Spinal motor neurones in murine muscular dystrophy and spinal muscular atrophy

A quantitative histological study

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SUMMARY Recent electrophysiological studies of human and mouse muscular dystrophy have prompted the hypothesis that both are of neurogenic rather than myogenic origin. A decreased number of spinal motor neurones might be expected if this hypothesis were correct. The total number of neurones in the anterior grey horns of seven normal mice, six Bar Harbor 129 strain dystrophic mice, and six mice suffering from genetically-determined spinal muscular atrophy have been counted. The number of neurones in the cell types believed to include the motor neurones was significantly reduced to 13 to 71% of normal in mice with spinal muscular atrophy. In mice with muscular dystrophy, the number of anterior horn neurones was higher rather than lower than normal. The significance of these findings is discussed.

Since the time of Duchenne (1868) and Erb (1890, 1891), muscular dystrophy in man has been believed to be a primary disease of muscle on the basis of the marked pathological change seen in the muscle, and in the absence of pathological change in the central and peripheral nervous systems. Some mental retardation is known to occur in a number of cases of Duchenne pseudohypertrophic muscular dystrophy, though there is dispute about the pathological basis of this (Rosman and Kakulas, 1966; Dubowitz and Crome, 1969; Rosman, 1970). An inherited disease showing pathological changes in the skeletal muscle similar to those of human muscular dystrophy exists in the Bar Harbor 129 strain of mice (Michelson, Russell, and Harman, 1955; West and Murphy, 1960). As in the human disease, initial pathological study indicated no abnormality of the central or peripheral nervous system (Michelson *et al.*, 1955). Further study of the intramuscular nerves in this condition showed a variety of changes thought to be secondary to the underlying muscle disease, though motor end plates were infrequent on the tiny dystrophic fibres with central nuclei (Harman, Tassoni, Curtis, and Hollinshead, 1963). Electromyographic studies of these animals showed fibrillation potentials and high frequency discharges similar to those seen in human dystrophia myotonica (McIntyre, Bennett, and Brodkey, 1959). The myotonic-like 'spasms' of the hind limbs of these animals may also be evidence for

the similarity between these diseases. However, fibrillation potentials and high frequency discharges are also seen in denervation, and the finding of a decreased frequency of miniature end plate potentials (Conrad and Glaser, 1966) and of supersensitivity to neostigmine and resistance to d-tubocurarine (Baker, Wilson, Olendorf, and Blahd, 1960) are additional evidence suggesting denervation. McComas and Mrózek (1967) elegantly demonstrated by direct electrophysiological means the presence of denervated fibres in murine muscular dystrophy.

Recently McComas and his colleagues have investigated patients with the Duchenne, limb-girdle, and facioscapulohumeral varieties of muscular dystrophy, as well as dystrophia myotonica (McComas and Sica, 1970; McComas, Sica, and Currie, 1970; Campbell, McComas, and Sica, 1970). They estimated the total number of motor units in the extensor digitorum brevis muscle of these patients by an electrophysiological technique, and found that the number was reduced four-fold in Duchenne muscular dystrophy, and to a similar extent in the other conditions. The remaining motor units were of normal amplitude in the Duchenne and myotonic dystrophy patients, while in the limb-girdle and facioscapulohumeral dystrophies they appeared enlarged rather than decreased as would have been expected from the classical views of the pathogenesis of muscular dystrophy. Moreover a study of the twitch tension developed

by the tibialis anterior muscle in dystrophic mice has shown a similar three-fold decrease in the number of motor units, though here the size of the units was reduced compared with normal (Harris and Wilson, 1971). The reduction of the number of myelinated nerve fibres in the nerve to tibialis anterior was concordant with this decreased number of motor units (Harris, Wallace, and Wing, 1971).

These findings have been taken to indicate that muscular dystrophy both in man and mouse is of neurogenic rather than myogenic origin. They have prompted a re-examination of the spinal cord at necropsy in cases of human Duchenne-type muscular dystrophy (Tomlinson, Kitchener, Rebeiz, and Walton, 1971), and in murine muscular dystrophy. The results of a comparison of the number of spinal motor neurones in dystrophic mice of the Bar Harbor 129 strain with those in normal mice is reported here. Mice of the 'wobbler' strain suffering from spinal muscular atrophy (Duchen and Strich, 1968) were also studied as an example of a condition with known degeneration of anterior horn cells.

METHODS

Nineteen male mice were used in these present studies, six dystrophic mice of the Bar Harbor 129 strain, six 'wobbler' mice, and seven normals. The latter group comprised three Bar Harbor 129 strain normal animals, and four normal litter-mates of the 'wobbler' animals. A number of the dystrophic mice and Bar Harbor 129 strain normal animals had been the subjects of the electrophysiological studies reported by Harris and Wilson (1971). In each of the groups, pairs of animals of 1 month, 3 months, and 6 months of age were studied. The clinical state of the animals was noted, and they were then killed by an overdose of ether. The skull and backbone were removed from the body, fixed in 10% formol calcium solution for three days, and the spinal cord dissected out under a dissecting microscope from the cervico-medullary junction to the cauda equina. The cord was straightened during double embedding in paraffin wax. Serial transverse sections of 10 μ thickness were taken from the cervicomedullary junction to the filum terminale. Sections were stained with cresyl fast violet. Sections at three levels of the upper limbs, lower limbs, head, ribs, diaphragm, heart, and bladder were also prepared from each animal (decalcifying where necessary in formic acid), and used for grading the histological severity of changes in the muscles of each animal.

The spinal cord sections were studied with no

knowledge of the diagnosis in each animal, the code being broken only at the completion of all counting. Sections were viewed using $\times 250$ magnification which allowed the whole of the anterior grey horn of one side to be seen within the microscopic field. The largest diameter of neurones was measured with an eye-piece graticule, increasing the magnification to $\times 500$ where necessary. Neurones anterior to a horizontal line passing through the midpoint of the central spinal canal were considered to lie within the anterior grey horn, though neurones of the intermediolateral column (sympathetic preganglionic neurones) were excluded from counts. The left and right horns were counted independently.

ARBITRARY CLASSIFICATION OF NEURONES In the mouse, the anterior horn motor neurones are not as clearly demarcated from other neurones as in man. All the neurones within the anterior grey horn were therefore classified into three arbitrary groups (see Figure):

Group I Neurones with extremely dense Nissl substance. These cells were frequently rather shrunken in outline.

Group II Neurones with dark prominent Nissl substance and with maximum diameter equal to or greater than 25 μ .

Group III All remaining neurones.

The method of counting neurones developed by Tomlinson (Tomlinson *et al.*, 1971) was adopted—that is, only those neurones whose nucleus contained a nucleolus within the thickness of the section were counted.

ESTIMATION OF COUNTING ERROR AND SAMPLING METHOD In order to estimate the counting error, the same four sections of spinal cord from one of the mice was counted 20 times at two-weekly intervals. In one animal every spinal cord section was counted for group I and group II neurones, and every tenth section for group III neurones. In the remaining animals every 10th section was used for counting group I and group II neurones, and every 40th section for group III neurones. The total number of sections involved in this study was more than 80,000.

RESULTS

In the animals in which every section was counted for group I and group II neurones and every 10th section for group III, the neurones were as expected more numerous in the cervical and lumbosacral areas. However, no evidence of preferential collection of neurones into spinal cord segments was detected. Similarly, no signifi-

FIGURE A representative field of the anterior grey horn of the spinal cord of a normal mouse (cresyl violet $\times 612$). Neurones classified (see text) as group I are shown with open broad arrows, as group II with closed broad arrows, and as group III with fine arrows. Only neurones with nucleoli seen under the microscope are marked.

cant difference was detected between the number of neurones within the right and left anterior grey horns. Therefore, it was felt justified to select every 10th section for counting group I and group II neurones and every 40th section for group III neurones. In the results described below the number of neurones on the left and right sides have been added together. Owing to difficulty in defining the cervicomedullary junction in many specimens, counts above the cervical enlargement have been excluded.

In 20 repeated counts of the same four sections, the standard deviation of counts of group I and II neurones was 3.3 and 4.9% of the mean for right and left anterior horns respectively, and that of group III neurones was 4.6 and 5.5% of the mean for right and left horns respectively.

The mean total number of anterior horn neurones of each group in the spinal cord of normal mice, and those with muscular dystrophy and spinal muscular atrophy is shown in Table 1.

TABLE 1
MEAN TOTAL NUMBER OF ANTERIOR HORN NEURONES
IN SPINAL CORD IN NORMAL MICE, AND THOSE WITH
MUSCULAR DYSTROPHY AND SPINAL MUSCULAR ATROPHY

Animal type	Neurone type	Cervical	Thoracic	Lumbo-sacral	Whole spinal cord
Normal	I	653	604	1,179	2,436
	II	7,816	7,549	12,471	27,836
	III	60,508	66,196	115,612	242,316
Dystrophic	I	947	660	1,480	3,087
	II	9,310	5,555	10,953	25,818
	III	71,532	70,692	129,688	271,912
'Wobbler'	I	88	110	687	885
	II	3,977	4,923	8,882	17,782
	III	56,128	67,320	101,288	224,736

In normal animals group III neurones outnumber group II by approximately 10-fold, and group I by approximately 100-fold. In dystrophic animals the number of each group of neurones exceeded the normal values at all levels by 10 to 45%, except for group II neurones at the

thoracic and lumbosacral levels. In 'wobbler' animals the numbers of all types of neurones at all levels were reduced to 13 to 71% of normal, with the exception of group III neurones in the thoracic cord.

A statistical comparison was made between the normal and dystrophic, and normal and 'wobbler' mice at the different levels and for the different groups of neurones using the Mann-Whitney U-test, and the probability values are shown in Table 2. It can be seen that no statistical

TABLE 2

SUMMARY OF STATISTICAL COMPARISON OF ANTERIOR HORN NEURONE COUNTS BETWEEN NORMAL AND DYSTROPHIC MICE, AND NORMAL AND 'WOBBLER' MICE

Statistical comparison	Neurone type	Cervical	Thoracic	Lumbo-sacral	Whole spinal cord
Normal <i>v.</i> Dystrophic	I	—	—	—	0.034
	II	—	—	—	—
	III	—	—	—	—
	I + II	—	—	—	—
Normal <i>v.</i> 'Wobbler'	I	0.002	0.002	0.034	0.002
	II	0.022	—	—	0.008
	III	—	—	—	—
	I + II	0.004	—	0.034	0.008

Figures quoted are the probability values applying the Mann-Whitney U-test to the data in Table 1. Values of P below 0.05 are marked —.

difference exists between normal and dystrophic animals with the sole exception of the total number of group I neurones in the whole spinal cord. Here the number in the dystrophic animals is higher than in the normals. In the comparison between normal and 'wobbler' animals the number of group I neurones is significantly reduced at greater than the 5% level in all areas of the spinal cord; group II neurones are also significantly reduced in number in the cervical region.

An examination of the anterior horn cells of the 'wobbler' mice revealed an occasional example of central chromatolysis and neuronophagia, though these changes were rare. The lipofuscin accumulation seen in human motor neurone disease was not present. Occasionally peripheral vacuolation of neurones similar to that reported by Andrews and Maxwell (1968) was seen. However, the significance of these changes was not known to the observer (T.A.P.) at the time of the study, and their presence was not thought therefore to have biased the observer during counting. No difference was detected between the anterior horn cells of dystrophic and normal mice.

DISCUSSION

The method developed by Tomlinson *et al.*, (1971) of counting the nucleoli of anterior horn cells was adopted in this study as the best method for enumerating neurones. Though an occasional nucleolus will be cut in two, and is therefore represented in each of two sections, this will be an infrequent event, and will occur equally in normal and pathological material. In the mouse as in the guinea-pig (Bradley, 1970) a small percentage of anterior horn cells have two nucleoli. No difference was detected between the frequency of these double nucleoli in normal and pathological material.

One major difficulty in the present study was the lack in the mouse spinal cord of a clear separation between motor neurones and the remaining neurones in the anterior grey horns. In man the situation is easier, for the motor neurones may be distinguished by size and the strikingly clumped character of the Nissl substance. It was therefore felt necessary in the mouse material to count all neurones in the anterior horns, dividing them into arbitrary groups. The dark, often shrunken neurones were almost certainly the result of fixation artefact (Cammermeyer, 1961), though the striking reduction in the number of these neurones in the 'wobbler' mice suggests that their presence may represent some significant *in vivo* feature of motor neurones. Neurones in group II were the largest cells with dark, clumped Nissl substance, and were thought likely to include most of the motor neurones. Group III comprised the remaining neurones, and were thought mainly to be interneurones. The decrease in group I and group II neurones with no significant decrease in group III found in 'wobbler' mice with spinal muscular atrophy was in keeping with this interpretation. In these animals, the decrease was greatest in the cervical region, which accords with the clinical and pathological signs of denervation predominating in the forelimbs. It is of interest that there was no decrease in the number of neurones with increase in age of these 'wobbler' mice, a finding which is at first sight surprising. It is, however, likely that a number of the younger animals were more severely affected, and would have died before reaching the age of the oldest mice in the study. The latter must have been suffering from the disease in a rather more benign form in order to live to the age of 6 months or more.

This study showed no evidence of a decrease in the number of anterior horn neurones in mice with muscular dystrophy, the numbers being, in part at least, significantly higher than normal. Moreover, no histological abnormality was seen in the neurones of dystrophic mice. The findings of this study do not therefore at first sight support the hypothesis of McComas and his colleagues. It is possible that a 'dying back' type of neuropathy (Cavanagh, 1964) exists in these animals, since Harris *et al.* (1971) found a decreased number of myelinated nerve fibres in the nerve to the tibialis anterior muscle in dystrophic mice. A study of the number of myelinated nerve fibres in other peripheral nerves is at present in progress in this department to amplify this finding.

Though no decrease in the number of neurones was demonstrated in dystrophic mice, the suggested abnormality of trophic influence of motor neurones on muscle cells might occur in the presence of a normal number of neurones. A considerable body of evidence exists for the presence of a trophic effect of nerve upon muscle. The reinnervation of a 'slow' muscle like the soleus by a nerve to a 'fast' muscle such as the flexor digitorum longus or vice versa changes the histochemical and physiological properties of contraction of the muscle towards that of the reinnervating nerve (Close, 1965; Romanul and Van der Meulen, 1967; Yellin, 1967; Guth, Watson, and Brown, 1968; Robbins, Kaparti, and Engel, 1969). In frog muscle, at least, a change of the type of contracture developed by the muscle may occur before effective neuromuscular transmission can be demonstrated (Elul, Miledi, and Stefani, 1968). The passage of protein from the motor nerve ending to the muscle has been demonstrated autoradiographically (Korr, Wilkinson, and Chornock, 1967). In adrenergic nerves, the adrenal medulla and the neurohypophysis, at least, a number of these proteins have been isolated and partially characterized (Bloom, Iversen, and Schmitt, 1970).

More direct evidence in support of the trophic influence of nerve in murine muscular dystrophy has been reported by Salafsky (1971). He found that dystrophic muscle transplanted into normal mice developed normal electrophysiological twitch characteristics. Regeneration in autotransplanted muscle has been well documented (Studitsky, Zhenevskaya, and Rummyantseva, 1963; Laird and Timmer, 1965, 1966), and it

has been shown in the frog that this regeneration probably occurs by means of satellite cells (Trupin, 1970). However, Laird and Timmer (1965, 1966), in contrast to Salafsky, found that dystrophic muscle retained its dystrophic morphology after regeneration when transplanted into normal histocompatible mice. Carlson (1970) has produced direct evidence of the rejection of homotransplanted normal muscle, with lymphocytic infiltration and a dramatic decrease in the size of the regenerate to considerably less than 50% of that seen in autotransplanted muscle. One possible explanation of Salafsky's finding is that regeneration of muscle in his material was occurring by means of myoblasts of the recipient animal derived from the bed of excised muscle. An autoradiographic investigation of this problem is at present in progress in this department.

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