STUDIES ON SPINAL MUSCULAR ATROPHY IN MICE

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SUMMARY

1. This study is based on material from two mouse mutants - the "wobbler" mutant, (which manifests a homozygous recessive lower motor neurone disorder which resembles human spinal muscular atrophy), and the ReJ/129 dystrophic mutant, (which manifests a homozygous recessive neuromuscular disease with certain similarities to human Duchenne type muscular dystrophy). Control material has been obtained from mice of the C57/BL colony as these animals are free from neuromuscular disease.

2. The work can essentially be divided into three main sections - clinical and genetic studies carried out largely on mice of the wobbler colony using the dystrophic colony as the control; biochemical studies carried out on mice from all three colonies; and histological and histochemical studies of which some were carried out on mice of all three colonies, but of which studies of muscle and spinal cord histology were primarily based on material from mice of the wobbler colony.

3. The clinical and genetic studies were carried out in order to verify the genetics of the "wobbler" mutant; also to consider the genetics and a method for possible early diagnosis of the late onset hindlimb paralysis disorder which occurred in several mice of the wobbler colony. These mice have been referred to as Wr/HLP mice throughout the study. Although no method for early diagnosis was found, the genetic studies showed that the Wr/HLP mouse could be a manifesting heterozygote for the mutant "wr" gene.

4. The biochemical studies were carried out on specimens of spinal cord. The free amino-acid content of the cervical and lumbar enlargements was assessed by means of column chromatography. The results indicated altered amino-acid profiles in mice from both mutant colonies. The most consistent finding was an increased homocarnosine level in wobbler mice cervical and lumbar enlargements. The possible role of

neurotransmitter amino-acids in the disease processes in both wobbler and dystrophic mice has been discussed.

5. The histological and histochemical studies can further be divided into three sections: studies of terminal innervation and end-plate morphology, histological and histochemical investigations of muscle, and studies of spinal cord histology.

6. The functional terminal innervation ratio, the end-plate size and end-plate morphology was studied in the gastrocnemius muscle. The combined acetylcholinesterase-silver technique was used for staining the muscle. Increased FTIR values were found in wobbler (wrwr) mice, in Wr/HLP mice and in dystrophic mice (dydy). The most prominent abnormality in the dystrophic mouse was found to be in the end-plate structure and not in the amount of collateral branching seen. Innervation studies in Wr/HLP mice showed some similarities to the changes seen in the wobbler mouse and suggest a lower motor neurone disease in these mice.

7. Histological and histochemical studies of muscle were also carried out on the gastrocnemius muscle. Measurements of muscle fibre size suggested that the disease in the Wr/HLP mouse was a slow progressing spinal muscular atrophy, although histochemical studies did not indicate any fibre-type grouping.

8. Histological studies of the spinal cord were carried out to confirm anterior horn cell degeneration in Wr/HLP mice and to observe whether any changes appeared similar to those found in wobbler mice. Some abnormalities in the motor-neurones of Wr/HLP were found, but no conclusion could be reached as to whether the same type of degeneration was occurring in Wr/HLP and wobbler mice.

9. The results of genetic studies as well as histological investigations of muscle and spinal cord of the Wr/HLP mice suggest that the late

onset hind-limb paralysis disorder could result from the "wr" gene manifesting in the heterozygote.

10. The final section of this study was carried out on human muscle. The acetylcholinesterase-silver technique, developed for use on mouse muscle, was modified and used on human biopsy and necropsy material. Terminal innervation was successfully studied in muscle specimens obtained from biopsies and from autopsies. The usefulness of this method has been discussed.

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CONTENTS

CHAPTER 1 - GENERAL INTRODUCTION

CHAPTER 1

Neuromuscular diseases have been conventionally divided into myopathies and neuropathies. Diseases such as muscular dystrophy, myotonic dystrophy and polymyositis have traditionally been regarded as primary diseases of muscle with no involvement of the central nervous system (Adams, Denny-Brown and Pearson 1962). Spinal muscular atrophy, human lower motor neurone disease and poliomyelitis have all been considered as primary diseases of the nervous system resulting in secondary involvement of the muscle.

Until recently the distinction between the two categories of diseases remained rigid, however certain clinical observations and laboratory findings have led to the suggestion that there may be a neurogenic component in dystrophy. It has been reported that a significant proportion of children with Duchenne muscular dystrophy are also mentally handicapped (Zellwengar and Hanson 1967), and electrophysiological evidence has suggested a reduction in the number of functional motor units early in the course of the disease (McComas, Sica and Currie 1970). However the idea that human dystrophy may have a neurogenic origin has been seriously questioned (Bradley 1974, Emery and Gosdon 1974).

Progress in research on neuromuscular diseases has generally been hampered by the lack of availability during life of the tissues of primary interest. The discovery of animal mutants with neuromuscular conditions resembling human diseases has therefore not surprisingly aroused considerable interest, in that such material can be extensively studied at various stages during the disease process. The present study has considered two such mouse mutants: wobbler mouse mutant, which suffers from a homozygous recessive lower motor neurone disorder and the ReJ/129 dystrophic mouse which at one time was considered to have a muscle disease similar to the Duchenne type of muscular dystrophy. These two mutants used in the study

could classically be considered as representing a neurogenic and a myopathic disorder respectively. The purely myopathic nature of murine muscular dystrophy has, however, been questioned for some time. Electrophysiological studies have suggested defective motor innervation (McComas and Mossaway 1965) and a reduction in the number of myelinated nerve fibres was also reported (Harris, Wallace and Wing 1972). More recently an abnormality in myelination was demonstrated at first in the sciatic nerve root (Bradley and Jenkison 1973) and later a similar abnormality was found at the roots of other cranial nerves (Biscoe 1974). There is now evidence which suggests that the primary lesion in muscular dystrophy is not in the muscle, as was originally believed, but lies actually in the Schwann cell. Although in man neurogenic involvement has been suggested in both Duchenne muscular dystrophy (McComas, Sica and Currie 1970) and myotonic dystrophy (Swash 1972), no similar aberrant myelination has been found in those human dystrophies. On the basis of current findings, therefore, it would be unwise to consider murine muscular dystrophy as analagous to any human condition. It remains however a valuable model for the study of neuromuscular disease, and the question of the interaction between the nerve and muscle.

There is now little to dispute the existence of a peripheral nerve abnormality in murine dystrophy, however, no motor neurone loss has been found (Papapetropoulos and Bradley 1972). The primary feature of the disease in wobbler mouse is the loss of anterior horn cells (Duchen, Strich and Falconer 1972). Hence it would appear that the two mutants used in this study should both really be considered as being affected by neurogenic disorders, although the primary lesions lie at different levels in the nervous system.

The dystrophic mouse mutant was first reported in 1955 (Michelson, Russel and Harman 1955) and has been very extensively studied since.

The wobbler mouse was first observed by Falconer in 1956 but was not described until several years later (Duchen, Falconer and Strich 1965). The wobbler mouse mutant has generally attracted much less attention than the dystrophic mouse, although at present evidence suggests that the wobbler mouse could be a useful model for the study of lower motor neurone diseases.

It is at first difficult to comprehend why these two mutants have attracted such a varying degree of attention among researchers interested in neuromuscular conditions. It may be that the general lack of understanding of the nature of pharmacological interactions in the central nervous system has resulted in the wobbler mouse being studied primarily from the histopathological angle with little consideration being given to the biochemical or pharmacological nature of the disease process.

The complex degenerative changes occurring in the central nervous system of mice with lower motor neurone disease have been described by Andrews (1975) but have proved difficult to interpret as none of the abnormalities found appear to be specific to this murine disorder. The degenerative features found in these mutant mice appear to be specific only in their relative frequency and distribution.

The wobbler mouse was used as a model for the study of axoplasmic flow but the interpretation of the results obtained was inconsistent. One study suggested impaired fast phase axoplasmic flow (Bird et al 1971). However another study did not demonstrate axoplasmic flow occurring at two rates - a fast rate clearly separated from a slow rate. Rather there appeared to be a smooth spectrum of the amount of transport occurring at different velocities and no variation of axoplasmic flow between healthy and diseased mice of the wobbler colony was demonstrated (Bradley, Murchison and Day 1971, Bradley 1972).

There have been very few biochemical studies of the central nervous system in wobbler mice. The finding of elevated acid phosphatase levels particularly in white matter of the wobbler central nervous system (Hirsch, Andrews and Parks 1974) led the authors to consider the mutant as a model for secondary demyelination. Increased activity of acid hydrolases have been reported in connection with Wallerian degeneration (McCaman and Robins 1959, Porcellati 1970). In wobbler mouse four acid hydrolases were found to be increased in cervical cord white matter (Hirch, Andrews and Parks 1974). Increases in lysosomal enzymes may be indirect evidence linking enzyme activity with the presence of such features as axonal dense bodies and myelin ovoids reported to occur in wobbler spinal cord tissue (Andrews and Maxwell 1969). The enzyme changes observed in wobbler mice, probably occur as the result of the degenerative process. The primary metabolic lesion resulting in those secondary changes remains to be elucidated.

In recent years animal material has been used extensively for the study of numerous transmitter substances within the nervous system (Phyllis 1970). Similar studies using human material have been limited, although free amino-acids, several of which are believed to function as neural transmitters, have been quantified in the CSF from patients with neurological conditions (Perry and Jones 1961, Logothentis and Bovis 1962). Amino-acids have also been estimated in material obtained from brain biopsy (Perry 1971a) and at autopsy (Perry 1971b). Studies of the function of chemical transmitters in the nervous system have resulted in certain pharmacological agents being used in treatment of certain neurological conditions (Hayashi 1966, Davidoff 1970).

As the true function of pharmacological substances in the central nervous system of healthy animals becomes better understood so such studies can be extended to diseased material. The use of such an approach in

studying animal analogues of certain human conditions could prove useful both in establishing the primary lesion and perhaps also in indicating a possible form of treatment. Although the interaction of all the various transmitter substances in the central nervous system are not yet fully understood, there is considerable evidence suggesting that amino-acid transmitters are important in the normal functioning of anterior horn cells (Curtis, Holsi and Johnston 1968, Curtis and Watkins 1960). The loss of interneurones has been observed to result in a reduction in the concentration of the amino-acids glycine, aspartate and glutamate in the spinal cord (Davidoff et al 1967). In the present study amino-acid concentrations in the spinal cords of mice from both wobbler and dystrophic colonies have been investigated in order to establish whether similar amino-acid changes could also be a feature of motor neurone loss or accompany other neurological conditions.

Anterior horn cell degeneration in the wobbler mouse leads to changes in peripheral innervation and hence to muscle atrophy (Duchen, Strich and Falconer 1968). It has already been mentioned that these changes resemble those which occur in human spinal muscular atrophy. An increase in peripheral nerve branching and in the functional terminal innervation ratio has been shown to be a feature of human SMA (Coërs and Woolfe 1959, Coërs, Telerman-Toppet and Gerard 1973b). In human Duchenne type muscular dystrophy no abnormality of innervation ratios has been found (Jedrzejowska, Johnson and Woolf 1965). No detailed study of murine peripheral innervation has until now been reported. The second part of this study has considered peripheral innervation and end-plate morphology in mice of the two colonies. In this part, rather greater emphasis has been placed on mice of the wobbler colony not only because previous work on this mutant is limited but also because of an unusual feature which

developed in some mice of this colony. During the process of maintaining the wobbler colony, several mice were found to develop paralysis in the hind-limbs at about the age of four months. These mice will, throughout the study, be referred to as Wr/HLP mice. As it was believed that these mice could be manifesting heterozygotes (carriers) of the wobbler gene, not only was peripheral innervation studied in these mice but also in several asymptomatic known heterozygote mice.

Muscle histology of the Wr/HLP mouse has been compared with the histology of affected wobbler mice and known heterozygotes as also has the histology of the spinal cord. Hence a considerable part of this study has been carried out in order to elucidate the nature of the disease in Wr/HLP mice. A limited number of genetic experiments have also been carried out in order to establish whether the Wr/HLP mouse does in fact represent the heterozygous state of the wobbler gene.

The major part of this study has been concerned with various aspects of spinal muscular atrophy in mice. However, human material has also been used in some studies in order to show how work carried out on animals might prove useful for the study of human diseases. Most studies of peripheral innervation in human muscle have used the vital staining techniques (Coërs 1952, Coërs and Woolf 1959). These techniques require the muscle to be very fresh, hence only biopsy material can be used. However an acetycholinesterase-silver technique developed for the study of mouse peripheral innervation was modified and used on both biopsy and autopsy human muscle specimens. The results of these studies indicate that this technique may prove particularly valuable in the study of innervation of human muscle.

CHAPTER 2 - MATERIAL AND METHODS

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CHAPTER 2

In this chapter the material and methods used in the study will be outlined briefly. Methods requiring detailed explanation and the methods used in collecting and analysing the data will be dealt with in full in the appropriate chapters.

I. Material

Mouse specimens were used primarily for this study but work on a few human specimens will also be reported.

i) Mouse Specimens

Three colonies of mice, bred in the Animal House of the Western General Hospital, Edinburgh, supplied the specimens used for this study. Details of these three colonies are given in Chapter 3. Mice from the C57/BL colony, known to be free from any form of neuromuscular abnormality were used as control animals in all experiments. Phenotypically healthy and affected mice from the Bar Harbor 129 ReJ/dy and the "wobbler" (WrWr) colony have supplied the tissues on which most of this study is based.

Spinal cord samples were used in the analysis of free animo-acid content of cervical and lumbar enlargements. A few cords were also used in histological preparations. Muscle samples were studied by means of histological and histochemical techniques. The gastrocnemius muscle was found to be most suitable for the study of peripheral innervation as sufficient numbers of muscle fibres remained, even in highly diseased specimens, for innervation to be studied in thick sections. This muscle was not found to be ideal for the histochemical study of muscle; however, it was thought desirable to study peripheral innervation and histology on the same muscle as different muscles may be affected to a varying degree by the disease process.

ii) Human Specimens

Specimens of human muscle were obtained from muscle biopsies and

from muscle taken at post-mortem examination. Very few of the specimens received proved suitable for the study of peripheral motor innervation. Specimens taken from various muscles have been used, as the muscle from which any biopsy is taken depends on the disease being studied. The deltoid muscle was used in the case of the patient with suspected lower motor-neurone disease, in two specimens taken from known Werdnig-Hoffmann carriers, and in necropsy specimens. The quadriceps was biopsied where patients were affected with diabetic amyotrophy. It was found that terminal innervation could be effectively studied in specimens obtained up to three days after death, but in the case of muscle biopsies it was desirable to freeze the muscle (see processing of specimen) within an hour of operation to prevent excessive drying and shrinkage of the tissue.

II. Methods

i) Processing of Specimens

a) For Amino-acid Analysis

The animals were killed by means of ether anaesthesia. The spinal column was removed immediately. The required area of the cord was extracted from the surrounding vertebrae with the aid of a dissecting microscope (Leitz wefzlar) magnification x 6.3 using bone forceps. Care was taken to prevent the contamination of the spinal cord with blood. The area of the cord selected for analysis was weighed and immediately homogenised in 1.5 ml of 10% sulphosalicylic acid at $4^{\circ}C$. The sample was centrifuged at 1,500 rev/min in a Gallenkamp junior centrifuge (A. Gallenkamp & Co Ltd). The supernatant was removed and stored in an ultrafreezer at $-70^{\circ}C$; storage was rarely for more than four hours. The time taken for preparing the sample from the time of death of the animal to the point of freezing of the sample was never greater than thirty minutes.

b) For Histology and Histochemistry

1. Muscle

Where mouse muscle was used the entire gastronemius muscle was removed and whenever possible the muscle was directly orientated on to the chuck either for longitudinal or transverse sections. The muscle was secured by means of Ames compound (Ames Co., Indiana) and frozen in isopentane chilled with liquid nitrogen. The chucks were stored in sealed containers at a temperature of between -20°C and -70°C until the tissue was sectioned. When tissue specimens were to be stored for more than one or two days the unmounted specimen was frozen carefully in chilled isopentane and stored in sealed tubes. The tissue was mounted on to the chuck before sectioning; when orientating frozen specimens on to the chuck care had to be taken to prevent the defrosting of the tissue. Frozen muscle specimens could be stored in well sealed tubes for up to a year without marked changes in muscle histology. Human muscle specimens were prepared for sectioning in the same way.

Sections were cut in a Slee retracting microtome (Slee Co Ltd), maintained at a temperature of between -25°C and -20°C and the sections were attached on to glass slides at room temperature. Transverse sections for use in histological and histochemical preparations were cut at a thickness of 10μ whereas longitudinal sections for use in preparations for the study of peripheral innervation were cut at thicknesses of between 20μ and 50μ for mouse specimens and between 50μ and 100μ for human specimens. Sections of less than 30μ thickness were dried at room temperature. Sections of over 30μ in thickness were dried in a current of cold air to prevent tissue shrinkage resulting from rapid drying. Sections of 100μ in thickness were not mounted very satisfactorily in this way and it

was found more appropriate to mount the section after previous fixation (for details see appendix).

2. Spinal Cord

Spinal cord specimens of either cervical or lumbar enlargement, whichever was not being used for amino-acid analysis, were frozen as outlined for muscle. All spinal cord specimens were mounted for transverse sections. These were cut at 20u thickness and fixed directly on to glass slides.

ii) Staining Techniques

a) Histological Stains (Carleton 1967)

1) Haematoxylin and Eosin (Muscle and Spinal Cord)

Muscle was stained for 2 minutes in haematoxylin and 30 seconds in 1% eosin, whereas spinal cord specimens required 3 minutes in haematoxylin and 15-20 seconds in eosin.

2) Toluidine Blue (Spinal Cord)

- Fix fresh frozen sections 1 hour in formol saline
- Rinse well in distilled water
- Stain 1% toluidine blue in 1% borax at 56°C for 1 minute
- Differentiate in 95% ethanol
- Dehydrate clear and mount.

3) Combined Luxol Fast Blue Cresyl Violet (Spinal Cord)

- Fix fresh frozen sections 1 hour in formol saline
- Stain overnight in 1% luxol fast blue in 95% alcohol and 5% acetic acid
- Wash in distilled water
- Differentiate 5-10 seconds in 0.05% aqueous lithium carbonate
- Continue differentiating in several changes of 70% alcohol until grey matter can be distinguished from white

- Rinse well in distilled water
- Stain for 6 minutes in 1% cresyl violet to which 5 drops of 10% acetic acid per 30 ml were added before use
- Rinse in distilled water
- Differentiate in 96% alcohol
- Dehydrate, clear and mount.

4) Gomori-Trichrome (Muscle)

Staining was carried out as described by Engel and Cunningham (1963).

b) Histochemical Stains

1) NaDH-diaphorase (Muscle)

The method used was Barka's modification (Barka and Anderson 1963) of a method developed by Scarpelli (Scarpelli, Hess and Pearce 1958). A stock solution was made up containing all the constituents except NaDH (see appendix); water was substituted for the NaDH solution. This stock solution could then be stored indefinitely at -20°C. The solution was stored in aliquots of about 2 ml, thus a small amount of the solution could be thawed as required and NaDH was added just before use to give a final concentration of approximately 1 mg/ml. Slides were dried for 15 minutes to prevent the sections becoming detached from the glass, and were then incubated in the above solution for one hour at 37°C. Control sections were incubated in the same solution from which NaDH was omitted.

After incubation the slides were rinsed in distilled water and placed in acetone for 5 minutes to remove unreduced tetrazolium. The sections were mounted either in 100% (w/v) aqueous polyvinylpyrrodine medium, as described by Burstone (1957) or in glycerine mountant.

2) Calcium-activated Myosin ATPase (Muscle)

A method for the localization of phosphatases was first described by Gomori (1941) and the method for studying myosin ATPase activity in situ has been developed from this. The basis of the method used in this study was described by Padykula and Herman (1955) with the modification that the 0.1 M sodium barbitone solution has been substituted by 0.1 M Tris.

For demonstrating myosin-ATPase activity at pH 4.3, the sections were pre-incubated for 10 minutes in Walpole's acetate buffer then rinsed in distilled water before being treated in the same way as slides for regular myosin-ATPase staining at pH 9.4. They were incubated for 40 minutes into the substrate solution at 37°C (see appendix). The sections pre-incubated at pH 4.3 often proved more successful than regular ATPase staining at a pH of 9.4 as at the lower pH, a surface precipitate and the wrinkling of tissue often obscured the true fibre-type; this could have been prevented by using an incubation medium of pH 9.3 containing 0.04 M calcium (Brooke and Kaiser 1969).

c) Stains for Demonstrating Peripheral Motor Innervation

Specimens of mouse muscle were stained directly using the combined acetylcholinesterase/silver technique. Human muscle specimens, where it was necessary to assess whether the sample being studied did encompass the motor point, were first stained with acetylcholinesterase alone to demonstrate the presence of end-plates.

1) Acetylcholinesterase Stain (Muscle)

A stain for demonstrating acetylcholinesterase activity in muscle was described by Koelle and Friedenwald (1949) and there are several methods in use at present (Pearse 1972). The method used in this study is outlined in the appendix.

2) Combined Acetylcholinesterase/silver Impregnation Stain

Silver impregnation techniques for peripheral innervation studies were first demonstrated by Palmgren (1960). Methods combining the visualisation of acetylcholinesterase activity with silver impregnation of the axons are now in common use. The method used in this study most closely resembles the one described by Namba (Namba, Nakamura and Grob 1967). The method is given in detail in the appendix. It has proved successful for use on both human and mouse tissue although certain adjustments were made for sections of 100μ thickness.

CHAPTER 3 - CLINICAL AND GENETIC STUDIES

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- ii) Methods
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CHAPTER 3

TABLES

CHAPTER 3

I. Introduction

Mice of the wobbler colony have not proved difficult to breed; however, the number of homozygous affected animals born has been shown to be significantly below the expected 25% of all animals born to proven heterozygous carrier parents (Duchen, Strich and Falconer 1968). From the original data on this colony the low percentage of 'wobbler' mice cannot be explained by an enhanced pre-weaning mortality. It has, however, been suggested that the available data on the disease pathology of wobbler mice, could also be compatible with other mechanisms of pathogenesis, with the role of the genetic factor being a contributory one rather than exclusive (Andrews et al 1974). The neuronal vacuolarizations reported in degenerating anterior horn cells of wobbler mice is a non-specific phenomenon which accompanies abnormal states of widely varying aetiology. It has therefore been suggested that in the'wobbler' mouse the genetic factor may be responsible for an enhanced vulnerability or an unusual response to an infectious agent or perhaps an atypical response to some other form of insult. Such an explanation would to some extent resolve the problem of the number of affected animals born to mice believed to be heterozygous for the condition, without the necessity of using prenatal mortality of animals as the explanation. Mice of the wobbler colony consistently have reasonably large litters and a number of affected animals may survive for a year or more, hence prenatal mortality would appear unlikely in a condition which is relatively benign. No infectious agent has however been found in affected wobbler mice (Andrews et al 1974), hence on present data one has to conclude that murine lower motor neurone disease is a homozygous recessive disease, but for some reason at present not understood, the number of affected animals born is somewhat below the expected value.

The purpose of this study has been to reassess the data on this colony and to compare the results previously reported with those obtained whilst a wobbler colony has been maintained at the Western General Hospital in Edinburgh. These results of breeding experiments using the wobbler colony have also been compared with those of the ReJ/129 dystrophic colony, as murine muscular dystrophy is also a homozygous recessive disorder and both colonies were maintained under identical environmental conditions.

In the process of studying the wobbler colony, the survival of affected animals was also observed. As has been mentioned, wobbler mice may survive for over a year (Duchen, Strich and Falconer 1968), although such a life-span is the exception rather than the rule, the condition more generally terminates in death at about three to five months of age. Such variability in survival in a condition believed to be caused by a single gene defect is difficult to interpret. In the human, spinal muscular atrophy has been classified into several conditions depending on age of onset, mode of inheritence and the rate at which the disease progresses (Emery 1971). In order to determine whether the disease occurring in the mouse is a single pathological condition, the life-spans of all affected animals within each sib-ship have been compared. The variability of life-spans in affected mice from the dystrophic colony were also observed, although in general the maximum life expectancy of dystrophic mice is not believed to be greater than six months (Michelson, Russel and Harman 1955).

The animals affected with both murine dystrophy and lower motor neurone disease are small in size compared with the healthy animal and this is due to small skeletal size and not solely to reduced muscle mass. It has, however, been shown that in the case of the dystrophic animal birth weight is not indicative of the likelihood of any animal being

affected (Harman et al 1963). Similar experiments have not been carried out on mice of the wobbler colony and it is not known whether birth weight or the growth rate of these animals up to a point of showing clinical symptoms could act as an indicator of early changes. In this study, therefore, birth weights and growth rates of mice from the wobbler, C57/BL, and dystrophic colonies have been observed.

During the course of maintaining the wobbler colony a phenomenon not previously reported was noticed in some of the mice. Several animals showing no disease symptoms early in life, developed a severe hind-limb paralysis at between four to five months of age. Hind-limb paralysis has been reported to occur in some mice with lower motor neurone disease, but onset was three weeks post-natally and the normal features associated with wobbler mice were also present in these animals (Harris 1975), whereas, in mice with the late onset of hind-limb paralysis the fore-limbs did not appear to be greatly affected. The clinical symptoms and genetics of the Wr/HLP mouse have been studied and will be discussed in the light of all the unusual findings associated with the wobbler colony.

II. Material and Methods

i) Material

The material on which the entire study is based has been obtained from two mutant mouse colonies, the ReJ/129 dy colony and the wobbler colony. Control data has been derived from studying mice of the C57/BL colony, as these animals are known to be free of neuromuscular disease. Although numerous strains of mice could have been used as control material the original 'wobbler' mutation occurred in an inbred strain of C57/BL Fa mice and was transferred to a non-inbred C57/BL background (Duchen, Strich and Falconer 1968). The study has been concerned primarily with mice of the wobbler colony as many of the clinical aspects of the disease in these

mice have not been clearly established. Clinical and genetic data on mice of ReJ/129 dy variety is readily available but some studies have also been carried out within this programme, primarily to monitor environmental conditions and to act as a control colony for genetic studies on mice of the wobbler colony.

ii) Methods

a) Animal House procedure

All the mice used in the study have been maintained under identical environmental conditions. Mice were caged with six to eight animals per cage, affected animals however were kept with only one or two littermates.

Wood shavings were used as the bedding and nesting material. The bedding was changed twice weekly, the cages were washed and autoclaved at least once a month. The animal diet consisted of Spratt's small animal diet no 1 (Spratt Patent Ltd, Barking, Essex), a supplement of grain mixture consisting of maize and oats was given twice weekly. A slice of fresh cucumber was placed daily into all cages where affected animals were present in order to prevent animals from becoming dehydrated, when they had difficulty in reaching the water bottles. Water was changed daily and the water bottles were washed thoroughly once a week.

The room temperature was maintained at between 21-25^{o}C. The animals were not however provided with an artificial light cycle; in winter, daylight hours were supplemented with electric lighting in the afternoons. The cages were checked daily for births, deaths, etc.

Ten breeding pairs for each of the mutant colonies and three C57/BL pairs were maintained at any one time. Both murine dystrophy and lower motor neurone disease are homozygous recessive disorders, hence matings were set up using littermates of affected animals. The breeding pairs were tested for heterozygocity by allowing each pair to have a minimum

eighteen sibs. If none of the animals from these sibships showed disease symptoms by six weeks of age, the breeding pair was discarded. Originally when such a pair was discarded all its offspring were also removed. However, after the original observation that some mice in the wobbler colony began to show abnormal features at four months of age all animals of this colony were observed until they were a minimum of six months old. A number of animals showing symptoms of hind-limb paralysis were also used for breeding purposes. These matings will be described in detail under the section covering genetic studies.

A number of other mouse colonies, eg Peruvian, Bar Harbor obese, Balb C, Swiss white, CBA/CA, were kept under identical environmental conditions. No animals from these colonies showed any symptoms similar to those observed for the Wr/HLP mice, nor was any infection noted in the animal house during the period that the wobbler colony was under observation.

b) Clinical Studies

The age of onset of clinical symptoms of disease was recorded for mice of both the wobbler and dystrophic colonies. All animals of the wobbler colony were also observed at regular intervals for symptoms of hind-limb paralysis. The age of onset of this syndrome in a mouse was also recorded. The progress of the disease and life-spans of all affected animals were also noted.

Animals from a total of eight litters born to mice of the wobbler, C57/BL, and dystrophic colonies were weighed daily from birth to weaning. Four wobbler litters and two from each of the other colonies were studied. Also a number of animals were weighed twice weekly from weaning until six months of age. The animals chosen for the second part of the weighing study were invariably the younger sibs of animals which were affected with hind-limb paralysis. C57/BL mice were also weighed as controls.

As muscle wasting invariably leads to some loss in weight it was important to establish whether weight loss was due to loss of muscle bulk or whether it preceded the onset of clinical symptoms.

Several other tests were devised in the hope of establishing a means of recognising Wr/HLP mice before the onset of clinical symptoms. Mice were observed climbing up the trellising of the cage when it was held vertically. Attempts at establishing muscle strength were made by hanging a mouse on the edge of a tall beaker and timing how long it clung before falling. Mice were also twisted vigorously by the tail and then the time that the animal required to recover a normal posture was recorded. Such tests have previously been useful in distinguishing both wobbler and dystrophic mice from their healthy littermates before the clinical symptoms are fully developed.

c) Genetic Studies

The data on the dystrophic colony was obtained over a period of two and a half years. Records of all matings were maintained together with the offspring that each produced. The number of affected animals and the pre-weaning mortality was carefully recorded.

The wobbler colony was set up at the Western General Hospital in Edinburgh in 1973 from eight pairs donated by Muscular Dystrophy Research Laboratories in Newcastle-upon-Tyne. This colony has, therefore, now been maintained for two and a half years and similar data was recorded as for the dystrophic colony.

In October 1973 some mice with an unusual hind-limb weakness were noticed in the wobbler colony. As the disease did not resemble any that result from an infectious agent and no environmental cause for the disorder could be established, certain breeding experiments outlined in Table 3.1 were set up. A full scale breeding programme could not be carried out as there was not enough material available at the time that this study was begun.

TABLE 3.1

The breeding experiments set up to test the genetics of Wr/HLP mice

Type of Mating	No	Total births	Pre-weaning Mortality	Wobbler offspring	Wr/HLP offspring
Wr/HLP x ♀ + Wr/HLP	5	-	-	-	-
Wr/HLP x ♀ + WrWr/Wrwr	3	38	3	-	7
Wr/HLP x ♀ + Wrwr	3	54	1	16	-
WrWr/Wrwr x ♀ + Wr/HLP	3	27	-	-	2
Wrwr x ♀ + Wr/HLP	1	12	2	-	-
WrWr x ♀ + Wr/HLP	1	18	2	-	11

III. Results

i) Clinical studies

The most characteristic early manifestations of murine dystrophy are the dragging of the hind legs, followed by a progressive paralysis of the hind-limbs; the fore-limbs are not affected to the same extent and movement is maintained by the mouse using its fore legs. The pectoral muscles become degenerate and the mouse assumes a hunchback appearance. Often the eyes become infected and close. This is probably as a result of poor grooming. A dystrophic mouse is illustrated in Plate 1.

The clinical symptoms of the wobbler mouse arise at about the same age as those of the dystrophic mouse, between three and four weeks after birth. The earliest recognisable feature being that of a fine tremor followed by muscle wasting primarily in the fore-limbs. The mouse is seen to have an unsteady gait and becomes unable to extend the fore-paw at the wrist, hence it is ultimately seen to walk on the dorsum of the paws. Both wobbler and dystrophic mice are seen to be smaller than healthy littermates from about the fourth week of age, but the disparity in size is somewhat greater in wobbler mice. The wobbler mouse is illustrated in Plate 2 .

It was mentioned in the introduction to this study that mice showing a hind-limb paralysis were observed in the wobbler colony. The earliest symptoms of this syndrome arise four to five months after birth. The mouse develops a slight tremor and is seen to have difficulty supporting itself on its hind-limbs. At very early stages of the disorder it is difficult to ascertain whether in fact the animal is diseased as there is a day to day variation in the severity of both tremor and of the visible weakness of the hind-limbs. As the disease progresses the abnormality becomes very striking with considerable wasting of the pelvic muscelature and progressive paralysis of the hind-limbs; occasionally paralysis of

the tail is also seen with the tail being permanently inclined forward. The fore-limbs remain comparatively unaffected particularly during the early stages of the disease process. However at about eight months, wasting of pectoral muscles can be seen and in the terminal stages of the disease there is in fact little to distinguish the Wr/HLP mouse from a wobbler mouse of about the same age, although paralysis of tail muscles in the wobbler mouse is unusual. Wr/HLP mice are illustrated in Plate 3.

The animals weighed at birth from each of the three colonies were between 1.2-1.8 grams. The average weight for animals from an individual litter depended primarily on litter size rather than on the colony of origin. The growth rate of mice from birth to weaning also depended primarily on litter size: it was found that during this part of the study several mice from wobbler litters died before weaning or very soon after, and although no wobbler mouse was diagnosed in any of the litters studied, the mice that died may have been affected. Wobbler mice do not stand up to stress very well and the daily weighings would have amounted to a form of stress. No pre-weaning deaths were encountered in litters from the C57/BL and dystrophic colonies. The mice from the wobbler colonies that did die appeared to lose weight one or two days prior to death. A much larger number of litters would be required to establish exactly the stage in their development at which growth becomes obviously stunted. However, at weaning the affected wobbler mouse is often the smallest of a litter, the difference in weight between healthy littermates and wobbler mice are rarely greater than 1.5 grams. At four weeks, however, affected animals weigh 6-8 grams, whereas healthy animals weigh between 16 and 19 grams. At four months affected animals weigh around 15 grams, and healthy littermates and C57/BL mice of the same age weigh between 25-35 grams. The differences are not found to be as great for the dystrophic colony.

Many animals from one wobbler colony were weighed twice weekly, three of which developed the late onset hind-limb paralysis. When the weights of these animals were compared with those of healthy littermates and C57/BL animals it was found that all fell well within the normal range for any given age, until the point at which muscle wasting was clearly visible. When growth rates were compared for the entire period that the animals were studied healthy animals showed a significant correlation between age and weight which was not found for Wr/HLP mice. When only the period after symptoms had occurred in Wr/HLP mice was used for statistical analysis, a significant correlation was found between the loss in weight and increase in age; no similar result was found for the healthy animals. The results are given in Table 3.2. These results indicate that the loss in weight is primarily due to loss of muscle bulk. The weight of mice could not be used as an indicator of the likelihood of a particular animal developing late onset muscle disease.

The attempts to assess developing muscle weakness by considering the climbing ability or clinging ability of the animal proved of little value as even affected animals were able to climb quite successfully. The hanging experiment proved only that the animals do not like being subjected to this form of stress and it is difficult to get the animal to cling to the edge of the beaker. Furthermore no two hanging times for a single animal showed any correlation.

One test, however, did prove useful, in confirming that an animal showing the earliest symptoms of weakness was in fact affected with the hind-limb paralysis disorder. After a mouse is vigorously twisted by the tail it invariably takes 15-30 seconds to recover and to begin using its limbs normally. However, it was found that Wr/HLP mice may take up to two minutes to recover and as they do so they often move backwards. This sort of backward movement is not frequently seen in healthy animals.

TABLE 3.2

Correlation between the increase in weight with age in Wr/HLP and healthy controls

		Age in days	r	P
Healthy Animals	1	58 - 214	0.8159	< 0.001
(WrWr/Wrwr)	2	58 - 189	0.8951	< 0.001
	3	58 - 189	0.9455	< 0.001
Affected Animals	1	58 - 189	0.1321	NS
(Wr/HLP)	2	58 - 214	-0.0432	NS
	3	58 - 214	-0.0994	NS
Healthy Animals	1	154 - 214	-0.6138	< 0.02
	2	154 - 189	-0.4527	NS
	3	154 - 189	0.8231	< 0.01
Affected Animals	1	154 - 189	-0.8767	< 0.01
	2	154 - 214	-0.8764	< 0.01
	3	154 - 214	-0.8832	< 0.01

In neurological diseases of mice the hind-limbs frequently become flexed and adducted instead of extended when the animal is raised by the tail. Although this is occasionally true for Wr/HLP mice, however, the test of lifting the animal by the tail cannot be used as a diagnostic procedure for the Wr/HLP mouse.

Several tests have been devised in an attempt to recognise Wr/HLP mice pre-clinically, particularly to enable more extensive genetic studies to be carried out; however none of the tests have been useful in establishing a method for early diagnosis, though some of the tests have proved useful in confirming early clinical symptoms.

ii) Genetic Studies

The results of the genetic studies are given in table 3.3. The results indicate that wobbler mice account for only 18.6% of total births from heterozygote breeders, however dystrophic mice account for 24.4% which is remarkably close to the 25% expected for a homozygous recessive disorder. The low numbers of wobbler mice cannot be accounted for by an increased pre-weaning mortality as the total percentage of pre-weaning deaths is the same for both wobbler and dystrophic colonies. These results confirm Falconer's earlier observations on the wobbler colony (Duchen, Strich and Falconer 1968).

The observations of the life-span of wobbler mice have shown that no genetic factor is involved in determining the rate of progress of the disease process in affected mice. It would appear that all affected animals undergo trauma shortly after weaning; those that do survive may live up to twelve months. Affected animals from an individual litter usually live for nearly the same number of days but this is not true of affected animals from an entire sibship. It would therefore appear that some environmental factor, as well as the litter size, determines the life-span of affected animals. Similar findings were also made on the

TABLE 3.3

The results of genetic observations on the wobbler and dystrophic colonies

Colony	Total no. of breeding Pairs	% hetero-zygote Pairs	Total births	Total pre-weaning mortality no.	%	Total affected animals no.	%
Wobbler	56	37.6	1105	111	10.0	185	18.6
Dystrophic	47	44.7	723	73	10.1	162	24.4

In the wobbler colony the number of affected animals is significantly different from the expected value $x^2 = 35.08$ $P < 0.001$

TABLE 3.4

Data on the genetics of Wr/HLP

Type of Mating	No.	Offspring			
		WrWr + Wrwr	wrwr	Wr/HLP	Total
Wrwr x Wrwr	17	750	169	22	941
Wr/HLP x WrWr	1	5	-	11	16
Wr/HLP x Wrwr	3	37	16	-	53

dystrophic mice, although these mice were not as vulnerable at weaning, whilst their maximum survival time was no greater than eight months.

Attempts to study the genetics of Wr/HLP mice have proved difficult because of the late onset of this disease and the small litters produced by affected animals. The results are given in Table 3.4.

The results have to be assessed using three hypotheses: firstly that the disease is an unusual manifestation of that occurring in wobbler mice; secondly that the heterozygote 'Wrwr' occasionally shows symptoms of a lower motor neurone disease which manifests itself in late onset hind-limb paralysis; and thirdly that the wobbler and hind-limb paralysis diseases are not connected genetically.

If the first hypothesis were true, the percentage of affected animals would increase to 22%. However, this is an unlikely explanation as Wr/HLP mice have occurred in litters where no wobbler mice have been diagnosed. Also an affected Wr/HLP female mated with a male, homozygous for the dominant 'Wr' gene has resulted in offspring with hind-limb paralysis.

There is evidence, however, that the second hypothesis may be true. A known heterozygote male when mated with a female showing hind-limb paralysis resulted in offspring showing the normal symptoms of wobbler mice. Further evidence for this hypothesis comes from a particular pedigree shown in Table 3.5. From this pedigree it would appear that the probability of hind-limb paralysis being associated with the 'Wrwr' genome is 72.3%, whereas the probability that this is not so is 27.7%. The results were calculated by the Bayesian method. Although the results are by no means conclusive because of the very small numbers involved, they are useful as an indicator for further study.

Not all heterozygote animals however show clinical manifestations. Moreover, histological studies on muscle and peripheral nerve (see chapters 5 and 6) confirm that many are free from any gross degenerative

TABLE 3.5

Evidence to support the hypothesis that Wr/HLP mice are carriers of the 'wr' gene

PR12 - The animals mated were obtained from	PR 6	x	PR 8
no. of offspring		57	
no. of wrwr		-	
no. of Wr/HLP		-	

PR26	PR 12	x	PR 12
no. of offspring		39	
no. of wrwr		-	
no. of Wr/HLP		1	

PR35	PR 26	x	PR 26/HLP
no. of offspring		8	
no. of wrwr		-	
no. of Wr/HLP		-	

PR46	PR 6	x	PR 35
no. of offspring		45	
no. of wrwr		5	
no. of Wr/HLP		3	

PR12

Genotypes	Wrwr x Wrwr	Wrwr x WrWr	WrWr x WrWr
Prior Probability	$4/9$	$4/9$	$1/9$
Posterior Probability	$(\frac{3}{4})^{57}$	$(1)^{57}$	$(1)^{57}$
Joint	0.0000000336	0.4444	0.1111
Rel. Probability is	0	0.80	0.20

$\therefore$ Probability that any offspring is Wrwr = $\frac{1}{2}(0.80) + 0(0.20) = 0.40$

PR26

Prior Probability	0.16	0.48	0.36
Posterior Probability	$(\frac{3}{4})^{39}$	$(1)^{39}$	$(1)^{39}$
Joint	0.00000215	0.48	0.36
Rel. Probability	0	0.57	0.43

$\therefore$ Probability that any offspring is Wrwr = $\frac{1}{2}(0.57) + 0(0.43) = 0.285$

PR35

Genotype	Wr/HLP(= Wrwr)	Wr/HLP($\neq$ Wrwr)
Prior Probability	0.285	0.715
Posterior Probability	$0.285((\frac{3}{4})^8-(\frac{1}{4})^8)$	$0.285(1-(\frac{1}{2})^8)$
/8 mice at least 1 is Wrwr/	$\propto 0.715(1-(\frac{1}{2})^8)$	+ 0.715(0)
	= 0.7407	= 0.2832

$\therefore$ Rel. Probability

Wr/HLP = Wrwr is

$$\frac{0.7407}{1.0239} = 72.3\%$$

Wr/HLP $\neq$ Wrwr

$$\frac{0.2832}{1.0239} = 27.7\%$$

changes other than those associated with ageing. It may therefore be the case that an epistatic gene is involved and the loss of the epistatic gene leads to the disease phenomenon.

All mice which have had offspring with hind-limb paralysis disease were directly related to Pair 8 which was one of the original pairs obtained from Newcastle. In addition 47.5% of the total Wr/HLP mice diagnosed were obtained from five brother/sister matings, whereas the remaining 52.5% of the mice were born from a further ten matings. Inbreeding would enhance any genetic abnormality such as the loss of an epistatic gene. Perhaps epistatic interaction could be useful in explaining the wide spectrum of severity of the disease in wobbler mice as well as the occasional occurrence of gross hind-limb involvement in murine lower motor neurone disease (Harris 1975).

The attempts to breed two Wr/HLP mice have proved unsuccessful, although such matings would have been very useful in determining the true genetic nature of this disease.

IV. Discussion

The study of the wobbler mouse from clinical and genetic aspects has confirmed earlier findings (Duchen, Strich and Falconer 1968), that the numbers of affected animals born falls short of the 25% level expected for a homozygous recessive disorder.

This shortfall cannot be accounted for by post-natal mortality, hence the deficiency must presumably be ascribed to pre-natal mortality. As the wobbler mice do show a lot of variability in the severity of the disease, perhaps some mice are very grossly affected and die before birth. It is, however, difficult to interpret the findings that diseased animals may live up to a year but over fifty per cent die before the age of two months. The wobbler mouse was firstly suggested as a model for human spinal muscular atrophy of the Werdnig-Hoffmann type, but was later

considered more in terms of an analogue for the Kugelberg-Welander disease because of the slow progress of the disease in the mouse. It would appear, however, that in the mouse this disease varies widely in severity. This does not appear to be genetically determined and for this reason perhaps it would be more useful purely to consider the mouse as a useful tool in the study of lower motor neurone disease rather than to consider it as an analogue of any human disorder.

The disease occurring in the Wr/HLP mouse creates further problems in interpretation. The results of the preliminary genetic investigations on this disorder suggest that Wr/HLP mice are manifesting carriers of the 'wr' gene. However the clinical symptoms in this disorder are very different from those of the wobbler mouse in that not only is the disease late in onset but different muscles appear to be involved. Manifesting carriers do occur in human Duchenne dystrophy (Moser and Emery 1974); however this is an x-linked condition and the variable abnormalities seen in these carrier females ranging from a barely raised creatine phosphokinase level to that of considerable weakness in hind-limb muscles is easily explicable by the Lyon hypothesis (Lyon 1962).

There are examples in human diseases where the heterozygous carriers of a homozygous recessive disease show symptoms. Sickle cell haemoglobin occurs to a variable extent in carriers of the sickle cell gene and the amount of sickling is dependent on such factors as the blood group of the patient and the amount of foetal haemoglobin present. Hence the system of epistatic genes, which form an integral part in the genetics of blood groups, determines the range of symptoms seen in the heterozygote (Giblett 1969).

Late onset hind-limb paralysis has not previously been described in mice of the wobbler colony, although Harris' (1975) study has demonstrated

a few wobbler mice with gross hind-limb involvement. From results of electrophysiological studies he has suggested that the phenomenon is due to an unusual distribution of muscle weakness rather than a separate disease entity. Although only one such mouse has been found in our colony it is possible that the same epistatic gene is involved in the late onset diesase and the unusual distribution of muscle weakness in the occasional wobbler mouse.

There is, however, a further possible explanation for Wr/HLP mice which cannot be entirely disregarded. A form of hind-limb paralysis similar to that shown in the Wr/HLP mouse has been shown to occur in mice as a result of Type C RNA virus infection. This infection, resulting in lower motor neurone disease, has an age of onset in the wild mouse of about four months. Paralysis is of the hind-limbs, the fore-limbs remaining relatively unaffected. This disease, however, is of a much shorter duration than that occurring in Wr/HLP (Gardner et al 1973). It is unlikely that the same virus would be responsible for the two diseases, but further investigations are required to eliminate the possibility of a viral agent being involved in the disease of Wr/HLP mice.

The data given in this study is compatible with the hypothesis that the Wr/HLP mouse is a possible carrier of the 'wr' gene and that a possible loss of an epistatic gene results in the manifestation of disease symptoms.

PLATE 1

a) An affected dystrophic mouse with a healthy littermate
Age 4 months

b) A mildly affected dystrophic mouse
Age 2 months

PLATE I

a

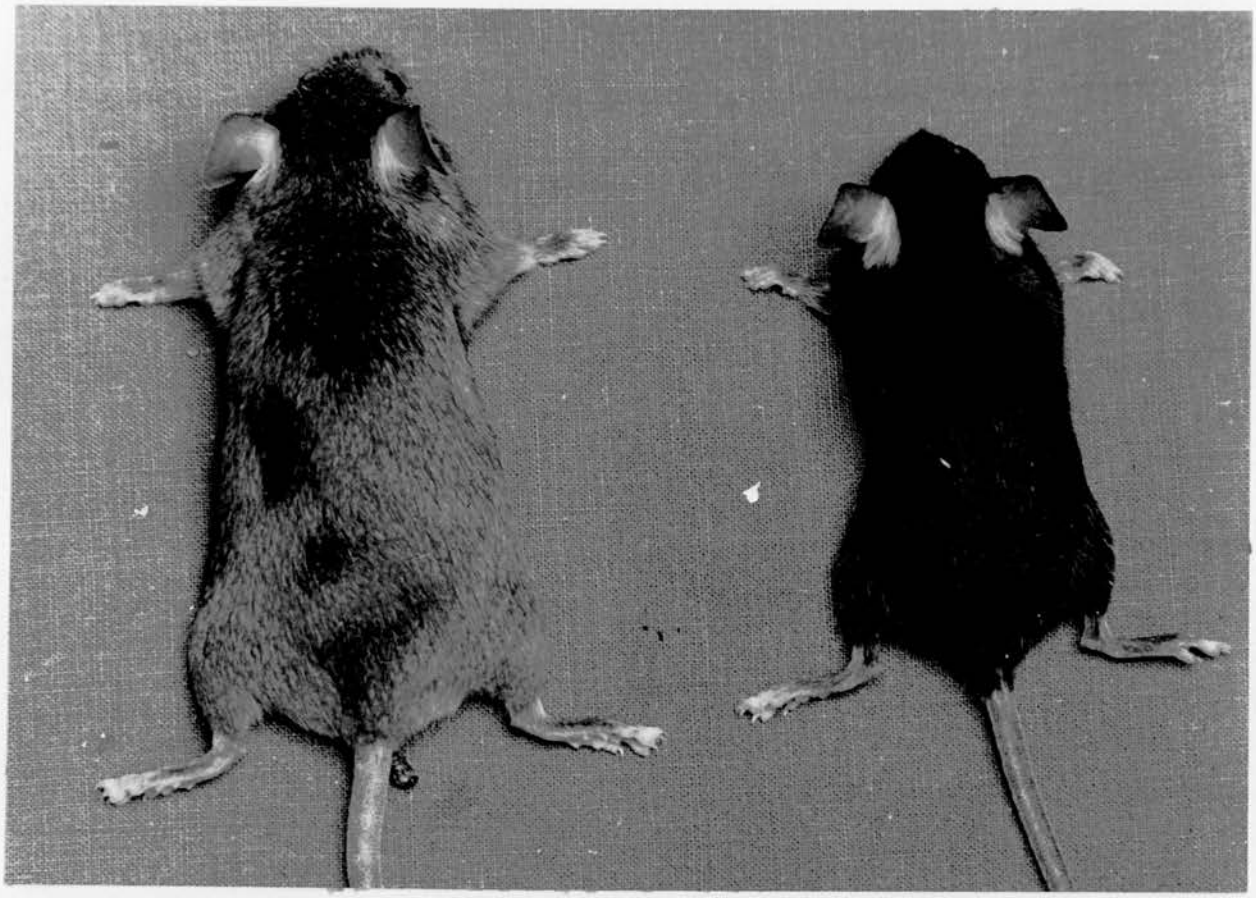
b

PLATE 2

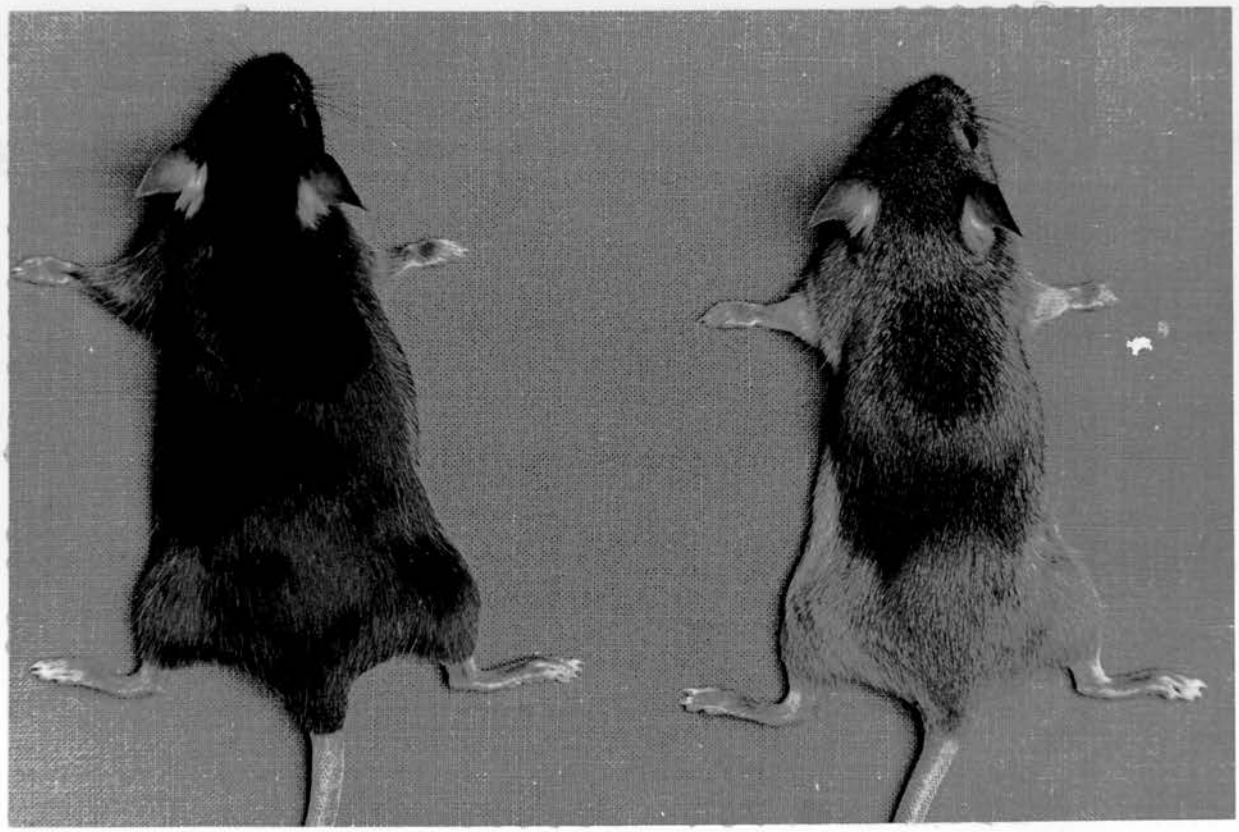
a) An affected wobbler mouse with a healthy littermate
Age 2 months

b) An affected Wr/HLP mouse with a healthy littermate
Age 6 months

PLATE 2



a

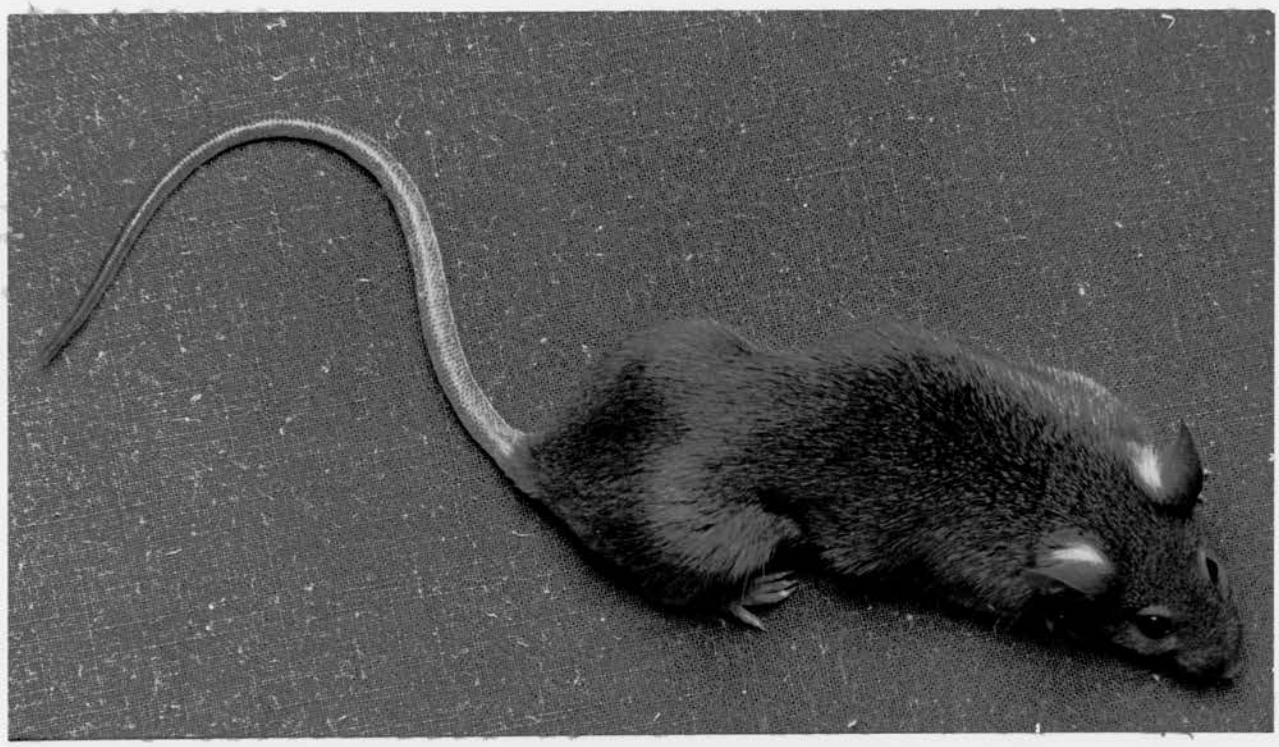


b

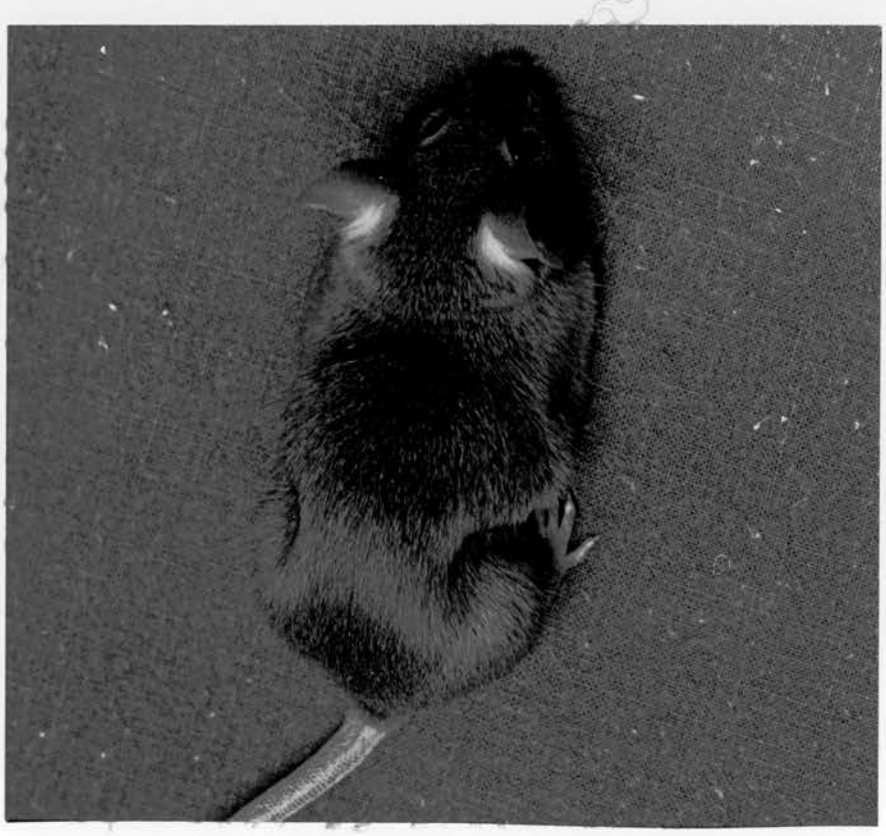
PLATE 3

a) and b) Both show a Wr/HLP mouse. Age 6 months. The loss of muscle from the pelvic area is clearly visible.

PLATE 3



a



b

CHAPTER 4 - FREE AMINO-ACIDS IN MOUSE SPINAL CORD

I. Introduction

II. Material and Methods
- i) Material
- ii) Methods
  - a) Preparation of samples
  - b) Amino-acid analysis
  - c) Calculation of amino-acid concentrations
  - d) Statistical analysis

III. Results
- i) Coefficient of variation for the technique
- ii) Coefficient of variation of amino-acids
- iii) Analysis of amino-acid concentrations
  - a) cervical region
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- iv) Analysis of amino-acid concentrations in each of the mutant colonies
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IV. Discussion

CHAPTER 4

TABLES

CHAPTER 4

I. Introduction

Free amino-acids are found in most tissues of the body as they serve as precursors for, and are the products of, protein metabolism. There are, however, certain tissues where a number of amino-acids are found in concentrations considerably higher than would be expected if the function was purely to participate in the amino-acid pool for protein metabolism. The tissues of the central nervous system have been found to contain particularly high concentrations of certain amino-acids such as glycine, aspartate, glutamate and glutamine (Tallan 1962). These amino-acids are also found in varying concentrations in other tissues, however, γ-amino-butyric acid (GABA) which appears in concentrations of between 1.5 and 6.0 micro moles per gram wet weight of brain tissue of all vertebrates is only found as a constituent of the nervous tissue. This would suggest that GABA and perhaps other amino-acids such as glycine and aspartic acid have a specific function within the central nervous system.

The development of the technique of iontrophoresis, which has enabled a drug to be placed directly into the extracellular environment of a single nerve cell (Nastuk 1953) has resulted in the identification of numerous pharmacological substances which influence neuronal excitability (Curtis & Watkins 1960, Curtis 1964). Two groups of structurally related amino-acids have been shown to influence neuronal activity when administered in the proximity of the cell. Several monocarboxylic ω-amino-acids related to glycine have a potent depressant effect on neuronal excitability (Curtis & Watkins 1960) whereas a number of acidic amino-acids related to aspartate have been shown to exhibit a pronounced excitatory effect on nerve cells in various regions of the brain and spinal cord (Curtis, Phyllis & Watkins 1960, Curtis & Watkins 1963). A table indicating the structure-

activity relationship of some excitant and depressant amino-acids is given in Table 4.1. Not all the amino-acids shown in the Table are found in the nervous tissue, whereas others only occur in minute concentrations. Those that are normally found in such concentrations that they can easily be measured have been indicated. They are, firstly, taurine, glycine and GABA which, when topically applied in the vicinity of the neurone have a depressant effect on that cell, and secondly, aspartate and glutamate which on iontrophoretic administration result in neuronal excitation. Glutamine has also been found in high concentration in the brain and spinal cord (Tallan 1962, Graham et al 1967). Glutamine does not influence neuronal excitability, however the metabolism of glutamine and glutamic acid are closely related.

There is some evidence that the five amino-acids - glutamic acid, aspartic acid, glycine, taurine and GABA - may function as transmitters within the nervous system. Nevertheless, the mere demonstration of a pharmacological substance having an effect on neurones does not irrevocably establish it as a transmitter.

In 1966 Werman set out certain criteria which he considered as the essential requirements to be satisfied by any substance before it could be defined as a neural transmitter. These are that the compound must be present in the nerve ending; the corresponding synthesizing system should be present in the neurone where the transmitter is believed to function; there ought to be a mechanism for termination of action of the compound; the compound should be released into the extracellular fluid when the neurone has been stimulated and, lastly, the action of the transmitter suspect must be identical in every way to that of the neural transmitters.

As GABA is an amino-acid found uniquely in the central nervous system, its significance has been intensively investigated (Curtis, Phyllis

TABLE 4.1 - STRUCTURE-ACTIVITY RELATIONSHIPS OF SOME EXCITANT AND DEPRESSENT AMINO-ACIDS ON CNS NEURONES

The table is taken from - J.W. Phillis 1970 - The Pharmacology of the Synapse

Inhibitory monocarboxylic and related amino acids	Structure	Relative potency	Corresponding excitatory amino acids	Structure	Potency
*Glycine	$H_2N.CH_2.COOH$	- -	DL-Aminomalonic acid	$HOOC.CH(NH_2).COOH$	+
β-Alanine	$H_2N.(CH_2)_2.COOH$	- - -	*L-Aspartic acid	$HOOC.CH_2.CH(NH_2).COOH$	+ + +
γ-Aminobutyric acid	$H_2N.(CH_2)_3.COOH$	- - -	*L-Glutamic acid	$HOOC.(CH_2)_2.CH(NH_2).COOH$	+ + +
δ-Amino-n-valeric acid	$H_2N.(CH_2)_4.COOH$	- -	DL-Aminoadipic acid	$HOOC.(CH_2)_3.CH(NH_2).COOH$	+
ε-Amino caproic acid	$H_2N.(CH_2)_5.COOH$	-	DL-Aminopimelic acid	$HOOC.(CH_2)_4.CH(NH_2).COOH$	+
*Taurine	$H_2N.(CH_2)_2.SO_3H$	- - -	*L-Cysteic acid	$HO_3S.CH_2.CH(NH).{}_2COOH$	+ + +
3-Aminopropane sulphonic acid	$H_2N.(CH_2)_3.SO_3H$	- - - -	DL-Homocysteic acid	$HO_3S.(CH_2)_2.CH(NH_2).COOH$	+ + + +
Glycocyamine	$H_2N.C(:NH).NH.CH_2.COOH$	- - -			
β-Guanidinopropionic acid	$H_2N.C(:NH).NH.(CH_2)_2.COOH$	-			
γ-Guanidinobutyric acid	$H_2N.C(:NH).NH.(CH_2)_3.COOH$	0			

* indicates the amino acids estimated in this study.

& Watkins 1959, Roberts & Eidelberg 1960, Tsukada et al 1963, Kravitz 1967). The action of GABA on spinal motor-neurones was shown not to be affected by strichnine (Curtis, Phyllis & Watkins 1959). It was believed that inhibition by GABA was not accompanied by membrane hyperpolarisation (Curtis & Watkins 1965); this led to the suggestion that GABA had a non-specific depressant activity on motor-neurones. It has since been demonstrated that GABA inhibition is accompanied by membrane hyperpolarisation, however not strychnine but picrotoxin and bicuculline are the specific inhibitors of GABA activity (Curtis, Hosli & Johnston 1967, Precht & Yoshida 1971, Curtis et al 1970). As strychnine effectively prevents the action of inhibitory transmitter on motor-neurones (Curtis & Watkins 1965), it is unlikely therefore that GABA is the transmitter responsible for hyperpolarising strychnine antagonised post-synaptic potentials of motor-neurones. Evidence is accumulating to suggest that GABA is not the primary inhibitory transmitter in the spinal cord; however it does appear to function in that capacity in other areas of the brain. Particularly well substantiated is the evidence that GABA functions as a transmitter in the substantia nigra, where the concentration of this amino-acid is higher than in any other area of the brain (Obata 1972, Okada & Hassler 1973). It has been demonstrated that cerebral cortex takes up radioactive GABA from external media leading to a large net increase of the amino-acid in the tissue (Inverson & Neal 1968).

As GABA is important in the central nervous system it is unfortunate that by the methods used in the present study it was impossible to estimate GABA concentrations in the spinal cord of mice. The purpose of the study, however, has been the investigation of concentrations of amino-acids in the spinal cord of wobbler mice, where the disease process is known to result in the loss of motor-neurones. GABA is the amino-acid least likely to be affected by such a disease process. It has been

demonstrated that mechanical damage to the cord which results in loss of interneurones affects the concentration of various amino-acid transmitter suspects but does not affect GABA concentration (Davidoff et al 1967).

There is plenty of evidence to show that glycine functions in the cord as an important inhibitory transmitter. Glycine distribution within different areas of the cord conforms with that expected for a post-synaptic inhibitory transmitter (Graham et al 1967). A high affinity uptake system for glycine has been demonstrated (Neal & Pickles 1969), and it has been suggested that this high affinity uptake system significantly contributes to the inactivation of synaptically released glycine at spinal inhibitory synapses (Johnston & Inversen 1971). The accumulation of glycine in the region of the synaptosomes was observed autoradiographically (Matus & Dennison 1972). The uptake of radioactive glycine by the cord has been shown to be two to six times that of the telecephalon (Aprison & McBride 1973). There is also little doubt that the action of glycine on the motor-neurones is identical with that of the natural occurring inhibitory transmitter (Werman, Davidoff & Aprison 1968, Phyllis 1970).

It was demonstrated by Davidoff in 1967 that glycine is associated with the interneurones in the spinal cord. When the spinal cord is damaged by surgical procedures, glycine, glutamic acid and aspartate concentrations are reduced; however, neither GABA nor glutamine are affected. The concentration of both glycine and aspartate have been shown to be directly correlated with the number of interneurones in the cord. A comparative study of various areas of spinal cord from seven different vertebrates has revealed that there is a higher concentration of glycine in regions of the cord supplying the nerves to limb musculature. In animals such as the snake where there are only vestigal limbs the concentration of glycine is the same along the whole length of the spinal cord, whereas the bird has a high concentration in the cervical

cord with high lumbar glycine being found in jumping animals such as the caimen (Aprison, Shank & Davidoff 1969). In order to establish glycine as the inhibitory post-synaptic transmitter in the spinal cord, in accordance with criteria set down by Werman, all that would be necessary would be the collection of the compound at the synaptic region in amounts proportional to the rate and extent of stimulation of the pre-synaptic nerve. Electrical stimulation *in vitro* does result in amino-acid release (Hammerstad, Murray & Cutter 1971). Glycine as well as GABA, taurine and glutamic acid are all released and there appears to be no regional specificity. Such an experiment cannot be entirely satisfactory as selective stimulation of a single synaptic pathway is almost impossible, hence, considering that many pathways are stimulated, the results of release experiments are difficult to interpret. The evidence that glycine is of considerable importance in the spinal cord is, however, sufficient to warrant an interest in its possible significance in lower motor neurone disease.

L-aspartic acid and L-glutamic acid have been postulated as the excitatory transmitters. Aspartate distribution in the different areas of the cord is in line with that expected for a polysynaptic excitatory transmitter (Graham *et al* 1967). Glutamate distribution is compatible with it being an excitatory transmitter at the terminals of primary afferent fibres (Duggan & Johnston 1970). A high affinity uptake system has been demonstrated for these two amino-acids (Logan & Snyder 1971, 1972). Although there is further circumstantial evidence to suggest transmitter function for aspartate and glutamate, the excitatory amino-acids have not been as thoroughly investigated as the possible inhibitory transmitters. Aspartate has been shown to be associated with interneurones (Davidoff *et al* 1967) and its concentration in cat spinal cord rises markedly during tetanus intoxication, possibly due to the increased

activity of excitatory spinal interneurones (Balcar & Johnston 1973). The activity of glutamate at crustacean synapses has been more extensively studied than its function in the vertebrate nervous system and there is evidence that in crustacea, glutamate does function in an excitatory capacity (Johnston 1972). It is possible that the same is true for neurones in the spinal cord. Glutamic acid diethylester reversibly blocks excitation evoked in the spinal cord and other areas of the brain and it is a more effective antagonist of glutamate induced neuronal activity than of excitation produced by other amino-acid transmitters (Haldeman & McLennan 1972).

Taurine is the only other amino-acid found in high concentrations in spinal cord and shown to influence neuronal excitability (Curtis & Watkins 1960). This amino-acid is also found in fairly high concentrations in other tissues and this is especially true for the mouse. Nevertheless, taurine has been found in nerve-ending particles isolated by centrifugation and within the particles there was also an enzyme for taurine synthesis (Kaczmarek, Agrawal & Davison 1970). Furthermore, taurine uptake by brain slices resembles that for GABA and glycine and it would also appear that taurine is released into extracellular fluid on stimulation of the rat cortex (Kaczmarek & Davison 1972). There is little further evidence; the distribution of taurine has not been studied and no specific inhibitor of taurine activity has been found. It has been suggested that taurine may function as a modulator influencing neuronal activity but not dependent on synaptic transmission. On present evidence, however, this hypothesis has little to support it or reject it.

As has already been mentioned, glutamine, although not associated with neuronal excitability, is nevertheless found in high concentrations in spinal cord. Glutamine and glutamate are metabolically closely

related in nervous tissue and the concentrations of the two amino-acids have been found to be approximately equal in the ventral cord but in the dorsal cord the glutamate concentration is rather higher (Graham et al 1967).

There are numerous other free amino-acids and several dipeptides found in the brain. The cerebral content of free amino-acids is labile and highly responsive to circumstances which affect body conditions. Subjecting an animal to malnutrition or excessive temperature would result in changes in some amino-acid profiles in the brain (McIlwain & Bachelard 1971). A progressive disease such as is seen in the wobbler mouse could influence the metabolic state of the brain and spinal cord.

The purpose of this study has been to investigate changes in transmitter suspect amino-acids in cervical and lumbar cord in two mutant mouse colonies, the first being the wobbler mouse where involvement of motor-neurones in the disease process has been well established (Duchen, Strich & Falconer 1968, Papapetropoulos & Bradley 1972) and in the dystrophic mouse where abnormalities in peripheral nerve have been reported (Bradley & Jenkison 1973), but there is still no universal agreement as to whether the disease is of a neurogenic or myopathic origin. Both diseases result in loss of muscle function although in the wobbler mouse the fore-limb is affected more than the hind-limb, whereas the reverse is true in the dystrophic mouse.

As the study of amino-acid transmitters was carried out by column chromatography, other amino-acids and ninhydrinpositive substances present could also be estimated, and the results of some of these have also been reported.

II. Material and Methods

i) Material

The cervical and lumbar enlargements of mouse spinal cord were analysed for free amino-acid content. Mice from the wobbler, dystrophic and C57/BL colonies were used. The number of samples from each of the groups of animals for both cervical and lumbar cord analysis are given in Table 4.2. Mice from the C57/BL colony were used as controls and their ages ranged from 48 to 268 days. The wobbler and dystrophic mutants were of varying ages showing the spectrum of severity of the disease. The healthy animals from the wobbler and dystrophic colonies were, whenever possible, littermates of the diseased animal but where no littermate was available an age-matched animal from the same colony was used.

In this study no animals from the wobbler colony showed any signs of the late onset neuromuscular disease described in Chapter 3.

The spinal cords of three C57/BL mice were pooled for the analysis of the coefficient of variation of the chromatographic technique. For the same purpose a pooled amino-acid standard (Sigma) was also used.

ii) Methods

a) Preparation of Sample

The method for the preparation of spinal cord samples for amino-acid analysis is given in Chapter 2, page 8 . Just before the sample was to be used it was allowed to thaw at room temperature. To 1 ml of the sample 0.1 ml of norleucine standard (BDH chromatographically homogenous norleucine diluted to 0.1 u moles/ml) was added.

The three C57/BL spinal cords were prepared in the same way, but they were homogenised in 10 ml of 10% sulphosalicylic acid. After centrifugation 9 ml of the supernatant was removed and to it was added 0.9 ml of the norleucine standard. The sample was then aliquoted to give

TABLE 4.2 - The Animals used in the Experiments

	Strain	Status	No. of cervical cords studied	No. of Lumbar cords studied
1	C57/BL	control	9	9
2	DyDy/Dydy	healthy	10	7
3	dydy	diseased	10	7
4	WrWr/Wrwr	healthy	9	8
5	wrwr	diseased	8	7

TABLE 4.3 - The Amino-acids found consistently in spinal cords

No.	Amino-Acid	No.	Amino-Acid
1	Cysteic Acid	9	Glycine
2	Phosphoethanolamine*	10	α-alanine
3	Taurine	11	Ornithine
4	Aspartic Acid	12	Hysine
5	Threonine	13	Histidine
6	Serine	14	Homocarnosine*
7	Glutamine	15	Arginine
8	Glutamic Acid		

* No. 2 - Phosphoethanolamine only estimated in cervical region

* No.14 - Homocarnosine and carnosine form one peak but most of the peak probably due to homocarnosine

individual samples of 1.2 ml. The aliquots were frozen and stored at -70°C. One ml of each sample was used for amino-acid analysis.

The amino-acid standard (Sigma Ltd) was prepared by diluting the commercial preparation by one in twenty-five with distilled water and 1.2 ml samples were stored at -70°C. Again, each sample was used for analysis.

b) Amino-acid Analysis

The free amino-acid content of the spinal cord samples was analysed by means of ion exchange chromatography using a single column gradient elution technique (Emery et al 1970). The technicon NC1 model autoanalyser was used which incorporates a column of 140 cm in length, 0.6 cm diameter. The sodium citrate buffer system was used. The ninhydrin reagent, which was maintained under an atmosphere of nitrogen to prevent oxidation, was made up freshly once a week.

c) Calculation of Amino-acid Concentrations

In calculating the concentrations of the individual amino-acids in a given sample, the peak area obtained for the known concentration of standard is compared with the peak area for each of the amino-acids in that sample. Not all amino-acids react in exactly the same way with the ninhydrin, hence a correction has to be made for the colourimetric intensity with which each amino-acid reacts with the reagent. Assuming the standard norleucine has an activity of 1 for a concentration of 0.1 u moles/ml, using the same concentration of each amino-acid one can calculate how the amino-acid reacts with the ninhydrin as compared to the activity of the standard. This value is known as the norleucine equivalent value (NLE).

i) $$\text{NLE} = \frac{\text{Area under peak of 0.1 u moles/ml norleucine}}{\text{Area under peak of 0.1 u moles/ml of a.a.x}}$$

For a sample where concentration of x is unknown:

ii) $$\text{Conc. in u moles/ml of x} = \frac{\text{Area under peak of a.a.x}}{\text{Area under peak of norleucine}} \times \text{NLE} \times 10.$$

This value was then converted to micromoles per gram wet weight of the spinal cord specimen. A slightly different method is used for calculating concentrations of amino-acids where two amino-acids form one peak. Here one uses the peak areas obtained for the two amino-acids at a wavelength of 570 Å and 440 Å. This method results in some inaccuracy where both amino-acids are of near equal concentrations. Using a sodium citrate buffer system glutamine and asparagine elute as one peak as do glutamic acid and proline. In spinal cord there is virtually no asparagine or proline and one could almost assume that peaks were made up entirely of glutamine and glutamic acid as both these amino-acids occur at high concentrations in the spinal cord. All calculations of amino-acid concentrations were carried out on the Olivetti calculator.

d) Statistical Analysis

The coefficient of variation was obtained for each of the amino-acids in the standard solution (Sigma Ltd) and for each amino-acid in the pooled spinal cord specimen. The average coefficient of variation was calculated for both methods.

The method was able to detect twenty-eight amino-acids, a list of which is given in the Appendix; several occur in the spinal cord at concentrations too low for reliable calculation. Fourteen amino-acids occur in most spinal cords at concentrations which can be easily measured and these have been used in analysing differences between the various mice. A list of the fourteen amino-acids are given in Table 4.3.

The coefficient of variation of several amino-acids has been calculated for the individual groups of mice and compared with the coefficient of variation for the machine.

Regression analysis was carried out for amino-acid concentrations on the age of the mouse for all the groups of animals. Student's t test was used to compare amino-acid concentrations in male and female mice of the control group of mice.

III. Results

i) Coefficient of Variation for the technique

The average coefficient of variation calculated using the standard was 6.7% whereas using the pooled spinal cord sample it was 2.5%. The coefficient of variation using the same technique on samples of amniotic fluid gave a value of 5% (Emery *et al* 1970).

ii) Coefficient of Variation of Amino-acids in the Mice

The coefficient of variation for seven of the amino-acids was calculated for each group of mice for both regions of spinal cord studied: it was noticed that in most cases the coefficient of variation of individual amino-acids for the mice was greater than that observed for the technique. The results suggest considerable variability of amino-acid concentrations in the spinal cord within all the groups of mice. Regression analyses have shown that there is no significant regression on age, nor is there a significant difference between the results obtained for the two sexes. There also appears to be a greater variability of certain amino-acid concentrations in mice from the two mutant colonies than is seen for the C57/BL mice.

iii) Analysis of Amino-acid Concentrations in the Mice

a) Cervical Region

The concentrations of amino-acids in cervical cord of control mice, wobbler mice and dystrophic mice are given in Table 4.4. It is interesting to note that all the amino-acids which differ significantly from the controls are possible transmitters with the possible exception of homocarnosine. Glutamine which has no effect on neuronal firing appears to be of much the same concentration in all the animals studied.

b) Lumbar Region

The concentrations of amino-acids in the lumbar cord for the three groups of animals is given in Table 4.5.

TABLE 4.4

Amino-acid concentrations in cervical cord of control (C57/BL), wobbler (wrwr) and dystrophic (dydy) mice

Results expressed as μ moles/gm wet weight (Mean ± SD) and the significance of the differences between affected animals and controls

	Controls	Wobbler	P	Dystrophic	P
Cysteic acid	0.22 ± 0.06	0.25 ± 0.18	-	0.20 ± 0.13	-
Phosphoeth.	0.62 ± 0.08	0.89 ± 0.30	-	0.63 ± 0.15	-
Taurine	5.74 ± 1.42	4.71 ± 1.80	-	3.70 ± 0.85	< 0.01
Aspartic acid	4.61 ± 0.62	2.40 ± 0.54	<0.001	2.78 ± 0.80	<0.001
Threonine	0.50 ± 0.20	0.38 ± 0.18	-	0.35 ± 0.18	-
Serine	0.94 ± 0.25	0.90 ± 0.19	-	0.90 ± 0.23	-
Glutamine	4.84 ± 0.84	5.19 ± 1.40	-	4.13 ± 1.20	-
Glutamic acid	7.58 ± 0.72	4.94 ± 1.10	<0.001	5.32 ± 1.10	<0.001
Glycine	6.92 ± 0.64	4.70 ± 1.30	<0.001	5.14 ± 1.00	<0.001
α-Alanine	1.00 ± 0.16	0.95 ± 0.17	-	0.91 ± 0.41	-
Ornithine	0.17 ± 0.04	0.17 ± 0.12	-	0.14 ± 0.05	-
Lysine	0.40 ± 0.06	0.44 ± 0.18	-	0.37 ± 0.10	-
Histidine	0.09 ± 0.05	0.18 ± 0.12	-	0.12 ± 0.04	-
Homocarnosine	0.44 ± 0.06	0.84 ± 0.12	<0.001	0.59 ± 0.14	-
Arginine	0.33 ± 0.07	0.23 ± 0.11	-	0.22 ± 0.08	<0.01

TABLE 4.5

Amino-acid concentrations in lumbar cord of control, wobbler and dystrophic mice

Results expressed as μ moles/gm wet weight (Mean ± SD), and the significance of the differences between affected animals and controls

A.A.	Controls	Wobblers	P	Dystrophic	P
Cysteic acid	0.32 ± 0.11	0.46 ± 0.20	-	0.34 ± 0.08	-
Taurine	5.09 ± 0.90	5.51 ± 0.65	-	4.92 ± 1.06	-
Aspartic acid	3.57 ± 0.22	4.44 ± 0.78	0.001	4.10 ± 0.81	-
Threonine	0.61 ± 0.13	0.59 ± 0.20	-	0.64 ± 0.16	-
Serine	1.17 ± 0.20	not estimated	-	0.89 ± 0.28	-
Glutamine	4.85 ± 0.58	6.52 ± 1.61	0.01	5.74 ± 1.75	-
Glutamic acid	6.85 ± 0.50	7.86 ± 1.65	-	8.25 ± 2.18	-
Glycine	6.72 ± 0.78	6.62 ± 1.23	-	6.72 ± 0.64	-
α-Alanine	0.88 ± 0.18	1.00 ± 0.27	-	0.90 ± 0.25	-
Ornithine	0.17 ± 0.10	0.21 ± 0.10	-	0.14 ± 0.10	-
Lysine	0.37 ± 0.07	0.42 ± 0.15	-	0.37 ± 0.07	-
Histidine	0.11 ± 0.05	0.14 ± 0.05	-	0.11 ± 0.03	-
Homocarnosine	0.44 ± 0.08	0.65 ± 0.11	0.001	0.60 ± 0.13	-
Arginine	0.18 ± 0.07	0.43 ± 0.27	-	0.19 ± 0.07	-

In the lumbar region very few amino-acids differ statistically from those found in the controls. In the lumbar region aspartic acid is increased in the wobbler mice, whereas in the cervical region this amino-acid concentration is lower in animals of the two mutant colonies. Homocarnosine is increased in the wobbler; it is similarly increased in the cervical region.

The concentrations of amino-acids obtained for the littermates of the affected animals are given in the Appendix. The littermates are not entirely valid as controls as they are of unknown genotype.

iv) Analysis of Amino-acid concentrations in each of the Mutant Colonies

a) The Wobbler Colony

When comparing statistically the results obtained for the affected animals and their littermates, the aspartic acid level in the cervical cord was found to be significantly reduced in the affected wobbler mice $P < 0.01$, whereas homocarnosine was raised in the cervical cord $P < 0.01$ and the lumbar cord $P < 0.05$.

b) The Dystrophic Colony

In the dystrophic colony there appears to be little difference between the affected animal and the littermates. Homocarnosine is slightly increased in the lumbar region of the affected animal but this is not reflected as a difference between the dystrophic mouse and the C57/BL mouse.

IV. Discussion

The results from the study of amino-acids in the spinal cords of mice from the C57/BL, wobbler and dystrophic colonies have proved rather more complex than was originally expected. It was believed that if differences in amino-acids did occur they would be between healthy and diseased animals regardless of the colony from which the healthy mice

originated. The results that have been obtained make any interpretation rather difficult. It could be suggested that variation between the strains of mice used in this study may account for the differences in amino-acid concentrations obtained for the C57/BL mice and those of the two mutant colonies. If this were the answer one would expect differences occurring between C57/BL and wobbler mice to be somewhat less than those between C57/BL and dystrophic mice. The wobbler mice originated as a mutation in an inbred strain of C57/BL mice whereas the dystrophic mice are a Bar Harbor strain. A further suggestion may be that inbreeding affects the physiology of the spinal cord of these mice. Both these suggestions may be in part correct; they do not, however, explain the extent of the differences in the concentrations of the amino-acids aspartate, glutamate and glycine between cervical regions of C57/BL mice and those of the wobbler and dystrophic mice. Several studies of brain amino-acids and a study of spinal cord glycine in various mammalian species have shown that the differences in concentrations between species are often less than those occurring between the strains of mice (Tallan 1962, Aprison, Shank & Davidoff 1969).

Loss of neurones from affected wobbler mice have been reported to be greater from the cervical region than from other regions of the cord (Papapetropoulos & Bradley 1972). It has also been shown that a loss of interneurones from damaged areas of the cord results in a reduction of aspartate, glutamate and glycine concentrations (Davidoff et al 1967). The low concentrations of these amino-acids in the cervical cord of wobbler mice may, therefore, be interpreted as being the result of the loss of neurones from the cord. The lumbar region asparate level is, however, raised above that of C57/BL mice even though some neurones are also lost from the lumbar cord in this mutant. This hypothesis fails to explain the amino-acid differences occurring between C57/BL mice and

the healthy littermates of the wobbler mice, in which motor-neurone loss, if any, is of a lower order than that occurring in affected mice. It in no way accounts for the raised homocarnosine levels occurring in both cervical and lumbar regions of most affected mice, nor can it explain the similarities in amino-acid concentrations occurring in mice of both wobbler and dystrophic colonies. There is, however, the possibility that two degenerative processes may result in similar changes in spinal cord amino-acids, but the low levels of certain amino-acids occurring in mice of both wobbler and dystrophic colonies cannot be explained in terms of motor-neurone loss.

It may be of greater significance to consider further the differences found in amino-acid profiles between the mice of the wobbler colony and the dystrophic colony. It has already been mentioned that the results of this study indicate that homocarnosine in the wobbler affected mouse cords is significantly elevated. In the cervical cord of mice from the dystrophic colony, taurine as well as glutamate, aspartate and glycine appears to be reduced. The profiles for amino-acid concentrations in dystrophic lumbar cord do not differ from those of the C57/BL mice, however there are some differences between C57/BL mice and dystrophic littermates for this region of the cord. Whether these variations in the healthy mice should be considered as a further abnormality or purely as a compensatory phenomenon resulting from differences in amino-acid distributions is not certain. In resolving this problem it may have been more useful to study the complete spinal cord of these mice. There has been a report suggesting that biochemical changes involving Kreb's cycle non-ketonic acids of various tissues including the brain are more readily reflected in heterozygotes than in homozygote affected mice of the dystrophic colony (Montalbo & Kabara 1974). In another study, the steady state and rate of turnover in the brain of a putative neurotrans-

mitter, acetylcholine, has been shown to be reduced in affected animals when compared with phenotypically normal mice; however, no reduction was seen in the steady state or rate of accumulation by the brain of δ-aminobutyric acid (Trabucchi et al 1974). It is not certain whether these results would also apply to the spinal cord. The results from this study indicate that further clarification of the role of neurotransmitters in murine dystrophy is required. Any further study should include genotypically normal as well as phenotypically normal and diseased animals from the dystrophic colony. It would also prove useful if various areas of the brain and spinal cord were assessed for amino-acid content and the active uptake by these tissues of certain transmitter suspects were studied.

In considering the results obtained in mice of the wobbler colony, a possible approach may be to look at them in the light of present knowledge of the effects of malnutrition on the nervous system. There is considerable evidence that malnutrition in both humans and animals results in neurological changes (Platt & Stewart 1971, Manocha & Olkowski 1973). These include tremor of the head, apathetic behaviour and stiffness and uncoordination of movement. Changes of this nature are not altogether surprising as although brain cells are not actively dividing the protein turnover of healthy nervous tissue is very high. Malnutrition leads to a change in the amino-acid profile of the blood, whereas many amino-acids found in the brain and spinal cord are taken up from the blood against a concentration gradient (Balcar & Johnston 1973); hence the reduction in concentration in the blood of any of these amino-acids would be rapidly reflected in the nervous tissue. Malnutrition in monkey results in a marked accumulation of histidine, 3-methylhistidine and homocarnosine in the brain, and associated with those changes are further marked distortions of other free amino-acid profiles (Enwonwu & Worthington 1973).

A protein-calorie deficient diet in monkey resulted in changes in all three areas of the brain that were studied (Enwonwu & Worthington 1974). Thus in the cerebrum, cerebellum and brain-stem, the amino-acid and two dipeptides already mentioned are increased, whereas two probable excitatory transmitters, L-glutamate and L-aspartate, are found in reduced concentrations; however, taurine, threonine and serine concentrations are also decreased. The amino-acid glycine and GABA appear to be remarkably unaffected by malnutrition. The amino-acids in the spinal cord, under conditions of malnourishment, have not been investigated. It would appear probable, however, that some of the changes mentioned above would also be reflected in the spinal cord. The results obtained from mice of the wobbler colony are not entirely consistent with those found in malnutrition in the monkey; however, one must take into account that different areas of the nervous system have been studied and that there may be variation between the specific changes that are likely to occur under the same conditions in monkey and mouse. In the cervical region of affected mice there is a marked increase in homocarnosine and a loss in aspartate, the concentrations of these two ninhydrin-positive substances being significantly different not only from the C57/BL control mouse but also between the affected mouse and its littermate. It would appear possible, therefore, that there may be a similarity between some of the biochemical changes occurring in malnutrition and those occurring in affected wobbler mutants. It has been suggested that neurones adapt to the changed environment of malnutrition by utilising structural proteins for their metabolism (Manocha & Olkowski 1973). Such changes would inevitably lead to a loss of neurones and it is a loss of motor-neurones from the spinal cord that characterises the disease process in the wobbler mouse. This study has produced insufficient evidence to either support or reject such a suggestion. It may be useful, however, if amino-acid

changes in mouse brain and spinal cord were studied under conditions of malnutrition and if further amino-acid analyses were carried out on the brain of healthy mice and mice with hereditary motor-neurone disease.

Throughout this study it has been assumed that the increase, in both cervical and lumbar cord in the wobbler mouse, is in the dipeptide homocarnosine even though it has been shown by Enwonwu, and confirmed using our amino-acid analyses, that homocarnosine and carnosine are recorded as one peak (Enwonwu & Worthington 1973). Homocarnosine is, however, found exclusively in tissues of the central nervous system, whereas carnosine which is primarily found in muscle, occurs in central nervous tissue in very low concentrations (Abraham, Pisano & Udenfriend 1961, 1962). There are suggestions that it is the same enzyme that synthesises both carnosine (β-alanyl-L-histidine) and homocarnosine (γ-aminobutyl-L-histidine), and competition between β-alanine and GABA for the same receptor-site on the enzyme carnosine synthetase has been shown (Skaper, Das & Marshall 1973). There is, therefore, a very close relationship between these two dipeptides in nervous tissue. In mouse carnosine has been found in high concentration in the olfactory bulb and this has led to the suggestion that carnosine is a potent neuroinhibitory substance in this area of the brain (Neidle & Kandera 1974). Homocarnosine is also suspected of being a neuroinhibitor. By using homocarnosine on both epileptic dogs and epileptic humans it has been demonstrated that homocarnosine is a special neuroinhibitory substance associated with seizures in the limbic motor system (Hayashi 1965, 1966). It would appear that whether the increase be of homocarnosine or carnosine it would have a marked effect on the central nervous system. An increase in either dipeptide accompanied by a decrease in aspartic acid, a probable neuroexcitatory amino-acid, could possibly result in membrane hyperpolarization of susceptible motor-neurones, resulting in their malfunction and eventual degeneration.

The results of this study have neither proved nor disproved an association between the disease phenomena in dystrophic or wobbler mice and changes in concentrations of transmitter amino-acids. It would appear, however, that the dipeptide, homocarnosine or carnosine, may in some way be linked with hereditary lower motor-neurone disease in mouse.

CHAPTER 5

THE STUDY OF PERIPHERAL INNERVATION IN HEALTHY MICE AND MICE WITH NEUROMUSCULAR DISEASE

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CHAPTER 5

TABLES

CHAPTER 5

I. Introduction

In recent years the improvement in techniques for histological preparations of peripheral nerve (Page 1970 and 1971) has been a contributing factor to the understanding of peripheral motor innervation in both man and animals.

Many human studies have primarily concentrated on distinguishing innervation changes occurring as a result of neuromuscular disease (Coërs and Woolf 1959; MacDermot 1961; Jedrzejowska, Johnson and Woolf 1965; Allen, Johnson and Woolf 1969) and on quantifying the extent of ramification of the terminal axon in both healthy and diseased muscle (Coërs, Reske-Nielson & Harmsen 1970; Coërs, Telerman-Toppet & Gerard 1973a & b).

In 1959 Coërs and Woolf defined the terms "actual terminal innervation ratio" (ATIR) and "functional terminal innervation ratio" (FTIR).

ATIR is defined as the ratio of the number of motor end-plates arising from a given number of terminal axons to that number of axons, whilst FTIR is defined as the ratio of the number of muscle fibres innervated by a given number of terminal axons to that number of axons.

$$\text{ATIR} = \frac{\text{No. of motor EP arising from x terminal axons}}{x}$$

$$\text{FTIR} = \frac{\text{No. of muscle fibres innervated by x terminal axons}}{x}$$

The difference in these two ratios is that, whereas ATIR is a measure of the overall sprouting from the terminal axon, FTIR is a measure of collateral innervation in response to partial denervation.

The study of these two ratios in normal and diseased muscle has proved useful in distinguishing between innervation changes resulting from damage to the lower motor neurones and other neurogenic changes

perhaps secondary to a myopathy. The study of the FTIR may be particularly useful where a distinction between a neuropathy and a myopathy may be difficult to establish purely on the basis of a histological and histochemical assessment of the muscle (Black et al 1974).

Mice have been used extensively in the study of neuromuscular disease (Banker & Denny-Brown 1959; Curtis, Abramas & Harman 1961; Meler 1969). Innervation in diseased muscle has been studied using both histological techniques (Harman et al 1963; Duchen, Strich & Falconer 1968) and electrophysiology (McIntyre, Bennett & Bradley 1959; McComas, Mrozek & Thomas 1968). No quantitative study of the functional terminal innervation in mouse muscle has been attempted, although it has been suggested that in murine muscular dystrophy the ATIR may be raised with the FTIR remaining normal (Harman et al 1963). Neither has there been any study of innervation in phenotypically healthy animals, which are obligatory heterozygotes for the gene associated with a neuromuscular disease. It was believed that the study of the functional terminal innervation ratio in mice of both the wobbler and dystrophic colonies would add to an understanding of the two hereditary murine disorders. It was also felt that such an approach to the study of murine muscular dystrophy in the light of present knowledge, which suggests a neurogenic component in this disease (Bradley and Jaros 1973; Bradley and Jenkison 1973), may be useful in resolving the controversy as to whether functional denervation is a feature of the disease and whether the condition closely resembles a primary neurogenic rather than myopathic disorder (Bradley 1971; Harris & Wilson 1971; Harris & Marshall 1973; Emery & Gosdon 1974; Law & Atwood 1974).

Changes in peripheral innervation which do not occur as a direct result of lower motor neurone disease or surgical denervation may not necessarily be reflected by an increased or reduced functional terminal

innervation ratio. Structural abnormalities of the subterminal axon or the motor end-plate may be more prominent. Changes in the myoneural junction as a result of disease have been studied mainly by means of electronmicroscopy. Dystrophic end-plates of both the 129 ReJ/dy mice and dy^{2J} mice have been shown to be abnormal (Ragab 1971; Gilbert, Steinberg & Banker 1973). There has, however, been a light microscopy study on growth and degeneration of motor end-plates in hind-limb muscles of cats of varying ages (Tuffery 1971). Mouse innervation and end-plate structures have been studied in muscles derived from animals with hereditary motor end-plate disease (Duchen 1970) and from animals previously treated with cardiotoxin (Duchen et al 1974). Although light microscopy has been employed for studies of mouse motor end-plates, under disease conditions, the changes in structure of the terminal arborizations occurring as a result of disease have not been previously quantified; nor have the types of end-plates found on muscle in healthy mice been classified. This study has used a similar system for the classification of end-plates to that suggested by Tuffery in his study of cat motor end-plates. The size and structure of end-plates in muscle from mice of varying ages and from each of the three colonies: the C57/BL, the wobbler and the dystrophic - were examined. The aim of the present study was to consider whether mouse nerve endings were affected by an ageing process similar to that described in cat (Barker and Ip 1965; Tuffery 1971) and to establish whether end-plate morphology in heterozygotes from either the wobbler or dystrophic colonies differed from that of control mice.

By studying both the innervation ratio and the variation of end-plate structure in the same muscle sample, it was hoped to establish how peripheral innervation in mice was affected by the wobbler and dystrophic genes and so obtain a better overall understanding of the effects of the two diseases on peripheral innervation.

II. Material and Methods

i) Material

The gastrocnemius muscle from a total of forty-one mice was used for the analysis of terminal innervation and motor end-plate morphology. Specimens from C57/BL mice aged between 80 and 274 days were compared with phenotypically healthy and affected animals from the dystrophic and wobbler colonies. Specimens from mice of the wobbler colony with a late onset hind-limb paralysis were also used in this study. The numbers of mice in each group are given in Table 5.1.

ii) Methods

a) Preparation of sample

The animals were killed by ether anaesthesia. The whole gastrocnemius was removed, frozen in liquid nitrogen and prepared for longitudinal sections as described in Chapter 2, page 9 . Sections of between 20-50 μ were cut and placed directly on to a slide. Extra caution was taken when drying sections of above 30 μ thickness. The slides were placed in a current of cool air to prevent excessive shrinkage of tissue. Sections of diseased wobbler muscle were cut at between 20-40 μ thickness whereas other samples were cut at between 40-50 μ thickness.

b) Methods for staining

Slides were stained using the combined acetylcholinesterase/silver technique detailed in the Appendix. Sections which were less than 40 u in thickness did not require the full two hour fixation in formol calcium; between one and one and a half hours was sufficient. It was also noticed that to obtain appreciable acetylcholinesterase activity in muscle from wobbler affected mice it was necessary to increase the time of incubation in substrate solution from the two minutes used normally to 4-5 minutes.

TABLE 5.1

Mice used in the Study of Peripheral Innervation

Genotype	No. studied	Age range in days
C57/BL	6	80-274
WrWr/Wrwr	6	54-180
Wrwr	4	237-428
Wr/HLP	7	182-320
wrwr	9	43-157
DyDy/Dydy	4	145-296
dydy	5	47-303

c) Method for assessing innervation

Stained preparations were observed using a Leitz Orthoplan Microscope (Leitz Optics Ltd) at a magnification of x(625). The length of the terminal axon was measured to the nearest 25 μ using a calibrated graticule inserted into the eye piece. The method used in the study of terminal innervation was based on the one used for human muscle (Coërs, Telerman-Toppet & Gerard 1973a). Certain adjustments were made to allow for the fact that mouse axons are considerably shorter than those found in human muscle; hence, whereas Coërs suggests that only axons greater than 200 μ in length should be included in the analysis, in this study axons of 100 μ in length and above have been used. All the axons measured were required to end in a terminal arborization on a muscle fibre; axons or branches of axons not seen to end in an end-plate were disregarded for the purpose of this study. Axons branching to give two end-plates on a single muscle fibre were treated in the same way as unbranched axons. Axons which branched collaterally, terminally or ultraterminally resulting in the innervation of more than one muscle fibre were all noted regardless of the total length of the axon.

In the majority of preparations a total of between forty and sixty axons could be used for the assessment of the FTIR. In specimens of wobbler mouse muscle the preparation was often such that no innervation ratio could be calculated whereas in others the ratio had to be based on small numbers of axons as very few met the specifications enabling them to be included in the calculation.

d) Method for calculation of results of terminal innervation studies

The FTIR was calculated using the formula given in the Introduction, page 46. FTIR was calculated for each of the mice studied and a mean FTIR for each of the groups of mice was obtained. The results are given in Table 5.2. The results for the animals in no way affected

with a neuromuscular disease were then pooled and analysed for the correlation between the increase in FTIR on age, and the mean FTIR for healthy animals was also calculated.

e) Methods for the study of motor end-plates

End-plate studies were carried out on the same preparations used for terminal innervation studies.

1. Size

The terminal arborization was located on a muscle fibre by its cholinesterase activity intensified with silver, as outlined in the staining method (see Appendix). The area of cholinesterase activity was assumed, for the purpose of this study, to represent the area of the motor end-plate. The longest diameter of this area was taken to be a reasonable estimation of end-plate size. The motor end-plates measured were all represented by a clearly defined area of cholinesterase activity, and were seen to be the termination of a motor axon, situated on a muscle fibre. This method of measuring the motor end-plates in acetylcholinesterase/silver stained preparations has various limitations. The intensity of staining of the motor end-plate is dependent on the cholinesterase activity in the area, which in turn depends on the time that the tissue is exposed to the substrate. Silver precipitation around the area of activity may be an additional cause of inaccuracy. Furthermore, the orientation of the tissue may not be entirely longitudinal and the end-plate not centrally placed on the muscle fibre, thus not exposing the entire length of the motor end-plate. It has also been suggested that the size of the subterminal apparatus should be related to the muscle fibre size (Zacks 1964). In this study only mean end-plate sizes were estimated for individual mice and for each of the groups of animals. Wherever possible the mean end-plate size was estimated from 100 measurements although in diseased animals, when fewer

end-plates were present, numbers were often smaller. Magnification used for measurement was x 625, and lengths were recorded to the nearest 4 μ.

2. Structure

End-plates were classified into different categories according to their structure. The guidelines for such a classification were suggested by Tuffery (1971). The 'T' number T 1-5 indicates the number of myelinated branches from the axon that contribute to the formation of a single end-plate. Such end-plates may be divided again into those that are just T_1, T_2, etc, and those that also have a 'sprout', defined as a non-myelinated outgrowth typically ending in a growth cone, or a 'contribution', a non-myelinated outgrowth ending not in a growth cone but in an axon terminal on the same sole-plate as those of their parent endings. A further type of end-plate can be distinguished: that of a 'duplex'. 'Duplex' innervation is defined as two branches of a single axon lying on separate sole-plates but on the same muscle fibre. Two hundred end-plates was the maximum number studied from any one specimen although in some poorer preparations and in samples from diseased muscle as few as 80 end-plates could be classified. In many of the samples numerous sprouts could be seen but it was difficult to decide from which axon they were branching or to see where the growth cone terminated. For this reason it was decided to consider sprouts only if they were nodal and appeared to be growing towards the muscle fibre on which the original end-plate was situated. In this way the emphasis on end-plate structure was maintained. Some of the end-plates classified may have been in various stages of degeneration but the degeneration of end-plates was not considered in this study.

f) Method for calculating results of end-plate studies

1. Size

The mean end-plate size was calculated for each specimen. The mean sizes for each group of animals were compared with the mean sizes for control C57/BL mice using Student's -t test. All mean end-plate sizes for healthy animals were pooled and a correlation of size with age was calculated. The mean end-plate sizes for affected mice were further compared with healthy animals from the same colony.

2. Structure

The number of end-plates in each of the categories outlined in section (e.2) was calculated as percentages of the total number of end-plates studied for each specimen. A mean percentage of end-plates within each category was calculated for the different genotypes. The percentages of end-plates classified into each of the following categories: 'T_1', 'T_2', 'T_3' and 'T_1 + contribution' for the control mice, were compared by Student's -t test to the percentages occurring in each of the other groups. The results for percentages of 'T_1' end-plates from all the phenotypically healthy animals were pooled and a correlation analysis of age with the percentage 'T_1' end-plates was calculated. The Student's -t test was also used for comparing the mean percentage of 'T_1' end-plates of healthy animals from a given colony with those of the affected animals.

III. Results

i) Terminal innervation

Table 5.2 indicates the mean FTIR for each group of mice. The mean FTIR for all healthy animals taken both from the C57/BL mice and littermates of affected mice has been found to be 1.04 with a standard deviation of 0.02. Using the criteria for the upper limit of normal as the mean $\pm$ 3 SD, the range for normal terminal innervation results

can be given as 0.98 to 1.10. There is, however, a small but significant correlation between FTIR and age ($r = 0.562$ $P < 0.02$). The mean terminal innervation ratio for all of the affected groups of animals was above the range for healthy individuals. The degree of terminal branching appeared to be least prominent in dystrophic mice and most obvious in the wobbler mice. The results for Wr/HLP mice were also high, the values lying between the controls and wobbler mice. The normal terminal innervation and innervation in animals of the affected groups is illustrated in Plates 1-4.

a) The wobbler colony

1. Phenotypically healthy animals

The FTIR of all phenotypically healthy animals, whether they were known to be heterozygotes for the wobbler gene or not, have terminal innervation ratios falling well within the normal range. The evaluation of FTIR does not therefore appear to be a method for distinguishing between genotypically normal and mice heterozygous for the 'wr' gene. The results are given in Table 5.2. Other features of innervation such as demyelination of axons and axonal degeneration without collateral branching have not been studied in detail. There does not appear to be any variation between innervation in C57/BL mice and phenotypically healthy animals of the wobbler colony with the possible exception of two animals, whose FTIR although still falling within the normal range appear to be somewhat above the value expected for that age of mouse. The individual results of FTIR are given in the Appendix. It is probable that the two results mentioned indicate a pre-clinical state of HLP.

2. Mice with hind-limb paralysis (Wr/HLP)

The results of terminal innervation ratio in these mice give little indication of a reason for paralysis, especially when one

TABLE 5.2

The Results of Functional Terminal Innervation Ratios (FTIR)

Genotype	No.	FTIR Mean	FTIR SD	Significance from normal
C57/BL	6	1.03	0.02	-
WrWr/Wrwr	6	1.05	0.02	NS
Wrwr	4	1.05	0.02	NS
Wr/HLP	7	1.20	0.05	$P < 0.001$
wrwr	7	1.64	0.30	$P < 0.001$
DyDy/Dydy	4	1.04	0.01	NS
dydy	5	1.12	0.01	$P < 0.001$

Using all healthy mice to obtain mean FTIR

Mean = 1.04 SD = 0.02

Hence using $M \pm 3SD$ as representing the normal range

$\therefore$ normal range = 0.98 - 1.10

TABLE 5.3

The Results of End-Plate Sizes

Genotype	No.	End-Plate Size Mean (μ)	SD
C57/BL	6	30.7	1.1
WrWr/Wrwr	6	30.2	1.0
Wrwr	4	30.7	0.8
Wr/HLP	7	31.1	2.0
wrwr	7	26.6	3.3
DyDy/Dydy	4	29.2	1.6
dydy	5	32.1	1.6

considers that in the wobbler mice the FTIR is much higher for the same muscle and yet no paralysis was seen in hind-limbs in any of the wobbler mice studied. The study of FTIR alone, however, does not give an adequate indication of the considerable abnormalities found in innervation of muscles in these mice; numerous fine fibres are seen both in the proximity of a group of sub-terminal axons, and wandering 'aimlessly' through the muscle. Although some of the fine fibres are found to be 'beaded' most do not show this characteristic of regenerative fibres (see Plate 2). The intramuscular nerve bundles have a mixture of normal appearing axons with some fine fibres, the number of normal axons becoming reduced as the disease progresses. There appeared to be an overall loss of axons in these mice; however, no count of intramuscular nerve fibres was attempted and the apparent loss of axons may be deceptive. The numbers of normal fibres is reduced with the substitution by fine fibres of a much smaller diameter. Some indications of demyelination of axons are seen, and nodal, subterminal and terminal sprouting are unmistakable features of innervation in this condition. In early stages of the disease, however, many areas of normal innervation can still be found. Such areas of normal innervation have not been seen in wobbler mouse muscle even in very young animals. Innervation in Wr/HLP is illustrated in Plate 2.

3. Wobbler mice

The results of FTIR in wobbler mice should be treated with caution as many of the mice studied showed extensive changes in terminal innervation with few axons ending in clear terminal arborizations. In each specimen large numbers of fine 'beaded' nerve fibres were seen 'wandering' among the muscle fibres, some ending in growth cones, whilst others were not seen to end at all. These fibres are often so numerous that they mask any normal appearing axons. In the intramuscular nerve

bundles a majority of the fibres appear to be very fine and unmyelinated but even in animals at an advanced stage of the disease a few normal myelinated fibres are still present. Some examples of 'dying-back' may also be found as well as Wallerian degeneration. Often it is in the older 'wrwr' mice that the terminal innervation characteristics for lower motor neurone disease, can be most clearly seen (Coërs and Woolfe 1959). The innervation ratio calculated for these mice is probably a gross underestimate of the true FTIR because few axons can be seen to terminate in clear end-plates. Innervation of wobbler muscle is shown in Plate 3.

b) The Dystrophic Colony

1. Phenotypically healthy animals

There appears to be no abnormality in phenotypically healthy animals of this colony; terminal innervation does not seem to be affected by the 'dy' gene in heterozygote mice but the number of mice studied from this group was very small.

2. The dystrophic mice

The functional terminal innervation ratio in dystrophic mice is slightly raised. Although a few regenerative fibres are seen they are not a common feature; axons in the intramuscular nerve bundles mostly appear normal but some subterminal axons do show features of repeated demyelination and remyelination (see Plate 4). Some axon swellings are found primarily close to terminal arborizations. The collateral branching seen in dystrophic mice has a few similar features to that seen in wobbler mice, but any axon ramification is mainly terminal or ultraterminal innervating muscle fibres close to the original terminal end-plate. Axonal branches mostly end in small poorly developed end-plates possibly resembling those described for human myotonic dystrophy (Allen, Johnson & Woolfe 1969). There is, however, no evidence

of several end-plates being found on a single muscle fibre. It may be that these small newly formed end-plates are not developing on atrophic fibres but on fibres occurring as a result of muscle fibre splitting, a well recognised feature of muscle degeneration in muscular dystrophy in both man (Isaacs, Bradley & Henderson 1973) and animals (Harman et al 1963; Bray & Banter 1970).

ii) End-plate Studies

a) Size

The mean end-plate sizes for the individual mice are given in the Appendix (Appendix 5 Table 1). The mean end-plate sizes for all phenotypically healthy animals are very similar (see Fig. 5.1). There is no correlation between end-plate size and age ($r = 0.27$ $p > 1$). The mean size of end-plates from mice with hind-limb paralysis is slightly, though not significantly larger than normal. The mean end-plate size is also slightly increased in dystrophic mice. Table 5.3 gives the mean sizes for the groups of animals.

It can be seen from Table 5.3 and Fig. 5.1 that only end-plates of wobbler mice differ in size from controls (wrwr e.p. size compared with C57/BL $P<0.02$). The mean size of wobbler end-plates is also significantly reduced when compared with phenotypically healthy animals from the wobbler colony (wrwr e.p. compared with WrWr/Wrwr + Wrwr $P<0.01$). There are, however, individual animals with lower motor neurone disease whose mean end-plate sizes fall within the normal range; these are the older animals which probably manifest a more slowly progressing form of the disease. There is no indication of large numbers of small end-plates innervating the dystrophic muscle but there appear to be numerous small end-plates in wobbler muscle.

b) Structure

The various types of end-plates found are illustrated in Plate

FIGURE 5.1

The distribution of mean end-plate sizes in the different groups of mice.

FIGURE 5.1

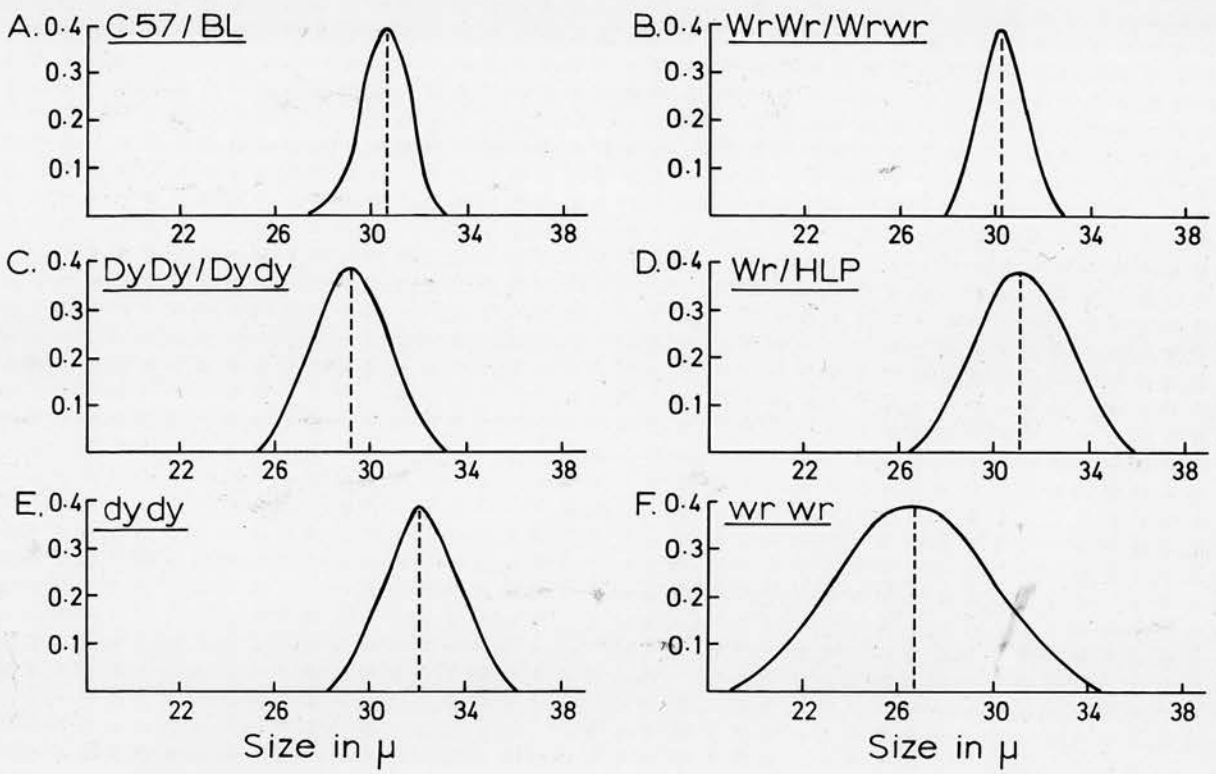

A. C57/BL
B. WrWr/Wrwr
C. DyDy/Dydy
D. Wr/HLP
E. dydy
F. wr wr
0·4
0·3
0·2
0·1
22
26
30
34
38
Size in μ

TABLE 5.4 - End-Plate Structure

Genotype	No. of EP observed	% of EP in each category											
		T_1	T_1+S	T_1+C	T_2	T_2+S	T_2+C	T_3	T_3+S	T_3+C	T_4	T_5	Duplex
C57/BL	1087	63.2	1.5	27.7	6.9	0.1	0.4	0.2					0.1
WrWr/Wrwr	823	69.6	2.0	21.7	5.7	0.1	0.1						0.7
Wrwr	514	62.1	2.0	25.3	7.8		1.6	0.6					0.6
Wr/HLP	906	56.3	2.1	24.6	11.4	0.1	2.8	1.1		0.5	0.2	0.2	0.7
wrwr	512	63.6	0.6	19.9	10.0	0.4	2.9			0.2			2.5
DyDy/Dydy	548	62.6	1.1	23.0	10.8		1.5	0.7					0.4
dydy	444	29.9	5.0	25.3	19.7	1.9	10.2	4.3		1.7	0.7		1.4

T = number of myelinated branches forming a single end-plate

C = 'contribution'

S = 'sprout'

5 . When the phenotypically healthy groups of animals were compared with one another no statistical significance was found between the percentages of 'simple' or 'T_1' end-plates in each of these groups. The results from all these animals were therefore pooled and a correlation analysis of the percentage of 'T_1' end-plates against the age of the mouse was carried out. There was a significant correlation between the reduction in the number of 'T_1' end-plates and the age of the mouse ($r = 0.6189$ $P < 0.01$). The percentages of end-plates within every classification for each of the seven groups of animals are given in Table 5.4. Fig. 5.2 shows the distribution of the percentage 'T_1' end-plates within each of the groups.

1. The wobbler colony

The percentages of end-plates classified into each type do not vary significantly between wobbler and healthy mice except in the case of the group 'T_2 + C' where in the wobbler the number is significantly raised (P 0.01); however the number of end-plates within this category is very small.

2. The dystrophic colony

The end-plate structure in dystrophic mouse muscle seems to be the most prominent abnormality of innervation. The percentage of 'T_1' or simple end-plates is reduced ($P < 0.01$). The percentage of 'T_1+ S' end-plates, those with one myelinated axon branch but also bearing a sprout, is increased ($P < 0.001$). The percentage of 'T_2' end-plates is increased ($P < 0.001$). The percentage of 'T_2 + S' end-plates is increased ($P < 0.02$) as well as the percentage of 'T_2 + C' end-plates ($P < 0.001$) and 'T_3' end-plates ($P < 0.01$). These comparisons are all with C57/BL control animals. There is also an increase in the percentage of duplex end-plates but these occur very rarely, hence percentages are very small in the order of 0 to 3%. However, only one duplex

TABLE 5.5

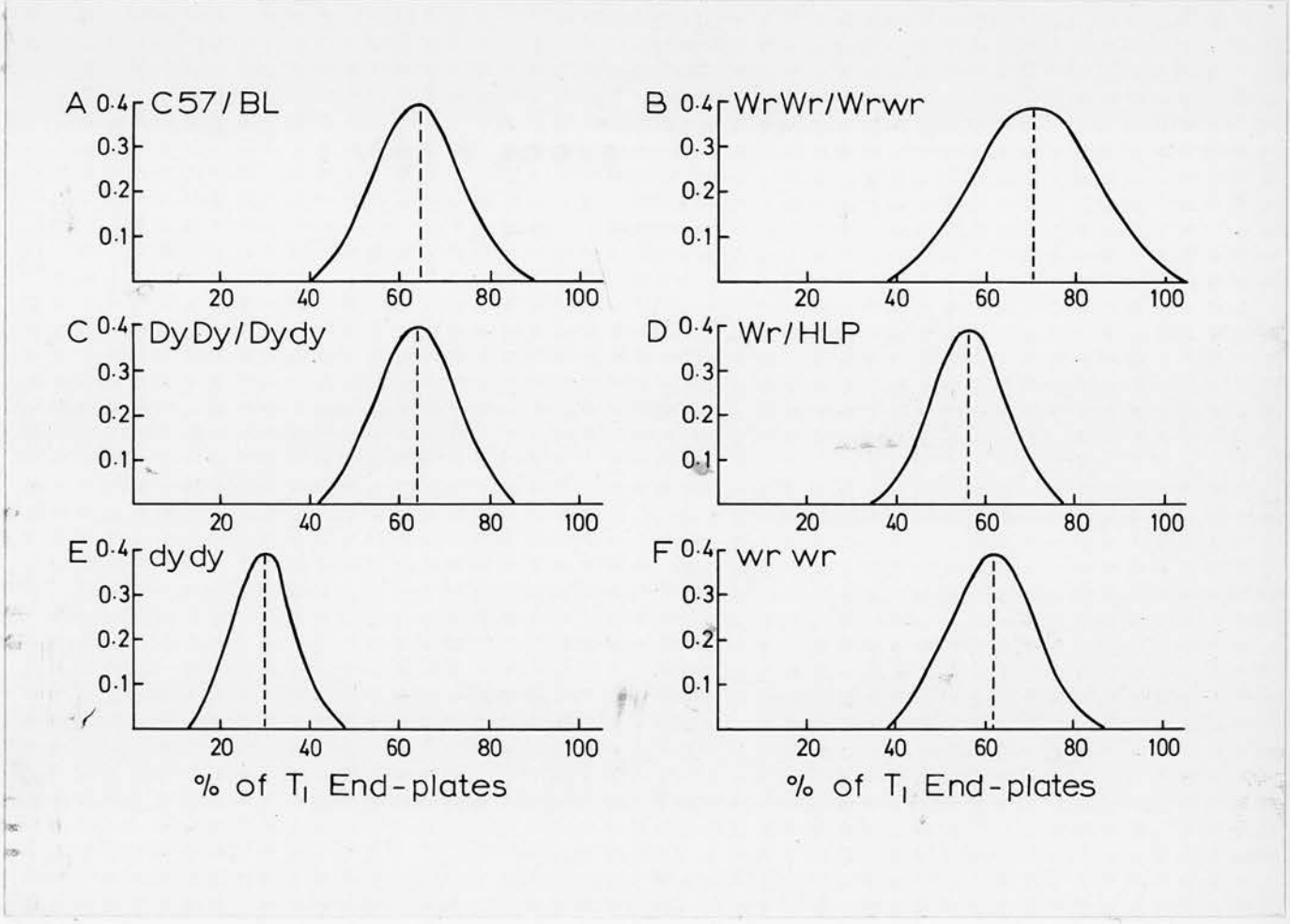
A statistical comparison using Student's -t test of end-plate size and structure of each mutant group against that of the control group

Statistical significance for the genotype	Results for the control (C57/BL) mice Mean ± SD EP size 30.7 ± 1.1	Mean ± SD % T_1 EP 63.6 ± 9.5	Mean ± SD T_1 +C EP 27.5 ± 4.9	Mean ± SD T_2 EP 6.8 ± 4.8	Mean ± SD T_3 EP 0.17 ± 0.4
WrWr/Wrwr	NS	NS	NS	NS	NS
Wrwr	NS	NS	NS	NS	NS
Wr/HLP	NS	$P < 0.1$	NS	NS	NS
wrwr	$P < 0.01$	NS	NS	NS	NS
DyDy/Dydy	NS	NS	NS	NS	NS
dydy	NS	$P < 0.01$	NS	$P < 0.001$	$P < 0.01$

FIGURE 5.2

The distribution of mean % of 'T_1' end-plates occurring in the different groups of mice.

FIGURE 5.2


A C57/BL
B WrWr/Wrwr
C DyDy/Dydy
D Wr/HLP
E dydy
F wr wr
0·4
0·3
0·2
0·1
20
40
60
80
100
% of T1 End-plates
% of T1 End-plates

end-plate was seen in the mice of C57/BL colony and two in healthy animals from the dystrophy colony.

iii) Summary of results from studies of peripheral innervation

Wobbler mice have an increased functional terminal innervation ratio with a decreased mean end-plate size. Small end-plates are consistently found in animals where the disease appears to be progressing rapidly. Many end-plates, however, may be near normal size in older animals where the progress of the disease appears to have slowed down. The reduced end-plate size would probably not be significant if it were correlated with muscle fibre size (Zacks 1964). The morphology of wobbler end-plates appears to be normal when studied using the outlined criteria. There may, however, have been some end-plates placed into the T_1' category which, although innervated by only one branch of an axon, may not have been true T_1' end-plates, in that the axon may have been unmyelinated.

The Wr/HLP mice have an increase in FTIR which is not as high as that of wobbler mice. They show degenerative changes of the subterminal axon, such as evidence of abnormality of axons and of Wallerian degeneration. The mean end-plate size is slightly but not significantly raised. End-plate size would be of little value as an indication of disease in these animals, as would analysis of end-plate structure, although slight increases in the number of the more complex end-plates such as T_2' and $T_2 + C'$ have been observed. These increases have also been found in one of the two mice in the WrWr/Wrwr group suspected of being an example of the pre-clinical changes in terminal innervation which presumably would subsequently result in hind-limb paralysis. The percentage of T_2' end-plates in this mouse as well as in most Wr/HLP mice is still within the normal range, therefore is not a significant diagnostic feature.

In dystrophic mice a slight increase in terminal innervation is observed, but many axons show either demyelination or fusiform swellings especially in the vicinity of the terminal arborization. The mean end-plate size is slightly though not significantly increased. Many end-plates appear to be undergoing degenerative changes. The number of simple 'T_1' end-plates is decreased, whereas the percentage of more complex end-plates is increased; most prominent are the relatively high percentages of 'T_2' and 'T_2 + C' type end-plates.

The study of peripheral innervation in heterozygote mice of both the wobbler and the dystrophic colony does not reveal changes in innervation associated with heterozygosity. There are, however, some changes in the pattern of innervation associated with increase in mouse age. The FTIR increases slightly with age whilst the number of 'T_1' end-plates reduces as a result of an increase in the more complex terminal arborizations.

These studies have only been carried out using the gastrocnemius muscle and it is not certain whether the results would apply equally to other limb muscles.

IV. Discussion

This study on terminal innervation in mice has been rather limited in that the number of animals studied was small. It has, however, provided further evidence to suggest that the ageing process is reflected in muscle by limited changes within the terminal innervation. Studies of limb muscles in certain small mammals have led to the suggestion that sprouting occurs under normal conditions, some of which could be accounted for as the result of growth and degeneration due to age (Barker & 1p 1965). An ageing process in human muscle has also been implied. Beyond the age of sixty a progressive fall in the number of motor units has been demonstrated (Campbell, McComas & Petito 1973) and so long as

the motor unit count does not fall below twenty percent, muscle size and strength is maintained as a result of functional compensation, thus masking the severity of motor unit loss (Brown 1973). Single fibre atrophy has been reported in humans over sixty (Adams 1969) and axonal degeneration with increased collateral ramification has also been demonstrated in the peroneus brevis muscle of elderly patients (Harriman, Taverner & Woolf 1970). Collateral branching with an increased terminal innervation ratio as a result of increase in age has not been demonstrated by Coërs and his co-workers. In their study of over thirty normal muscles, the specimens examined were not taken from distal muscles of the legs and the methods used for scrutinising controls in which those with histological changes in the muscle were disregarded, would probably account for the inconsistency in their results with those of other studies as muscles showing ageing changes would have been discarded (Coërs, Telerman -Toppet & Gerard 1973). A comprehensive study of the effect of ageing on the growth and degeneration of motor end-plates in hind-limb muscles of the cat has revealed that two separate phenomena occur as a consequence of ageing, a growth effect resulting in an elaboration of the end-plate structure, and the degeneration of end-plates in response to muscle fibre loss (Tuffery 1971). The results of the present study appear to confirm these observations for the gastrocnemius muscle of the mouse. Both an increase in collateral branching and end-plate 'elaboration' has been demonstrated to correlate with the increased age of the animals studied. There appears to have been no previous study of age effect on innervation in mouse muscle. The results of the present study, however, will require confirmation using a larger number of animals, a wider age range, and various proximal and distal limb muscles.

From the observations on terminal innervation ratio and end-plate structure no variation was detected between the results in control

C57/BL mice and those of heterozygotes from either the wobbler or the dystrophic colonies. The number of animals within each group has however been a limiting factor. Much larger numbers would be required to consider whether the rate in the ageing process in muscles of heterozygote mice of either colony would be indicative of any detrimental effect of the heterozygote gene. There are suggestions that heterozygote carriers of lower motor neurone disease in humans do show some abnormalities in muscle (Emery, Anderson & Noronha 1973). Innervation in human carriers has not been studied in great detail although observation on a single male and female carrier of SMA will be reported in Chapter 8.

The study of functional terminal innervation ratio in the wobbler mouse muscle has confirmed that extensive axon sprouting and collateral innervation is a feature of the disease (Duchen, Strich & Falconer 1968). It has, however, shown that FTIR in these mice is of little value as an indicator of the rate of progress of the disease. In animals where the disease is most benign one would expect a lower FTIR than in those where the disease progresses more rapidly; this is not always true since in those animals where the muscle is less atrophied the number of axons is greater and hence they can be more easily and accurately measured. The results for this aspect of the study have demonstrated that the FTIR innervation ratio alone is not always very reliable in estimating disease prognosis, possibly even less so where the muscle chosen for study is believed to be only mildly affected by the disease process.

End-plate sizes of wobbler mice are smaller than in healthy animals; such a result would be expected if one does not correct for muscle fibre size (Zacks 1964), and where the area of cholinesterase activity is used as a measure of end-plate size. Muscle fibres innervated by collateral

branches may have small underdeveloped end-plates, whilst degenerating end-plates may have a low cholinesterase activity. More surprising perhaps is the relatively small number of complex end-plates found in the affected mice. It would appear that in these animals the 'dying-back' phenomenon is not associated with an elaboration of the terminal arborization structure.

Innervation studies on gastrocnemius muscle from mice with hind-limb paralysis (Wr/HLP) demonstrate clearly the involvement of a neurogenic factor in the pathological process seen in these animals. The FTIR is increased, thus indicating a form of neurogenic atrophy. An electrophysiological study on the extensor digitorum longus muscle of the wobbler mice displaying a hind-limb paralysis has demonstrated the similarity between results for this muscle and those found in the proximal fore-limb muscles of affected mice with no hind-limb paralysis (Harris 1975). Harris suggests that wobbler mice with the hind-limb paralysis demonstrate an abnormal distribution of affected muscles. The mice with late onset hind-limb paralysis which have been used in this study would require electrophysiological investigation to enable further clarification of the disease pathology.

The Wr/HLP mice show no differences in mean end-plate size and, although there is a slight increase in the complexity of end-plate structure, the abnormal terminal innervation ratios and the large numbers of fine axons in the muscle are much more striking features of the disease pathology. Electronmicroscopic studies of end-plate in these mice may prove more revealing.

Terminal innervation studies in dystrophic mouse muscle suggest that a neurogenic factor may be involved in the disease process. There is mounting evidence regarding the neurogenic involvement in murine muscular dystrophy (McComas, Sica & Currie 1970; Bradley 1971; Harris,

Wallace & Wing 1972; Bradley & Jenkison 1973). The dystrophic process in mouse is nevertheless not equivalent to the neurogenic atrophy found in wobbler mice (Harris & Ward 1974) nor to atrophy resulting from surgical denervation (Law & Atwood 1972). The observations from the study of terminal innervation have further confirmed that the peripheral nerve in the dystrophic mouse does not resemble that of wobbler mice and that the pattern of innervation differs from that found in mice with hind-limb paralysis. The functional terminal innervation ratio is slightly elevated but it does not represent the primary neural abnormality in the muscle. The structure of some subterminal axons, which show evidence of a thickening of axons ,or fusiform swellings, and the grossly abnormal appearance of many end-plates are much more prominent features of the abnormalities in peripheral innervation seen in dystrophic mice.

The increase in FTIR in dystrophic mice may be due to a slight neurogenic atrophy. The collateral sprouting of axons, however, may be in response to fibre splitting; the axon branches innervate small 'atrophic-like' fibres which appear similar in size to those found in neurogenic atrophy. The branching axons do not resemble those found in atrophic conditions. Electrophysiological results from human Duchenne type muscular dystrophy suggest that existing axons innervate new fibres and fibres which result from longitudinal fibre splitting rather than collateral branching occurring in response to neurogenic atrophy (Desmedt & Borenstein 1973).

The extent of damage to the peripheral nerve resulting from the disease in murine muscular dystrophy is not suggestive of a purely myopathic nature of the disease, although the pathological changes in the sub-terminal nerve are most prominent at the neuromuscular junction; consequently it could be argued that they are the direct result of an

impairment in the muscle. However, a myopathy stimulated by the administration of cardiotoxin into an area of mouse muscle results in permanent changes of end-plate structure but such a myopathy does not cause axonal damage (Duchen et al 1974). The administration of the neuromuscular blocking agent, botulinum toxin, into mouse gastrocnemius has been found to result in similar damage to peripheral nerve and muscle as that found in murine muscular dystrophy (Duchen 1971). This result indicates a possible neuromuscular block or an abnormality of some neural 'trophic' factor, resulting in pathological changes of both nerve and muscle. The concept of a 'trophic' factor being involved in the pathogenesis of certain neuromuscular diseases is not new. Experiments involving the transplantation of muscle from dystrophic mice to healthy ones and vice versa have been designed to demonstrate whether the dystrophic nerve is a prerequisite of the disease (Salafsky 1971; Laird-Ralston 1972; Neerunjun & Dubowitz 1975). The results of these studies are contradictory and often the limitations of transplantation experiments, such as the immune response of the host to the inserted muscle and even such changes as result from transplantation of muscle from one area to another within the same animal, are not taken into account when interpreting these results (Carlson 1970). The results from in vitro experiments are also inconsistent (Gallup & Dubowitz 1973; Moore M.J. 1975), therefore they have so far proved of little value. It has been suggested that the denervation of dystrophic muscle does not result in a cessation of the dystrophic process (Banker & Denny-Brown 1959) and an attempt to induce impairment of a possible 'trophic' effect of the nerve by repeated denervation and reinnervation experiments does not result in muscle changes similar to those found in dystrophy (Bradley 1972). These experiments indicate that a neural 'trophic' effect is

not responsible for the disease process in dystrophy. On the other hand, however, dystrophic muscle does not respond to cross reinnervation in the same way as normal muscle (Law & Atwood 1972); an abnormality in axoplasmic flow in murine dystrophy has been reported (Bradley & Jaros 1973), and altered neural proteins have also been demonstrated (Salafsky & Sterling 1973). Such evidence would be indicative of a neural involvement. Also an abnormality in the ventral roots in animals with mouse muscular dystrophy has been reported (Bradley & Jenkison 1973). It would be unlikely that such changes in the nerve would be dissociated from those found in muscle. The abnormalities of peripheral nerve at the level of the subterminal axon and terminal arborization reported in this study may be a consequence of the changes in the nerve root. There is, therefore, considerable evidence implicating a neural factor in the pathogenesis of murine muscular dystrophy. The disease, however, may be the result of a more generalised metabolic condition manifesting itself in both muscle and nerve (Emery & Gosdon 1974). The results of this study, although they have outlined the morphological features involved in the degeneration of the subterminal axon in murine dystrophy, do not resolve the present confusion regarding the importance of the nerve in the dystrophic process.

A further observation made on the preparations of dystrophic muscle may be of some value. The considerable interest in the pathogenesis of murine muscular dystrophy has resulted from the resemblance of this disease, primarily in muscle histology, to that observed in human dystrophies. Evidence;of a neural involvement in human dystrophy is not very substantial (Emery & Gosdon 1974). The original suggestion of denervation in Duchenne type muscular dystrophy based on electrophysiological results (McComas, Sica & Currie 1971) is now being questioned (Scarpalezos & Panayiotopoulos 1973). It is therefore surprising that

some features described for innervation changes in Duchenne muscular dystrophy (Jedrzejowska, Johnson & Woolf 1965) resemble those noted in this study for murine muscular dystrophy. Although comparisons between species, especially where a disease process is involved, are not entirely justified, it is however possible that the disease in man and mouse have a common origin. It seems possible that in both mouse and man the variable manifestations in nerve and muscle result from a more generalised, metabolic abnormality (Rifenberick, Gamble & Max 1973; Montalbo & Kabara 1974; Emery & Gosdon 1974).

PLATE 1

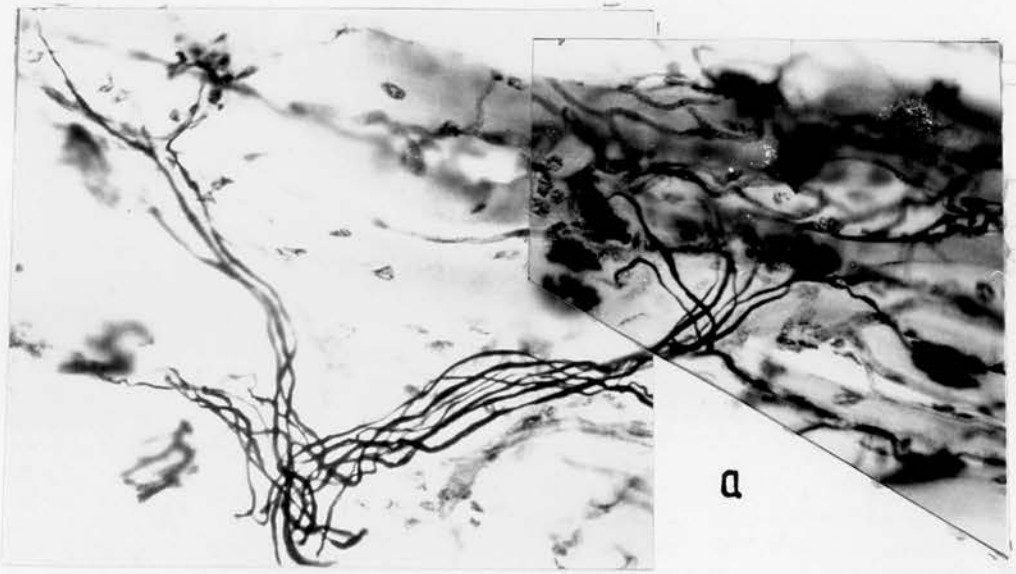
a) Normal innervation in the gastrocnemius muscle from a wobbler littermate (WrWr/Wrwr). Age 2 months. Ache/Silver impregnation x 400

b) Normal innervation in gastrocnemius muscle from a wobbler littermate (WrWr/Wrwr). Age 4 months. Ache/Silver impregnation x 400

c) Normal innervation in gastrocnemius muscle from a C57/BL mouse. Age 4 months. Ache/Silver impregnation x 160

d) An intramuscular nerve bundle in the gastrocnemius muscle from a wobbler littermate (WrWr/Wrwr). Age 4 months. Ache/Silver impregnation x 400

PLATE I



a

b

c

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PLATE 2

a) Innervation in the gastrocnemius muscle of a Wr/HLP mouse.
Age 6 months
Ache/Silver impregnation x 400

b) Innervation in the gastrocnemius muscle of a Wr/HLP mouse
Age 7 months
Ache/Silver impregnation x 160

c) Axonal branching in the gastrocnemius muscle of a Wr/HLP mouse.
Age 6 months.
Ache/Silver impregnation x 160

PLATE 2

a

c

b

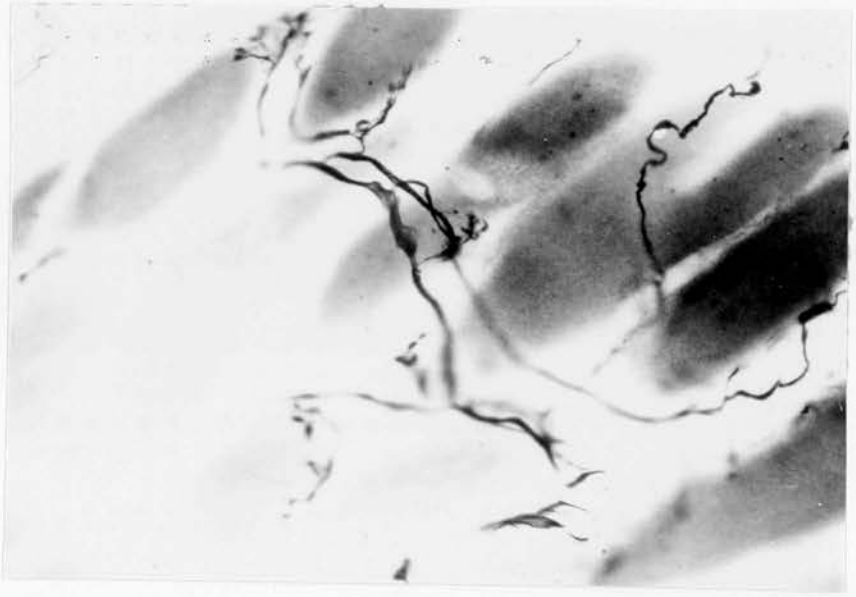
PLATE 3

a) Innervation in the gastrocnemius muscle of a wobbler (wrwr) mouse. Age 2 months
Ache/Silver impregnation x 400

b) Innervation in the gastrocnemius muscle of a wobbler (wrwr) mouse. Age 5 months
Ache/Silver impregnation x 400
Axonal branching and "beaded" axons are visible

c) An intramuscular nerve bundle in the gastrocnemius muscle of the wobbler (wrwr) mouse. Age 5 months
Ache/Silver impregnation x 400
Demyelinated axons and a loss of axons can be seen.

PLATE 3



a

b

c

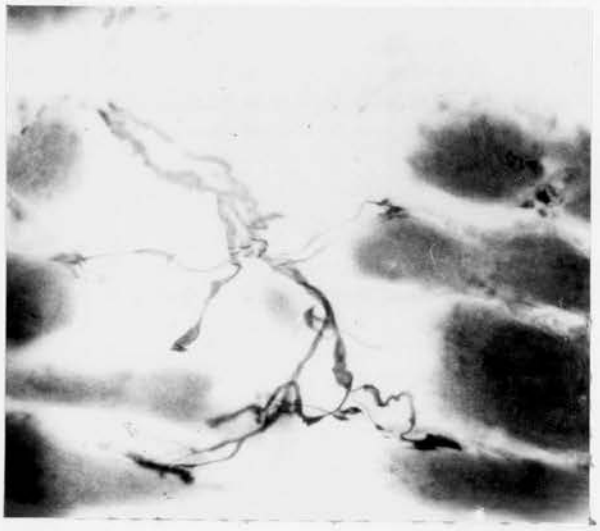
PLATE 4

a) Innervation in the gastrocnemius muscle of a dystrophic (dydy) mouse. Age 3 months
Ache/Silver impregnation x 400
Axonal branching can be seen

b) Innervation in the gastrocnemius muscle of a dystrophic (dydy) mouse. Age $3\frac{1}{2}$ months
Ache/Silver impregnation x 400
Axonal branching can be seen

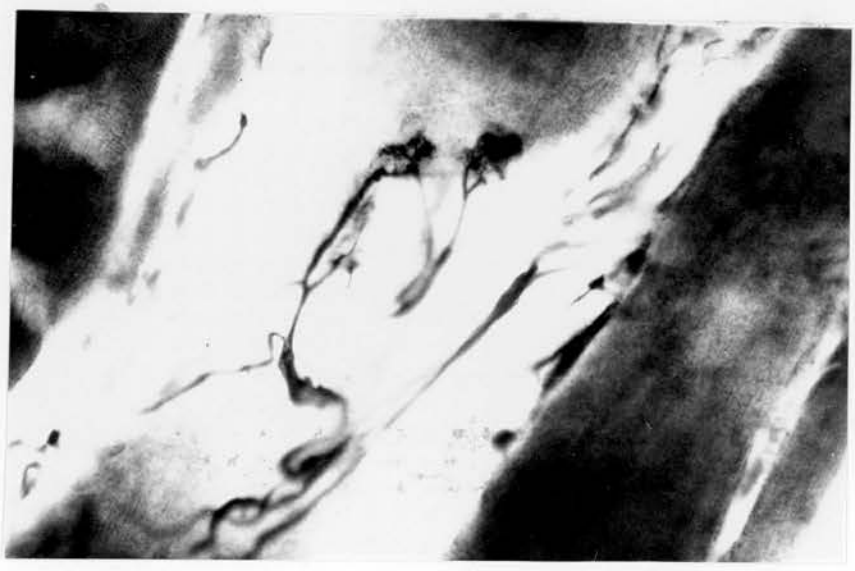
c) Innervation in the gastrocnemius muscle of a dystrophic (dydy) mouse. Age 3 months
Ache/Silver impregnation x 400
Sprouting from the end-plate can be seen

PLATE 4



a

c



b

PLATE 5

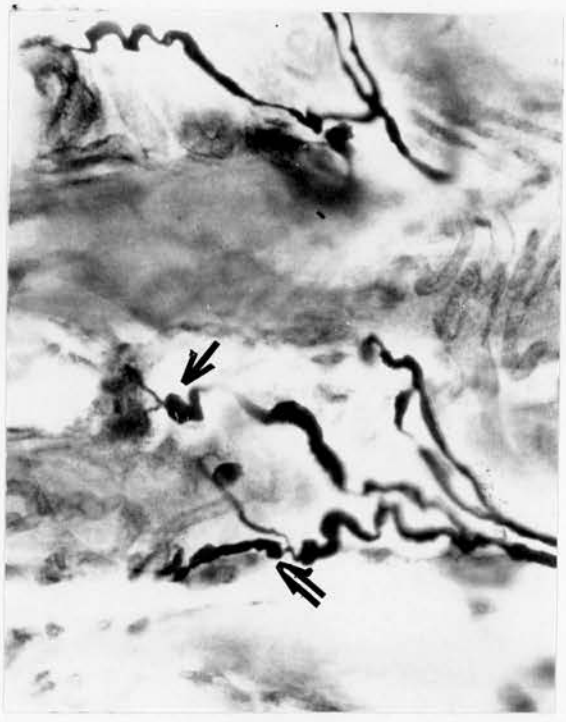
a) An end-plate innervated by two myelinated branches from a single axon ('T_2' end-plate) from the gastrocnemius muscle of a C57/BL mouse
Ache/Silver impregnation x 625
The T_2 end-plate is indicated by a single arrow.
A branching axon is indicated by a double arrow.

b) A 'T_2' and possibly also a 'T_3' end-plate from the gastrocnemius muscle of a wobbler heterozygote (Wrwr) mouse
Ache/Silver impregnation x 625
The 'T_2' end-plate is indicated by a single arrow
The 'T_3' end-plate is indicated by a double arrow

c) Multiple myelinated branches to a single end-plate in the gastrocnemius muscle of a Wr/HLP mouse
Age 7 months
Ache/Silver impregnation x 625

d) A grossly abnormal end-plate, from the gastrocnemius muscle of a dystrophic (dydy) mice. Age $3\frac{1}{2}$ months
Ache/Silver impregnation x 525.

PLATE 5



a

b

c

d

CHAPTER 6

HISTOLOGICAL AND HISTOCHEMICAL STUDIES OF MUSCLE

I. Introduction

II. Material and Methods

(i) Material

(ii) Methods

a) Staining techniques

b) Observation tissue

1. Histology

2. Histochemistry

III. Results

(i) Histology

(ii) Histochemistry

IV. Discussion

CHAPTER 6

TABLES

CHAPTER 6

I. Introduction

In laboratory investigations of neuromuscular disease, histological and histochemical examinations of skeletal muscle specimens are of primary importance (Engel 1962). Histological observations are of value in determining gross pathological changes in muscle such as fibre atrophy, scattered or grouped, hyalin or vacuolar degeneration, phagocytosis, fibre necrosis, fibre splitting etc. Measurements of muscle fibre diameter may be of further value in determining whether fibre atrophy or hypertrophy can be regarded as a significant feature of the disease. Pathological changes in muscle vary in different diseases, but there are certain characteristics which distinguish myopathies from neurogenic atrophies (Dubowitz & Brooke 1973).

Histological studies alone often yield insufficient information to command an accurate diagnosis of a particular disease and additional histochemical studies may be required. The particular enzymatic activity of individual muscle fibres determines their type. There have been suggestions that there may be as many as eight different fibre types in mammalian skeletal muscle (Romanul 1964). Brooke and Kaiser (1970) however, studying the enzyme myosin ATPase, have shown that at a pH of 9.4 two main fibre types can be distinguished in both human and animal muscle. Fibres of low activity at this pH are denoted as Type I fibres, the remaining as Type II. The type II fibres can be further subdivided in three subsections according to the pH range at which the myosin ATPase remains active. There is experimental evidence to suggest that muscle fibre type is determined by the motor neurone and that fibres of a single motor unit are of one type (Brooke, Williamson & Kaiser 1971). There appears to be agreement now that there are three important fibre type groups, Type I, Type IIA and Type IIB. In the study of human muscle, the classification of fibres

into the two main fibre types has been suggested as being sufficient. Animal muscle requires typing into three fibre types because of the much larger proportion of intermediate fibres.

It is now well established that a feature of muscle pathology resulting from peripheral nerve damage is the altered spatial distribution of muscle fibre types. In experimental reinnervation the mosaic pattern which is the normal feature of muscle, becomes altered, the amount of grouping varying from a few fibres in one group to a large number of fibres within others. Experimental denervation and reinnervation has been found to lead to the motor unit being enclosed within a smaller area although involving the same number of fibres (Kugelberg, Edstrom & Abbruzzese 1970). In lower motor neurone disease fibre type grouping occurs as a result of collateral branching with the motor unit size becoming increased. Alteration of the histochemical profile of muscle may occur as a result of disease (Johnson et al 1973) or in connection with the ageing process of the muscle (Jennekens, Tomlinson & Walton 1971).

In this study only mouse muscle has been used. Histological studies on mouse muscle have largely been concerned with examining diseased muscle such as is found in murine muscular dystrophy (West & Murphy 1960, Pearce & Walton 1963, Goldspink & Rowe 1968). Although histochemical studies of normal muscle are numerous, few have concentrated on normal mouse muscle. In mouse diaphragm muscle fibres have been shown to be predominantly of the type II variety when classified using myosin ATPase; however, all fibres are high in oxidative enzymes (Davies & Gunn 1972). Although this is not entirely true for skeletal muscle in general, many muscles are made up of largely one fibre type. Such a pattern inevitably creates problems when investigating possible changes of fibre type distribution as a result of disease.

The purpose of this study has been to establish the extent to which changes in the subterminal nerve of Wr/HLP mice (see Chapter 5) are reflected in altered muscle pathology and to compare these findings with the histological and histochemical profiles observed in wobbler mice.

II. Material and Methods

i) Material

The study is largely based on investigations of the gastrocnemius from control mice (C57/BL) and mice of the wobbler colony. The number of mice in each group is given in Table 6.1.

ii) Methods

Mouse muscle was removed from the animal and prepared for sectioning as detailed in Chapter 2. Transverse sections of 10 μ thickness were cut using the retracting microtome. An attempt was made only to use sections from near the centre of the muscle and not from the tendon regions.

a) Staining techniques

Each specimen was stained with the histological stains haematoxylin/eosin and Gomori-trichrome. The histochemical procedures used were NADH-diaphorase activity and myosin ATPase activity at pH 9.4 and 4.3 (for details of staining procedures used, see Chapter 2).

b) Observation of tissue

1. Histology

Each muscle specimen was examined for evidence of necrosis, phagocytic infiltration and the existence of granular fibres. The percentage of fibres showing central nuclei was obtained by counting 500 fibres at random. The diameter of 200 fibres was measured for each sample. Measurements were made directly from the slides to the nearest 2.5μ using 400 x magnification.

TABLE 6.1 - THE ANIMALS USED IN THE STUDY

Genotype	No. studied	Age range in days
C57/BL	6	80 - 160
WrWr/Wrwr	3	54 - 124
Wrwr	3	237 - 300
Wr/HLP	5	182 - 266
wrwr	3	98 - 136

2. Histochemistry

Slides were observed after histochemical staining for evidence of fibre-type grouping.

III. Results

i) Histology

The results of the histological studies are given in Tables 6.2 and 6.3. The mean fibre diameters for all mice, except the wobbler affected animals were found to be remarkably similar. However, there was a correlation between fibre size and age in the C57/BL mice $r = 0.79$ $P < 0.05$. In the Wr/HLP mice the correlation, though not statistically significant, reflects a decrease in fibre size with age $r = -0.72$ $P > 0.1$. These results are indicative of an increasing number of small atrophic fibres as the disease progresses.

The coefficient of variation of fibre size in wobbler mice is much higher than in healthy mice. There is no overlap of results between these two groups (see Figure 6.1). The mean coefficient of variation for the Wr/HLP mice is greater than for the control animals. Although there is no overlap of results between Wr/HLP and C57/BL mice, the control animals are considerably younger; there is, however, some overlap with the heterozygotes which are more closely age-matched.

Muscle from Wr/HLP mice does show a greater number of necrotic foci than are seen in healthy animals. Granular fibres and fibres with central nuclei were found to be uncommon in both healthy and Wr/HLP mice; central nuclei are a feature of wobbler mouse muscle, although these occur mainly in the atrophic fibres, whereas few are visible in the more normal appearing areas of the muscle. Muscle histology is illustrated in Plate I.

ii) Histochemistry

The histochemical investigations did not prove very successful

TABLE 6.2 - RESULTS OF HISTOLOGICAL STUDIES

Genotype	Fibre Size (u) Mean	SD	Significant difference from controls	Coefficient Variation(%)	Significant difference from normal
C57/BL	45.6	3.0	-	21.5	-
WrWr/Wrwr	43.5	2.0	NS	22.4	NS
Wrwr	50.3	4.5	NS	23.2	NS
Wr/HLP	43.8	3.5	NS	30.6	$P < 0.001$
wrwr	29.5	5.4	$P < 0.001$	43.9	$P < 0.001$

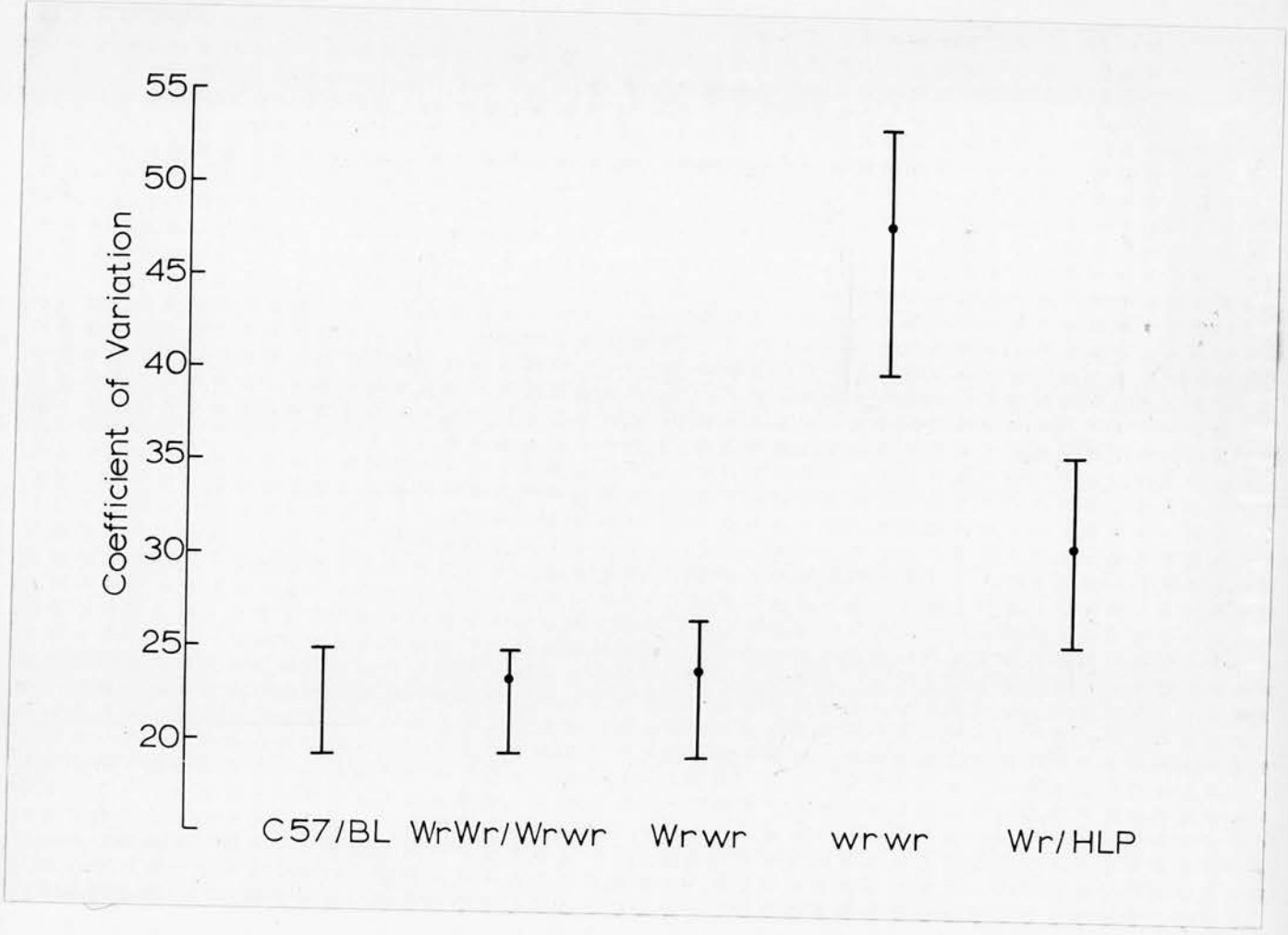
TABLE 6.3 - RESULTS OF HISTOLOGICAL STUDIES FOR THE INDIVIDUAL MICE

Mutant		1	2	3	4	5	6
C57/BL	Age in days	80	90	90	106	120	160
	Mean fibre size(μ)	43.67	45.63	45.46	46.55	41.35	51.12
	SD	8.7	9.8	9.7	11.6	8.9	9.8
	Coeff. variation	19.9	21.5	21.3	24.9	21.5	19.2
WrWr/Wrwr	Age in days	54	74	124			
	Mean fibre size(μ)	40.82	45.59	44.01			
	SD	9.5	11.3	8.5			
	Coeff. variation	23.3	24.8	19.3			
Wrwr	Age in days	237	266	300			
	Mean fibre size(μ)	43.97	54.50	52.47			
	SD	11.7	13.0	11.3			
	Coeff. variation	26.6	23.8	19.3			
Wr/HLP	Age in days	182	188	203	266	?	
	Mean fibre size(μ)	45.98	49.64	42.36	41.43	39.76	
	SD	16.3	15.3	12.4	13.6	15.7	
	Coeff. variation	35.4	30.6	29.2	32.8	25.1	
wrwr	Age in days	98	111	136			
	Mean fibre size(μ)	25.76	25.60	37.20			
	SD	12.3	13.6	11.5			
	Coeff. variation	47.7	53.1	30.9			

FIGURE 6.1

The range of coefficients of variation
of muscle fibre size in the five groups of mice studied

FIGURE 6.1


Coefficient of Variation
55
50
45
40
35
30
25
20
C57/BL
WrWr/Wrwr
Wrwr
wrwr
Wr/HLP

because the gastrocnemius muscle is primarily composed of one fibre type. Only the deep-head of the muscle is composed of mixed fibres. Although fibre-type grouping can be readily seen in the wobbler mouse, no similar feature could be distinguished for the Wr/HLP mouse. Histochemical profiles of muscle in wobbler and Wr/HLP mice are illustrated in Plates 2 and 3.

IV. Discussion

The wobbler mice used in these investigations were all free from paralysis of the hind-limbs, however both muscle atrophy and fibre-type grouping are clearly visible. These features which are in agreement with those expected for a spinal muscular atrophy are not prominent in the Wr/HLP mice, even though these animals show a paralysis of the hind-limbs. The features of muscle histology and histochemistry of mice with late onset hind limb paralysis resemble in many ways those suggested for myopathic conditions (Dubowitz & Brooke 1973). Muscle fibre sizes, although showing an increased variance, do not appear as two distinct peaks, the normal fibres and the atrophic fibres, but there appears to be a wider spectrum of fibre sizes. There is also no overlap in coefficients of variation for muscle fibre size between wobbler and Wr/HLP mice. The coefficient of variation is probably the most useful measure of abnormality in muscle as it has been shown to be largely independent of age and sex effects, and the muscle or area of muscle used (Lumb, Vassilopoulos & Emery 1976). Observations of histological and histochemical preparations of muscle from patients with diabetic neuropathy have led to the suggestion that changes customarily attributed to myopathies may also occur in chronic neuropathic conditions (Vassilopoulos - personal communication). An important histological characteristic of myopathic degeneration is the migration of nuclei into the centre of the fibre; Wr/HLP mice show very few

fibres with central nuclei. The histological changes seen in Wr/HLP muscle may therefore be compatible with a chronic neurogenic condition.

The results of histochemical studies of Wr/HLP gastocnemius muscle do not indicate any fibre type grouping. These observations are surprising when one considers that the FTIR in these animals is raised. It has, however, been demonstrated in human muscle that collateral branching may be well developed and yet no fibre type grouping is seen with NADH-diaphorase (Morris & Raybould 1971). In fact a branching axon has been shown to innervate two muscle fibres with different NADH-diaphorase activity (Morris & Woolf 1970). Cross reinnervation experiments have shown that very few fibres convert from one type to another, hence such results have questioned the importance of the nerve in muscle protein synthesis (Samaha, Guth & Albers 1970).

Therefore, there may be no inconsistency with the results for terminal innervation studies and the histochemical profile in Wr/HLP mice. Furthermore it has been demonstrated that after nerve crushing, although collateral branching can be observed, no histochemical modification can be distinguished for at least twenty-eight days after injury and evidence of fibre-type grouping does not occur until about the forty-second day (Warszawski et al 1975). It is possible that in a slow progressing neuropathy, such as is seen in mice with the late onset neuromuscular disease, the changes in the histochemical profile of the muscle are minor and occur relatively slowly. Although it also appears likely that the gastrocnemius muscle in Wr/HLP diseased mice is relatively mildly affected compared to other muscles of the hind-limb and pelvic musculature. The changes visible in the gastrocnemius muscle do not explain the paralysis seen in these mice, particularly as in the wobbler mouse where the muscle appears to be more seriously affected, the hind-limbs maintain considerable mobility.

A much more detailed histological and histochemical examination of all hind-limb muscles from Wr/HLP mice would be necessary in order to distinguish the muscle primarily affected by the disorder. The present data does not disprove the hypothesis that paralysis in the Wr/HLP mouse is the result of an abnormal manifestation of the 'wr' gene. Nevertheless if all hind limb muscles in these mice appeared mildly affected as compared to the wobbler, the extent of paralysis in Wr/HLP mice would be difficult to explain in terms of a spinal muscular atrophy.

PLATE 1

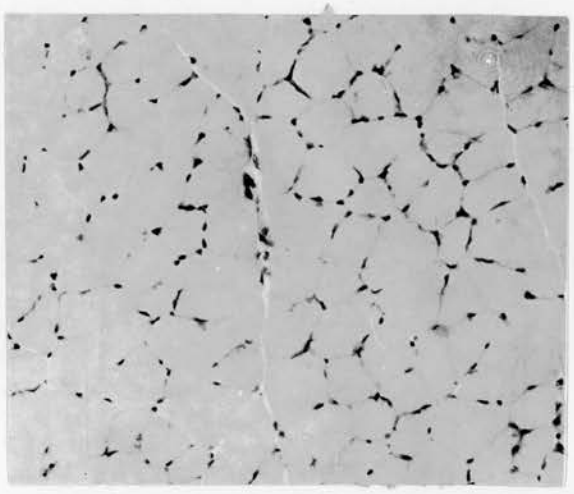
a) A transverse section of gastrocnemius muscle from a wobbler littermate (WrWr/Wrwr). Age 3 months
Haematoxylin/Eosin x 160

b) A transverse section of gastrocnemius muscle from a wobbler (wrwr) mouse. Age 4 months
Haematoxylin/Eosin x 160

c) A transverse section of gastrocnemius muscle from a dystrophic (dydy) mouse. Age 3 months
Haematoxylin/Eosin x 160

d) and e) Transverse sections of gastrocnemius muscle from a Wr/HLP mouse. Age 5 months
Haematoxylin/Eosin x 160
Some foci of atrophic fibres and some possible "wedged" fibres can be seen
Atrophic foci indicated by a single arrow
Possible "wedged" fibres indicated by a double arrow.

PLATE I



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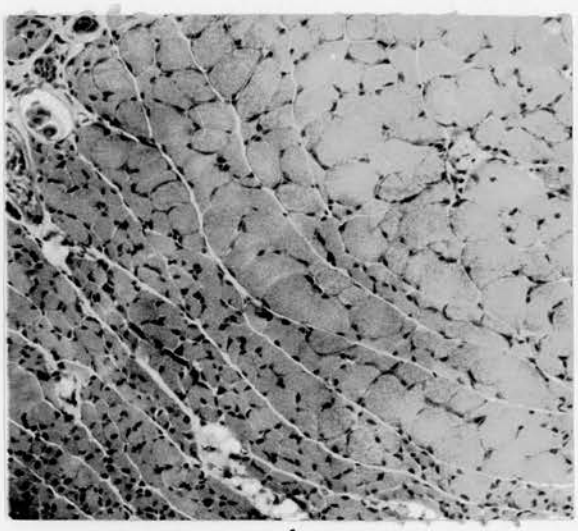

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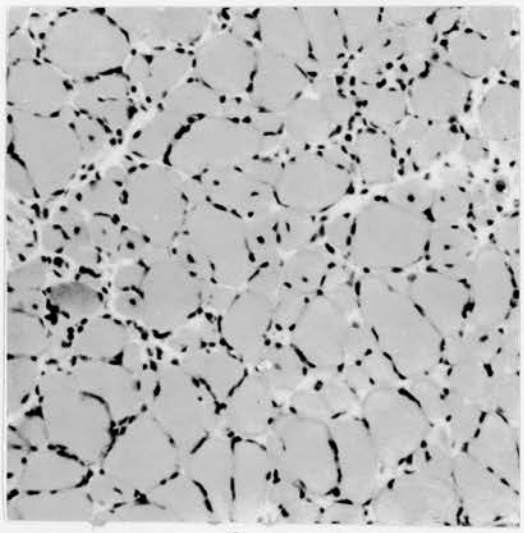

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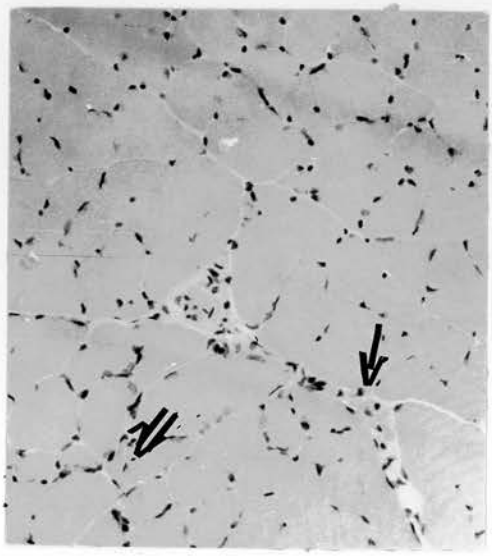

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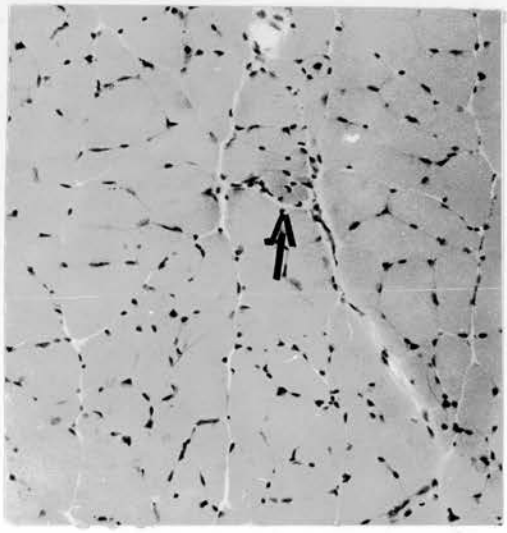


e

PLATE 2

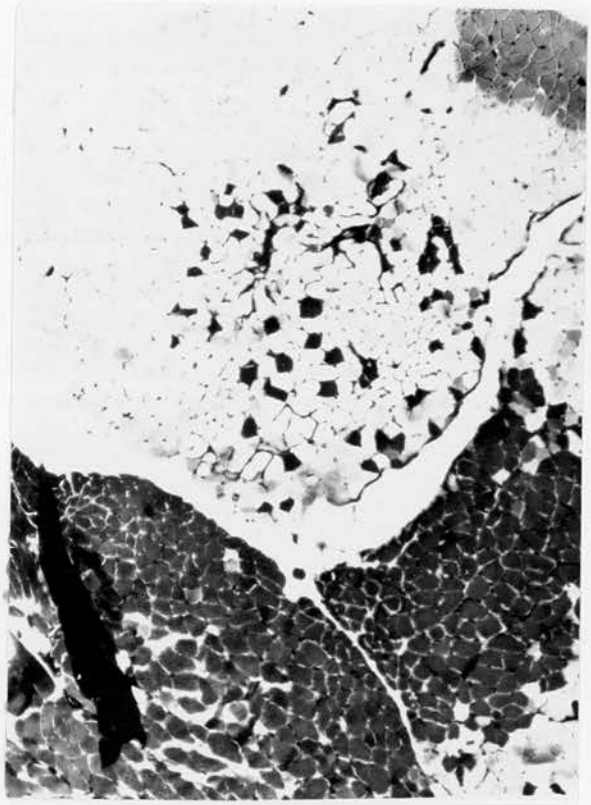
a) Transverse section of gastrocnemius muscle from a wobbler (wrwr) mouse. Age 3 months
Myosin ATPase pH 4.3 x 63

b) Transverse section of gastrocnemius muscle from a wobbler (wrwr) mouse. Age 3 months
Myosin ATPase pH 9.4 x 63

c) Transverse section of gastrocnemius muscle from a wobbler (wrwr) mouse. Age $3\frac{1}{2}$ months
Myosin ATPase pH 4.3 x 160
Fibre type grouping is clearly visible

d) Transverse section of gastrocnemius muscle from a Wr/HLP mouse. Age 5 months
Myosin ATPase pH 4.3 x 160
No fibre type grouping can be seen

PLATE 2



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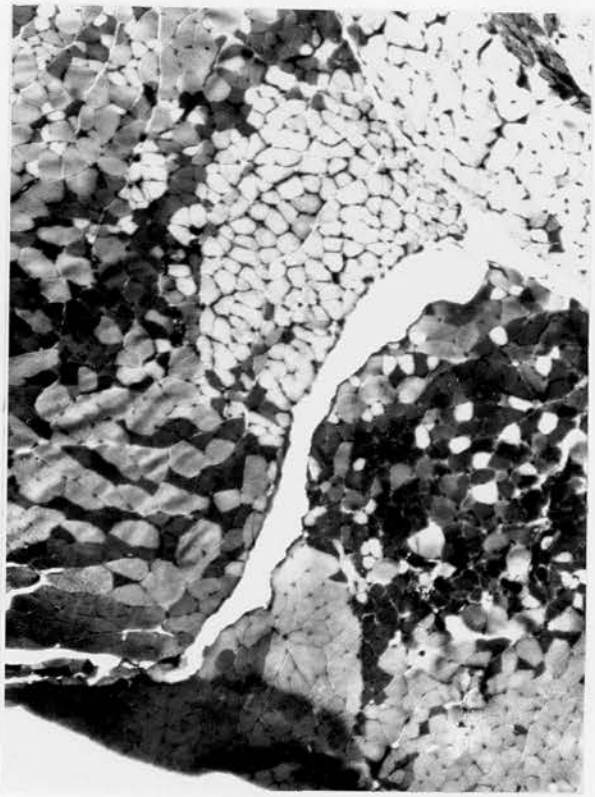

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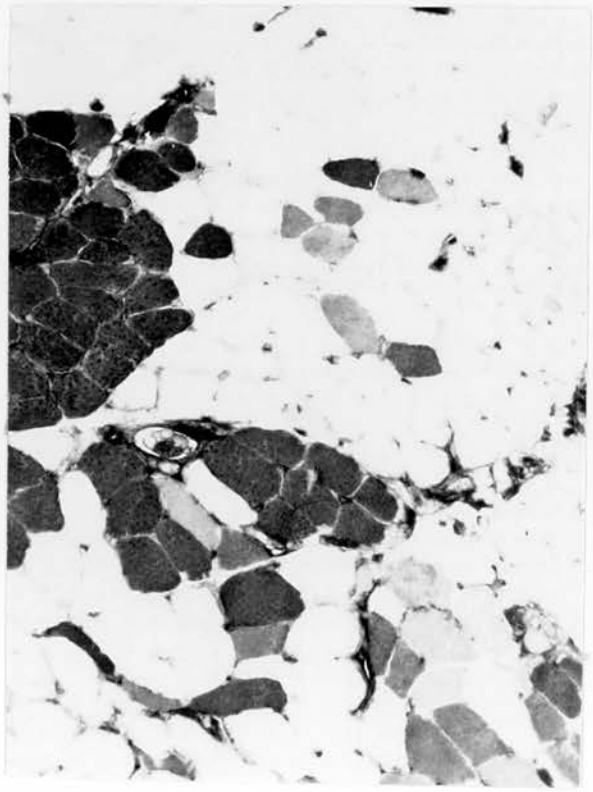

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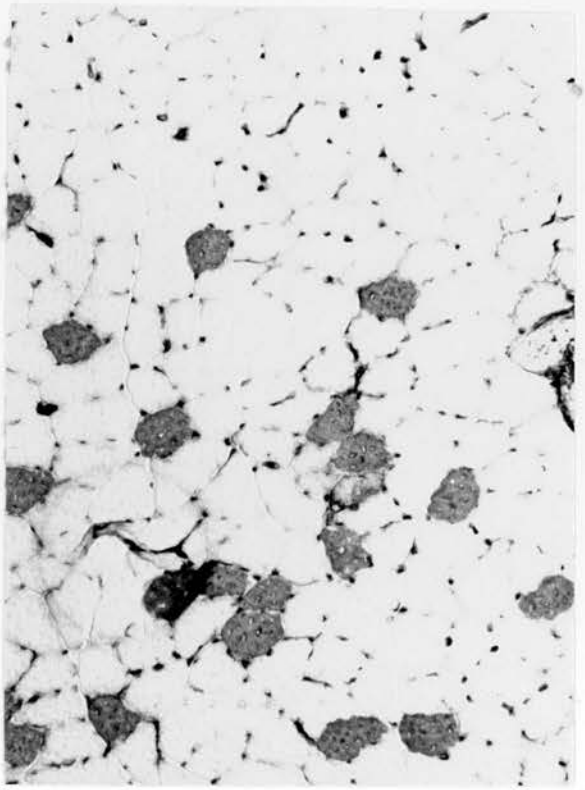

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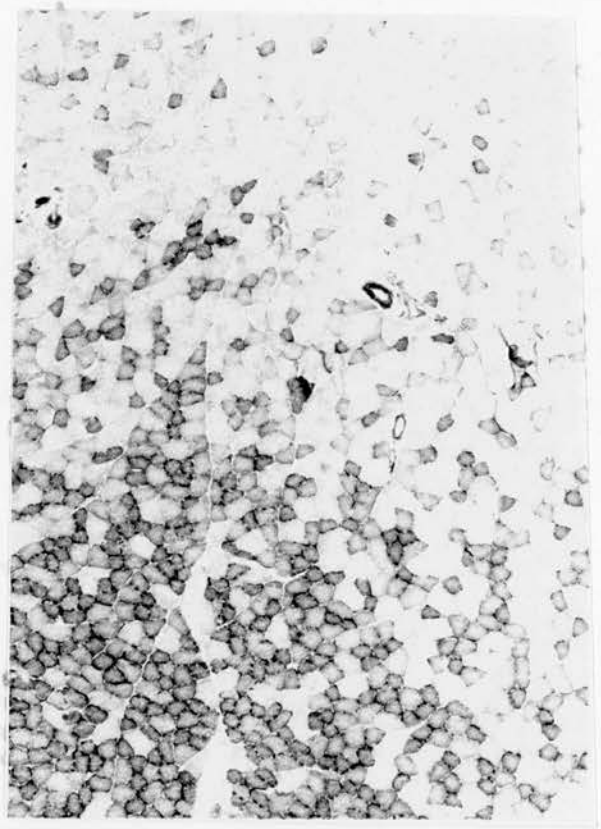
PLATE 3

a) Transverse section of gastrocnemius muscle from a wobbler littermate (WrWr/Wrwr). Age 3 months
NADH - diaphorase x 63

b) Transverse section of gastrocnemius muscle from a wobbler (wrwr) mouse. Age 3 months
NADH - diaphorase x 160

c) Transverse section of gastrocnemius muscle from a Wr/HLP mouse. Age 5 months
NADH - diaphorase x 160

PLATE 3



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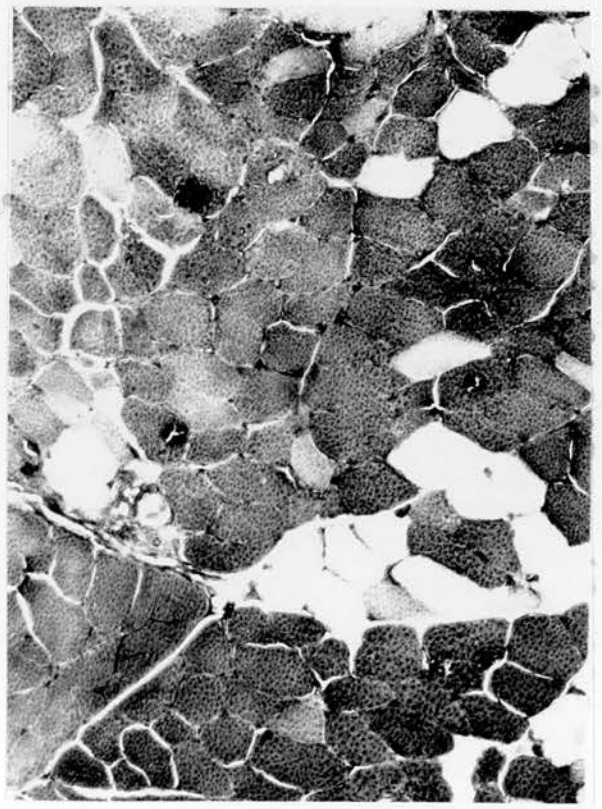

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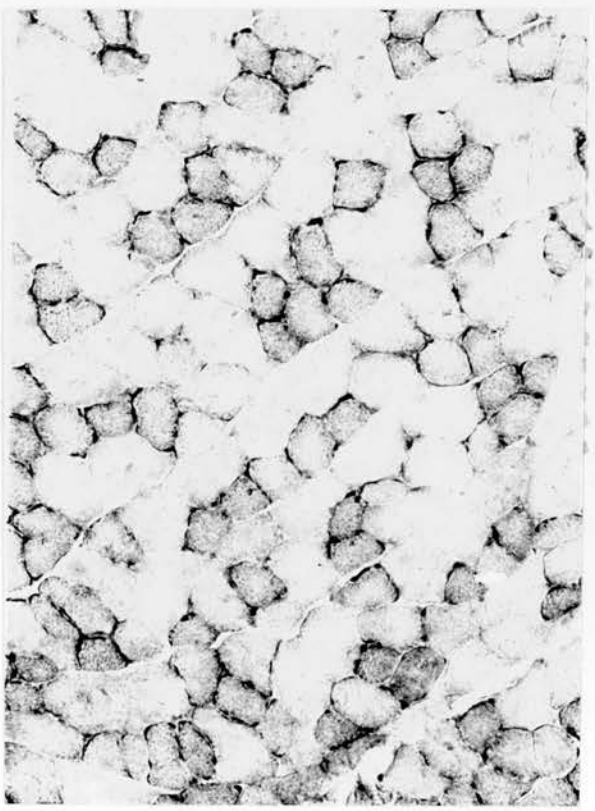

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CHAPTER 7

HISTOLOGICAL STUDIES ON THE SPINAL CORD

I. Introduction

II. Material and Methods

i) Material

ii) Methods

III. Results

IV. Discussion

CHAPTER 7

I. Introduction

The pathological changes in anterior horn cells of wobbler mice have been studied using both light and electronmicroscopic techniques. Duchen (1968) reported that in early stages of anterior horn cell degeneration the nerve cell enlarges, occasionally becoming rounded. The Nissl substance of the perikaryon stains poorly whilst the nucleus with its prominent nucleolus remains in the central position as in normal cells. As degeneration progresses the cell body becomes filled with vacuoles and in some cells the nucleus loses its central position. In the final stages of degeneration the nerve cell is barely recognisable, the nucleus being surrounded by an ill-defined mass of vacuoles. Andrews & Maxwell (1969) confirmed these findings and studied the fine structure of degenerating motor neurones. They suggested that the vacuoles seen in the electronmicroscope were of two kinds. The first type resulted from an enlargement of Golgi cisternae, which were usually to be found in a perinuclear location; other organelles in such affected cells remained normal. The second type of vacuole found to be generally distributed throughout the perikaryon appeared to be located in neurones with no identifiable Golgi complex and were found to be focal cystic expansions of other membrane systems, predominantly the endoplasmic reticulum. These vacuoles did not appear to take up any of the commonly used histological and histochemical stains.

Further studies regarding degenerating neurones in spinal cord of wobbler mice have shown that in affected animals the number of neurones becomes reduced by between 13-71%. The loss of motor neurones appears to be greater in the cervical cord than in the lumbar and lumbosacral cord, particularly in the early stages of the disease (Papapetropoulos

& Bradley 1972). More detailed electronmicroscopic studies have suggested a wider distribution of abnormality in the central nervous system of these mice (Campbell 1972). A study of both neuronal ultrastructure and axoplasmic flow, in which intraneuronal aggregates of tubular elements were found as well as impaired axoplasmic flow (Bird et al 1971), led to the suggestion that an abnormal neurotubular protein was involved in the disease process.

A more recent study of the cervical spinal cord and ventral roots of wobbler mice has been carried out on a wider spectrum of affected animals (Andrews 1975). This study confirms that cytomembrane vacuolarization is the most readily detectable abnormality in the neurones of affected wobbler mice. However, further abnormal changes are also detectable such as non-vacuolar dissolution of the granular endoplasmic reticulum, proliferation of cytoplasmic microtubules and neurofilaments, lipid accumulation as well as increased numbers of lysosomes and autophagic vacuoles. Occasionally these abnormalities have been found to occur together, although more frequently one or more of these features becomes the prominent pathological abnormality in any one cell. The control mice used in Andrews' study, which were genetically undefined littermates of wobbler mice, did show some degenerative changes, such as dilation of the Golgi complex and of the granular endoplasmic reticulum, some rare phagocytic cells and scattered Wallerian degeneration. These changes were however more common in older mice and the frequency never approached that encountered in wobbler mice.

The feature which makes the degenerative changes observed in the wobbler mouse peculiar to it, is not that these changes occur uniquely in this mutant but rather it is the relative frequency and distribution with which they occur and the near absence of other changes which would normally occur simultaneously in other neurological abnormalities (Lieberman 1971, Raisman & Matthews 1972).

A lower motor neurone disease in mice associated with a type C RNA virus infection has also been reported (Gardner et al 1973). The most striking neuropathological changes in these diseased mice have been observed in the lumbosacral spinal cord. Vacuoles have been found in both grey and white matter, also a varying degree of vacuolarization was seen in the neurones. Other changes included mild to severe gliosis found primarily in,and adjacent to,anterior horn cells and the presence of mononuclear cells of uncertain origin with swollen cytoplasm. Neuronal vacuoles which develop in CRNA infected mice appear as multifocal dilations of the rough and smooth endoplasmic reticulum. Some loss of motor neurones from the ventral horn of severely affected mice has also been suggested (Andrews & Gardner 1974).

Although the pathological features in the spinal cord of wobbler mice and CRNA infected mice are not the same, there is a degree of similarity between vacuolated neurones in these two groups of mice (Andrews et al 1974). The clinical manifestations seen in the Wr/HLP mouse were described in Chapter 3 and to some degree appear to be similar to those resulting from the viral induced neurogenic atrophy. The study of innervation and muscle histology of Wr/HLP mice are indicative of a slowly progressing neurogenic atrophy. In order to establish the nature of the disease it is necessary to demonstrate degeneration of anterior horn cells. It is unlikely that a light microscopy study of the spinal cord alone could verify whether the disease more closely resembled that seen in wobbler mice or in the CRNA infected mice; however it could be useful in the further understanding of the late onset hind-limb paralysis of the Wr/HLP mice.

II. Material and Methods

i) Material

The lumbar and cervical enlargements of spinal cords from a total of twenty mice were examined: C57/BL (5), WrWr/Wrwr (6), Wr/HLP (3) and wrwr (6).

ii) Methods

Spinal cords were frozen and mounted for transverse sectioning. When previously stored frozen material was being prepared for sectioning, extra care was taken to prevent the defrosting of the tissue during the process of mounting. Sections of 20 μ thickness were cut and the tissue stained with toluidine blue and the combined luxol fast blue/cresyl violet stain (for methods see Chapter 2). The preparations were examined for evidence of anterior horn cell degeneration and for the presence of other visible abnormalities.

III. Results

Two preliminary observations made with regard to the gross appearance of the spinal cord were firstly that the diameter of both the lumbar and cervical enlargements of wobbler mouse appeared to be considerably smaller than that of other mice examined. Secondly that the cords of both wobbler and Wr/HLP appeared to be firmer in texture and easier to handle. The small diameter of the wobbler cord is not surprising as the mouse is invariably smaller than an age matched littermate. The firmer texture of the cord and the fact that it appeared less susceptible to freezing artefact may reflect an increase in fibrous tissue as a result of anterior horn cell loss.

At low magnification fewer anterior horn cells were seen in the cervical cord in wobbler mice than in controls. A loss of motor neurones could not be seen at this magnification in Wr/HLP mice. In the lumbar cord, at a magnification of x 63 an actual reduction in

number of cells could not easily be seen in either wobbler mice or Wr/HLP mice. See Plate 1.

Observations on cords from wobbler mice at magnifications of between x 125 and x 625 demonstrate neurones in various stages of degeneration. The most easily recognisable factor was the increased size of motor neurones. Although the presence of vacuoles in some neurones was seen, the tissue appeared to be very susceptible to freezing artefact thus making interpretation difficult. Some neurones which have distinguishable nucleus and nucleolus appear to have little nissel substance surrounding them, suggesting that these cells are in a late stage of degeneration. Anterior horn cells in various stages of degeneration have been found in both cervical and lumbar cord of wobbler mice. See Plate 1.

The study of spinal cords of the Wr/HLP mice has proved difficult because of the problems of distinguishing anterior horn cell degeneration from damage caused by freezing. The cervical cord of all affected mice closely resembled those of controls, except for the possibly increased size of some neurones. In the lumbar cord, however, the presence of some exceptionally enlarged neurones was the most easily recognisable feature. As in the case of wobbler mice a number of neurones with very sparsely staining Nissl substance could be seen. A number of cells appeared to have nuclei that were not centrally placed and some possibly degenerate neurones were also visible. Features of the Wr/HLP cord are shown in Plate 2 . The number of possibly degenerating or degenerate anterior horn cells in any one section of the wr/HLP mouse was much less than in wobbler, but the actual loss of cells in diseased animals was not quantified.

It would therefore appear from the results of the present study that there is motor neurone degeneration in the spinal cord of wr/HLP

mice, particularly in the lumbar region of the cord as would be expected from the clinical distribution of weakness. However, further investigations on the spinal cord should be carried out not on fresh frozen material but using paraffin embedded tissue.

IV. Discussion

The results of the present investigation of the histological features of the spinal cord of Wr/HLP mice act as further evidence for the neurogenic origin of paralysis in this disease. They have, however, not been of value for determining whether the degeneration seen in the cord is sufficiently similar to that found in wobbler mice to act as evidence for the hypothesis (discussed in Chapter 3) that the hind-limb paralysis is the result of manifestations in the heterozygote of the mutant 'wr' gene. A detailed electronmicroscopic study would be necessary. However, even such a study may not be entirely satisfactory as varying degrees of dilation of endoplasmic reticulum have been reported to accompany processes as varied as ageing (Andrews 1975) and exposure to toxins (Yates & Yates 1972) as well as the transmitable agent such as the CRNA virus (Gardner et al 1973). It would, however, indicate whether an infectious particle such as the CRNA virus could be seen in the tissues of the central nervous system (Andrews & Gardner 1974). If such a particle were not found and attempts at transmitting the disease from affected animals to healthy ones by inoculation of homogenates of diseased nervous tissue were not successful (Andrews et al 1974), this would be suggestive that in fact a genetic factor was involved.

Ultimate proof that the 'wr' mutant gene is a prerequisite for the hind-limb paralysis could only come from detailed genetic studies. However because of the age of onset of the disease and the sporadic nature in which it occurs, such experiments could prove difficult.

If, however, ultrastructural studies were carried out on large numbers of wobbler mice, Wr/HLP mice and known heterozygotes showing no hind-limb paralysis, and if a spectrum of similar degenerative patterns was demonstrated in spinal cord, this would be very suggestive that Wr/HLP mice are manifesting heterozygotes.

The results of this preliminary study, although partially confirming that muscle weakness occurring in Wr/HLP mice could be the result of anterior horn cell degeneration, further evidence is required to establish whether the disease in the Wr/HLP mouse has a similar origin as that seen in wobbler mice.

PLATE 1

a) Transverse section of lumbar spinal cord from a wobbler littermate (WrWr/Wrwr). Age 3 months.
Stained with luxol fast blue/cresyl violet x 63

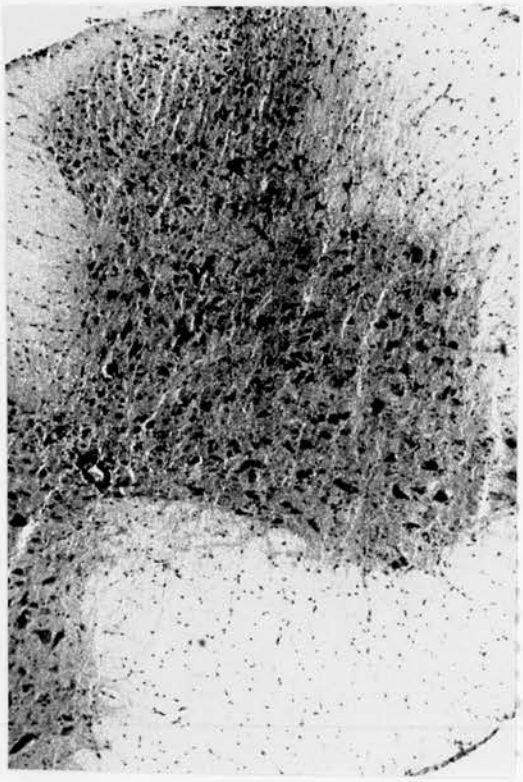
b) Transverse section of lumbar spinal cord from a wobbler littermate. Age 3 months.
LFB/CV x 400

c) Transverse section of lumbar spinal cord from a wobbler littermate. Age 3 months.
LFB/CV x 625
The anterior horn cells show no degenerative changes at this magnification

d) Transverse section of wobbler (wrwr) cervical spinal cord.
Age 3 months.
Toluidine blue x 63

e) Transverse section of wobbler cervical spinal cord.
Age 3 months.
T.B. x 625
Anterior horn cells in various stages of degeneration can be seen.

PLATE I



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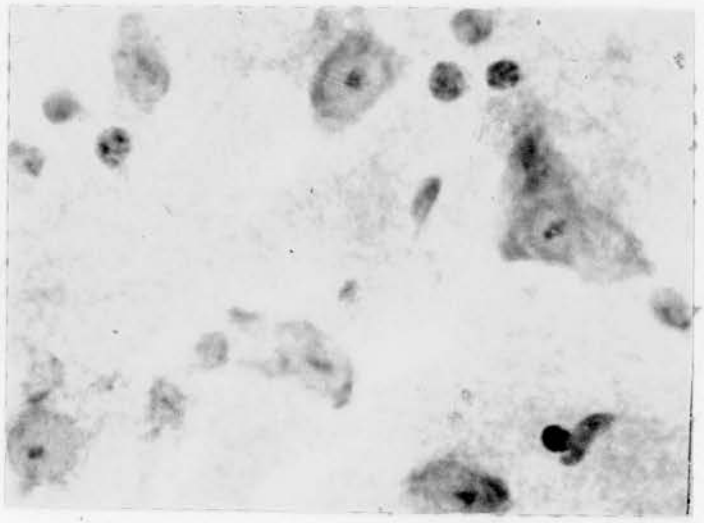

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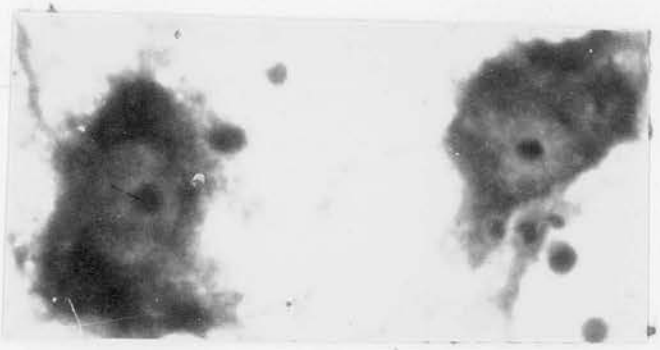

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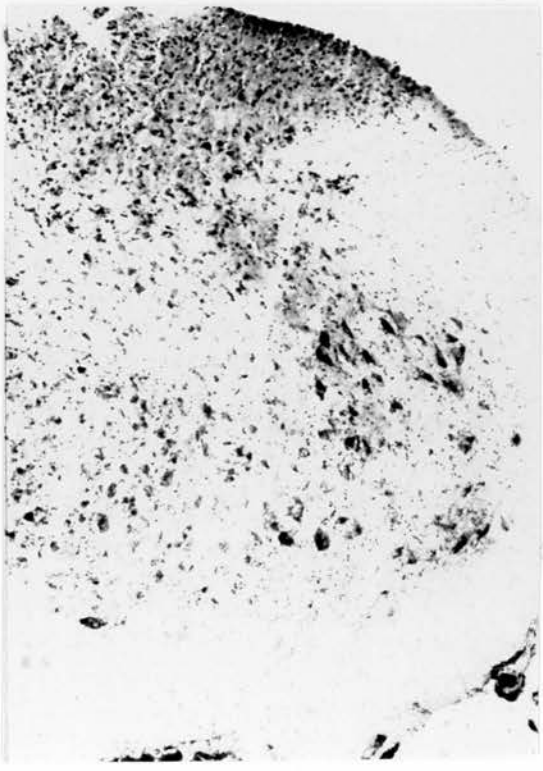

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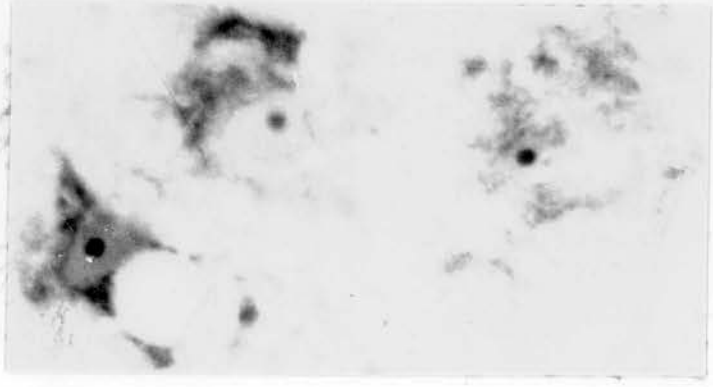

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PLATE 2

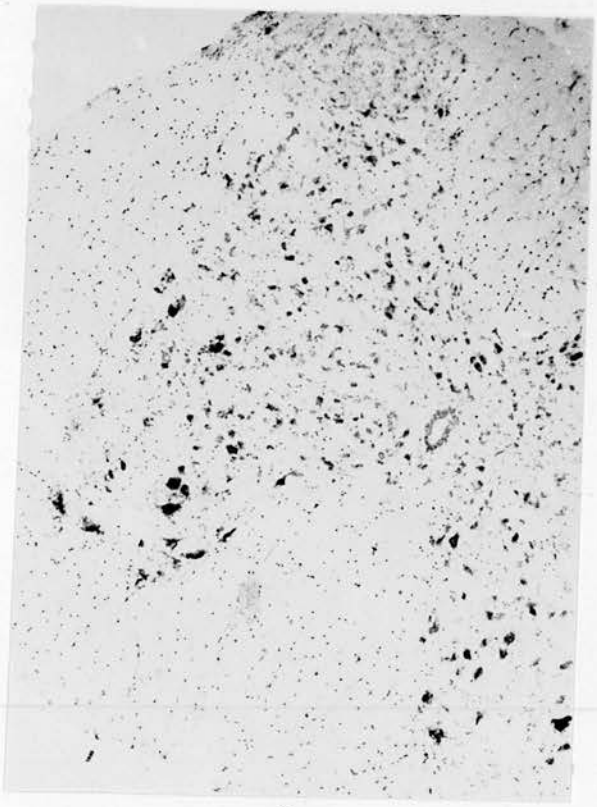
a) T.S. section of lumbar spinal cord from a Wr/HLP mouse
Age 7 months
Toluidine blue stain x 63

b) T.S. section of cervical spinal cord from a Wr/HLP mouse
Age 7 months
Toluidine blue stain x 160
No degenerative changes in the anterior horn cells can be seen at this magnification

c) T.S. section lumbar spinal cord from Wr/HLP mouse
Age 7 months
T.B. stain x 625
There appears to be some vacuoles in the nissel substance in the anterior horn cells

d) T.S. section lumbar spinal cord from a Wr/HLP mouse
Age 7 months
T.B. stain x 625
A grossly enlarged anterior horn cell can be seen.

PLATE 2



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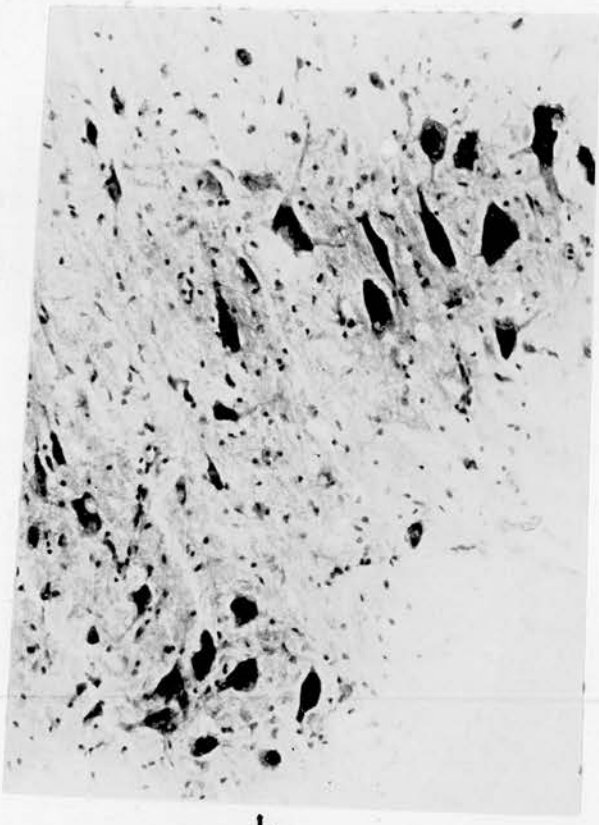

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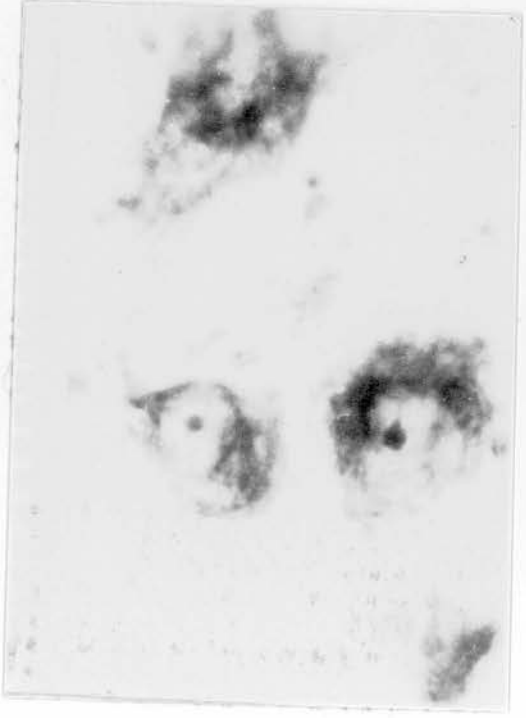

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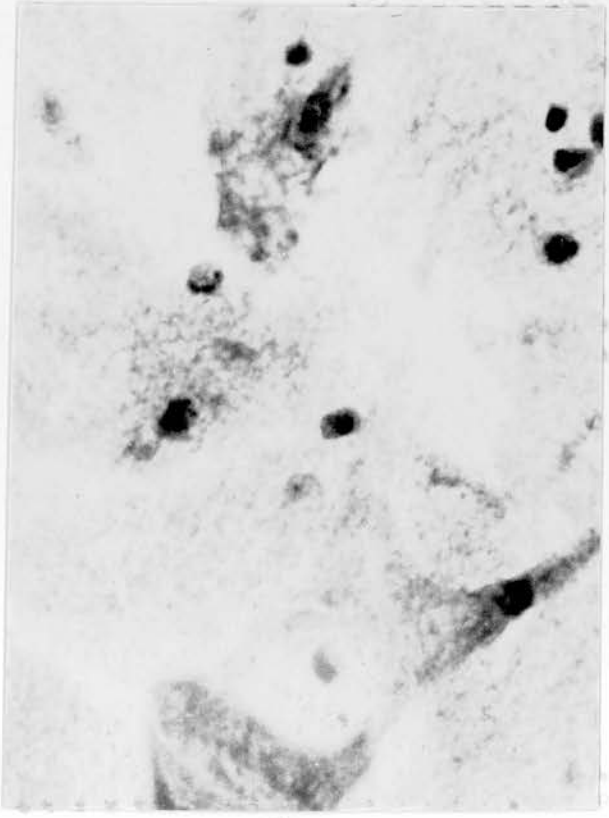

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CHAPTER 8

STUDIES ON TERMINAL INNERVATION IN HUMAN

I. Introduction

II. Material and Methods

i) Material

ii) Methods

III. Results

IV. Discussion

CHAPTER 8

TABLES

CHAPTER 8

I. Introduction

Most terminal innervation studies involving human material have utilised the vital staining technique developed by Coërs (1952). This method has one major disadvantage in that it requires very fresh tissue. It would be of great advantage for the study of peripheral innervation for a staining method to be equally successful on fresh tissue from biopsy and on post-mortem material. An acetylcholinesterase/silver technique was developed in this department for use on human foetal material which can be used on either fresh tissue or on material several days after death (Toop 1974). This technique did not prove very successful on adult tissue. However a similar technique to the one used in the study of mouse terminal innervation (see Chapter 5) has, with some modifications, been used successfully on both fresh and post-mortem human muscle tissue.

This technique could prove very valuable in further studies on terminal innervation in neuromuscular disease. Its advantage over the vital staining methods is that it allows terminal innervation to be studied in a variety of muscles obtained at autopsy from a single individual. Hence the effect of age in different muscles could be assessed. There is evidence to suggest that some muscles undergo changes in peripheral innervation more readily than others (Harriman, Taverner & Woolf 1970). It would therefore be possible to establish a normal range of Functional Terminal Innervation Ratios (FTIR) for various muscles and perhaps also for different age groups. This would be particularly useful in the study of benign forms of motor-neurone disease and in establishing, along with electrophysiological studies, possible neurogenic involvement in diseases believed to be primary myopathies (Stalborg, Trontelj & Janko 1970; Takahashi, Nakamura & Nakashima 1974).

A method which can utilise post-mortem material has, in fact, many possibilities for the study of human innervation. A high affinity of the post-synaptic membrane for divalent metal cations has been demonstrated (Nakamura, Namba & Grob 1967); various abnormalities are associated with poisoning by metals, especially lead. Post-mortem studies on all patients exposed for some time to high levels of lead would prove very useful. It would also be of great value for the understanding of the dynamic nature of the peripheral innervation in humans, to examine the effects on innervation of tetanus intoxication (Duchen 1973), of strychnine poisoning (Doller, Crawford & Conner 1973) and of long-term use of certain drugs especially those administered in neurological conditions (Tallan 1962). These studies could be more ethically carried out on post-mortem material.

The results given in this study indicate the success of the acetylcholinesterase/silver technique for the study of human innervation.

II. Material and Methods

i) Material

Human specimens were obtained from two sources. Some muscle specimens were acquired at post-mortem, some at biopsy operations, where muscle was taken, for routine histology for diagnostic purposes. Post-mortem muscle was always taken from the deltoid muscle, whilst fresh tissue was taken either from the deltoid or the quadricep muscle, depending on the disease of the patient being studied. Four out of a total of seven muscle samples prepared for terminal innervation studies proved suitable for FTIR estimations, whilst the remaining three could only be photographed. Although numerous other specimens were obtained they were found to have been of muscle outside the motor point area. The details of each specimen to be reported in this study are given in Table 8.1.

TABLE 8.1 - The Patients used in Terminal Innervation Studies

Patient No.	1	2	3	4	5	6	7
Age	28	29	32	52	65	43	37
Sex	F	F	M	M	M	F	M
Diagnosis	? SMA ? Scapulo-humoral MD	Heterozygote for SMA	Heterozygote for SMA	Diabetic	Diabetic	Malignant hypertension Duodenal Ulcer	Cardiac failure Psychosis
Muscle Studied	Deltoid	Deltoid	Deltoid	Quadriceps	Quadriceps	Deltoid	Deltoid
Biopsy/PM	Biopsy	Biopsy	Biopsy	Biopsy	Biopsy	PM	PM

ii) Methods

As soon as the muscle specimen was received it was prepared for sectioning (see Chapter 2).

A few muscle sections of 20 μ thickness were tested for cholinesterase activity (for method see Appendix). Specimens where the presence of end-plates was demonstrated by this method were then sectioned for innervation studies. Muscle slices of between 50-100 μ were cut using the retracting microtome (Slee Co Ltd). Sections of 50 μ thickness were mounted directly on to slides and dried in a current of cold air. Tissue slices of 75-100 μ were placed as floating sections into formol-calcium fixative at 4°C for two hours. They were then carefully transferred into distilled water and mounted on to clean slides. After removing all the excess water the slides were dried very slowly. It was essential that the slides were very clean and that drying was allowed to proceed slowly as pre-fixed tissue is not easily mounted, whilst the practice of coating slides with albumen to facilitate mounting resulted in poorer staining of the material. The method for the Ache/silver stain is given in the Appendix.

The specimens where no end-plates were demonstrated by the initial acetylcholinesterase method were not discarded immediately. They were sectioned in the same way, but every ten sections a 20 μ section was cut and tested for cholinesterase activity. Only when it was clear that the entire specimen was of no value for innervation studies was it discarded. Such a precaution was particularly necessary when the tissue had been obtained from a biopsy operation and FTIR studies were important for diagnostic purposes.

The slides were examined and the FTIR estimated by the same method as for mice (see Chapter 5), with one minor adjustment - that in human tissue only axons greater than 150 μ in length were used in calculating

the final result. Two estimates of the FTIR were calculated for each specimen, one using all the available axons, a second using only axons over 200 μ in length. It has been suggested that the probability of subterminal branching occurring at a distance greater than 200 μ is less than 5% (Coërs, Telerman-Toppet & Gerard 1973).

Those specimens where the area of innervated muscle was too small to calculate the TIR were examined microscopically and a photographic record was made of any unusual appearance in the innervation.

III. Results

The results of the FTIR for each of the specimens studied are given in Table 8.2. The highest innervation ratio was found in a patient with a suspected benign form of lower motor neurone disease and, even though results of histological and histochemical examination of this tissue did not prove to be very satisfactory, the innervation results suggest that the muscle weakness reported in this patient was of neurogenic rather than myopathic origin. Plate 1 indicates collateral branching seen in this patient.

Although terminal innervation was studied in two heterozygotes of Werdnig-Hoffmann type spinal muscular atrophy, the FTIR could not be obtained. However some unusual features of innervation were found (Plate 2). The significance of such abnormal features of innervation cannot be clarified until more is known about the ageing phenomenon in muscle. This has been discussed in connection with mouse muscle (see Chapter 5).

Only one specimen from patients with diabetic amyotrophy could be used for assessing FTIR although a second one was stained and will be commented on below. There is obvious collateral branching in the specimen analysed, with a high innervation ratio. There are differences in the type of collateral ramification observed as a result of lower motor

TABLE 8.2

The Results of Terminal Innervation Studies in Humans

The results of functional terminal innervation ratio for healthy individuals (Coërs, Telerman-Toppet & Gerard 1973). The normal values are 1.11 ± 0.05 (Mean ± SD), ie. the normal 95% confidence limits are 1.01 to 1.21.

Patient No.	No. of axons 150 μ	FTIR	No. of axons 200 μ	FTIR
1	68	1.30	28	1.45
4	62	1.23	28	1.35
6	105	1.18	65	1.23
7	53	1.23	32	1.30

neurone disease and that found in this specimen. In the diabetic patient branching was primarily pre-terminal or terminal (see Plate 3) whilst in the case of the spinal muscular atrophy patient and the two post-mortem biopsies to be discussed later most collateral branching was nodal.

The second diabetic patient did show cholinesterase activity but no terminal innervation was found; the muscle was also of no value for histochemical or histological study. The cause of the considerable muscle degeneration in this specimen is not clear. However, cholinesterase activity is known to remain for several weeks after effective denervation (Brzin & Zajicek 1958).

Two post-mortem specimens of muscle are reported in this study. Histochemical examination of both specimens demonstrated an obvious Type II fibre atrophy. A slightly elevated TIR could be demonstrated in both specimens. In patient 6, however, the innervation ratio is not elevated much above the normal range but there is an unusually large number of duplex end-plates (see Plate 3). In the case of patient 7, who died of heart failure, the terminal innervation was definitely raised above the accepted normal range. The patient, however, also had a history of psychosis, and an association between psychosis and collateral branching has been reported (Meltzer & Crayton 1974).

Histochemical studies would normally have ruled out the above-mentioned two specimens as being inappropriate for controls in any study of innervation; however they have been used primarily to demonstrate the use of the same Ache/silver method for both fresh biopsy material and post-mortem material up to 3 days after the patient's death.

IV. Discussion

The results of this study confirm that the acetylcholinesterase/silver technique can be used successfully to study terminal innervation of human muscle. In these investigations, however, it has been necessary to compare the results obtained for FTIR with the normal range for human innervation obtained by the vital staining method (Coërs, Telerman-Toppet & Gerard 1973). If the Ache/silver technique were to be used regularly it would be essential to establish a normal range using this method of staining.

An attempt at innervation studies in two volunteer heterozygous carriers of the gene for Werdnig-Hoffmann disease was undertaken as part of a study of the effect of the heterozygote gene on muscle and peripheral nerve structure. There is some suggestive evidence from electrophysiology that in female heterozygotes some manifestations can be found in certain muscles (Emery, Anderson & Noronha 1973; Mawatari & Katayama 1973). The study of muscle histology and histochemistry of the specimen from the two volunteers has not revealed any striking difference between these and specimens of control muscle, although there is an indication of increased variance in the size of Type II fibres in the two heterozygotes (Lumb 1975, personal communication). There are also a few abnormal axon terminals (Plate 1), but as was mentioned earlier, these may be due to an ageing phenomenon (Harriman, Taverner & Woolf 1970).

In attempting any estimation of a normal range for functional terminal innervation ratios it is essential to realise how many conditions result in atrophy of muscle, not to mention the loss of motor units due to ageing (Campbell, McComas & Petito 1973). In this preliminary study the only two specimens of muscle obtained at autopsy from patients under fifty years of age would both have been discarded

from a study of normal terminal innervation because of a pronounced Type II fibre atrophy. One such patient was found to have suffered from psychosis and it has been suggested that psychosis also has a pronounced effect on motor innervation (Meltzer & Crayton 1974).

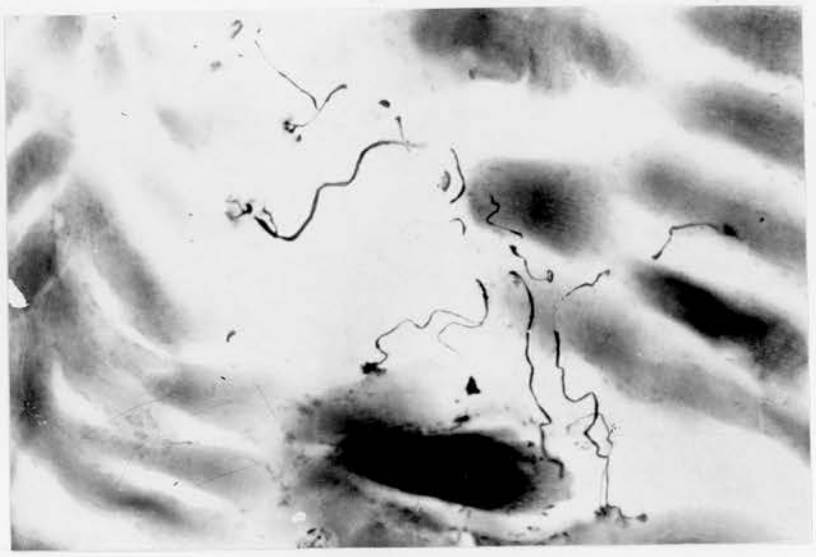
The study of terminal innervation from certain diabetic patients who have developed muscle weakness was intended as part of a larger study of myopathic and neurogenic changes in this disease. Although several biopsy specimens were received only the two reported here were taken from the area of the motor point. It is impossible to discuss terminal innervation in diabetic amyotrophy on the basis of these two specimens. There is evidence to suggest that muscle weakness in diabetes is a result of a neuropathy (Christenson 1972; Brown & Feasby 1974) and it would appear from these observations that in terms of peripheral innervation the resultant collateral ramification is easily distinguishable from that found in lower motor neurone disease.

This study of innervation in human muscle has not been comprehensive. It has, however, demonstrated clearly that the acetylcholinesterase/silver technique can be used for terminal innervation studies in human as well as animal muscle. One can use both fresh and post-mortem material and it has the further advantage of not being time-consuming. A specimen obtained in the morning can be analysed by the following morning. The method is therefore most useful in those laboratories where only the occasional terminal innervation study is a diagnostic procedure for neuromuscular diseases.

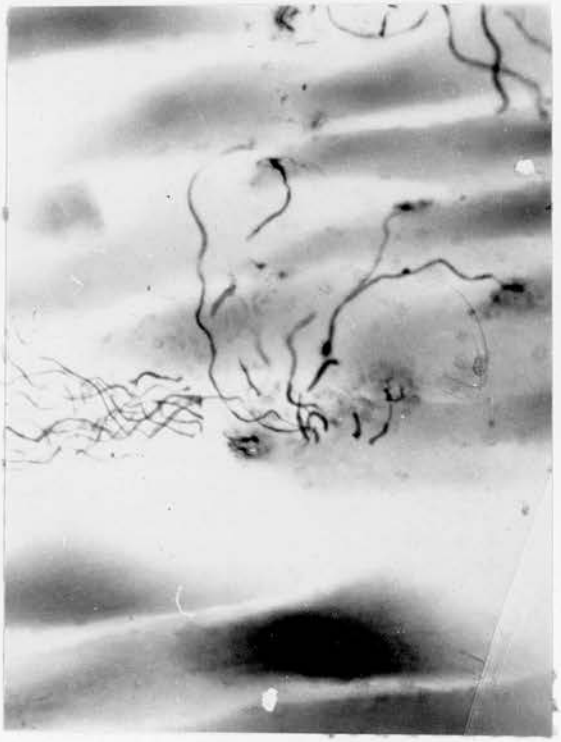
PLATE 1

a) and b) Innervation in the deltoid muscle from a patient with spinal muscular atrophy. Patient 1.
Ache/Silver impregnation x 525
Axonal branching can be seen.

PLATE I



a



b

PLATE 2

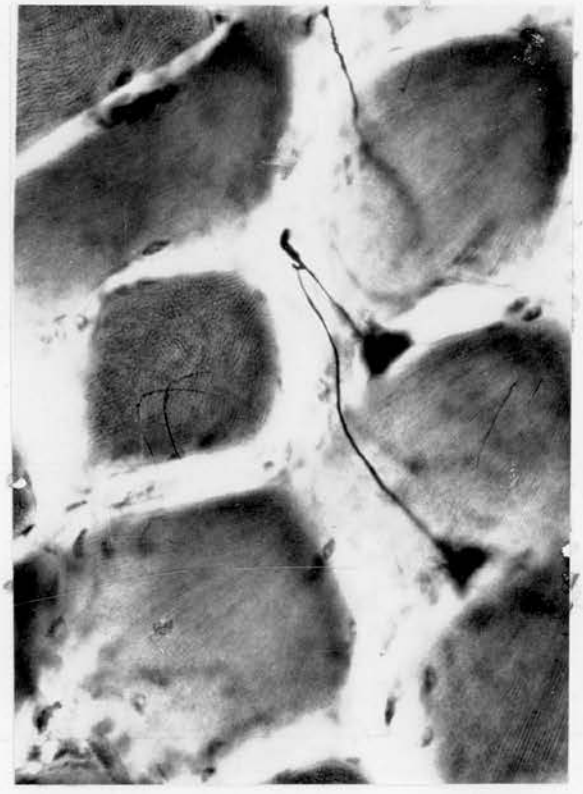
a) Innervation in the deltoid muscle in a male carrier of Werdnig-Hoffmann type spinal muscular atrophy. Patient 2.
Ache/Silver impregnation x 400
A possibly branching axon can be seen

b) Innervation in the deltoid muscle of Patient 2
Ache/Silver impregnation x 400
Axonal swelling and an unusual end-plate structure can be seen

c) Innervation in the deltoid muscle in a female carrier of Werdnig-Hoffmann type spinal muscular atrophy. Patient 3.
Ache/Silver impregnation x 400
A 'T_2' end-plate can be seen

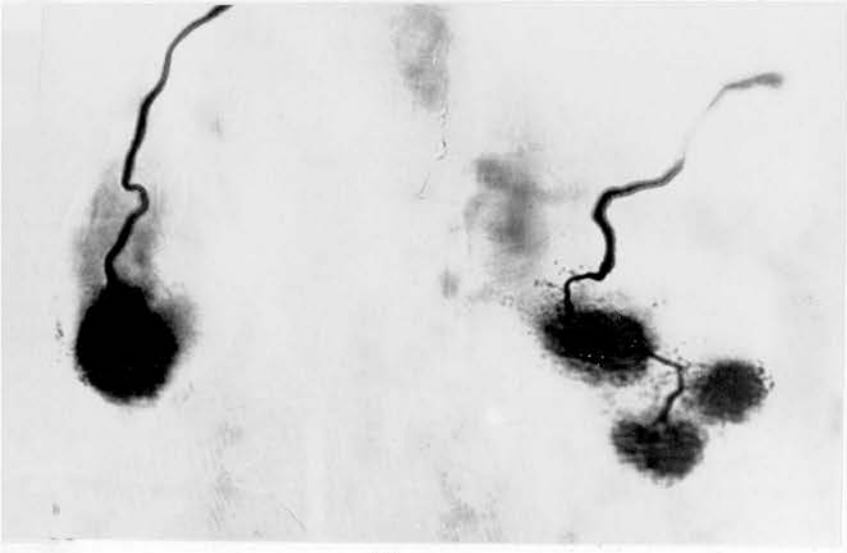
d) Innervation in the deltoid muscle in Patient 3
Ache/Silver impregnation x 400
? A Duplex end-plate or possibly a branched axon.

PLATE 2



a

b



c

d

PLATE 3

a) and b) Innervation in the quadriceps muscle from a patient with diabetic amyotrophy. Patient 4
Ache/Silver impregnation x 525
Terminal branching can be seen

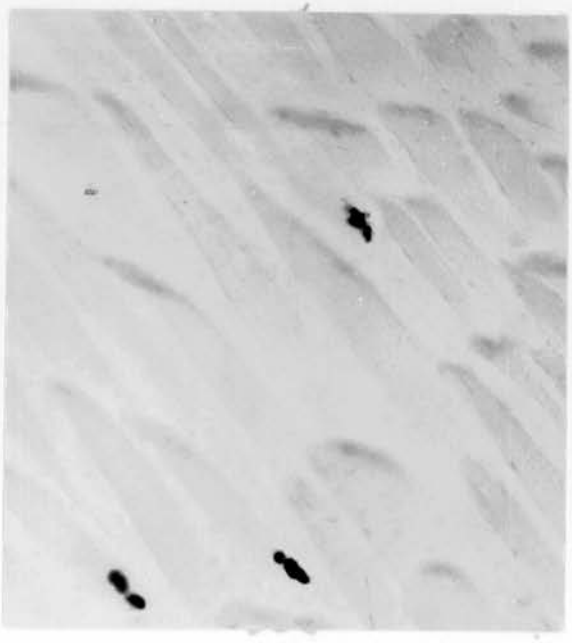
c) A longitudinal section of quadriceps muscle showing the end-plates on muscle. Patient 7
Ache-intensified with Silver/Light green counter stain x 160
The normal appear and distribution of end-plates can be seen

d) Section of quadriceps muscle showing areas of cholinesterase activity in Patient 5 with diabetic amyotrophy
Ache-intensified with Silver/Light green counter stain x 160

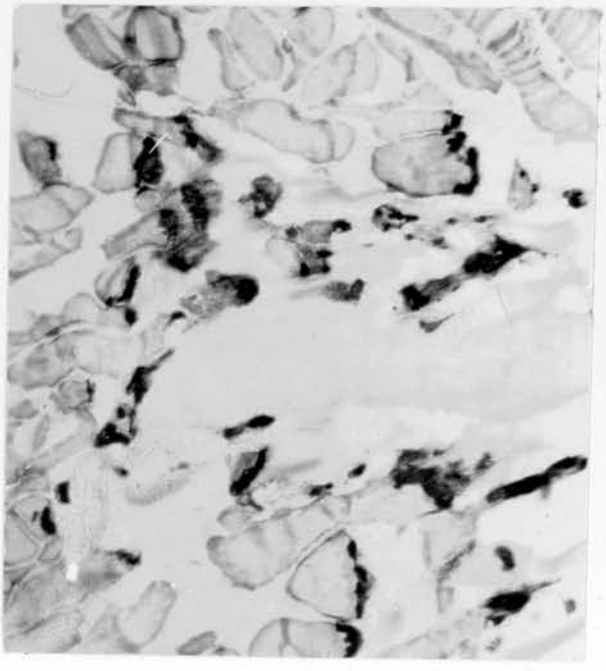
PLATE 3

a

b



c



d

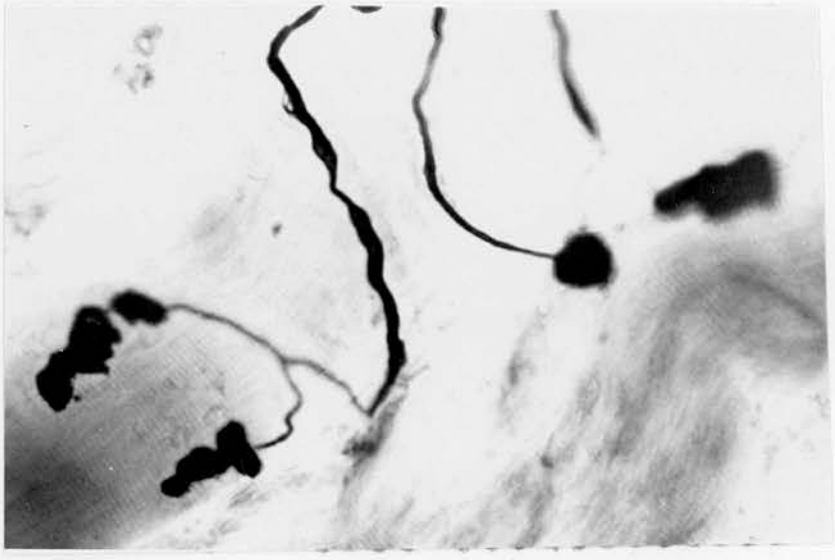
PLATE 4

a) Innervation in quadriceps muscle from Patient 6
Ache/Silver impregnation x 625
A duplex end-plate can be seen

b) Innervation in quadriceps muscle from Patient 6
Ache/Silver impregnation x 400
A branching axon can be seen

c) Innervation in quadriceps muscle from Patient 7
Ache/Silver impregnation x 400
A branching axon can be seen

PLATE 4



a

b

c

CHAPTER 9

Final Discussion

CHAPTER 9

The present study has considered several aspects of spinal muscular atrophy in mice. The investigations have been carried out primarily on mice of the wobbler colony, although in parts of the study mice from the dystrophic colony (ReJ/129dy), have also been used. The disease occurring in 'wobbler' mice is a hereditary neurological condition resulting in anterior horn cell loss and muscular atrophy (Duchen, Strich & Falconer 1968). The loss of motor neurones from wobbler mouse spinal cord does not occur uniformly at all levels of the cord. At early stages in the disease process, the degeneration of anterior horn cells is principally in the cervical region, resulting in the degeneration of fore limbs and pectoral musculature. Although motor neurones may also be lost from the lumbar and lumbo-sacral regions of the cord, it is uncommon for paralysis to occur in the hind limbs of affected mice. At present it is not possible to give any suggestions as to the biochemical lesions which might result in this form of selective anterior horn cell loss. In this study, however, free amino-acids have been estimated in cervical and lumbar regions of the cord of both wobbler and dystrophic mutants. There is substantial evidence that a number of naturally occurring amino-acids function as neural transmitters in the spinal cord (Curtis & Watkins 1960, Curtis 1974). It has also been demonstrated that a loss of interneurones from the spinal cord results in the loss of concentration of the amino-acids glycine, aspartic acid and glutamic acid, all of which are believed to function as neural transmitters (Davidoff *et al*). Numerous forms of stress such as malnutrition and excessive temperatures have been shown to result in changes of free amino-acid concentrations in the brain (McIlwain & Bachalard 1971). It was therefore

considered that a phenomenon of motor neurone degeneration may also affect the amino-acid profiles particularly in those areas of nervous tissue predominantly affected by the disease process.

The results which have been discussed in detail in Chapter 4 indicate that the neuromuscular diseases occurring in both wobbler and dystrophic mice do give rise to changes in amino-acid concentrations, particularly in the cervical region of the spinal cord. The amino-acids which are found in significantly lower concentrations in diseased animals than in control (C57/BL) mice are three transmitter suspects; aspartic acid, glutamic acid and glycine. It must be stressed, however, that these amino-acids occur in the spinal cord in considerably higher concentrations than most other amino-acids. It is therefore possible that differences in these amino-acids are more easily detected. Nevertheless, the results do indicate that free amino-acids in the nervous system may be important in neuromuscular disease.

The results of amino-acid studies in the wobbler mouse further suggest a possible involvement of a neurological transmitter substance in the disease process. There are a number of pharmacological substances believed to function as transmitters in the central nervous system (Phillis 1971). The inter-relationship between the various transmitters and their specificity for the neurone receptor-sites is not fully understood. However, a number of pharmacologically active substances such as γ-amino-butyric acid and homocarnosine have been used in the treatment of neurological conditions (Tallan 1962, Hayashi 1965, 1966). It is interesting to find that it is the level of homocarnosine that is raised in cervical and lumbar regions of wobbler mouse cord. The manner in which homocarnosine functions is not known, but this dipeptide appears to be specific to the central nervous system (Abraham, Pisano & Underfriend 1961, 1962), and it is synthesised from

GABA and L-histidine, both these amino-acids have been shown to give rise to neuronal excitability when topically applied. Furthermore, homocarnosine has been suggested as a strong inhibitor in the limbic motor system (Hayashi 1965, 1966). It may be unwise to speculate on the manner in which homocarnosine functions in the nervous system and whether increased levels of this dipeptide could lead to degeneration of motor neurones. It is possible, however, that there is a direct link between these two phenomena, particularly as the level of homocarnosine is more significantly raised in the cervical region of the cord of wobbler mice. Before any suggestions can be put forward as to how homocarnosine may affect anterior horn cells, its true role in the nervous system must be clarified.

Throughout the study the wobbler mouse has been considered as a possible analogue of human spinal muscular atrophy and suggestions have been made that certain aspects, such as the amino-acids be followed up using human material. If, however, the study is considered as a whole, the problems which result from using animals as models for human diseases can be seen very clearly. The use of the dystrophic mouse as an analogue for Duchenne dystrophy has been questioned for some time. There is increasing evidence that the neurological changes found in the dystrophic mouse (Bradley & Jenkinson 1974, Biscoe 1974, Bradley 1975) do not occur in the human disease (Emery & Gosdon 1974, Bradley 1974). A large survey of peripheral innervation in Duchenne dystrophy was carried out in 1965 (Jedrzejowski, Johnson & Woolf 1965) and no abnormality in terminal innervation was noted. If a similar study had been carried out on the dystrophic mouse, the abnormalities reported in Chapter 5 of this study may have been noticed. In murine dystrophy although peripheral branching is not the primary abnormality of innervation it is, however, a significant feature in the hind-limb

musculature. Therefore, although muscle histology in both the murine and human diseases does show remarkable similarity, the innervation studies are indicative of a variable disease process.

A considerable part of this study has been devoted to describing the Wr/HLP mouse which arose spontaneously in the wobbler colony. Genetic evidence reported in Chapter 3 suggests that the disease may be associated with the heterozygous 'wr' gene. The results of muscle and spinal cord histology given in Chapters 5, 6 and 7 suggest that paralysis in the Wr/HLP mice may be the result of a lower motor neurone disorder, which may possibly arise as an unusual manifestation of the heterozygous wobbler gene. This hypothesis gains support from a report by Harris (1975) of the occurrence of hind limb paralysis in several wobbler mice, which he attributes to an unusual distribution of muscle weakness in these animals rather than to a different disease process. The existence of such variability in a single disease does, however, raise the question of whether murine spinal muscular atrophy is the result of a single gene abnormality. The possibility of a different form of pathogenesis has been raised previously from the result of earlier genetic findings in wobbler mice (Andrews et al 1974). There is therefore substantial evidence to imply that the disease in animals of the wobbler colony is vastly more complex than was originally believed and hence, it would appear unwise to draw too many analogies between the murine and human forms of spinal muscular atrophy. There is increasing evidence to suggest that as work continues on the wobbler mouse a similar situation will arise as is already seen with the dystrophic mouse mutant, and numerous differences will be found between the human and mouse forms of the disease. There are those who already feel that the lower motor neurone disease resulting from C-RNA lymphoma virus infection is a better model for the study

of human lower motor neurone diseases (Munsat 1975, Personal Communication). The apparently variable manifestations of the disease process in the genetically inherited anterior horn cell disease seen in the wobbler colony may further substantiate this argument. The studies of both wobbler and dystrophic mice indicate that caution must be taken in discussing animal diseases in relation to known human disorders. Animal models can, however, be useful in the furthering of knowledge of a number of human conditions. Chapter 8 indicates how the technique developed for assessing terminal innervation in mice was modified for use on human material. This technique could prove valuable as it enables the study of innervation in autopsy material. Further use of the technique could give an indication of terminal innervation changes which result from ageing or conditions known to affect the nervous system but which would not normally warrant the taking of a muscle biopsy.

Animal models have to be used for biochemical investigations of those tissues which cannot be readily obtained from humans. The study of amino-acid levels in human brain or cerebro-spinal fluid cannot be carried out unless there is evidence that such a test is likely to be of value to the patient. It is therefore important that animals with conditions similar to a form of human disease be extensively studied in order to act as indicators for possible metabolic lesions. However, it appears very unwise to approach the study of a mouse mutant considering it as an analogue of a human disease,when the similarities are purely morphological.

The evidence given in the study is that murine spinal muscular atrophy is a much more complex disease than was originally thought. Not only do there appear to be variable forms of the disease in the one colony, but also there are changes in amino-acid profiles in the

spinal cord which cannot be easily explained. It would therefore appear that until such a time that more is known about the disease in the mice of the wobbler colony, any reference to it being an analogue of human spinal muscular atrophy should be approached with caution.

APPENDIX

APPENDIX TO CHAPTER 2

1. **Substrate Solution for NaDH-diaphorase Staining**

DPNH solution (2-3 mg/ml)	0.7 ml
Nitro B.T. (0.2%)	2.5 ml
Phosphate buffer 0.2 M (pH 7.2 - 7.4)	1.0 ml
Ringer solution	0.8 ml

2. **Substrate Solution for Myosin-ATPase Staining**

Tris buffer 1.0 M	4 ml
Calcium chloride $6.H_2O$ 0.18 M	2 ml
ATP (disodium salt)	30 mg
Distilled water	14 ml

Final solution adjusted to pH 9.5 with 0.1 N HCL

3. **The Method for Localisation of Cholinesterase Activity**

a) **Muscle**

- Fix 20 μ sections in formal calcium 1 hr
- Wash in distilled water
- Incubate at room temperature in cholinesterase solution 5 min
- Wash well in distilled water
- Place $\frac{1}{2}$ min in 1% $AgNO_3$
- Wash well in distilled water
- Place $\frac{1}{2}$ min in 10% thio
- Wash well
- Counter stain with 1% light green
- D.C.M.

b) **Spinal Cord**

As above but leave for 1 hr in cholinesterase solution

4. Technique for combined staining of cholinesterase and nerve fibres

a) Mouse Muscle

- Cut longitudinal sections 40-50 μ thick
- Rapidly dry on to slide with cold air from hair drier
- Fix in cold Formal calcium 2 hrs
- Wash in distilled H_2O
- Incubate at room temperature in cholinesterase solution $1\frac{1}{2}$ min
- Wash several times in distilled H_2O
- Leave 1 hr in distilled H_2O in fridge
- 0.25% Potassium Ferricyanide 8-10 min
- Wash several times in distilled H_2O
- Leave 1 hr in absolute alcohol
- Rehydrate 1 min in distilled H_2O
- Silver solution at 37°C - 45 min to 1 hr
- Wash in distilled H_2O - 2-5 min. Use a test slide: place into developer and see how quickly it darkens - if too rapidly leave sections longer in water
- Place into reducing solution - one slide at a time agitating the slide in the solution for the first min. Place into second jar of solution, leave until the section darkens to a lightish brown or lightish green
- Wash several times in distilled H_2O
- D.C.M.

b) Human Muscle

- Cut longitudinal sections 100 μ thick
- Fix free-floating in formal calcium at 4°C 2 hrs
- Remove sections from formal calcium to water
- Mount on to slides and allow to dry
- Incubate at room temperature in cholinesterase solution 2-$2\frac{1}{2}$ min

Continue as for mouse but reduce the time in 0.25% Potassium Ferricyanide to 4-5 min

All solutions except the formal calcium fixative are made up freshly before use.

Solutions

Formal Calcium

10 gms Ca. Acetate

100 ml Formalin

900 ml Distilled water

Cholinesterase Solution

100 mg Acetyl - thiocholine iodide

+ 4 ml Distilled H_2O

+ 7 ml $^{M}/_{10}$ $Cuso_4$ $5H_2O$ - very slowly

(6.25 gms in 100 ml)

leave 10 min - filter

+ 62 mg glycine

+ 3 ml $^{M}/_{1}$ Sodium acetate (136 g litre) - pH about 5.1

made up to 50 ml with distilled water

Silver Solution

10 gms $AgNO_3$

0.05 g $CuSO_4$ $5H_2O$

0.10 g $CaCo_3$

100 ml Distilled H_2O

Reducing Solution

0.5 g Hydroquinone

10 g $Na_2SO_3 7H_2O$

100 ml Distilled H_2O

APPENDIX TO CHAPTER 4

TABLE 1

A List of the Amino-Acids that are estimated on the Amino-Acid Analyses

An indication is given whether the amino-acids are found in spinal cord samples.

Amino-acid		Amino-acid	
Cysteic acid	**	Isoleucine	*
P-ethanolamine	* **	Leucine	*
Hydroxypholine	O	Phenylalanine	*
Aspartic acid	***	Ethanolamine	*
Threonine	**	BIAB	O
Serine	**	Ornithine	**
Glutamine	***	Lysine	**
Asparagine	O	Histidine	**
Glutamic acid	***	Tryptophan	O
Alanine	** ***	Homocarnosine/ Carnosine	**
Glycine	***		
Cystane	*	Arginine	**
Valine	*		
Methionine	O		

* = Trace

** = Concentrations < 1.0 μ moles/gm)

*** = Concentrations > 1.0 μ moles/gm)

Results for these are given in Chapter 4

Results for 14 amino-acids given in Chapter 4.

APPENDIX TO CHAPTER 4

TABLE 2

Amino-Acid Concentrations in Cervical and Lumbar Cord of the Wobbler and the Dystrophic Littermates

Results given as μ moles/gm wet wht (M ± SD)

	Cervical cord		Lumbar cord	
	WrWr/Wrwr	DyDy/Dydy	WrWr/Wrwr	DyDy/Dydy
Cysteic acid	0.14 ± 0.06	0.15 ± 0.07	0.34 ± 0.16	0.31 ± 0.06
Phosphoeth.	0.69 ± 0.22	0.57 ± 0.17	-	-
Taurine	4.54 ± 0.72	3.84 ± 0.85	4.25 ± 0.65	5.12 ± 0.77
Aspartic acid	3.10 ± 0.38	2.96 ± 0.55	3.86 ± 0.51	4.27 ± 0.85
Threonine	0.34 ± 0.08	0.36 ± 0.11	0.49 ± 0.11	0.52 ± 0.12
Serine	0.92 ± 0.22	0.89 ± 0.26	-	1.03 ± 0.18
Glutamine	4.09 ± 1.00	4.40 ± 1.80	4.64 ± 1.83	5.72 ± 1.66
Glutamic acid	5.40 ± 0.96	5.49 ± 0.89	7.30 ± 1.12	8.30 ± 1.52
Glycine	4.75 ± 0.99	4.91 ± 0.59	5.94 ± 0.58	6.60 ± 0.81
α-Alanine	1.03 ± 0.27	1.01 ± 0.33	0.72 ± 0.19	0.91 ± 0.14
Ornithine	0.16 ± 0.06	0.19 ± 0.11	0.13 ± 0.04	0.10 ± 0.04
Lysine	0.33 ± 0.07	0.37 ± 0.08	0.34 ± 0.07	0.37 ± 0.09
Histidine	0.11 ± 0.04	0.16 ± 0.05	0.11 ± 0.03	0.09 ± 0.03
Homocarnosine	0.60 ± 0.18	0.55 ± 0.13	0.54 ± 0.09	0.48 ± 0.09
Arginine	0.28 ± 0.14	0.33 ± 0.18	0.23 ± 0.09	0.18 ± 0.06

APPENDIX TO CHAPTER 5

TABLE 1

RESULTS OF FTIR STUDIES IN MICE

Mutant		1	2	3	4	5	6	7	8	9
C57/BL	Age in days	80	90	106	120	160	274			
	Tot.no.of axons	52	58	58	60	49	49			
	FTIR	1.02	1.02	1.03	1.05	1.00	1.04			
WrWr/ Wrwr	Age in days	74	54	135	124	156	180			
	Tot.no.of axons	50	47	62	51	48	36			
	FTIR	1.04	1.05	1.08	1.04	1.04	1.08			
Wrwr	Age in days	300	266	237	428					
	Tot.no.of axons	53	53	63	51					
	FTIR	1.06	1.04	1.03	1.08					
Wr/HLP	Age in days	182	188	203	266	-	320	220		
	Tot.no.of axons	50	67	50	53	52	39	51		
	FTIR	1.12	1.20	1.14	1.26	1.17	1.23	1.27		
wrwr	Age in days	55	136	111	98	157	145	80	43	70
	Tot.no.of axons	16	22	-	-	24	29	31	19	17
	FTIR	2.06	1.63			1.70	1.40	1.42	1.30	2.00
DyDy/ Dydy	Age in days	145	172	296	296					
	Tot.no.of axons	49	51	50	50					
	FTIR	1.04	1.02	1.06	1.04					
dydy	Age in days	146	173	303	90	47				
	Tot.no.of axons	56	48	40	46	42				
	FTIR	1.13	1.13	1.10	1.11	1.14				

APPENDIX TO CHAPTER 5

TABLE 2

SIZES OF END-PLATES (μ)

100 end-plates in each individual mouse

Mutant		1	2	3	4	5	6	7
	Age in days	80	90	106	120	160	274	
C57/BL	Mean	30.3	29.4	30.1	30.4	31.1	32.6	
	SD	6.9	7.4	6.7	8.5	9.2	8.1	
	Age in days	74	54	135	124	156	180	
WrWr/Wrwr	Mean	30.2	29.2	29.5	32.0	30.0	30.4	
	SD	7.2	6.4	9.0	7.7	7.2	8.6	
	Age in days	300	266	237	428			
Wrwr	Mean	30.0	30.2	30.9	31.8			
	SD	7.8	8.3	6.9	7.6			
	Age in days	182	188	203	266	-	320	220
Wr/HLP	Mean	31.8	32.0	31.2	34.3	29.0	28.4	30.9
	SD	9.5	8.0	11.2	8.3	9.2	6.9	8.6
	Age in days	55	136	157	145	80	43	70
wrwr	Mean	22.2	28.8	31.1	29.3	24.5	23.4	27.2
	SD	6.8	8.5	10.5	8.3	7.5	5.8	7.1
	Age in days	145	172	296	296			
DyDy/Dydy	Mean	27.2	31.0	29.7	28.7			
	SD	7.6	7.4	9.1	7.3			
	Age in days	146	173	303	90	47		
dydy	Mean	29.3	33.2	32.6	32.6	32.7		
	SD	11.4	11.8	10.7	10.2	9.3		

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I declare that the composition is entirely my own and that the work herein has been carried out by myself

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