The effect of age on motor neurone death following axotomy in the mouse

M. M. POLLIN1,2,3, S. McHANWELL1 and C. R. SLATER1,2

1Division of Neurobiology, School of Neuroscience, The Medical School, Framlington Place, Newcastle-upon-Tyne, NE2 4HH, UK
2Muscular Dystrophy Group Research Laboratories, Newcastle General Hospital, Westgate Road, Newcastle-upon-Tyne, NE4 6BE, UK
3Present address Central Veterinary Laboratory, New Haw, Weybridge, Surrey KT15 3NB, UK

Summary

The ability of mouse motor neurones to survive axotomy during the first month of life was studied. The motor neurones that lie in the dorsolateral columns of spinal segments C7 and C8 and supply the flexor muscles of the forepaw were axotomized by cutting and removing part of the median and ulnar nerves above the elbow. The number and position of cell bodies with axons in these nerves were confirmed by retrograde labelling of the cut axons with horseradish peroxidase.

The ability of these neurones to survive axotomy varies with the age of the animal at the time of axotomy. When the axons are sectioned within the first four postnatal days, 80—90 % of the cell bodies will die, more than half of this death occurring in less than one week after axotomy. If the animals are one week old at the time the nerves are cut, a significantly smaller number (50 %) die (P=0.013), and the time-course of death is different, with eight to ten days elapsing before half the death has occurred. 40 % of the neurones will die if sectioned at two weeks of age, and it is not until four weeks of age that more than 90 % of the cells can survive axotomy.

We conclude, therefore, that the kinetics of motor neurone death, as well as the final extent of neuronal loss, are affected by the age at which the animal is axotomized.

Key words: spinal cord, median nerve, ulnar nerve, horseradish peroxidase, quantitation, mouse.

Introduction

Motor neurone death occurs naturally during early embryonic development, apparently as a result of failure in a competition between motor neurones for a limited supply of putative trophic factors derived from muscle. In rats and mice, although this naturally occurring death is largely complete at birth (Lance-Jones, 1982; Schmalbruch, 1984, 1988; Oppenheim, 1986), the survival of motor neurones continues to be dependent on support from the muscle for some time. Thus, if muscle nerves are cut soon after birth and reinnervation is prevented, a large number of the motor neurones die (Torvik, 1972; Kashihara et al. 1987; Luthman et al. 1988; Schmalbruch, 1988; Crews and Wigston, 1990). In contrast, extensive neuronal death does not result from cutting adult peripheral nerves (Kashihara et al. 1987; Schmalbruch, 1988). It seems, therefore, that the normal maturation of motor neurones involves a change from a state of dependence on muscle to one of relative independence.

Although the phenomenon of neonatal neuronal susceptibility to axonal damage has been extensively documented, the precise time course of this aspect of neuronal maturation remains unclear. Many discrepancies in the literature can be accounted for by the differing developmental age of the various species at birth, but opinions also vary regarding the extent of cell death at a given age within a single species. For example, Luthman et al. (1988) reported that crushing rat sciatic nerve at 7 days of age led to an 80 % loss of motor neurones, as assessed morphologically, but Lowrie et al. (1982) demonstrated no significant cell loss and nearly complete reinnervation of muscle, following sciatic nerve crush in rats 5–6 days old. The time taken for susceptible neurones to die is also unclear; Kashihara et al. (1987) found that after cutting at 4 days of age in rats, motor neurones survived for almost 2 weeks before the onset of cell death. Romanes (1946), on the other hand, showed that cutting the nerve at 3–5 days of age in mice led to a 20–30 % deficit in motor neurone number within 6 days. (This author gives no data for rats, but states that they are similar to mice.) Romanes' work on mice also indicates that nerves to distal muscles are more susceptible to sectioning than nerves to proximal muscles, a phenomenon that may be attributable to the earlier differentiation of motor columns for the proximal musculature, or to the proximity of the lesion to the cell body (Lieberman, 1974).

In addition to the age and species of the animal, and the nature and site of the lesion, the specific neuronal population involved may also affect the response to axotomy. For example, it is generally agreed that most spinal motor neurones are capable of surviving disconnection by 4 weeks of age in several species, but in rats...
one third of hypoglossal neurones are lost following section of the nerve in adults (Snider and Thanedar, 1989).

As a result of the varied conditions of previous studies, it is difficult to assemble, from the literature, an accurate account of both the extent and kinetics of cell death in any single species. The present study was undertaken to provide such an account for a single population of neurones in relation to a specific lesion in mice during the first postnatal month. The information gained will provide a basis for subsequent studies of factors influencing the survival of immature motor neurones.

Materials and methods

Animals

48 CD-1 mice were used. These mice were bred in the Muscular Dystrophy Group Research Laboratories, New-castle-upon-Tyne, from breeding stock purchased from Charles River UK Ltd, Margate, Kent.

Choice of neuronal population

The flexor muscles of the forepaw are innervated by a group of motor neurones whose cell bodies lie in the dorsolateral columns of the ventral horns of spinal segments C5–T1—predominantly C7 and C8 (Parry and Falconer, 1986; Pollin et al. 1990). The axons of these neurones emerge from the brachial plexus as a common trunk, divide to form the median and ulnar nerves (Greene, 1968), which lie alongside the brachial artery and vein in the upper arm, and are readily accessible to surgical intervention in that region.

Only the ability to grip is lost as a result of sectioning these nerves—weight-bearing, locomotion, feeding and grooming all remain unaffected.

Surgical procedure

The choice of anaesthetic was determined by the age of the mouse; ether (M & B) was used for neonates, methanefourane (Metofane; C-Vet) for mice 4 days old, and halothane (Fluothane, ICI) and nitrous oxide for animals 1 week of age or older.

At all ages, the animals were anaesthetised and placed on a Harvard homeothermic blanket. The median and ulnar nerves were exposed in the right arm via median approach, and the nerves cut just below the point at which they emerge from beneath the teres major muscle. This was 6 mm from the spinal cord in neonates, 10 mm in animals 1 week of age and 13 mm in 4-week-old animals. In each case, the lesion was half (50±1 3%, n=3) the distance from the spinal cord to the wrist. At least 2 mm of the distal stump (or more, where the size of the animal allowed) was removed to prevent reinnervation. The skin incision was closed with Mersilk 6/0 sutures.

Age of animals and survival periods

Fifteen animals were axotomized within 24h of birth, and allowed to survive for either 3, 5, 9 or 28 days.

Fifteen animals were axotomized at 4 days of age (i.e. 4 days from birth date), and allowed to survive for either 6, 12, 17, 22 or 28 days.

Four animals were axotomized at 7 days of age, and allowed to survive for 7, 14 or 28 days.

Three animals were axotomized at 14 days of age, and at 28 days of age, and allowed to survive for 28 days.

At each of these time points three animals were examined, with the exception of the neonatal axotomy 9-day and 28-day survival points, when four and five animals, respectively, were used.

Dissection of cord and section cutting

Each mouse was anaesthetised with pentobarbitone (Sagatal; RMB Animal Health Ltd) administered intraperitoneally, the left ventricle cannulated and the animal killed by perfusion with 0.9% saline (30 ml) and fixing with a mixture containing 1.25% glutaraldehyde and 1% paraformaldehyde (final concentrations) in 0.1 M phosphate buffer, pH 7.4, administered continuously for 30 min at a rate of 2 ml min⁻¹ by an infusion pump. The cervical spinal cord was exposed by dorsal laminectomy, each spinal segment identified by the caudal border of its corresponding nerve root, and a small hole made at this point on the left side of segments C4 to T1 using a fine glass probe (McHanwell and Biscoe, 1981). The spinal cord from C3 to T2 was removed and placed in fixative for at least a further 24h. It was then placed in 30% sucrose in 0.1 M phosphate buffer overnight prior to sectioning. Serial 40 μm horizontal sections were cut using a freezing microtome, mounted on gelatine-coated slides and air-dried for at least 60 min before being stained with galloycyanine (Einarson, 1932) overnight at room temperature.

Labelling of axotomized motor neurones using retrogradely transported horseradish peroxidase

The median and ulnar nerves were exposed, as described above, in three 3-month-old mice. Each nerve was cut above the elbow, the proximal stumps dissected free from under-lying structures and the cut ends placed in a small cup containing 30 μl of 30% horseradish peroxidase (HRP) (Boehringer) made up in 0.9% saline containing 2% lysophosphatidylcholine (Sigma), for 15 min (Hardman and Brown, 1985).

24h later, each mouse was anaesthetised with pentobarbitone and killed by perfusion fixation (as described above). Immediately after fixation, the spinal segments were identified and the cervical cord dissected and placed in 30% sucrose on 0.1 M phosphate buffer overnight. Serial 40 μm frozen horizontal sections were mounted on gelatinised slides and the HRP visualised using tetramethylbenzidine (TMB) according to the method of Mesulam (1978), as described fully in Pollin et al. (1990). The number of HRP-labelled cell bodies in each segment of each section was counted, then the sections stained with galloycyanine (see below) for total cell counts to be made.

Counting procedures

The majority (74.2%) of HRP-labelled neurones were found in C7 and C8 (Fig. 1), and all other counting was confined to those segments.

The large neuronal perikarya in each segment were counted on both the axotomized and control side of each section. The minimum diameter of neurones included in this category varied depending on the age of the animal, and ranged from 12 to 15 μm in animals less than one week old to 20 μm in 2- to 3-month-old animals. The position of neurones within a distinctive motor column and the size of the perikarya relative to surrounding cell bodies were also taken into consideration when deciding whether or not to include a particular neurone.

No correction was made for double counting of cells due to nuclear or nucleolar splitting at any age, since contralateral
Axotomy-induced motor neurone death

controls were used throughout and all comparisons between animals were based on proportional rather than absolute values.

Data analysis
The serial sections were cut and counted from ventral to dorsal. The first section to contain motor neurones was numbered 'Section 1', and subsequent sections labelled sequentially. This was done independently for each segment on each side of the cord.

When neuronal number was plotted as a function of section number (Fig 2), a distinct bimodality was seen in both C7 and C8, the right-hand peak representing the dorsolateral motor column of the ventral horn. Taking the lowest point between the two peaks as their boundary, the proportion of sections forming the right-hand peak is unaffected by age (37.5±5.64% in C7 and 48.8±5.65% in C8).

When this mean proportion is applied to individual animals, the calculated low point is within one section of the natural low point in 90% of animals, and so the natural low point was used as the boundary for further calculations. In the remaining five animals, the calculated point was within one or two sections of the natural low point, so the section adjacent to the calculated mean and nearest the natural low point was used as the boundary for further calculation in these animals.

This calculated boundary was then taken as the ventral boundary of the dorsolateral column and all neurones in this section, and all sections dorsal to it, formed the total count.

Neuronal loss in each animal was calculated as the absolute difference between the axotomized and control sides in C7 and C8 combined. However, only 75 % of the neurones in these segments are labelled retrogradely by HRP (Figs 1 and 2). Consequently, the number of neurones lost was expressed as a percentage of the susceptible population i.e.

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\frac{\text{control count} - \text{axotomized count}}{75 \% \text{ of control count}} \times 100
\]

Statistics
All values are presented as the mean (±s.d.), unless otherwise stated. A paired Student's two-tailed t-test was used for the comparison of data, and statistical significance was set at the 95 % level of confidence.

Results
The dimensions of the dorsolateral motor column
The number of motor neurones in the dorsolateral column of spinal segments C7 and C8 (as defined in the Materials and methods) is not affected by age, the mean number being 602±90 (n=47).

75 % of these motor neurones can be shown to send their axons to the median and ulnar nerves by retrograde labelling with HRP (Fig. 2).

Morphology of neurones following axotomy
The intracellular morphology of neurones stained with gallo cyanine in 40μm sections is not very distinct, and no morphological changes related to axotomy-induced
cell death could be identified in these preparations (Fig. 3), even at times when rapid cell loss was known to be occurring (see below).

There was no indication that neuronal loss could be overestimated as a result of cell body shrinkage, rather than death. As can be seen in Fig. 3, the surviving neurones on the axotomized side of the cord appear to be a similar size to those in controls, so no detailed morphometric analysis was undertaken. There are no other visible perikarya in the vicinity; only nuclear profiles, as seen on the control side of the cord.

The time course of neuronal death following axotomy at different ages

The time course of neuronal death following axotomy at various ages is summarised in Fig. 4.

When the nerves are cut at 1 day of age, the number of neurones falls rapidly, reaching a final value approximately 20% of that on the control side (19.5 ± 33.2 at 9 d; 21.3 ± 19.8 after 4 weeks). Half of the neuronal loss occurs within the first 4 days. (The variation in groups axotomized at 1 day of age is much greater than that in any of the other groups. This may be due to the technical difficulty of performing axotomies at exactly the same point on the nerve in very small mice at a time when the nerves are still translucent.)

A similar timecourse is observed when the mice are axotomized at 4 days of age. The rate of loss appears slightly more gradual in the second week, after axotomy at 4 days, than it does after axotomy at 1 day of age, but these differences are not statistically significant. (There is no significant difference between the 12- and 28-day survival points.) As with the animals operated on at 1 day old, 50% of the neuronal loss has occurred within 4–5 days after axotomy.

When axotomy is not performed until the animals are 1 week old, a different pattern of loss emerges (Fig. 4). The final deficit is not so great, and 9 days are required for 50% of the total loss to occur i.e. twice as long as in animals 1–4 days old at the time of axotomy. As with the younger animals, however, the final deficit is established well within the maximum survival period of 28 days.

The effect of age at axotomy on the extent of neuronal death

The proportion of neurones surviving 4 weeks after axotomy at different ages is summarised in Fig. 5.

When the nerve is cut within a day of birth, only 21.3% (±19.8) of the axotomized neurones have survived after 4 weeks. A very poor survival rate is also seen after cutting the axons at 4 days of age (9.6% ± 3.59). However, if the animals are 1 week old before the nerves are cut, then 49.6% (±15.5) of the neurones survive after 4 weeks. This is significantly different from the outcome in 4-day-old animals (P=0.013).

A steady increase in the number of neurones surviving axotomy is seen from 1 to 4 weeks of age, and by 4 weeks of age nearly all the neurones (91.4% ± 7.05) have acquired the ability to survive axotomy.

Discussion

Postnatal death of some neurones in the motor pool supplying the flexor muscles of the forepaw in mice can be induced by cutting the axons at any time within the first four weeks. The extent of this death decreases with increasing age of the animal, so that by 4 weeks of age less than 10% of the neurones die following axotomy.

The estimates of total motor neurone number were based on counts of all large neurones in the dorsolateral motor column. The loss in any individual was expressed as a proportion of the control side prior to any comparison being made either with an animal from the same experimental group or with any other animal. Therefore, no prior correction for any difference that might be introduced by variation in animal size or neuronal size was required. Using the same counting technique (Pollin et al. 1990), the number of motor neurones in the cervical spinal cord was similar to counts of cervical ventral root myelinated axons in the same strain of mouse (Biscoe and Lewkowicz, 1982). Therefore, we believe that our results represent a reasonable estimate of motor neurone number in the mouse cervical spinal cord.

The results of any method attempting to estimate the size of a neuronal population are strengthened by verification using a different technique. Much previous work with HRP has shown that it can be a reliable quantitative tracer (Burke et al. 1977; Oppenheim, 1981; McHanwell and Biscoe, 1981; Lance-Jones, 1982; Hardman and Brown, 1985) and, in the present study, little variation was seen in the three animals labelled with HRP (Figs 1 and 2). The very close correlation between the HRP-labelled cells and the total count in the most dorsal sections of C8 (Fig. 2B) strengthens the assumption that both methods provide a reasonable estimate of total population size. The marked difference between the number of labelled cell bodies and the estimate of neuronal number in gallocyanine-stained sections in C7 (Fig. 2A) can be accounted for by the presence, in the dorsolateral motor column of C7, of cell bodies with axons found in the radial nerve (Jenny and Inukai, 1983). These observations led to the final adjustment of the total number in the dorsolateral column of C7 and C8 in order to obtain a reasonable estimate of susceptible population size, i.e. those cell bodies with axons in the median and ulnar nerves only.

Our findings, on the extent of neuronal loss resulting from cutting the nerve at various ages, are very similar to those of Schmalbruch (1984, 1988) who counted the myelinated fibres in L4–L6 ventral nerve roots of the rat following sciatic nerve section. When his figures of final deficit are expressed as a proportion of the axons cut, a pattern closely resembling that in Fig. 5 is seen: cut at birth, 100% loss; 7 days, 65%; 14 days, 26%; 28 days, 4.6%.

This is in contrast to the work by Luthman et al.
Fig. 3. Galloycyanine-stained 40 μm frozen horizontal section of spinal cord (dorsolateral column, C7/C8 boundary) from an animal axotomized at one day of age and allowed to survive for 4 weeks. (A) Control side, (B) axotomized side. Bars=200 μm. (C) and (D) Higher power magnification of the areas outlined in A and B. Bars=100 μm. It can be seen that the number of large motor neuronal perikarya (arrows) is reduced on the axotomized side. There is no indication that this reduction in number might be due to shrinkage rather than death of the affected neurones.
week, the number of neurones surviving also increases, but 50% have acquired the ability to survive by 1 week of age. As the age at axotomy increases beyond 1 week, the number of neurones surviving also increases, until at 4 weeks of age there is no significant loss. (Mean±s.E.M.).

One of the most extensive studies of axotomy-induced death was carried out by Romanes (1946) in mice. He showed that amputation of a hindlimb at birth led to a 90% loss within one week, with half that loss occurring in 3-4 days. He also demonstrated an 'increasing resistance of cells' appearing within the first week of life. However, he possibly underestimates the final loss after cutting at 6 days of age (17%) by looking only 6 days after axotomy. Our present results suggest that, while this may be adequate to show the ultimate loss after cutting at birth, a longer survival time is probably required in older animals to assess the full extent of axotomy-induced death.

Kashihara et al. (1987) working in rats axotomized at 4 days of age also confirm the extensive loss of motor neurones after cutting the nerve and preventing reinnervation (18% survival). They also demonstrated that allowing reinnervation 'rescued' many of the motor neurones which would otherwise have died (77% survival). In agreement with our results, the loss of neurones occurred over a 2-week period but, unlike our findings, Kashihara and his colleagues found a delay of almost 2 weeks following axotomy before the onset of neuronal death. This delay may be accounted for by the fact that they cut the nerve very close to its target muscle. There is extensive evidence in the literature (discussed in detail by Lieberman, 1974) that axonal lesions close to the cell body in adults are followed by a more rapid perikaryal response, a more intense reaction and greater neuronal degeneration than is the case with distal lesions.

Our findings thus agree with previous studies on the duration of the period of susceptibility of immature neurones to axonal damage. While our results clearly show that the survival of motor neurones following axotomy depends upon the age at which the neurones were axotomized, the interpretation of these results is less clear. One hypothesis is that this age-related change reflects a qualitative maturation of the motor neurone or its target. Another hypothesis is that the age-related changes reflect an increase in the distance of the site of injury from the cell body as the animal grows. It is well established for some neuronal populations that when axonal lesions are made closer to the cell body there is a more rapid response, a more intense reaction, and greater neuronal degeneration (Lieberman, 1974). In the present study, axotomy within 1 day of birth results in rapid neuronal loss with 50% of that loss occurring within the first 4 days. In contrast, if axotomy is performed 1 week after birth then 9 days are required for half the neuronal loss to occur. Over this period, the distance of the site of the injury from the cell soma increases from 6 mm to 13 mm. Taken together, these observations would suggest that the increased length of the proximal stump is responsible for the timing of motor neurone death, and they would be consistent with the suggestion that a signal passes from the site of injury to the cell soma at less than 1 mm per day. However, since axotomy of motor neurones in adult animals does not result in extensive neuronal death, it seems unlikely that the more extensive neuronal loss seen in younger animals is simply due to the shorter length of the proximal stump.

An alternative view would be that the inability to induce motor neuronal death by axotomy is the result of a maturation of the motor neurone itself, or altered...
interactions between motor neurones and putative trophic factors. These trophic factors could come from muscle or nerve or both. Sendtner et al. (1990) have shown that ciliary neurotrophic factor (CNTF), present in high concentrations in nerve can prevent axotomy-induced motor neurone death in neonatal rats. They have also shown that the level of CNTF in peripheral nerve is low at birth, and increases to adult values within the first 2 weeks of life (Stöckli et al. 1989). Since uninjured neonatal motor neurones do not normally die in spite of low levels of CNTF, it may be that other trophic factors essential for motor neurone survival are present in the periphery.

A clear distinction between the effects of proximity of the lesion to the cell body and qualitative maturation have also shown that the level of CNTF in peripheral nerve is low at birth, and increases to adult values within the first 2 weeks of life (Stöckli et al. 1989). Since uninjured neonatal motor neurones do not normally die in spite of low levels of CNTF, it may be that other trophic factors essential for motor neurone survival are present in the periphery.

The authors would like to thank Mr B. White and Mr J. Battong for their care of the animals, and Ms M. A. McGrane for helpful discussions. This work was supported by the MRC.

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(Accepted 18 January 1991)