “type 4” SMA should now be added to the three childhood onset forms of the disease.

ISSUES ARISING FROM THE CLINICAL COURSE

SMA has an unusual clinical course that has attracted the attention of clinicians for many years. The onset of weakness is either subacute or insidious, as children fail to gain strength with development (Fig. 1). Once the disease manifests itself, clinical experience (Dubowitz, 1964, 1995) and recent trials (Iannaccone *et al.*, 1993) suggest that children with the more mild forms of SMA deteriorate more slowly or not at all. A number of pathophysiologic and clinical implications arise from this unusual course.

One striking aspect of this “up-front” course of SMA, where the steepest decline in power occurs at the outset of the disease, is its distinction from the course of most other neurodegenerative disorders. Neurodegenerative disease is generally heralded by insidious onset and increasingly rapid or at least steady decline, a course which mirrors the exhaustion of the available

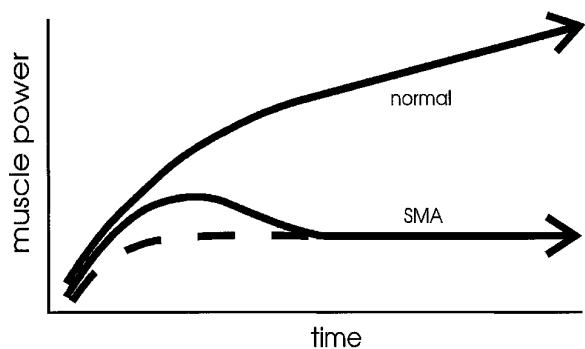


FIG. 1. Schematic of strength with age in normal children and those with SMA. Children with SMA commonly develop at normal or near-normal strength, then begin to manifest weakness, either by a subtle loss of strength or by a failure to gain strength (dashed line), and then enter a phase of relatively stable muscle power over an extended time. This pattern of development is common to all types of SMA, but the time scale changes: infants with SMA type 1 generally manifest weakness around or before the third month; those with milder forms manifest weakness at later times.

cellular and system-wide compensations before signs and symptoms manifest. In contrast, the course of SMA suggests that substantial early losses of functioning motor neurons are followed by increasing stability of the surviving neurons. This pattern of cell loss resembles the kinetics of developmental apoptosis, although exaggerated in degree and delayed in timing. The developmental appearance of SMA and the absence of a distinctive cellular pathology has led others to suggest that SMA represents a disorder of apoptosis (Sarnat, 1984; Oppenheim, 1991); this observation about the clinical course may provide further corroboration. If SMA is a disorder of unregulated apoptosis, SMA may be likened more to a birth defect than to a degenerative disorder.

A purely clinical corollary of the unusual clinical course is the important distinction between function and muscle power. Most longitudinal studies of the clinical course describe progressive loss of function over time (Iannaccone *et al.*, 1993; Carter *et al.*, 1995; Zerres & Rudnik-Schöneborn, 1995). For a variety of reasons, children with SMA suffer from a number of medical complications due to their weakness, which in turn place greater demands upon the available muscle strength. The development of scoliosis and other contractures, repeated episodes of pneumonia, and difficulties with nutrition, sleep, and disuse atrophy all increase functional disability, apart from the underlying function of the surviving motor neurons. Many of these complications are treatable, at least in part. The

assumption that this is a progressive disorder may have a stifling effect on vigorous evaluation for occult complications of weakness and become self-fulfilling. Whether SMA is truly a progressive disease at the level of the primary pathology, rather than a progressive disorder at the level of function, is yet to be established.

Finally, the timing of motor neuron loss in SMA need not parallel the course of the weakness. During normal development, after apoptosis diminishes the motor neuron population, the surviving motor neurons and muscle fibers have a unique relationship with one another. Each muscle fiber maintains innervation by multiple motor units, while each motor neuron innervates many more muscle fibers than in the adult. With further development, around Postnatal Week 2 in rats, this expanded motor unit size and polyneuronal muscle fiber innervation is lost (Kelly & Rubinstein, 1994). The reduction in motor unit size proceeds, although at a slower pace, in muscles that have an experimentally reduced number of axons, even if it means that some muscle fibers will be vacated of innervation. (Brown *et al.*, 1976). Thus, the timing of muscle denervation need not coincide with that of neuronal loss. Shrinkage of the motor neuron pool may occur well before this shrinkage of motor unit size leads to denervated muscle, producing clinical weakness. The timing of withdrawal of polyneuronal innervation and motor unit shrinkage is unknown in humans, but is an attractive hypothesis to explain the curious up-front natural history of the weakness of SMA. Neonatal motor nerve injury in rats produces a muscle with many of histologic features of SMA (Schmalbruch, 1990, 1988).

PHYSIOLOGY

The outstanding feature on electromyographic (EMG) evaluation of children with SMA is evidence of profound muscle denervation with diminished numbers of surviving motor units (Hausmanowa-Petrusewicz, 1988). The EMG studies of severe cases during infancy differ significantly from those of milder cases studied at later ages. EMGs of milder cases are dominated by very large motor units and the absence of abnormal spontaneous activity suggesting that surviving axons have sprouted to reinnervate neighboring orphaned muscle fibers. Denervated muscle fibers have been either lost or gathered into the surviving motor units. In contrast, EMGs of severely affected infants tend to

TABLE 1
Neuropathology of Spinal Muscular Atrophy

Author	Cases	Tissue	Technique	Comment
Matsumoto <i>et al.</i> (1993)	5 SMA 1	Spinal cord	Ubiquitin ICC	Granular ubiquitin deposits found within ballooned neurons. Appearance differs from skein-like ubiquitinated deposits found in ALS neurons.
Murayama <i>et al.</i> (1991)	2 SMA 1	Brain Spinal cord DRG	NFs ICC Ubiquitin ICC EM	Ballooned neurons found in anterior horn, Clarke's column, DRG, and thalamus. In ballooned neurons neurofilament ICC stained the peripheral perikarya and ubiquitin stained the central perikarya. EM showed central accumulation of mitochondria and vesicular and membranous profiles and peripheral accumulation of neurofilaments.
Kato & Hirano (1990)	1 SMA 1	Oculomotor nuclei	NF ICC Ubiquitin ICC	Oculomotor nuclei showed chromatolytic ballooned cells, with NF immunoreactivity in the periphery and ubiquitin in the center.
Fidzianska <i>et al.</i> (1990)	1 SMA 1	Muscle	LM EM	Muscle biopsy and autopsy specimens show clumped nuclear pattern and inclusions interpreted as consistent with apoptosis, along with features of immaturity in residual muscle fibers.
Chien & Nonaka (1989)	4 SMA 1 2 SMA 2	Muscle Intramuscular Nerves	LM EM	Selective loss of large myelinated fibers in intramuscular nerve bundles. Changes resemble those seen in Wallerian degeneration, including axonal degeneration, myelin breakdown with phagocytosis, axonal sprouting, and remyelination.
Biral <i>et al.</i> (1989)	20 SMA	Muscle	myosin ICC and immunoblot	Fetal myosins expressed in subpopulation of atrophic fibers.
Lippa & Smith (1988)	2 SMA 1	Brain Spinal cord DRG	NF ICC	Chromatolytic neurons found in ventral horn, DRG, Clarke's column, motor cranial nerve nuclei, and thalamus. Accumulation of phosphorylated neurofilament immunoreactivity within neurons.
Peress <i>et al.</i> (1986)	1 SMA 1	Brain Spinal cord	LM EM	Chromatolytic neurons identified in the lateral geniculate body.
Walsh & Moore (1986)	3 SMA 1 9 SMA 2/3	Muscle	5.1H11 ICC	5.1H11 immunoreactivity on atrophic cells of all SMA cases suggests persistent fetal features, not characteristic of acute (less than 6 weeks duration) denervation.
Towfighi <i>et al.</i> (1985)	4 SMA 1	Brain Spinal cord DRG	LM EM	Multifocal appearance of chromatolytic neurons in DRG, Clarke's column, scattered primary sensory cranial nerve nuclei, lateral thalamus. Multisystem involvement may be greater in younger, more severely affected cases.
Brock & McIlwain (1984)	2 SMA 1	Ventral root Dorsal root	2-D protein Electrophoresis	Two-dimensional electrophoresis showed increased GFAP in the protein composition of ventral and dorsal roots.
Shishikura <i>et al.</i> (1983)	5 ? SMA 1	Brain Spinal cord	LM	Multifocal appearance of chromatolytic neurons in ventral horn, Clarke's column, and lateral thalamus. Glial bundles in ventral root and 2/5 dorsal roots. Scattered involvement of other centers.
Nakazato & Ishida (1983)	1 SMA 1?	Cerebellum	LM EM	Lamellar bodies found in heterotopic neurons within the cerebellum.
Mitsumoto <i>et al.</i> (1982)	1 SMA 1	Spinal cord	LM GFAP ICC	Glial bundles in spinal cord not as prominent as expected in severe case of SMA 1.

TABLE 1—Continued

Author	Cases	Tissue	Technique	Comment
Chou <i>et al.</i> (1982)	5 SMA 1	Onuf's nucleus	LM	Central chromatolysis found in the Onuf's nucleus.
Kuzuhara & Chou (1981)	4 SMA 1	Phrenic motor Neurons	LM	Phrenic motor neurons relatively spared.
Fitzsimons & Hoh (1981)	12 SMA 1	Muscle	Myosin Electrophoresis	Muscles characterized by high concentration of fetal myosin isoenzymes.
Sung & Mastro (1980)	1 SMA	Onuf's nucleus Spinal cord	LM	Normal Onuf's nucleus and intermediolateral column suggest relative sparing of autonomic related motor neurons.
Hausmanowa-Petrusewicz <i>et al.</i> (1980)	12 SMA 3	Muscle	LM EM	Features of immaturity apparent in small muscle fibers.
Steiman <i>et al.</i> (1980)	2 Infantile neuronal degeneration	Brain Spinal cord	LM	Two infants with clinical features of SMA 1 found to have spinal cord and brainstem motor neuron loss as well as in thalamus, cerebellum; one had motor/sensory peripheral nerve demyelination. Onuf's nucleus is normal in SMA.
Iwata & Hirano (1978b)	2 SMA 1 1 SMA	Spinal cord	LM	
Chou & Nonaka (1978)	6 SMA 1	Spinal cord Roots	LM EM	Morphometric analysis of spinal cord and roots in six patients disclosed severe axonal atrophy/immaturity or selective loss of large myelinated fibers. Glial proliferation in bundles seen in all. Authors hypothesize a centrifugal degeneration secondary to pathology in proximal root.
Carpenter <i>et al.</i> (1978)	5 SMA 1 2 SMA 2	Sural nerve DRG	LM EM	Sural nerve biopsy in seven cases of SMA showed Wallerian degeneration. In a single autopsy case the DRG showed chromatolytic neurons.
Robertson <i>et al.</i> (1978)	1 SMA 1	Spinal cord	LM	Morphometric counts in lateral column of ventral horn shows selective loss of large motor neurons.
Ghatak (1978)	2 SMA 1	Spinal roots	LM EM	Glial bundles demonstrated along ventral roots. Such processes always enclosed by a basal lamina.
Ohama & Ikuta (1977)	1 SMA 1	Spinal roots Sciatic nerve	LM EM	Nerves demonstrated polyaxonal pockets, a characteristic of early development.
Szliwowski & Drochmans (1975)	4 SMA 1 2 SMA 2	Muscle	LM EM	Six muscle biopsies with features of denervation. End plates in one case and muscle spindles were normal.
Marshall & Duchen (1975)	6 SMA 1 3 SMA 2	Spinal cord DRG	LM EM	In six of nine SMA 1 cases involvement of sensory system is demonstrated as DRG degeneration and pallor of dorsal columns in older cases.
Fidzianska (1974)	7 SMA 1/2	Muscle	LM EM	Features of immaturity of muscle fibers suggest arrest in maturation rather than muscle atrophy.
Chou & Fakadej (1971)	1 SMA 1	Spinal cord Roots	LM EM	Chromatolytic neurons show increased mitochondria, clumps of ribosomes, and vesicles in the central region of the cell soma and accumulation of filamentous material peripherally.
Mastaglia & Walton (1971)	8 SMA 3	Muscle	EM	Muscle features are predominately those of denervation.
Huttenlocher & Cohen (1966)	5 SMA 1	Spinal cord	LM	Histochemistry of ballooned chromatolytic neurons similar to that following axotomy.
Roth <i>et al.</i> (1965)	5 SMA 2/3	Muscle	EM	Muscle shows ultrastructural features of atrophy, dissolution of contractile elements.
Coërs & Woolf (1959)	SMA	Muscle	LM	Abnormal beading of intramuscular nerves.

Note. EM, electron microscopy; LM, light microscopy; ICC, immunocytochemistry; NF, neurofilament; GFAP, glial fibrillary acidic protein.

be dominated by fibrillation potentials of acutely denervated muscle fibers while the residual motor units are of normal size. There is thus an association between disease severity and the capacity of surviving motor neurons to respond to neighboring muscle fiber denervation by terminal axonal sprouting. This qualitative difference in the capacity for sprouting presents an alternative hypothesis to account for the difference between severe and mild forms of SMA, in contrast to the prevailing hypothesis that the range of severity is due to quantitative differences in the number of residual motor neurons or the timing of motor neuron loss (Crawford *et al.*, 1995a).

Two groups have called attention to abnormal rhythmic motor unit discharge during sleep, a characteristic not found in control infants or children (Buchthal & Olsen, 1970; Hausmanowa-Petrusewicz & Karwanska, 1986). The significance of this finding is unknown.

BIOCHEMICAL FINDINGS

Although the pathology of SMA lends no clues to suggest a specific disorder of intermediary metabolism, hints of a nonspecific abnormality of fatty acid oxidation have emerged from analysis of patient specimens from several different laboratories. (Kelley *et al.*, 1986; Tein *et al.*, 1995; Harpey *et al.*, 1990). Infants with type 1 SMA develop a significant dicarboxylic aciduria in response to fasting that is comparable in severity to the dicarboxylic aciduria of children with primary defects of fatty acid β -oxidation when fasted. In contrast to these patients with known metabolic defects, however, infants with SMA 1 also develop a moderate ketonuria. Random plasma samples from these infants have an abnormally high C12/C14 ratio (Crawford *et al.*, 1995b). The nature of the abnormality is complex and cannot be explained by any single metabolic defect or pathway, but does not appear to be a consequence of extreme debility or a property of denervated muscle because these changes do not appear in disease-control patients. These abnormalities may be pathogenic, epiphenomenal, or related to a non-SMA gene defect, but precedent for selective vulnerability of subsets of neurons to mutation of constitutively expressed housekeeping gene exists in the superoxide dismutase mutations responsible for some cases of familial ALS (Rosen *et al.*, 1993) and possible abnormalities in glycolysis in Huntington's disease (Burke *et al.*, 1996).

PATHOLOGY

Most neuropathological studies of the central nervous system in SMA have involved limited numbers of patients, in groups that were either clinically poorly characterized or heterogeneous in presentation, and usually concentrate on a single neuropathologic issue (Table 1). Most of the autopsy studies involve infants with SMA 1 because fatalities are concentrated in this form. The premiere neuropathological change in SMA is the paucity of motor neurons in spinal cord and lower brainstem, with the few surviving motor neurons characterized by swelling of the perikarya and chromatolysis. Ultrastructural analyses have shown that mitochondria and clumps of vesicular or membranous bodies collect in the cell soma of chromatolytic neurons, with adjacent accumulation of neurofilaments (Chou & Fakadej, 1971; Murayama *et al.*, 1991). Immunocytochemical studies have disclosed concentrations of phosphorylated neurofilaments in the periphery of the perikarya and ubiquitination of the central region of neurons undergoing chromatolysis (Matsumoto *et al.*, 1993; Murayama *et al.*, 1991; Kato & Hirano, 1990; Lippa & Smith, 1988).

Other subtle neuropathologic features, which are more widespread in the CNS but are either subclinical or obscured clinically by weakness and young age, have been described in infants who come to autopsy. These features, which may be greatest among the most severely affected infants (Towfighi *et al.*, 1985), include chromatolysis of sensory neurons and nodules of Nagoette (remnants of clusters of satellite cells) in dorsal root ganglia; there may be a small number of degenerating sensory fibers in the sural nerve (Marshall & Duchon, 1975; Carpenter *et al.*, 1978). Within the central nervous system chromatolytic neurons may be seen in Clarke's column and among subsets of neurons in the lateral geniculate and posteroventral or ventrolateral nuclei of the thalamus (Marshall & Duchon, 1975; Towfighi *et al.*, 1985; Peress *et al.*, 1986; Murayama *et al.*, 1991). The significance of these subtle findings is not clear, but they suggest that the primary defect of SMA, while highly concentrated in the large motor neurons, is not exclusive to these cells.

Ventral nerve roots are frequently invaded by parallel glial bundles that extend out from the spinal cord and are enclosed by a basal lamina, a finding that generated substantial early discussion about possible pathogenesis. (Chou & Fakadej, 1971; Chou & Nonaka,

1978; Ghatak, 1978). However, this glial reaction has subsequently been found in other early disorders of the motor neuron and is thus likely a secondary phenomenon (Iwata & Hirano, 1978a).

A few studies of intramuscular nerves in SMA suggest there may be specific and important distal motor nerve pathology. Two case studies report nerve bundles with multiple unmyelinated axons invested in a single Schwann cell or even multiple axons within a single pocket of a Schwann cell (Ohama & Ikuta, 1977; Fidzianska, 1974). Such Schwann cell pockets containing multiple axons, although normal in the early development of the peripheral nerve, are not seen as a pathologic change in any known disease or during regeneration. In another study these polyaxonal pockets were not seen in the intramuscular nerves of six patients (Chien & Nonaka, 1989); this investigation instead noted surviving motor axons of diminished size, suggesting selective loss of large or atrophy of surviving axons. An early anatomical study of intramuscular nerves, which used Evans blue staining of nerves in preparations of squashed or teased muscle fibers, demonstrated an unusual beading of intramuscular nerve fibers not previously seen (Coërs & Woolf, 1959).

These findings, sketchy as they are, raise the question of the locus of primary neuronal pathology in SMA. The long-held prejudice of many has been that SMA is primarily a neuronopathy principally involving the cell soma, with secondary degeneration of axons. However, there is some reason to believe the neuronopathy may follow from pathology expressed initially in the distal axon. There are similarities between the "pathogenic" chromatolytic cell of SMA and the chromatolysis seen following distal axotomy (Huttenlocher & Cohen, 1966), although others have highlighted their differences (Murayama *et al.*, 1991). Detailed electrophysiologic and pathologic reviews of two animal models of motor neuron disease suggest a precedent for primary abnormalities in the distal axon of motor neurons (Pinter *et al.*, 1995; Cork *et al.*, 1989; Schmalbruch *et al.*, 1991). Finally, abnormalities in muscle culture derived from patients with SMA suggest that the primary pathology of SMA may result from a disturbance in trophic interaction between muscle and nerve (Henderson *et al.*, 1987; Braun *et al.*, 1995). These findings constitute sufficient evidence to warrant careful evaluation of whether SMA is, indeed, a primary neuropathy or whether instead the initial focus of pathology is in the distal axon.

THE SMA CRITICAL REGION: CLASSIC AND MOLECULAR GENETICS

In the years following the linkage of SMA to chromosomal region 5q, interest quickly narrowed to a region within the 5q13 region (Brzustowicz *et al.*, 1992; Morrison *et al.*, 1992, 1993; Cobben *et al.*, 1993; Francis *et al.*, 1993; Kleyn *et al.*, 1993; Melki *et al.*, 1993; Soares *et al.*, 1993; Thompson *et al.*, 1993; Wirth *et al.*, 1993; Brahe *et al.*, 1994; Burghes *et al.*, 1994a; Clermont *et al.*, 1994). As the SMA-critical region narrowed, its complexity increased, as did confusion over the physical map of markers placed within this region. It became apparent that individual genetic markers occur in different copy number and orientation within each individual chromosome (Burghes *et al.*, 1994b; DiDonato *et al.*, 1994; McLean *et al.*, 1994; Theodosiou *et al.*, 1994; Roy *et al.*, 1995b; Wang *et al.*, 1995). Moreover, an unusually large number of expressed genes in this region are of a peculiar form of *unprocessed* pseudogene, i.e., a partially degraded gene homolog of parent genes most of which appear elsewhere in the genome (Thompson *et al.*, 1995). Thus, the conventional task of classic genetics, to establish a physical map of markers within a region, is undermined by this heterogeneity since it appears that any physical map must be individual chromosome specific.

The original cause of the repetition and disorder of these sequences within the SMA critical region is unknown, but once such heterogeneity is established the consequent genetic instability leads to a proliferation of even more duplications and deletions. This appears to be the molecular "niche" responsible for the high incidence of the disorder. *De novo* deletion mutations occur frequently during meiosis because of the difficulty in arranging for equal crossing over between highly heterogeneous sister chromatids (Theodosiou *et al.*, 1994). A new deletion mutation causing one of the two pathogenic chromosomes in the affected child is responsible for as many as 10% of all cases of SMA (Melki *et al.*, 1994; Rodrigues *et al.*, 1995; Wirth *et al.*, 1995). Because this is a recessive trait, the implication is that these rearrangements occur at a similar frequency in all meioses but are obscured from phenotypic expression by the normal companion allele. This is an extraordinarily high frequency for *de novo* mutation producing a recessive trait and has implications for the natural history of the mutation at the population level. Recessive disorders are generally thought to be formed at a low *de novo* mutation rate because there is no selection against the asymptomatic carrier of a single

copy of the mutation and hence no selection against the mutation until prevalence is sufficiently high that homozygote individuals manifesting the trait appear. If spontaneous mutation were a frequent event—as the evidence suggests it is in SMA—a mutant allele would be expected to proliferate quickly until the selection pressure manifested by the incidence of homozygous lethals balanced the mutation rate. However, in SMA the reduction in reproductive fitness to carriers is at least one order of magnitude smaller than the spontaneous mutation rate (at a heterozygote carrier frequency of 1 in 60 the probability of any one carrier having an affected child is equal to the one-fourth the probability of parenting with another asymptomatic carrier or $1 \times 1/60 \times 1/4 = 1/240 = 0.4\%$). Because the prevalence of the SMA mutation is likely stable (given the roughly equal incidence of SMA worldwide), forces other than selection pressure must balance the high rate of *de novo* mutation. The most reasonable hypothesis would be that the high rate of *de novo* mutation is balanced by a comparable rate of corrective reversion mutations. Presumably, deletions within the region can be repaired by the same process of unequal crossing over during meiosis that is responsible for production of deletions in the first place.

THE TWO CANDIDATE GENES

Survival Motor Neuron

The French group led by Judith Melki and Arnold Munnich identified their candidate gene within a large duplicated 500-kb region of DNA (Lefebvre *et al.*, 1995) in the donor chromosome used to construct the YAC contig that they searched. The duplicated stretches lie adjacent and inverted to one another. The *SMN* gene lies in the telomeric duplicated region; in addition, a highly homologous copy of *SMN* (termed ϵ BCD541, and later ϵ *SMN*, by other authors) lies within the centromeric region. Polymerase chain reaction (PCR) products of the eight exons and surrounding intronic DNA from the two genes differ at only five nucleotides. One of these differences, in the coding region of the gene, produces a synonymous mutation; another is in the 3' noncoding region; the other three are in unexpressed intronic DNA. PCR-amplified products from the two genes can be distinguished by single-strand conformational polymorphism (SSCP) analysis, and the absence of *SMN* can be detected by specific PCR (van der Steege *et al.*, 1995).

Of a total of 229 children with SMA, Melki and

co-workers detected deletion of all or part of *SMN* in 226. The 3 remaining patients had disabling point mutations (Lefebvre *et al.*, 1995). Deletion mutations of comparable frequency have been demonstrated by SSCP in other populations of SMA children and range from 84 to 100% (mean, 93.5%) (Lefebvre *et al.*, 1995; van der Steege *et al.*, 1995; Rodrigues *et al.*, 1995; Cobben *et al.*, 1995; Aubry *et al.*, 1995; Bussaglia *et al.*, 1995; Hahnen *et al.*, 1995; Chang *et al.*, 1995). One other important example of a nondeletion *SMN* mutation was reported subsequently from a different group of patients. A frameshift mutation and premature stop codon found in four unrelated Spanish children, each with a similar haplotype background, suggests a founder effect for the mutation within this population. This lends substantial credence to *SMN* as a SMA-determining gene (Bussaglia *et al.*, 1995).

Homozygous deletion of *SMN* was not detected in any of the French group's original 246 controls (Lefebvre *et al.*, 1995), but has subsequently been found in 11 asymptomatic relatives of SMA-affected children (Cobben *et al.*, 1995; Hahnen *et al.*, 1995). According to classic genetic theory, this would be persuasive evidence that *SMN* is not the pathogenic gene for SMA. However, these cases of genotype/phenotype mismatch have been found almost exclusively in families with SMA type 3, suggesting instead that the difference in disease expression is due to some relatively subtle aspect of pathogenesis. If *SMN* were a true bystander gene, and not a cause of SMA, no such bias toward the more mild SMA types would be expected in the few described phenotype-discordant, genotype-concordant families. Further supporting the idea that these normal relatives with *SMN* deletions owe their health to a subtle downstream alteration in pathogenesis is the finding of enlarged motor units, suggesting partial denervation, in one apparently normal SMA type 3 sibling with homozygous deletion of *SMN* (Brahe *et al.*, 1995).

One of the biggest quandaries raised by the genetic studies of *SMN*, however, is the lack of correlation between genotype and phenotype. Although a weak correlation exists between the frequency of homozygous deletion of *SMN* and severity of disease (in the above combined published reports the frequency of homozygous *SMN* deletion in type 1, 2, and 3 SMA is 97, 96, and 86%), most patients with severe and mild disease share the same deletion mutation of *SMN*. The most likely explanation for differences in phenotype involves the existence of a disease-modifying gene and, because most affected siblings share a similar

phenotype, such a gene would need to be tightly linked to the pathogenic gene. Supporting this hypothesis is the observation that deletion of neighboring markers generally correlates with severity of disease (McLean *et al.*, 1994; Melki *et al.*, 1994; Daniels *et al.*, 1995; Wirth *et al.*, 1995; Burlet *et al.*, 1996). One attractive candidate for the disease-modifying gene is the other candidate gene for SMA, *NAIP*. In the setting of homozygous *SMN* deletion, deletion of exon 5–6 markers for *NAIP* correlate with disease severity, but still many severely affected infants appear to have an intact copy of *NAIP* (Hahnen *et al.*, 1995; Burlet *et al.*, 1996). Unfortunately for this analysis, as noted below, identification of *NAIP* mutations is complicated by the presence of numerous degraded *NAIP* pseudogene copies.

The other leading candidate for the disease-modifying gene is ϵ *SMN* (ϵ BCD541). Although the predicted protein product for ϵ *SMN* is identical to that of *SMN*, ϵ *SMN* produces both a full-length and an alternatively spliced transcript (Lefebvre *et al.*, 1995; Gennarelli *et al.*, 1995). This alternatively spliced form of ϵ *SMN*, expressed at equal or greater concentrations to the full-length transcript, produces a new c-terminus of the protein and probably has a different tertiary structure (Gennarelli *et al.*, 1995). Homozygous deletion of ϵ *SMN* is common, seen in 4% of normal individuals (Lefebvre *et al.*, 1995); however, it is noteworthy that homozygous deletion of both *SMN* and ϵ *SMN* has not been detected in any SMA patient, suggesting both that this combination is an embryonic lethal and that ϵ *SMN* influences the biology of *SMN*. The total copy number of ϵ *SMN* on both chromosomes, as well as individual or tissue-specific influences on ϵ *SMN* isoform transcription, may be important in determining the severity of SMA phenotype in the setting of homozygous *SMN* deletion.

Northern blots demonstrate that the *SMN* gene, its homolog ϵ *SMN*, or both are expressed in a wide range of tissues (Lefebvre *et al.*, 1995; Gennarelli *et al.*, 1995). The sequence of *SMN* predicts a protein product 294 amino acids long, but manifesting no known homology with any other described gene or gene product. Unfortunately, few clues about function emerge from the sequence.

Neuronal Apoptosis Inhibitory Protein

A portion of the SMA-candidate gene *NAIP*, identified by Alex MacKenzie's group in Ottawa Canada, is homozygously deleted in 45% of type 1

SMA patients and in 18% of patients with SMA types 2 or 3 (Roy *et al.*, 1995a). Unfortunately, genotype assessment is difficult because the molecular definition of *NAIP* is clouded by multiple degraded pseudogene copies for *NAIP* within the SMA-critical region of chromosome 5q. Like every other identified sequence within this region the numbers of these pseudogene copies vary greatly from chromosome to chromosome. The exon 5–6 region of *NAIP* is the only region of the gene that is usually found to be missing from the pseudogene copies. Thus, the actual percentage of SMA type 1 patients with homozygous deletions of the full-length *NAIP* gene would perhaps exceed 45% if the partially degraded pseudogene copies of *NAIP* contain exons 5 and 6 and so invalidate the use of the exon 5–6 probe.

MacKenzie and co-workers did not detect homozygous deletion of exons 5 and 6 of *NAIP* in any of 800 random normal controls. As with the studies of *SMN*, however, several asymptomatic parents of children with SMA were found to have no full-length copy of *NAIP*. But in contrast to the *SMN* findings, these genotype-concordant, phenotype-discordant families showed no tendency to manifest only the milder forms of SMA. Again, classic genetic theory would consider this fact persuasive evidence that *NAIP* is not the pathogenic gene for SMA. But to preserve the candidacy of *NAIP* as the SMA-pathogenic gene, it is suggested that the very pseudogenes that confound genotype assessment have partial activity. These are in fact *unprocessed* pseudogenes; that is, they resemble gene homologs in expression and regulation, but sequence analysis frequently reveals mutations which disrupt translation into a stable protein. The 5–6-deleted form of *NAIP* is proposed to retain partial function (it is an in-frame deletion) in cases of full-length *NAIP* deletion and thus to possess the capacity to rescue a normal phenotype. Given the background of a variable number of degraded *NAIP* pseudogenes, each with unknown potential for translation into functional protein, this hypothesis will be difficult to verify at the level of DNA or RNA. More severely affected patients, however, do tend to have fewer copies of *NAIP* (Wirth *et al.*, 1995; Burlet *et al.*, 1996).

Neuronal apoptosis inhibitory protein is so-named because the exon 5–6 portion of the gene is homologous to a portion of a baculoviral gene product (inhibitor of apoptosis protein, *IAP*) that suppresses apoptosis within the insect cells it infects. Among insects, apoptosis is an efficient anti-viral defense mechanism that disables the cellular nucleic acid-

replicative apparatus before productive viral infection can spread to the rest of the organism. Viruses appear to have acquired a number of *IAPs* that are homologous to natural apoptotic regulatory genes within higher species. *NAIP* is unlike *IAP* in that it lacks the zinc finger motif that is important to the anti-apoptotic effect of *IAP*. However, *NAIP* does have demonstrable anti-apoptotic activity in a variety of mammalian cells and models of apoptosis, equal in some cases to that of Bcl-2, the first described mammalian anti-apoptotic regulatory gene (Liston *et al.*, 1996). Other mammalian *IAPs* have been found recently, and their association with the cytoplasmic domain of the tumor necrosis factor receptor suggests a role for these and presumably the family of *IAP* genes in intracellular signal transduction of extracellular signals (Rothe *et al.*, 1995).

Taken together, the deletion of *NAIP* in a substantial portion of children with SMA, the homology of portions of *NAIP* to *IAP*, and the demonstrated anti-apoptotic effect of *NAIP* raise the intriguing possibility that SMA is a disorder of unrestrained apoptosis. This of course is a recapitulation of the same hypothesis developed from clinical observations. The strongest argument for *NAIP* as a candidate gene for SMA is thus one of biologic plausibility and chance: the finding of a protein of such interesting biologic properties within the small unstable SMA-critical region would seem an unlikely accident. Perhaps the strongest argument against *NAIP* is the presence of equal or stronger genetic arguments in favor of *SMN*. A promising compromise hypothesis, which would resolve the weaknesses of each candidate, would be that deletion of *SMN* is necessary for the disorder, but that *NAIP* regulates severity of the phenotype.

RESOLVING THE PUZZLE

The search for the cause of SMA will continue at the level of molecular genetics, but confirmation of one, both, or neither of these two candidate genes will probably evolve from an understanding of gene function. There is clearly a role for further exploration at multiple levels, including the study of tissue and developmental gene expression, the study of the cellular biology of these genes, and the generation of animal models. One natural strategy would be to knock out each gene in a transgenic animal. The *SMN* gene is likely the easier gene to manipulate because it exists in lower copy number, where *NAIP* is a more difficult target because of its multiple pseudogene copies.

Generation of a true animal model of SMA would be invaluable in developing an understanding of the pathophysiology of the disorder and may possibly lead to a means to modify its course. A true animal model may also bring general insights into the ontogeny of the nervous system. As the identity of the SMA gene emerges, we can anticipate gaining a comparable multilevel understanding of SMA, from the gene and gene product to the biology of neuronal cell death, the inborn vulnerabilities of motor neurons during development, and, with good fortune, a delineation of trophic effects or other related aspects that might positively affect the course of the disease in these children.

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