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UNDERSTANDING MOTONEURONE DEVELOPMENT EXPLAINS SPINAL MUSCULAR ATROPHY

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INTRODUCTION

Studies of the organisation and function of the adult central nervous system (CNS) elucidated many basic properties of neurones and their interactions with each other. The present meeting illustrates the progress made during the past years in understanding the mechanisms involved in the processing of information by the CNS. Developmental biologists and neuroscientists are attempting to understand how this complex system has developed and what are the essential factors for establishing the networks seen in the adult. Recently much work has concentrated on studying the signals that induce the commitment of cells to the neuronal lineage, establish the identity of neurones and their initial connections with each other.

Regarding the development of motoneurones and their connections with afferent signals recent results of Jessel and his colleagues has provided new and important information about the signalling molecules involved in this process (5). However, most of these studies are concerned with early developmental events, a period when the basic layout of the CNS and the connectivity between neurones as well as their peripheral targets is being established. Surprisingly in recent years there has been relatively little progress in understanding the events that take place after the basic layout of the CNS has been established. Yet, the developmental events during these periods are perhaps the most important for the evaluation of functional disturbances responsible for neuromuscular disorders that involve the motoneurone. One example of such a disorder is spinal muscular atrophy (SMA). In children suffering from this condition the basic 'body plan' at birth is not affected. It is possible that in cases where the development of the foetus is severely affected survival is impossible. Yet although some motoneurones die before birth, other components of the CNS are present, and their correct connections with each other are established, there is a devastating deterioration of the function of the neuromuscular system after birth. Here the possible implications of developmental events of the neuromuscular system that occur shortly before and immediately after birth for the pathogenesis of SMA will be considered.

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NEUROMUSCULAR DEVELOPMENT

Motoneurones

Motoneurones are among the first cells to be generated in the spinal cord. As in other neuronal populations, not all of these neurones survive and a proportion of them die (12, 26). This event is referred to as 'naturally occurring cell death' and the extent of it depends on the availability of target muscle. This finding indicated that motoneurone-muscle interactions are of great importance for motoneurone survival and development. The naturally occurring cell death takes place during embryonic development of the nervous system. Nevertheless, even later i.e. during postnatal life, motoneurones remain critically dependent on contact and active interaction with their target.

Studies on early postnatal development of the neuromuscular system in rats, which are born less mature than human babies, show that temporary disconnection of motoneurones from their target muscles results in the death of a proportion of motoneurones (20) (Fig. 1). However, even those motoneurones that survive after temporary loss of contact with the target do not develop normally. The nature of the changes in these surviving motoneurones may be useful in providing a clue as to the cause of death of those that had failed to survive. They have a smaller cell body (19) and their firing pattern is abnormal, i.e. they discharge more readily and display long-lasting activity (23) indicating that they are more excitable. The increased activ-

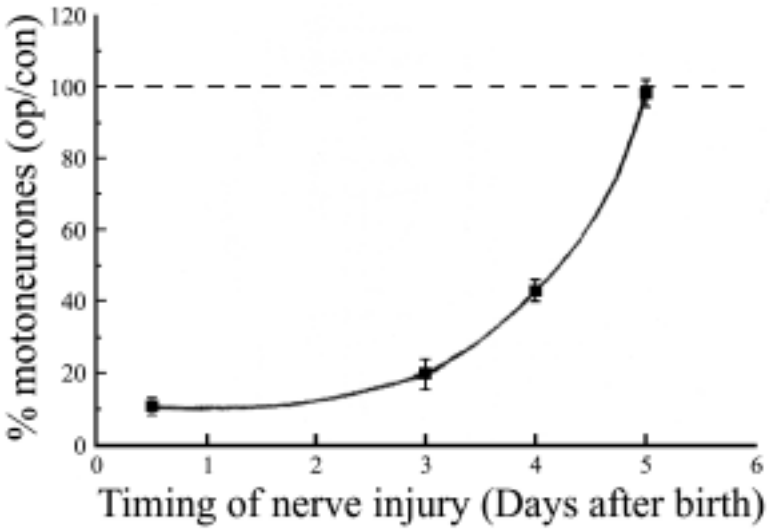

Fig. 1. - *The importance of age on axotomy induced motoneurone death.*
The percentage of surviving motoneurones to soleus muscle is plotted against postnatal age at which the sciatic nerve was injured. All groups of animals were examined 3 months after nerve injury. Note: loss of motoneurones is greatest when axotomy is carried out shortly after birth and decreases with age. Injury inflicted at 5 days or later does not cause any motoneurone death.

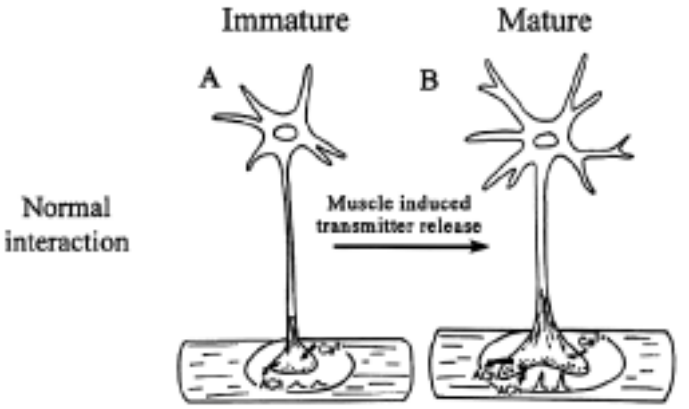

Fig. 2. - *The development of motoneurone proceeds normaly when interaction of the terminal and muscle occurs.*

ity of these motoneurones compared to normal motoneurones is unlikely to be caused by a larger number of synaptic inputs, for after axotomy there is massive death of sensory neurons in the dorsal root ganglia, and also considerable loss of cells that send descending inputs from the brain to the motoneurones (25). Therefore the increased activity noted in cells that survive axotomy is most likely caused by their greater excitability, which is unfavourable to their survival.

The level of excitation of axotomized motoneurones is an important factor in regulating motoneurone death. In the postnatal rat, motoneurones destined to die can be rescued by agents that reduce their excitability (11) (Fig. 3) and more motoneurones can be induced to die by imposing increased activity upon them (29).

We therefore propose that the death of motoneurones after neonatal axotomy is caused by the inability of the motoneurone to adjust to the increased strength of afferent inputs that converge onto motoneurones as the CNS undergoes further development and more activity is imposed upon the motor system.

How does the target induce the maturation of the motoneurone? A possibility frequently mentioned is that the target provides trophic factors that enable motoneurone survival. However, most studies show that the rescue of axotomized motoneurones by trophic factors is temporary, and permanent rescue has so far not been found. Another explanation relates to the changing function of the motoneurone during development. Initially the axons and dendrites of motoneurones grow and only gradually do they acquire their normal function, i.e the dendrites become targets for afferent inputs and the axon terminals start transmitting impulses. It is the acquisition of this function, i.e. the change from a growing neurone to a transmitting one that is the most likely candidate for the survival of the motoneurone (Fig. 2). Evidence to show that preventing transmitter release from nerve endings causes motoneurone to die and premature induction of transmitter release leads to their resistance to axotomy support this idea (Fig. 4).

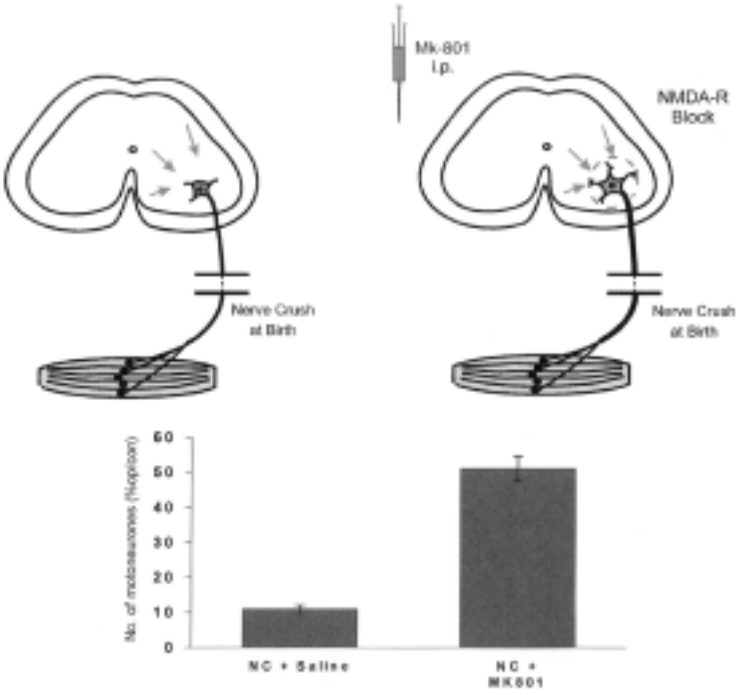

Fig. 3. - *The effect of blocking NMDA receptors on motoneurone survival after neonatal nerve injury.* The top diagrams illustrate the afferent inputs (arrows) to an axotomized motoneurone in the spinal cord. The left diagram illustrates saline treated and the right diagram MK801 treated spinal cords where the NMDA receptors were blocked. The block diagrams show the % loss of motoneurones to soleus muscles from the saline (left) and the MK 801 (right) treated animals. The vertical bars are SEMs.

The developmental changes involved in changing a growing neurone into a transmitting one include the replacement of growth associated proteins with those involved in transmission. Together with this change other immature protein isoforms that regulate the neurones excitability are replaced by their more mature counterparts.

Development and maturation of muscle

The development of muscle fibres can be divided into the following sequential events: (1) commitment of mesenchymal cells to myogenic lineages, (2) fusion of myoblasts to form primary and secondary myotubes and (3) separation of the secondary myotubes from the primary myotube and differentiation of muscle fibres into various fibre types under the influence of the motoneurone to become part of the motor unit. The first 2 steps of muscle development have been recently reviewed (22) but the third stage is poorly understood.

In rats, the most important changes in protein composition of developing muscles that enable them to carry out the contractile activity elicited by the motoneurones occurs during the first 15 days postnatally when the less mature protein isoforms are

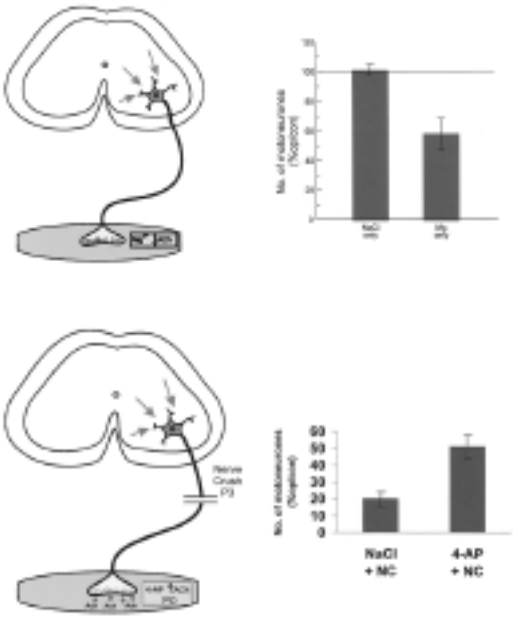

Fig. 4. - The diagrams illustrate motoneurones and their inputs (arrows) in rats that where treated with (a) Mg^{2+} shortly after birth, and (b) 4-AP. The 4-AP treated rats had their sciatic nerve subsequently crushed. The numbers of motoneurones to soleus was established 3 months later. The block diagrams show a loss of motoneurones after Mg^{2+} treatment at birth (a), and rescue of motoneurones when 4-AP treatment preceded the nerve injury.

replaced by their more mature counterparts (19, 32). This developmental step requires molecules that can facilitate these changes and in their absence the muscles are vulnerable.

Evidence from experiments in rats, where muscle maturation was delayed by denervation, provides some insight into the importance of the appropriate timing of muscle fibre maturation in relation to function. Immature muscle fibres survive denervation for considerable periods of time, but their development into mature muscle fibres is critically dependent on functional innervation (19). Although skeletal muscle fibres denervated during a critical period of postnatal development (in rats day 5 to 16) survive, on reinnervation 60% of them die. This does not happen if they are left denervated (19). What is the reason for the devastating effect of reinnervation on the survival of previously denervated muscle fibres? The explanation we favour is that muscle fibre differentiation and maturation was arrested during the period of denervation, a critical time for muscle fibre development. During this period of arrested muscle development, the spinal cord circuitry continues to develop and motoneurone activity assumes a more mature pattern, i.e. they fire more often and at higher frequencies (32). On reinnervation by axons of these motoneurones, many muscle fibres that had their development arrested are unable to withstand this new pattern of activity and die (23).

Thus like motoneurones, immature muscle fibres must become competent to cope with functions that are changing with the development of the locomotor system.

These findings on the development of the neuromuscular system clearly show that the survival of both motoneurone and muscle depends on the accurate timing of changes of their molecular composition that allow them to carry out the functions required.

How does this knowledge help us to understand the pathogenesis of SMA?

SPINAL MUSCULAR ATROPHY

SMA is an inherited neuromuscular disorder. It is one of the most common autosomal recessive diseases, with an incidence of 1 in 10000 live births and a carrier frequency of 1 in 40-60 adults (27).

The phenotype is extremely variable and patients are classified clinically into four groups: severe (SMA I), intermediate (SMA II), mild (SMA III), and adult (SMA IV). This classification is based on the age at onset of symptoms, age at death, and age at the achievement of certain motor control milestones, e.g. sitting or walking without support (15).

The clinical symptoms in all four groups of SMA include: (i) hypotonia, (ii) symmetrical muscle weakness and atrophy (predominantly of proximal muscles of the shoulder and pelvic girdle), (iii) tremor of fingers and hands, (iv) fasciculation of the tongue muscles, (v) hyporeflexia and contractures of some muscle groups. The diaphragm and extraocular muscles remain unaffected until late stages of the disease and there is little or no impairment of sensory systems (15).

The symptoms are thought to be due to selective degeneration of motoneurones in the spinal cord and cranial motor nuclei leading to progressive muscle weakness, atrophy and paralysis (10).

The genetic defect in all 4 forms of SMA has been localized to chromosome 5q11.2-13.2 using linkage analysis (18). This region might be unstable, resulting in a high frequency of inherited and *de novo* deletions and rearrangements. The gene that localizes to this region and is altered in patients with SMA has been identified and named ***smn*** (survival motor neurone) gene (18, 24).

In humans there are 2 copies of the *smn* gene – *smn* 1 and *smn 2*; *smn* 1 is localized at the telomeric region and *smn* 2 at the centromeric region of the chromosome. *Smn* 2 differs from *smn* 1 by only 5 nucleotides. This difference does not alter the amino acid sequence of the protein (SMN) they encode for. However, *smn* 2 can undergo alternative splicing of exon 7 giving rise to a truncated transcript which encodes for a protein that lacks the last 16 residues at the C-terminal. Therefore the full-length transcripts are predominantly produced by *smn* 1 and not by *smn* 2 genes (24).

In SMA patients the *smn* 1 gene is missing or altered. The *smn* 2 gene is present, but since it lacks exon 7 it encodes for a truncated form of the SMN protein.

Nevertheless the *smn* 2 gene does give rise to a small number of the full-length SMN transcript and due to this the number of copies of the *smn* 2 gene present modulates the severity of the disease.

The full-length *smn* transcript, i.e. SMN protein is essential for the survival of motoneurones, for in its absence their survival is compromised and many die. What then is the cellular function of the full length SMN protein?

FUNCTION OF SMN PROTEIN

SMN protein is expressed in most tissues (28, 31). It is part of a macromolecular complex that directly regulates the assembly of a specific class of RNA-protein complexes, the so-called spliceosomal U snRNPs (9, 31). It has been termed the master RNA assembler (28, 31) and is an essential component of the spliceosome that catalyses pre-mRNA splicing (9, 31). Morphologically these complexes can be identified as nuclear and cytoplasmic inclusions referred to as gems (33). Thus the function of SMN protein is linked to the control of protein synthesis, and possibly to the expression of new protein isoforms. It is these functions of the SMN protein that may be particularly important during development.

In most organisms, changes of protein composition of their tissues occur throughout life but these events are particularly evident during development and maturation. During development, protein isoforms typical of immature cells are replaced by their adult counterpart. It is likely that the spliceosomal complex of the U snRNPs, of which SMN protein is an essential part, is particularly important during this time. The finding that SMN protein is highest during development and is down-regulated in later life is consistent with this suggestion (3, 17).

IN THE ABSENCE OF SMN PROTEIN MATURATION OF THE MOTOR UNIT IS SLOW

An indication that SMN protein is important for transitional events involved in maturation is provided by a more detailed analysis of the changes in the different components of the motor unit in SMA patients as well as from some results obtained from animal models where the SMN protein was altered (14).

In SMA patients the sizes of the surviving motoneurones are smaller with less developed Nissl substance then in normal individuals of comparable age (8, 10). Moreover, motor units of SMA patients are continuously active (2, 13). This increased activity is likely to be due to the increased excitability of motoneurones in SMA patients and is similar to that seen in animals in which maturation of motoneurones was delayed by axotomy at a young age (see above) (23).

Motor axons in SMA patients also show signs of immaturity. Axons in the ventral roots are small (10) and in peripheral nerves have longer latency to electrical stimu-

lation and reduced conduction velocity compared to normal individuals of comparable age (16).

Perhaps the best explored signs of immaturity are in skeletal muscles. They contain large numbers of extremely small fibres with centrally placed nuclei. The fetal character of these cells is indicated by their high RNA content, and presence of desmin and vimentin, molecules that are down-regulated during normal development (7, 8).

Animal studies also show that development is severely affected by the absence, or reduction, of the *smn* gene and its protein product. Knockout *smn* mice embryos fail to develop and die before birth (30). They can survive if some copies of the human *smn* gene are inserted into the mouse, but the phenotype of these animals is not yet documented. Unlike these KO mice, larvae of transgenic drosophila flies that lack the *smn* gene do survive, probably because they have some maternal SMN protein, however they do not enter pupation or develop into a fly. The small amount of the SMN protein allows survival of the larvae but the neuromuscular junctions of their muscles show signs of immaturity in that their axon terminals release smaller amounts of transmitter. Moreover, the development of the animal fails to continue, i.e. the transformation of the larva into a pupa and finally a fly with a completely novel body plan does hot occur (4). In zebrafish down-regulation of the SMN protein is either lethal or it results in aberrant guidance of axons with excessive axonal branching (21). Since guidance is a result of interaction of the growth cone with environmental cues, it is possible that the growth cones fail to express the appropriate molecules that allow this interaction. However, the relevance of this finding to the human disease is questionable, for in SMA children no misdirection of axons can be detected and the innervation pattern of muscles is correct, suggesting that the axons do find their way to the appropriate targets.

The expression of new proteins in primary cultures of cerebellar granule cells in response to glutamate also seems to depend on the presence of SMN protein. Exposure to glutamate induces the expression of glutamate receptors in granule cells from normal mice, and this coincides with an increased expression of the SMN protein (1). Taken together, these findings indicate that SMN protein and its macromolecular complex are essential for transitions of the cell phenotype that occur during development, and possibly during later life.

These observations show that the SMN protein could be particularly important during development and maturation. In the total absence of SMN protein the *smn* KO mouse dies during embryonic development, and this is probably so in humans too. With low levels of the protein, the human embryo is able to complete most of its development but the neuromuscular system is severely affected.

SPECIAL INVOLVEMENT OF MOTONEURONES

Why is the motoneurone affected more than any other cell? To account for this unique need of motoneurones for the SMN protein it may be useful to list the particular features of the motoneurone:

(a) *The motoneurone is a cholinergic cell*, i.e. the transmitter it synthesizes and releases is acetylcholine. However, other cholinergic cells in the CNS including those with large projections and relatively long axons, such as neurones in the Meynert nucleus, are not affected, for the cognitive behaviour, i.e. memory and intelligence of SMA children, is if anything above average, and no morphological changes in this part of the brain had been reported. Thus neither the cholinergic nature of the motoneurone nor its exceptionally large projections can account for its special need for the SMN protein.

(b) *The axon of the motoneurone is outside the CNS.* The location of motoneurone axons in the periphery distinguishes it from many other neurones. Nevertheless, sensory neurones too have their long process outside the CNS and they are not dramatically affected by reduction of the SMN protein. Moreover, even cholinergic cells with axons in the periphery, such as many neurones of the autonomic nervous system, are not critically dependent on the presence of the SMN protein and are not compromised in patients with SMA.

(c) *Special relationship of motoneurone and muscle.* The last and probably most unique feature of the motoneurone is its special relationship with skeletal muscle fibres. Indeed, it appears that this feature distinguishes the motoneurone from all other neurones of the central and peripheral nervous system and therefore points to the cause of its unique involvement in SMA. Why does a neurone that interacts with skeletal muscle fibres have a special requirement for the *SMN* gene and protein?

During normal development, the motoneurone undergoes a change of phenotype in which many of the immature molecules are replaced by their more mature counterparts. The timely transition is necessary for the survival of the motoneurone and is induced by its interaction with the muscle. In SMA the reduced rate of splicing due to a reduction of SMN protein, slows the rate of this muscle induced change and delays the appearance of the more mature protein isoforms. It is this delay that condemns the motoneurone to death.

CONCLUSIONS

It is known that contact with muscle during early development is necessary for maturation of the motoneurone, which includes the replacement of many of its ‘immature’ constituents with their more mature counterparts. If this transition fails to take place during a critical time of development, the motoneurone is doomed and dies. What better candidate then the SMN protein with its crucial housekeeping function (6) to participate in these developmental transitions? Observations on SMA patients who have reduced amounts of SMN protein and on some animal models of SMA suggest that they retain many immature features of their neuromuscular system.

Hopefully further research such as a detailed analyses of the phenotype of animal models of SMA will elucidate the question of the unique involvement of the motor unit in SMA.

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