J. exp. Biol. **132**, 93–109 (1987)
Printed in Great Britain © *The Company of Biologists Limited 1987*

STRUCTURAL CHANGES IN INTERCOSTAL MOTONEURONES FOLLOWING AXOTOMY

BY T. A. SEARS

Sobell Department of Neurophysiology, Institute of Neurology, Queen Square, London, WC1N 3BG, UK

SUMMARY

Motoneurone disease (MND or amyotrophic lateral sclerosis) is a paralysing disease of unknown cause involving progressive, widespread muscle atrophy due to degeneration of spinal and other motoneurones and an accompanying loss of Betz cells in the motor cortex. A current hypothesis attributes the disease to the loss of a muscle-derived neurotrophic factor acting in concert with the normal age-related deterioration and loss of motoneurones. The roots of this hypothesis are traced through research based mainly on the developing neuromuscular system, and in particular on the age-related processes of natural motoneurone death during embryogenesis: the neonatal reduction of polyneuronal innervation and the age-dependent variations in motor nerve terminal sprouting in response to partial denervation. A consideration of the disease process itself in association with the review of earlier work provide the background for the present work which re-examines ultrastructurally the chromatolytic and later responses to axotomy and the muscle-dependent factors responsible for the reformation of the Nissl bodies.

INTRODUCTION

The study of respiratory movement and of its nervous mechanism is of interest from many points of view. In the 'awake' state the movements are broadly of two kinds. The most 'automatic' ones occur rhythmically, without the need for conscious intervention and being chemically driven, satisfy the homeostatic need for oxygen and the elimination of CO_2. The least automatic ones generate the pressures which sustain, for example, straining, bearing down and vocalization so that in the case of human speech and song these could be regarded as the most voluntary of all human movements (Sears, 1974).

Automatic respiratory movements are unique in that they persist during sleep, under anaesthesia and also following decerebration, so permitting a mammalian motor system to be investigated at the behavioural, cellular and membrane levels of organization during the course of natural movement. The diaphragm and intercostal muscles of small mammals have also provided important *in vitro* muscle preparations which have allowed a broad range of questions to be answered which are of interest to both basic and clinical neuroscientists. In particular, the study of biopsied human intercostal muscle has contributed enormously to our understanding of human

Key words: motoneurones, axotomy, Nissl body, motoneurone disease.

neuromuscular diseases such as myasthenia gravis, the Eaton Lambert syndrome and muscular dystrophy. The research on intercostal motoneurones outlined below is also at the interface between basic and clinical science insofar as it aims to advance basic knowledge that might bear on the problem of motoneurone disease (MND), a distressing paralysing disease which has juvenile and adult forms (see Tandan & Bradley, 1985*a*,*b* for a recent review). The key features of MND are distal weakness and muscle atrophy, an onset in middle age (mean age of onset 66 years) and an unrelenting course (typically 2 years) with fatal outcome due usually to a severe impairment of respiratory function (Appel, 1981). The degenerative process is restricted to two groups of neurones: motoneurones innervating most skeletal muscles (although some are spared, e.g. occulomotor and certain sacral motoneurones) and the large motoneurones (Betz cells) in the motor cortex through which the voluntary commands for movement are executed. The loss of these 'upper' motoneurones and the accompanying degeneration of the laterally situated corticospinal tract explain the alternative name for the disease amyotrophic lateral sclerosis (ALS) and also account for the hypereflexia and attendant weakness of the upper motoneurone type. Symptoms and signs may occur first in cranial motoneurones (pseudobulbar palsy) but it is generally assumed that the unknown etiological factor(s) is probably the same. Here I shall not consider all of the possible etiological mechanisms; this has been done comprehensively by Tandan & Bradley (1985*b*). Instead, I shall briefly emphasize certain fields of experimental neurobiology the findings and concepts from which are embodied in the hypothetical etiology of this disease as proposed by Appel (1981) and further developed by Gurney, Belton, Cashman & Antel (1984).

NATURAL MOTONEURONE CELL DEATH

The work of Hamburger (see Hamburger, 1977) and more recently Oppenheim and colleagues (see Oppenheim, 1985) has highlighted the natural process of neuronal cell death during embryogenesis. At the time of birth the surviving motoneurones comprise perhaps only 50% of the original population of differentiated motoneurones which, earlier in embryonic life, can be labelled retrogradely following intramuscular injection of horseradish peroxidase (HRP) (Lance-Jones, 1982). The number of surviving motoneurones is modifiable, since early removal of the 'target' muscle results in a loss of virtually all the motoneurones destined to innervate that muscle. Conversely, an experimental reduction in the number of motoneurones competing for a target (Lance-Jones & Landmesser, 1980) or the provision of excess target (Hollyday & Hamburger, 1976) results in the 'rescue' of ones destined to die and a corresponding increase in the number of motor nerve terminals (Strihari & Vrbova, 1980). Blockade of neuromuscular transmission during the period of normal cell death can also rescue virtually all those motoneurones that would have died (Pittman & Oppenheim, 1978; see also Oppenheim, 1985). These and related results, lead to the same general conclusion that, for their survival, embryonic motoneurones normally need a factor(s) that is associated in some way

with muscle innervation. For the rat Harris & McCaig (1984) have provided clear evidence of an inverse relationship between motoneurone death and muscle innervation, the former being completed at the time the maximum number of nerve terminals per motoneurone has been achieved. To explain this they advance the hypothesis that, for their survival, embryonic motoneurones require a trophic substance released by muscle cells. The amount taken up depends on the number of nerve terminals possessed by each motoneurone. Muscle contractile activity would inhibit release of the factor while it would be released in excess in paralysed muscle. Motoneurones bearing the most terminals would thus have a selective advantage for survival when competing for a limited amount of the factor. The finding that electrical stimulation of muscle actually enhances motoneurone death (Oppenheim & Nunez, 1982) would be explained by this hypothesis by reduced production of trophic substance by the activated muscle. Harris & McCaig (1984) believe that intrinsic control mechanisms govern the number of terminals established by embryonic motoneurones, otherwise muscles would eventually contain a few very large motor units regardless of their functional role in the animal.

The question of whether the natural death of motoneurones occurs postnatally in the mammal was originally studied by Romanes (1946) who reported a substantial loss (20 %) of lumbosacral motoneurones in the first few days postnatally in the mouse, although a later study using HRP labelling (Lance-Jones, 1982) established that the number of motoneurones was stable from E18 onwards. Motoneurone counts based on Nissl-stained material by Nurcombe, McGrath & Bennett (1981) also revealed a 45 % loss of brachial motoneurones between postnatal days 1 and 6, but more recent experiments by Oppenheim (1986) led him to conclude that there is no significant loss of brachial or lumbar motoneurones in the postnatal rat. He explained his different results as probably being due to differences in the size criteria for categorizing cells as alpha motoneurones. Furthermore, he attributed the loss of polyneuronal innervation of muscle, which also mainly occurs postnatally, solely to the elimination of axons. Similarly, Jenq, Chung & Coggeshall (1986) assign the postnatal loss of axons in the rat sciatic nerve to the elimination of axon branches rather than to motoneurone death, as Fladby (1987) has done for the mouse.

MOTONEURONE DEATH STUDIED IN TISSUE CULTURE

The target dependence of embryonic motoneurones for survival is also clearly demonstrated in tissue culture. Such experiments mostly deal with 'factors' which promote neurite extension, rather than survival *per se*, but Bennett, Lai & Nurcombe (1980) were the first clearly to demonstrate in culture that HRP-identified motoneurones depended on skeletal muscle for their survival, as was also confirmed by Slack & Pockett (1982) and Tanaka & Obata (1983). An alternative approach has been to label embryonic motoneurones *in situ* by the retrograde transport of fluorochromes (McPheeters & Okun, 1980), prior to cell dissociation, thus enabling the motoneurones in enriched cultures subsequently to be identified by fluorescence microscopy in fixed tissue (Smith, Vaca, McManaman & Appel, 1986) or *in vitro*

(Calof & Reichardt, 1984), preferably using an image-intensified video-camera (Fruns, Krieger & Sears, 1987). Such techniques have yielded clear evidence that the survival of labelled motoneurones *in vitro* is greatly enhanced by the presence of skeletal muscle or muscle-conditioned medium. This occurred whether or not a fluorescent-activated cell sorter (FACS IV) had been used, which enabled an estimated recovery of 21–26 % of the motoneurones in the lateral motor column of the chick (O'Brien & Fischbach, 1986*a*). Evidently, uninnervated myotubes can prevent the major phase of natural cell death from occurring on schedule *in vitro*. Interestingly, sorted motoneurones (i.e. identified ones which could be cultured in the absence of other neurones and of glia) degenerated in the second week in culture, even when plated on muscle, suggesting that myotubes may stop producing survival factors as they become innervated or as they age. The enhanced survival achieved with heterogeneous cultures, in the presence of muscle, suggests that during development the source of essential survival factors probably shifts from muscle to interneurones. In support of this O'Brien & Fischbach (1986*b*) show that in addition to regulating the morphology of motoneurones in culture the presence of the interneurones also governs the glutamate sensitivity of motoneurone neurites. Such a shift in dependence could explain the relative difficulty in demonstrating cell death in *adult* motoneurones, even with double lesioning of their axons (see Romanes, 1946), once the vulnerable early postnatal period has passed (Schmalbruch, 1984; Kashihara, Kuno & Miyata, 1987).

The isolation and identification of factors which promote neurite extension and survival of motoneurone-enriched cultures has been pursued by several workers, notably Smith *et al.* (1986), Flanigan, Dickson & Walsh (1985) and most recently by Dohrmann, Edgar, Sendtner & Thoenen (1986). The latter describe a soluble form of laminin which, presented alone, had no effect on survival of motoneurone-enriched cultures of chick spinal cord but enhanced survival when bound to polyornithine-coated substrates. The relationship between this factor and the three proteins described by Smith *et al.* (1986) has yet to be clarified, but one of these, a 12–15 kDa peptide, selectively augmented motoneurone survival and the capacity for acetylcholine (ACh) synthesis. Another factor (55 kDa) did not enhance cell survival but did promote neurite growth and ACh synthesis. This action resembled that of the 56 kDa factor derived from denervated muscle which promotes neurite sprouting and enhances survival of spinal cord neurones in culture (Gurney & Apatoff, 1984).

SPROUTING OF MOTOR NERVE TERMINALS

Normally, terminal sprouting probably occurs continuously to maintain the integrity of the neuromuscular junction (Barker & Ip, 1966). When motoneurones are lost through disease, as in MND or poliomyelitis (see Stalberg, Hilton-Brown & Rydin, 1986), or when motor axons are traumatically injured, the remaining axons sprout to reinnervate the denervated muscle fibres (for a review, see Brown, Holland & Hopkins, 1981; or Edds, 1953 for the earlier literature). This process is enhanced

by procedures which interfere with cholinergic transmission and result in denervation-like changes, such as following botulinum toxin (Duchen & Strich, 1968) or, secondarily, in response to impulse blockade by tetrodotoxin (Pestronk, Drachman & Griffin, 1976; Brown & Ironton, 1977). Conversely, when denervation changes induced by botulinum are prevented, by implantation of a foreign but unpoisoned nerve, then the terminal outgrowth that normally occurs is inhibited (Duchen & Tonge, 1977). Daily injections of the postsynaptic blocking agent α-bungarotoxin also cause ultraterminal sprouting in mouse soleus muscle, according to Holland & Brown (1980), although this was not confirmed by the finding that such blocking of postsynaptic receptors actually inhibited botulinum-induced sprouting in rat soleus muscle (Pestronk & Drachman, 1978). However, Pestronk & Drachman (1985) repeated these studies, using an osmotic pump to provide a continuous supply of α-bungarotoxin to the endplate regions. This caused the expected functional denervation, but by itself induced little or no terminal growth, in either the mouse or the rat (as estimated by nerve terminal branching, endplate length and ultraterminal sprouting). It did, however, inhibit botulinum-induced sprouting, thus confirming the suggestion that the extrajunctional acetylcholine receptors play an important role in mediating the muscle-regulated outgrowth of motor nerve terminals.

Another important aspect of sprouting in relation to MND is its age dependence. In partly denervated, adult rat soleus muscles, the surviving motor units sprout to produce twitch tensions of to four times their normal size, whereas in similar experiments on immature animals the motor units actually become smaller, presumably as a result of the normal processes which eliminate polyneuronal innervation (Brown, Jansen & Van Essen, 1976; Thompson & Jansen, 1977). Similar, but less dramatic, changes occur in rat lumbrical muscles (Betz, Caldwell & Ribchester, 1980). Re-examining this problem, Fladby & Jansen (1987) conclude that factors intrinsic to the motoneurone govern the maximum number of terminals that it can sustain (see Harris & McCaig, 1984), while competition regulates which muscle fibres are innervated so as to determine the motor unit territories. Of considerable interest, in relation to adult MND, is the finding that the motoneurones of senescent rats (24 months) have a greatly reduced capacity to sprout in response to botulinum toxin or to regenerate axons following a crush (Pestronk, Drachman & Griffin, 1980).

The sprouting of mature nerve fibres *in vivo*, following intoxication with botulinum, has been used to assay serum factors from patients with MND that might exert inhibitory effects on nerve growth (Gurney, 1984). Thus Gurney *et al.* (1984) have described a serum antibody in patients with MND that both inhibited the botulinum-induced sprouting in mouse skeletal muscle and reacted with the 56 kDa antigen derived from denervated muscle (Gurney & Apatoff, 1984). They emphasize that it is unknown whether this antibody is responsible for the destruction of motoneurones seen in MND, but they conclude that sprouting and trophic maintenance of the motoneurone may be related, through the action of a hypothetical single muscle-derived factor which at low concentrations provides trophic support and at high concentrations induces terminal sprouting (see Brown *et al.* 1981; Slack, Hopkins &

Pockett, 1983). Analogous propositions, including factors that would inhibit terminal growth or the production of target-derived factors, have been advanced for the regulation of sprouting in sensory nerve fibres (Diamond, 1979).

MOTONEURONE DEATH DUE TO AXOTOMY

While natural motoneurone death is completed before birth, sectioning of the sciatic nerve in the early postnatal period is known to cause massive degeneration of lumbar motoneurones (Schmalbruch, 1984). This accords with the classical literature which recognized that cell death is most prominent in young animals, as detected by cell loss (Gudden, 1870) or the chromatolytic appearance of diffuse basophilia, eccentric nucleus and swollen cell body in Nissl-stained tissue. However, in the later literature motoneurone death was not a feature when nerves were sectioned near to the muscle (Bennett & Pettigrew, 1974; Brown *et al.* 1976). This important question has been re-examined in rats by Kashihara *et al.* (1987) whose work demonstrates that the majority of medial gastrocnemius motoneurones, axotomized 4 days after birth, survived up to 2 weeks without target contact (see O'Brien & Fischbach, 1986*a*) but thereafter died if peripheral reinnervation was prevented. Hence, the target-dependence of motoneurone survival extends from the embryonic phase into the early postnatal period. Curiously, however, forelimb amputation by ligation in 12- to 24-h neonatal rats led to no reduction in the number of ventral root myelinated fibres (estimated 4 weeks later), indicating no substantial loss of motoneurones (Heath, Coggeshall & Hulsebosch, 1986). In the absence of explicit information as to the fate and growth of the terminals in the distal nerve stumps, it is difficult to relate this to the results discussed above.

The position concerning the extent of motoneurone death following axotomy in the *adult* is confused, for, in the early literature, chromatolysis observed in the light microscope represented a degenerative response which proceeded to cell death whereas, currently, it represents a regenerative one aimed at reconstituting the axon (see Kreutzberg, 1982; Grafstein & McQuarrie, 1978). A morphometric analysis of permanent peripheral axotomy in the adult cat, when motoneurones were denied access to muscle-dependent signals, showed no significant reduction in the number of motoneurones in the lateral cell columns (Carlson, Lais & Dyck, 1979), as was the case for sciatic nerve section in older rats (Schmalbruch, 1984). Nevertheless, differences in motoneurone types, age and species confound this field and further work is necessary to define the extent of cell death following axotomy of young, adult and ageing motoneurones.

The above brief review of natural motoneurone death, sprouting and the effects of axotomy reveal some elements of the natural history of the hypothesis advanced by Appel (1981) concerning MND and, more broadly, other degenerative diseases of the ageing nervous system. Its essence is that MND reflects a slow and progressive loss of extrinsic, muscle-derived neurotrophic factor(s) that regulates the normal, intrinsically determined, age-related deterioration of the motoneurone. The extrinsic factor would exert its action at the cell body level following uptake in the presynaptic

terminals and retrograde axonal transport. Much of the supporting evidence and subsidiary concepts derive from the study of developing motoneurones and their synaptic contacts by *in vivo* and *in vitro* experiments and this highlights the need for complementary studies on the fully differentiated adult as well as ageing nervous systems. Indeed, Brown (1984) has concluded that sprouting of motor nerves in adult muscles 'represents a replay – modified by the effects of age and size – of embryonic and neonatal events'.

If *lack* of a muscle neurotrophic signal is the etiological agent, then it follows that after a period of inadequate maintenance of the terminals each would retract, being deprived of its growth-maintaining stimulus. At this stage the motoneurone somata might be expected to display a sequence of changes resembling chromatolysis due to acute axotomy. However, only a small number of chromatolytic motoneurones die as part of the axon response in adult animals, as reviewed above, even with two consecutive lesions of limb peripheral nerves (Romanes, 1951), as also was the case in comparable studies on cat intercostal motoneurones in work done in this laboratory (Coffey, 1971). It cannot, therefore, be assumed that chromatolysis *per se* is a necessary intermediate morphological stage in the events leading to cell death in MND. Although chromatolytic motoneurones are present in the spinal cords of patients who have died from MND, they can be distinguished from the motoneurones showing argentophilic spheroids containing neurofilaments, fragments of rough endoplasmic reticulum (rER) and microtubules; there are also accumulations of dense granular material resembling that of Bunina bodies, which are also present, as described by Hirano (1984). Elsewhere, we have likened these appearances more to those that result from the primary inhibition of protein synthesis that occurs when motoneurones (intercostal) are intoxicated by diphtheria toxin retrogradely transported from a peripheral site of uptake following intramuscular injection or application to the central end of a divided intercostal nerve (Sears, Pullen & Johnson, 1984). This inhibition is highly specific since the toxin inhibits protein synthesis by inactivating EF2, the elongation factor essential to the movements of ribosomes along mRNA in eucaryotic cells (Pappenheimer, 1982). Interest in the use of such toxins has heightened since the discovery by Lee & Iglewski (1984) of a cytosolic enzyme which recognizes the same modified amino acid, 2-[3-carboxy-amido-3-(trimethylammonio)propyl]-histidine (dipthamide), on EF2, as utilized by diphtheria toxin, to inactivate EF2 by ADP-ribosylation (Ueda & Hayaishi, 1985) and thus to provide an important normal mechanism for controlling protein synthesis at the translational level. Appel, Glenn-Smith, Vaca & McManaman (1984) have further supposed that it is the depletion of neurotrophic hormone over many years that could account for the different morphological features of MND and those resulting from axotomy. In this context, a search of the literature reveals one careful morphometric analysis of the lateral column motoneurones in two adult humans, aged 54 and 60, who died after 4·5 and 9 years, respectively, following amputation of lower limb and in whom a substantial loss of motoneurones was detected (Kawamura & Dyck, 1979). Both the age of the animal and the duration of the axotomy are therefore factors that will need more consideration in future animal-

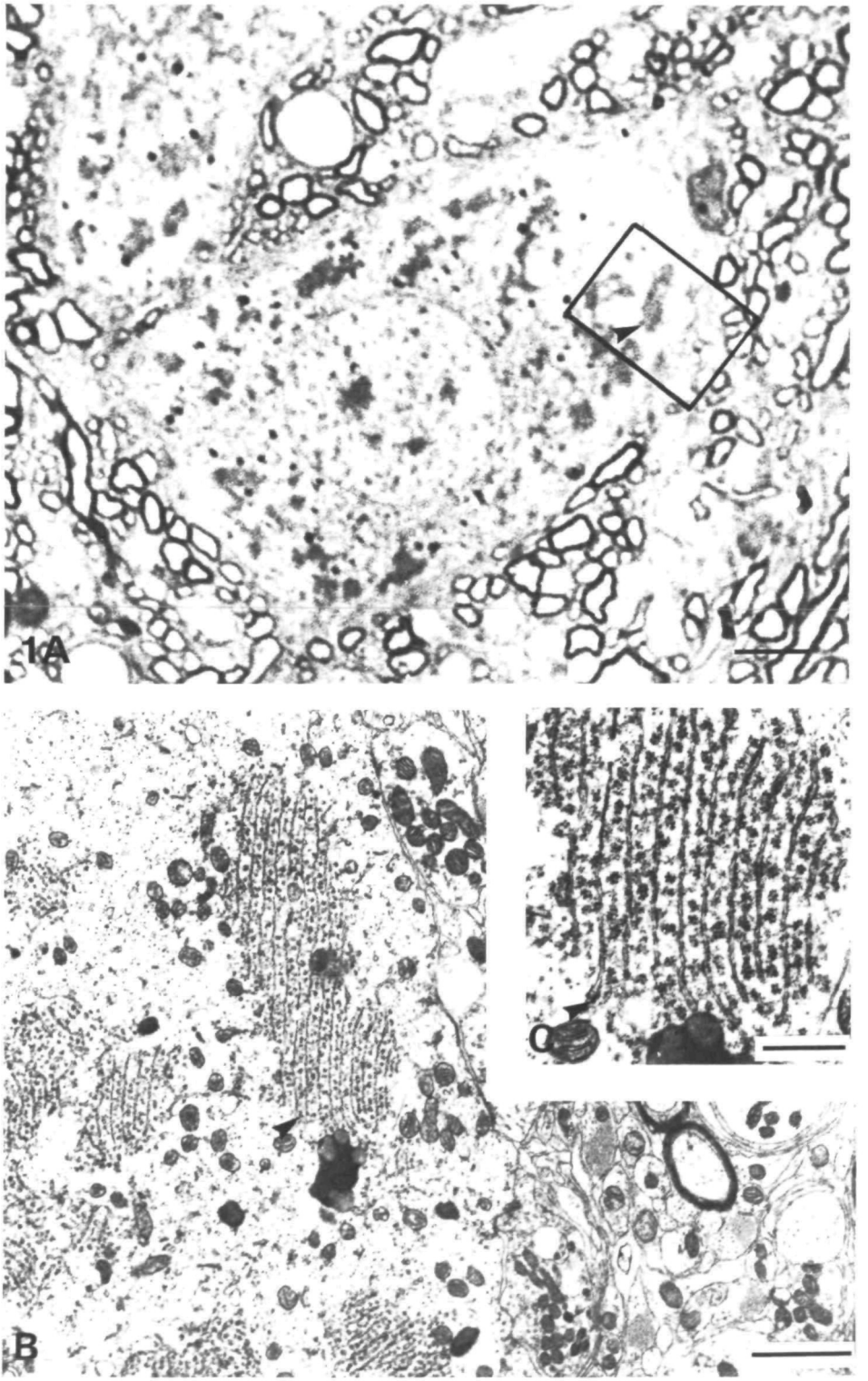
based experiments that explore Appel's hypothesis. Our own experiments on adult – though not aged – cats have a different natural history, being based originally on our need better to understand the factors governing the size of the discrete Nissl bodies that are topographically associated with the C-type synapses on intercostal (and other) motoneurones. These synapses proliferated in number and size in intercostal motoneurones which were caudal to a chronic hemisection of the cat spinal cord (Pullen & Sears, 1983); being of segmental, interneuronal origin they are possible candidates for the recovery of respiratory motoneurone activity in such animals (Kirkwood, Sears & Westgaard, 1984). We therefore set out to explore ultrastructurally the long-term effects of axotomy, with or without target influences, on the Nissl bodies at large since it is well known that axotomy causes a dispersal of the polyribosomes and rER during chromatolysis (Lieberman, 1971; Grafstein & McQuarrie, 1978).

CHRONIC AND REVERSIBLE AXOTOMY OF INTERCOSTAL MOTONEURONES

Johnson, Pullen & Sears (1985) have studied the effects of 'reversible' axotomy induced by intercostal nerve crush and compared them with those due to 'permanent' axotomy induced by nerve section with ligation of the proximal stump, to promote neuroma formation, and removal of the distal stump, to minimize regeneration. To identify normal and axotomized motoneurones, they were labelled by retrograde transport of HRP in an experiment 24 h before the animal was killed; this procedure allowed the identification of axotomized motoneurones independently from their morphological appearance in the light microscope. As the results show, light microscopy (LM) does not detect that motoneurones have failed to regenerate to their muscle targets. HRP-labelled motoneurones were identified by LM in transilluminated 70 μm thick plastic sections; the same motoneurones were identified in 0·5 μm thick sections and subsequently analysed in ultra-thin sections by electron microscopy (EM).

Using classical criteria, Nissl bodies were identified by LM as irregular patches of cytoplasmic basophilia (Fig. 1A) and shown in adjacent ultra-thin sections to consist of highly ordered stacks of rER and linear arrays of polyribosomes between individual lamellae (Fig. 1B); at still higher magnification the single ribosomes studding the ER can be distinguished (Fig. 1C). The designation 'lamellae-associated polyribosomes' (see Pullen & Sears, 1983) is used to distinguish them from polyribosomes elsewhere in the cytoplasm and the single ribosomes on the ER. In normal intercostal motoneurones of the adult cat, the great majority of polyribosomes appear to be lamellae-associated. In the light microscope apparently 'normal' Nissl bodies were present 4 days after reversible axotomy, but in the electron

Fig. 1. (A) Oil-immersion light micrograph of a normal motoneurone at T9. Within the box, a large, prominent Nissl body (arrowhead) can be seen. 0·5 μm thick sections stained with Toluidine blue. Scale bar, 10 μm. (B) Electron micrograph of the area enclosed by the box in A. The arrowheads in A, B and C identify the same Nissl body. Scale bar, 1·0 μm. (C) High-magnification electron micrograph of the same Nissl body. Note the orderly arrangement of rough endoplasmic reticulum and polyribosomes. Scale bar, 0·5 μm.


1A
B

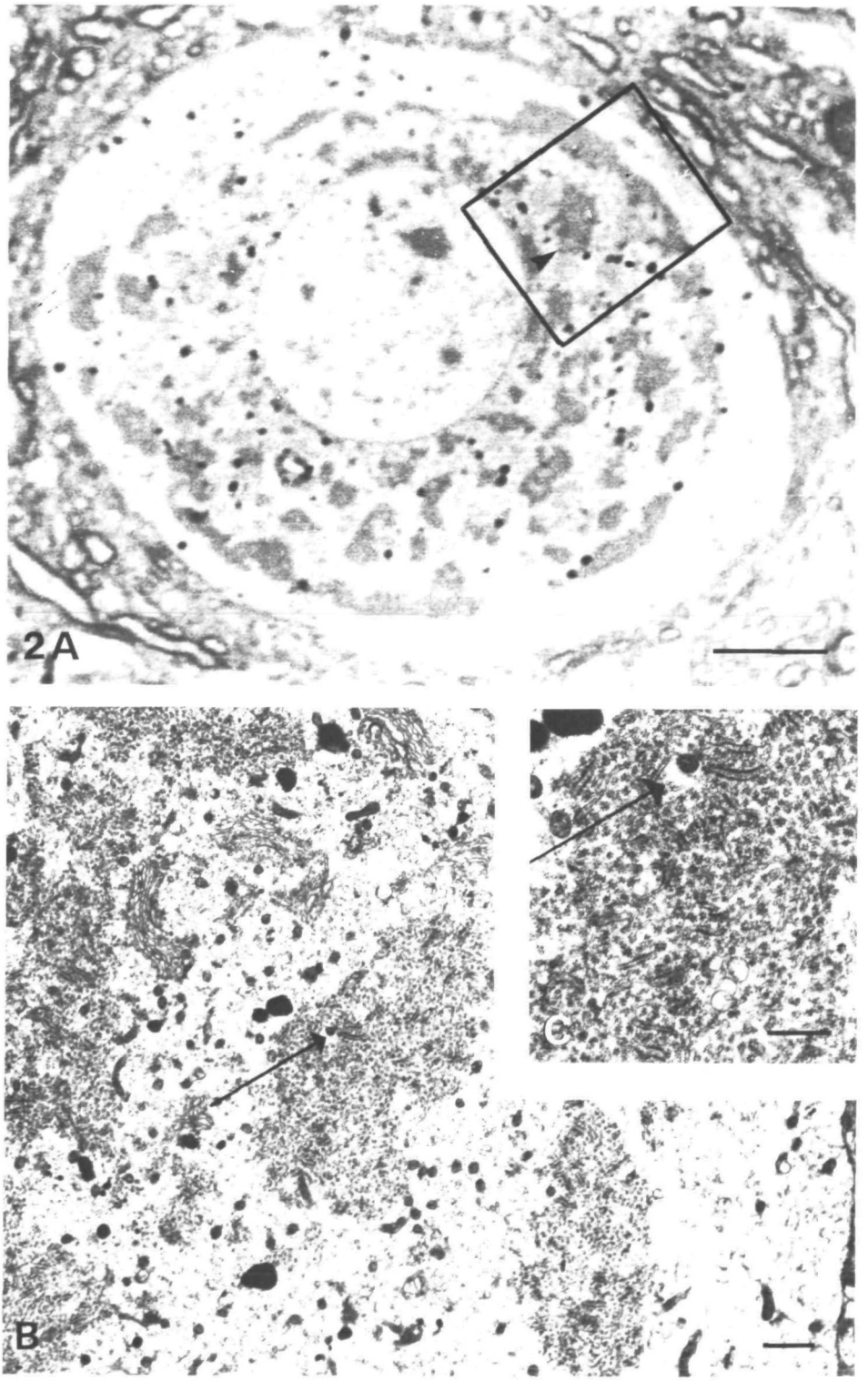
microscope these had already lost their orderly infrastructure: they lacked the parallel stacks of rER and, instead, were composed of polyribosomal aggregates within which only short fragments of rER were present. By 4 days the changes in the 'permanently' axotomized motoneurones were much further advanced, since diffuse basophilia was seen in the light microscope, due to Nissl fragmentation and dispersal, while in the electron microscope the Nissl fragments consisted entirely of small polyribosomal aggregates devoid of lamellae: changes that applied equally to the Nissl bodies associated with the C-type synapses. Chromatolysis was seen in the light microscope to be fully developed between 8 and 33 days after axotomy, but during this period the two types of axotomized motoneurones were indistinguishable ultrastructurally.

Between 64 and 208 days, some motoneurones remained chromatolytic following axotomy while others had reformed apparently normal Nissl bodies (Fig. 2A). Indeed, only our use of HRP as an independent label allowed them to be distinguished from normal motoneurones, labelled or not. However, ultrastructural examination of the same and other Nissl bodies established that in the case of permanent axotomy they were composed of dense clusters of polyribosomal aggregates (Fig. 2B) interspersed with only short fragments of randomly oriented rEr (Fig. 2C). In contrast, with reversible axotomy (in which regeneration of the nerve to the peripheral target had been permitted), the Nissl bodies regained their normal, highly ordered infrastructure, indistinguishable from that illustrated in Fig. 1. Examination of the distal nerve stumps confirmed that axonal regeneration had, or had not, occurred following reversible or permanent axotomy, respectively.

In the original group of experiments described by Johnson *et al.* (1985) we concluded that the 'normal' infrastructure of a Nissl body (i.e. one comprised of the linear stacks of rER) is dependent for its integrity on either the nerve terminal itself, with the formation of functioning synapses, or the muscle which it innervates, but not the process of axonal regeneration *per se*. Thus, we showed that motoneurones which had been permanently axotomized for 49 days subsequently developed normal Nissl bodies when the neuroma was removed and the central nerve stump was allowed to regenerate for 64 days into the distal stump of the internal intercostal nerve of the same segment. No normal Nissl bodies were present in control, permanently axotomized motoneurones in the adjacent-but-one segment.

To distinguish between the essentially pre- and postsynaptic neuromuscular source of the signal that enables reconstitution of normal Nissl bodies, we have now used the paradigm based on the failure of an innervated muscle to accept foreign innervation (Jansen, Lomo, Nicholaysen & Westgaard, 1973). The external intercostal nerves were sectioned in two non-adjacent segments. In one, the cut end of the

Fig. 2. (A) Oil-immersion light micrograph of an HRP-labelled motoneurone at T9, 64 days following permanent axotomy. Within the box, a large, prominent Nissl body (arrowhead) can be seen. 0·5 μm thick sections stained with Toluidine blue. Scale bar, 10 μm. (B) Electron micrograph of the area enclosed by the box in A. The arrows in A, B and C identify the same Nissl body. Scale bar 1·0 μm. (C) High-magnification electron micrograph of the same Nissl body. Note the lack of ultrastructural orderliness. Scale bar, 0·5 μm.


2A
B
C

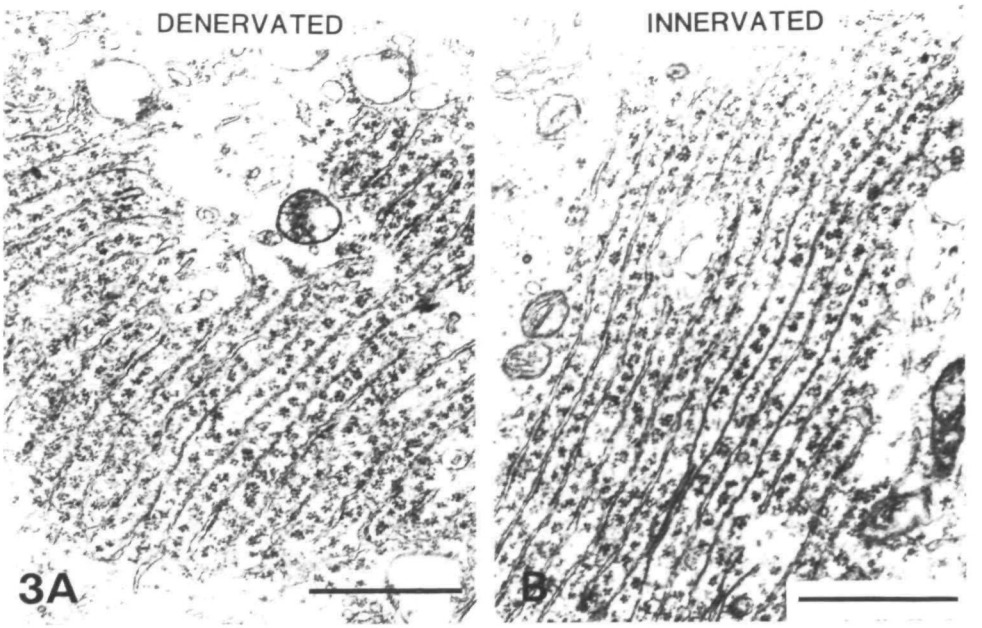


Fig. 3. High-magnification electron micrographs of representative Nissl bodies in external intercostal motoneurones of two adjacent-but-one segments. The motoneurones were axotomized by sectioning the external intercostal nerves. The cut central end of each nerve was inserted into the external intercostal muscle (host) of the adjacent segment. In A the innervation of the host segment was intact, whereas in B the host muscle was denervated by sectioning the external intercostal nerve at its origin from the ventral spinal ramus. Scale bars, 1·0 μm.

central stump was inserted into the external intercostal muscle of the adjacent segment whose innervation was left intact. The external intercostal nerve from the second segment was inserted into its adjacent external intercostal muscle which, in this case, was completely denervated by sectioning the external intercostal nerve at its origin from the ventral spinal ramus (Sears, 1964). Control experiments in other animals had shown that with these procedures any CO_2-driven inspiratory-phased electromyographic activity recorded from these muscles could be attributed with confidence either to the intact, normal innervation or to the foreign nerve when its host muscle had been denervated. Fig. 3A,B shows that in either case there was a full restoration in the normal ultrastructural appearances of the Nissl bodies.

Whatever the nature of the muscle-dependent signal(s), it would appear to control two separate processes that reflect the relative stability of the motoneurone–muscle relationships in the adult cat. First, there is the need for synthesis of the additional endoplasmic reticulum (ER) with its various receptors (ribophorins, signal recognition proteins etc.) that allow binding of polyribosomes and their associated mRNAs for the cotranslational insertion and vectorial discharge of membrane and secretory proteins (see Sabatini, Kreibich & Morimoto, 1982; Wickner & Lodish, 1985). Second, the highly ordered structure of the normal Nissl body presumably must depend on appropriate cytoskeletal elements to maintain the parallel arrays of

ER lamellae. Cross-linking neurofilaments could provide this stability. This would require the synthesis of a protein analogous to the high molecular weight polypeptide (H, 195 kDa) whose delayed appearance in the developing axon (rabbit optic nerve) marks the transition from a plastic to a stable state of axonal growth (Willard *et al.* 1984) during development.

The nature of the signal itself is unknown, but is most likely to be a muscle-derived diffusible factor taken up by the motor nerve terminals and retrogradely transported to the cell body to regulate the processes described above. Alternatively, a muscle-derived extracellular matrix or surface membrane molecule encountered by the motor nerve terminal could modulate an intrinsic signal molecule in the terminal that would be retrogradely transported to the cell body (see Ingoglia, Zanakis & Chakroborty, 1984).

GENERAL REMARKS

The intercostal motoneurones of the thoracic spinal cord are eminently suited for the kind of chronic experiments described above. In particular, the multisegmental organization (with its repeated distribution of inspiratory and expiratory motoneurones and their respective muscle layers within), allows several experimental as well as control procedures in the same animal, thus minimizing experimental variability. These factors, coupled with our use of HRP to provide an independent label of axotomized motoneurones, have permitted a clearer picture than hitherto to emerge of the target-dependence of the normal ultrastructure of the Nissl bodies. It is now clear that the presence in light micrographs of Nissl bodies depends solely on the basophilia associated with aggregated polyribosomes and not on how the polyribosomes and other structures are spatially organized within the aggregates. The light microscope cannot detect whether such aggregations are accompanied by the highly organized arrays of ER lamellae that characterize the majority of cytosolic Nissl bodies in normal motoneurones. That the reformation of the normal orderly structure can be achieved without *functional* reinnervation has wider implications. It suggests mechanisms for the maintenance of motoneurone integrity under a wide variety of circumstances relating to the dynamic processes discussed earlier of motoneurone death during embryogenesis, relating to the shaping of motor unit territories during the reduction of polyneuronal innervation, and relating to the process of sprouting in response to partial denervation.

With regard to MND itself, the results can be said to contribute to knowledge of the basic mechanisms through which the etiological agents might operate to cause the disease. Appel's hypothesis, and this review, highlight the need for more research on nervous systems comparable in age to those of patients suffering from this disease.

I wish to thank A. Pullen and I. Johnson for their major contribution to the work cited in this article. This research is supported by grants from the Medical Research Council, The Brain Research Trust and The International Spinal Research Trust.

REFERENCES

APPEL, S. H. (1981). A unifying hypothesis for the cause of Amyotrophic Lateral Sclerosis, Parkinsonism, and Alzheimer disease. *Ann. Neurol.* **10**, 499–505.

APPEL, S. H., GLENN-SMITH, R., VACA, K. & MCMANAMAN, J. (1984). Neurotrophic factors and the pathogenesis of amyotrophic lateral sclerosis. In *Neuromuscular Disease* (ed. G. Serratrice, D. Cros, C. Desnuelle, J. L. Gastaut, J. F. Pelissier, J. Pouget & A. Schiano), pp. 347–359. New York: Raven Press.

BARKER, D. & IP, M. C. (1966). Sprouting and degeneration of mammalian axons in normal and de-afferented skeletal muscle. *Proc. R. Soc. Ser.* B **163**, 538–544.

BENNETT, M. R., LAI, K. & NURCOMBE, V. (1980). Identification of embryonic motoneurones *in vitro*: Their survival is dependent on skeletal muscle. *Brain Res.* **190**, 537–542.

BENNETT, M. R. & PETTIGREW, A. G. (1974). The formation of synapses in reinnervated and cross-reinnervated striated muscle during development. *J. Physiol., Lond.* **241**, 547–573.

BETZ, W. J., CALDWELL, J. H. & RIBCHESTER, R. R. (1980). The effects of partial denervation at birth on the development of muscle fibres and motor units in rat lumbrical muscles. *J. Physiol., Lond.* **303**, 265–279.

BROWN, M. C. (1984). Sprouting of motor nerves in adult muscles: a recapitulation of ontogeny. *Trends Neurosci.* **7**, 10–14.

BROWN, M. C., HOLLAND, R. L. & HOPKINS, W. G. (1981). Motor nerve sprouting. *A. Rev. Neurosci.* **4**, 17–42.

BROWN, M. C. & IRONTON, R. (1977). Motor nerve sprouting induced by prolonged tetrodotoxin block of nerve action potential. *Nature, Lond.* **265**, 459–461.

BROWN, M. C., JANSEN, J. K. S. & VAN ESSEN, D. (1976). Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. *J. Physiol., Lond.* **261**, 387–422.

CALOF, A. L. & REICHARDT, L. F. (1984). Motoneurons purified by cell sorting respond to two distinct activities in myotube-conditioned medium. *Devl Biol.* **106**, 194–210.

CARLSON, J., LAIS, A. C. & DYCK, P. J. (1979). Axonal atrophy from persistent peripheral axotomy in adult cat. *J. Neuropath. exp. Neurol.* **38**, 579–585.

COFFEY, G. L. (1971). The distribution of respiratory motoneurones in the thoracic spinal cord of the cat. Thesis, University of London.

DIAMOND, J. (1979). The regulation of nerve sprouting by extrinsic factors. In *The Neurosciences*: 4th Study Program (ed. F. O. Schmidt & F. F. Worden), pp. 937–955. Cambridge, MA: MIT Press.

DORHMANN, U., EDGAR, D., SENDTNER, M. & THOENEN, H. (1986). Muscle-derived trophic factors that support survival and promote fibre outgrowth from embryonic chick motoneurones in culture. *Devl Biol.* **118**, 209–221.

DUCHEN, L. W. & STRICH, S. J. (1968). The effects of botulinum toxin on the pattern of innervation of skeletal muscle in the mouse. *Q. J. exp. Physiol.* **53**, 84–89.

DUCHEN, L. W. & TONGE, D. A. (1977). The effects of implantation of a foreign nerve on axonal sprouting usually achieved by botulinum toxin in skeletal muscles of the mouse. *J. Anat.* **124**, 205–215.

EDDS, M. V. (1953). Collateral nerve regeneration. *Q. Rev. Biol.* **28**, 260–276.

FLADBY, T. (1987). Postnatal loss of synaptic terminals in the normal mouse soleus muscle. *Acta physiol. scand.* **129**, 229–238.

FLADBY, T. & JANSEN, J. K. S. (1987). Postnatal loss of synaptic terminals in the partially denervated mouse soleus muscle. *Acta physiol. scand.* **129**, 239–246.

FLANIGAN, T. P., DICKSON, J. G. & WALSH, F. S. (1985). Cell survival characteristics and choline acetyl transferase activity in motor neurone-enriched cultures from chick embryo spinal cord. *J. Neurochem.* **45**, 1323–1326.

FRUNS, C., KRIEGER, C. & SEARS, T. A. (1987). Electrical activity of identified embryonic rat motoneurones in culture. *2nd IBRO Congress, Budapest.* (Abstract, in press).

GRAFSTEIN, B. & MCQUARRIE, I. G. (1978). Role of the nerve cell body in axonal regeneration. In *Neuronal Plasticity* (ed. C. W. Cotman), pp. 155–195. New York: Raven Press.

GUDDEN, B. (1870). Experimentaluntersuchungen uber das peripherische und centrale Nervensystem. *Arch. Psychiat. Nervenkrankh.* **2**, 693–723.

GURNEY, M. E. (1984). Suppression of sprouting at the neuromuscular junction by immune sera. *Nature, Lond.* **307**, 546–548.

GURNEY, M. E. & APATOFF, B. (1984). Activity of a muscle-derived growth factor for spinal neurons *in vivo* and *in vitro*. *Soc. Neurosci. Abstr.* **10**, 1051.

GURNEY, M. E., BELTON, S. M., CASHMAN, N. & ANTEL, J. P. (1984). Inhibition of terminal axonal sprouting by serum from patients with amyotrophic lateral sclerosis. *New England J. Med.* **313**, 933–939.

HAMBURGER, V. (1977). The developmental history of the motor neuron. *Neurosci. Res. Prog. Bull.* **15** (Suppl.), 1–37.

HARRIS, A. J. & MCCAIG, C. D. (1984). Motoneuron death and motor unit size during embryonic development of the rat. *J. Neurosci.* **4**, 13–24.

HEATH, D. D., COGGESHALL, R. E. & HULSEBOSCH, C. E. (1986). Axon and neuron numbers after amputation in neonatal rats. *Expl Neurol.* **92**, 220–233.

HIRANO, A. (1984). The fine structure of motor neurone disease. In *Research Progress in Motor Neurone Disease* (ed. F. C. Rose), pp. 328–356. London: Pitman Press.

HOLLAND, R. L. & BROWN, M. C. (1980). Postsynaptic block can cause motor nerve terminal sprouting. *Science* **207**, 649–651.

HOLLYDAY, M. & HAMBURGER, V. (1976). Reduction of the naturally occurring motoneurone loss by enlargement of the periphery. *J. comp. Neurol.* **170**, 311–320.

INGOGLIA, N. A., ZANAKIS, M. F. & CHAKROBORTY, M. F. (1984). Transfer RNA-mediated postranslational aminoacylation of proteins in axons. In *Axonal Transport in Neuronal Growth and Regeneration, Advances in Neurochemistry*, vol. 6 (ed. J. S. Elam & P. Cancalon), pp. 119–136. New York: Plenum Press.

JANSEN, J. K. S., LOMO, T., NICHOLAYSEN, K. & WESTGAARD, R. H. (1973). Hyperinnervation of skeletal muscle fibres: dependence on muscle activity. *Science* **181**, 559–561.

JENQ, C. B., CHUNG, K. & COGGESHALL, R. E. (1986). Postnatal loss of axons in normal rat sciatic nerve. *J. comp. Neurol.* **244**, 445–450.

JOHNSON, I. P., PULLEN, A. H. & SEARS, T. A. (1985). Target dependence of Nissl body ultrastructure in cat thoracic motoneurones. *Neurosci. Letts* **61**, 201–205.

KASHIHARA, Y., KUNO, M. & MIYATA, Y. (1987). Cell death of axotomised motoneurones in neonatal rats and its prevention by peripheral reinnervation. *J. Physiol., Lond.* **386**, 135–148.

KAWAMURA, Y. & DYCK, P. J. (1981). Permanent axotomy by amputation results in loss of motor neurons in man. *J. Neuropath. exp. Neurol.* **40**, 658–666.

KIRKWOOD, P. A., SEARS, T. A. & WESTGAARD, R. H. (1984). Restoration of function in external intercostal motoneurones of the cat following partial central deafferentation. *J. Physiol., Lond.* **350**, 225–251.

KREUTZBERG, G. W. (1982). Acute neural reaction to injury. In *Repair and Regeneration of the Nervous System* (ed. J. G. Nicholls), pp. 57–69. Berlin: Springer-Verlag.

LANCE-JONES, C. (1982). Motoneuron cell death in the developing lumbar spinal cord of the mouse. *Devl Brain Res.* **4**, 473–479.

LANCE-JONES, C. & LANDMESSER, L. (1980). Motoneurone projection patterns in embryonic chick limbs following partial deletions of the spinal cord. *J. Physiol., Lond.* **302**, 559–580.

LEE, H. & IGLEWSKI, W. J. (1984). Cellular ADP-ribosyltransferase with the same mechanism of action as diphtheria toxin and *Pseudomonas* toxin A. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2703–2707.

LEIBERMAN, A. R. (1971). The axon reaction: a review of the principal features of perikaryal responses to axon injury. *Int. Rev. Neurobiol.* **14**, 49–124.

MCPHEETERS, M. & OKUN, L. M. (1980). Identification and isolation *in vitro* of presumptive motoneurones marked by retrograde transport of a new fluorescent tracer. *Soc. Neurosci. Abstr.* **6**, 247.

NURCOMBE, V., MCGRATH, P. A. & BENNETT, M. R. (1981). Postnatal death of motor neurons during the development of the brachial spinal cord of the rat. *Neurosci. Letts* **27**, 249–254.

O'BRIEN, R. J. & FISCHBACH, G. D. (1986*a*). Isolation of embryonic chick motoneurons and their survival *in vitro*. *J. Neurosci.* **11**, 3265–3274.

O'BRIEN, R. J. & FISCHBACH, G. D. (1986*b*). Modulation of embryonic chick motoneurone glutamate sensitivity by interneurones and agonists. *J. Neurosci.* **6**, 3290–3296.

OPPENHEIM, R. W. (1985). Naturally occurring cell death during neural development. *Trends Neurosci.* **8**, 487–493.

OPPENHEIM, R. W. (1986). The absence of significant postnatal neuronal death in the brachial and lumbar spinal cord of the rat. *J. comp. Neurol.* **246**, 181–286.

OPPENHEIM, R. W. & NUNEZ, R. (1982). Electrical stimulation of hindlimb increases neuronal cell death in chick embryo. *Nature, Lond.* **295**, 57–59.

PAPPENHEIMER, A. M. (1982). Mechanisms of cell injury by certain protein toxins. In *Neuronal–Glial Cell Interrelationships* (ed. T. A. Sears), pp. 229–250. *Life Science Research Report* **20**, *Dahlem Konferenzen*. Berlin: Springer-Verlag.

PESTRONK, A. & DRACHMAN, D. B. (1978). Motor nerve sprouting and acetylcholine receptors. *Science* **199**, 1223–1225.

PESTRONK, A. & DRACHMAN, D. B. (1985). Motor nerve terminal outgrowth and acetylcholine receptors: Inhibition of terminal outgrowth by α-Bungarotoxin and anti-acetylcholine receptor antibody. *J. Neurosci.* **5**, 751–758.

PESTRONK, A. & DRACHMAN, D. B. & GRIFFIN, J. W. (1976). Effect of muscle disuse on acetylcholine receptors. *Nature, Lond.* **260**, 352–353.

PESTRONK, A., DRACHMAN, D. B. & GRIFFIN, J. W. (1980). Effects of aging on nerve sprouting and regeneration. *Expl Neurol.* **70**, 65–82.

PITTMAN, R. H. & OPPENHEIM, R. W. (1978). Neuromuscular blockade increases motoneurone survival during normal cell death in the chick embryo. *Nature, Lond.* **271**, 364–366.

PULLEN, A. H. & SEARS, T. A. (1983). Trophism between C-type axon terminals and thoracic motoneurones in the cat. *J. Physiol., Lond.* **337**, 373–388.

ROMANES, G. J. (1946). Motor localization and the effects of nerve injury on the ventral horn cells of the spinal cord. *J. Anat.* **80**, 117–131.

ROMANES, G. J. (1951). The motor cell columns of the lumbosacral spinal cord of the cat. *J. comp. Neurol.* **94**, 313–363.

SABATINI, D. D., KREIBICH, G. & MORIMOTO, T. (1982). Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* **92**, 1–22.

SCHMALBRUCH, H. (1984). Motoneuron death after sciatic nerve section in newborn rats. *J. comp. Neurol.* **224**, 252–258.

SEARS, T. A. (1964). The fibre calibre spectra of sensory and motor fibres in the intercostal nerves of the cat. *J. Physiol., Lond.* **172**, 150–161.

SEARS, T. A. (1974). The afferent regulation of learnt movements. *Brain Res.* **71**, 465–473.

SEARS, T. A., PULLEN, A. H. & JOHNSON, I. P. (1984). New perspectives on motor neurone disease: Investigations on cat respiratory motoneurones following chronic partial central deafferentation or intoxication by diphtheria toxin. In *Research Progress in Motor Neurone Disease* (ed. F. C. Rose), pp. 141–162. London: Pitman.

SLACK, J. R., HOPKINS, W. G. & POCKETT, S. (1983). Evidence for a motor nerve growth factor. *Muscle & Nerve* **6**, 243–252.

SLACK, J. R. & POCKETT, S. (1982). Motor neurotrophic factor in denervated adult skeletal muscle. *Brain Res.* **247**, 138–140.

SMITH, R. G., VACA, K., MCMANAMAN, J. & APPEL, S. H. (1986). Selective effects of skeletal muscle extract fractions on motoneuron development *in vitro*. *J. Neurosci.* **6**, 439–447.

STALBERG, E., HILTON-BROWN, P. & RYDIN, E. (1986). Capacity of the motor neuron to alter its peripheral field. In *Recent Achievements in Restorative Neurology*, 2, *Progressive Neuromuscular Diseases* (ed. M. R. Dimitrijevic, B. A. Kakulus & G. Vrbova), pp. 237–253. Basel: Karger.

STRIHARI, T. & VROBOVA, G. (1980). Effects of neuromuscular blocking agents on the differentiation of nerve–muscle connections in slow and fast chick muscles. *Dev. Growth Differ.* **22**, 645–657.

TANAKA, H. & OBATA, K. (1983). Survival of HRP-labelled spinal motoneurons of chick embryo in tissue and cell cultures. *Devl Brain Res.* **9**, 390–395.

TANDAN, R. & BRADLEY, W. G. (1985*a*). Amyotrophic lateral sclerosis. Part 1. Clinical features pathology and ethical issues in management. *Ann. Neurol.* **18**, 271–280.

TANDAN, R. & BRADLEY, W. G. (1985*b*). Amyotrophic lateral sclerosis. Part 2. Etiopathogenesis. *Ann. Neurol.* **18**, 419–431.

THOMPSON, W. & JANSEN, J. K. S. (1977). The extent of sprouting of remaining motor units in partially denervated immature and adult skeletal rat soleus muscles. *Neurosci.* **2**, 523–535.

UEDA, K. & HAYAISHI, O. (1985). ADP-ribosylation. *A. Rev. Biochem.* **54**, 73–100.

WICKNER, T. W. & LODISH, H. F. (1985). Multiple mechanisms of protein insertion into and across membranes. *Science* **230**, 400–407.

WILLARD, M., PATE SKENE, J. H., SIMON, C., MEIRI, K., HIROKAWA, N. & GLICKSMAN, M. (1984). Regulation of axon growth and cytoskeletal development. In *Axonal Transport in Neuronal Growth and Regeneration, Advances in Neurochemistry*, vol. 6 (ed. J. S. Elam & P. Cancalon), pp. 171–183. New York: Plenum Press.