

THE DEVELOPMENT OF IDENTIFIED MAMMALIAN SPINAL
MOTONEURONES IN CULTURE

By

Manuel Fruns, M.D.

Thesis submitted for the degree of Doctor of Philosophy to the Faculty of Science
of the University of London.

1991

Institute of Neurology
Queen Square
London WC1N

ProQuest Number: U049295

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest U049295

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

UNIVERSITY OF MICHIGAN
LIBRARY
MEDICAL CENTER
ANN ARBOR, MI 48106-1064

THESIS
13.652

DATE DEC 1992

This work is dedicated to my wife

"Solomon saith: There is no new thing upon the earth.
So that as Plato had an imagination, that all knowledge
was but remembrance, so Salomon giveth his sentence,
that all novelty is but oblivion."

Francis Bacon

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor T. A. Sears for his help and encouragement to all my work over the past years in the Sobell Department. I would also like to thank Dr. C. Krieger for his advices at the beginning of this work and for the use of his electrophysiological studies on labelled motoneurons. I am also indebted to Dr. P. Doherty for refer to his studies on neurite growth using NCAM transfected cells. I want to express my deep gratitude to all the members of the Sobell Department for their helpful advices and many discussions, and especially to Mss. A. Hunter, and Mss. J. Savides, for their valuable help. Finally, I would like to thank Dr. E. Rodillo for the long and critical discussions related to this work.

I gratefully acknowledge the financial support of the International Spinal Research Trust, Motoneurone Disease Association, and Brain Research work.

ABSTRACT

Motoneurons are one of the first type of neurone to be generated in the mammalian spinal cord during development. After generation and migration they settle in the lateral part of the ventral spinal cord and produce axons that will innervate specific muscles. In vivo and in vitro studies have well established that during embryonic life the survival of motoneurons and their final number depend critically on their muscle-targets. It has been suggested that other neurones and non-neuronal cells may affect their survival, but whether they also regulate the development and growth of motoneurons, is not known. In the present study, the development, growth, and survival of spinal mammalian embryonic motoneurons in culture were studied, paying special attention to changes in size, neurite growth and branching. The effects of different non-neuronal cells on growth and survival were studied. For these purposes a culture system of dissociated well identified embryonic motoneurons from rat spinal cord was firstly developed. Embryonic spinal motoneurons were retrogradely labelled with fluorochromes, and labelled motoneurons were identified by the use of a low-light level video camera. It was possible to keep rat spinal motoneurons in culture for up to 5 weeks when co-cultured with myotubes and up to 7 days when cultured over glia monolayers. In contrast, the survival of motoneurons on fibroblasts was poor. Myotubes gave the best conditions to neurite growth and development of motoneurons in vitro. Glial cells were also a permissive environment for motoneurone development, although with a reduced rate of growth, for only a few days, and mainly of proximal dendrites. The results of this study showed that motoneurons in culture can survive in the absence of muscle, and that glia cells are a permissive environment for a time that in the embryo goes well into the period of natural cell death, and

that the interaction with non-neuronal cells affects different types of neurite growth. These results are discussed in terms of possible influences that cell-cell contact, and cell adhesion molecules, may exert on the development of spinal motoneurons.

CONTENT

	Page
Title page	1
Dedication	2
Acknowledgments	3
Abstract	4
Table of Contents	6
List of Figures and Tables	9
Abbreviations used in the text	11

CHAPTER I INTRODUCTION

A. General Background of the Study	12
B. Development of Mammalian Spinal Cord Motoneurones	14
1. Neurogenesis	14
2. Cell lineage	18
3. Migration	20
4. Morphological differentiation	21
5. Columnar organization	23
6. Phenotypic differentiation	24
C. Motoneurone Cell Death During Development	25
1. Motoneurone cell death	26
2. Motoneurone cell death in rodents	31
D. In Vitro Studies	32
1. Survival and differentiation of motoneurones	33
2. Labelled motoneurones in culture	37
E. Summary of Introduction and Aims of the Study	39

CHAPTER III METHODS

A. Animals	42
B. Labelling of Spinal Motoneurones	42
1. Anaesthesia and surgery	43
2. Labelling of spinal cord motoneurones	45
3. Injection techniques and incubation of lumbar cord limb segments	46
4. Identification of labelled motoneurone in the spinal cord	49
C. Identification of Labelled Motoneurones in Culture	49
1. Video-microscopy	50

LIST OF FIGURES AND TABLES

FIGURE	Page
1. Schematic diagram of retrograde labelling and preparation of motoneurone cultures.	44
2. Grid pattern.	56
3. Immunoreactivity to fibronectin and GFAP in fibroblasts and glia monolayers.	58
4. Diagram of Scholl's concentric circles.	63
5. Retrograde labelling of spinal motoneurons with RITC.	66
6. Retrograde labelling of spinal motoneurons with HRP.	67
7. Immunoreactivity to neurofilament protein.	69
8. Labelled motoneurons in culture.	71
9. Labelling of motoneurons in culture. Enriched versus non-enriched cultures.	74
10. Effect of embryo incubation time on viability, seeding efficiency, labelling, and survival of motoneurons.	76
11. Decay in fluorescence of labelled motoneurons cultured on CPDL and over glia monolayers.	78
12. Labelled motoneurons in culture. Morphology.	80
13. Histograms of motoneurone diameter, area, and primary neurites at 12h in culture.	82
14. Morphology of motoneurons in culture. Shape and neurites.	84
15. Development of motoneurons in long-term cultures.	85
16. Motoneurone charging curve.	88
17. Voltage recordings from identified motoneurons.	89
18. Membrane current records from identified motoneurons.	91
19. Motoneurone survival. No. of cells and relative survival.	94
20. Effect of serum on motoneurone survival.	97
21. Effect of seeding density on motoneurone survival.	100

22. Survival of E13-14 motoneurones cultured on CPDL, non-neuronal cells, and with DRG neurones.	102
23. (A-B). Survival of E18-20 motoneurones on CPDL, glial cells, and with myotubes.	104
23. (C-D) Survival correlation curve (C); and motoneurone half-lives.	106
24. Growth of motoneurones cultured on CPDL, fibroblast, and glia cells. Computer-aided reconstruction of motoneurone images.	109
25. Growth of motoneurones cultured with myotubes.	110
26. Primary neurites at 0.5d, 2d, and 6d in culture.	112
27. Changes in cell body size versus time in culture.	114
28. Histograms of cell body diameter of motoneurones cultured over glia cells, and with myotubes, at different times in culture.	116
29. Shape (aspect ratio) versus time in culture.	120
30. Neurite branching at 0.5d-6d in culture.	122
31. Peak branching at 0.5d and 6d in culture.	124
32. Relative number of motoneurones with longest neurites at different times in culture.	126
33. Branching density and branching density/primary neurites.	128
34. Branching density at 100 μm / primary neurites.	129
35. Neurite length and neurite growth rate.	131

TABLE

1. Motoneurone labelling in culture and lumbar cord.	75
2. Morphological data of identified motoneurones at 12h in culture.	81
3. Survival of E13-14, E15, and E18-20 motoneurones.	95
4. Cell body size of motoneurones at 0.5d.	111
5. Cell body size of motoneurones at 2-14d.	117

ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholine esterase
BSA	Bovine serum albumin
cDiam	Compound diameter ($\sqrt{[d1 \cdot d2]}$)
CGRP	Calcitonin gene related peptide
ChAT	Choline acetyltransferase
CM	Conditioned media
CMF	Calcium magnesium free
CNS	Central Nervous System
CPDL	Collagen-poly-D-lysine
DRG	Dorsal root ganglia
Dx	Dextran
E	Embryonic age (days)
FACS	Fluorescence activating cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillar acidic protein
HBSS	Hank's balanced salt solution
HBSS-HO	HBSS plus hydrogen peroxide
HRP	Horse radish peroxidase
HS	Horse serum
Ly	Lucifer yellow
mAb	Monoclonal antibody
NCAM	Neural cell adhesion molecule
PBS	Phosphate buffer
PDL	Poly-D-lysine
PN	Postnatal age (days)
PNeur	Primary neurites
RITC	Rhodamine isothiocyanate
RT	Room temperature
TeTx	Tetanus toxin
³ H-Thy	Tritiated thymidine
TIS	Trypsin inhibition solution
TRITC	Tetramethyl Rhodamine isothiocyanate
TTx	Tetrodotoxin

INTRODUCTION

A. General Background of the Study

The CNS is a complex system composed of numerous types of neurones generated at different times during development. Neuronal precursors migrate, generally as post-mitotic cells, to their final position where they settle, die or differentiate, and finally mature to their adult form (Cowan, 1979). During these different phases neurones progressively acquire their definite phenotype by mechanisms that probably include changes in the expression of many structural and regulatory genes (Alberts, Bray, Lewis, Raff, Roberts, & Watson, 1989). Whether epigenetic mechanisms or genetic instructions are the signals responsible for these selective changes in gene expression and regulation, which culminate in the different neuronal phenotype, is unknown (Purves & Lichtman, 1985).

In the mammalian spinal cord motoneurones are among the first type of neurone generated (Altman & Bayer, 1984). In a few days the number of motoneurones rapidly increases, subsequently to be halved during the period of natural cell death (Hamburger, 1977). Before this period of cell death, motoneurones migrate, send axons to establish neuromuscular connections, and start to develop dendrites (Jacobson, 1978). At this time, motoneurones receive no afferents from other central neurones or from the periphery (Altman & Bayer, 1984). Furthermore,

motoneurons at that time have a close relation with ventricular cells, neuronal precursors and radial glial cells (Jacobson, 1978), and blood circulation has not yet developed. Thus during this short period each spinal segment can be considered as a small compartment of high cellular density, with cells in active replication, migration, determination and early differentiation into neurons or glia, and interacting with peripheral mesenchymal tissues. During and after the period of cell death, motoneurons settle in their final position, begin to aggregate in clusters, establish connections with skeletal muscles and, as their mature phenotype is acquired, their final number is defined (Jacobson, 1978). With vascularization, afferents and other neurons appear in the neural tube, astrocytes and other glial cells start to multiply and differentiate, and the limb bud becomes organized into its constitutive tissues (Altman & Bayer, 1984).

The conditions at this time are therefore ideal for the study of cell-cell interactions, and local inductive or permissive phenomena that could control the early development and growth of motoneurons. The close relation of motoneurons to radial glial cells in the neural tube, and to mesenchymal cells from the limb bud, suggest that these cells may have an important role in the development of motoneurons. In this Introduction I review the early development of spinal motoneurons up to the period of natural cell death, including studies using cell culture models. Particular attention is given to the differentiation of motoneurons, cell death and survival, and the influences that other cells, neurons and non-neurons, may have over these processes.

B. Development of Mammalian Spinal Cord Motoneurones

Whenever possible I have reviewed the data from rodents, but in those cases where none is available, or too inconclusive, data from other mammalian or avian studies has been quoted in order to give a more coherent picture of motoneurone development. The terminology used for the neural tube is that adopted by the Boulder Committee (1970). In this thesis I use the term determination to mean the time at which the specialized fate of a cell is fixed although the overt demonstration and realization of that fate may not yet be apparent (Maclean & Hall, 1987); and by differentiation I mean that process by which cells become different in terms of structure and function (Purves & Lichtman, 1985; Maclean & Hall, 1987). In relation to spinal motoneurones I assume that this specialization is already established, although not completed, by the time functional neuromuscular connections are formed. The complete development of the adult traits of a neurone I refer to as maturation.

B.1. Neurogenesis

Motoneurones located in the ventral horn of the spinal cord arise from the ventricular zone of the basal plate (Hamburger, 1948; Holley, Wimer & Vaughn, 1982). They are generated earlier than dorsal horn neurones which arise from the alar plate (Fujita, 1964; Langman & Haden, 1970). Using tritiated thymidine (³H-Thy) incorporation, Nornes and Das (1974) showed that in the rat this generation pattern also was related to the size of the cell, in that large-size neurones (e.g., motoneurones) arise earlier than small-size neurones (intermediate zone and dorsal horn). Motoneurone generation also presents a temporal gradient in the rostro-

caudal axis of the spinal cord, the first arising in the cervical segments followed by those in the thoracic and lumbar regions, sequence also followed in the chick (Holliday and Hamburger, 1977). In Nornes and Das' study, the generation span extended from day 11 to 13 along the rostrocaudal axis. In my view this period should be corrected to day 10.5 and day 12.5 of gestation, since the period of mating used in that study was 20 h and the authors defined the day of mating as the first day of gestation, which really should be 12h on the first day. At any level of the spinal cord the time of generation was around 48h. In the cervical segment, 24% of motoneurons were generated on day 11 of gestation and 76% on day 12; in the thoracic level 86% arose on day 12, while in the lumbar region 61% of motoneurons were generated on day 12 and 39% on day 13. These generation times are probably accurate since a complete mitotic cycle in the ventricular zone of the mouse spinal cord is around 12h (Atlas & Bond, 1965; Kauffman, 1968). The generation cycle of 12h in the rat spinal cord, which is in agreement with studies in the chick embryo (Fujita & Fujita, 1964; Martin & Langman, 1965), suggests that around four or five divisions are probably needed to generate the total number of motoneurons prior to the phase of natural cell death (Jacobson, 1985; Stern, Fraser, Keynes & Primmitt, 1989). This idea of 4-5 cycles between the commitment of a ventricular stem cell to the motoneurone lineage and the postmitotic state is interesting because it is the same as found for other type of cells (Quinn, Holtzer & Nameroff, 1985; Temple & Raff, 1986), thus arguing in favour of an intrinsic clock or counting mechanism for regulating the number of differentiated cells. But this assumption is only reasonable if the cell lineage is symmetrical. If that is the case, the total number of precursors necessary to produce the initial number of motoneurons would be very low (200-400/lumbar segment in the rat). This small number of progenitors, also observed in other

type of neurones (Williams & Herrup, 1988), would mean that the ventricular epithelium is composed of small subsets of cells either pre-specified or activated by specific factors to a defined cell lineage. Moreover, the total number of motoneurones produced for the different segments of the spinal cord is fairly constant among individuals of the same species, and is not modified by ablation or addition of limb buds (Prestige, 1970; Hamburger, 1958) suggesting a strict control of this process. Nevertheless these latter experiments do not rule out the possibility of inductive influences from the mesoderm at earlier stages of development.

In general, the data of Nornes and Das, is in agreement with that of by Altman and Bayer (1984), also in the rat, although in their study the time span of motoneurone production was between 4 days in the cervical and 3 days in the lumbar segments. Apart from the rostrocaudal gradient of motoneurone generation, they also showed for the brachial region a lateral-to-medial gradient (gestational day 12 and 13, respectively), but not for the remaining segments of the spinal cord. Special mention is necessary of the generation of motoneurones from the spinal nucleus of the bulbocavernosus in the rat, which innervates striated perineal muscles in the male. Using ³H-Thy, Breedlove, Jordan and Arnold (1983), showed that motoneurone generation in this nucleus was between E12 and E14, when analysis was confined only to the heavily labelled cells. In complete agreement with Nornes and Das, and Altman and Mayer, this study of a sexually dimorphic nucleus showed that the generation of motoneurones is not controlled by hormonal factors, since testosterone is not produced until E15, androgen levels between sexes do not differ until day 18, and there is no difference in motoneurone generation between sexes.

Most studies of neuronal generation have used ^3H -Thy as a marker for replicating cells. Except for the work of Nornes and Das (1974), none specify the criteria or definition of "marked" cells, so it is unclear whether or not the presence of such labelling represents a post-mitotic cell, ie. neurone or glial cells. The heaviest labelled cells are in their last division, but for the remainder no firm conclusion is possible. Thus motoneurone generation may occupy a longer time span as suggested from the work of Altman and Bayer (1984). All such studies suffer from the absence of independent specific markers for each type of neurone. Nevertheless the overall picture of neuronal generation in the mammalian spinal cord, particularly with regard to motoneurons, fits with that found in other regions of the central nervous system (Cowan, 1979). There is a morphogenetic pattern (rostro-caudal) of generation, with sequential gradients in time and space (Hamburguer, 1948; Altman & Bayer, 1984). The constancy of this picture points to a strict control of the factors that regulate the number and type of motoneurons throughout the spinal cord.

Nornes and Das (1974) also noted a correlation between the ventrodorsal gradient of neurone generation and the regression of the ventricular zone in any given region of the neural tube. This observation shows that cells migrate in an orderly pattern from the ventricular zone, that each type of neurone displays different areas of generation, and that for most of them cell migration occurs in a restricted region. It also seems likely that each area of the ventricular zone is activated at different times under different control mechanisms i.e., functioning as an autonomous compartment. The time of migration from the ventricular zone and the final place of settlement, probably extends between 24h and 48h. Nornes and Das suggest that in the cervical level, two clusters of motoneurons observed at

day 15 segregate from a single, homogeneous column of cells observed at day 13 in the most ventral part of the intermediate zone. It is possible then that over this same period all the somatic and visceral motor neuroblasts in the thoracic spinal cord originate from, and are initially organized in a homogeneous column along, the ventral part of the intermediate zone throughout the rostrocaudal axis, as has been shown for the chick (Levi-Montalcini, 1950; Angulo y Gonzalez, 1940). In the mouse, the visceromotor column assume their final location in the medio-lateral part of the neural tube (Levi-Montalcini, 1950).

B.2. Cell lineage

Most studies on the cell lineage of neurones and glia cells have been done in nematodes or other invertebrates, but also in amphibians and avians (see Jacobson, 1978), but little is known about this in mammals (see Herrup, 1987). The work done in avians refer mainly to neural crest cells (Le Douarin, 1980). Working in primate brain, Rakic (1981, 1984) established that at the onset of neurogenesis a pool of proliferative cells with glial phenotype, glial fibrillary acidic protein (GFAP) (+) cells, coexist already with a pool of GFAP(-) dividing cells (see also Levitt, Cooper & Rakic, 1981 and 1983). They propose that glia and neurones derive from two separate proliferative precursors, the cells of which would be committed to each phenotype before the last mitotic cycle. Whether these precursors are generated in the ventricular zone from a common progenitor cell which diverges early in two lines or that there are different progenitors, is unknown. Tapscott, Bennett and Holtzer (1981a) demonstrated the presence of neurofilament protein in mitotic cells, probably the precursors of neurones in the ventricular zone of the chick spinal cord. More recently, using Rat401 a

monoclonal antibody (mAb) that reacts with an antigen in radial glial cells between E10 and E18, Hockfield and McKay (1985) showed that almost 100% of cells in the neural tube present this antigen between E11 and E12, with a rapid decline subsequently. Given the fact that most cells express this marker for radial glial cells, Hockfield, Frederiksen and McKay (1986) also suggest that ventricular cells may first differentiate into radial glial cells, which then generate post mitotic neurones, including motoneurones. However, at present the specificity of this mAb may need to be questioned as it stains almost 100% of the cells in the neural tube. As at this stage all cells would derive from radial glia or ventricular cells, and all neuronal precursors have this antigen, this information contributes little to our knowledge of the lineage of specific cells. Studies in rat retinal and brain cells, using recombinant retrovirus as vectors, suggest a common precursor of glia and neurones (Price, Turner & Cepko, 1987). In a similar study, Leber, Breedlove and Sanes (1990) observed that, in the embryonic chick spinal cord, motoneurones were clonally related to interneurones and autonomic preganglionic neurons in the column of Terni. Motoneurones were also related to astrocytes and possibly oligodendrocytes. All motoneurone clones observed were restricted to the same rostrocaudal position, but not necessarily to the same motor pool. In agreement with Hockfield's studies, these results suggest that motoneurone progenitors are multipotential, generating neurones and glia cells, but do not rule out the possibility of a vector infection to two or more progenitors restricted to the same area.

B.3. Migration

To the best of my knowledge there is no work concerning the specific route of migration of motoneurons. Most of our knowledge is based on the chick, and even in this case the facts available are insufficient to give a clear picture of motoneurone determination and migration, which routes are followed, whether the different waves of migration mean different subsets of motoneurons or their precursors, etc. All that can be said is that after an appropriate number of divisions, some postmitotic cells move towards the lamina externa, and immediately generate an axon. At this stage, precursors, and ventricular cells, are indistinguishable from each other, even at the ultrastructural level (Lyser, 1968). It is generally believed that young neurons or their precursors migrate through guiding pathways already established and that in several areas these are provided by radial glial cells (Sidman & Rakic, 1973; Rakic, 1981). It is possible that motoneurons migrate using the radial glial processes, given the fact that these extend from the ventricular zone to the external lamina (Hockfield & McKay, 1985). But, it is also possible that another type of the kind described by Moody and Heaton (1983), for trigeminal motoneurons, provides the tangential migration of motoneurons in the ventral area of the spinal cord. Again, it is possible that motor neuroblasts do not really migrate as do cortical neurons but that through a translocation of the nucleus the cells reach a more external location in the neural tube, as suggested by the work of Wentworth and Hinds (1978) (see below). Hockfield's suggestion (see above) that radial glial cells may generate motoneurons is interesting because the migration of young motoneurons could be understood as nuclear migration (see Cowan, 1979) or somatic translocation as in the case of trigeminal (Moody & Heaton, 1983).

B.4. Morphological differentiation

The description by Cajal (1929) of the morphological differentiation of spinal neuroblasts on the chick embryo, remains one of the most accurate and detailed available. He observed three phases: first, a stage of bipolarity, as the cell migrates; secondly, a stage of unipolarity when the ventricular process withdraw; and thirdly, a phase of multipolarity that appears after the cells settle in their definitive location. Frequently the stage of unipolarity was missing as the ventricular process became an early dendrite. A similar description has been made by Wentworth (1984a) for the mouse embryo, observing at E9 a preaxonic neuroblast followed by a bipolar neuroblast, then a secondary bipolar neuron, and finally a multipolar neuron from E10. Therefore, as early as E9 and as late as E11 (E13-E14 in the rat) there are bipolar cells (neuroblasts and neurones, respectively) with an axon growing through the basal external lamina. Wentworth and Hinds (1978), in a serial E.M. study at E10, showed clearly that unipolar or bipolar motor neuroblasts present axons that cross the external basal lamina even before they reach their final position. An early rough endoplasmic reticulum was present, and dendrites started to appear from the apical process. Cajal also stated that motor neuroblasts do not differentiate simultaneously but in waves. The first step is the withdrawal of the apical process from the ventricular zone and the basal processes are transformed into axons. Sometimes an axon grows out when the perikaryon is still within the ventricular layer, which suggests that the basal processes of the ventricular cells never lose their contact with the external basal lamina, but transform into an axon. This pattern of axon outgrowth during migration but before the appearance of dendrites is not particular to spinal motor neuroblasts, as has been observed in other central neurones (Sidman, 1970;

Shoukinas & Hinds, 1978). Dendrites may grow from the axon hillock (Cajal, 1929; Tennyson, 1970), and may appear before axons reach the periphery (Wentworth & Hinds, 1978) or even in the absence of periphery (Oppenheim, Chu-Wang & Maderdrut, 1978). Motor neuroblasts migrate with their axons crossing a layer of axons, from commissural interneurons (Cajal, 1929; Altman & Bayer, 1984), that are generated in the rat at E10-E11, previous to motoneurons (Altman & Bayer, 1984). From the work of Cajal, it seems that motor axons freely cross the lamina externa to enter the mesoderm, and from the 5th day the mesodermal nuclei multiply, and embrace neurite fascicles. In the chick most of the axons have left the spinal cord before E3, not more than 24 h after the onset of migration. At this stage, motor neuroblasts have a voluminous nucleus with 1 or 2 large nucleoli, and some bipolar cells persist to the fourth day. Around 48 h after migration, motor neuroblasts approach the definitive multipolar type, with dendrites having dorsolateral and ventrolateral directions. The axons emerge from any point of the cell body, more often from a large dendrite. In one of the few studies using HRP retrograde labelling, Smith and Hollyday (1983) found, in the rat thoracic spinal cord, that at E13.5 most labelled motoneurons were multipolar, but some of them were bipolar, as described by previous authors (see above, and Windle & Baxter, 1936). Dendrites had two main trunks, one dorsomedial and other ventrolateral, but ^{were} otherwise not well developed. At this stage, only dorsal and ventral ramus nerves were observed, localized at the dorsal part and inner surface of the myotome, respectively. At later stages, the main directions of dendrites still remain, but with profuse ramification.

B.5. Columnar organization

Studying the development of the cervical ventral horn in albino rat fetuses, Angulo y Gonzalez (1940) found that at E14 motoneurons are not yet segregated in motor columns. At this stage motor cells form a cytological/homogeneous and compact mass. At E15, there is a clear separation of this homogeneous mass into a medial and lateral groups, the former subdivided into two subgroups. This columnar segregation also presents a rostrocaudal development, especially at early stages, in which rostral segments segregate earlier than the caudal ones. From E16 the lateral group begins to segregate into several columns, progressing continuously for the rest of the foetal life, with around 12 columns of motor cells in the newborn. Afterwards this columnar organization increases at the expense of cell size and separation of each group. There is some evidence that the segregation into medial and lateral columns is already established at the time of neural tube closure, and that the lateral motor columns for limb regions are also determined (Wenger, 1951). In the thoracic spinal cord of the rat embryo (Smith & Hollyday, 1983), motoneurons already showed some organization in ventromedial and ventrolateral groups at E13.5, when labelled with HRP from the dorsal and ventral ramus, respectively. This is probably the result of some kind of selectivity of innervation rather than being the result of motoneurone cell death which starts later, as observed in studies of motor fibres growth (Landmesser, 1978; Lance-Jones & Landmesser, 1981). This suggests that muscles are innervated by clusters of motoneurons with a specific location in the ventral horn, but does not resolve whether this early motoneurone organization is an intrinsic feature or the result of myotome induction. Transplantations of neural tube have shown that the type of lateral motor column (brachial or lumbar) is also determined (Narayanan &

Hamburger, 1971; Straznicky, 1963). Holliday (1980) suggests that regions of the ventricular zone produce particular sets of motoneurons destined to innervate specific types of muscles and to form lateral motor columns. However, this suggestion does not agree with lineage studies using retrovirus vectors (see Leber et al., 1990) showing that motoneurone clones belong to different motor pools, and so the possibility that motor pools are established by segmental influences cannot be ruled out.

No clear correlation between the segregation of columns and the reflex activity of the embryo has been observed (Angulo y Gonzalez, 1940). Proximal forelimb reflexes appear at E16, with individual reflexes at E19. The timing of reflex activity has probably more to do with the arrival of sensory fibres to the ventral horn from the DRG and interneurons afferents than with the segregation in columns itself (see Smith & Hollyday, 1983). In an *in vitro* system of foetal rat spinal cord, Saito (1979) found segmental reflex response following stimulation of the skin or the spinal dorsal root at E15.5. Plantar skin flexor reflex has been evoked at E17.5, and monosynaptic stretch reflex in the rat triceps surae at E19.5 (Kudo & Yamada, 1985).

B.6. Phenotypic differentiation

Few studies have been done on the phenotypic differentiation of spinal motoneurons, and in most of these only the position and size were used as criteria for identification. In a descriptive study of changes in acetylcholine esterase (AChE) and cholineacetyl transferase (ChAT) activities during embryonic development in homogenates from the ventral rat spinal cord, Burt (1973)

demonstrated a different pattern of activity for each type of enzyme. Whereas AChE showed a steady nineteenth-fold increase from E13 to PN12, ChAT activity remained low and unchanged until E17, but from this point to PN12 increased nine-fold. Both enzymatic activities reached a plateau at PN12-PN14. The increase in both enzymes from E16-E17 correlates with the appearance and increase in spontaneous and reflex activity in rat embryos (Narayanan & Hamburger, 1971). Using immunocytochemical techniques, Phelps, Barber, Brennan, Maines, Salvaterra and Vaughn (1990) showed that ChAT appears first in motoneurons, which were also the first cholinergic neurons to be generated in the cervical cord of the rat embryo. ChAT appeared at E13 one or two days after motoneurone migration, and before the establishment of neuromuscular connections and the onset of the natural cell death period. Calcitonin gene-related peptide (CGRP), a peptide present in spinal motoneurons that may exert trophic effects on the neuromuscular junction, is expressed at E6 in chick embryos (Fontaine, Klarsfeld, Hokfelt & Changeux, 1986). Recently Tessier-Lavigne and Mudge (1987) demonstrated CGRP(+) and CGRP(-) rat motoneurons, early in development, which suggests that motoneurons may form a heterogeneous population from early stages in development.

C. Motoneurone Cell Death During Development

Natural motoneurone cell death, as an example of developmental cell death, is an accepted phenomenon that occurs throughout phylogenesis and has been recognized to be a prominent feature of nervous system development for the last fifty years or so (see Hamburger, 1977). It is considered to be a mechanism which during development controls the final number of motoneurons and matches the

size of motoneurone population to the target muscles. Also, it has been conceptually important because on the one hand it has been the background of the target-dependent trophism concept in the last years, and on the other, it is assumed that understanding of its mechanisms at the cellular level would give new insights on the neuronal cell death that occurs in different diseases of the CNS (Wisniewski, 1987).

C.1. Motoneurone cell death

Motoneurone cell death occurs during a defined and short period of time in the embryonic development of vertebrates (Hamburger, 1977; Cowan, 1979; Oppenheim, 1985). This period occurs after young motoneurons have migrated from the ventricular zone to their final ventrolateral position in the neural tube, and coincidentally with axonal growth through the limb bud and the establishment of neuromuscular connections (Hamburger, 1977). It is a phenomenon that occurs rather abruptly, rapidly reaches a peak, and ends after a few days, at a time when most of the connections to their target muscles have been established (Oppenheim, 1985). Roughly 40-60% of the initial population of motoneurons in the lumbar cord die during this period (Lance-Jones, 1982; Oppenheim, 1986). It seems to be a random phenomenon (but see Lamb, 1979), and there is no evidence at the ultrastructural level, as for the ciliary ganglion (Pilar & Landmesser, 1976), that those cells that ultimately die are different from those that survive (Chu-Wang and Oppenheim, 1978). This does not rule out, however, the possibility of biochemical or phenotypic differences between the cells that die and those which survive, so that some type of "programed or genetic" cell death could occur. Interestingly Carr and Simpson (1982) showed that about 15% of chick embryo DRG neurones that

had incorporated ^3H -Thy showed signs of degeneration 2h after the injection, supporting the idea that some neurones die in a programmed way. In the absence of their targets, it seems that motoneurones are generated, migrate, cluster and produce axons and dendrites before cell death, but do not differ from those motoneurones that have made target contact (Oppenheim et al., 1978). However, the possibility of epigenetic mechanisms acting during that time has not been tested directly. It is now well known that target presence affects the extent of motoneurones' cell death. Almost all motoneurones die after limb ablation (Hamburger, 1958; Prestige, 1967; Oppenheim et al., 1978) or in a limbless mutant chick (Lanser & Fallon, 1984), whereas the addition of a supernumerary limb (Hollyday & Hamburger, 1976), or a wing to the limbless mutant chick (Lanser, Carrington & Fallon, 1986), increases the number of surviving motoneurones. Also, the number of motoneurones surviving correlates with the number of target myotubes during the period of cell death (McLennan, 1982), supporting the classical idea that motoneurone death is a process that matches the size of the neuronal pool with the size of the synaptic target, regulating the final number of motoneurones.

A linear relationship between motoneurone number and target size was shown by Tanaka and Landmesser (1986) in chick-quail chimeras embryos. In four types of chimeras, they found a coefficient correlation of 0.996 between the number of motoneurones that survive the period of cell death and the number of myotube clusters. Lanser and Fallon (1987) on the wingless mutant chick, confirmed the existence of a linear relationship between the extent of cell death, or better the number of motoneurones, and the size or volume of limb muscle. Interestingly, with zero limb volume there is still a remnant of motoneurones that does not die

after the period of cell death. Also, in the wingless chick, the number of motoneurons is less than at any stage of development, even at E5 before the period of cell death. Motoneurons die rapidly in this mutant prior to E8 at a time when little death is seen in the normal chick (and when motoneurone migration and production are still under way), a phenomenon also observed in surgical ablations (Laing, 1982; Oppenheim et al., 1978). The latter has been explained by one or all of the following mechanisms: 1) decrease in motoneurone generation; 2) reduced or delayed migration; 3) increased cell death during the period of motoneurone determination. As a larger number of dead cells at E5 was not observed, but instead fewer than normal, it was suggested that a decreased generation or migration of motoneurons occurs in wingless chick embryos (Lanser & Fallon, 1987). This same phenomenon was also observed in the limbless chick mutant (Lanser et al., 1986). In these embryos cell death was accelerated prior to E6, and their rescue by a wing graft was mainly exerted before that day. The previous data suggest that motoneurone death in the absence of periphery is not a simple enhancement of the normal cell death, that some changes might occur in earlier phases of motoneurone differentiation, and even that some motoneurons can survive in the absence of limb-bud muscles. An important point to consider, when chick mutants or limb chimeras are used for studies of motoneurone cell death, is that there is not only a reduction or enhancement of target-muscles, but also of other ectodermal and mesenchymal tissues that can influence cell death. For instance the number of DRG may have changed in these studies, affecting secondarily the final number of motoneurons. Lanser and Fallon (1987) also showed a biphasic rate of cell death in normal chicks, with an acceleration of cell death in the early phases, suggesting that more than one mechanism might operate in motoneurone cell death, as also suggested by Lamb (1979). Considering that

some 40-50% of motoneurons die in the chick embryo (Oppenheim, 1985), it is possible to speculate that the total number is therefore given by the last cell cycle of mitotic precursors at a time when cell death begins (E6), and that the cell cycle might be regulated by the periphery. In fact, recent studies using transplant of muscle tissue and notochord, suggest that early in the development of the chick neural tube, peripheral mesenchymal tissues may be involved in the regulation of proliferation and differentiation of neuroepithelial cells (Fontaine-Perus, Chanconie & Le Douarin, 1989; Van Straaten, Hekking & Beurgsgens, 1989). Although there is no knowledge about the mechanism of natural motoneurone death, classically this relation between neuronal number and target size has been explained in terms of a competition between the growing axons for an unknown factor from their targets either a secreted and diffusible factor, or as synaptic sites in the muscle (Oppenheim, 1985; Tanaka & Landmesser, 1986). Although the evidence in favor of this contention, is strong, as described above, there are some unexplained facts. It is not yet proved that the size of the available target (initial number of sites or amount of factor) is smaller than the number of the initial axons that arrive at the beginning of the cell death period. In the chick limb at the onset of the period of cell death most of the motoneurone axons have failed to penetrate very far into the developing muscle masses (Tosney & Landmesser, 1985). So, the initial death of the first few axons is not completely explained (Oppenheim & Chu-Wang, 1977). Also, it remains to be proved that the size of the available target is always smaller than the number of ingrowing axons. However, in the chick, the differentiation of the limb bud takes place after most motor axons have entered the limb, and before muscle cleavage (Hamburger, 1975; Tosney & Landmesser, 1985), making possible a difference in the size of both pool of cells. Also it is unclear whether motoneurons are really an homogenous cell

population before the period of cell death, although there is evidence that early during development motoneurons are not homogeneous (Tessier-Lavigne & Mudge, 1987). Why is there a polyneuronal innervation during the formation of the connections? If there is a kind of competition, the latter might suggest that this occurs between different cell population types. Working on Xenopus laevis tadpoles, Lamb (1979) removed the lateral motor column of rostral segments that innervate the future knee flexor muscles before the period of cell death. Normally, motoneurons in the caudal segments temporarily project to the presumptive knee flexors, but those are the first to die, leaving only the rostral motoneurons connected with those muscles. There were no HRP-labelled cells in caudal segments, nor was there labelling of the well-defined group that normally innervate the extensors. This study suggests that there is no competition between caudal and rostral segments for flexors muscles. Lamb suggests that perhaps the specificity of innervation might explain these results, especially considering that the motoneurone pool to each muscle maintains the same relative position as in normal animals (Morris, 1978). Nevertheless, most motoneurons establish their connections with considerable precision (Lance-Jones & Landmesser, 1981a), although wrongly projecting motoneurons do not inevitably die (Lance-Jones & Landmesser, 1981b). Another unexplained fact is that the rescue of motoneurons by a supernumerary limb, experimental or spontaneous, goes only from 7% to 25%, even though the limb mass increase is by 100% (Hollyday & Hamburger, 1976). Nevertheless, blockade of muscle acetylcholine receptor or neuromuscular activity can rescue up to 100% of the motoneurons destined to die (Laing & Prestige, 1978; Pittman & Oppenheim, 1978), suggesting that the latter are not different from those who survive, and that motoneurons are not "programmed" to die. However, pharmacological blockade does not necessarily support the concept of competition,

but is an argument that favors a relation between the rescue of motoneurons and the activity of the cholinergic receptor. It is possible that during embryonic development motoneurons depend on muscle for their survival, although an additional mechanism may act at the same time. The electrical activity of neurons and/or muscles also may play a role in motoneuron death (Harris & McCaig, 1984; Oppenheim, 1987), as motoneuron number is increased in embryos in which neuromuscular activity has been abolished, presynaptically or postsynaptically by pharmacological agents or neurotoxins. Increased activity, by electrical stimulation of the limb musculature also enhances the magnitude of motoneuron cell death (Oppenheim & Nunez, 1982). It is still unknown whether this correlation with the electrical activity is an epiphenomenon, or is the regulative element per se.

C.2. Motoneuron cell death in rodents

Using counts of the fifth cervical ventral root fibers in rat embryos at different gestational ages, Harris and McCaig (1984), established that the great loss (65%) of axons occurs between E15 and E16 (from 6900 to 2400 fibers). A slower reduction to 1200 fibers was completed by E21. About 95% of the final loss has occurred by E18. The maximal number of nerve terminals per motoneuron was established when motoneuron death was complete (E18). They conclude after using tetrodotoxin (TTX) muscle paralysis to rescue motoneurons from cell death, that the first motoneurons were committed to die at E14, before any structural features of endplates could be recognized. A reduction of 45% and 41% of motoneurons in the brachial and lumbar segments, respectively, between E15 and PN1 (Oppenheim, 1986) has been observed in the rat. The number of motoneurons at PN3 was not significantly different from PN1, discarding the

possibility of motoneurone death in postnatal life. In a more detailed study in the mouse, Lance-Jones (1982) showed similar figures to those for the rat. She counted a peak number of 5000 cells at E13 (2-3 days after motoneurone generation), a fall of 65% between E13-E18, with 85% of motoneurone death (50% of motoneurone number) occurring between E13-E15. No cell death occurs after E18. Most of L1 and L6 motoneurons die, and there was a slight temporal rostro-caudal gradient of cell death, but the distribution of motoneurons through the different segments remained practically the same. In embryos injected with HRP, most motoneurons at E13 (previous to cell death) had sent axons to the limb bud (73%-97%). So, the period of motoneurone death in the rat lasts about 3 days, starting after the migration, growth of axons and initial differentiation of motoneurons, as in the chick embryo.

D. In Vitro Studies

The many different cell types in the CNS and the complex relationships they present pose great difficulties for developmental studies at the cellular level. A common approach has been the use of in vitro systems of explants or dissociated neural cells in culture (see Fedoroff & Hertz, 1977). Although an in vitro system of dissociated cells in culture is artificial, and has little resemblance with its in vivo counterpart, it is the ideal system to dissect the effects of single or a limited number of factors on cellular properties under a variety of conditions. Nevertheless, any conclusions relate only to the in vitro situation, and eventually their validation in vivo is clearly necessary. In the past neural cell cultures have been frequently used to study the type of problems discussed above, particularly

relating to neuronal survival and differentiation, as now reviewed and discussed.

D.1. Survival and differentiation of motoneurons in culture

Spinal cord cultures from avian or rodent embryos have been used as explants or dissociated cultures (Varon & Bunge, 1978), in studies ranging from the electrophysiology of the neuromuscular synapses (Ransom, Neale, Henkart, Bullock & Nelson, 1977), to survival and putative growth factors for motoneurons (Godfrey, Schrier & Nelson, 1980; Riopelle & Cameron, 1984). They have in common the use of whole or ventral spinal cord at an age after the generation of motoneurons. In many studies conclusions were drawn as if they related to motoneurons alone, although no special procedure for isolating such neurons had been employed nor were specific markers used. In some studies density-gradient techniques were used, separating neurons by size (Berg and Fischbach, 1978), others relied on ChAT as marker (Masuko, Kuromi & Shimada, 1979), but without previously defining neither motoneuron size nor the specificity of the cholinergic marker. The use of spinal cord cultures of dissociated cells as a simplified model of the in vivo situation for motoneurons rest on the following findings: spinal cord neurons can establish functional synapses with muscle cells in culture (Fischbach, 1972; Masuko, et al, 1979); the electrophysiological activity of these cells is comparable to that found in vivo (Ransom et al, 1977); it is possible to find neurons with large cell bodies, like those of motoneurons (Berg & Fischbach, 1978); some neurons are cholinergic, expressing ChAT and AChE activity (Masuko et al., 1979; Berg & Fischbach, 1978) and can secrete ACh (Berg, 1978). In a small neuronal population from rodent spinal cord cultures, developmentally regulated cholinergic receptors have been shown through the

specific binding of α -Bungarotoxin (Schaffner & Olek, 1986). These findings show that dissociated cells from embryonic spinal cord behave and present features resembling those in vivo, so it has been assumed then that other cellular functions of spinal cord neurones behave in a similar way.

Most of the studies using different in vitro models are related to the question of motoneurone or spinal neurone survival, or neurotrophic activity in muscle extracts or conditioned medium (CM) from different types of cells. Dribin and Barret (1980) showed that CM from muscle cells and other non-neuronal cells increased neurite outgrowth from spinal cord explants of E15-E16 rat embryos, and that this effect was not modified by the presence of serum. The increase of ChAT activity in dissociated E12-E14 mouse spinal cord cultures was observed with the addition of mouse muscle CM (but not chick), and with CM from heart, kidney and liver primary cultures (Godfrey et al., 1980). Henderson, Huchet and Changeux (1981) studied the effect of myotubes CM on the neurite growth of dissociated chick ventral spinal cord cells at E4.5 showing a clear effect at 20 hours in culture on the number of cells with neurites. They found that the active(s) factors were macromolecules of 40,000 and 500,000 D. They also found activity in CM from liver and skin cells, but not from lung, heart or C6 glioma cells, nor in horse (HS) or foetal calf (FCS) serum, or insulin. They suggested that these active factors possibly act upon motoneurones. Using a similar assay, Henderson, Benoit, Huchet, Guenet and Changeux (1986) reported the survival-promoting effect of muscle extracts from the mutant "Paralyse" mice and tenotomised rats, reinforcing previous suggestions (Pittman & Oppenheim, 1978 and 1979) that skeletal muscle activity might regulate motoneurone cell death. It is not possible to decide with this type of assay, in which the controls had a very low survival, whether these effects were the result of specific or unspecific muscle factors. Using a different assay, Tanaka,

Sakai and Obata (1982) observed a dose-dependent effect of FCS on neurite growth in chick E6-E8 spinal cords explants, which was abolished by heat treatment. Skeletal muscle extract had no such effect unless FCS was present in the medium. A substratum-dependent effect was also observed with heart, liver and brain extracts, and CM from heart, skeletal muscle, brain and C6 cells. All these effects were substrate dependent. Similar results were obtained using dissociated cells (Tanaka & Obata, 1982). The possibility that specific and non-specific factors released from neurones affect neurite growth of spinal cord cultures has been suggested by Riopelle and Cameron (1984), although their spinal cord cultures were not purely neuronal. Smith, McManaman and Appel (1985) showed in cultured rat ventral spinal neurones the presence of two types of soluble protein factor from skeletal muscle extracts, one that promotes neurite extension (33,000 to 37,000 D), and the other acetylcholine synthesis (with broad range in molecular size). Both activities were also observed with extracts from cerebral cortex, and from lung and cardiac muscle for the cholinergic activity. The neurite promoting factor decreased with the age of muscle and increased with denervation. In contrast, the cholinergic factors were unchanged with the age of muscle, but decreased with denervation. The neurite-promoting factor affected more than 60% of the cells, and is similar to a neurite factor found in bovine brain (Kligman, 1982). As part of the same study, McManaman, Smith and Appel (1985) described a small peptide (1300-1500 D) with cholinergic trophic activity present in muscle extracts, supporting the idea (Müller, 1984) that small peptides can also present trophic activity.

^b
Substratum molecules regulate neuronal adhesiveness and growth in culture (Letourneau, 1975), depending on cell-surface properties. In contrast to what

happens in peripheral neurones, fibronectin appears to have no effect on neurite growth of spinal neurones, either as a soluble factor added to the medium (Driblin & Barret, 1980) or as substratum (Rogers, Letourneau, Palm, McCarthy & Furcht, 1975). In the latter study, substratum-bound laminin, a component of basal lamina, induced longer neurites in dissociated spinal neurones from E6 chick embryo, but not more branching than poly-L-lysine. These results are in agreement with findings in embryonic development, where fibronectin has been found along the paths of neural crest cell migration (Newgreen & Thiery, 1980), but not associated with neurones or glia of the CNS (Linder, Vaheri, Ruoslahti & Wartiovara, 1975), and only in reduced amounts in myoblastic regions of limb buds (Sanes, 1982). Laminin, instead, is distributed in all germinal layers of the embryo and in basement membranes (Timpl, Rohde, Robey, Rennard, Foidart & Martin, 1979), including lamina externa and limb-bud (Bonner-Fraser & Lallier, 1988). Together with the finding that these molecules are secreted by muscle cells (Lander, Fujii & Reichardt, 1985), the previous results suggest that components of the basal lamina act not only as substratum-bound molecules supporting axon elongation, but also trophic activity. Monolayers of non-neuronal cells like glia or fibroblasts promote neurite growth of spinal cord explants and of dissociated central neurones (Fallon, 1985a). Fixed monolayers did not however support neurite growth, even in the presence of CM, suggesting that soluble glial factors alone are insufficient for neuronal survival.

The different methodology and bioassay employed, the use of cell number as a measure of neurite growth, and the different embryonic ages and culture conditions makes the interpretation and comparison of the above results difficult. Furthermore, the fact that motoneurones constitute only a very small proportion of

the cells in cultures of the spinal cord (Schnaar & Schaffner, 1981; Smith, Vaca, McManaman & Appel, 1986), precludes relating those results to motoneurons. A specific method of identification is therefore essential when it is intended to study specific populations of neurons *in vitro*, particularly motoneurons. In 1980 Bennett, Lai and Nurcombe reported the use of retrograde labelling of E6 chick spinal motoneurons with HRP, followed by the dissociation of the spinal cord and, after culture for a few days identification of the labelled motoneurons in fixed preparations. At the same time Okun (1981) communicated a similar, but potentially more useful method of using fluorochromes, instead of HRP, to label chick spinal motoneurons. After the dissociation of the spinal cord, motoneurons were identified *in vitro*, and fluorescent cell sorting (FACS) was applied to obtain pure populations of motoneurons. Subsequently, this method has been used by others, with or without cell sorting, mostly in the chick embryo (Calof & Reichardt, 1984; Smith et al., 1986).

D.2. Labelled motoneurons in culture

Bennett et al. (1980) observed that less than 20% of labelled motoneurons survived after 48 hours in control medium, but most of them survived in muscle CM. Later they reported that myotube CM increased motoneurone survival and neurite outgrowth in a dose-dependent manner, and greater than with myoblast or fibroblast CM (Nurcombe, Tout & Bennett, 1985). Denervated adult rat muscle CM increased the number of motoneurons that survive in relation to innervated muscle, but this increase was lowered by previous muscle stimulation (Hill & Bennett, 1986). Interestingly, 28% of motoneurons survive in control medium (without CM) for several days. Either some were not motoneurons or a certain

amount of glia cells contaminated the cultures, or both. Similarly, motoneurone survival was increased by chick embryo extract and denervated adult mouse muscle extract from the endplate zone, but not when the latter was obtained from innervated extrajunctional muscle (Slack & Pockett, 1982). Tanaka and Obata (1983) also using HRP labelling described an increase in survival with chick extract embryo, HS and muscle CM. Calof and Reichardt (1984) working with fluorescent sorted E6 chick motoneurons showed a substratum-bounded neurite promoting factor, and a survival factor from myotubes CM. They also showed that CM from dividing cells in the spinal cord and fibroblasts presented survival-promoting activity, although less than muscle CM, especially after 48 hours. Later on, the neurite growth factor was demonstrated to be identical to laminin (Calof & Reichardt, 1985), a finding in agreement with the identification of laminin in spinal cord ventral roots during development (Rogers et al., 1975; Rogers, Edson, Letourneau & McLoon, 1986). An increased survival of E6 chick HRP-labelled motoneurons has also been observed with glia CM or when motoneurons cultured over glia monolayers (Eagleson, Raju & Bennett, 1985). Motoneurone survival presents a similar increase with muscle as with glia CM at 24 and 48 hours, but no difference between glia CM or glia monolayer (but see Fallon, 1985a). This trophic activity affected survival but not neurite growth, although a doubling of cell body size was observed. A shift in motoneurone trophic dependence on glia at an early stage (E5) to muscle CM later (E7) in embryonic development has been reported in chick motoneurons cultures, suggesting a different responsiveness to non-neuronal cells at different embryonic ages (Eagleson & Bennett, 1986). In that study no effect of muscle CM on survival at E5 was observed, although by then motoneurons would already have sent axons to the periphery. A decline of the glia survival-promoting effects on motoneurons occurs

after E8. O'Brien and Fischbach (1986) showed that a small fraction of pure E5 chick motoneurons survive for up to 14 days with skeletal myotubes or muscle CM. In heterogeneous cultures, however, labelled motoneurons survive up to 3 weeks if muscle CM or skeletal myotubes are added, suggesting that other neurons or non-neuronal cells may also be important for motoneurone survival.

D. Summary of Introduction and Aims of the Study

This review indicates that the final number of motoneurons during development and their survival in vitro depend on the muscle-targets. Although it is not yet completely established, the available evidence suggest that they do not depend, however, on the periphery for their generation and early differentiation. Soon after their generation motoneurons migrate and, in close contact with radial glial cells, produce an axon, develop a basic pattern of dendrites, and express ChAT. During this period, and previous to cell death, motoneurone development could then be regulated by cell-cell interactions with other cells such as glia cells or other neuronal precursors. During and after the period of cell death there is some evidence that muscle may not be the only factor which regulates the survival or death of spinal motoneurons. In these later periods it is possible that glia cells and other neurons, particularly interneurons, play an important role in the development of motoneurons.

The influences of other cells on motoneurone development and growth have not been studied. A common approach to the study of these problems has been the

use of cell cultures. Most of these studies refer to the avian embryo. Nevertheless, from the review of the literature, comparison of studies using the spinal cord or motoneurons in culture is difficult because of the different methodologies and assays used. The use of cultures of spinal cord cells without specific identification of motoneurons, makes difficult any interpretation.

Therefore, this project was mainly directed to study the development of mammalian motoneurons and the effect of non-neuronal cells and other neurons on their survival, morphological development and growth. For these purposes, I have chosen, as a simplified model, cultures of dissociated rat spinal motoneurons. The development of rat motoneurons is well known (Nornes & Das, 1974; Altman & Bayer, 1984), several morphological and physiological studies using embryonic spinal cord in this species have been reported (Saito, 1979; Kudo & Yamada, 1985), and spinal cord cultures of the rat have been used frequently in the past (Schnaar & Schaffner, 1981; Smith et al., 1985). I have given special attention to the development of a culture system containing well identified mammalian motoneurons able to survive for long periods in culture. For the identification of motoneurons I have used the retrograde axonal transport of fluorescent labels (Okun, 1981), and a system of video-microscopy to facilitate the identification and study of motoneurons in culture. The morphology of labelled motoneurons was studied using phase-contrast microscopy, and their electrophysiological features through the cell patch-clamp method. To study the effect of non-neuronal cells on motoneurone development and growth, labelled motoneurons were prepared from embryos at an age soon after their generation and previous to cell death (E13-14). These motoneurons were then cultured over glia or fibroblasts, and co-cultured with myotubes, and the morphological

development and pattern of neurite growth were followed during several days. For the studies concerning the survival of motoneurons, E13-14 motoneurons and motoneurons at an age after the cell death period (E18-20) were prepared and cultured in those conditions, but also in co-culture with DRG neurons, and at high density of interneurons.

M E T H O D S

A. Animals

Sprague-Dawley rats were used throughout all the study. Time-mated pregnant rats (250 - 350) at different gestational ages were obtained from a commercial breeder (Bantin & Kingman, Hull, UK). The day of detection of a sperm positive vaginal smear was designated as the first gestational day. The embryonic age (E) was determined in each embryo under microscopic observation following the method and criteria of Christie (1964), who defines stages 16 to 32 corresponding to 9.5 and 18.5 embryonic days. Special attention was given to the limb bud changes described by Christie to define between 13 and 15 days of embryonic age. Experiments were done in embryos between E13-14 as the group before cell death, and in older embryos, E18-E20, as the group after the cell death period.

B. Labelling and Identification of Motoneurons in Culture.

This section summarizes the whole procedure of labelling and identification in culture to provide an overview prior to the detailed description of each techniques. In this study, lumbar segments were used because they develop between E12-E14, later than brachial segments (see Altman & Bayer, 1984).

Pregnant rats were anaesthetized and embryos exposed by laparotomy. Younger embryos (E13-E15) were staged, decapitated and eviscerated under microscopic observation. Fluorescent labels were pressure-injected into hindlimb-buds. In older embryos the injection with fluorochromes was done in situ. Segments of lumbar spinal cord with hindlimbs were incubated in an oxygenated medium at 30° C usually for 10-12h to allow the retrograde transport of the label. Following this incubation, spinal cord segments were either fixed and sectioned for histological identification of labelled cells, or ventral cord pieces were freed from meninges and DRGs, incubated in trypsin at 37° C for 30 min, and mechanically dissociated. Cells were then plated on coverslipped-dishes coated with collagen and poly-D-lysine (CPDL), over non-neuronal cell monolayers, or co-cultured with DRG neurones or myotubes. After 4-6 hours, labelled motoneurones were identified in an inverted microscope coupled with a low-light level video camera. Images of labelled cells were stored in a video-recorder and analysed, afterwards, using an image analysis system (fig. 1.).

B.1. Anaesthesia and surgery

Pregnant rats were anaesthetized with ^{Sodium} Pentobarbital (Salgatal, May & Baker Ltd., UK) at a dose^e of 40 mg/Kg-w im. This procedure gave a good level of anaesthesia for about 7h, sufficient for the entire surgery. In E18-20 embryos the labelling was done in situ and the longer period of anaesthesia was achieved by small supplements of ^P Pentobarbital ^{one} 10 mg/Kg-w every 3h. In this way anaesthesia was maintained for up to 20h, without evidence of reduced reflex activity of the foetuses. To obtain an adequate and steady level of anaesthesia it was necessary to maintain the temperature of the mother by continuous warming throughout the entire procedure. Nevertheless, with periods of anaesthesia beyond 10h there was

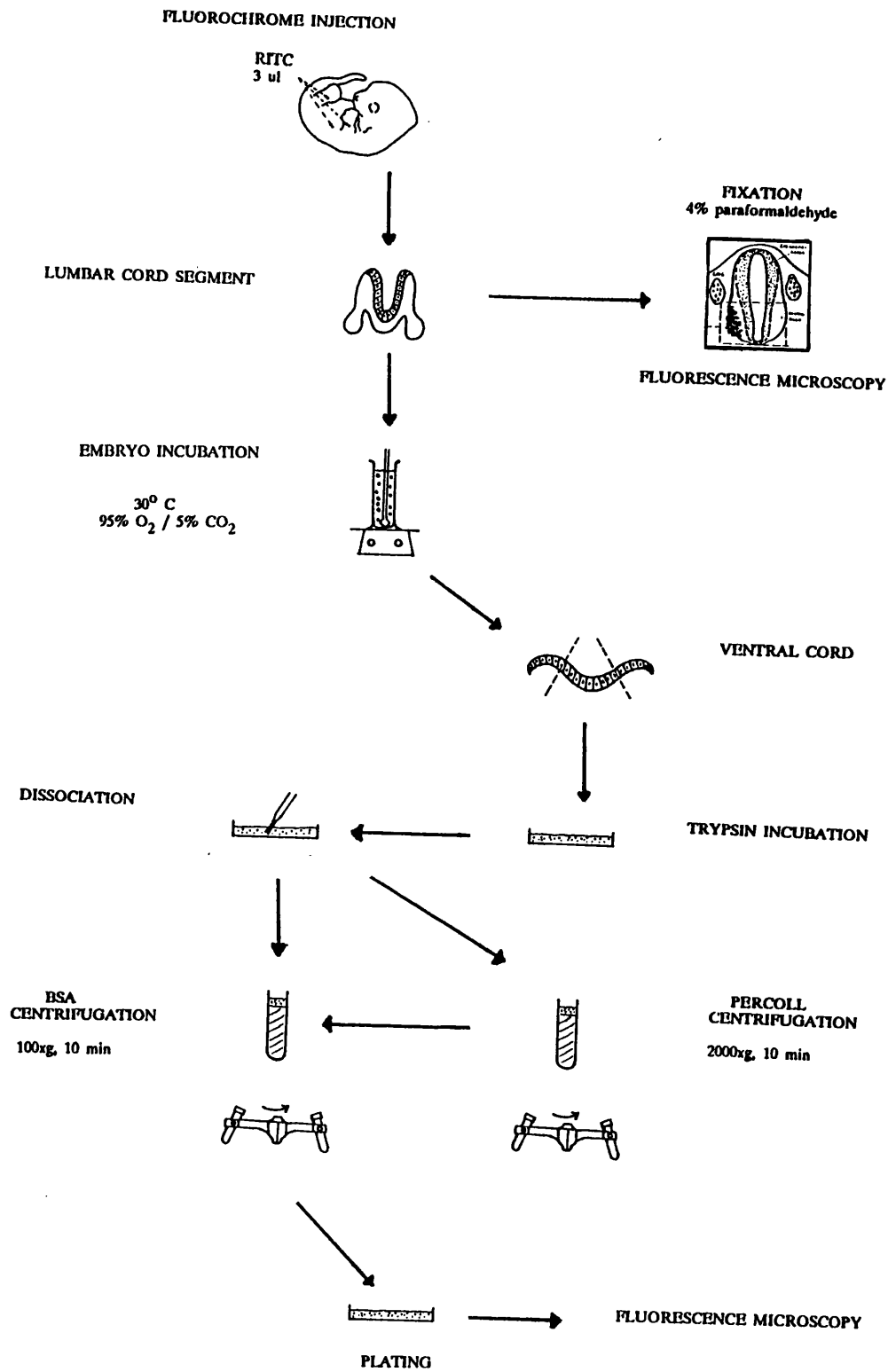


FIGURE 1. Retrograde labelling and preparation of motoneurone cultures (see text).

an increasing number of deaths due to respiratory insufficiency.

All the surgical procedures were performed aseptically. The abdominal wall of anaesthetised pregnant-rats was shaved, cleaned, sterilized with ethanol, and a medial longitudinal laparotomy performed.

B.2. Labelling of spinal motoneurones

As most of the studies were done using embryos at E13-E14, the description of the methods used in this group will be given in detail and as the standard procedures, and only in the appropriate instances reference to those applied in the older group (E18-E20) will be given.

Several different preparations were used for the labelling of motoneurones in rat embryos. We first injected the label through the uterine wall, in situ. This was succesful at E17 or above but, completely unreliable at E13-E15. At this earlier age, the thickness of the uterine wall, and the higher ratio between the amniotic volume and embryo size preclude a reliable and accurate injection. We also tried to inject exteriorized embryos while keeping them within the abdomen over the viscerae. Although this procedure facilitated the injection, the embryos survived only for few hours (< 3h) as they were compressed by the rest of the viscerae. Another preparation tried was the injection of the label in situ through a small incision of the uterine and amniotic walls and making a type of pouch with a circular suture through which only the posterior limb was exteriorized, and then reintroduced to the uterus. Although in this case the injection was feasible, the procedure was long, difficult, and with a low survival, making this preparation

unpractical. The exteriorization and maintenance of embryos in a saline bath at room temperature (RT) (Smith & Hollyday, 1983) was also inefficient because survival, as based on persistence of the heart beat, was no more than 3h. Finally, two different procedures were adopted for the two groups of embryos: 1. In older embryos (E18-E20) the label were injected in situ, through the uterine wall, into distal areas of the lower limb, and the pregnant-rat was kept anaesthetized during the required period for the retrograde transport of the label (see below). 2. For younger embryos (E13-E15) an in vitro system were developed. They were exteriorized from the uterus, keeping intact the embryonic membranes and placenta, and as a whole placed in a dish with Hank's balanced salt solution (HBSS; Flow Co.,U.K.) plus 0.03 % H₂O₂ (HBSS-HO), 33 mM glucose, Benzylpenicillin (100 µg/ml, Glaxo, UK) and Streptomycin sulphate (50 µg/ml; Sigma, UK) (HBSS-HO plus) at RT. Using a Wild stereomicroscope, embryonic membranes were open^{ed} and embryos staged. Embryos were then decapitated, eviscerated, flattened and pinned on a dish with a cushion of Sylgard (Dow-Corning, Belgium) (see figure 1), and covered with HBSS-HO plus at RT. At this point both groups were prepared to receive the injection of the appropriate label.

The use of H₂O₂ in HBSS during the dissections procedures was based in the work of Walton and Fulton (1983), in which they showed an increase of neonatal rat spinal cord viability in an in vitro preparation for several hours using 0.03% of hydrogen peroxide.

B.3. Injection techniques and incubation of lumbar cord-limb segments

Several fluorochromes were tried: wheat germ agglutinin (WGA) conjugated with Lucifer yellow (WGA-Ly) or rhodamine iso-thiocyanate (RITC), RITC,

sulphorhodamine, dextran-RITC. RITC (Sigma 9877, UK), prepared according to Thanos and Bonhoeffer (1983), gave the best results, and so it was used throughout all the present study. Four mg was dissolved in 20 μ l in dimethyl sulphoxide (DMSO), and further diluted in 1 ml dH₂O with vigorous shaking for 2 min shortly before use.

Injections of fluorochromes were performed using glass micropipettes (EC 120F-10, Clark Electromedical Instr. Ltd., UK) made with a micropipette puller (Moving-coil Microelectrode puller Model 753, Campden Instruments, UK), in which the tips were given an external diameter of 20-30 μ m and 40-60 μ m, for the younger and older embryos respectively. Pipettes were siliconized with Sigmacote ^{kk} (Sigma, UK), sterilized and mounted on a holder in a Leitz micromanipulator. The micropipette was connected through a sterile tubing filled with silicone fluid (Dow-Corning, Belgium) to an Aglar microsyringe (Welcome Lab., UK) attached to the micromanipulator. Micropipettes were retrogradely filled with the RITC solution previous to the injection procedure. Under microscopic observation a total volume of 1-3 μ l of label per limb were pressure injected into the hindlimbs. This volume was delivered in three or four smaller volumes, as this gave better results than one delivery, by minimizing the diffusion of the label to other areas. The label was usually injected in both hindlimbs, and care was taken to place the injection in mid and distal areas of each limb, in younger and older embryos respectively. If the injection was too proximal, the label frequently spread around the external lamina of the spinal cord in which case these were discarded because of the possibility of diffusion within the spinal cord. Throughout these procedures the embryo was continuously moistened or covered with medium, which also facilitated skin puncture without breakage of the micropipette.

After the injection, the spinal cord was opened dorsally through a longitudinal section with an ophthalmic scissors, the embryo sectioned at the level of the last four thoracic segments and at the end of sacral segments, and removed together with the hindlimbs, rinsed in HBSS and placed in an incubating bath.

Older embryos were injected in situ through the uterine wall, using the same system described above, but the micropipette was driven manually. The latter gave better results than the use of a micromanipulator. The uterus was fixed by one end and the label injected in both hindlimbs. Those cases in which there was spread of label to the amniotic fluid were discarded. Embryos were selected from the distal end of uterine horns. After the injection, uterine horns were replaced in the peritoneal cavity and the abdominal wall resutured, and secured with collodion. Pregnant-rats were kept warmed and anaesthetized for the period of the retrograde axonal transport of the label (10-20h).

Spinal cord-limbs segments were placed in a glass flask with incubation medium (M-I) based on a mixture of DMEM/F12/1:1 (GIBCO, UK), 33 mM glucose, 0.3% bovine serum albumin, fraction V (BSA; Sigma, UK) and 20 mM HEPES (Flow; UK), bubbled with a mixture of 95% O₂/ 5% CO₂ at 30° C for different times. The flask were kept in a temperature-controlled bath during the incubation. In order to maximise the O₂ in the medium I used a long and narrow flask and delivered the oxygen through a sytaglass bulb (No.1, Gallenkap, UK) at the end of the O₂ tube.

B.4. Identification of labelled motoneurons in the spinal cord

Labelling was certified in fixed sections of the spinal cord after the injection of the fluorescent label and appropriate incubation time. Spinal cord-limb preparations were fixed with 4% paraformaldehyde at 4 C° for 12h, followed by cryoprotection with 10% and then 30% sucrose in phosphate buffer (PBS) plus 4% paraformaldehyde, during 12h each. Spinal cord with surrounding tissues were then embedded in OCT and sectioned in a cryostat by M^s. A. Hunter. Sections 10-12 μ m thick were mounted with 10% glycerol-gelatine in subbed slides, and observed immediately under fluorescence microscopy using rhodamine filters, and with a 40x/0.75 neofluar-phase objective.

C. Identification of Labelled Motoneurons in Culture

After the organ incubation, dissociation procedures, and plating (see below), cells were allowed to settle and attach to the dish for 4 hours. Labelled cells were then identified by their fluorescence observed using an inverted microscope equipped for epifluorescence. From the beginning it was clear that the amount and intensity of labelling depended not only on the injection and incubation time, but also on the microscopy system. The influence of the magnification was important, as lower magnification gives lower brightness (see Inoue, 1986). Some fluorescent cells were not easy to detect through the eyepiece, usually needing several observations and comparisons with the corresponding phase images to identify the type of cells. Consequently, the cells were exposed several times to the exciting light with the risk of damage. The latter was the main reason to use a video-microscopy and video-recording system, with a low-light video camera, and to facilitate the process

of identification. Labelled cells were then identified directly through a video monitor that received the images from the video-camera coupled to the microscope. Primary images were stored in a video recorder, or digitized, processed and enhanced, as necessary, previous to recording. The system also consists of a computer with an image analysis processor for posterior analysis of the recorded images. This complete system was used as the regular method of identification and observation during the entire study.

C.1. Video-microscopy system

The system consists in a Zeiss inverted microscope (ICM 405) equipped for transmitted and phase microscopy, and with a 50 W Hg lamp for epifluorescence with a filter house for two set of filters (FITC and RHO). A long-working distance condenser and a 10x eyepiece were used during all the observations. During the study most of the observations were performed with a 40x/0.75 phase Neofluar objective. Also 16x/.25 and 25x/.25 phase Neofluar objectives were used. A low-light level video camera (Panasonic 1900, Japan) was permanently connected to the microscope through a 1x lens connecting tube, and to a video monitor (M1; Hitachi VM 910E/K, Japan), which was connected to a video cassette recorder (Sony U-matic VO-5800, Japan). The video monitor M1 was also connected to a video processor and image digitizer, and to an image analyzer (Sight Systems, UK) and a BBC computer. The image analyzer was connected to a second video monitor (M2; Hitachi VM-1220, Japan) that gave the processed images, to a printer (Epson FX-80, Japan), and to a termal video-copy processor (Mitsubishi P60B, Japan). Finally, the image analysis system was connected to a second channel of the video recorder, from which the image was displayed onto a third

monitor (M3; Panasonic WV-5320, Japan), mounted in a hood to which a Nikon F3 SRL camera was attached to obtain pictures from the primary or processed images. Using the 40x/0.75 objective, a total magnification of 400x was obtained from the eyepiece, and 640x and 890x through the monitors M1 and M2, respectively.

C.2. General procedure

Cells were exposed to the exciting light for periods of less than 3 seconds, to avoid possible damaging effects (see O'Brien and Fischbach, 1986). Images were captured in the video monitor and simultaneously recorded in the VCR, followed by the recording of the corresponding phase image. Morphological analysis and number of total and labelled neurones were determined later from recorded images. Each dish was examined in a similar and previously defined pattern, scanning through two complete and perpendicular dish diameters, and recording fluorescent and phase images of every other field, but not less than 15 fields/dish. A labelled neurone was considered ^{to be} any fluorescent cell with one or more processes longer than one cell body diameter. To reduce sampling bias, the brightness, phase type, and the morphological features, other than the presence of neurites, were not considered to define a labelled neurone as motoneurone. The identity of glia and fibroblast cells was established by the morphology and immunocytochemistry.

D. Motoneurone Cultures

After incubation the spinal-cord limb preparation was transferred to a sterile dish with cold oxygenated HBSS-plus, and under a stereomicroscope pieces of lumbar cord, free from meninges and DRGs, were dissected using a fine ^{watchmaker's} forceps and tungsten needles. In this work the lumbar segment is defined as the spinal cord included between the last thoracic segment and the caudal six segments. The segmental levels were determined at the time of the dissection by counting the DRGs. Two longitudinal cleaned strips of spinal cord attached to each other by the ventral surface was then obtained, and placed, with the ventricular surface up, over a white Millipore filter disk submerged in HBSS-plus. With the help of a knife made with a small piece of razor blade, the two strips of spinal cord were sectioned longitudinally following an imaginary line between the dorsal and ventral halves (see fig. 1).

Both ventral halves of each spinal cord were placed in a dish with oxygenated HBSS-plus, at RT, and after collecting an appropriate number, they were cut in small pieces and incubated in 0.25 % Trypsin (Flow Co.) in HBSS with 33 mM glucose and 40 μ g/ml DNase (Type I, Sigma) at RT, for 20 min. Enzymatic activity was blocked with a cold solution (Trypsin inhibition solution, TIS) composed of HBSS plus + 10% FCS + 0.3% BSA and 0.04% DNase. Ventral cord pieces were collected by slow centrifugation, resuspended in TIS and mechanically dissociated by 10 strokes with a wide-bore pasteur pipette (around 1000 μ m diameter), and followed by 5 strokes using a 600-800 μ m bore pasteur pipette. The cell suspension (\pm 2 ml) was then centrifuged over a cushion of 4 ml of cold 4 % BSA in HBSS $\text{Ca}^{++}/\text{Mg}^{++}$ free (CMF) at 50 x g, the supernatant discarded and the cells resuspended in complete neurone medium, based in

DMEM/F12, 1:1 with 10% horse serum (HS, Flow Co., UK), 33 mM glucose, 0.1 U/ml Insulin (Wellcome Co., UK), 0.3% BSA, 2.5 mM glutamine, and 20 mM HEPES). All solutions and media used were cell culture grade, had a pH 7.2-7.4, except the trypsin solution (7.6-7.8), and a similar osmolarity (310-315 mOsm/Kg H₂O).

In some experiments the fraction of motoneurons was enriched by adding 2 ml of the cell suspension on top of a 5 ml of cold Percoll solution (LKB-Pharmacia, UK) at density of 1.048 prepared in DMEM in a 15 ml centrifuge tube. Tubes were then centrifuged at 2000xg for 15 min in a Beckman GPKR rotor GH-3.7 at 4°C. The fraction from the top of the column, containing most of fluorescent neurons, was recovered with a Pasteur pipette and added to 10 ml of ice cold DMEM, mixed carefully, centrifuged over a cushion of BSA, as above, and resuspended in complete neurone media. The density selected was taken from the work of Schnaar and Schaffner (1981) as the most appropriate for obtaining an enriched fraction of spinal rat motoneurons.

The final cell suspension was plated on 60-mm plastic (Nunc, Denmark) plain culture dish for 45 min, to eliminate non-neuronal cells from the suspension by differential cell adhesion. After this pre-plating, the medium was recovered, and cells counted using Trypan blue (0.4% in HBSS) in a hemocytometer (Freshney, 1983). Viable cells were plated at a concentration of $10-40 \times 10^4$ cells/cm² or $0.7-2 \times 10^4$ cells/ μ l, in 300 μ l of neurone media, to which another 300 μ l was added 24h later. Three days later half of the medium was changed, and completely each three days onwards. Cultures were incubated at 36.5 C° in 6% CO₂/air and almost 100% humidity.

The viability of the cell suspension was defined as the fraction of viable cells per ml (unstained by Trypan blue). The seeding efficiency was obtained calculating the proportion of attached cells from the total number of viable seeded cells. A Labelling Fraction was obtained as the ratio of fluorescent neurones to the number of seeded cells. Assuming a similar attachment for labelled and non-labelled cells, the number of fluorescent cell in the viable fraction of the total dissociation volume was calculated, and from this an estimate of the number of labelled cells per half lumbar spinal cord was obtained.

D.1. Culture dish modification

A 16 mm-hole was drilled in 35-mm plastic culture dishes and after smoothing the edges, a cleaned glass coverslip, 22 mm² (No. 1, Chance Proper, UK) was glued to the outside bottom of the dish with Sylgard. These dishes were then placed for at least 12h in the oven at 60 °C. The Sylgard sets, seals the coverslip to the plastic dish and creates a well of 16 mm of diameter and 1-2 mm depth, with an area of 200 mm².

Before use coverslips were cleaned in xylene for 24h, acetone 4h, absolute alcohol for another 24h, and then boiled for 2h in distilled water, and finally oven dried. Once glued to the culture dish, these were sterilized by exposure to U.V. or in 75% ethanol, and rinsed 5x in distilled water before use.

D.2. Grid pattern

An E.M. copper grid (Sira 200, Agar, UK) was placed on the center of a 22 mm clean glass coverslip, and using a vacuum-sputter (Edwards, UK), carbon particles

were sputtered over the surface until a heavy brown negative pattern was obtained. The area of the grid pattern was around 1.7 mm^2 with dark-squares of $100 \text{ }\mu\text{m}$ side (fig. 2) separated each other by clear strips of $40 \text{ }\mu\text{m}$. These coverslips were glued to the 35-mm dishes and sterilized as above. A notch on one side of the pattern served as reference for the identification of the position-number in the grid under the microscope. The carbon coating was sufficiently transparent to permit morphological studies and only slightly reduced the brightness of fluorescent cells. Labelled motoneurons were given a number corresponding to the square occupied, starting from the side of the notch. Motoneurons were then followed by their phase images at different times in culture.

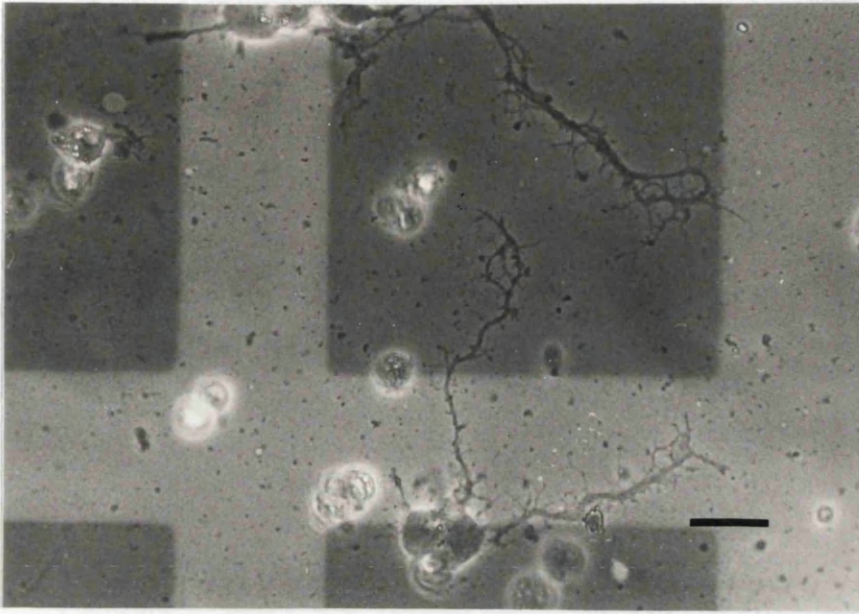
D.3. Substrate

A few drops of a sterile Collagen (Calf Skin Type I; Sigma) solution in glacial acetic acid were added to the well of the 35-mm dish and spread carefully. Dishes were subsequently dried at RT in a sterile, air-vented hood overnight, and washed 3x in sterile dH_2O . Dishes were then incubated at 37° C overnight with $50 \text{ }\mu\text{g}/\text{ml}$ of poly-D-lysine hydrobromide in dH_2O (PDL; 70000 mw; Sigma), and finally rinsed 3x with sterile dH_2O . The dishes were used immediately or allowed to dry and used later.

E. DRG Cultures

Dorsal root ganglion (DRG) neuronal cultures were prepared from E16-E18 embryos by a modification of the method of Bzyczko and Horwitz (1986). DRGs were dissected from the lumbar segment, placed in HBSS CMF with 33 mM

A.



B.



FIGURE 2. Carbon-coated grid pattern. **A.** phase contrast, and **B.** fluorescence micrographs of dissociated RITC labelled E13-14 motoneurons 6h after plating, and cultured over collagen-poly-D-lysine. Bar = 20 μ m.

glucose, cleaned, chopped and incubated in 0.25% trypsin solution for 30 min at 37°C. After blocking the enzymatic activity with TIS solution, DRG pieces were resuspended in complete media, triturated in a small-bore glass pipette, and filtered through a 25 μ m nylon mesh. The cell suspension was then preplated in a plain 60-mm dish (5×10^6 cells/dish) in neurone media and incubated at 36.5°C. After 1h the media with the cells in suspension was collected, centrifuged at 100xg, resuspended and plated in CPDL coated coverglassed 35-mm dishes in neurone media. Cultures were incubated at 36.5° C and 6% CO₂/air, and after 24h they were ready to receive motoneurons.

F. Fibroblast Cultures

Skin fibroblast cultures were obtained from E19-E20 days old embryos. Pieces of tissue were minced and incubated in 0.25 % Trypsin solution + 0.03 % Collagenase (Sigma, UK) at 37° C for 30 min, the suspension being frequently swirled during this period. This procedure was repeated twice or three times, all the supernatants collected and after blocking the enzymatic activity with TIS, they were centrifuged at low speed and resuspended in DMEM + 10 % FCS. The remaining pieces of tissue were washed in TIS, mechanically dissociated, centrifuged, resuspended in complete medium (DMEM + 10 % FCS), and then mixed with the cells from the supernatants. This cell suspension was sieved through a sterile nylon mesh (50 μ m) in a twinex filter holder, plated on collagen coated 60-mm dishes, cultured in DMEM + 10% FCS, and maintained at 36.5° C and 6% CO₂/air. Primary cultures were subcultured after confluency, expanded, and used, after the second or third passages, to obtain fibroblast monolayers on coverglassed dishes. These monolayers were almost 100% fibronectin (+) (see fig. 3, A-B).

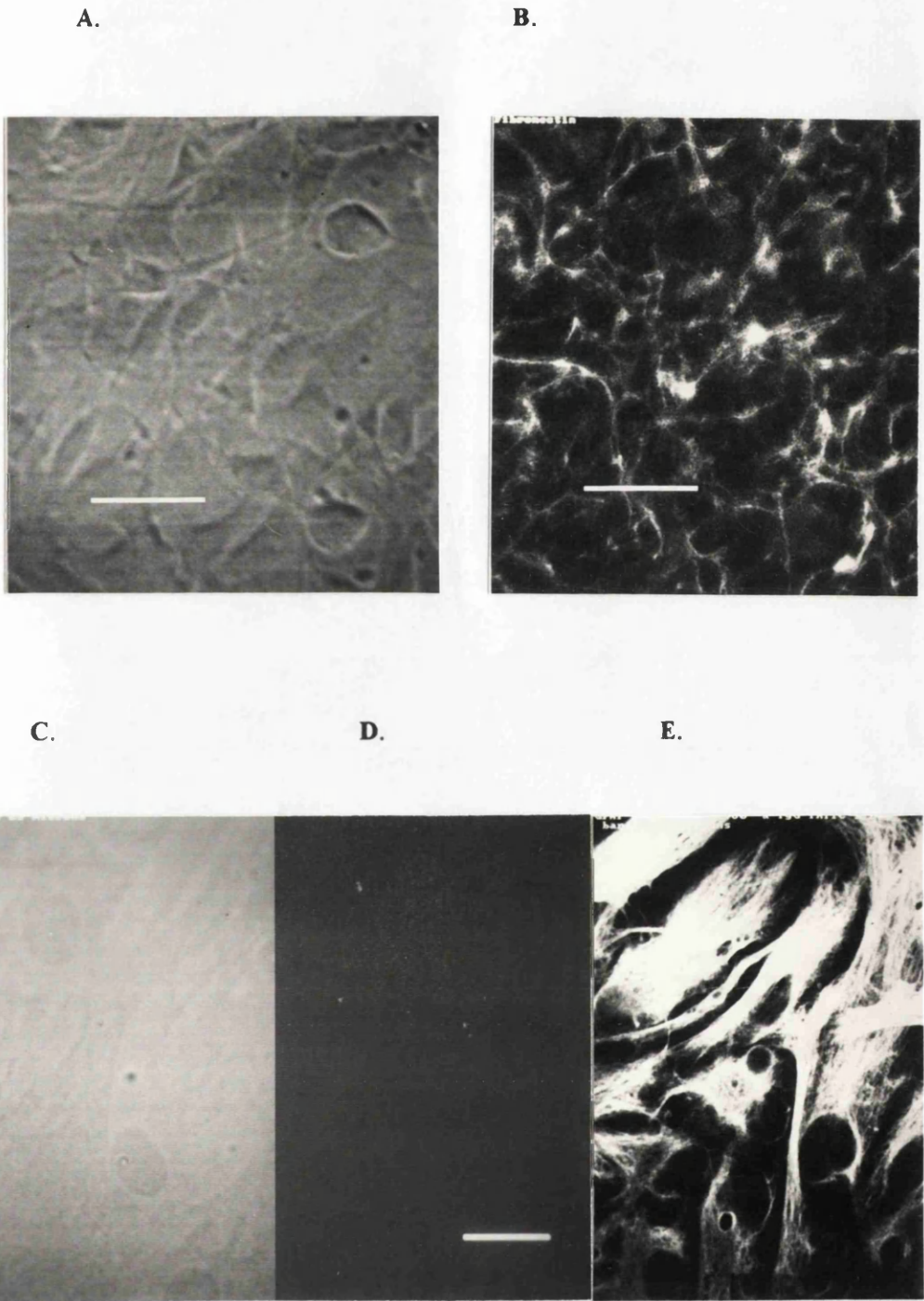


FIGURE 3. Fibroblast (A-B) and glia monolayers (C-E). A. phase, and B. fluorescent images of rat fibroblast monolayers stained with anti-fibronectin antibody and anti-IgG-TRITC as secondary antibody. C. phase, and D., E., fluorescent images of glia monolayers incubated with rabbit serum plus anti-IgG-TRITC conjugate (D, control), and with anti-GFAP antibody and anti-IgG-TRITC conjugate (E.). Bars = 25 μ m.

G. Astrocyte Cultures

Glia cultures were obtained following the method of McCarthy and DeVellis (1980) with some modifications. E19-E21 embryos from an anaesthetized pregnant rat were decapitated and the brain exposed. Pieces of cerebral cortex from 4 cerebral hemisphere or 6 pieces of spinal cord were freed from vessels and meninges, kept in cold HBSS-plus, cut in small fragments, and incubated in 0.25% Trypsin sol. for 30 min at 37° C. Enzymatic activity was blocked with complete media, centrifuged at low speed, resuspended in 3-5 ml of complete media, and dissociated mechanically with a large bore Pasteur pipette. The cellular dissociate was filtered through a 50 μm nylon sieve in a swinex holder and viable cells counted in an hemocytometer using Trypan blue. Cells were plated at 1×10^6 viable cells in a 60-mm petri dish, coated with CPDL, and cultured in DMEM + 10% FCS + 33mM glucose at 36.5° C and 6% CO₂/air. The medium of these initial mixed cultures were changed 6 or 8 days after plating, once a confluent layer of flat cells was formed, leaving neurones and oligodendrocytes at the top. Dishes were washed in HBSS CMF, secured and shaken 3x for 5 min on a vortex, set at the lowest speed, changing the HBSS each time. Finally, cultures were treated with 0.05% Trypsin + 0.02% EDTA (Flow Co., UK) for 3-5 min at RT, shaken as before for another 5 min or until neurones and oligodendrocytes detach from the surface of the culture. Cultures were subcultured, and $1-5 \times 10^5$ viable cells plated on CPDL coated coverslashed dishes in complete media. After a monolayer was formed, the medium was replaced by neurone media, and after 1-2 days they were ready to receive motoneurones. These monolayers, that were $\pm 95\%$ GFAP (+) flat astrocytes (fig. 3, C-E), remained stable, without significant cell proliferation, for several weeks.

H. Muscle Cultures

Following a modification of Konnisberg's method (1979), rat skeletal myoblast and myotube cultures were obtained from 19-20 days old embryos. Embryos were quickly removed, and pectoral or thigh muscles were transferred to a 60-mm dish with cold HBSS-plus, and cleaned carefully, taking special attention to remove connective and vascular tissues. Cleaned muscles from 2-4 embryos were transferred to a new 60-mm dish with HBSS-plus, minced, collected and centrifuged at low speed, and then incubated with 0.25% Trypsin + 0.04 % Collagenase for 30 min at 37° C. Enzymatic activity was blocked with complete media plus 0.04% DNase, and fragments were pelleted at 200xg, resuspended, and dissociated with a flamed pasteur pipette (800-1000 μm). The suspension was filtered through a nylon sieve (25 μm mesh), counted in a hemocytometer, and pre-plated in 60-mm plain plastic cultures dishes ($2\text{-}3 \times 10^6$ cells/dish) for 30-45 min. Cells that remained in suspension were collected, counted, and plated on collagen-coated 60-mm dishes (0.5×10^5 cells/dish) with DMEM + 10% FCS and 5% chick embryo extract (Flow Co., UK), to promote myoblast proliferation, and incubated at 36.5° C and 6% CO₂/air. After 16-18h these primary cultures were used to prepare secondary suspensions using the same procedure, but without sieving, and cells plated on CPDL coated coverglassed-35-mm dishes for culture with motoneurons. Myoblasts were seeded at a low density (5×10^4 cells/dish) and cultured as above. After 1-2 days, when the required number of myoblasts was obtained, the medium was changed DMEM plus 10% Horse serum, without FCS, to promote fusion. Myotubes started to form about 24h later, and when they reached an appropriate size, cultures were treated with 10^{-5} mM cytosine arabinoside (ARA C; Sigma, UK) for 24-48h to kill proliferating cells, and the neurons were then added to these cultures.

Fibroblast and glia monolayers were allowed to reach confluency, and then kept in medium without FCS or in serum-free medium to stop proliferation as required for long-term motoneurone cultures. Myotubes cultures were more difficult to maintain without excessive fusion and the formation of large myotubes which made it difficult to visualize neurones. We generally prepared short myotubes cultures by promoting fusion early after the second passage.

I. Immunocytochemistry

The identity and purity of glia and fibroblast monolayers, and motoneurones cultures were confirmed through indirect immunofluorescence. Cultures were prepared in 13 mm glass coverslips, fixed in 5% acetic acid/95% ethanol at -20°C for 10 min, for GFAP and neurofilament immunocytochemistry, and in 4% paraformaldehyde at RT for 20 min for fibronectin immunoreactivity. After incubation in serum and BSA solution for 60 min at RT to block unspecific binding, cultures were incubated with rabbit anti-GFAP (1:80; DAKO) and rabbit anti-fibronectin (1:100; ICN) polyclonal antibodies, and with mouse anti-Neurofilament 200 kD (1:50; Boehringer) monoclonal antibody, overnight at 4°C. After several washes with PBS, cultures were incubated with anti-mouse or anti-rabbit IgG-TRITC antibodies (1:200; Sigma) for 60 min at RT. Coverslips were then washed in PBS, mounted in Citifluor (AGAR, UK), and analyzed using rhodamine filters.

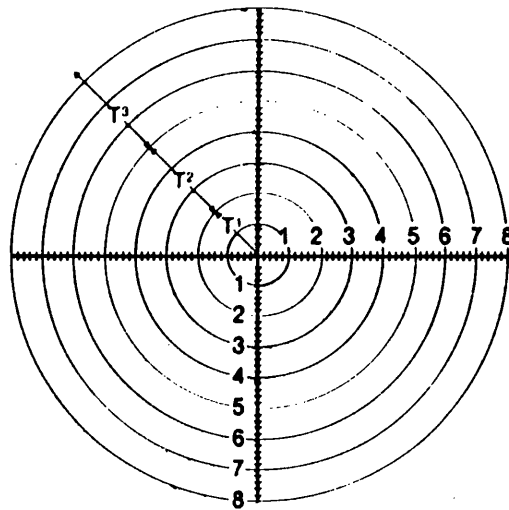
J. Electrophysiological Recordings

Whole-cell patch-clamp recordings, performed by C.Krieger, were made from the identified motoneurons using a List EPC-7 amplifier (List Medical, Darmstadt, FRG). The headstage probe amplifier was mounted on a Leitz micromanipulator which was firmly bolted to a steel plate supported on a concrete block to which the inverted microscope was so bolted rested on the antivibration table described above. Fire polished Sylgard-coated microelectrodes (1.5 mm o.d.) with tip diameters of 1-2 μm and resistances of 1-10 $\text{M}\Omega$ were made from glass capillaries (Clark Electromedical Instruments, Pangbourne) using a Nagarishe PP-83 microelectrode puller. The microelectrodes were advanced onto the cell under phase-contrast microscopy. The seal resistance was greater than 5 $\text{G}\Omega$ before breaking the patch. Recordings were corrected for the fast capacitative transients of the electrode, and the data was filtered (Bessel, 2.5 kHz, - dB), recorded on a FM tape recorder and the signals were displayed on a digitizing oscilloscope and plotted on an x-y recorder.

K. Morphometry

The morphology of labelled motoneurons under conditions and times in culture were studied from the video-recorded images using the different routines of an image analysis system (Sight System, UK). These enabled cell body area, perimeter and diameters, and length of processes to be studied. Changes in shape was studied through comparison of the computer based drawings of the cells. Cell body of motoneurons were outlined (without including the stem of neurites) using a drawing routine, and cell body areas, perimeters and diameters obtained. Cell

A.



B.

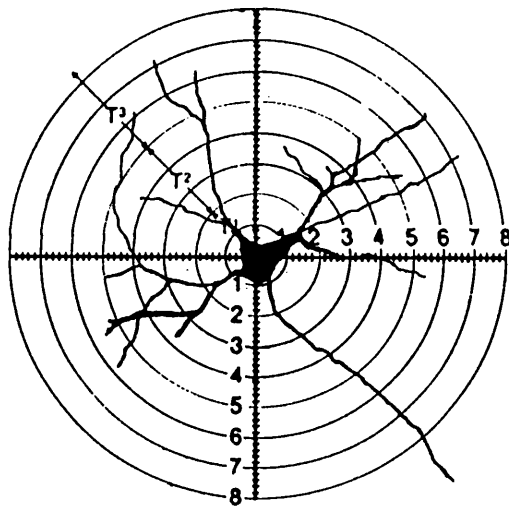


FIGURE 4. A. Scholl's concentric circles up to 160 μm ; 1-8 radii, separated each other by 20 μm ; T^1 = area of primary neurites; T^2 = area of proximal and peak branching; T^3 = area of distal branching. B. Concentric circles projected over a computerized image of a motoneurone.

diameters were also obtained directly from video-copy images of motoneurons. The compound diameter ($d = \sqrt{d_1 \cdot d_2}$), that includes the minor diameter, was used as a measure of cell body size. Cell shape were studied calculating simple relationships (see Inoue, 1986) between area (A), diameters (d) and perimeter (P): A/P ; d_2/d_1 ; circularity ($2A/[P \cdot d_1/d_2]$); and rectangularity ($A/d_1 \cdot d_2$ or $P/[d_1 + d_2]^2$). In the latter indices, a factor of 1 describes a circular or rectangular shape. Neurite length was obtained after the drawing of each neurites using a one pixel cursor. The perimeter for each neurite was automatically obtained, and the length was calculated as $perimeter/2$ as the thickness of each drawn neurite was negligible in relation to the length. The length of each neurite was then added and the Total neurite length obtained. To study neurite branching, Scholl's technique (1953) was applied to video-copy images of motoneurons (see Fig. 4). The number of crossings was determined at concentric circles drawn from the center of the cell and separated each other by $20 \mu\text{m}$ up to $200 \mu\text{m}$ from the cell center. The total number of crossings at each circle, and branching density as the total crossings (branching) per area, and also per neurite, were calculated in motoneurons in each condition.

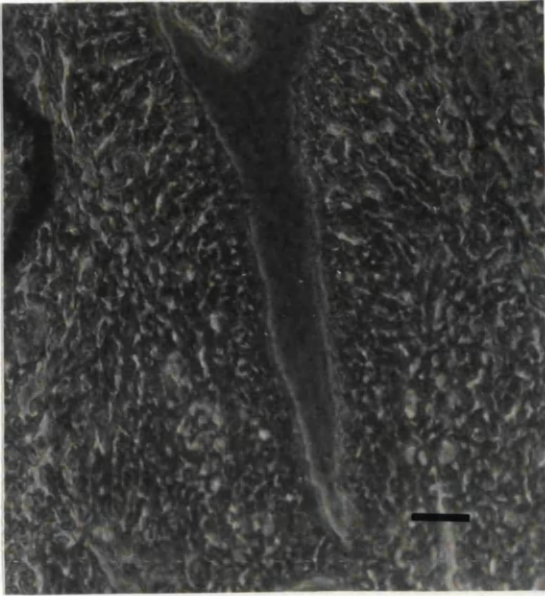
J. Statistical Analysis

In this study the data ^{are} described by the means, standard deviation (SD) and/or standard error of the mean (SEM), and the student t-test is used for the analysis of this ^{ese} data. However, the results from the studies on morphometry and growth are analysed and discussed only in terms of trends of the mean data, given the relative low number of motoneurons studied.

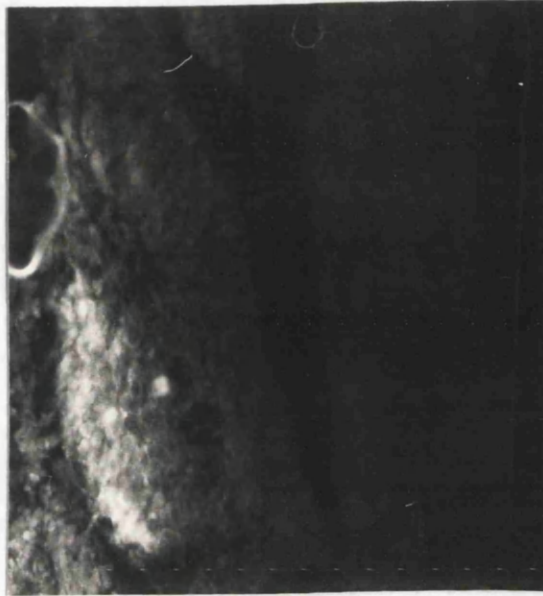
SECTION I. Labelling and General Features of Motoneurones in Culture**A.1. Retrograde labelling of lumbar motoneurones**

To identify motoneurones after their dissociation in single cells, fluorochromes were injected in the hindlimb buds of rat embryos. The label was retrogradly transported to the spinal cord after an appropriate period of incubation. As figure 5 (A-B) shows, 10h after the injection of 0.4% RITC into one hindlimb of E14 rat embryo almost all the neurones of the ventral and lateral part of the spinal cord were labelled. In this case 3 μ l of RITC were injected at several sites in the hindlimb. But when 1 μ l was injected at two distal sites in the hindlimb, only a fraction of the lateral part of the ventral lumbar spinal cord was labelled (fig. 5,C). A clear relation therefore was observed between the volume and sites of injection in the hindlimb and the distribution of the fluorochrome in the ventral part of the spinal cord. This ventral and lateral area of the spinal cord labelled by RITC corresponds to the area in which, at this embryonic age, the recently generated motoneurones are distributed. When those labelled lumbar spinal cord sections were observed at higher magnification, it was seen that the fluorochrome was distributed in large neurones of the ventrolateral area. Embryos older than E16 required larger volumes of fluorochromes and longer incubation times, but in other respects the labelling of lumbar motoneurones were similar to that seen in younger embryos. To obtain labelling similar to that of figure 5, an incubation

A.



B.

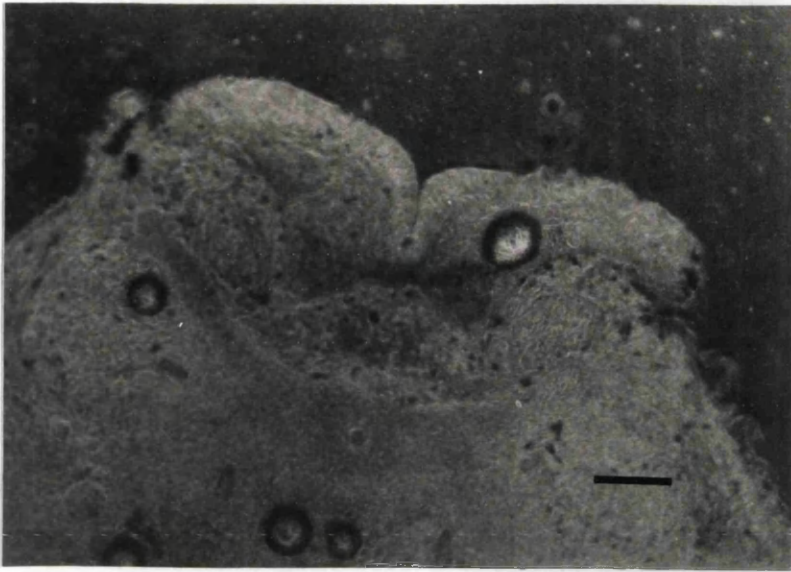


C.



FIGURE 5. Retrograde labelling of E13.5 lumbar motoneurons. Cryostat transverse sections of 4% paraformaldehyde fixed spinal cords 10h after the injection of RITC in the hindlimb bud. A. phase, and B. fluorescent micrographs after a 3 μ l injection of RITC. C. fluorescent image of lumbar cord after 1 μ l injection of RITC. Epifluorescence microscopy with rhodamine filters. Bar = 50 μ m.

A.



B.

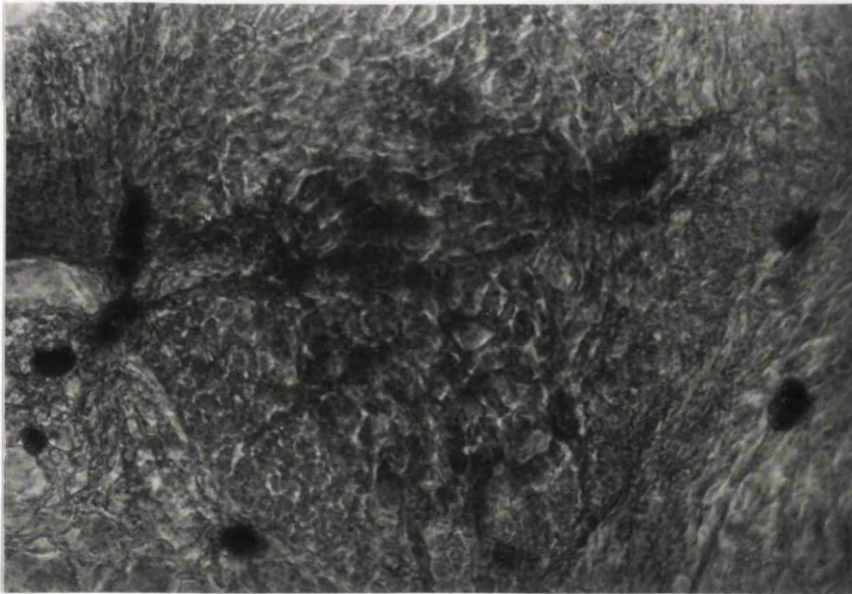


FIGURE 6. Retrograde labelling of E15 lumbar motoneurons. Phase images of a paraffin transverse section of 2% glutaraldehyde fixed lumbar neural tube 10h after HRP injection to the left hindlimb. The roof of the neural tube has been opened. **A.** lower magnification. Bar = 300 μ m. **B.** higher magnification of the left ventral horn observed in A. Bar = 50 μ m.

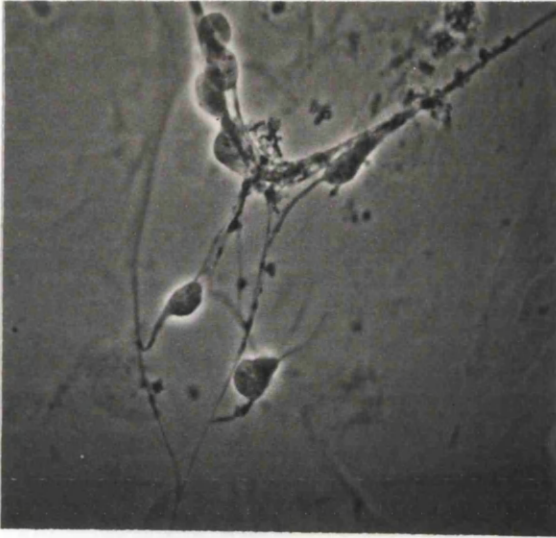
time of 8h or more was required in E14-E15 embryos, and longer than 12h with embryos older than E16. The volume or concentration of fluorochrome injected in the limbs did not affect significantly the intensity of fluorescence observed subsequently in the labelled cells in culture. Other fluorochromes (see methods), like Sulphorhodamine, WGA-RITC or Ly, and Dx-RITC were also tried, but RITC gave the best results in terms of brightness with short incubation times. DiI gave a good labelling in the spinal cord and in culture with the advantages of a longer duration of the fluorescence and the labelling of processes, but requires longer incubation time than RITC. In the present results only those obtained with RITC are included.

To verify the distribution of the retrograde labelling observed with RITC, HRP was used as an independent marker for spinal motoneurons. Using the same procedure and with similar volumes, 3 μ l of 5% HRP (in PBS) was injected in hindlimbs of E15 rat embryos. These embryos were then decapitated, eviscerated, and lumbar cord-hindlimb segments were obtained. After 10h of incubation the lumbar cord-hindlimb segments were fixed in 2% glutaraldehyde, incubated in DAB, and paraffin sections from the lumbar spinal cord were obtained (Landmesser, 1978). A similar distribution of labelling was observed (fig. 6), confirming and validating the retrograde labelling of spinal motoneurons using RITC. In this thesis motoneurone will mean labelled, i.e., identified motoneurone, and the terms will be used interchangeably.

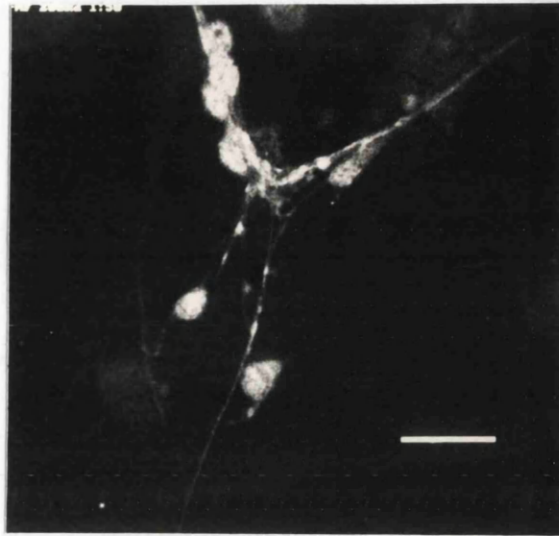
A.2. Use of video-microscopy

The great majority of labelled cells observed in the ventral cord cultures presented a neuronal morphology and electrophysiological characteristics (see below). These

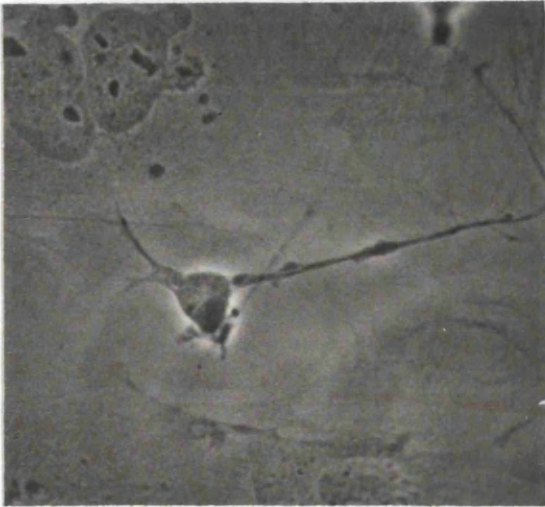
A.



B.



C.



D.

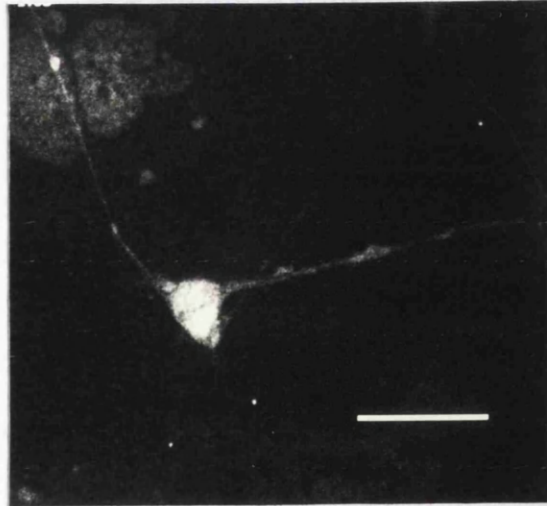


FIGURE 7. Spinal motoneurons from E13-14 rat embryos after 2d in culture over fibroblast (A-B) and glia monolayers (C-D). Immunoreactivity to neurofilament protein (200 kD), using anti-mouse IgG-TRITC as secondary antibody. A., C. phase, and B., D. fluorescent images. Bars = 25 μ m.

ventral cord cultures also presented immunoreactivity to a monoclonal antibody against the 200 kd form of the neurofilament protein (fig. 7, A-D), that is specific for neurones. The labelled neurones had different sizes and more than one process longer than one cell body diameter.

The identification of labelled motoneurones in culture was greatly facilitated by the use of video-microscopy with a low-light level (image-intensified) video-camera. Motoneurones differed in the intensity of their fluorescence (fig. 8), some having a weak fluorescence few hours after plating or after 24h in culture (see fig. 12, C-D), and others were imperceptible through the eyepiece. Nevertheless, they were clearly visualized in the video monitor (figs. 8 and 12), allowing ready identification of more labelled cells in culture. This advantage was also important because 2-3 hours after plating cultures were mainly composed of rounded cells with short processes. The latter complicated the use of morphology as a criteria of identification. Moreover, the presence of fluorescent cellular debris, and blood-borne cells with autofluorescence in older embryos, made the process of identification of labelled neurones through the eyepiece more difficult and slow requiring sometimes long exposure to the exciting light. The duration of observation was greatly reduced using video-microscopy, which was particularly useful when motoneurones were selected for patch-clamping, and for morphological analysis. The exposure of cells to the exciting light for 1-2 seconds, with immediate capture and storage of many images of different fields of view, greatly reduced the possibility of damage and the adverse effects on viability secondary to long periods of observation. This system also made possible the examination of cells without fixation, facilitating the follow-up of individual motoneurones in culture. Finally, the recording system facilitated further quantitative analysis of labelled

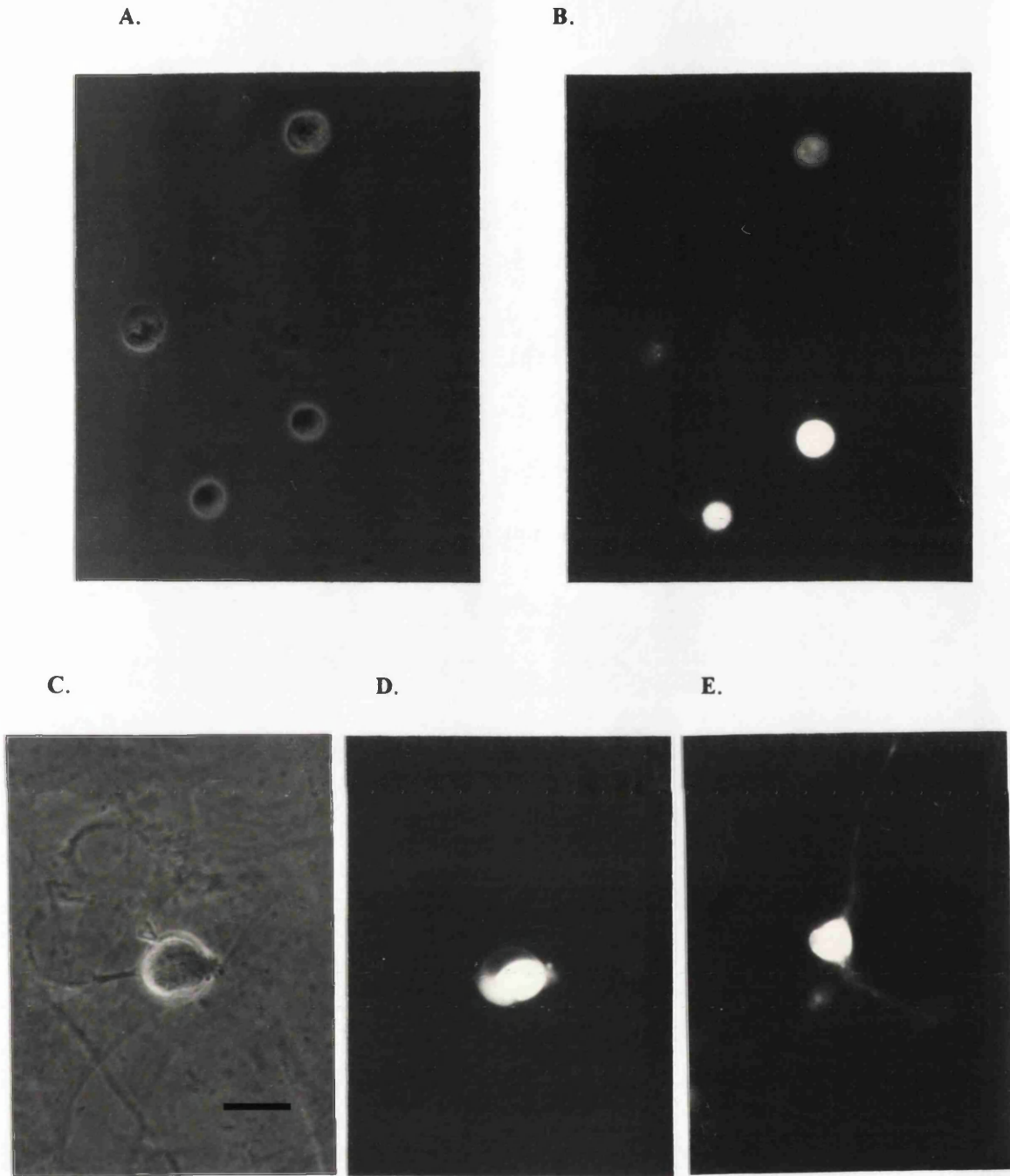


FIGURE 8. Labeled motoneurons in culture. **A.** phase, and **B.** fluorescent images of labelled motoneurons 2h after plating, and cultured on CPDL. **C.** phase, and **D.** fluorescent images of a dying labelled motoneurone after 2d in culture over a monolayer of fibroblasts. Note the retraction of a neurite. **E.** fluorescent micrograph of a labelled motoneurone at 12h in culture on CPDL showing the labelling of neurites. Bar = 20 μm .

motoneurones.

Usually the fluorescence was granular with a typical perinuclear distribution (fig. 12), but homogeneous fluorescence, and a few examples of neurones with labelled neurites were also observed (see fig. 8, E). Few non-neuronal labelled cells (<1%) were encountered which means a low diffusion of fluorochrome in the spinal cord or during the dissociation. Non-neuronal cells were large and flat with no processes. By morphological features and immunoreactivity they were most probably glia and/or fibroblasts, and on morphological grounds some were macrophages (identified as large and rounded cells). Few autofluorescent objects were seen with FITC or Rhodamine filter sets, as they were significantly reduced by the centrifugation of the dissociated cells through a solution of albumin. Most of them were cellular debris and, in older embryos, some were red blood cells, all of which were easy to identify by their morphology, small size, and intense fluorescence. Such nonspecific fluorescence was more frequently observed in cultures from E18-E20 embryos, but again identification was greatly facilitated by the use of the video-camera and video-recording system.

In some instances motoneurones became autofluorescent, or their fluorescence increased during the process of cell death (fig. 8, C-D). These cells were easy to recognize, as they became rounded, more birefringent, and increased in size with retraction of their neurites. They also could show a foamy and vacuolated appearance, with short or no processes before they disappeared. These degenerating or dying cells were loosely attached, presented no electrophysiological activity, and were also more frequently seen in older embryos, or after 24-48h in culture. They were observed in labelled and non-labelled preparations, and no

differences were found between different fluorochromes.

A.3. Number of labelled motoneurons in culture

To estimate the proportion of labelled cells in culture, dissociated cells were plated at a seeding density of 15,000 cells/cm², and labelled and non-labelled cells counted after 4-6 h. In general, cultures from E13-E14 embryos presented labelling from 0.8-30 % (No. labelled motoneurons/total No. cells per dish). But when experiments without enrichment procedures were considered alone, as figure 9 (A) shows, the average labelling was 3.1 %. The same figure (9, A) shows that the enrichment procedures of BSA centrifugation and pre-plating, significantly increased the labelling to 19.3 % (S.D.= 6.4, n= 6; p <0.01, by F-test), with an enrichment factor of 5.5. When a step-gradient centrifugation with Percoll was added, the enrichment factor increased to 10-15. Table 1 and figure 9 (B) compare the results from the two group of embryos (cultures without enrichment procedures), showing that older embryos presented a significant lower labelling (1.7%; p <0.05). Although no significant differences were observed in viability in this group of experiments (without enrichment procedures; but see below), cells from older embryos presented a lower seeding efficiency (S.E.), although with a p barely over 0.05. The latter, together with the absolute and relative decrease in the number of spinal motoneurons at that age, may have accounted for the lower % of labelling. The greater scattering of the data for younger embryos, observed in figure 9 (B), probably reflects variability in the procedure of injection of fluorochrome and isolation of the ventral cord at that age. The estimate of labelling per half lumbar cord shown in Table 1 was obtained from the data observed in culture (without enrichment procedures) with account being taken of

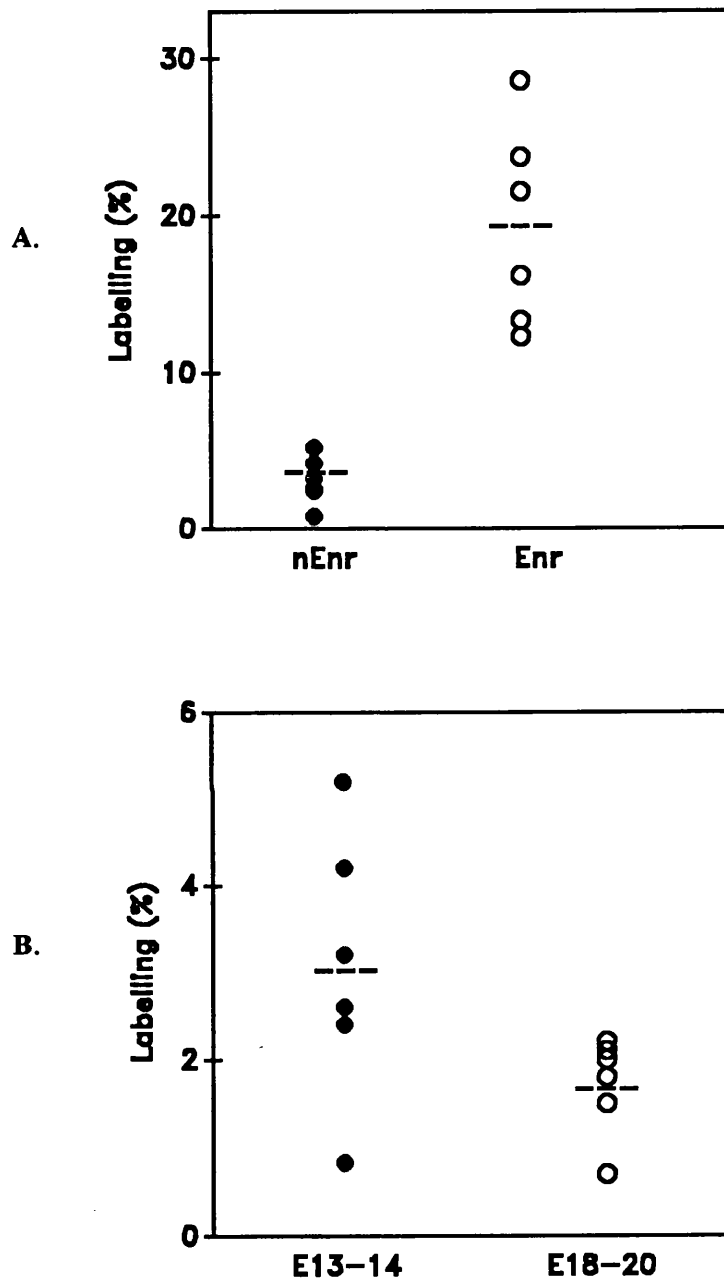


FIGURE 9. Number of fluorescent cells/total cells/dish (labelling %; means of at least 6 dishes) 4h after plating. RITC retrograde labelling. A. enriched (Enr) and non-enriched (nEnr) cultures, from E13-14 embryos. Transverse lines indicate means of experiments. B. Non-enriched cultures from E13-14 and E18-20 embryos.

the corresponding seeding efficiency, viability and number of embryos. For this estimate, it was assumed that at E13-E14 and E18-E20 a rat embryo has 6000 and 3500 motoneurons per half lumbar cord respectively (Oppenheim, 1986; see also Lance-Jones, 1982 who gives similar figures for the mouse). In both groups of embryos a similar labelling of over 60% was obtained showing that the majority of motoneurons was labelled.

Table 1.
% Motoneurone Labelling in Culture and Lumbar Cord

	Seed. Eff. %	Viab. %	Labell ¹ %	Estimate Mn ² No.	Mn ³ %
E13-14					
(n=6 exp. 24 emb.)	71.4 (±8.9)	77.4 (±3.3)	3.1 (±1.5)	3,793 (±1,125)	63.2 (±18.7)
E18-20					
(n=6 exp.; 16 emb.)	62.3 (±5.5)	73.8 (±4.7)	1.7 (±0.5)	2,158 (±558)	61.7 (±15.9)
p	0.057*	n.s.*	<0.05**	--	n.s.*

Values are means ± S.D.

(1) = % of labelled motoneurons in culture to total cells.

(2) = No. labelled motoneurons/half lumbar cord.

(3) = % labelled motoneurons, considering 6,000 and 3,500 motoneurons/half lumbar cord for E13-14.5 and E18-20, respectively (see text).

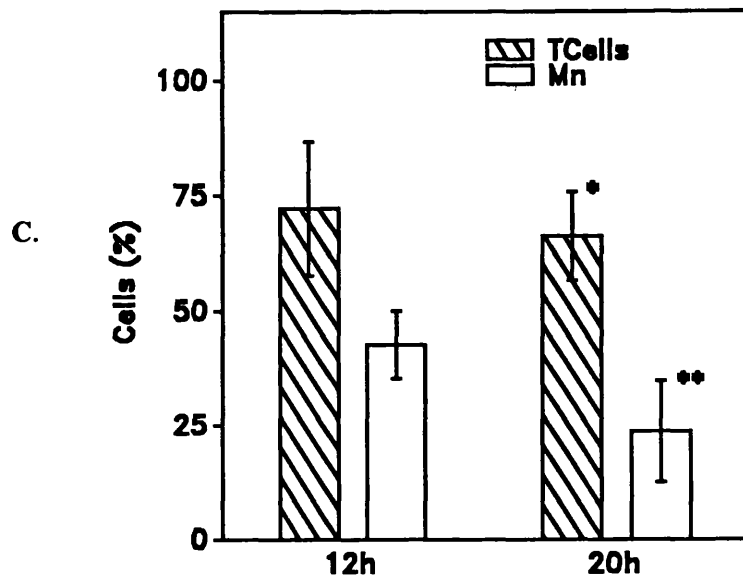
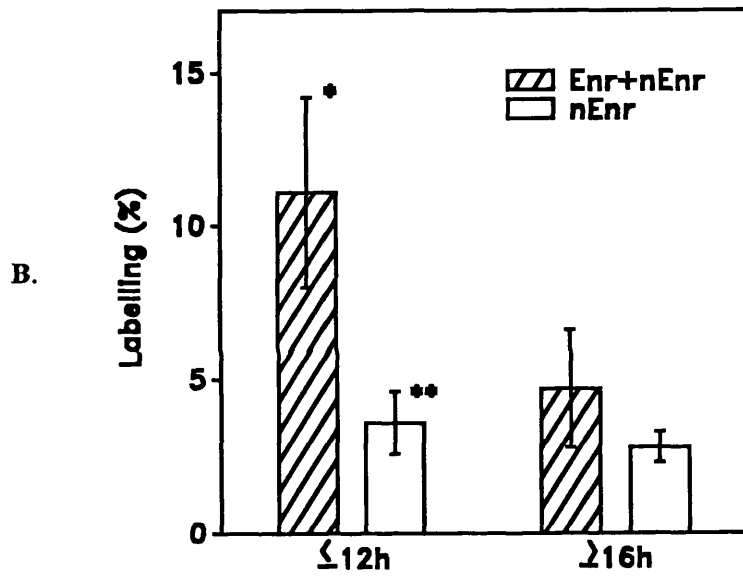
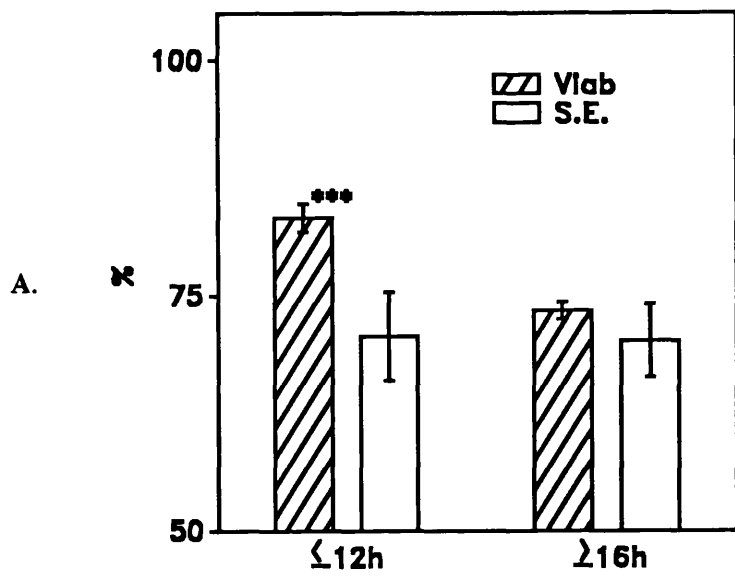
(*) = t-test.

(**)= F-test.

Mn = motoneurons

n = experiments and total embryos.

FIGURE 10. Effect of embryo incubation time on viability, seeding efficiency, labelling, and survival after RITC injection. **A.** % viable cells (Viab) and seeding efficiency (S.E.) ([No. attached cells/No. seeded cells] \times 100) of total dissociated labelled ventral lumbar cords, from embryos incubated for 6-12h or 16-20h. Means (\pm SEM) of 5 exps.. (***) $p < 0.001$. **B.** labelling % (as for fig. 9) in Enr and nEnr cultures from embryos incubated for 6-12h or 16-20h. Means (\pm SEM) of at least 5 exps. (**) $p = 0.05$; (*) n.s. **C.** relative survival at 48h ([No. cells/No. cells at 6h] \times 100) of total cells (TCells) and motoneurons (Mn) from embryos incubated for 12h or 20h, and cultured on CPDL. Means (\pm SEM) of 3 exps., (**) $p < 0.05$; (*) n.s.



A.4. Motoneurone cultures: viability and seeding efficiency

To investigate the viability of the ventral cord cultures after the dissociation procedure, cells were incubated in Trypan blue (see Methods), and unstained, viable, cells counted in a hemocytometer and expressed as a fraction from the total cells. To obtain the seeding efficiency (S.E.) of these cultures, 4-6h after plating attached cells were counted and the number expressed as a fraction from the total seeded cells. Viabilities and S.E. of motoneurone cultures in collagen-poly-D-lysine (CPDL) were in the range of 70-90 % and 50-85 %, respectively. Cultures from older embryos presented a significant reduction in viability ($p < 0.05$) as shown in Table 1, but without differences in S.E.. Figure 10 (A) shows that in E13-14 embryos longer incubation times (≥ 16 h) produced a significant reduction in cell viability ($p < 0.001$), with values around 70 % when embryos were incubated for 20 h. No difference was observed in S.E.. The effect of incubation time over the ^{percentage} % of labelling at 4-6 h after plating was not evident. Although there was a reduction, this was barely significant when only cultures without enrichment procedures were compared (fig. 10, B). In experiments with non-enriched procedures higher ^{percentage} % labelling was associated with viabilities above 80 %, but this correlation was not significant (C.C. = 0.549, $p > 0.05$).

To ascertain whether the incubation time affected the survival of motoneurons, dissociated cells were prepared from E13-14 embryos incubated for 12 and 20 hours after the injection of RITC and plated in dishes coated with CPDL. Although the viabilities of the dissociated cells were 82.8 % (S.D. \pm 4.4) and 72.3 % (S.D. \pm 4.2; $p < 0.001$) for 12h and 20h of incubation time, respectively, no difference were observed, as expected, in the survival of the total population of cells in culture. Nevertheless motoneurons from embryos incubated for 20 hours

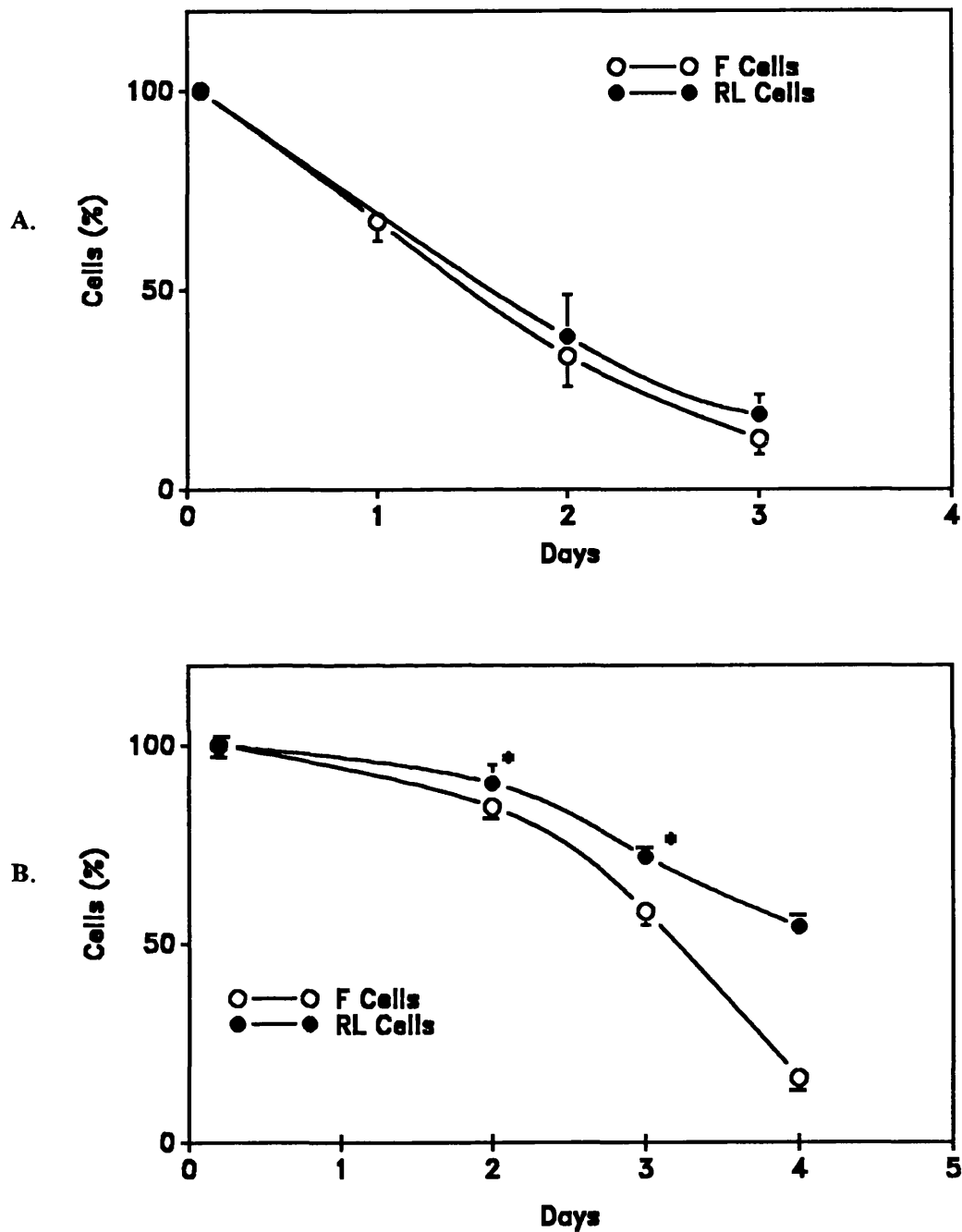


FIGURE 11. Decay in fluorescence of labelled motoneurons. Relative survival to 4h observation of fluorescent cells (Fcells) and same cells relocated (RLcells) on a grid (see methods) versus time in culture. E13-14 embryos. A. cells plated on CPDL. Means (\pm SEM) of 3 expts.. B. cells plated over glia monolayers. Means (\pm SEM) of 3 expts. (*n.s.

presented a significant reduction in the survival at 48h in culture (fig. 10, C).

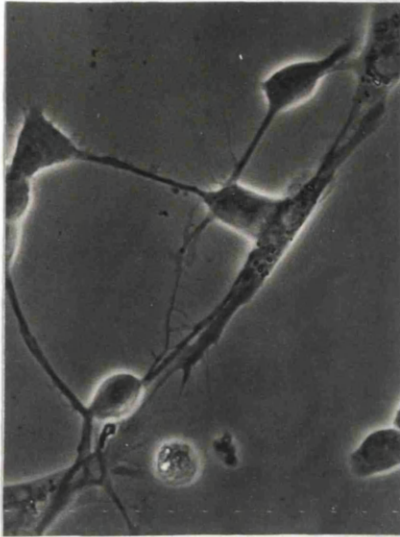
A.5. Decay in fluorescence in labelled motoneurones

When cells were grown over CPDL there was a ^{vapid} fast reduction in the ^{percentage} % of fluorescent neurones, with almost no cells at 3 days in culture (fig. 11, A) . The same relocated cells (see methods) followed a similar curve. To be certain that this decay in the ^{proportion} % of fluorescent motoneurones represented a true reduction in survival and not merely a loss of fluorescence, similar cultures were prepared but cells were plated over glia monolayers (fig. 11, B). These experiments showed clearly that given the right conditions motoneurones survived longer, but also that RITC fluorescence decayed significantly after three days in culture. In cultures with longer survival, ~~only~~ few fluorescent motoneurones were found after 4 days in culture. It is clear from these results that in short-term studies (24-48h) the number of labelled cells is a reliable parameter for assessing survival, but not in studies longer than 3 days.

B. Morphology of Labelled Motoneurones

A few hours after plating E13-14 motoneurones appeared either phase-dark or phase-bright, had an ovoid shape with a thin rim of cytoplasm, and had several short and thin processes (see figs. 2 and 12, A-B). Table 2 summarizes the mean data from 60 labelled motoneurones after 12h in culture over CPDL. Above 50% of motoneurones presented a major diameter of $\geq 16 \mu\text{m}$ and a cell body area of $\geq 110 \mu\text{m}^2$ (fig. 13, A and B). More than 20% were larger with diameters between 18 and 24 μm , and cell body areas of 130 to 180 μm^2 . At this time the majority

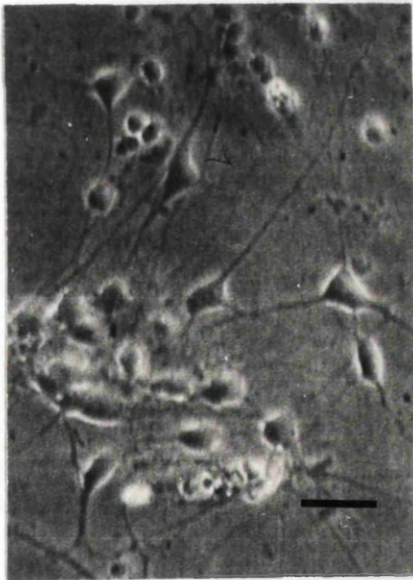
A.



B.



C.



D.



FIGURE 12. E13-14 labelled motoneurons in culture. **A.** phase, and **B.** fluorescent images of motoneurons cultured on CPDL, 12h after plating. Bar = 40 μm . **C.** phase, and **D.** fluorescent images of motoneurons cultured over glial cells, 24h after plating. Bar = 20 μm .

of cultured motoneurons had 3 or more primary neurites, with 4-5 in 50% of them, but bipolar motoneurons were also seen (6%; see figs. 12 and 13, C). The nucleus had an eccentric position, usually with one large neurite emerging from the opposite pole. Under these conditions motoneurons, at this time, had a mean of 6 branches/cell, but neurites longer than 100 μm were rarely seen.

Table 2.

**Morphological Data of Identified Spinal Motoneurons
at 12 h in Culture (n=60)**

Cell Body	<u>mean (\pm S.D.)</u>
Area (μm^2)	116.3 (\pm 23.8)
Perimeter (μm)	45.1 (\pm 5.8)
Diameter 1 (μm)	15.8 (\pm 2.4)
Diameter 2 (μm)	10.2 (\pm 1.6)
Diameter c (μm)	12.6 (\pm 1.4)
Neurites	
Primary (No./cell)	3.5 (\pm 0.8)
Branches (No./cell)	6.2 (\pm 2.3)
Shape	
Aspect ratio	0.66 (\pm 0.12)
Circularity	0.66 (\pm 0.11)
Rectangularity (a)	0.76 (\pm 0.05)
(b)	0.88 (\pm 0.05)

diameter 1 (d1)= diameter major.

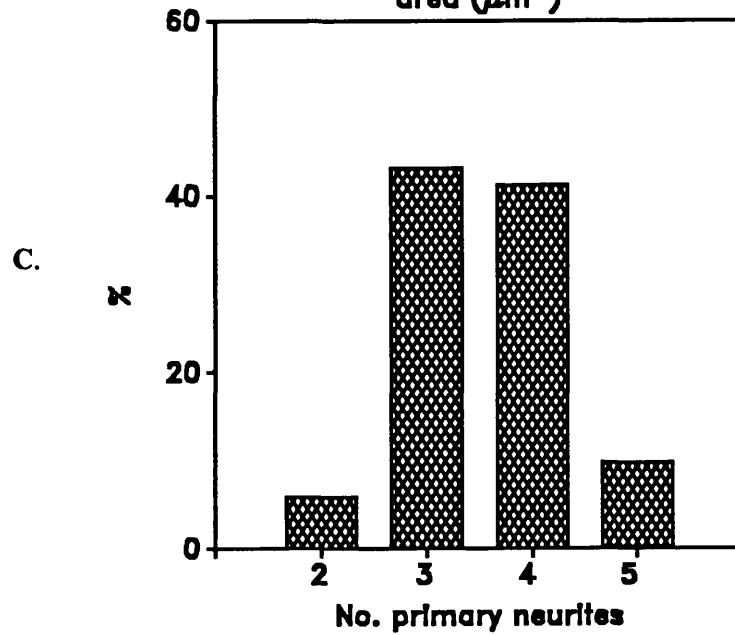
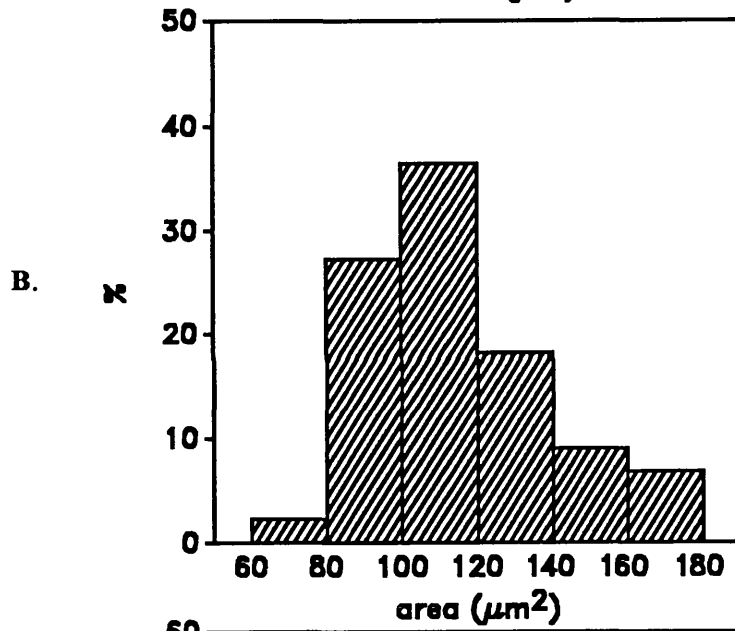
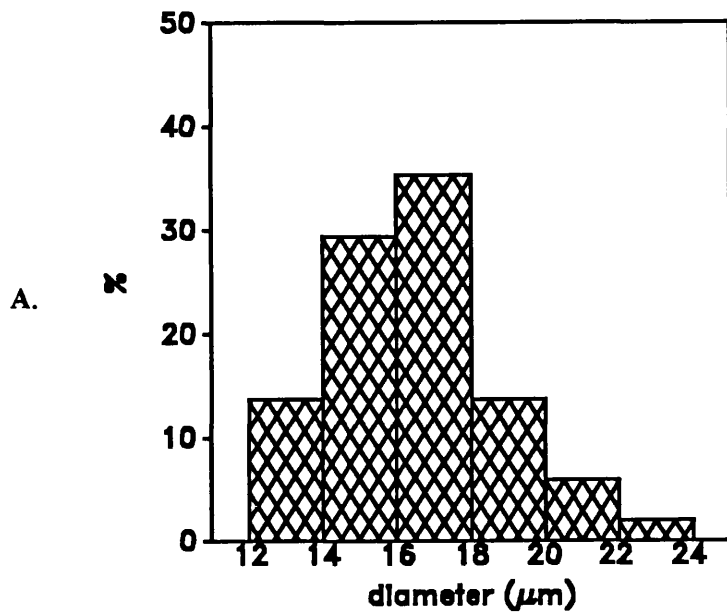
diameter 2 (d2)= perpendicular to d1 at cell center.

diameter c= compound diameter ($\sqrt{d1 \cdot d2}$)

Aspect ratio = $d2/d1$; Circularity = $2 \cdot \text{Area}/(\text{Per} \cdot d1/d2)$;

Rectangularity = (a) $\text{area}/d1 \cdot d2$; (b) $\text{Per}/(d1+d2)^2$ (see Inoue, 1986).

FIGURE 13. Morphological parameters of E13-14 labelled motoneurons at 12h, cultured on CPDL-coated dishes (n = 60 motoneurons). **A.** histogram of major diameter (μm). **B.** histogram of cell body area (μm^2). **C.** distribution of primary neurites.



There was a moderate positive correlation between size and number of primary neurites. Those with larger perimeter and compound diameter had more neurites (Corr. coeff. = 0.45 $p < 0.01$, and 0.53 $p < 0.001$, respectively). Although motoneurons were mostly multipolar from early stages, the stellate shape of the cell body was more evident later in culture (see fig. 12, C-D). At early stages their cell bodies could be better represented with a rectangular shape (see Table 2). Although above 30% of motoneurons were best represented with a rectangular shape (R index > 0.80), others (17%) were better defined as rounded (C index > 0.8). As expected, these latter were smaller in size, as the major diameter and perimeter correlated negatively with the circularity index (Corr. Coeff. = -0.76 $p < 0.001$ and -0.51 $p < 0.001$, respectively). As motoneurons enlarged, the cell body flattened and became phase-dark, the nucleus occupied a more central position, and usually 2 larger and thicker processes projected from opposite sides (see fig. 14, C and D). They were probably dendrites because of the progressive reduction of their calibre. Thinner neurites appeared in the next hours, either from the cell body or from the stem or more distal segments of neurites. Occasionally it was possible to define one or more thin neurites without branches, that resembled axons, emerging from the cell body or the stem of a thicker neurite.

After 24h in culture the basic multipolarity was established, and after 2-3 days the basic morphology of motoneuron was established (fig. 14, A and C). Thereafter there was a continuous growth of motoneurons in culture, with increase and reshaping of neurites and branches. Motoneurons co-cultured with muscle or overglia monolayers not only survived longer but presented higher branching and longer processes which could exceed 200 μm (see below and fig. 14). When cultured under those conditions on coverslips bearing the grid reference it was possible to follow the growth and differentiation of individual motoneurons for

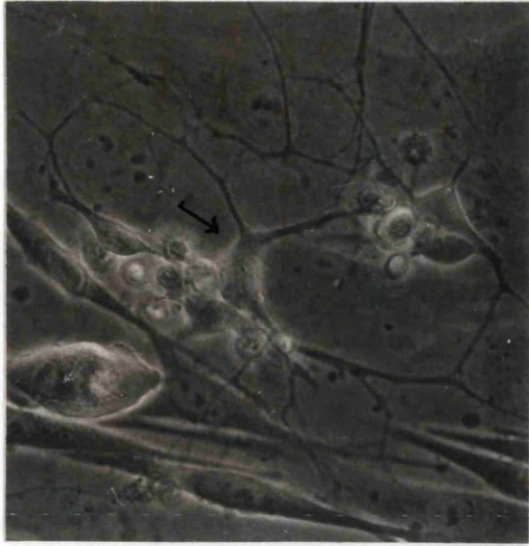
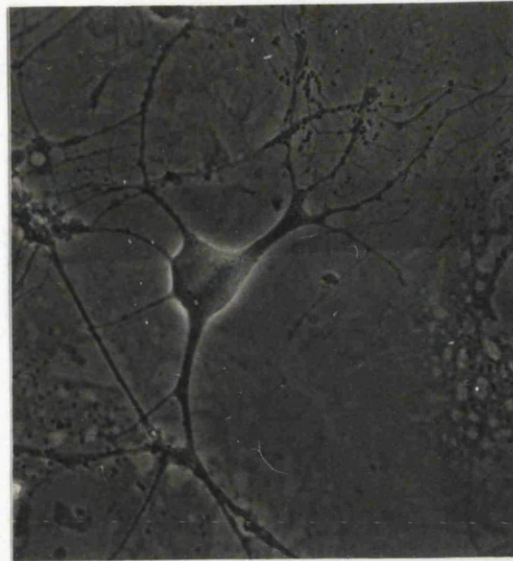
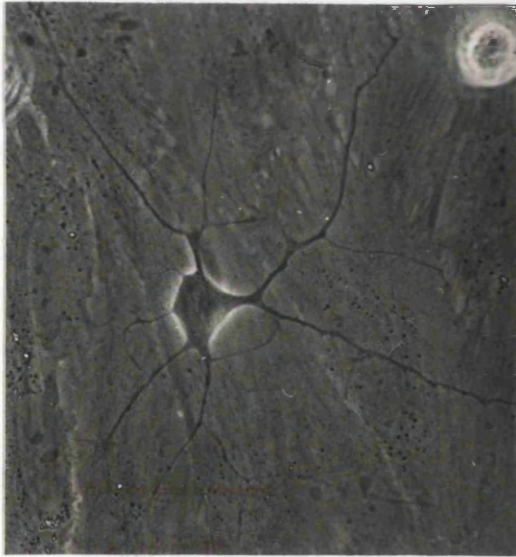
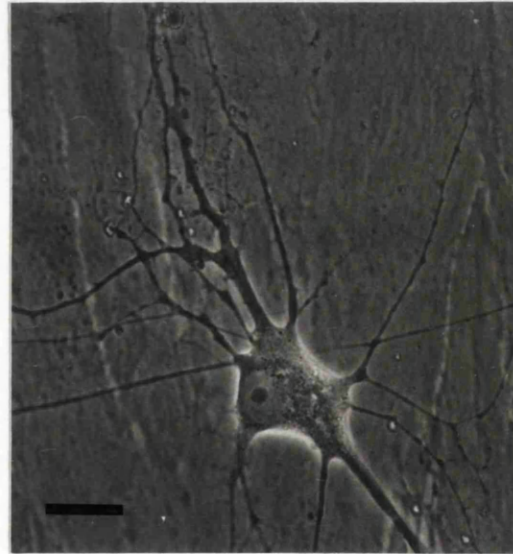
A.**B.****C.****D.**

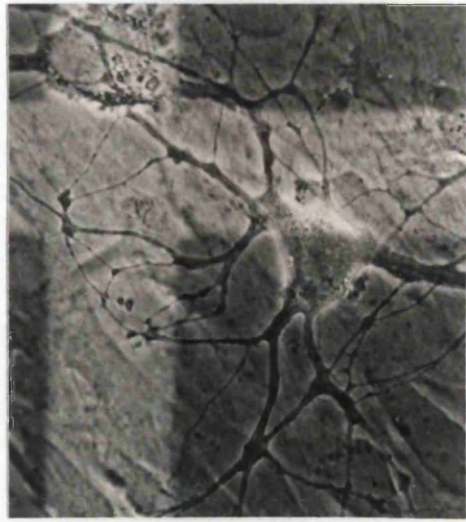
FIGURE 14. Morphology of E13-14 labelled (relocated) motoneurones in culture. Phase images. **A.** motoneurone (arrow) cultured with myotubes, 2d after plating. **B.** and **C.** motoneurones cultured over glia monolayers, 6d and 4d after plating respectively. **D.** motoneurone cultured for 5d over glia monolayer plus myotube conditioned media. Bar = 30 μm (**A**); 20 μm (**B-D**).

FIGURE 15. Development of identified individual E13-14 motoneurons in long-term cultures with myotubes. Phase images. **A.** micrograph of a motoneurone at 3d, and **B.** after 7d in culture. **C.-D.** images of two motoneurons at 6d (**C**) and 20d (**D**) after plating. **E.** image of a motoneurone after 4 weeks in culture. Bar = 20 μ m.

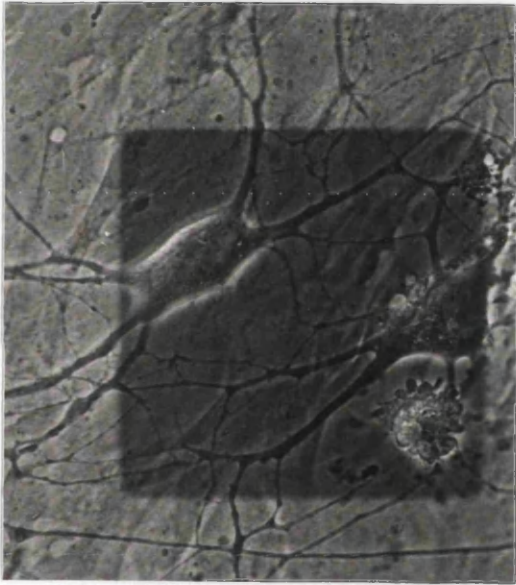
A.



B.



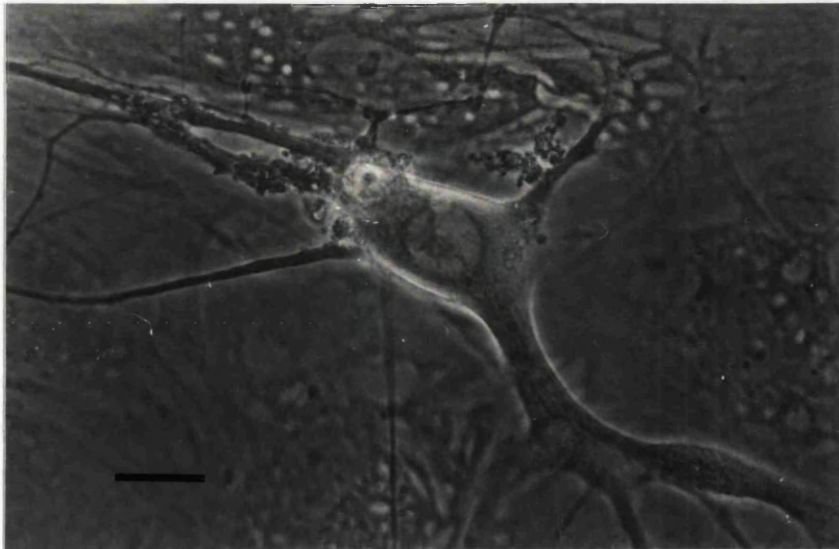
C.



D.



E.



several days. Their shape was slightly and continuously modified (fig. 15, A-B and C-D) for the next 1 or 2 weeks, depending on cell density. There was an increase in the number of secondary processes and in the thickness of primary neurites. Some neurites regressed as others grew out. Nevertheless, the number of primary neurites remained essentially unmodified in the first few days, but increased after 3-4 days when motoneurons were cultured with myotubes. Figure 15 (E) shows a motoneuron cultured in the presence of myotubes for more than 4 weeks, presenting as a large, multipolar cell body with a thick dendrite. In this motoneuron, it is possible to observe a reduction in the proximal branching of neurites.

Under less favourable conditions for survival, as when grown on CPDL or fibroblast monolayers, most motoneurons died during the first 48h in culture. Although initially they presented neurite outgrowth, after several hours in culture motoneurons stop growing, their processes regressed, and the cell body, with a foamy aspect, became rounded and increased in size before they disappeared (see fig. 8, C-D). In some cases this process occurred very quickly, without it being possible to define the intermediate stages. In others, growth cones became globular, neurites stopped extending and thickening, and increased in contrast or fragmented before changes in the cell body became evident.

C. Electrophysiological Recordings

For electrophysiological recordings, cultures with labelled motoneurons were prepared using the procedures described in Methods from E13 to E19 embryos.

Labelled motoneurons were identified using fluorescence video-microscopy, followed by immediate capture of phase and fluorescence images. Selection of the labelled cells for whole cell patch-clamping was done by comparing the two sets of recorded images, and electrophysiological studies performed directly under phase contrast microscopy. All the electrophysiological recordings and analysis of this study was performed by Dr. C. Krieger.

The mean input impedance of 10 identified motoneurons cultured over 1 to 3 days was $635 \pm 369 \text{ M}\Omega$ (\pm S.D.) and the mean resting membrane potential was $-65.6 \pm 15.1 \text{ mV}$ (using KCl filled pipettes). The membrane capacitance was determined from the area under the capacitive portion of the current recording under voltage-clamp conditions and the mean value was $19.1 \pm 9.6 \text{ pF}$. In some labelled motoneurons time constants were determined by the relation between the rate of change of voltage in response to current injection against time. This is illustrated for the passive voltage responses shown in figure 16. A semi-logarithmic relation is shown with a time constant of 19 ms. Plots of the rate of change of potential for 4 motoneurons demonstrated curves fitted by a single exponential corresponding to a mean membrane time constant of $18.6 \pm 7.1 \text{ ms}$. Some motoneurons had spontaneous, slow irregular fluctuation of membrane potential (fig. 17, A), but these fluctuations did not generate action potentials, except in one instance during the recording of 10 motoneurons in which a spontaneous action potential was observed.

Action potentials were produced in response to applied depolarizing currents (fig. 17, B) or anode-break stimuli (fig. 17, C) in 7 of 10 recordings. These action potentials had a mean amplitude of $59.1 \pm 14.9 \text{ mV}$ with a mean overshoot of $7.2 \pm 12.3 \text{ mV}$. The spike duration at half-maximum amplitude was $2.4 \pm 0.7 \text{ ms}$. In

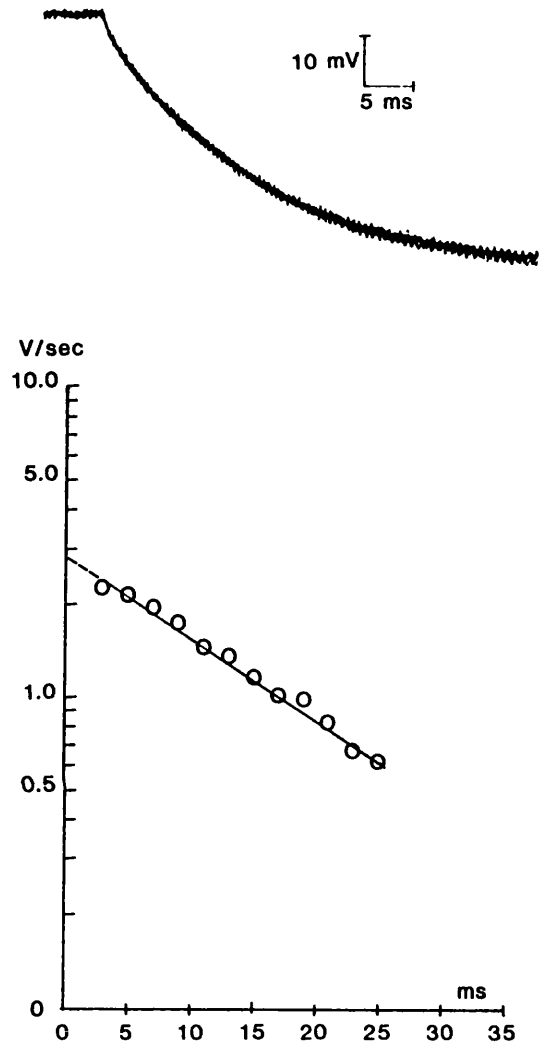


FIGURE 16. Motoneurone charging curve. Upper shows 5 superimposed voltages traces in response to a current pulse of 0.75 mA. Lower, semilogarithmic plot of dV/dt versus time for the above data; time constant 19 ms.

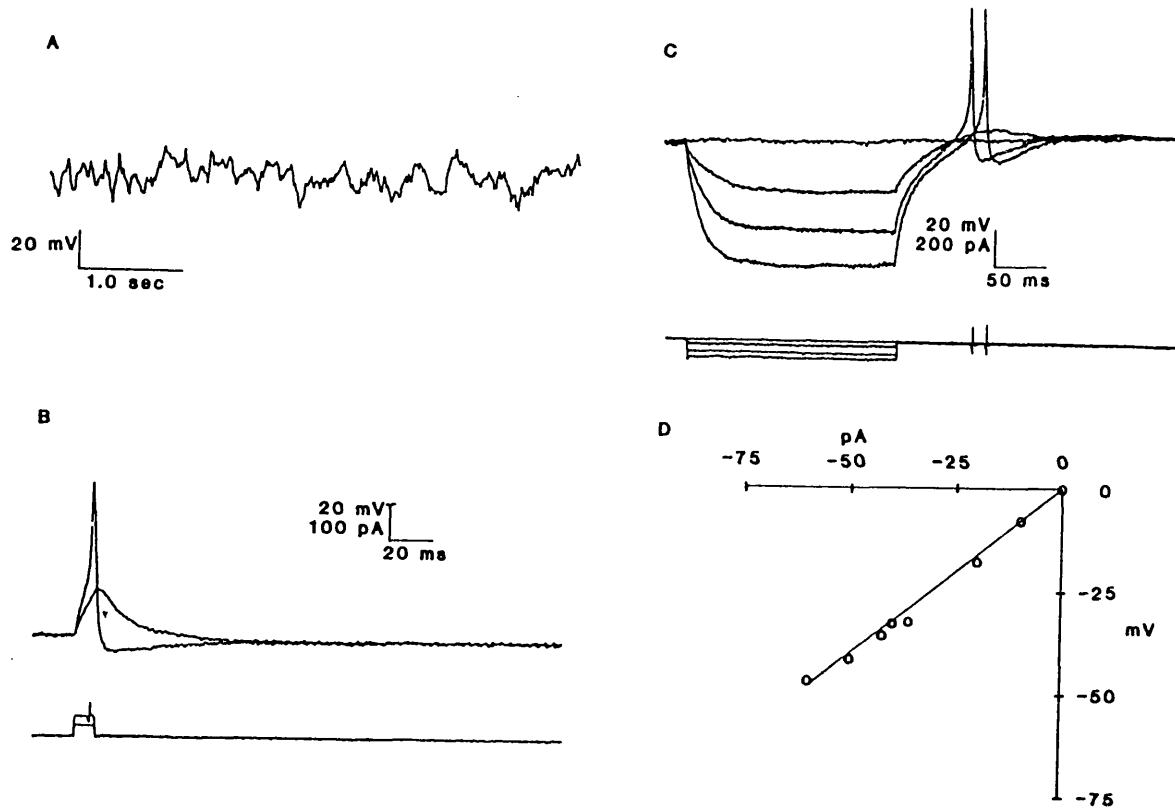


FIGURE 17. Voltage recordings from E13-15 labelled motoneurons. **A.** membrane potential recordings demonstrating spontaneous voltage fluctuations. **B.** membrane potential changes to depolarizing current pulses of 10 ms duration. **C.** membrane potential changes to hyperpolarizing current steps. **D.** I-V plot obtained from recordings shown in C. Voltage scale is with respect to resting membrane (-55 mV). Connecting lines drawn by eye.

3 of 10 motoneurons action potentials were followed by afterhyperpolarizations (fig. 17, B and C), having a mean duration of 57.3 ± 3.7 ms and a mean peak amplitude of 11.3 ± 5.4 mV. The resistance of these motoneurons remained constant during hyperpolarizing current pulses indicating the absence of inward rectification (fig. 17, D).

When motoneurons were voltage-clamped in an external medium containing K^+ , Na^+ and Ca^+ , using a K^+ concentration in the pipette similar to that of the intracellular concentration, depolarization from a holding potential of -75 mV evoked both inward and outward currents in 7 of 10 motoneurons (fig. 18, A). The other three motoneurons demonstrated outward current only. The current-voltage relation uncorrected for leak resistance is shown in figure 18 (B). The inward current (open circles) deviated from the leak current at about -50 mV, it peaked at around -15 mV and had an extrapolated reversal potential of about +25 mV. A late, outward current was also present, having a threshold of -30 mV and this increased in magnitude with larger command voltages (filled circles). In two motoneurons examined, potassium currents were abolished by inclusion of Cs^+ in the pipette solution allowing the inward current to be observed in the absence of an outward current as illustrated in figure 18 (C). The current-voltage relationship for this inward current (fig. 18, D) showed that the current had a threshold of about -50 mV, it peaked at -25 mV and had an extrapolated reversal potential of about +50 mV. Calcium-dependent currents were not observed in the control solution and were not evaluated using elevated external calcium concentrations.

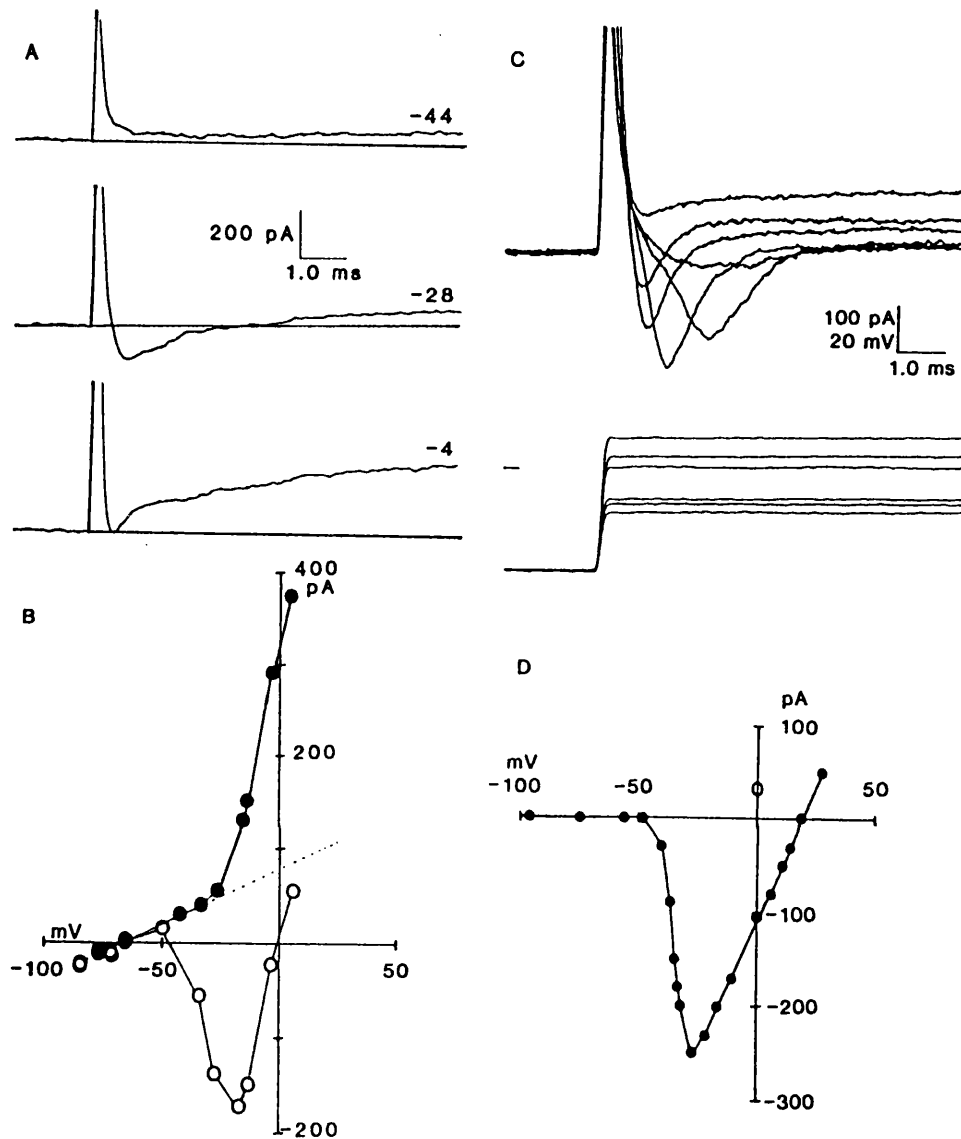


FIGURE 18. Membrane current records from E13-15 labelled motoneurons. **A.** representative current recordings evoked by depolarizations from a holding potential of -75 mV to values indicated to the upper right of each trace. **B.** I-V plot of the data shown in **A.** Open circles, minimum values of the currents within 2 ms of the stimulus; filled circles, current values 20 ms after the stimulus. Points are uncorrected for leak resistance (interrupted line). **C.** current records (upper panel) produced by depolarizing voltage commands from a holding potential of -90 mV (lower panel). **D.** I-V relation of the peak inward current for the data in **C.** Data point represented by filled circles, not corrected for leak. Pipette solution in **A.**, predominantly 140 mM KCl, in **B.**, 140 mM CsCl, control external solution.

C. Summary Section I

In the present study spinal motoneurons were labelled through the retrograde transport of RITC, previously injected into limb muscles. These labelled motoneurons were then identified in dissociated cell cultures by the use of fluorescence and video-microscopy. The purpose of these experiments was to develop heterogeneous cultures containing well identified embryonic mammalian spinal motoneurons thus to facilitate the study of motoneurone development in vitro.

This part of the study showed that it was possible to label spinal motoneurons of mammalian embryos at different ages. By this method motoneurons were specifically labelled as shown in fixed sections of the spinal cord. Only ventral portions of the spinal cord were dissociated, which reduced the dilution of labelled motoneurons, and further enrichment could be obtained by step centrifugation and differential attachment. In the younger embryos, motoneurone labelling was effectively achieved through the incubation of a hindlimb-spinal cord preparation during a period long enough to label up to 60% of the motoneurons from half spinal cords, and to obtain 3-5% of labelled motoneurons per total number of cells (per dish). In older embryos motoneurone labelling was done through in situ injection of fluorochrome into hindlimbs. In this case the % of labelling was also about 60% for the spinal cord, but only 1.7% of cells in culture. The identification and quantitative analysis of labelled motoneurons were greatly facilitated by the use of video-microscopy. Labelled motoneurons were either dark or, less common, bright-phase, multipolar cells with up to 5 primary neurites at 12h in cultures. These motoneurons presented cell bodies with mean values of

15.8 x 10 μm for diameters, and 116 μm^2 for area, with 50% of them with major diameter $\geq 16 \mu\text{m}$ and an area $\geq 110 \mu\text{m}^2$. Whole-cell patch-clamp recordings of these motoneurons showed a mean input resistances of 635 MOhm and resting membrane potentials of -65 mV. Action potentials were evoked and, under voltage clamp, inward and outward currents were present.

SECTION II. SURVIVAL OF MOTONEURONES IN CULTURE

A. Survival of Identified Motoneurons

Motoneurons presented poor survival in culture. Although they attached to a substratum of CPDL and displayed neurite out-growth, most of them died within few days after plating. Immediately after plating some labelled motoneurons remained as rounded cells with short processes, some had a foamy appearance or others were in the process of cell death (see above); all of these 'abnormal' motoneurons disappeared within a few hours of plating. This early death depended critically on the quality of the dissociation of the ventral cord. Prolonged trypsin treatment or mechanical dissociation increased greatly the early disappearance of motoneurons. Figure 19 (A) shows the survival of E13-14 motoneurons and non-labelled neurones cultured over CPDL. There was a great reduction in the number of motoneurons at 48h, with only few present at 72h. Nevertheless, the group of non-labelled neurones showed better survival, with most of the cells still present at 72h. The small increase in the number of non-labelled neurones at 18h is a reflection of neurite growth and our definition of neurone (at least one neurite $\geq 2x$ cell body diameter). Figure 19 (B) shows the same data but

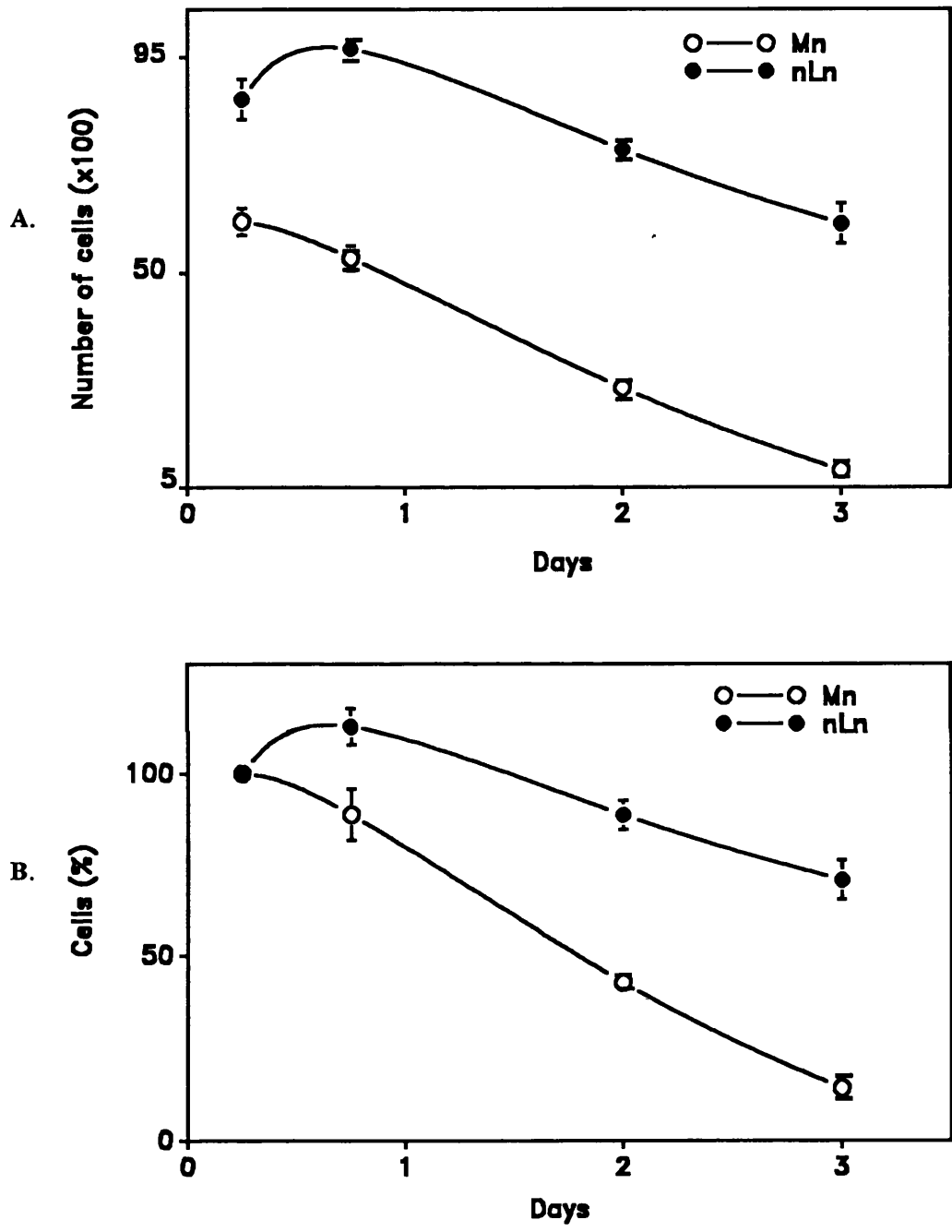


FIGURE 19. Survival of E13-14 labelled motoneurons (Mn) and non-labelled neurons (nLn) versus time in culture. Cells plated on CPDL coated dishes. **A.** Number of motoneurons. Means (\pm SEM) of 5 expts.. **B.** Relative survival (expressed as % of the No. of Mn relative to that at 6h after plating). Means (\pm SEM) of 5 expts..

normalized as relative survival in relation to the 6h observation. This figure demonstrates more clearly that the loss of cells affected ^{preferentially} ~~specially~~ the group of motoneurons, and that the reduction in motoneurons is more than 50% at 48h and 80% at 72h in relation to the motoneurons observed at 6h. To see whether motoneurons from different embryonic ages differ in terms of survival in vitro, cultures were prepared from embryos at E13-14 (previous to the period of natural cell death), E15 (beginning of motoneurone cell death), and E18-20 (end of cell death), and the relative survival determined (Table 3).

Table 3.

Survival of E13-14, E15 and E18-20 motoneurons

		6h	18h	48h	72h
E13-14					
(n=5)	Mn No.	6,076 ±628	5,313 ±581	2,588 ±380	851 ±84
	%/6h	100 -	88.6 ±15.8	42.7 ±5.5	14.1 ±1.5
E15					
(n=2)	Mn No.	7,100 ±778	5,425 ±248	2,575 ±177	588 ±53
	%/6h	100 -	76.7 ±5	36.6 ±6.5	8.4 ±1.6
E18-20					
(n=6)	Mn No.	9,003 ±951	5,284 ±877	2,889 ±370	575 ±126
	%/6h	100 -	59 ±9.8	32.2 ±4.5	6.5 ±1.9

Values are means ± SD.
For student t-test see text.

Although there was a similar trend in survival for the three groups of motoneurons, those from E18-20 embryos presented a significant reduction in survival of about 30% and 25% at 18h and 48h respectively ($p < 0.01$; t-test), and about 50% at 72h ($p < 0.001$; t-test) compared to E13-14 motoneurons. Motoneurons from E15 embryos presented a similar survival to E13-14 motoneurons up to 48h in culture, but 40% less at 72h ($p < 0.01$; t-test). At 18h in culture E15 motoneurons survived slightly better than motoneurons from older embryos ($p = 0.054$), but no significant differences in survival were observed between both groups at 48h and 72h in culture.

A.1. Effect of Serum

To study whether it was possible to increase the low survival of motoneurons in culture by changes in the serum-component of the media (DMEM plus glucose 33 mM, Insulin 0.1 U/ml), 10% foetal calf serum (FCS) and 0.3% bovine serum albumin (BSA) were compared with 10% horse serum (HS). In these experiments all dishes were incubated with complete media plus HS for 1h at 37°C before plating to eliminate differences in attachment. Figure 20 (A) shows the survival of non-labelled neurones and motoneurons as percentages of controls (HS) at 48h. In the absence of any serum no cells survived at 48h. When BSA was added to HS a non significant increase over the controls was observed, but BSA alone could not replace completely the effect of HS. Motoneurons presented a much lower survival than the controls when cultured with FCS ($p < 0.02$), and this reduction was greater than that observed in the group of total cells ($p < 0.05$). There was no significant difference between both population of cells with BSA. No difference was observed in the relative survival of motoneurons when cultured either with

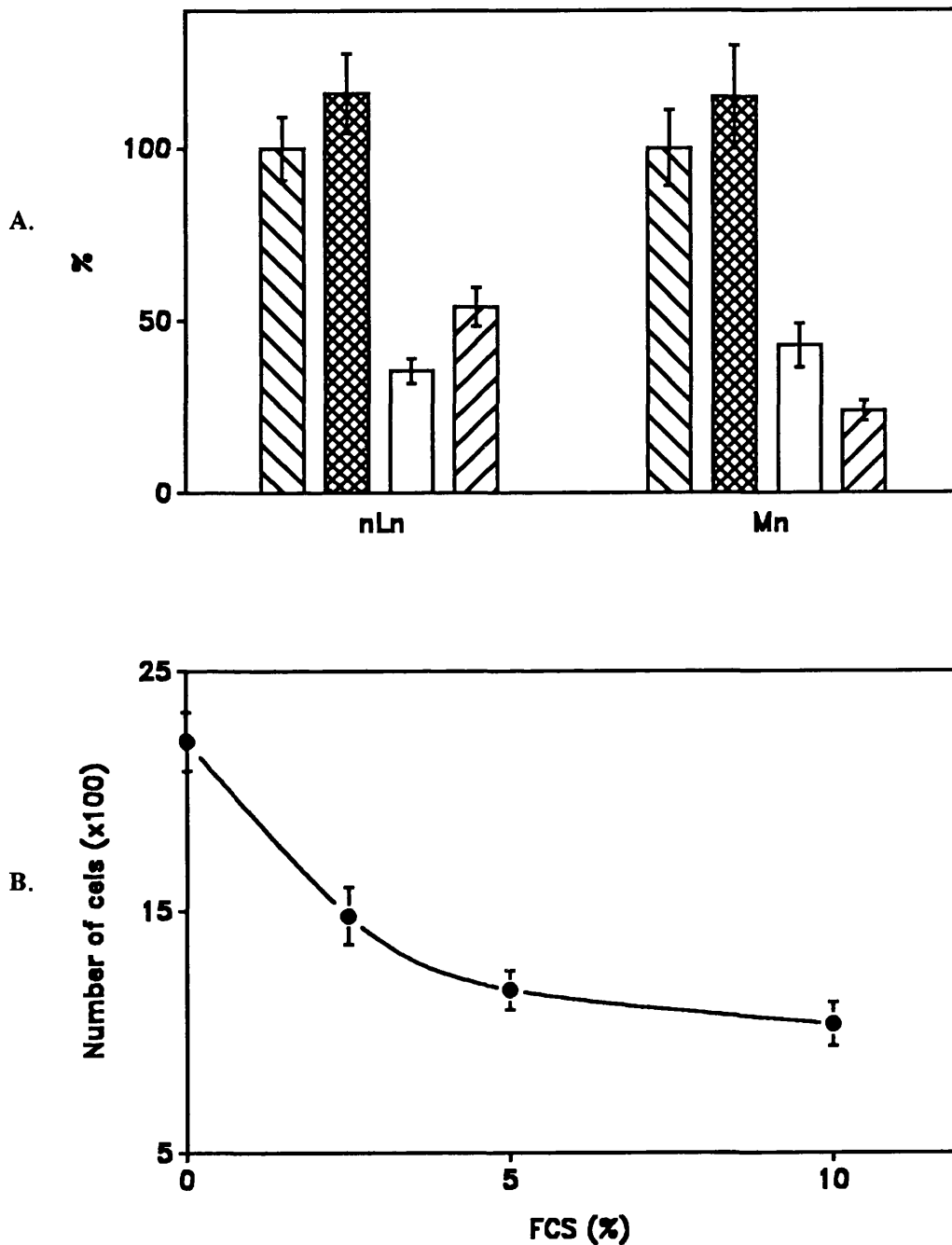


FIGURE 20. Effect of serum and other media components on the survival of E13-14 motoneurons cultured on CPDL. **A.** Survival of motoneurons (Mn) and non-labelled neurons (nLn) as % of controls (cultures with HS) at 48h in culture. Bars : left diagonal stripes = HS (10% horse serum); crosshatch = HS+BSA (10% HS plus 1.5% bovine serum albumin); blank = 1.5% BSA; right diagonal stripes = FCS (10% fetal calf serum). Means (\pm SEM) of at least 3 expts.. **B.** Number of motoneurons at 24h in culture versus FCS concentration (% of media, v/v). Means (\pm SEM) from 2 expts..

5% or 10% HS (data not shown). In two other experiments cultures were prepared, maintained with 2% HS plus 0%, 2.5%, 5% or 10% of FCS, and motoneurone survival determined 24h after plating (fig. 20, B). There was a significant concentration-dependant reduction in motoneurone survival with 2.5% ($p < 0.001$) and 5% ($p < 0.0001$) FCS added to the media.

These results showed that different types of serum affected similarly every type of neurone in these cultures, that the detrimental effect of FCS was greatest on motoneurons; and that BSA produced a small, nonsignificant increase in survival. As a consequence all studies related to survival and growth were done using media with HS (10%) and BSA (0.3%).

A.2. Effect of Interneurones

Interneurones are already present in the neural tube when motoneurons are generated (see Altman & Bayer). Labelling of the intersegmental associative interneurons were tried by the injection of 0.5-1 μ l of RITC into the ventral half of the lumbar spinal cord from E15-16 embryos. After 10h of incubation using the same *in vitro* technique as for motoneurone labelling, the thoracic spinal cord at least 5 segments above the lumbar cord was isolated and dissociated. Although it was possible to identify labelled interneurons later in culture, they constituted a small population, and their isolation as pure population would have required FACS techniques, so this was not pursued. However, by plating ventral cord cells at different densities, it was possible to modulate the level of neurone-neurone interaction from almost none (1×10^4 cells/cm²) to a very high chance of contact (3×10^4 cells/cm²). Consequently, dissociated cells from previously labelled E13-14

ventral cords were prepared and plated at densities of 1×10^4 , 2×10^4 , and 3×10^4 cells/cm² over CPDL coated coverslipped 35mm-dishes. Figure 21 (A) shows the relative number (% of cells to No. of seeded cells) of motoneurons, at different time in culture. Although there was a slight decrease in the population of total cells at 48h (see fig. 21, B), no significant differences were observed with the seeding densities studied. In contrast, motoneurons presented a clear reduction in their number at 36h and 48h with lower cell densities, but those cultured at higher seeding density (3×10^4 cells/cm²) survived significantly better at 36h ($p < 0.05$; t-test) and 48h ($p = 0.05$) than at the intermediate density, and with this latter survival was better than for those cultured at the lowest cell density ($p < 0.01$ at 36h and 48h; t-test). For comparison, figure 21 (B) shows the relative survival at 48h (referred to that observed at 6h) for motoneurons and total cells. Note that there was no significant difference in the decay of the total population of cells, but about 50 % of motoneurons still survived after 48h at the highest cell density compared to less than 20% at the lowest seeding density tested. However at longer times in culture, survival decreased significantly even at higher cell densities. At this higher densities motoneurons presented also a higher branching and length of neurites at 48h. Motoneurons cultured at seeding densities below 1×10^4 cells/cm² survived only for few hours. Although it was evident that they survived longer at cell densities higher than 3×10^4 /cm², motoneurons were difficult to count because of the tendency to aggregate in clumps at such densities.

In conclusion, these experiments demonstrated that motoneurons were more sensitive to the density of cells in culture than the population of total cells, and suggest that the number of interneurons may be important for motoneuron survival in culture. In studies related to survival cell densities of 2×10^4 /cm² were

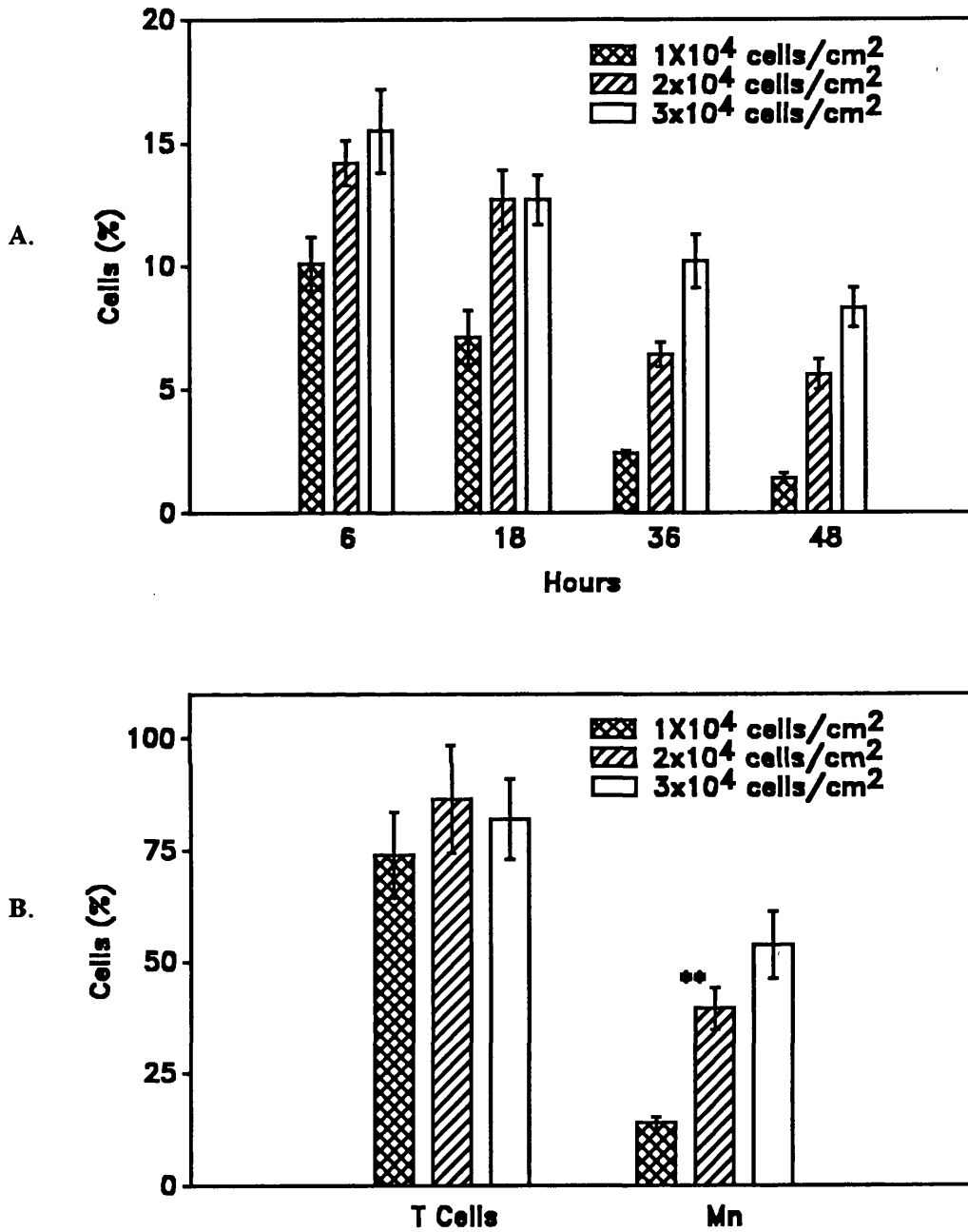


FIGURE 21. Effect of seeding density (No. interneurons) on the survival of E13-14 motoneurons cultured on CPDL. A. Survival ([No. Mn/No. seeded cells]x100) versus time in culture, at different seeding densities. Means (\pm SEM) of 3 exps. B. Comparison of relative survival (as % of 6h observation) at 48h, and at different seeding densities. TCells = total cells; Mn = motoneurons; (**) $p < 0.05$.

used, but for those on growth slightly lower densities were used to permit a better visualization of individual motoneurones.

C. Effect of Non-neuronal cells and DRG neurones

To investigate the effect of non-neuronal cells on motoneurone survival in culture, monolayers of fibroblasts or glia cells were prepared, as well as myotubes/cultures, as described in Methods. Glia monolayers were prepared from embryonic spinal cord (E19-20), although in preliminary experiments no difference^s were found with brain glial cells. Subsequently, dissociated labelled motoneurones from E13-14 or E18-20 embryos were added, and the relative survival determined for up to 3-4 days in culture (see Methods). E13-14 motoneurones cultured over CPDL, as shown in figure 22 (A), presented a low survival, with greater reduction in the number of cells in the first 24h, and a relative survival of only 33.8% (± 10 , S.D.) and 9.9% (± 2.2) at 2 and 3 days in culture, respectively. The same figure shows that the survival of motoneurones over fibroblasts was similar to that observed in CPDL, with a small but significant increase at 2d (43.1% \pm 4.3; $p < 0.05$) and 3d (17% \pm 1.5). In contrast, both glia monolayers and myotubes/cultures produced a marked increase in the survival of E13-14 motoneurones compared with those observed in CPDL and fibroblasts monolayers. Motoneurones were able to survive up to 7d on glia monolayers and up to 5 weeks with myotubes or over glia monolayers plus myotube conditioned media (CM). After 4d E13-14 motoneurones presented a relative survival of 59.2% (± 6) and 66.7% (± 4.4) when cultured over glia and myotubes respectively (fig. 22, B). This difference was non-significant, with a similar pattern in both conditions, but with a trend towards higher survival on myotubes, especially at 3d and 4d in culture. Figure 22 (B) also shows the

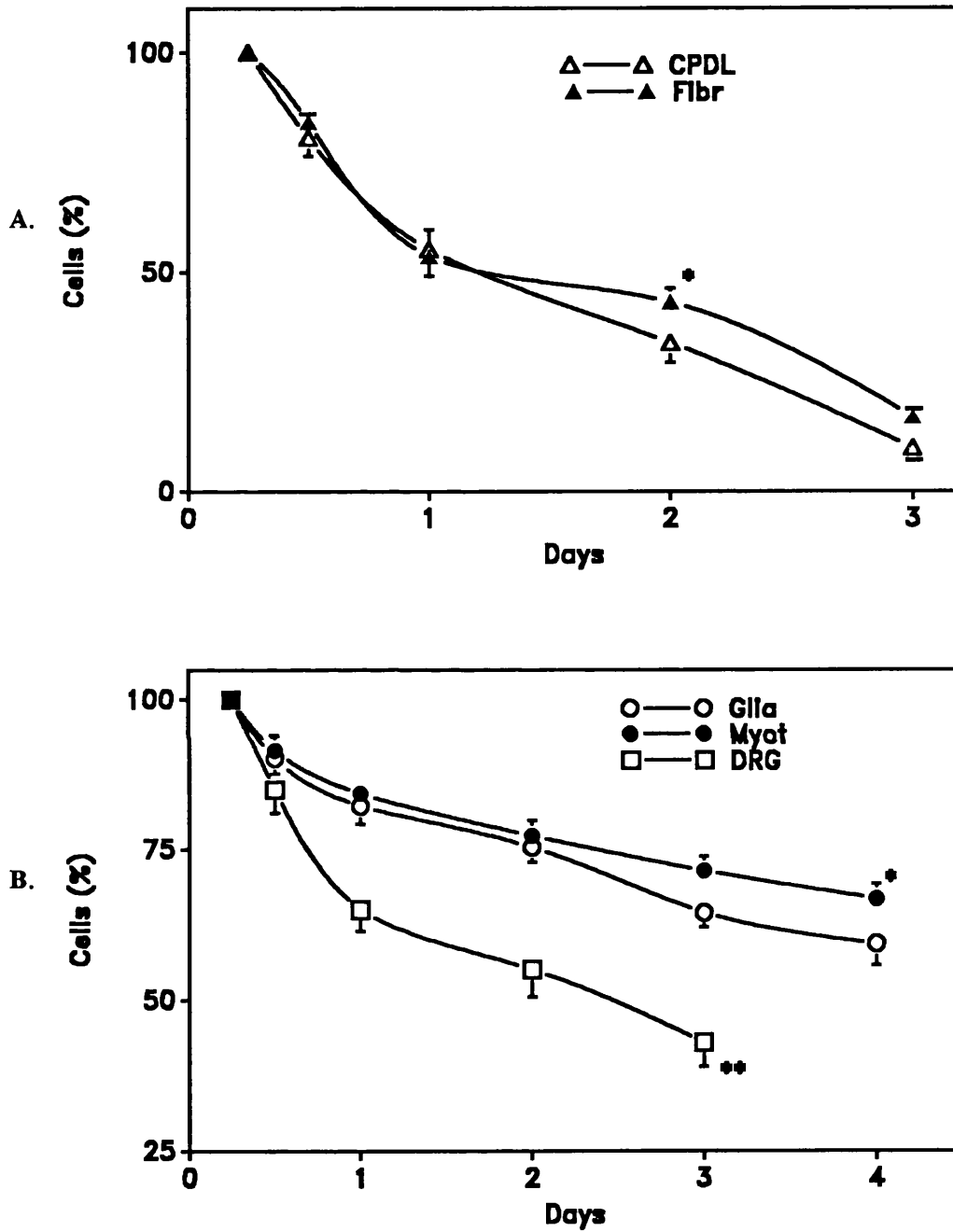


FIGURE 22. Relative survival (as % of 6h observation) of labelled E13-14 motoneurones versus time in culture. **A.** Motoneurones plated over CPDL or fibroblast monolayers (Fibr). Means (\pm SEM) of at least 4 expts. **B.** Motoneurones plated over glia monolayers, co-cultured with DRG neurones or myotubes (Myot). Means (\pm SEM) of 3 expts..

results when E13-14 motoneurons were co-cultured with DRGs/neurons (see Methods). Under these conditions motoneurons presented a relative survival of 43% (± 7) at 3d in culture, which was clearly greater than that observed with CPDL or fibroblasts, but lower than that over glia ($p < 0.02$) or myotubes. The pattern of motoneuron survival on DRGs was similar to that observed on CPDL and fibroblasts with a greater reduction in the first 24h.

When E18-20 motoneurons were cultured over CPDL, the survival was similar to E13-14 motoneurons, with almost no cells ($6.9\% \pm 1.7$) at 3d in culture. Also, as with the younger motoneurons, during the four days those from E18-20 embryos presented higher survival when cultured with myotubes or over glia monolayers than over CPDL (fig. 23, A). During this period E18-20 motoneurons survived better in myotube co-cultures than over glial cells, with a significant increase at 3d, with survivals of $37.6\% (\pm 3.8)$ and $60.6\% (\pm 5.8)$ over glia and myotubes respectively ($p < 0.01$). There were no significant differences in survival between E13-14 and E18-20 motoneurons cultured over myotubes, but as shown in figure 23 (B), E13-14 motoneurons presented a higher survival than E18-20 motoneurons at 3d when cultured over glial cells ($p < 0.01$).

Under all the conditions tested the loss of motoneurons was greater during the first 24h, and also in the last 24h when motoneurons were cultured over CPDL or fibroblast monolayers. After the first 24h, the loss of motoneurons was rather regular with differences either in the level or rate of that reduction. Figure 23 (C) shows a semi-log plot of the negative regression lines of survival versus time when E13-14 motoneurons were cultured over glia or myotubes. It is clear from these regression lines that the decay of survival presented by motoneurons cultured over glia is higher than when cultured over myotubes. The regression line of the

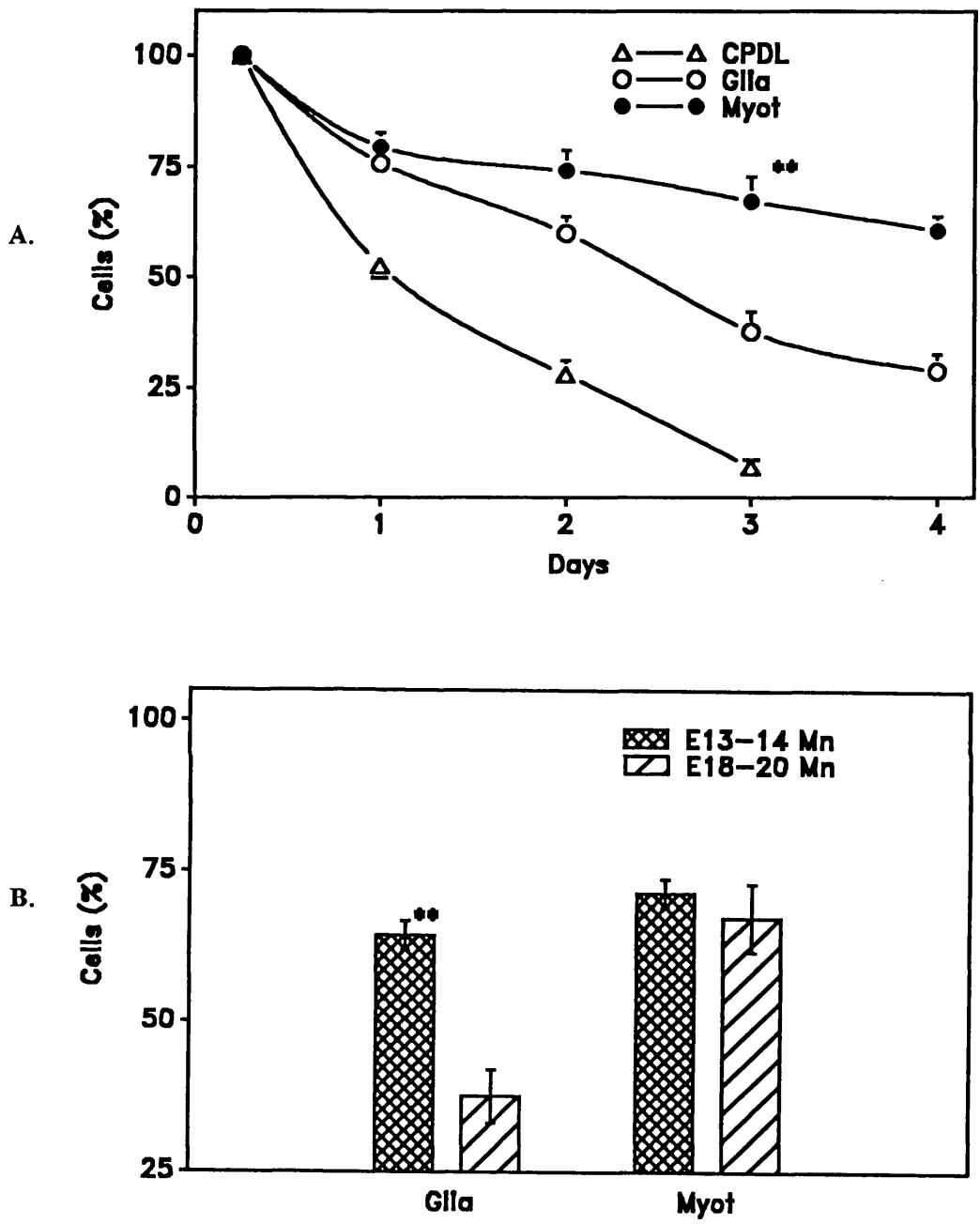


FIGURE 23. Survival of E18-20 motoneurons. **A.** Relative survival (% of 6h) of E18-20 motoneurons versus time in culture. Motoneurons plated on CPDL, over glia monolayers or in co-cultures with myotubes (Myot). Means (\pm SEM) of 3 expts. **B.** Comparison of the survival of E13-14 and E18-20 motoneurons cultured over glia monolayers or with myotubes, at 3d in culture. Means (\pm SEM) as for figs. 22B and 23A.

relative survival for E18-20 motoneurons when cultured over CPDL, glia or myotubes were similarly obtained. These older motoneurons presented a higher decay in survival than E13-14 motoneurons, particularly when cultured over glia monolayers. Assuming a constant rate of cell loss through the whole period of survival, the half-lives (T_{50}) of motoneurons under the different conditions were obtained from the regression lines. E13-14 motoneurons presented a T_{50} of 16 days in glial cells and 22 days in myotubes, compared with a T_{50} of 3, 4, and 7.8 days when motoneurons were cultured over CPDL, fibroblasts and DRGs, respectively. E18-20 motoneurons presented a T_{50} of 2.7 days in CPDL, similar to younger motoneurons, but a shorter T_{50} of 7d and 18.7d when cultured over glia and myotubes respectively (see fig. 23, D). Except for the T_{50} of motoneurons cultured over myotubes, these cell lines do not correspond with the observed survival because it is improbable that the cell loss remain constant during the period of survival in culture. Nevertheless, they show clearly the differences in the rate of motoneurone loss under the different conditions.

From these results it is clear that myotubes provided the best conditions for the survival of motoneurons in culture at both ages, with only a significant difference at 4d in culture. Nevertheless, although the half life of motoneurons on myotubes was close to one week, it was possible to maintain E13-14 motoneurons for up to 4-5 weeks (see fig. 15). In contrast, E18-20 survived less than 15d. More interestingly, the results show that glial cells not only supported motoneurone survival for about one week in the absence of their targets, but under these conditions permitted E13-14 motoneurons to survive longer and in greater number than E18-20. DRG, fibroblasts and CPDL were unable to maintain motoneurons in culture for more than few a days. The possibility to maintain motoneurons for

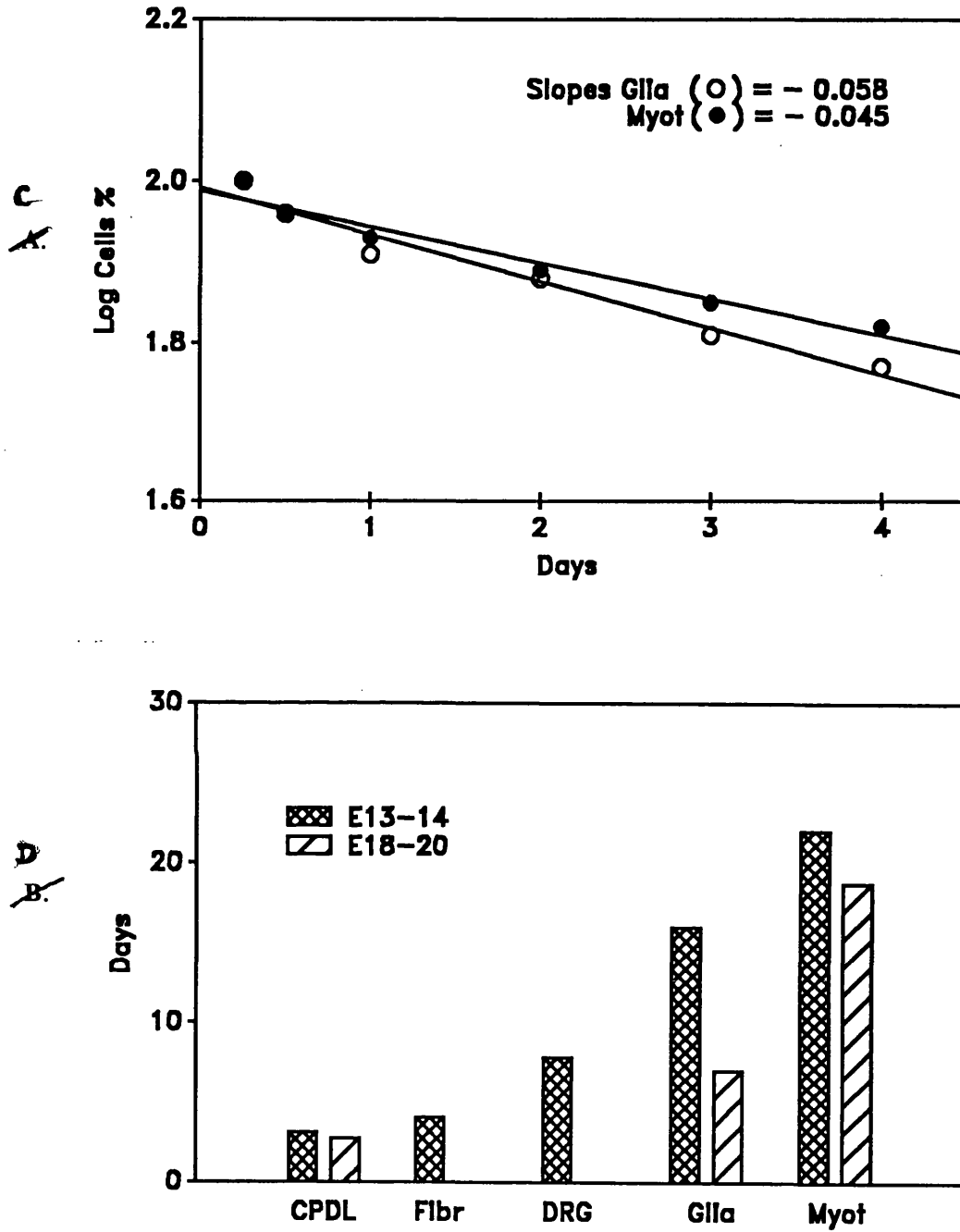


FIGURE 23. C. Linear regression of log relative survival versus time i.c. of E13-14 motoneurons cultured over glia monolayers and with myotubes. D. Half-lives (T_{50}), in days, of E13-14 and E18-20 motoneurons cultured on CPDL, non-neuronal cells and with DRGs, and derived from the relative survival regression lines.

several weeks in culture is an important advance that would facilitate future studies on motoneurone differentiation in vitro that may go up to the postnatal life. The survival of motoneurons for 1 week on glia monolayers, without myotubes, is also important because shows that motoneurons are not necessarily dependent on muscle for their survival early in development, and up to the end of the cell death period.

D. Summary Section II

In these studies the survival of identified motoneurons cultured with other neurons and with different non-neuronal cells were investigated. Motoneurons presented a reduced survival when cultured over CPDL. This survival was age dependent, in that E18-20 motoneurons presented a lower relative survival than E13-14 motoneurons, and those from E15 embryos had a similar reduced survival than that from older embryos after 48h in culture. The effect of interneurons was studied ^{by} modulating the level of cell density. The number of E13-14 motoneurons at 48h was clearly dependent on cell density, with about 50% of survival at 48h when cultured at 3×10^4 cells/cm². However, the number of motoneurons decreased significantly at longer times in culture, even at that high cell density. When cultured over fibroblast monolayers E13-14 motoneurons presented a similar survival to that on CPDL. In contrast when cultured over glia cells or with myotubes, motoneurons not only presented a higher survival but also for longer time. E13-14 motoneurons were able to survive for up to 7d on glia cells and up to 4-5 weeks with myotubes or over glia monolayers plus myotube CM. However, E18-20 motoneurons only survived for few days over glia cells, but for at least

15d with myotubes. When co-cultured with DRG neurones, E13-14 motoneurones had an intermediate survival between that presented on fibroblast and that on glia cells.

These studies showed that motoneurone survival is age dependent and is influenced by other neurones and non-neuronal cells, particularly glia cells and myotubes. Myotubes provided the best environment for long-term survival of E13-14 motoneurones.

SECTION III DEVELOPMENT AND GROWTH OF MOTONEURONES IN CULTURE

To ascertain whether this differential effect of non-neuronal cells on motoneurone survival also implied analogous effects on motoneurone growth, or whether non-neuronal cells only have a permissive role on growth, E13-E14 labelled motoneurones were plated over CPDL, fibroblast or glia monolayers, and myotubes cultures as for the survival studies. Changes in cell body size, primary neurites, cell shape, and neurite ramification and length were measured and followed on individual motoneurones between 0.5 and 6 days (see Tables 4 and 5). Motoneurones were identified by their position on the grid established at 12h or 24h in culture from their fluorescent images (see figs. 2, 15, 24, and 25).

A. Changes in Cell Body Size and Shape

There was no difference in cell body size of motoneurones at 0.5d when cultured over CPDL or fibroblast monolayer, with perimeters of 35 - 57 μm , areas from 78

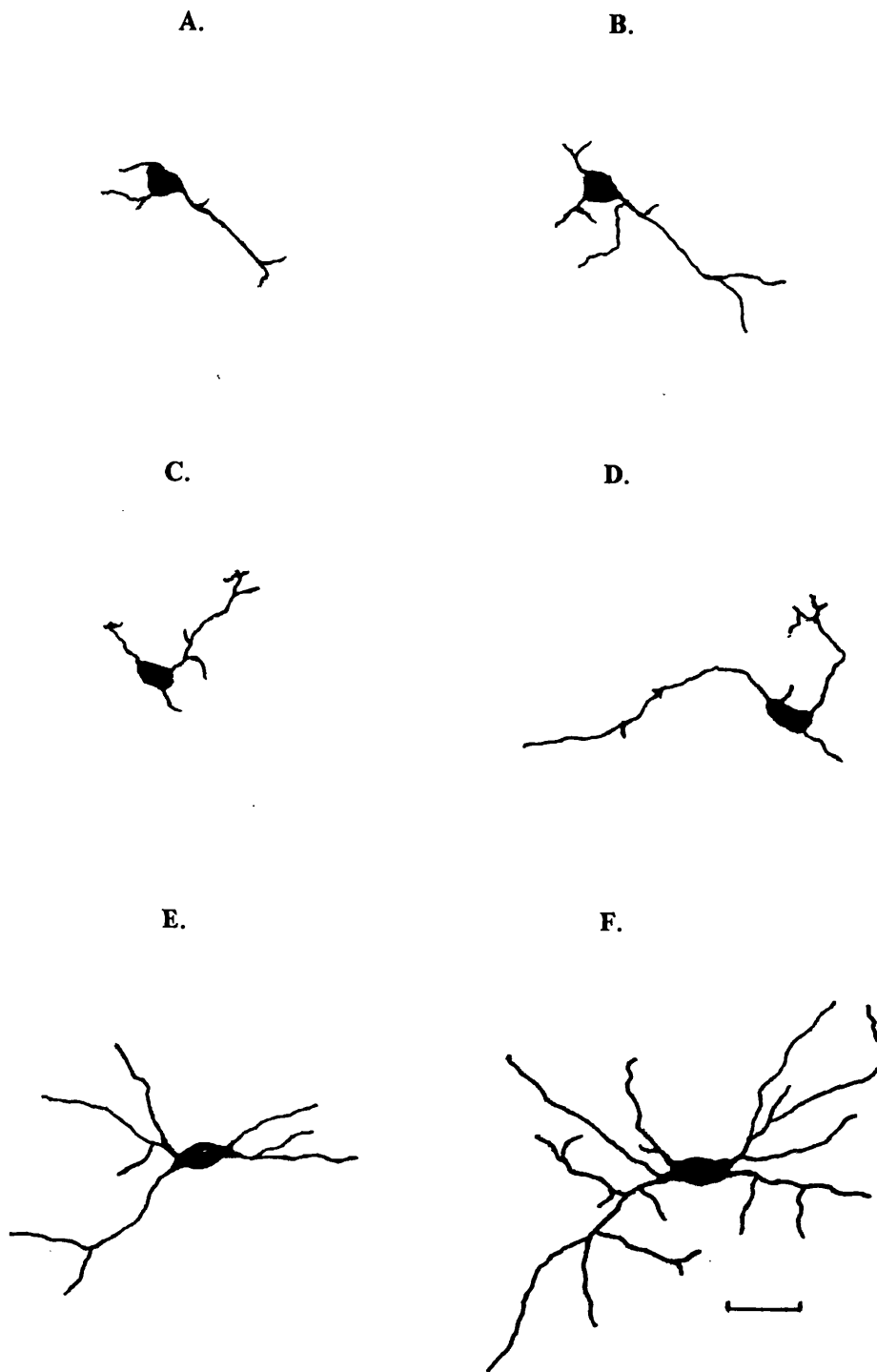


FIGURE 24. Development and growth of individual identified E13-14 motoneurons cultured on CPDL (A-B), over fibroblast (C-D) and glia monolayers (E-F). Computerized-aided reconstruction of digitized images. A. 0.5d, and B. same motoneurone at 1.5d in culture on CPDL. C. 0.5d, and D. 2d on fibroblast cells. E. 1d, and F. same motoneurone at 5d in culture on glia cells. Bar = 30 μm .

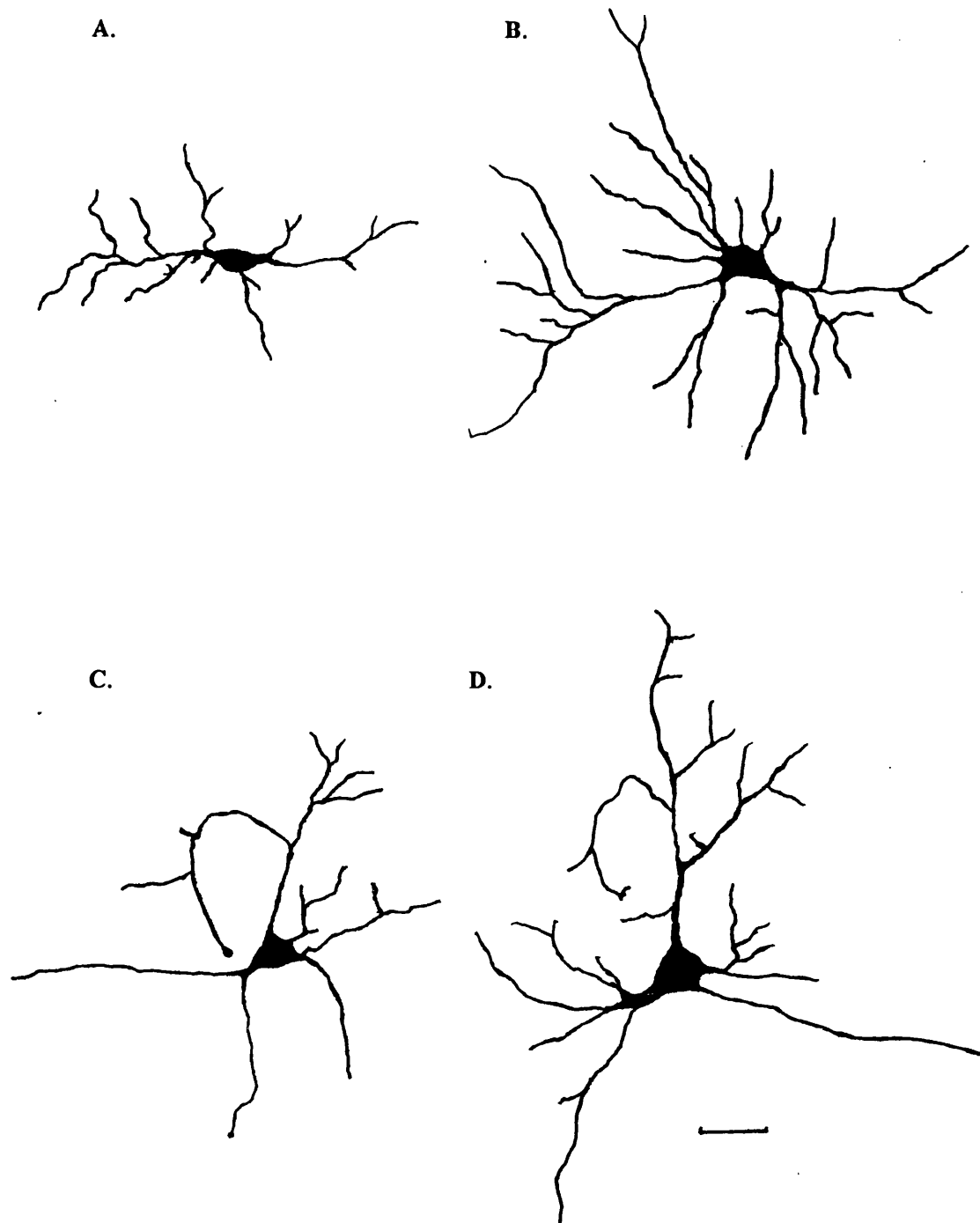


FIGURE 25. Development and growth of individual identified E13-14 motoneurons cultured with myotubes. Computer-aided reconstructed images as for fig. 24. The thickness of neurites are not real, and the end of long neurites are not shown. A. and B. motoneurone 1d and 6d after plating, respectively. C. and D. motoneurone at 2d and 7d in culture, respectively. Bar = 30 μm .

to 180 μm^2 , and compound diameters (cDiam) of 10.5 - 16 μm . But when cultured over glial cells or myotubes motoneurons presented a significantly ($p < 0.001$) larger cell body than over CPDL or fibroblasts at this early stage (Table 4).

Table 4.

Cell Body Size of Motoneurons at 0.5d

(μm , means \pm S.D.)

	Per	Area	Diam1	Diam2	cDiam	Pneur
CPDL	45	116	15.8	10	12.6	3.5
(n=60)	± 5.8	± 23.8	± 2.4	± 1.6	± 1.4	± 0.8
FIBR	44	112	15.3	9.8	12.2	3.7
(n=60)	± 5.2	± 22.9	± 2.1	± 1.7	± 1.3	± 0.9
GLIA	51	148	16.2	11.9	13.9	3.6
(n=45)	± 10.2	± 45.8	± 2.7	± 2.0	± 1.8	± 1.0
MYOT	58	189	17.5	11.8	14.3	3.8*
(n=52)	± 7.7	± 36.6	± 2.0	± 1.6	± 1.4	± 0.9

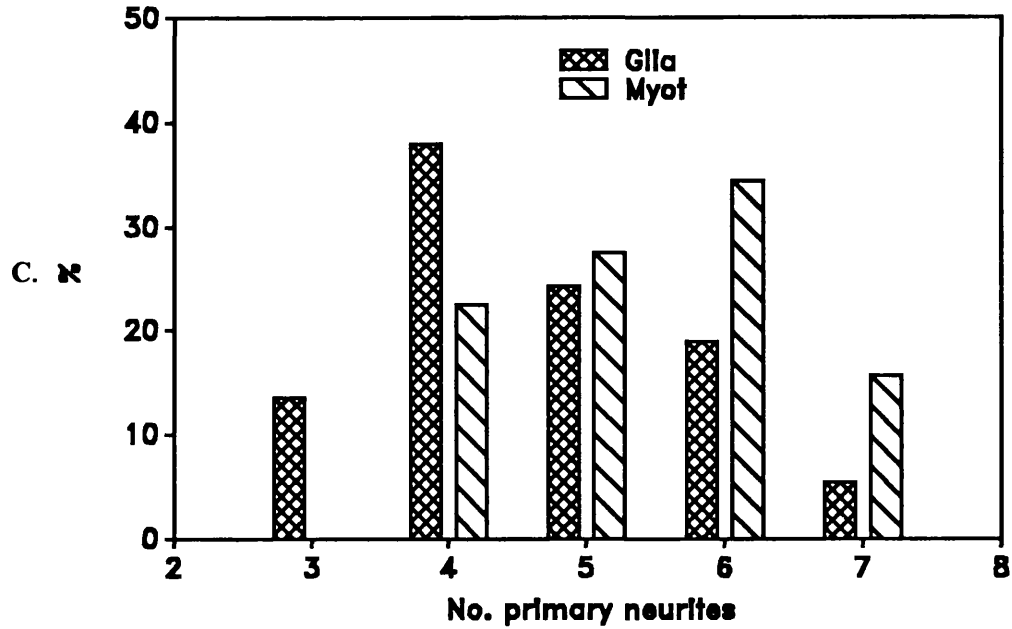
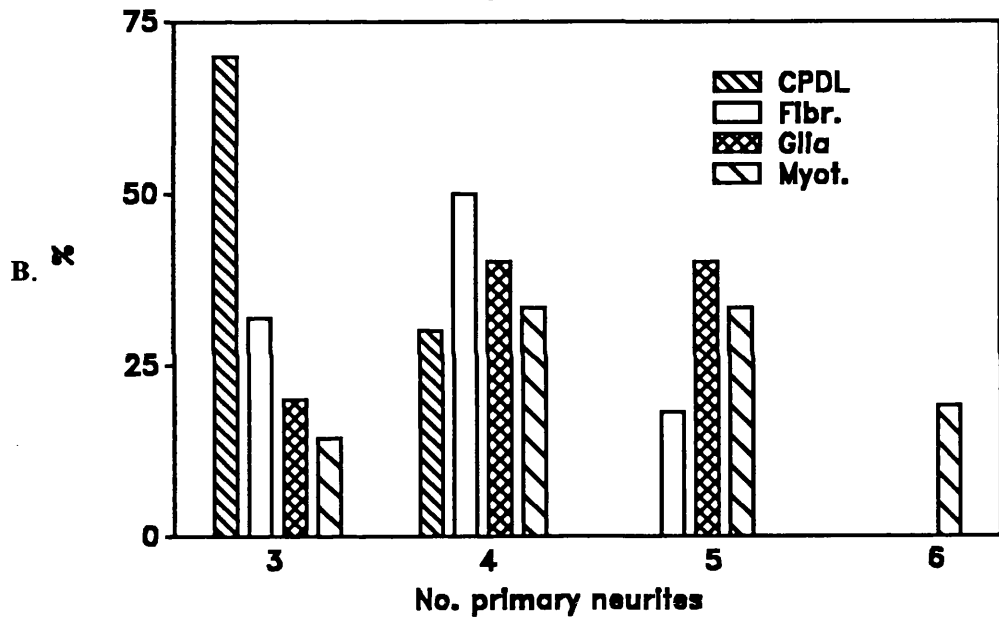
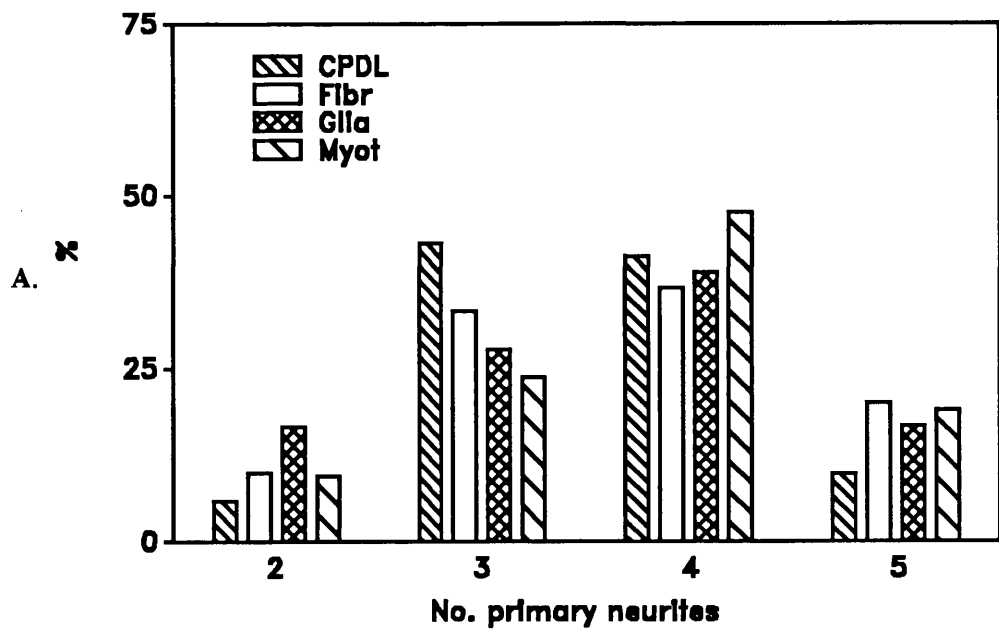
area (μm^2); n = No. motoneurons.

Pneur = primary neurites.

(*) $p < 0.001$ to CPDL.

However, at 0.5d there was no significant difference in cDiam (that correlates well with cell body size) between motoneurons cultured over glia or muscle. Also at this time there were no differences in the number of primary neurites of motoneurons (Table 4) cultured over the different non-neuronal cells, except over muscle motoneurons presented a slight but significantly greater number of

FIGURE 26. Changes in multipolarity of E13-14 motoneurones cultured on CPDL and over non-neuronal cells. Frequency distribution of primary neurites at different time in culture (Fibr = fibroblast; Myot = myotubes). For n see Table 4. A. 0.5d. B. 2d. C. At 6d in culture, over glia monolayers and with myotubes.



neurites when compared with those over CPDL. The distribution of the number of primary neurites at 0.5d given in figure 26 (A), shows that motoneurons with 2 and 5 neurites increased to 10-17% and 18-20% respectively, when they were cultured over any non-neuronal cells, compared to those observed in CPDL. It is possible to observe also a shift to the right in the distribution of primary neurites in motoneurons culture over non-neuronal cells, with more motoneurons with 3 neurites when on CPDL.

At 2d in culture, motoneurons presented a similar growth when cultured over CPDL or fibroblasts, with a small but significant increase in all parameters (9% and 14% in cDiam, 25.7% and 26.9% in area, and 11.6% and 14.8% in perimeter, respectively) as Table 5 shows, and no significant change in primary neurites over fibroblasts, but a reduction of 6% when cultured over CPDL. The greater increase in area was probably more related to a change in shape than in size (see below). Although the cell body size of motoneurons cultured over glial cells or with myotubes at 2d was much greater than that over CPDL and fibroblasts, the relative increase in the first 48h was similar except for area and neurites (10% and 14% in cDiam, 16% and 18% in area, 8% and 15% in perimeter, and 17% and 21% in the number of primary neurites, respectively over glia cells and myotubes). At this stage, by all the parameters analysed, motoneurons grew better with myotubes than over glia cells (Table 5).

No bipolar motoneurons were observed at this time in culture under any of the conditions (fig. 26, B). But when cultured over CPDL or fibroblasts motoneurons with 3-4 primary neurites were predominant at this stage. In contrast, there was an increase in the % of motoneurons with 4 and 5 neurites when cultured over

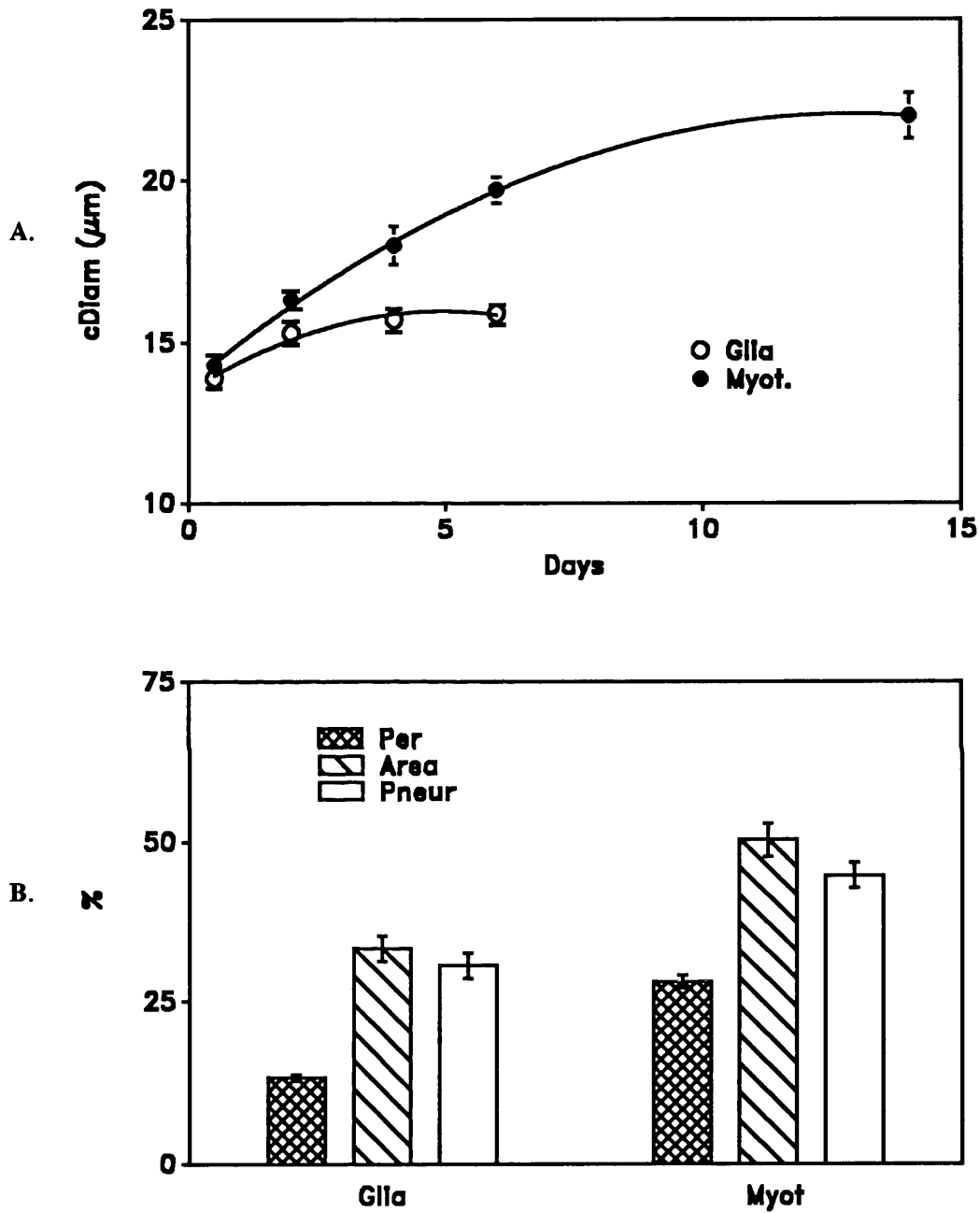


FIGURE 27. Changes in size primary neurites of E13-14 motoneurons in culture over glia monolayers and with myotubes. **A.** Cell body diameter (cDiam) versus days in culture. Means \pm SEM. **B.** Changes (% from 0.5d) in cell body perimeter, area, and primary neurites at 6d in E13-14 motoneurons cultured over glia cells and with myotubes (n=55 and 50 motoneurons, respectively). Means \pm SEM.

glial cells, and with 4-6 neurites when cultured with myotubes. Motoneurons cultured over glia and muscle presented a significant increase ($p < 0.001$) in the number of primary neurites compared to those presented at 0.5d in culture, and also compared with those observed over CPDL (and fibroblasts) at 2d. Motoneurons in co-culture with myotubes also presented a slightly higher number of primary neurites than over glia monolayers at this stage.

Not only were there few motoneurons in the dish after the first two days when plated on CPDL or over fibroblasts, but the remaining ones presented signs of degeneration, neurite retraction or they were not growing. Motoneurons cultured over glia presented only a small growth with 13% and 14.4% increase in cDiam at 4 and 6 days in culture respectively (15.7 and 15.9 μm), whereas over myotubes they presented a larger increase in cDiam, with 25.9% and 37.8% (18 and 19.7 μm , cDiam) at 4d and 6d, respectively, and 53.9% (22 μm) after 14d in culture (see Table 5). Fig. 27 (A) shows the increase in cDiam up to 6d and 14d for motoneurons cultured over glia and myotubes, respectively. It is clear from this figure that cultured over glial cells the cell body diameter of motoneurons stops growing at about 2d, but over myotubes the diameter increases for at least the first two weeks in culture. Fig. 27 (B) shows that there were large increases in area, perimeter, and number of primary neurites in the first week when cultured with myotubes, but these changes were less clear when cultured over glia cells. This increase in cell size was also shown by a clear shift to motoneurons with higher cDiam after 6d over glial cells (fig. 28, A) and myotubes, that was even more evident after 14 days in myotubes co-cultures (fig. 28, B). However, motoneurons with small cell body size were still observed.

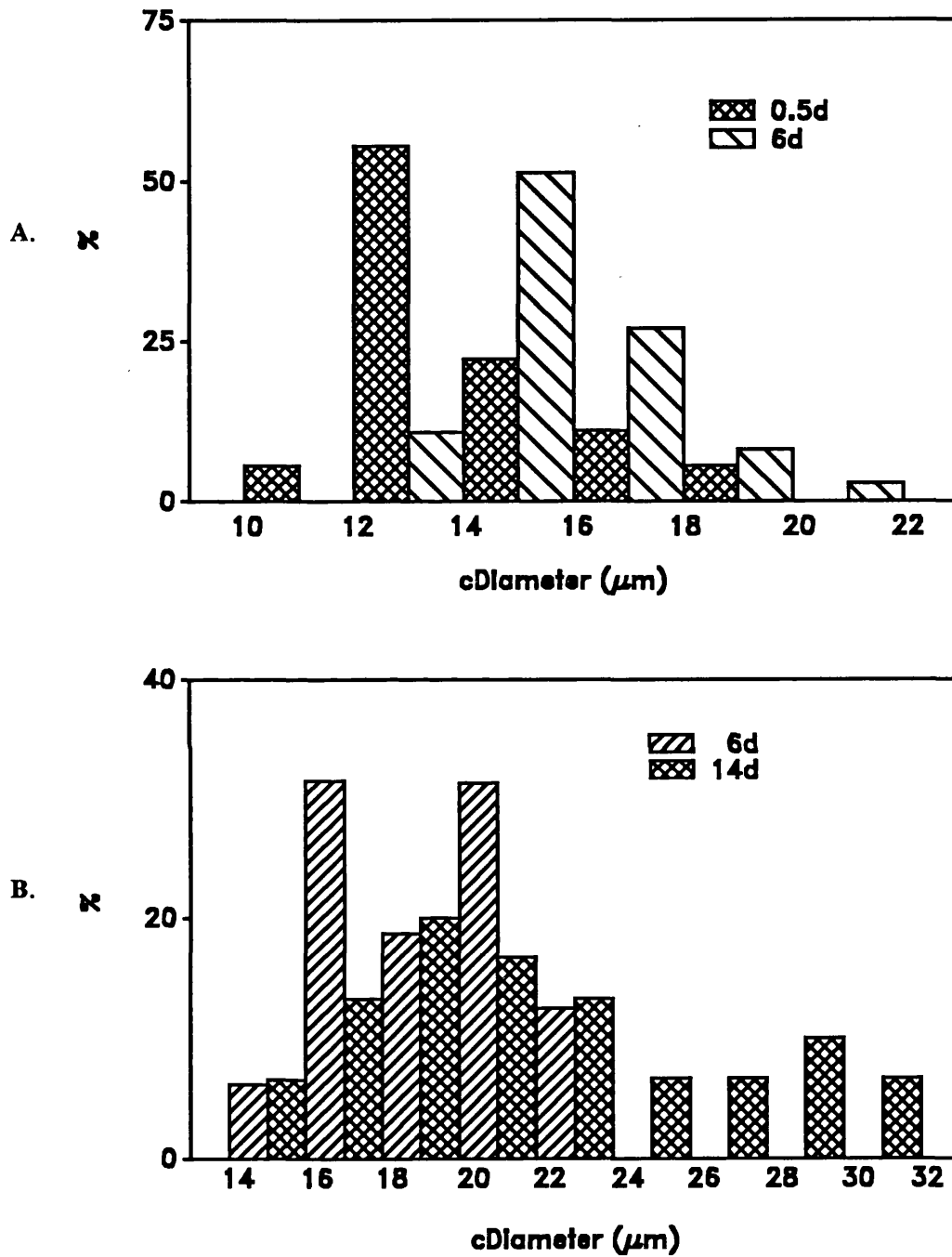


FIGURE 28. Changes in the distribution of E13-14 motoneurone sizes with time in culture (i.c.) **A.** Frequency histogram of cDiam at 0.5d and 6d i.c. of motoneurones cultured over glia monolayers. For n see Table 5. **B.** Frequency histogram of cDiam for motoneurones at 6d and 14d i.c., and cultured with myotubes. For n see Table 5.

Table 5.

Cell Body Size of Motoneurones at 2-14d

(μm , means \pm S.D.)

	Per	Area	Diam1	Diam2	cDiam	Pneur
CPDL						
2d (n=50)	50 ± 6.3	146 ± 33.1	16.1 ± 1.9	11.7 ± 1.7	13.7 ± 1.7	3.3 ± 0.5
FIBR						
2d (n=44)	50 ± 7.1	142 ± 34.5	17 ± 1.9	11.6 ± 1.7	13.9 ± 1.3	3.9 ± 0.7
GLIA						
2d (n=40)	55 ± 7.6	171 ± 34.9	17.8 ± 1.5	13.2 ± 1.3	15.3 ± 1.3	4.2 ± 0.8
4d (n=40)	-	-	18.5 ± 2.6	13.4 ± 1.8	15.7 ± 1.8	4.8 ± 1.3
6d (n=55)	58 ± 8.7	197 ± 56	18.8 ± 2.4	13.5 ± 2.2	15.9 ± 1.9	4.7 ± 1.2
MYOT						
2d (n=45)	66 ± 7.8	223 ± 36.8	19.6 ± 2.0	13.7 ± 1.2	16.3 ± 1.1	4.6 ± 1.0
4d (n=40)	-	-	20.2 ± 6.7	16.0 ± 2.9	18.0 ± 4.0	-
6d (n=50)	74 ± 16	284 ± 93	23.6 ± 3.3	16.6 ± 3.3	19.7 ± 3.1	5.5 ± 1.1
14d (n=50)	-	-	24.7 ± 5.4	19.7 ± 4.4	22.0 ± 4.7	5.4 ± 1.0

(n)= No. motoneurones
area= μm^2

At 0.5d about 70% of motoneurons cultured over glial cells presented cDiam of 12-14 μm ; with only 20% with cDiam $>18 \mu\text{m}$ at 6d in culture. In contrast, after 0.5 days in culture with myotubes 47.6% of motoneurons already had cDiam of 12-14 μm ; at 6 days 62.5% had cDiams of 18-30 μm , with none presenting cDiams below 14 μm ; after 2 weeks in culture 80.1% of motoneurons had cDiams between 18 and 35 μm , and 30% between 24 and 35 μm . Although the size of motoneurons cell body cultured over glia stopped increasing at around 48h in culture, a small but significant increase in the number of primary neurites continued to the 4th day in culture (from 4.2 ± 0.8 at 2d to 4.8 ± 1.3 at 4d; $p < 0.001$) with no further change at 6d (4.7 ± 1.2 neurites). But cultured over myotubes motoneurons presented significant increase in the number of primary neurites between the 2nd and the 6th day (from 4.6 ± 1 at 2d to 5.5 ± 1.1 at 6d, $p < 0.001$; and 5.4 ± 1 at 14th day), an increase greater than that observed with glia cells ($p < 0.001$). This increase in the number of primary neurites correlated well with the general pattern of growth of motoneurons when cultured over myotubes, so that although the final number was established between 2d and 4d the later growth involved the secondary and tertiary branches. Nevertheless when cultured over both glia and myotubes a still further increase in the number of high multipolar motoneurons was observed at 6d, with 80% and 50% of motoneurons with ≥ 5 neurites, over myotubes and glia respectively (fig. 26, C).

Motoneurons were mostly multipolar (≥ 3 neurites), but as described in the previous section bipolar motoneurons were present under all conditions of culture especially on the first day. The general shape of motoneurons was established in the first 2-3d, and although motoneurons continue to increase the number of primary neurites and branches when cultured over glia or myotubes, the basic shape of the cell body was established early in their development in culture.

Motoneurons presented a complex shape, and have their definition depended greatly on the primary neurites. Therefore for the study of shape only simple and basic formulas were applied based on measurements of area, perimeter and diameters, and intended to define motoneurone cell body in terms of its approximation to a circular or rectangular shape.

Although the shape of motoneurons cultured over CPDL tended to be simpler than when plated over non-neuronal cells, because of fewer proximal branches, at 0.5d motoneurone cell bodies presented a similar aspect ratio (d_2/d_1 and Area/Per) than motoneurons cultured over fibroblasts (0.65-0.64 and 2.6-2.5, respectively). In these two conditions motoneurons presented also similar indexes of circularity and rectangularity (0.66-0.67 for circularity, and 0.87-0.88 for rectangularity). These latter indexes were far from unity so they did not describe adequately motoneurone shape at this stage. Nevertheless at this stage and under these conditions the larger motoneurons were closer to a rectangular shape, the smaller ones to a sphere. When motoneurons were cultured over glia or with myotubes, at 0.5d they presented higher aspect ratios, particularly Area/Per and rectangularity indexes when cultured with myotubes (2.8 and 0.90 over glia, and 3.3 and 0.99 with myotubes, respectively). The latter factor correlated with a rectangular shape especially in those of larger size. Motoneurons cultured over CPDL or fibroblasts presented no major differences in either of the shape factors after 2 days in culture except for an increase in the Area/Per (3.2 and 2.9, respectively), a change that was consistently present in all conditions tested as motoneurons became older. Nevertheless, motoneurons cultured over glia or with myotubes showed a greater increase in Area/Per at 2 and 6 days in culture (fig. 29, A), with a rectangularity factor that remained high at 2d (0.90 and 0.99,

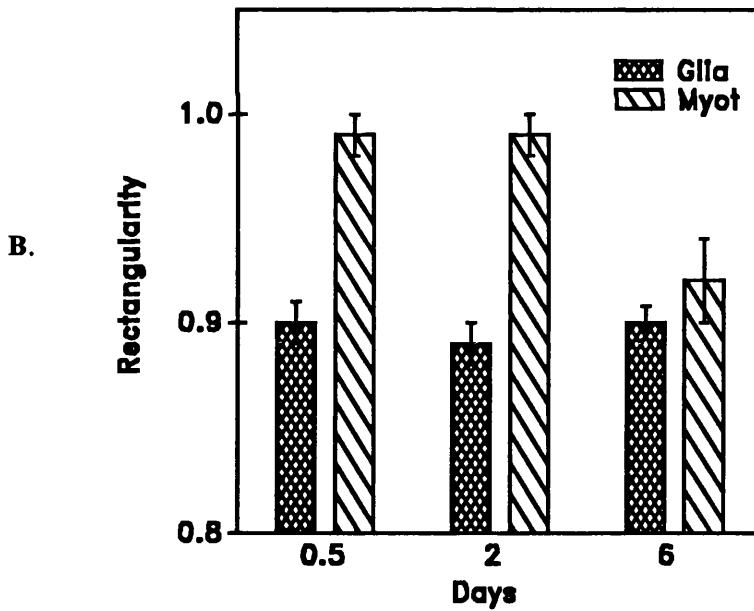
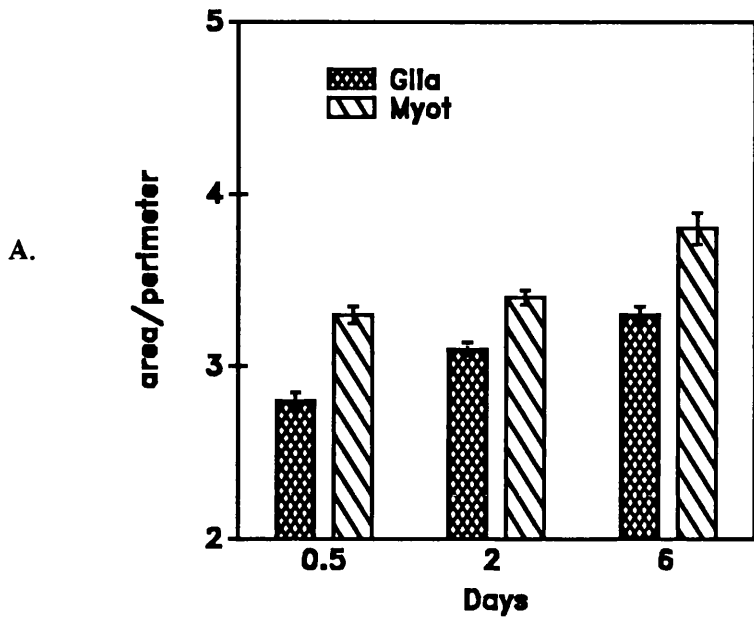


FIGURE 29. Shape of E13-14 motoneurons versus time in culture. **A.** Aspect ratio (area/perimeter) versus days i.c. in motoneurons plated over glia monolayers or with myotubes. Means \pm SEM; data from Table 5. **B.** Rectangularity factor ($Per/[d1+d2] \times 2$) versus days i.c. in motoneurons cultured over glia monolayers or with myotubes. Means \pm SEM; data from Table 5.

for glia and myotubes, respectively), but was smaller in motoneurons with myotubes at 6d in culture (0.92; see fig. 29, B). This evolution of the rectangularity factor correlated with the observed images of motoneurons cultured over myotubes. In the first few days these presented a shape closer to a rectangle, but this was progressively lost as motoneurons acquired more proximal branches, and primary neurites became longer and thicker. Both in motoneurons cultured over glia or myotubes the Area/Per and the rectangularity factor had no correlation with cell body size (cDiam) and number of primary neurites.

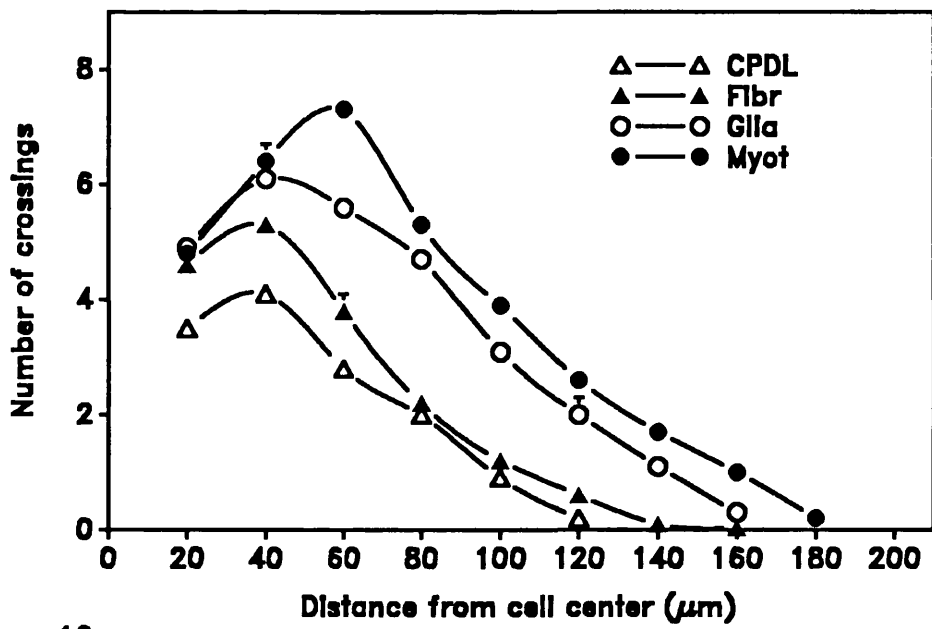
B. Branching and Length of Neurites

In the previous section the morphological analysis showed that motoneurone cell body increased in size (cDiam) steadily for at least 2 weeks when cultured with myotubes and only for about 2 days over glia monolayers although in the latter case, the number of primary neurites continued to increase for up to the 4th day. Therefore question arises as to whether glia cells support the growth of neurites better than cell body size, and also whether branching as opposed to length is affected differentially by non-neuronal cells.

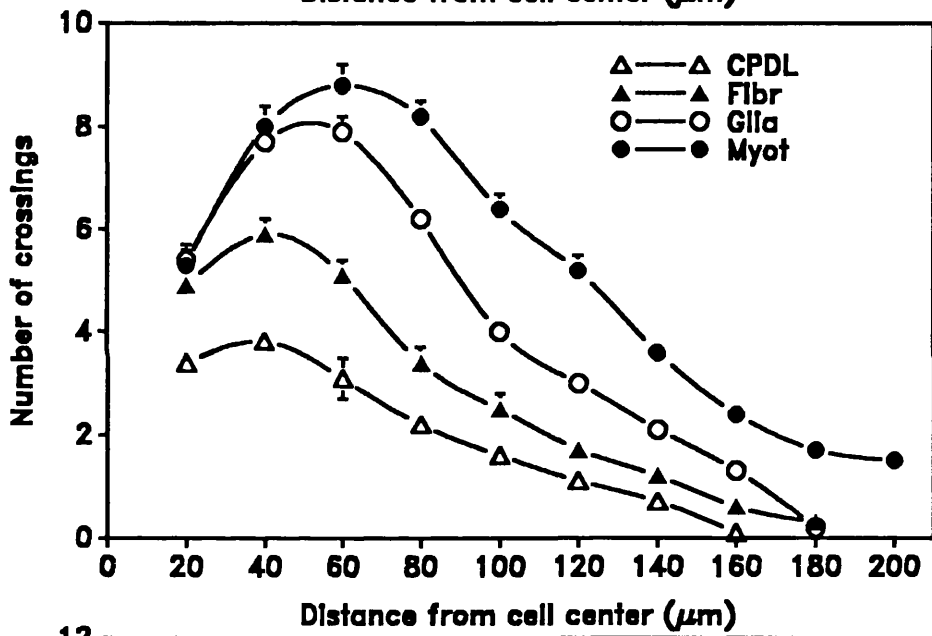
Neurite ramification or branching was studied as the number of intersections (crossings) observed at concentric circles traced over motoneurones figures (drawn from stored digitized images) at 20 μm intervals and up to 200 μm from the center of the cell body (fig. 4, A-B) at 0.5d-6d. The distribution of crossings, the circle or distance with the peak of crossings, and the percentage of cells with neurites at each distance were analysed.

FIGURE 30. Distribution of neurite branching (number of crossings or neurite intersections at each circle; see Methods) at different distances from the cell body and time i.c., in E13-14 motoneurons cultured on CPDL and over non-neuronal cells. Data from Table 5. Means \pm SEM. A. 0.5d. B. 2d. C. 4d-6d.

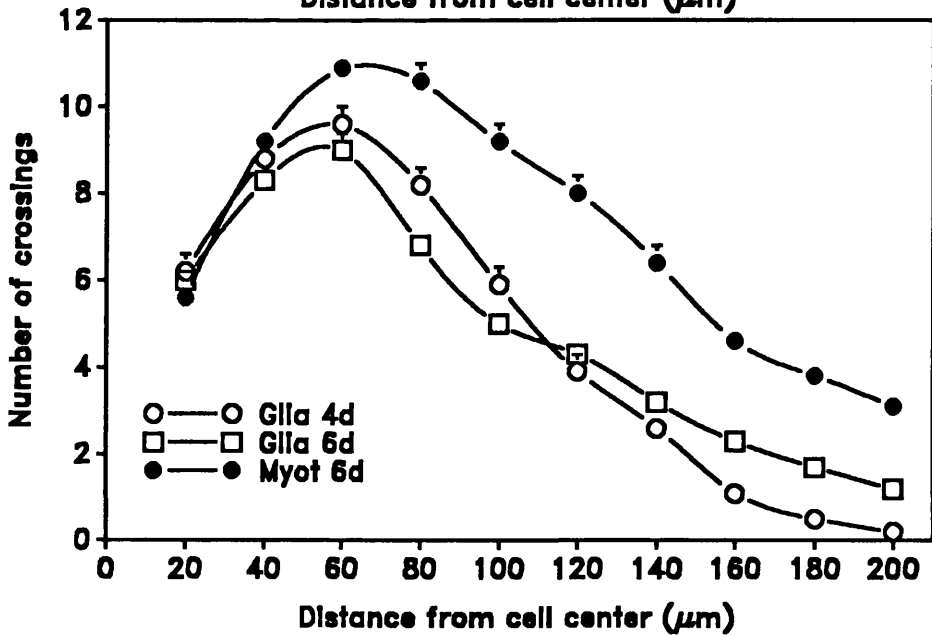
A.



B.



C.



The pattern of distribution of neurite ramification (No. of crossings at each radius or circle) was similar in all conditions as shown in figure 30 (A-C), with a peak in the mean of crossings at 40-60 μm from the center of the cell body, and a progressive and marked reduction in the number of crossings thereafter and up to 200 μm , depending on the conditions in which motoneurons were cultured. This peak was closer to the cell body in CPDL and fibroblast and glia monolayers (40 μm), with a mean of 4.1-5.3 (SD \pm 1.0-1.7) at 0.5d (fig. 30, A) and 3.8-5.9 (\pm 1.5-1.7) at 2d in culture (fig. 30, B). Almost no increase in ramification of motoneurons was observed on CPDL after 2d in culture (see fig. 30 A and B) except for a small increase in the length of neurites (20% of cells with neurites at 120 μm at 0.5d compared with 60% at 2d, and with 10% at 160 μm). Although motoneurons cultured over fibroblast monolayers presented an increase in branching compared to CPDL, neurite growth after two days was minimal. The peak in ramification of motoneurons cultured over glia monolayers at 0.5 days was also at 40 μm , but shifted to 60 μm away from the center of the cell body at 2d, 4d, and 6d in culture (see fig. 30, A-C). Under these conditions motoneurons not only presented a greater number of branches at all distances at 0.5d in culture (with 6.1 ± 1.4 crossings at 40 μm and 28.6% cells with neurites at 160 μm), but also the growth in length and branching of neurites was clearly greater than in CPDL at 2d (7.9 ± 1.8 crossings at 60 μm), but only in branching when compared with fibroblast at this time in culture. After 4d motoneurons cultured over glia monolayers still presented an increase in branching (9.6 ± 2.8 crossings at 60 μm) with some motoneurons with neurites up to 200 μm (16.7%). At 6d in culture over glia monolayers, motoneurons did not grow significantly and even presented a small reduction in the peak number of crossings (see fig. 30, C). When motoneurons were co-cultured with myotubes (see fig. 30, A-C) the mean peak in branching was at 60 μm at any time in culture. Under these condition

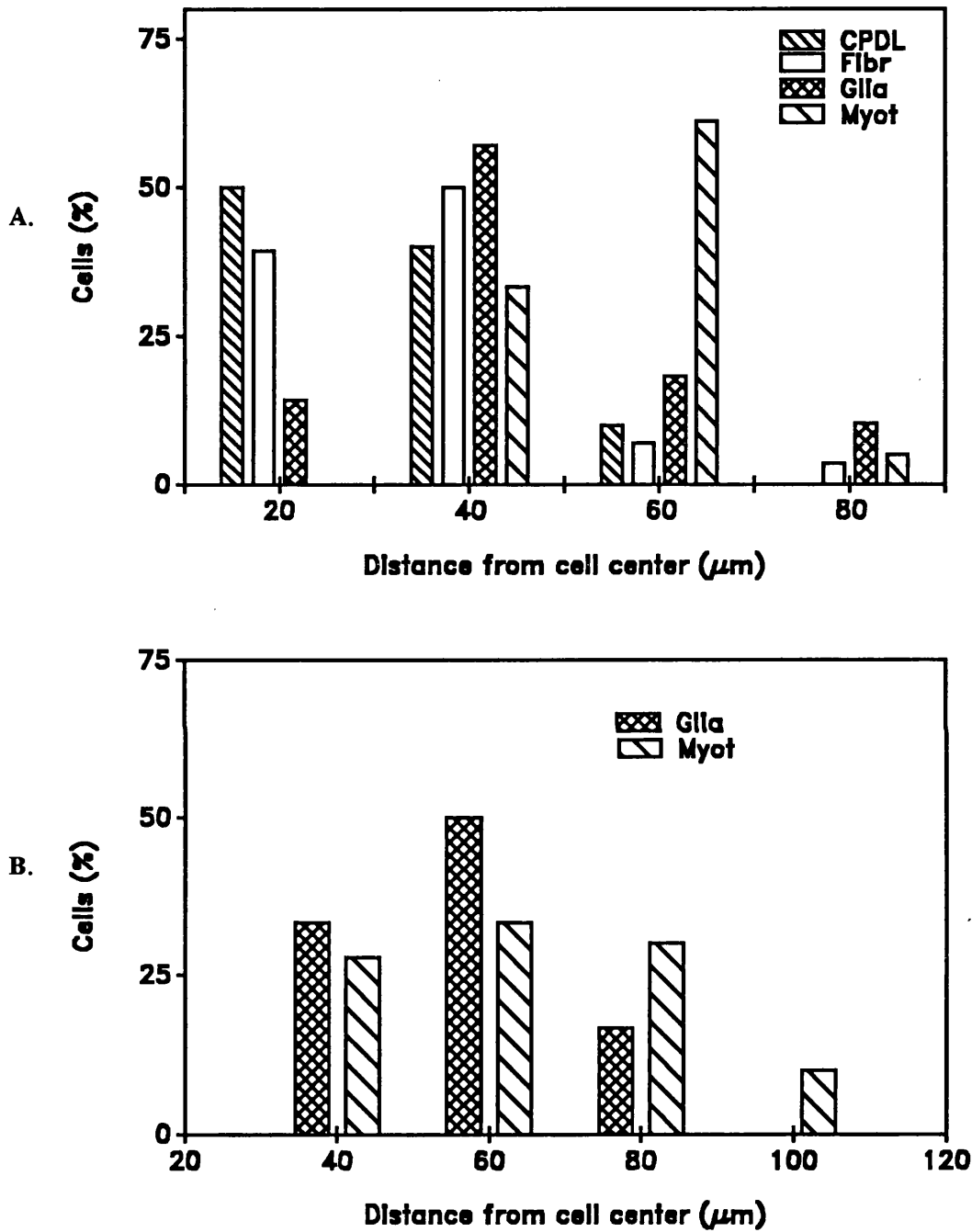
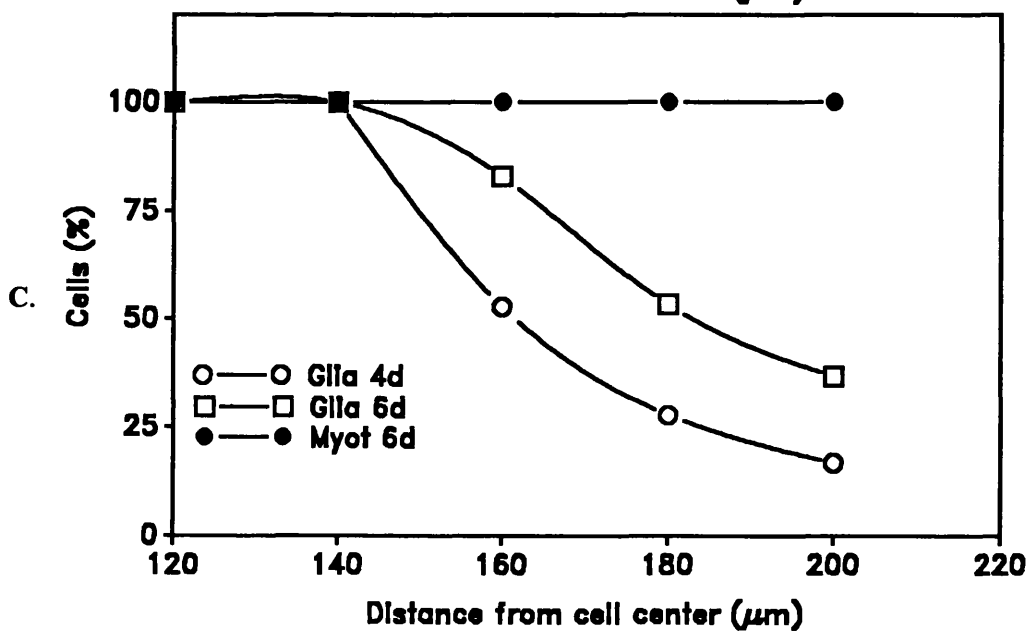
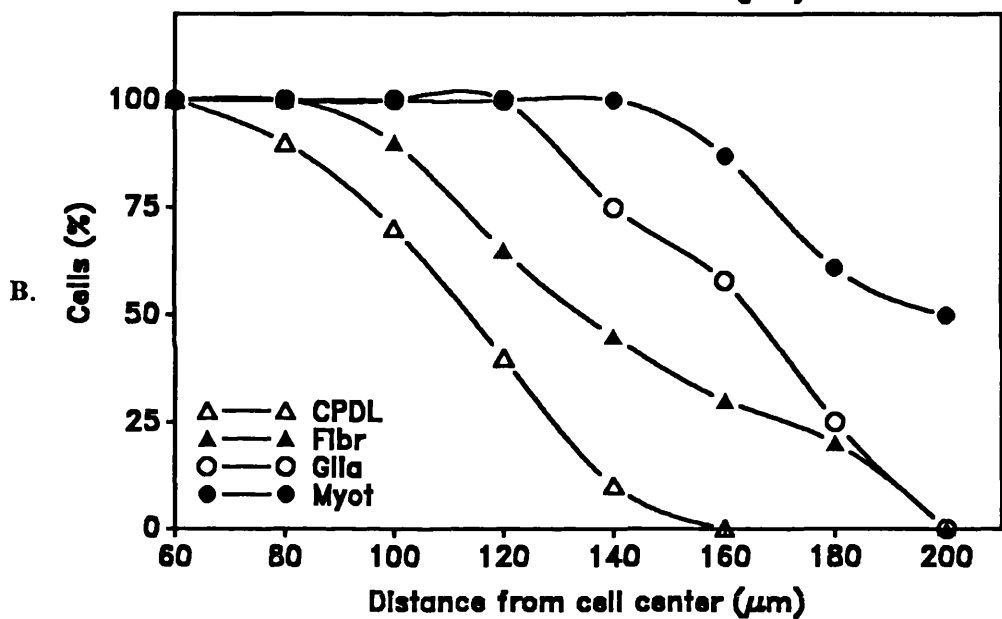
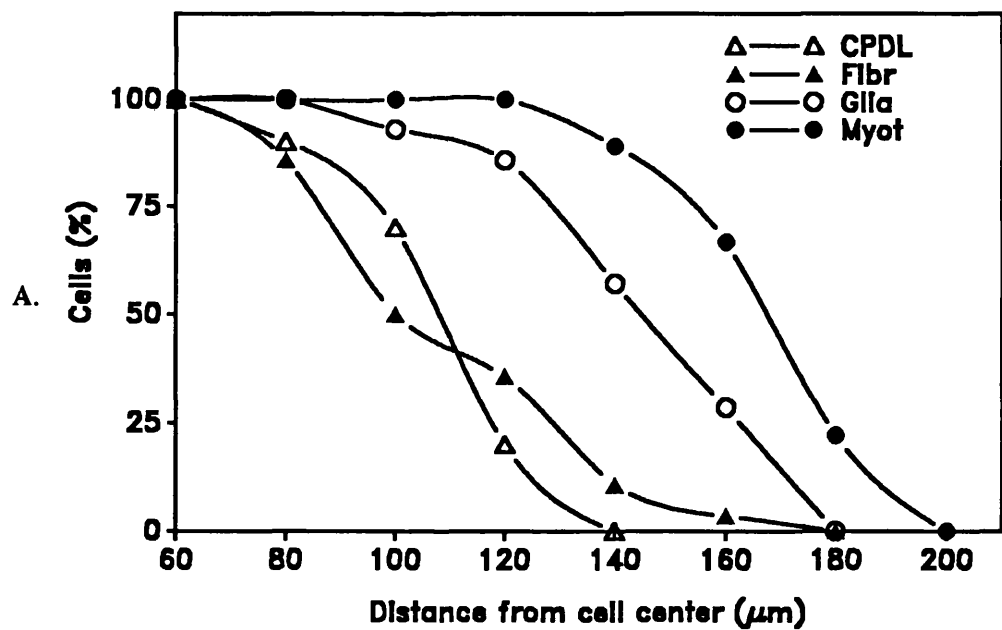


FIGURE 31. Distribution (%) of peak branching (concentric circle with the greatest No. crossings) versus distance from cell body, and at different time i.c., in E13-14 motoneurones cultured on CPDL and over non-neuronal cells (Fibr = fibroblasts; Myot = myotubes). A. 0.5d. B. 6d.

motoneurons presented the largest number of crossings at all distances from the cell body with a peak of 7.3 (± 1.3) and 10.9 (± 2.2) crossings at 0.5d and 6d, respectively, and greater number of cells with longer neurites (22% at 180 μm at 0.5d, and 100% at 200 μm at 6d; see fig. 32, A-C). It is clear from previous figures that fibroblasts provided only a slightly better substratum for branching than CPDL, the greater branching occurring only in the region of 40-60 μm from the cell body center. Glial monolayers not only produced more branching in this same region (peak branching), but also further from the motoneurone cell body, with maximum growth at 4 days. They also produced longer neurites. Myotubes gave the best conditions for the growth of motoneurons in terms of branching, with even greater ramification at the peak region (40-60 μm) only a few hours after plating and over the next few days a significant increase at this region, and at greater distances from the cell body, with more cells having longer neurites. Under these conditions motoneurons continue to grow for at least the first 6 days, but with only a small growth on glia cells. Figure 31 (A-B) shows the % cells with peak branching at different distances at 0.5d and 6d, respectively. It is clear from these figures that when cultured over glia and myotubes a greatest number of motoneurons presented peak branching at greater distances from the cell body and that the % of motoneurons with higher branching at further distances also increased as the cell grew (see fig. 31, B). Motoneurons cultured over fibroblasts or CPDL presented no change in the peak branching nor in the distribution of this peak. That an increase in neurite length was a clear effect when motoneurons were cultured with glia or myotubes is shown in figure 32 (A-C), in which are plotted the percentage of cells with the longest neurites. This increase in length was more evident after 80 μm from the cell body, with 100% of cells with at least one neurite longer than 200 μm at 6d in culture with myotubes, but less than 50% when motoneurons were cultured over glia

FIGURE 32. Number (as %) of E13-14 motoneurons with longest neurites at different distances from the cell body, when cultured on CPDL and over non-neuronal cells. A. 0.5d i.c. B. 2d i.c. C. 4d-6d i.c.



monolayers. These figures also show that although at 6d motoneurone grown over glia presented a reduction in branching there were more motoneurones with long neurites (fig. 32, C). A similar phenomenon was observed for motoneurones cultured over fibroblasts at 2d with an increase in length and in the number of cells with long neurites (fig. 32, B) in spite of an almost no increase in branching.

These results show that branching density falls greatly with distance from the cell body. After 100 μm the growth was mainly by length rather than branching except when motoneurones were cultured with myotubes. In figure 33 (A) the density of branches (total crossings/total circular area covered by neurites) was plotted, showing that motoneurones presented no increase in branching when cultured over CPDL, fibroblasts or glial cells (with even a reduction on CPDL). But cultured with myotubes, motoneurones increased the branching in 24% and 39% at 2d and 6d in culture, respectively. Figure 33 (B) shows the effect of considering the number of primary neurites on branching-density. It shows more clearly that only when cultured over myotubes did motoneurones present an increase in branching density per neurite. Under all other conditions there was no real growth with even a reduction in CPDL at 48h and over glia after 2d in culture (although this latter reduction is mainly given to the greater area covered by neurites over glia monolayers). If now consideration is given to the changes in branching density/neurite restricted to 100 μm from the cell body, then motoneurones cultured over fibroblasts increased their branching-density in this area at 48h (fig. 34, A), and also when cultured over glia cells up to the 4th day in culture. Nevertheless, when motoneurones were cultured with myotubes the branching-density over this area increased with a similar level at 6d with that observed in motoneurones on glia at 4d (fig. 34, B). These figures demonstrate that motoneurones cultured over fibroblasts or glia cells presented mainly a growth in

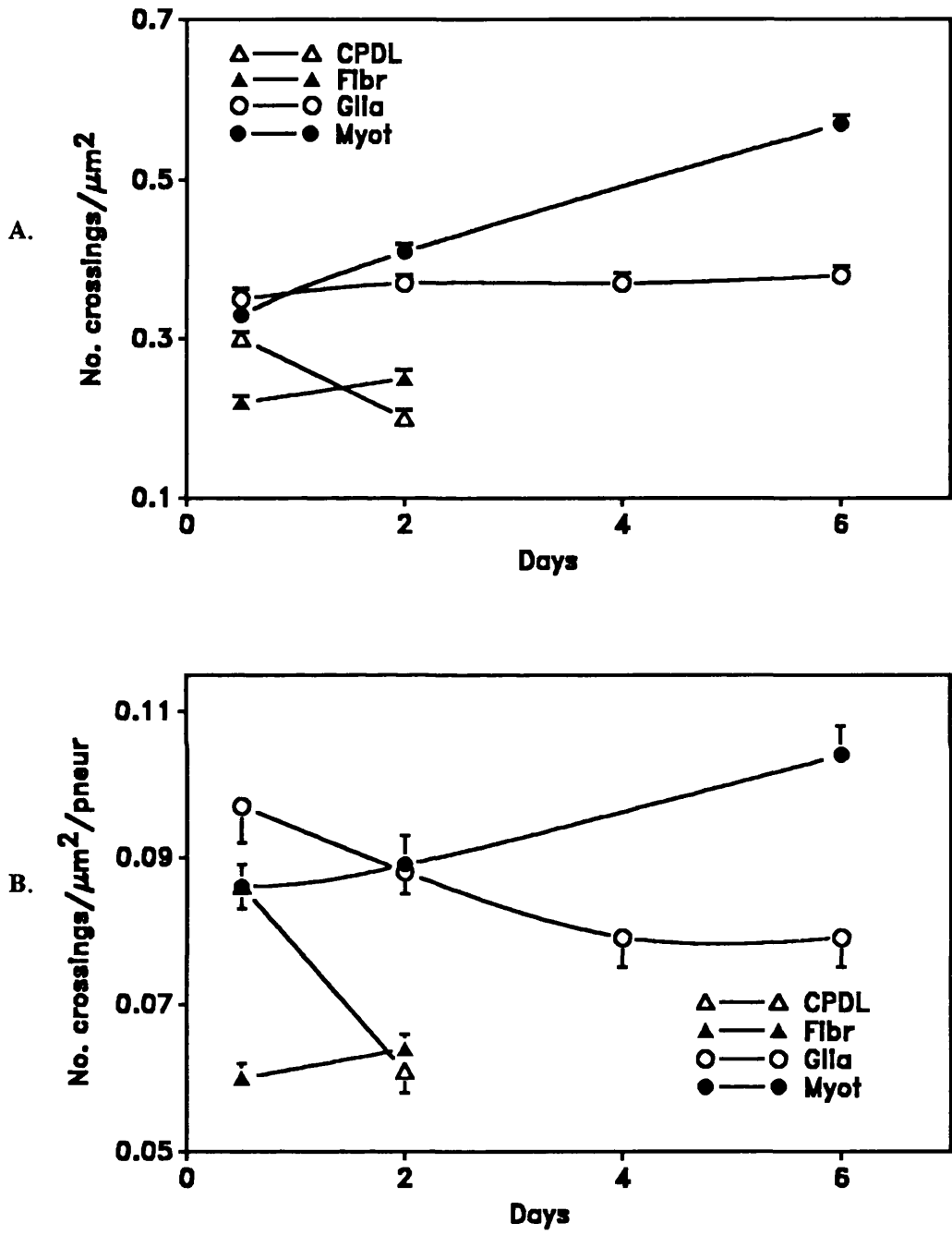


FIGURE 33. Branching density (No. of crossings per total neurite area in each cell) versus time in culture. E13-14 motoneurons cultured over CPDL, fibroblast and glia monolayers, and with myotubes. **A.** Branching density per total neurite area. Means \pm SEM. **B.** Branching density per primary neurites versus time in culture. Means \pm SEM.

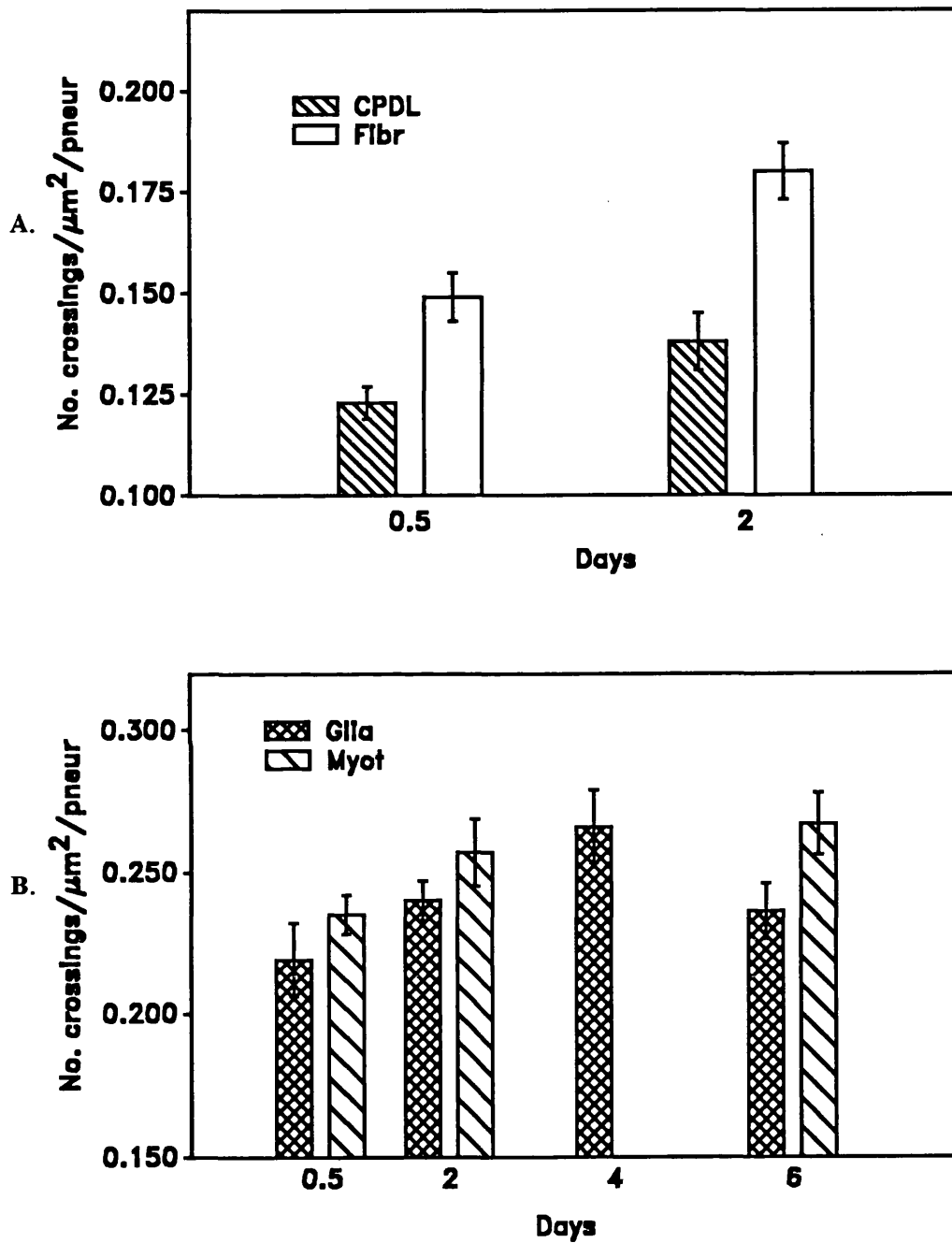


FIGURE 34. Branching density per primary neurites up to 100 μm from the cell body versus time in culture. E13-14 motoneurons cultured, **A.** Over CPDL and fibroblast monolayers. Means \pm SEM.; and **B.** Over glia and with myotubes. Means \pm SEM.

proximal branches. Also shows that after 2d in culture, in motoneurons grown with myotubes the increase in branching occurred mainly at distances greater than 100 μm from the cell body even though the area covered by neurites was larger.

The distribution of branches within each circle (around 360°) were not analysed quantitatively, but most branches were concentrated in two quadrants in each circle, frequently opposite to each other, from the first hours in culture, but could change to other quadrants at a later time. This suggests that some polarity was established in motoneurons early in culture.

Total neurite length was measured from digitized images of motoneurons with the help of an ~~image~~ image analysis system (see Methods). Although there was a good positive correlation between the length of the longest neurite and total neurite length, for motoneurons the total neurite length was preferred because of the multipolarity and branching of cells. Figure 35 (A) shows that there was a significant growth of total neurite length at 2d in culture in all conditions, but greater in myotubes co-cultures (22%, 46%, 34%, and 59%, for CPDL, fibroblasts, glia cells, and myotubes, respectively). The differences in growth between fibroblast and glia cells at 2d were not significant, nor was the increase observed in glia cells between 4d and 6d in culture. This figure shows that neurites from motoneurons cultured over glia cells stop growing in length at around the 4th day in culture, but continue to do so for at least 6d when motoneurons were cultured with myotubes. This pattern of growth correlated well with the growth in branching. Figure 35 (B) shows the absolute growth rate for neurite length of motoneurons cultured over glia and myotubes up to 6d in culture. In both conditions there was a similar declining growth rate curve, but with a significant increase ($p < 0.001$) when motoneurons were cultured over myotubes.

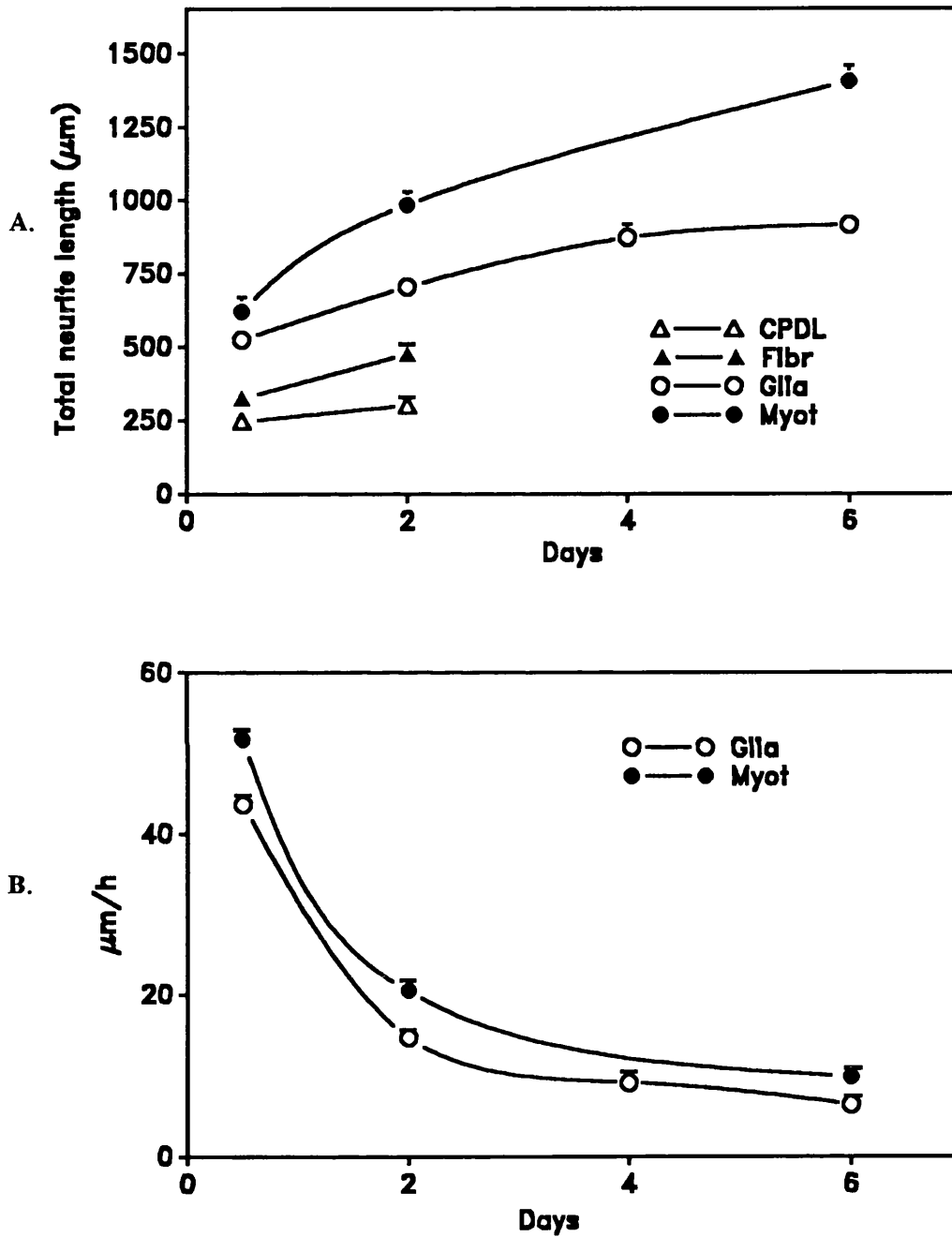


FIGURE 35. Neurite growth versus time in culture. E13-14 motoneurons (No. motoneurons as in Table 5 for different groups). A. Total neurite length versus days in culture in motoneurons plated on CPDL, over fibroblast and glia monolayers, and with myotubes. Means \pm SEM. B. Absolute neurite growth (total neurite length/time) versus time in culture. Motoneurons cultured over glia monolayers and with myotubes. Means \pm SEM.

C. Neurite Growth of Cerebellar Neurons Cultured over Fibroblasts Transfected with the cDNA for NCAM Isoforms

The study of specific molecules on neurite growth has mainly relied on the use of competitive assays or blocking effects of monoclonal antibodies against the specific molecule of interest. While these studies have given invaluable information of possible relationships between specific components of the cell membrane or substratum with neurite growth (Neugebauer, Tomaselli, Lillien, & Reichardt, 1988) they suffer from the uncertainties of the specificities of the antibodies used, the expression of the antigenic molecules, etc. For those reasons the possibility to transfect genes (cDNA) on recipient cells that would express the molecule of interest makes an useful model for the study of membrane molecules that may participate in the growth of neurons. As an attempt to apply this model to study the role of muscle and other isoforms of N-CAM on the growth of motoneurons, as a first study, using a simple model, early postnatal cerebellar neurons were cultured over fibroblasts (3T3) transfected with the gene of the different isoforms of N-CAM. After 24h the cultures were fixed, labelled with specific anti-neuronal antibodies and anti-IgG-TRITC antibodies, and fluorescent images analysed by the same method used for the study of motoneurone development. This study showed that neurite length and branching were directly related to the amount of NCAM expressed in the membranes of the transfected fibroblasts, and that after a threshold there was a steeply increase both in neurite length and branching. This study also showed that the technique used to quantitate branching and neurite length is useful and appropriate when applied for studies on the effect of specific molecules on neurite growth. This work on cerebellar cells was communicated and published recently (Doherty, Fruns, Seaton, Dickson, Barton, Sears & Walsh,

1990). In future the same model will be applied to study the effect of these neural cell adhesion molecules on motoneurone growth.

D. Summary of Section III

In this section changes in cell body size, shape, and length and branching of neurites of well identified embryonic motoneurons in culture were described. For these purposes ventral cord cells were cultured over CPDL, fibroblasts and glial cell monolayers, or with myotubes. Motoneurons were then identified, initially by their fluorescence, and subsequently by their position on a grid created on the culture dish. Neurite branching was studied as the number of crossings observed at concentric circles traced over the stored and digitized images of motoneurons cultured on CPDL and over non-neuronal cells from 0.5d to 6d in culture.

Different sizes of motoneurons were observed depending on the conditions in which they were cultured, with diameters of 12.6 to 14.3 μm in the first few hours in culture. In general, although the distribution was initially similar, more larger motoneurons were observed when cultured with myotubes and glia than in other conditions. Small motoneurons ($\leq 12 \mu\text{m}$ cDiam) tended to present a round cell body, and larger motoneurons ($\geq 16 \mu\text{m}$) a rectangular one. As motoneurons became older, shape depended not only on size but also on the thickening and growth of primary neurites, and the outgrowing of proximal neurites. After few days motoneurons cultured with myotubes lost their rectangularity. In any condition tested, irrespective of size, older motoneurons presented an increase in the aspect ratio (A/P). Initially motoneurons presented similar number and distribution of primary neurites (3.5-3.8 primary neurites/ motoneurone) in any of

the conditions tested. In general a positive correlation was observed between cell body size and number of primary neurites. After few days a small growth in size, but not in the number of primary neurites, was observed when motoneurons were cultured in CPDL or over fibroblasts. In contrast, when cultured with myotubes, motoneurons presented a greater growth, especially of the second diameter, and the size increased continuously for two weeks in culture (with mean diameters of $24.7 \times 19.7 \mu\text{m}$). Nevertheless, the number of primary neurites increased and then stabilized in the first few days in culture. Motoneurons cultured over glia cells presented a small increase in size for 2d, and in number of primary neurites up to 4d in culture (mean diam. of $18.5 \times 13.4 \mu\text{m}$ at 4d). Multipolarity after a week in culture was higher in motoneurons cultured with myotubes (5.5 pneur/Mn) than over glia cells (4.7 pneur/Mn), and the distribution of cell size was skewed to the larger cells in the former condition.

Branching had a similar general pattern in all conditions with a peak at 40-60 μm , with a progressive reduction from thereafter at farther distances from the cell body. The peak in branching was at greater distance from the cell body in motoneurons cultured with myotubes than in the other conditions in the first few hours in culture. At this time, motoneurons cultured over glia cells and myotubes had longer neurites. No increase in ramification, but a small increase in neurite length, was observed in motoneurons on CPDL and fibroblasts, after two days in culture. Motoneurons cultured on glia cells increased their number of proximal branches and length of neurites after two days in culture, but with only a small increase at 4d and none after 6d in culture. When cultured with myotubes, motoneurons presented the largest number of branches at all distances from the cell body, as well as longer neurites, with a continuous growth in both parameters. As motoneurons became older, when cultured over glia and with myotubes, the

peak branching point occurred progressively further from the cell body. Total branching density only increased in motoneurons cultured with myotubes, and remained unchanged in those on glia cells and fibroblasts, with a reduction on CPDL. Total branching density/pneur again only increased in motoneurons cultured with myotubes, and even decreased when cultured over glia cells. However, branching density/pneur restricted to the proximal 100 μm presented a small increase in motoneurons on CPDL and fibroblast for 2 days, but a similar greater increase on glia cells and myotubes and up to 4 days in culture. A mean total neurite length of about 250 μm was observed in motoneurons on CPDL, and less than 500 μm over fibroblasts, at 2 days in culture. When cultured over glia cells motoneurons presented a total neurite length of about 750 μm at 4d, but increased continuously in motoneurons cultured with myotubes to about 1500 μm at 6 days in culture.

DISCUSSION

A. Introduction

The development of neurones depends on intrinsic as well as extrinsic or inductive factors. Several lines of evidence indicate that cell-cell interactions play an important role in the development, migration, differentiation and survival of the different type of neurones (Purves & Lichtman, 1985). Spinal motoneurones constitute a particular case of central neurones, in that their axons exit from the spinal cord to connect with specific target-muscles and thus directly interact with peripheral cells (neuronal and nonneuronal) as well as other cells within the CNS. This special feature of spinal motoneurones makes them ideal for the study of cell-cell interaction and environmental factors on development. It is now well established that target-muscles control the number of motoneurones (see Lanser & Fallon, 1987), but they may also regulate their phenotypic differentiation (McManaman, Oppenheim, Prevetie & Marchetti, 1990). Whether other cells (neurones and/or nonneuronal cells) participate in motoneurone differentiation and development is not yet known, nor is anything known about the specific molecular mechanisms regulating these phenomena. The present study was designed to address whether other cells regulate the development of motoneurones. The generation of spinal motoneurones from 2 to 3 days before the establishment of neuromuscular connections (Altman & Bayer, 1984; Dennis, Ziskind-Conhaim & Harris, 1981), their close relationship with radial glial cells during the period of

generation, migration and initial differentiation (Wentworth & Hinds, 1978; Henrikson & Vaughn, 1974), and the appearance of glial cells after motoneurons are generated and during the period of cell death (Jacobson, 1978) suggest that glial cells, as well as muscles, may play an important role in the initial development of spinal motoneurons. Several studies, using in vivo and in vitro models, have shown the importance of muscle on the survival of motoneurons (McManaman et al., 1990). But there are no studies on the factors that influence the development and growth of identified motoneurons. Previously such studies have been hampered by the low survival of motoneurons in vitro and the absence of specific markers that would permit their isolation and identification. In the present study these problems were circumvented by the fluorescence labelling of motoneurons to enable a study of the development of embryonic rat motoneurons in culture. The effect of non-neuronal cells on motoneurone survival and specifically on motoneurone growth were tested and compared with the effect of myotubes on those same processes. Heterogeneous cultures with well identified embryonic spinal lumbar rat motoneurons were established. The identification of motoneurons rested upon the retrograde transport of a fluorochrome from the hindlimb bud muscles to motoneurons of the spinal cord. This method, pioneered by Okun (1981) and extended by Smith et al. (1986) and O'Brien and Fischbach (1986), was originally established for the chick embryo and it was necessary, therefore, to modify it for application to the rat (Fruns, Krieger & Sears, 1987). Such labelling is based on the specific feature of spinal motoneurons that, at an early stage of development, these are the ones whose axons innervate muscles (Altman & Bayer, 1984). The definition of motoneurone in this study as labelled neurones was therefore anatomical and established in situ previous to the dissociation of the tissue. In the absence of a specific marker for motoneurons,

this type of identification is critical. Even though it is possible to identify motoneurons by the presence of ChAT (Smith et al., 1986), they comprise only a small fraction of the cholinergic cells in the spinal cord (Schnaar & Schaffner, 1981), and the phenotype of spinal interneurons (commisural and association neurons) has yet to be established. Changes in phenotype with culture conditions have been shown in neurons from sympathetic ganglia (Patterson, 1978), and it remains to be shown that spinal motoneuron phenotype is stable in dissociated cultures. Therefore until these issues are solved (specific marker, phenotype stability), an independent label is the ideal way to identify motoneurons either in heterogeneous or in pure populations in culture. Okun (1981) and subsequently others (Calof & Reichardt, 1984; O'Brien & Fischbach, 1986) have also used the retrograde labelling technique in conjunction with fluorescence activated cell sorting (FACS) to prepare almost pure populations of motoneurons. Although cell separation using FACS techniques is extremely specific, motoneurons present a reduction in viability and survival when compared with non-sorted preparations (O'Brien & Fischbach, 1986; Martinou, Le Van Thai, Cassar, Roubinet & Weber, 1989a), and because of the need of a high selective window to avoid contaminants (St. John, Kell, Mazetta, Lange & Barker, 1986; Schaffner, St. John & Barker, 1987), cultures prepared by this technique may represent a selected population of spinal motoneurons. Consequently, for the present study on the growth and development of motoneurons, heterogeneous cultures were preferred. The use of the ventral segments of the spinal cord and the careful dissection of the DRG, permitted an increase in the percentage of labelling after dissociation and eliminated the possible contamination with other labelled neurons.

B. Retrograde Labelling of Spinal Motoneurones

The identity of the labelled cells after the retrograde injection of RITC in the embryo hindlimb buds was clearly established. RITC labelled large neurones in the spinal cord and all of them were positioned in the lateral and ventral half of the neural tube, that at E13-14, is occupied by the recently generated motoneurones (Nornes & Das, 1974). This distribution was the same when retrograde HRP was used as the tracer, confirming the position of motoneurones. In older embryos, labelled motoneurones occupied the lateral and ventral parts of the ventral horn as in the adult spinal cord. Although some DRG neurones were also labelled, particularly in older embryos, no instances of labelling in the dorsal horn or in medial parts of the neural tube were observed. DRG were removed before the dissociation of the ventral cord. These results provide evidence that labelling of ventral motoneurones is specific and not simply the result of diffusion of the fluorochrome. The conclusion is supported by the fact that when a smaller volume of RITC was injected at one place of the E14 limb bud, only few neurones in a localised group were labelled in the spinal cord. A mean estimate of about 60% motoneurones (50-80%) at E13-14 and E18-20 from each hemi-lumbar cord (assuming 6000 and 3500 lumbar motoneurones per hemi-cord at E13-14 and E18-20, respectively; see Oppenheim, 1986; Lance-Jones, 1982) were labelled in the present study. This may be a low estimate considering the fading of fluorescence and the loss of cells during the dissociation and plating. But this percentage of labelling means that most motoneurones were labelled and that then no selective element was introduced apart from that given by the existence of axons in the vicinity or innervating the muscle masses of the limb bud at that time. O'Brien and Fischbach (1986) gave an estimate of labelling with WGA-LY between 66%

and 95%, similar to our own. It is possible then to assume that, at this stage of development (E13-E14), most axons have already penetrated into the primordial limb muscle masses or that their terminal axons are close to them. This is consistent with previous studies showing that motoneurons produce axons outside the spinal cord soon after they settle in the ventro-lateral part of the neural tube (Cajal, 1929; Altman & Bayer, 1984; Wentworth, 1984a).

In culture the labelled cells presented a neuronal morphology and electrophysiology (see below), and most of the neurones presented immunoreactivity to neurofilament protein. Only a few labelled flat non-neuronal cells (< 3%) were observed, and this was further evidence against diffusion of the fluorochrome in the spinal cord or its incorporation in other cells during dissociation. In summary, the technique used in this study provided specific labelling of most motoneurons of the lumbar embryonic spinal cord.

Other fluorochromes were tested, but RITC (either as crystals or in suspension) gave the most consistent results, and similar to those reported by Dorhmann, Edgar, Sendtner & Thoenen (1986) for the chick embryo. Dextrans (Dxs) conjugated with rhodamine (Rho) or fluorescein (FITC) were also retrogradely transported, but they were not well fixed and so diffusion from motoneurons was observed. Nevertheless, fibroblasts in culture incorporated Dx-Rho that remained inside the cell for about 2 weeks. The new generation of Dxs (Fritsch, Dubuc, Ohta & Grillner, 1989) coupled to amines (Dx-lysine-Rho or FITC) that permit their fixation, together with the feature of being non-metabolized by cells, make them very useful as retrograde tracers, as well as intracellular labels and would be tried in future work. Wheat germ agglutinin (WGA) conjugated with different

fluorochromes (RITC, FITC or Lucifer yellow), as used by Okun (1981), Calof and Reichardt (1984) and O'Brien and Fischbach (1986) in the chick embryo, was also tested but not with as good a result as with RITC in our system. They required more time to be transported to the spinal cord (Okun, 1981; see also Schaffner et al., 1987), and as a consequence an increase in the duration of embryo incubation, jeopardising motoneurones viability (see below). In addition the intensity of the labelling was not as high as with RITC. Also, a greater number of labelled non-neuronal cells was observed with WGA-RITC, probably because of the easy incorporation of lectins into cells. Another disadvantage of WGA is the possibility of transneuronal transport (Borges & Sidman, 1982). I obtained comparable results to RITC with Sulphorhodamine. This dye is useful in double labelling when used with FITC filters, but it has the disadvantage of being more expensive. Other new fluorochromes used as retrograde markers are the lipidic membrane ionophores carbocyanines DiI¹ and DiO², that have been shown to be very good as tracers and for labelling cells in culture (Honig & Hume, 1986). These fluorochromes present the advantage that they are incorporated into the cell membrane, giving an intense labelling of the whole cell, so it is possible to visualize neurites for the first few days before the label accumulates in the cytoplasm where they remain for a long time (Honig & Hume, 1986). Although, in my experience, the fluorescence of cultured cells labelled with DiI last for about one week, others have reported for up to 10 days (Martinou et al., 1989a). Fluorochromes used as retrograde markers differ mainly in the brightness of labelling, and the distribution and time they remain inside the cell, but also in the time required for retrograde transport. In this study differences in brightness

¹ DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate)

² DiO (3,3'-dioctadecyloxacarbocyanine perchlorate)

were not critical as the use of an image-intensification video-camera facilitated the visualization of labelled cells. The most important factors that determined the extent and quality of labelling were the volume of the injected fluorochrome, and the incubation time necessary for the retrograde transport. With long incubation times (16-18h) more motoneurons were labelled, but these longer incubation were associated with reduced viability of neurones after the dissociation of the spinal cord as determined by the Trypan blue test and the survival of cells after 48h in culture. The isolation of the limb bud-lumbar cord segment from the rest of the embryo, and an inadequate oxygenation of the preparation, may have contributed to this reduced viability.

No important differences in motoneurone labelling between E13-14 and E18-20 embryos were observed, either in the distribution or in the amount of labelling, even though different procedures were used. In both cases a similar estimate of motoneurone labelling in the spinal cord was obtained. The in utero injection in older embryos was a more physiological procedure permitting longer incubation times and better viability of the spinal cord than the incubation method used for the younger embryos. And although larger volumes of fluorochrome were required in older embryos, the entire procedure was easier, accounting for the reduced variation dispersion in the percentage labelling in this group of embryos. Unfortunately, the in situ injection was an unreliable technique in embryos younger than E17, because at that age this is a blind procedure (see methods).

C. Identification of Motoneurons in Culture

The identification of labelled cells in culture through fluorescence microscopy was straightforward, particularly when the cells were already fixed. Nevertheless, the comparison with phase images, or the selection of neurones for patch-clamping could take a long time. Cells were then exposed to the incident light for long periods, with the risk of damage. Cells exposed for longer than 2 minutes retracted their neurites, became rounded and lost their transparency. Electrophysiological activity was absent or disappeared from these neurones, confirming the damaging effect of long periods of exposure to the fluorescent light. Similar observations were made by O'Brien and Fischbach (1986). Smith et al. (1986) also reported similar morphological changes although they did not record from these cells electrophysiologically. However, cells with similar signs of damage, but not exposed to the incident light, were also found in the present study. Although the possibility that the fluorochrome has a toxic effect cannot be ruled out, the fact that they were observed in labelled, as in non-labelled cultures, argues in favour of a process secondary to cell death or to damage by the dissociation procedure. Most of these problems were circumvented by the use of video-microscopy that reduced significantly the duration of study and the exposure time to the incident light. Another important advantage arising from the use of the low-light level camera was the possibility of visualizing faintly labelled motoneurons. Labelled cells that could not be seen through the eye piece were easily identified in the video-monitor. Several other factors helped in the identification of labelled cells. The technique of contrast enhancement, the use of objectives with high numerical aperture but final low magnification (see Inoue, 1986), and the use of a video-recorder. The latter allowed immediate capture of phase and fluorescent images

in fixed sequences in a short time. Recorded images were then available for later analysis. Apart from the use of video-camera by O'Brien and Fischbach (1986) we have not seen other studies with a systematic use of these systems.

Few labelled non-neuronal cells were observed in these cultures, supporting a low diffusion of the label in the spinal cord. The number of such cells did not increase with the time in culture, which argues against a leakage of fluorochrome from the labelled motoneurons or from dying motoneurons. Nevertheless, in older embryos the increase in labelled non-neuronal cells and the appearance of macrophage-like cells were probably related to the increase in the number of glial cells after E16, and the establishment of blood circulation in the spinal cord after E15, respectively (Jacobson, 1978). The virtual absence of labelled non-neuronal cells in cultures prepared from ventral cords can be explained by the fact that at E13-14 no major proliferation of glial cells is observed. Other important factors were: the use of differential cell attachment previous to the final plating of ventral cord cells which helps to eliminate non-neuronal cells (Varon & Rainbow, 1969); the replacement of FCS by horse serum; the low density of our cultures; and the low CO₂, that reduces the number of proliferating cells. For this last, cytosine arabinoside was also tried in few instances but a toxic effect on neuronal cells was observed.

Labelled cells had a neuronal morphology and relevant electrophysiological activities, and after a few hours, neurites longer than one cell diameter, and immunoreactive to neurofilament protein, appeared. Some hours after plating labelled motoneurons were either phase-dark or phase-bright, but after two days in culture, and in particular when cultured over non-neuronal monolayers, most motoneurons were phase-dark. The latter is probably explained by the increase

in cell body area, a less prominent nucleus, and a more extended morphology with stronger adhesion to the substratum. Usually, a phase-bright appearance has been used as sign of neuronal identity for cells in culture, but the present study shows that the appearance in phase, as far as motoneurons in culture is concerned, is not a good indication of the neuronal nature of a cell. These observations are in agreement with those of Dohrmann et al. (1986) for labelled motoneurons and with those of Schnaar & Schaffner (1981) for a motoneurone enriched fraction, in which most motoneurons were phase-dark.

C. Number of Labelled Motoneurons

Motoneurons were only a small fraction of the cells in culture. Considering only those experiments in which no enrichment procedures were carried out, motoneurons usually accounted for only 2-3% of the total cells in culture. These figures are even more surprising taking into account that only ventral portions of the spinal cord were used for preparation of the cultures. These percentages of labelled motoneurons are in agreement with those found by Smith et al. (1986) for the E14 embryo rat (2.3% of total cells per dish, and 3.3% of ventral cord cells) and O'Brien and Fischbach (1986) for the chick embryo (2% per total cells) using WGA-Ly. Calof and Reichardt (1984) and Dohrmann et al. (1986) obtained higher values for the chick embryo, but only after enrichment through FACS and gradient-centrifugation, respectively. These higher figures reflect in part the greater proportion of spinal motoneurons in chick than in rodent embryos (see Hamburger, 1975; and Lance-Jones, 1982). Also, data from cell sorting studies are more variable as a consequence of different thresholds and windows used (Calof

& Reichardt, 1984; Schaffner et al., 1987). In the present study, the use of ventral half cords and the pre-plating of the dissociated cells gave a mean 5.5x enrichment. By adding a step gradient-centrifugation with Percoll, a 10-15x enrichment was obtained, similar to that of Dohrmann et al. (1986) in the chick embryo using a Metrizamide gradient. Nevertheless, at these levels of enrichment these cultures still have to be regarded as heterogeneous. The fact that motoneurons constitute a small population of cells from the spinal cord stresses the importance of a marker or labelling in studies with heterogeneous cultures. The lower fraction of motoneurons obtained from older embryos (1.7%) is probably explained by the difficulties found in the dissociation of the spinal cord at that age, the likelihood of greatest damage to neurons with long processes, and the reduction observed in the seeding efficiency of those cultures. Moreover, at this embryonic age (E18-20) the period of motoneuron cell death is almost complete (Oppenheimer, 1986) and interneurons and glial cells have already arisen (Altman & Bayer, 1984), decreasing both the absolute and relative number of motoneurons in the spinal cord, respectively.

Although dissociated cells from older embryos presented lower viabilities, they were still in the usual ranges for primary cultures. The lower viability was probably the result of the same factors as for the reduction in labelling in these older embryos, even though the preparation in older embryos was a more physiological procedure. Although dissociated cells from E13-14 embryos incubated for more than 16h presented lower viabilities, labelling was not significantly affected. Viability was studied through the Trypan blue test, which measures permeability of the cell membrane to a dye (Patterson, 1979), so this may over or underestimate the true number of viable cells (cells that will survive). This was

clearly shown when comparing the relative survival of cells at 48h after plating from E13-14 embryos incubated for 12 and 20 hours. The population of total cells presented no differences in survival at 48h, although the viabilities of the dissociated cells were significantly lower in embryos incubated for a longer time. These results were expected because a defined number of viable cells was plated and survival was relative to the 6h observation. However, labelled motoneurons from embryos incubated for 20h showed a reduction in relative survival at 48h. These results showed that viable motoneurons from E13-14 embryos incubated for longer periods (>12h) may show a reduced survival later in culture.

The decay in fluorescence of labelled cells reflected the cell death of motoneurons when cultured on CPDL or fibroblasts monolayers. However, after three days in culture over glia monolayers, the fluorescence was no longer useful as a marker because it did not identify all the motoneurons that survived. In this case the decay of RITC fluorescence had a half life of 3d, that is to say, faster than motoneurone death in culture. Consequently, few labelled motoneurons were observed after four days in culture. Thus using RITC, studies longer than three days in culture needed a different type of identification. The location of labelled motoneurons on a grid made on the culture dish gave good results although some of them could be lost by translocation between periods of observation especially when motoneurons were cultured over monolayers. Similar results were observed by O'Brien and Fischbach (1986) using WGA-Ly. They showed that when spinal cord cells were cultured over collagen the decay of fluorescence represented cell death but when cultured with myotubes the reduction in number of labelled motoneurons represented the decay in fluorescence. In their experiments WGA-Ly had a half life of only 2d in culture with myotubes.

E. Electrophysiological Recordings of Identified Motoneurons

Labelled embryonic E13-14 and E18-20 motoneurons were electrically excitable. No activity was obtained from non-neuronal cells. Motoneurons showed input impedances similar to those observed in embryonic chick motoneurons (O'Brien & Fischbach, 1986), but they were much higher than those obtained by conventional intracellular recording from *in vitro* preparations of isolated hemisected neonatal rat spinal cord (Fulton & Walton, 1986). The smaller size of motoneurons and the apparently less traumatic procedure of whole cell patch-clamping were probably the reason for these higher impedances. Nevertheless, resting membrane potentials were similar to those observed in the hemisected neonatal rat spinal cord preparation (Fulton & Walton, 1986; Harada & Takahashi, 1983). Spontaneous fluctuations of the membrane potential were observed in some motoneurons in the absence of morphological or physiological evidence of synaptic contacts. Such spontaneous activities are analogous to those observed in gonadotrophic cells from ovine pituitary cultures (Sikdar, Waring & Mason, 1986) and also in isolated hemisected neonatal rat spinal cords (Fulton & Walton, 1986; Harada & Takahashi, 1983). After-hyperpolarizations were observed in some motoneurons. Motoneurons presented inward currents and a late potassium-dependent outward current, as voltage-clamp studies showed. Although the ionic dependence of this inward current was not investigated with tetrodotoxin, it is likely to be sodium-dependent as it has a reversal potential of about +50 mV and has similar kinetics to the sodium-dependent inward current observed in mouse spinal cord neurones in culture (Krieger & Sears, 1986 and 1988; MacDermott & Westbrook, 1986). These results are in agreement with a recent report showing the presence of Na⁺ and K⁺ currents early in motoneurone

differentiation in labelled motoneurons in culture from E4 chick embryo 24h after plating (McCobb, Best & Beam, 1990). Calcium currents were not found in the present recordings from E13-15 motoneurons, which is probably explained by a later maturation than Na^+ and K^+ currents, as has been shown for mouse spinal neurons (Krieger & Sears, 1988) and in labelled chick motoneurons (McCobb, Best & Beam, 1989).

F. Survival of Motoneurons in Culture

The lower survival of E13-14 and E18-20 motoneurons cultured on CPDL was not due to general components of the serum. Although horse serum was an obligatory requirement for neuronal survival, concentrations from 2% to 10% made no difference in motoneurone survival. Bovine serum albumin produced a non-significant increase in survival, and the addition of KCl did not modify the survival of motoneurons. Although FCS was detrimental to all neurons, especially for motoneurons, as has been mentioned by others (Dorhmann et al., 1986), in its absence, motoneurons still presented a lower survival. These results showed that the absence of specific factors or other cells in the culture system may have been the cause of this poor survival of motoneurons in culture.

Three important observations were made from these studies. Firstly, between E13 and E20, motoneurons were unable to survive in CPDL (even in the presence of interneurons). Secondly, that in the absence of muscle, motoneurons can survive over a monolayer of glia cells for several days (up to one week), and thirdly, that motoneurons can survive for several weeks (≥ 4) in the presence of myotubes or

in previous studies (Smith et al., 1986; O'Brien & Fischbach, 1986) motoneurons survived for no more than 7-10 days, even in the presence of muscle. Differences in the embryonic age, dissociation technique and media used, type and number of myotubes in conjunction with a glia monolayer, could account for this longer survival, or it may be species specific. An important factor in this longer survival was the use of E13-14 embryos. The quality and viability of cells from older embryos (E15, E18-20) were always reduced, and a lower survival of motoneurons was observed. Whether this was related to a change in survival requirements, or to the dissociation procedures, is not known (but see below). The possibility of maintaining motoneurons for long durations in culture allows studies on the development of motoneurons for periods that extend to the onset of their adult life. Using similar techniques, Schaffner et al. (1987) have also obtained survival of up to 5 weeks.

The half lives obtained for motoneurons show very clearly the effects on survival of the different culture conditions. No major differences were observed between CPDL, at any age, and fibroblasts. But when cultured over glia monolayers a clearly longer T_{50} of E13-14 compared to E18 motoneurons was observed. Although these half lives represent the linear regression for the first 4d there was an initial higher loss of motoneurons in the first 12h or 24h under all conditions, though more pronounced in CPDL, but only slightly evident on glia or myotubes. This initial greater loss of motoneurons was probably caused by the trauma of the dissociation procedures, and by a reduced seeding efficiency, especially on CPDL, but less so with glia or myotubes.

F.1. Effect of interneurons and DRG neurones

In the experiments designed to investigate the effect of other neurones (afferents) on motoneurone survival either E15-16 DRG neurones were added to ventral cultures or interneurons increased by plating at different cell densities. Although in the latter case motoneurons and interneurons were increased in the same proportion, the increase in overall cell density permitted a greater interaction between motoneurons and interneurons. The labelling of intersegmental interneurons with RITC (see Results) from the ventral half of the lumbar at E16 cord showed that associative intersegmental neurones reach the ventral lumbar cord early in development. However, few labelled cells were obtained, so no attempt was made to work with pure populations of interneurons. Future studies would require cell sorting methods to obtain pure populations of both types of neurones.

At the cell densities used in this thesis ($\pm 15 \times 10^3$ cells/cm²), in which neurones remained largely isolated from each other, motoneurons had a low survival. When interactions were permitted at higher cell densities, only about 50% survived at 48h, and few motoneurons were observed after 4d in culture. This increase in the number of surviving cells in culture is commonly observed (Banker & Cowan, 1977). However, studies using FACS show that pure populations of motoneurons have a poorer survival than motoneurons in heterogeneous cultures (O'Brien & Fischbach, 1986; Martinou et al., 1989a), so it is unlikely that the increase in the number of motoneurons was an important factor. Either a higher concentration of soluble trophic factors, or cell-cell interaction with interneurons, or both, may explain this density effect. Furthermore, it is also possible that at very high cell

densities ($>30 \times 10^4$ cells/cm²) an increase in non-neuronal cells could have increased survival. Nevertheless the increase in survival was only transient indicating that interneurons alone were unable to support the survival of E13-14 motoneurons. It could be that interneurons can support dendritic growth in other conditions, as in the presence of muscle, but this was not pursued in the present study, as it would have required work on purified populations of interneurons. Commissural neurones, distributed along the lateral surface of both dorsal and ventral portions of the neural tube, have been clearly shown to be generated before spinal motoneurons in the chick embryo (Holliday & Hamburger, 1977), and during the same period of spinal motoneurons in the rat and mouse embryos (Windle & Baxter, 1936; Holley, 1982; Wentworth, 1984b). Windle and Baxter (1936) found the first commissural and association neurones at 12.5d in the cervical rat spinal cord with axons in the lateral-ventral and floor plate regions appearing at 13d. These interneurons were frequently found at 14.5-15.3 embryonic days with close linking to motoneurons. This latter period would correspond to E15-16 in the lumbar cord if a similar rostro-caudal gradient occurs in the rat as observed in the chick embryo (see Yaginuma, Shiga, Homma, Ishihara & Oppenheim, 1990). This data means that although these interneurons are generated before the period of motoneurone cell death their development and relationship with motoneurons appears to be later, when motoneurone cell death has already started. Previous to the period of cell death in the chick embryo most contacts in the lateral funiculus of the lumbar cord are of the axo-glial type (Oppenheim et al., 1978), although axo-dendritic synapses are also observed. Axo-glial contacts are progressively replaced, so by the end of the period of cell death only axo-dendritic synapses exist. These observations are in agreement with studies in which spinal deafferentation, early in the development of chick embryos (Okado

& Oppenheim, 1984), produces an increase in the loss of motoneurons but only at the end of the natural cell death period. All this data, together with those from the present study, suggest that interneurons probably do not play an important role in the survival of motoneurons early in development before the period of normal cell death, but they may be important later in development not only influencing survival but also the dendritic growth of motoneurons.

DRG neurons produced a small increase in motoneurone survival in relation to the control group (CPDL). Admitting a possible effect of cell density (see above), and assuming that a sufficient number of neurons were present, these results suggest that DRG neurons are not critical for motoneurone survival in culture at this stage of development. Considering that in these latter experiments DRG neurons and interneurons were present at the same time, and that no additive effect on motoneurone survival was observed, the idea that at this stage of development motoneurons do not depend greatly on those neurons for their survival is reinforced. An important consideration for these experiments is the timing of the development of DRG afferents in relation to lumbar motoneurons. In fact, HRP studies in the rat lumbar cord have shown that sensory axons already exist in the hindlimb at E13.5-14.5, and that central afferent axons extended to the dorsal horn from E15.5 onwards, but it is not until E17.5 that afferent axons started to appear in the vicinity of motoneurone dendrites, and by E18.5 into the vicinity of motoneurone somata (Smith & Hollyday, 1983). This later development of DRG afferent-motoneurone interactions is in agreement with the finding that the removal of virtually all primary afferents from the DRG in the chick embryo several days previous to the period of cell death produced no effect on the survival of motoneurons up to E10-12, but increased significantly the loss of

motoneurons at the end of the period of cell death (Okado & Oppenheim, 1984). This data, as discussed for interneurons, support the suggestion from the present study that afferents are not primarily involved in motoneurone survival early in development and at the beginning of the period of cell death.

F.2. Effect of non-neuronal cells

From the results of these experiments it is possible to conclude firstly, that in the absence of muscle, glial cells are able to support the survival of spinal motoneurons in culture early in their development and for a period up until the end of the cell death phase. Secondly, that myotubes are sufficient to support the survival of motoneurons from early in their development and for a period that goes well into the postnatal life, so that myotubes are able to rescue most of the motoneurons that would otherwise die. Thirdly, that fibroblasts, at least in the absence of muscle cells, seem to have no important influence on the survival of motoneurons. It is important to remember that under all conditions used in the present work, motoneurons were in the presence of interneurons.

F.2.1. Motoneurone survival on fibroblast monolayers

Early after their migration spinal motoneurons are not in contact with mesenchymal cells in the neural tube (Altman & Bayer, 1984). And although their axons grow through the mesenchyme outside the neural tube, containing cells of a fibroblastic nature, the mesenchyme is regarded by some as a nonspecific,

permissive terrain for axonal growth and guidance (Tosney, Watanabe, Landmesser & Rutishauser, 1986; Yip & Yip, 1990). Also, it is a well known observation that non-neuronal cells of glial origin provide a better environment and surface than fibroblasts for the growth of neurites from central and peripheral neurones (Fallon, 1985a and 1985b). Nevertheless, fibroblasts, as well as glia cells, produce fibronectin and other matrix substances (Akiyama & Yamada, 1987; Gallagher, Lyon & Steward, 1986), which probably increase the cell adhesion of neurones in culture. And it has recently been reported that fibroblasts from denervated muscle fibers not only synthesize matrix substances but also cell adhesion molecules (Gatchalian, Schachner & Sanes, 1989), which could provide more specific cues to nerve regeneration. But whether fibroblasts promote neurite growth during normal development is unknown. In the present study, neurones presented higher seeding efficiency when cultured over fibroblast or glia monolayers, particularly the latter, in agreement with other studies (Noble, Fok-Deang & Cohen, 1984). Therefore, the small effect of fibroblasts on motoneurone survival over controls (CPDL) probably reflects this increase in cell adhesion as seen in other neurones (Seilheimer & Schachner, 1988). In fact, motoneurones, as well as non-labelled neurones, tended to remain over flat fibroblasts cells, with the neurites growing over the fibroblast layer and rarely on the glass substratum. High protein concentration of conditioned media from mesenchymal cells and fibroblasts produced only a small increase in survival of chick embryo motoneurones in heterogeneous cultures (Nurcombe, Tout & Bennett, 1985), and a small but significant increase in the percentage of cells with neurites (Calof and Reichardt, 1984). Consistent with those studies, our results show that the fibroblast cell surface with its conditioned media have only a small and short-lived effect on motoneurone survival. This also shows that although an improvement in the

general conditions may produce a transient increase in survival, it is not sufficient to prolong the survival of motoneurons.

F.2.2. Motoneurons survival on glia monolayers, in the absence of myotubes

It is unlikely that the increase in motoneurone survival observed on glia monolayers was secondary to contamination with other cells. These monolayers were prepared from late embryonic spinal cord, separated from DRGs and mesenchymal tissue and used after the third passage following removal of neurones and oligodendrocytes. Such glia cell monolayers were $\pm 95\%$ GFAP positive. Although there is evidence that astrocytes may be a heterogeneous population (Wilkin, Marriot & Cholewinsky, 1990), motoneurons survived equally well on cortical and spinal astrocytes, suggesting that the survival-promoting effect was not region specific, but this point was not pursued.

The increase in E13-14 spinal motoneurons survival in culture by astrocytes has several implications. First, it is possible that early in development, after migration, motoneurons may depend on glial cells for their survival. Second, that although muscle cells or myotubes may affect motoneurons very early in development, this influence is not strict and other cells may be important at this stage. Third, that in the absence of muscles, glia are sufficient to support the survival and growth (see following sections) of motoneurons in culture for a period corresponding up to the end of cell death in vivo. It is possible that in this latter effect the presence of interneurons play an important role, but one that itself is favoured by the presence of glia.

Although the currently accepted view is that glial cells are important for the survival and growth of neurones (Varon & Bunge, 1978), based mainly on in vitro studies, it is generally believed that this influence occurs late in development and that, apart from migration, glia cells are not importantly involved in the early stages of motoneurone development. This is so mainly because autoradiographic studies indicate that glia cells (astrocytes) are generated later than neurones during development (Altman & Bayer, 1984). In the spinal cord this idea has also been reinforced by the existence of the period of natural cell death and its dependence on muscle. The fact that almost all motoneurones die or are rescued in experiments in which limb buds have been removed or added, respectively (Hamburger, 1977), has been interpreted as implying that target relationships are a strict requirement for motoneurone survival. Results using cell culture models (Bennett et al., 1980) have again reinforced this conclusion. Nevertheless, several lines of evidence, together with the present results indicate that this may not be the case for the early development of spinal motoneurones.

During migration and for a few days after the settlement in the ventro-lateral region of the neural tube, motoneurones are in close relation with radial glial cells (Wentworth & Hinds, 1978). Henrikson and Vaughn (1974) have observed frequent axon bundles within channels formed by radial processes, motoneurone dendrites grouped around the radial processes in the marginal zone, axo-glial contacts between radial glia cells and motoneurones, and puncta-adherens between dendrites and radial processes, in the ventro-lateral region of brachial neural tube up to E14 in the mouse embryo (that corresponds to E16-17 in the lumbar rat spinal cord). Similar contacts have been observed up to E10 in the chick embryo neural tube (Oppenheim et al., 1978). The disappearance of these contacts

coincides with the end of the cell death period, and with the slow appearance of astrocytes, which in the rat spinal cord is mainly postnatal (Frederiksen & McKay, 1988). There is also evidence that radial glial cells are precursors to astrocytes (Schmechel & Rakic, 1979; Levitt et al., 1981). These data show that spinal motoneurons are in close relation to glial cells early in development and during the period of cell death.

In vitro studies also support the idea that glial cells are important for the survival and development of neurones. Astroglia and its conditioned media increase the survival and growth of hippocampal neurones (Banker, 1980; Müller et al., 1984), Schwann cells induce the morphological maturation of sensory neurones in vitro (Mudge, 1984), and glia monolayers are an appropriate substrate for the survival and growth of cerebellar and spinal cord neurones in culture (Noble et al., 1984). There is also evidence of a specific effect of glia cells on the survival of embryonic motoneurons, even in the absence of muscle. Eagleson et al. (1985), working with E6 chick embryo motoneurons labelled with HRP, in heterogeneous cultures, showed that most motoneurons (80%) survived for 48h either in glia CM or monolayers of flat glia cells from E7-10 embryo spinal cord. There was no difference with muscle CM at the same time in culture. This study is consistent with our results in the sense that motoneurons survive on glia cells. But they did not observe neurite outgrowth, except in a few motoneurons (< 5% over monolayers and \approx 20% using glia CM), which casts some doubt about the viability of the motoneurons, and together with the use of older embryos (at E6 chick embryos are already in the period of cell death; see Hamburger, 1977), may account for the shorter survival. In the present study, motoneurons cultured over CPDL in the presence of glia CM had very poor survival. This is consistent with the results from Noble et al. (1984) and Fallon (1985a) on spinal cord cells, and

from Martinou et al. (1989b) on E14 sorted rat motoneurons, showing that survival and neurite growth were much better on astrocyte monolayers than with glia CM. A longer survival (6d) in the absence of muscle or muscle CM has also been observed in a small percentage of RITC labelled E6 chick motoneurons over poly-ornithine-laminin as substrate in the presence of a spinal cord cells CM (Dorhmann, Edgar & Thoenen, 1987). The effect of this CM was not related to brain derived neurotrophic factor (BDNF), NGF, and acidic and basic FGF as these growth factors had no effect on motoneurone survival. Studies with sorted motoneurons also indicate that cells other than muscle are important for a longer survival. Rat motoneurons survived longer (1 week) in heterogeneous cultures over astrocyte monolayers, in the absence of myotubes, than sorted motoneurons in similar conditions (Martinou et al., 1989b). Although no quantitative estimate of motoneurone survival was provided by most of these studies, all of these data are consistent with the present results that glia monolayers provide a better environment than glia CM for the survival of motoneurons, and that the presence of glia can support the survival of motoneurons in culture for at least one week in the absence of muscle. Not surprisingly, in the present study and in Schaffner's et al. (1987), the combination of glia monolayers with myotubes, or with myotube CM, were able to support for ≥ 5 weeks spinal rat motoneurons in culture, showing that both conditions are critical to simulate the in vivo situation. This long survival is in agreement with the observation by Dorhmann et al. (1987) that a spinal cord cell CM had a synergistic effect with myotube CM on motoneurone survival.

Therefore it is possible to suggest that glia cells (in the presence of interneurons) may be critical for the regulation of survival early in motoneurone development, and even through the period of cell death.

It has been suggested (Davies, 1990), in relation to target-relationship, that the longer the distance between neurone and target the longer the time until the cell death period. In other words, until the neurone contacts its target, its survival is independent of it. This may be one reason explaining the longer survival in the present study when cultured on glia cells. Therefore in the absence of target, but in the appropriate environment (glia plus interneurons), motoneurons can survive longer, but only up to a certain age (?end of cell death). This would explain the failure in survival in CPDL and fibroblast. Then why does this not occur in vivo when limb buds are removed? It could be that astrocytes produce a factor that is not in radial glia cells. But a time factor appears also to be involved, because cell death almost always starts at the same time, so that motoneurons may have a clock in cell differentiation, which makes them target dependent. And therefore the timing for the removal of limb buds may be critical.

F.2.3. Change in motoneurone survival with embryonic age

In the present study an age dependent effect on motoneurone survival was observed when ventral cultures were prepared over astrocyte monolayers. E13-14 motoneurons not only presented an increase in survival, but they were able to survive longer than E18-20 motoneurons. If this age dependent difference in survival was not the result of the dissociation procedures, then it means that motoneurons must change their requirement for survival with the embryonic age. Although it is not possible to rule out an effect due to cell dissociation, the similar survival of motoneurons at both ages when cultured with myotubes makes this an unlikely factor. Several possibilities could explain a change in the requirement

for motoneurone survival during development. Glia cells could change after the period of cell death, and cease their ability to support motoneurones survival. There is evidence indicating that astrocytes originate from radial glia cells (Choi, Kim & Lapham, 1983; Pixley & De Vellis, 1984), and that the latter disappear at the end of foetal life and are replaced by astrocytes (Tapscott, Bennett, Toyama, Kleinbart, & Holtzer, 1981b). At E10 in the chick and at E17 in mouse embryos there is a loss of contact between radial glia cells and motoneurones (see above), coincidentally with the appearance of the first astrocytes and vascular elements (Bignami & Dahl, 1975; Tapscott et al., 1981b; Frederiksen & McKay, 1988). Eagleson et al. (1985) showed that E6 chick motoneurones survived better in "immature" (embryonic) spinal cord glia monolayers than in astrocytes from neonatal spinal cord. Also Martinou et al. (1989b) have observed that newborn rat astrocytes were a relatively non-permissive condition for ChAT expression in rat motoneurones. The normally restricted capacity of adult neurones to regenerate has been also explained as a change in glia and substratum pathways (Aguayo, 1985). However, it is also possible that a stronger dependence of motoneurones on muscle is established during and after the period of cell death, and after the establishment of neuromuscular connections. Eagleson and Bennett (1986) showed that there was a shift in chick motoneurone dependence from astrocytes to myotubes from early to later stages in embryonic development. Although these were only short term survival studies, their results are in general agreement with ours which showed that most E18-20 motoneurones were rescued by myotubes, but only survived for few days on glia monolayers. In the present study, E15 and E18-20 motoneurones showed a greater reduction in survival after 48h than E13-14 motoneurones when cultured on CPDL, suggesting that the survival of motoneurones in culture changes after E15, and supporting the idea of a change

in motoneurone requirements for survival during the period of natural cell death.

F.2.4. Survival of motoneurons cultured with myotubes

The effect of myotubes or myotube CM on motoneurone survival has been clearly shown both in in vivo and in vitro models. Multiple components of myotube CM may increase the survival and morphological differentiation of identified chick and rat motoneurons in culture (Tanaka & Obata, 1983; Calof & Reichardt, 1984; Smith et al., 1986; Dohrmann et al., 1986; MacManaman, Crawford, Clark, Richter & Fuller, 1989), and the survival of embryonic chick motoneurons in vivo (Oppenheim, Haverkamp, Prevet, MacManaman & Appel, 1988). Studies have identified laminin activity as an essential component of muscle CM to promote neurite outgrowth on motoneurons (Calof & Reichardt, 1985; Dohrmann et al., 1986). A factor from rat skeletal muscle has recently been purified which increases the level of ChAT in rat E14 spinal cord neurons (MacManaman, Crawford, Stewart & Appel, 1988). This factor, a 22 kd acidic polypeptide named ChAT development factor (CDF), also produced an increase of 20% in the number of lumbar chick motoneurons at the end of the period of cell death in an in vivo model (MacManaman et al., 1990). Another partially isolated factor from rat skeletal muscle CM also increased the level of ChAT but without increasing the survival of E14 rat motoneurons cultured over glia monolayer (Martinou et al., 1989b). The presence of different components with different effects, and the use of different type of cultures, probably explains the variability observed in the response to myotube CM. Although it was clearly shown that myotubes and myotube CM contains survival-promoting substances, and one of them rescues

motoneurons from cell death in vivo, those studies did not address the problem of a possible age-dependent effect. In general, the results in this thesis are in agreement with all those studies, in the sense that myotubes promote the survival of motoneurons. Although myotubes were a sufficient condition for the longer survival of E13-14, and a necessary condition for the survival of E18-20 motoneurons, a similar level of "rescue" of motoneurons in the presence of myotubes was observed at both embryonic ages. The observation that motoneurone survival, early in development, appears not to be strictly dependent on muscle is also consistent with the effects of limb bud removal (Oppenheim, 1978; Hamburger, 1977) which indicate that the presence of limb muscle is not essential for the generation and early differentiation of motoneurons up to the period of cell death. The fact that in the rat embryo, motoneurons establish connections with myotubes at E14.5-15 (Dennis et al., 1981) in the thoracic spinal cord (probably corresponding to E16 in the lumbar cord), which coincides with the beginning of the cell death period (see Harris & McCaig, 1984), is consistent with the above suggestion. It is important also to note that in the rat hindlimb, primary myotubes start to appear at E16 coincident with the decrease in motoneurone number, and that secondary myotubes do not appear until E19, at the end of the period of cell death (Ross, Duxson & Harris, 1987). So it is not unreasonable to suggest that both phenomena may be related. Be this as it may, still more recent evidence suggests that mesenchymal tissue may affect the development of early neural tube cells (Heaton, Paiva & Swanson, 1990) and the number of cells in neuroepithelium early in development (Fontaine-Perus et al., 1989), making more complex the possible influence of non-neuronal cells on motoneurone development. The finding in the present study that E13-14 motoneurons are already responsive to myotubes is consistent with this latter evidence.

In the present study only about 75% of the initial (plated) motoneurons survived when cultured with myotubes. Other authors have shown figures close to 100% (O'Brien & Fischbach, 1986). Although it was not possible to control the number of myotubes formed before adding the motoneurons, they were in relatively low number to permit better visualization of motoneurons which could account for the lower rescue of motoneurons in the present study, apart from differences in the preparation of the cultures. O'Brien and Fischbach (1986) also observed that sorted motoneurons from E5 chick embryo survived no more than 1 or 2 days when cultured over collagen or poly-D-lysine, respectively; when myotube CM was added, the number of motoneurons declined after 3-4 days, but longer survival was observed when motoneurons were co-cultured with myotubes, and even longer (7d) when myotube CM were added to motoneurons in heterogeneous cultures. This observation, together with the present study on heterogeneous cultures, suggests that interneurons may play an important role in motoneuron survival and development (see above) after the connection with their target-muscle has been established.

G. Development and Growth of Motoneurons in Culture

One of the main purposes of this thesis was to study the development and growth of motoneurons in culture, and the effect of non-neuronal cells upon this growth. As discussed in previous sections, great attention has been paid in other studies to the effect of muscle on motoneuron survival, but we are not aware of any studies on the morphological development and growth of identified motoneurons under different conditions. Although some previous studies refer to neurite

outgrowth (Calof & Reichardt, 1984; Smith et al., 1986), this was estimated in terms of the number of cells with neurites \geq 1-2 cell diameters in length. However, such criterion is more a measure of survival than neurite growth because it depends on the number of cells present and neurite outgrowth is established early in culture (see below).

To study the development and growth of motoneurons in culture, the positions of fluorescent labelled motoneurons were established on a grid at 12h, and the same cells followed at different times after plating. By the use of this method we were able to identify motoneurons clearly for extended periods in culture independently of fluorescence fading. Although the morphological analysis was done by one observer, bias in the selection of motoneurons for study was reduced as much as possible by strict adherence to predetermined criteria (see METHODS). Technical and other factors, however, meant that this analysis of growth and development could only be completed on a limited number of motoneurons (40-60/condition) under different experimental conditions and durations in culture. Unlike the studies on survival and viability in the short terms, when the results could be evaluated statistically, these on growth and development are discussed only in terms of trends in the mean data.

F.1. Morphology of motoneurons

When cultured over CPDL at relatively low cell densities, the contact of motoneurons between themselves and with other neurones was severely limited, so that overall the influence of cell-cell contact is negligible. Under these conditions therefore, the development of motoneurons could be considered mainly

the result of intrinsic determinants, interacting or not with presumably soluble elements of the 'conditioned' media.

The study of motoneurons at 12h in culture, at which neurite outgrowth has already been observed, gave the earliest picture of the basic morphology a few hours after plating. Although motoneurons made the largest neurons in ventral cord cultures a few hours after plating, some being 20-24 μm in diameter, small motoneurons were also found (12-16 μm). Large lumbar motoneurons ($>30 \mu\text{m}$) are generated during the 12th and 13th day of gestation in the rat embryo (Nornes & Das, 1974), so differences in generation time at the time of dissociation (E13-14) may explain the range in motoneuron size. Nevertheless, a population of small motoneurons (14-18 μm , cDiam), in comparison with the largest motoneurons (30-35 μm , cDiam), was still observed at 14d in culture with myotubes. Whether these smaller motoneurons represented gamma motoneurons, is unknown. Medium sized and some small motoneurons (20-30 μm and $< 20 \mu\text{m}$ in diameters, respectively) in the rat ventral lumbar cord are generated between the 13th and 15th day of gestation (Nornes & Das, 1974), and if some of these were gamma motoneurons they could have been retrogradely labelled in the present study. Smith and Holliday (1983) also observed small ventral neurons labelled with HRP between E13-E15. But a clear bimodal distribution, as found in adult mouse hindlimb motoneurons (McHanwell & Biscoe, 1981) was not observed, except that at 14d in culture (corresponding to 7 days postnatal) with myotubes a main peak at 18-20 μm of cDiam was observed together with a small peak at 28-30 μm .

Most motoneurons were already multipolar, and at 12h half presented 4-5 primary neurites, close to the final number of primary neurites (± 6). Nevertheless, some

bipolar motoneurons were also found (6%), consistent with findings in the chick (Cajal, 1929) and mouse (Wentworth, 1984a) embryos, that after migration there is a period in which it is possible to find bipolar motoneurons with axons exiting the spinal cord. Such bipolar motoneurons disappeared rapidly over the next few hours, and probably corresponded to the last wave of motoneurons that had been generated and migrated to the lateral neural tube. Although there was a moderate positive correlation between size and number of primary neurites, most bipolar motoneurons were smaller in size ($< 14 \mu\text{m}$) suggesting their recent generation. After 24h they were transformed into multipolar motoneurons and none was less than $14 \mu\text{m}$. The possibility that culture conditions would have selected a particular type of motoneuron was ruled out by following the growth of individual motoneurons in culture. Invariably, new neurites grew out from bipolar motoneurons and increased in size and branching. In agreement with these observations, bipolar motoneurons were not present after E15 in the thoracic rat embryo spinal cord (Smith & Holliday, 1983). Some multipolar motoneurons followed for several days did not increase their initial number of primary neurites but did increase the number of secondary neurites. Assuming that for E13-14 12h in culture would correspond to 14-15 days of embryonic life, these observations suggest that some motoneurons have already attained their basic morphology early in development and before the period of cell death. This also means that even under the culture conditions of CPDL alone, motoneurons acquire a similar morphology to that observed in the embryo.

It is not possible to rule out that interneurons present in the ventral cultures may have affected the basic morphology of motoneurons, but under the conditions used this is unlikely. Studies of pure populations of sorted chick motoneurons (O'Brien & Fischbach, 1986) show no differences in the set of primary neurites,

but the dendritic pattern and branching was simpler than in non-sorted motoneurons. In the present study, most neurites presented morphological features of dendrites. Long and unbranched axon-like neurites were not always seen at this stage, but when they were observed, they emerged from the cell body or, more frequently, from the stem of a dendrite. This absence of axons or their variable emergence, which is also observed when motoneurons were cultured with myotubes or over glia, seems to be characteristic of neurones in vitro, as they have been observed in explants as well as in dissociated neurones (Hendelman & Marshall, 1978; Banker & Cowan, 1979). Interestingly Cajal (1929) described a similar phenomenon in the early stages of motoneurone development in the chick spinal cord embryo.

Although a variety of patterns of dendrite-like neurite formation were observed, after few days one, or more frequently, two thick primary dendrites emerging from opposite sides with secondary branching developed in most motoneurons, unrelated to multipolarity and culture conditions. This pattern has been observed by Schaffner et al. (1987) in sorted rat motoneurons, and also in neuronal cultures from hippocampus (Banker & Cowan, 1979) and cerebellum (personal observations). The neurite branching, under all conditions tested, and early in culture, was usually distributed in two 45° quadrants of the branching circle area.

It is possible, therefore, that given the random distribution of the other components of the culture system, this pattern of growth, is an intrinsic feature of motoneurons and other neurones. In this respect one might speculate that the phase of bipolarity observed in embryonic motoneurons (Cajal, 1929; Wentworth, 1984a) establishes that this dendritic pattern is established early in development. It seems unlikely that in dissociated cultures interneurons could have been

responsible for this basic polarity of neurites in motoneurons, but it may be that interneurons provide the appropriate signals to the growth and direction of distal branches (see below). At this stage although it was not possible to identify axon-like neurites in some motoneurons, others presented with more than one axon-like neurite, as has been observed in ciliary neurons (Role & Fischbach, 1987).

In agreement with the present study, O'Brien and Fischbach (1986) observed that labelled motoneurons from E5 chick embryo were all invariably multipolar (≥ 3 primary neurites) shortly after plating. They also found a similar number of primary neurites between sorted (pure population) and unsorted (heterogeneous) motoneurons (5.1 and 5.7, respectively) cultured with myotubes. Their data, together with our result that in the first few days motoneurons presented similar number of primary neurites independent of the culture conditions, suggests that the number of primary neurites is a property determined mainly by intrinsic signals. Nevertheless, the fact that the final number of primary neurites increased slightly and became established by about 4d, when motoneurons were cultured with glia or myotubes, indicate that these conditions are necessary for the full expression of this property. The idea that the number of primary neurites and their polarity are neuronal features regulated by endogenous signals is also suggested from observations on hippocampal neurons in cultures (Banker & Cowan, 1977; Dotti, Sullivan & Banker, 1988) and from studies with neuronal cell lines (Solomon, 1979; Solomon, 1981). These show that neurons not only tend to retain the same number of neurites and polarity as *in vivo*, but daughter neurons from precursors or parental cells also present the same morphological pattern.

The basic shape of motoneurons was defined in the first few days in culture, at the same time at which the number of primary neurites was established (3-4d). In

the first few hours the correlation of cell body size and shape, i.e. that small motoneurons tended to be round and larger ones rectangular, was probably the result of neurite deletion or loss of cytoplasm during the dissociation procedures. In the next few days the cell body of motoneurons cultured over myotubes tended to adopt a rectangular shape given by the emergence of more dendrites and flattening of the cell body, and as a consequence an increase in the second diameter. The latter did not occur on glia monolayers, so that in the absence of any change in aspect ratio, this provides evidence of a reduced growth of motoneurons when cultured over glia cells. In analysing the shape of motoneurons it is important to consider the method of determining the cell profile. In the present study the perimeter of motoneurons was drawn without considering the emerging neurites from the cell body. This obviously changed the relationship between area and cell body perimeter, and the shape obtained. As multipolarity of motoneurons increased, a reduction in the Area/Perim. index would have been expected. The fact that the reverse was obtained is explained by the criteria used to determine the outline of the cell body. In contrast, the rectangularity factor was reduced in motoneurons older than 2d due probably to the increase in the number and particularly the thickness of primary neurites. In any case, these shape indexes gave an inadequate idea of changes in shape of motoneurons. A more complex analysis taking into account the changes in dendritic stem as motoneurons grow, for instance, an integration of successive radii from the cell centre to the cell periphery would probably have given a more accurate account of motoneurone shape during development.

Apart from the number of primary and secondary neurites, the pattern of branching changed continuously over the following days with retraction and out-

growth of branches and axon-like neurites. The latter is comparable to that described by Role and Fischbach (1987) for ciliary neurones, but different from hippocampal neurones in culture in which only one axon-like neurite develops (Dotti et al.,1988). This suggests that motoneurones may have the capacity to produce several axons before the definitive pattern of connections is established. In fact, a similar growth is observed in adult motoneurones after axotomy (Hawton & Kellerth, 1987). The continuous reshaping, observed both over glia or with myotubes, means that for a long time motoneurones are exposed to factors or conditions that permit or regulate the growth of neurites. Although no quantification was possible in cultures of high densities over CPDL, nevertheless it was clear that the number of secondary neurites increased and, more particularly, the branching increased so changing the overall pattern. This suggests that conditions in which greater interneuron-neuron contact was allowed are important for the growth and branching of neurites. In cultures with CPDL and at the low cell densities which permitted morphological analysis, motoneurones tended to present a rather sparse dendritic tree mainly with primary and few secondary neurites. O'Brien and Fischbach (1986) injected Lucifer yellow intracellularly in chick motoneurones cultured with myotubes, and observed that at 6d motoneurones were multipolar and highly branched when cultured in heterogeneous cultures but much less so in sorted motoneurones. They observed that sorted motoneurones presented only 65% of branch-points and 62% of total neurite length of unsorted motoneurones. These observations were obtained from chick embryos at E5 motoneurones and after 6d in culture. However, in the present study, the basic shape and neurite pattern in E13-14 motoneurones was already observed only a few days after plating. Taken together, these observations and the present results, all support the idea that the basic structure of

motoneurone (primary and secondary neurites, polarity) depends mostly on intrinsic signals.

No detailed study has been reported on the basic morphological features and size of well identified embryonic motoneurones either in in vivo or in in vitro , but the scant available data is in agreement with our results. In cultures prepared from E14-15 rat embryos Smith et al. (1986) described labelled motoneurones at 6h after plating with an ovoid somata and diameters of 15-25 μm x 10-15 μm , similar to our results. Schaffner et al. (1987), also in E13-15 rat embryos, studied sorted motoneurones cultured over glia and with myotube conditioned media. They give diameters of 15-25 μm at 2 weeks in multipolar motoneurones, similar to the size of motoneurones at 14d (18-30 μm) cultured over myotubes observed in the present study. O'Brien and Fischbach (1986), in labelled motoneurones from E5 chick embryos, give diameters of 10-12 μm , but without giving the time in culture. At 24h in culture they observed that motoneurones were invariably multipolar, with 25% of dye coupling, a phenomenon that was not observed in the present study.

Whether motoneurones in culture show similar features to the in vivo situation is an important requirement to the use of a culture system as an in vitro model for the study of motoneurone development. The morphology, the transition from a bipolar to a multipolar motoneurone, and the presence of two main trunk dendrites observed in motoneurones in the present study, are features described by Cajal (1929) for chick, by Wentworth for mouse (1984a), and Smith and Holliday (1983) for rat embryonic motoneurones. The latter authors report that early in development E14-15 HRP labelled embryonic rat motoneurones extend their primary dendrites in two main groups, dorsolaterally and dorsomedially in the rat spinal cord, which is in agreement with our findings that in culture,

motoneurons organize their dendrites into two main groups and that these are localized usually in two quadrants. The fact that these features were observed under all conditions, and established early in culture similar to the embryonic development, suggest strongly that they are part of a basic developmental programme which can be expressed in an in vitro environment. Although no detailed studies have been reported related to cell body size and development of dendrites and branches in embryonic motoneurons, studies on postnatal and adult motoneurons are consistent with the findings on labelled motoneurons. Barber, Phelps, Houser, Crawford & Salvaterra (1984) studying the adult rat spinal cord by immunocytochemical methods, find that ventral motoneurons present 3-8 primary neurites. Bernstein and Standler (1983) using Golgi techniques, find that thoracic adult rat motoneurons present a mean of 5 (4-7) primary neurites, with a mean maximal dendritic field of 275 μm from the cell soma, a mean number of dendritic segments (branching) of 37 (27-45), and a peak in dendritic segments at 50-100 μm from the cell body. Sakla (1959) studying the postnatal growth of mouse cervical motoneurons by the Golgi technique and using Scholl's technique (1953), shows that the dendritic field of motoneurons stabilizes at around 30 days postnatal. The total dendritic length at postnatal day 1 (PN1) varied between 250 and 1500 μm , and in the adult mouse 4500-5500 μm . The number of dendritic branches changed from 10-30 at PN1 to 45-65 in the adult mouse. The dendritic density fell exponentially with distance from the cell body, with a mean peak of crossings at 40 μm from the cell body at PN1, and at 150 μm in the adult mouse. In the present study, after one week in culture (corresponding to PN1), motoneurons presented 3 to 7 primary neurites (mean 5.5) when cultured either over glial cells or with myotubes, with no changes at 14d in culture. The maximal neurite field was over 200 μm , with a shift in the peak of branching from 40-60 to 60-100 μm from the cell body when cultured on myotubes, due probably to the

growth of the stem of primary neurites. A total neurite length of about 1500 μm , after one week in culture with myotubes and a similar exponential decline in branching per unit area with distance from the cell body were also observed. Nevertheless, cultured motoneurons had a simpler dendritic and branching pattern compared with embryonic motoneurons (see Smith & Holliday, 1983), even when cultured with myotubes. Available evidence suggests that there is a relationship between motoneuron size and motor unit or target size (Culheim, 1978; Holliday & Mendell, 1976; and Sperry and Grobstein, 1985). It is not unreasonable, therefore, to expect differences in size between cultured and embryonic motoneurons, apart from those differences originated between a bidimensional and a tridimensional system. Barber et al. (1984) reported that large and medium size adult rat motoneurons presented diameters of 55x40 μm and 45x26 μm , respectively. Mouse hindlimb motoneurons present mean compound diameters of 16.4 μm at PN1-3, 20 μm at PN6-7, and 26 μm in adult life (Boulac & Meininger, 1983). In the present study, at 14d in culture (equivalent to PN7) with myotubes, motoneurons had a mean compound diameter of 22 ± 4.7 μm , and 24.7×19.7 μm as mean major and minor diameters. From the available data it is possible then to see that the range of cell body sizes and branching pattern found in the present study are of comparable magnitudes to those reported for the postnatal and adult rat and postnatal mouse spinal motoneurons. So, together with the resemblance in morphology and development (see above), it is possible to conclude that in a permissive environment like myotubes, and to some extent glia cells, the *in vitro* development of motoneurons is similar to that of embryonic motoneurons.

G.2. Growth of motoneurons in culture

It is unlikely that a selection of motoneurons accounted for the differences in motoneurone growth observed when cultured over glia or myotubes. A similar survival above > 50% was observed in both conditions after 4d in culture. However, a selection bias of motoneurons cannot be ruled out when cultured over fibroblasts, and this probably occurred after 2d on CPDL. In the latter condition only 30% survived at 2d, and a loss of high multipolar motoneurons was observed. The reduced number of motoneurons with 5 primary neurites on CPDL might have been the result of the death of the larger motoneurons. As the size and number of primary neurites showed a positive correlation, it is possible that the older motoneurons at the time of plating (generated at E12) might already have been dependent on muscle, and therefore lost soon after plating. Nevertheless, under all conditions tested, motoneurons presented a similar morphological development during the first 12h in culture.

Several observations were obtained from these studies. CPDL and fibroblast monolayers are non-permissive substrata for the survival and growth of motoneurons. Aside from a small increase in cell body size, motoneurons did not grow when cultured on CPDL during 2d of survival. At 48h there was already retraction of neurites and therefore a reduction in branching in most cells. Although motoneurons presented a similar behaviour on fibroblasts, a small increase in neurite length and proximal branches was observed after 2d. Glia cells and myotubes were better permissive substrata and environments for motoneurone development and growth. Although motoneurons survived for a week over glia cells, cell body size, and the number of primary neurites and proximal branching

increased for 2-4 days. In contrast, neurite length increased for almost the whole time of survival, but with a reduction in branching density for the total neurite area. Myotubes were the most appropriate environment for growth, not only in size and neurite length, but also in branching. Although in this condition motoneurons grew continuously for at least 14d, the number of primary neurites and proximal branching stabilized at the same level and time (2-4d) as observed with glial cells.

These results provide clear evidence that glia cells and myotubes are critical for the morphological development and growth of motoneurons. They suggest that factors that regulate motoneurone survival may be different from those that affect the pattern of neurite growth, but also that the different type of neurite growth observed may be regulated by different signals. Finally, that different signals or conditions may be required to unfold, in a timed sequence, the features of the morphological development of motoneurons. The number of primary neurites, basic cell body shape, and proximal branching and polarity are probably expressed early during development, previous to cell death, and target independent, and controlled mainly by endogeneous and glial (radial) signals. In contrast, cell body size, neurite length and distal branching are features probably more dependent on environmental instructions, particularly from their targets and afferents, and occur after the period of cell death.

As shown in previous sections, non-neuronal cells affected motoneurone survival, but whether non-neuronal cells affect the growth and neurite pattern of motoneurons had not been shown before. Several studies have referred to the effect of CM or CM fractions on neurite outgrowth, but they have either used the % cells with neurites (Dorhmann et al., 1986) as index of growth (see above)

and/or have used non-identified spinal cord cell preparations (Henderson et al., 1981). Motoneurons cultured over glia cells, and particularly with myotubes, presented a clear increase in cell body size, and in the number, length and branches of neurites. Some of these features, like neurite elongation, were clearly related to survival, as it increases during the whole period of survival under each condition.

That this effect on morphological development is more than a consequence of increased survival, is suggested by two observations. Motoneurons cultured on fibroblasts presented a similar low survival to that on CPDL, nevertheless, a small increase in neurite length and percentage of cells with long axons together with a small increase in proximal branching were observed after 2d. Although fibroblasts do not normally support neurite growth in central neurones, a small but consistent growth of a few unbranched neurites was observed in E15 spinal cord rat neurones by Fallon (1985a). This small increase in neurite length may be mediated by an increase in motoneurone adhesion on fibroblasts. These cells express fibronectin (Akiyama & Yamada, 1987), which promote neurite growth (Rogers et al., 1985), but accordingly, this effect is less marked or unsustained on central neurones (Rogers et al., 1987). The fact that glia monolayers supported the survival of motoneurons for one week but the morphological growth for only 2-4 days, is further evidence suggesting that different signals regulate survival and morphological development. Such stabilization in growth after a few days was not simply a consequence of a reduced cell viability of motoneurons as shown by the continuous neurite growth (in length and % cell with long neurites) observed for at least 6d. The differentiation between survival and neurite growth factors has been difficult (Calof & Reichardt, 1984) because the first is a pre-requirement for

the second, and because neurite growth is dependent on attachment and adhesion. Also, previously inappropriate indices have been used to determine neurite growth (see above). Nevertheless, the findings that different fractions and peptides from muscle CM may independently regulate survival and the level of ChAT in motoneurons (Smith et al., 1986; Martinou et al., 1989a), suggest that different signals for neurite growth may also exist. In culture, glial cells not only express fibronectin but also laminin (Price & Hynes, 1985). Laminin is a component of muscle CM (Lander et al., 1985), and promotes the survival and neurite growth of sympathetic neurons (Edgar, Timpl & Thoenen, 1984); it is also an essential component of the neurite-promoting activity of myotube CM on motoneurons (Calof & Reichardt, 1985). Recent studies suggest, however, that laminin may have a neurite growth activity independent of cell adhesion and of survival (see Lander, 1990). Several mechanisms may account for this dissociation between survival and morphological development and/or neurite length, but it is possible that survival factors and cell adhesion molecules (Millaruelo, Nieto-Sampedro & Cotman, 1988) acting cooperatively at different levels and times during development, may be responsible for the different effects on survival and neurite growth observed in motoneurons.

The observation that for a similar duration glia cells promote the development of certain morphological features (i.e., primary neurites, proximal branching) to a similar degree as when motoneurons are cultured with myotubes, suggest that given a permissive environment for survival, intrinsic signals are also important for the development of these features. The fact that a small increase in proximal branching occurred in motoneurons cultured over fibroblast supports this suggestion. The permissive effects of glia cells on cell body size persist for 2d,

whereas the effect on neurite length persists for the entire survival time, but both effects occurred at a lower level than in the presence of myotubes. This means that different events in the morphological development of motoneurons are regulated differently, and also that certain environmental signals are required for their full development. On morphological grounds, one can assume that neurites with proximal branching corresponded to dendrites, and long neurites to axons (see Banker & Cowan, 1979). Distal branching was the result of terminal axonal branching, but distal dendritic branching also occurred as observed in cultured chick motoneurons (Role, Matossian, O'Brien & Fischbach, 1985; Role & Fischbach, 1987). Taken together these results suggest that the basic dendritic pattern of motoneurons is developed early probably through intrinsic and glial signals, but different from those responsible for axonal elongation and branching and distal dendritic branching, that appear to be target dependent. The importance of glial cells on dendritic growth is also supported by similar observations on sympathetic and mesencephalic neurons (Tropea, Johnson & Higgins, 1988; Dennis-Donini, Glowinski & Prochiantz, 1984). Although dendritic growth is mainly a postnatal phenomenon, there is evidence that, prior to cell death and the establishment of neuromuscular connections, motoneurons have already developed a basic dendritic pattern (Windle & Baxter, 1936; Smith & Hollyday, 1983; Wentworth, 1984a).

Little is known about the signals that regulate the development of dendrites, but several lines of evidence suggest that different types of neurone use different strategies. Studies of normal development and experimental deafferentation suggest that afferent inputs regulate the growth of dendrites in some central neuronal systems (Rakic, 1972; Berry, Bradley & Borges, 1978; Deitch & Rubel, 1984). In

others, like sympathetic ganglion neurones, dendritic growth appears largely independent of preganglionic inputs (Voyvodic, 1987). Indeed, in the latter study, after denervation sympathetic ganglion neurones presented a similar dendritic growth as in controls up to the first postnatal month, and thereafter a continuous but slower growth. No changes in primary neurites, dendritic branching and cell body size were observed 3-6 months after denervation. A correlation between somatic and dendritic growth was found suggesting that target signals primarily influence dendritic growth in sympathetic ganglionic neurones. In the present study a target dependence of distal dendritic branching was observed, but the presence of interneurones may have had some influence on the growth of dendrites. Nevertheless, Standler and Bernstein (1984) showed that dendritic length in adult spinal motoneurones was relatively insensitive to the disruption of descending central connections, although dendrites became shorter after axotomy (Standler & Bernstein, 1982).

Studies on extracellular matrix and cell adhesion molecules suggest that in addition to retrograde factors, local signals may also participate in neurite growth (see Lander, 1990). In a recent study (Hantaz-Ambroise, Vigny & Koenig, 1987) laminin promoted an increase in the branching of short neurites around the perikarya of rat spinal cord neurones in culture, while heparan sulphate proteoglycan (HSPG) increased neurite elongation. Laminin and HSPG are components of muscle basal lamina and are already present in pre-muscle masses in chick embryo hindlimb (Godfrey, Siebenlist, Wallskog, Walters, Bolender & Yorde 1988). Although those molecules may have influenced dendritic growth and neurite elongation when motoneurones were co-cultured with myotubes the fact that glia cells also produce laminin (see above) suggest that other factors or

signals may be involved in the type of dendritic growth observed in motoneurons cultured over astrocytes. Chamak and Prochiantz (1989) observed that dendritic growth in mesencephalic neurones was strongly dependent on cell adhesion. It is possible therefore, that cell adhesion molecules influence the development of dendrites as well as neurite growth. To explore this on a simpler system before application to motoneurons, cerebellar neurones were cultured over fibroblast monolayers transfected with different isoforms of NCAM (Doherty, Fruns, Seaton, Dickson, Barton, Sears & Walsh, 1990). An increase both in neurite elongation, and branching, was observed. Both types of neurite growth were related to the amount of NCAM expressed on fibroblasts, with a steep increase after a threshold level. These results suggest that, by increasing cell adhesion or by other mechanisms, NCAM may regulate neurite growth (see Jessel, 1988). The presence of NCAM isoforms on embryonic myotubes at the end of the period of cell death in the chick embryo (Grumet, Rutishauser, & Edelman, 1982) and the NCAM-mediated strong adhesion between spinal cord neurites and muscle cells (Rutishauser, Grumet & Edelman, 1983), suggest that NCAM may participate in the development of nerve muscle connections. Also, recent observations suggest that neurite growth over astroglial surfaces depends on several adhesion systems, like NCAM, N-cadherin, and integrins (Neugebauer et al., 1988) and that these may change with the developmental age of neurones (Tomaselli, Neugebauer, Bixby, Lilien & Reichardt, 1988). Other studies have shown that NCAM, through cell-cell contact, regulates ChAT in sympathetic neurones (Acheson & Rutishauser, 1988), and that through cell adhesion molecules astrocytes not only influence neurite growth but may regulate the pattern of neurites and neuronal organization (Crossin, Prieto, Hoffman, Jones & Friedlander, 1990; Grierson, Petroski, Ling & Geller, 1990).

H. Prospective Studies

I have shown that the motoneurone cultures established in the present study is an appropriate in vitro model to investigate motoneurone development. Future work using this model but with pure populations of labelled motoneurons (cell sorting) would allow the study of other important extrinsic factors, like interneurons and Schwann cells, on the development and dendritic growth of motoneurons. At the same time, the use of fibroblasts transfected with the cDNA of different isoforms of NCAM and other cell adhesion molecules could be of great value to provide a better understanding of the molecular mechanisms underlying the different type of neurite growth of motoneurons. The use of specific markers for dendrites and axons (see Caceres, Banker & Binder, 1986) will be critical to the establishment of the different signals that regulate the different type of neurite growth observed in motoneurons in the present study. Also, to confirm the present findings on the early development of motoneurons, the need for specific markers is critical for their appropriate isolation. Another approach to study the cellular and molecular signals that regulate early events in motoneurone development, that arose from the work of this thesis, is the use of neuroepithelial or precursors cell lines using oncogene transfection, a line of research currently in development in our Department. These future studies together with the present work will also be important to obtain a better insight on the possible basic mechanisms underlying the motoneurone diseases and spinal muscular atrophies.

SUMMARY

1. In this work the survival and development of rat spinal motoneurons in culture were studied. The effects of non-neuronal cells on the growth of motoneurons was also investigated. For these purposes heterogeneous cultures of well identified lumbar embryonic rat motoneurons were established, after the retrograde transport of Rhodamine from muscles. Motoneurons were identified early in culture by their fluorescence through video-intensification microscopy and images stored in a video-recorder.
2. Electrophysiological recordings from labelled motoneurons, using the whole cell-patch clamp technique, demonstrated mean input resistances of $635\text{ M}\Omega$ with resting membrane potentials of -65 mV . Evoked action potentials, and inward and late outward currents were present under voltage clamp, probably corresponding to I_{Na} and I_{K} , respectively.
3. Stored images of labelled motoneurons and of motoneurons later in culture, after their location on a grid, were analysed with the help of an image analysis system, to study their morphology and development. Motoneurons were mainly phase-dark multipolar neurones, with up to 5 primary neurites, and a mean cell body diameter of $16 \times 10\ \mu\text{m}$ at 12h in culture. Cultured over glia and myotubes, motoneurons developed dendrite-like neurites and one or more axon-like long neurites. Branching presented a peak at around $60\ \mu\text{m}$ from the cell body. Early in culture dendrite-like neurites were organized in two main trunks similar to the polarity observed in embryonic motoneurons.

4. Motoneurons presented a low survival in collagen-poly-D-lysine and over fibroblast monolayers. In contrast, when cultured over glia monolayers, E13-14 motoneurons were able to survive for 1 week. However, E18-20 motoneurons survived only for about 3d. When cultured with myotubes or over glia monolayers with myotube conditioned media, motoneurons were able to survive for several weeks. Interneurons and DRG neurons, induced a moderate increase in motoneuron survival, and only for few days.

5. When cultured on CPDL or fibroblast monolayers, motoneurons develop poorly, but in the latter condition a small increase in proximal branching and in length of axon-like neurites were observed. Glia cells were a permissive substratum for the development of motoneurons up to 2-4d in culture with an increase in cell body size, proximal branching and number of primary neurites, and also with a continuous but slower growth in neurite elongation during the week of survival. However, a decrease in total branching density/primary neurites was observed in this condition. Cultured with myotubes, motoneurons grew continuously in cell body size, neurite elongation and total branching density/primary neurites for at least 14 days. The number of primary neurites and proximal branching density, however, increased up to 4d in culture and reached a similar level to that observed in motoneurons cultured over glia monolayers.

6. This study demonstrated that identified embryonic mammalian motoneurons presented similar electrophysiological and morphological features to embryonic motoneurons. Motoneurons were independent from the targets for their survival for at least 2-4 days in culture, that in the rat embryonic life goes up to the beginning of the period of natural cell death and the establishment of

neuromuscular connections. During this period motoneurons, cultured over glia cells or with myotubes, were able to develop a basic set of primary neurites and a proximal dendritic pattern, suggesting that intrinsic signals may be important for the development of these morphological features. Motoneuron size, the length of neurites and the distal branching, in contrast, appear to be more dependent on the presence of motoneuron targets.

8. Future studies using the culture system developed in the present work, or the use of pure sorted motoneurons would allow the study of the role of interneurons in motoneuron development, particularly in neurite growth. The use of specific markers for dendrites and axons, as well as the use of transfected fibroblast with cDNA of cell adhesion molecules, as in the study on cerebellar neurons, should permit the study of specific cellular and molecular signals important for the development and growth of dendrites and axons in embryonic mammalian motoneurons.

REFERENCES

- ACHESON, A., & RUTISHAUSER, U. (1988). Neural cell adhesion molecule regulates cell contact mediated changes in choline acetyltransferase activity of embryonic chick sympathetic neurons. *J. Cell Biol.*, 106, 479-486.
- AGUAYO, A.J. (1985). Axonal regeneration from injured neurons in the adult mammalian central nervous system. In : *Synaptic Plasticity*, C.W. Cotman, ed.. The Guilford Press, New York, N.Y.
- AKIYAMA, S.K., & YAMADA, K.M. (1987). Fibronectin. *Adv. Enzymol. Relat. Areas Mol. Biol.*, 57, 1-57.
- ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K., & WATSON, J.D. (1989). *Molecular Biology of The Cell*. Garland Publishing, Inc., New York.
- ALTMAN, J. & BAYER, S.A. (1984). The development of the rat spinal cord. *Adv. Anat. Embryol. Cell Biol.*, 85, 1-166.
- ANGULO y GONZALEZ, A.W. (1940). The differentiation of the motor cell columns in the cervical cord of Albino rat fetuses. *J. Comp. Neurol.*, 73, 469-488.
- ATLAS, M., & BOND, V.P. (1965). The cell generation cycle of the eleven day mouse embryo. *J. Cell Biol.*, 26, 19-24.
- BANKER, G.A., & COWAN, W.M. (1977). Rat hippocampal neurons in dispersed cell culture. *Brain Res.*, 126, 397-425.
- BANKER, G.A., & COWAN, W.M. (1979). Further observations on hippocampal neurons in dispersed cell in culture. *J. Comp. Neurol.*, 187, 469-494.
- BANKER, G.A. (1980). Trophic interactions between astroglia cells and hippocampal neurons in culture. *Science*, 209, 809-810.
- BARBER, R.P., PHELPS, P.E., HOUSER, C.R., CRAWFORD, G.D., & SALVATERRA, P.M. (1984). The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: and immunocytochemical study. *J. Comp. Neurol.*, 229, 329-346.
- BENNETT, M.R., LAI, K., & NURCOMBE, V. (1980). Identification of embryonic motoneurons in vitro: Their survival is dependent on skeletal muscle. *Brain Res*, 190, 537-542.
- BERG, D.K. (1978). Acetylcholine synthesis by chick spinal cord neurones in dissociated cell cultures. *Dev. Biol.*, 66, 500-512.
- BERG, D.K., & FISCHBACH, G.D. (1978). Enrichment of spinal cord cell cultures with motoneurons. *J. Cell Biol.*, 77, 83-97.

BERNSTEIN, J.J., & STANDLER, N.A. (1983). Dendritic alteration of rat spinal motoneurons after dorsal mince: computer reconstruction of dendritic fields. *Exp. Neurol.*, 82, 532-540.

BERRY, M., BRADLEY, P., & BORGES, S. (1978). Environmental and genetic determinants of connectivity in the central nervous system. An approach through dendritic field analysis. *Prog. Brain Res.*, 48, 133-148.

BIGNAMI, A., & DAHL, D. (1975). Astroglial protein in the developing spinal cord of the chick embryo. *Dev. Biol.*, 44, 204-209.

BONNER-FRASER, M. & LALLIER, T. (1988). A monoclonal antibody against a laminin-heparan sulfate proteoglycan complex perturbs cranial neural crest migration in vivo. *J Cell Biol.*, 106,1321-1329.

BORGES, L.F. & SIDMAN, R.L. (1982). Axonal transport of lectins in the peripheral nervous system. *J. Neurosci.*, 2, 647-653.

BOULAC, M., & MEININGER, V. (1983). Postnatal development and cell death in the sciatic motor nucleus of the mouse. *Exp. Brain Res.*, 50, 107-116.

BOULDER COMMITTEE (1970). *Anat.Rec.*, 166, 257-261.

BOZYCZKO, D. & HORWITZ, A.F. (1986). The participation of a putative cell surface receptor for laminin and fibronectin in peripheral neurite extension. *J. Neurosci.*, 6, 1241-1251.

BREEDLOVE, M.S., JORDAN, C.L., & ARNOLD, A.P. (1983). Neurogenesis of motoneurons in the sexually dimorphic spinal nucleus of the bulbocavernosus in rats. *Dev. Brain Res.*, 9, 39-43.

BURT, A.M. (1973). Choline acetyltransferase and neuronal maturation. In: *Progress in Brain Research* (Ford, D.H., ed.), vol. 40,pp 245-252. Elsevier, Amsterdam.

CACERES, A., BANKER, G., & BINDER, L. (1986). Immunocytochemical localization of tubulin and microtubule-associated protein 2 during development of hippocampal neurons in culture. *J.Neurosci.*, 6, 714-722.

CAJAL, S.R. (1929). *Studies on Vertebrate Neurogenesis* (translated by Guth, L., 1960). Chapter 1; Charles C. Thomas, Springfield, Illinois, USA.

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

CALOF, A.L. & REICHARDT, L.F. (1985). Response of purified chick motoneurons to myotube conditioned medium: Laminin is essential for the substratum-binding, neurite outgrowth-promoting activity. *Neurosci. Lett.*, 59, 183-189.

CARR, V.M. & SIMPSON, S. (1982). Rapid appearance of labeled degenerating cells in the dorsal root ganglia after exposure of chick embryos to tritiated thymidine. *Dev. Brain Res.*, 2, 157-162.

CHAMAK, B. & PROCHIANTZ, A. (1989). Influence of extracellular matrix proteins on the expression of neuronal polarity. *Development*, 106, 483-491.

CHOI, B.H., KIM, R.C. & LAPHAM, L.W. (1983). Do radial glia give rise to both astroglial and oligodendroglial cells? *Dev. Brain Res.*, 8, 119-130.

CHRISTIE, G.A. (1964). Developmental stages in somite and post-somite rat embryos, based on external appearance, and including some features of the macroscopic development of the oral cavity. *J. Morphol.*, 114, 263-283.

CHU-WANG, I-W. & OPPENHEIM, R.W. (1978). Cell death of motoneurons in the chick embryo spinal cord. I. A light and electron microscopic study of naturally occurring and induced cell loss during development. *J. Comp. Neurol.*, 177, 33-58.

COWAN, W.M. (1979). Selection and control in neurogenesis. In: *The Neurosciences. Fourth Study Program.* F.O. Schmitt and F.G. Worden (eds.); pp 59-79. The MIT Press. Cambridge, Massachusetts.

CROSSIN, K.L., PRIETO, A.L., HOFFMAN, S., JONES, F.S., & FRIEDLANDER, D.R. (1990). Expression of adhesion molecules and the establishment of boundaries during embryonic and neural development. *Exp. Neurol.*, 109, 6-18.

CULHEIM, S. (1978). Relations between cell body size, axon diameter and axon conduction velocity of cat sciatic motoneurons stained with horseradish peroxidase. *Neurosci. Lett.*, 8, 17-20.

DAVIES, A. (1990). Personal Communication.

DEITCH, J.A. & RUBEL, E.W. (1984). Afferent influences on brainstem auditory nuclei of the chicken: time course and specificity of dendritic atrophy following deafferentation. *J. Comp. Neurol.*, 229, 66-79.

DENNIS, M.J., ZISKIND-CONHAIM, L. & HARRIS, A.J. (1981). Development of neuromuscular junctions in rat embryos. *Dev. Biol.*, 81, 266-279.

DENIS-DONINI, S., GLOWINSKI, J., & PROCHIANTZ, A. (1984). Glial heterogeneity may define the three-dimensional shape of mouse mesencephalic dopaminergic neurons. *Nature*, 307, 641-643.

DOHERTY, P., FRUNS, M., SEATON, P., DICKSON, G., BARTON, CH., SEARS, T.A. & WALSH, F.S. (1990). A threshold effect of the major isoforms of NCAM on neurite outgrowth. *Nature*, 343, 464-466.

DORHMANN, U., EDGAR, D., SENDTNER, M. & THOENEN, H. (1986). Muscle-derived factors that support survival and promote fiber outgrowth from embryonic chick spinal cord motor neurons in culture. *Dev. Biol.*, 118, 209-221.

- DOHRMANN, U., EDGAR, D. & THOENEN, H. (1987). Distinct neurotrophic factors from skeletal muscle and central nervous system interact synergistically to support the survival of cultured embryonic spinal motor neurons. *Dev. Biol.*, 124, 145-152.
- DOTTI, C.G., SULLIVAN, C.A., & BANKER, G.A. (1988). The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.*, 8, 1454-1468.
- DRIBIN, L.B. & BARRET, J.N. (1980). Conditioned medium enhances neuritic outgrowth from rat spinal cord explants. *Dev. Biol.*, 74, 184-195.
- EAGLESON, K.L., RAJU, T.R., & BENNETT, M.R. (1985). Motoneurone survival is induced by immature astrocytes from developing avian spinal cord. *Dev. Brain Res.*, 17, 95-104.
- EAGLESON, K.L. & BENNETT, M.R. (1986). Motoneurone survival requirements during development: the change from immature astrocyte dependence to myotube dependence. *Dev. Brain Res.*, 29, 161-172.
- EDGAR, D., TIMPL, R., & THOENEN, H. (1984). Heparin-binding domain of laminin is responsible for its effect on neurite outgrowth and neuronal survival. *EMBO J.*, 3, 1463-1468.
- FALLON, J.R. (1985a). Preferential outgrowth of central nervous system neurites on astrocytes and schwann cells as compared with nonglial cells in vitro. *J. Cell Biol.*, 100, 198-207.
- FALLON, J.R. (1985b). Neurite guidance by non-neuronal cells in culture: preferential outgrowth of peripheral neurites on glial as compared to nonglial cell surfaces. *J. Neurosci.*, 5, 3169-3177.
- FEDOROFF, S. & HERTZ, L. (1977). *Cell, tissue and organ culture in neurobiology*. Academic Press, New York.
- FISCHBACH, G.D. (1972). Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev. Biol.*, 28, 407-429.
- FONTAINE, B., KLARSFELD, A., HOKFELT, T., & CHANGEUX, J.P. (1986). Calcitonin gene-related peptide, a peptide present in the spinal cord motoneurons, increases the number of acetylcholine receptors in primary cultures of embryo chick myotubes. *Neurosci. Lett.*, 71, 59-65.
- FONTAINE-PERUS, J.C., CHANCONNIE, M., LE DOUARIN, N., GERSHON, M., & ROTHMAN, T. (1989). Mitogenic effect of muscle on the neuroepithelium of developing spinal cord. *Development*, 107, 413-422.
- FREDERIKSEN, K. & MCKAY, R.D.G. (1988). Proliferation and differentiation of rat neuroepithelial precursor cells in vivo. *J. Neurosci.*, 8, 1144-1151.
- FRESHNEY, I.R. (1983). *Culture animal cells. A manual of basic techniques*. Alan R. Liss, Inc., New York.

- FRITZSCH, B., DUBUC, R., OHTA, H., & GRILLNER, S.J. (1989). Efferents to the labyrinth of the river lamprey (*Lampetra fluviatilis*) as revealed with retrograde tracing techniques. *Neurosci. Lett.*, 96, 241-246.
- FRUNS, M., KRIEGER, C., & SEARS, T.A. (1987). Identification and electrophysiological investigations of embryonic mammalian motoneurons in culture. *Neurosci. Lett.*, 83, 82-88.
- FUJITA, S. (1964). Analysis of neuron differentiation in the central nervous system by tritiated thymidine autoradiography. *J. Comp. Neurol.*, 122, 311-327.
- FUJITA, H. & FUJITA, S. (1964). Electron microscopic studies on neuroblast differentiation in the central nervous system of domestic fowl. *Z. Zellforsch. Mikrosk. Anat.*, 60, 463-478.
- FULTON, B.P. & WALTON, K. (1986). Electrophysiological properties of neonatal rat motoneurons studied *in vitro*. *J. Physiol. (Lond.)*, 370, 651-678.
- GALLAGHER, J.T., LYON, M., & STEWARD, S.P. (1986). Structure and function of Heparan Sulfate Proteoglycan. *Biochem. J.*, 236, 313-325.
- GATCHALIAN, C.L., SCHACHNER, M., & SANES, J.R. (1989). Fibroblasts that proliferate near denervated synaptic sites in skeletal muscle synthesize the adhesive molecules Tenascin (J1), N-CAM, Fibronectin, and a Heparan Sulfate Proteoglycan. *J. Cell Biol.*, 108, 1873-1890.
- GODFREY, E.W., SCHRIER, B.K., & NELSON, P.G. (1980). Source and target cell specificities of a conditioned medium factor that increases choline acetyltransferase activity in cultured spinal cord cells. *Dev. Biol.*, 77, 403-418.
- GODFREY, E.W., SIEBENLIST, R.E., WALLSKOG, P.A., WALTERS, L.M., BOLENDER, D.L., & YORDE, D.E. (1988). Basal lamina components are concentrated in premuscle masses and at early acetylcholine receptor clusters in the chick embryo hind limb muscles. *Dev. Biol.*, 130, 471-476.
- GRIERSON, J.P., PETROSKI, R.E., LING, D.S.F., & GELLER, H.M. (1990). Astrocyte topography and tenascin/cytotactin expression: correlation with the ability to support neuritic outgrowth. *Dev. Brain Res.*, 55, 11-19.
- GRUMET, M., RUTISHAUSER, U., & EDELMAN, G.M. (1982). Neural cell adhesion molecule is on embryonic muscle cell and mediates adhesion to nerve cells *in vitro*. *Nature*, 295, 693-695.
- HAMBURGER, V. (1948). The mitotic patterns in the spinal cord of the chick and their relations to histogenetic processes. *J. Comp. Neurol.*, 88, 221-283.
- HAMBURGER, V. (1958). Regression versus peripheral control of differentiation in motor hypoplasia. *Am. J. Anat.*, 102, 365-410.
- HAMBURGER, V. (1975). Cell death in the development of the lateral motor column of the chick embryo. *J. Comp. Neurol.*, 160, 535-546.

HAMBURGER, V. (1977). The developmental history of the motor neuron. *Neurosci. Res. Prog. Bull., MIT*, 15 (suppl), 1-36.

HANTAZ-AMBROISE, D., VIGNY, M., & KOENIG, J. (1987). Heparan sulfate proteoglycan and laminin mediate two different types of neurite outgrowth. *J. Neurosci.*, 7, 2293-2304.

HARADA, Y. & TAKAHASHI, T. (1983). The calcium component of the action potential in spinal motoneurons of the rat. *J. Physiol. (Lond.)*, 335, 89-100.

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

HAWTON, L. & KELLERTH, J.O. (1987). Regeneration by supernumerary axons with synaptic terminals in spinal motoneurons of cats. *Nature*, 325, 711-714.

HEATON, M.B., PAIVA, M., & SWAONSON, D. (1990). Comparative responsiveness of early chick neural tube neurons to muscle medium, laminin, NGF and fibronectin. *Dev. Brain Res.*, 52, 113-119.

HENDELMAN, W.J. & MARSHALL, K.C. (1978). HRP characterization of neurons in organized cultures of cerebellum. *Neurosci. Abstr.*, 3, 591.

HENDERSON, C.E., HUCHET, M., & CHANGEUX, J-P. (1981). Neurite outgrowth from embryonic chicken spinal neurons is promoted by media conditioned by muscle cells. *Proc. Natl. Acad. Sci. USA*, 78, 2625-2629.

HENDERSON, C.E., BENOIT, P., HUCHET, M., GUENET, J-L, & CHANGEUX, J-P. (1986). Increase of neurite-promoting activity for spinal neurones in muscles of 'paralyse' mice and tenotomised rats. *Dev. Brain Res.*, 25, 65-70.

HENRIKSON, C.K. & VAUGHN, J.E. (1974). Fine structural relationships between neurites and radial glial processes in developing mouse spinal cord. *J. Neurocytology*, 3, 659-675.

HERRUP, K. (1987). Roles of cell lineage in the developing mammalian brain. *Curr. Top. Dev. Biol.*, 21, 65-97.

HILL, M.A. & BENNETT, M.R. (1986). Motoneurone survival activity in extracts of denervated muscle reduced by prior stimulation of the muscle. *Dev. Brain Res.*, 24, 305-308.

HOCKFIELD, S. & McKAY, R. (1985). Identification of the major cell classes in the early mammalian neural tube. *J. Neurosci.*, 12, 3310-3328.

HOCKFIELD, S., FREDERIKSEN, K., & McKAY, R. (1986). The Differentiation of cells types in the vertebrate CNS. In: *Molecular Aspects of Neurobiology*. R. Levi-Montalcini, P. Calissano, E. Kandel, and A. Maggi (eds.); pp 121-131. Springer-Verlag, Berlin, Heidelberg.

HOLLEY, J.A. (1982). Early development of the circumferential axonal pathway in the mouse and chick spinal cord. *J. Comp. Neurol.*, 205, 371-382.

- HOLLEY, J.A., WIMER, C.C., & VAUGHN, J.E. (1982). Quantitative analyses of neuronal development in the lateral motor column of mouse spinal cord. III. Generation and settling patterns of large and small neurones. *J. Comp. Neurol.*, 207, 333-343.
- HOLLYDAY, M. & HAMBURGER, V. (1976). Reduction of naturally occurring cell motor neuron loss by enlargement of the periphery. *J. Comp. Neurol.*, 170, 311-320.
- HOLLYDAY, M. & MENDELL, L. (1976). Analysis of moving supernumerary limbs of *Xenopus Laevis*. *Exp. Neurol.*, 51, 316-324.
- HOLLYDAY, M. & HAMBURGER, V. (1977). An autoradiographic study of the formation of the lateral motor column in the chick embryo. *Brain Res.*, 132, 197-208.
- HOLLYDAY, M. (1980). Motoneuron histogenesis and the development of limb innervation. *Curr. Top. Dev. Biol.*, 15, 181-215.
- HONIG, M.G. & HUME, R.I. (1986). Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. *J. Cell Biol.*, 103, 171-187.
- INOUE, S. (1986) Video microscopy. Plenum Press, New York.
- JACOBSON, M. (1978). *Developmental Neurobiology*. 2nd edition, Plenum Press, New York.
- JACOBSON, M. (1985). Clonal analysis and cell lineages of the vertebrate central nervous system. *Ann. Rev. Neurosci.*, 8, 71-102.
- JESSEL, T.M. (1988). Adhesion molecules and the hierarchy of neural development. *Neuron*, 1, 3-13.
- KAUFFMAN, S.L. (1968). Lengthening of the generation cycle during embryonic differentiation of the mouse neural tube. *Exp. Cell Res.*, 49, 420-424.
- KLIGMAN, D. (1982). Isolation of a protein from bovine brain which promotes neurite extension from chick embryo cerebral cortex neurones in defined medium. *Brain Res.*, 250, 93-100.
- KONNISBERG, I.R. (1979). Skeletal myoblast in culture. In: *Methods in Enzymology*. Vol. LVIII (W.B. Jakoby & I.H. Pastan, eds.), pp. 511-527.
- KRIEGER, C. & SEARS, T.A. (1986). The development of voltage-dependent conductances in murine spinal cord neurones in culture. *J. Physiol. (Lond.)*, 381, 70P.
- KRIEGER, C. & SEARS, T.A. (1988). The development of voltage-dependent ionic conductances in murine spinal cord neurones in culture. *Can. J. Physiol. Pharmacol.*, 66, 1328-1336.

KUDO, N. & YAMADA, T. (1985). Development of the monosynaptic stretch reflex in the rat: An *in vitro* study. *J. Physiol.*, 369, 127-144.

LAING, N.G. & PRESTIGE, M.C. (1978). Prevention of spontaneous motoneurone death in chick embryos. *J. Physiol.*, 282, 33-34P.

LAING, N.G. (1982). Motor projection patterns in the hind limb of normal and paralysed chick embryos. *J. Embryol. Exp. Morphol.*, 72, 269-286.

LAMB, A.H. (1979). Evidence that some developing limb motoneurons die for reasons other than competition. *Dev. Biol.*, 71, 8-21.

LANCE-JONES, C. & LANDMESSER, L. (1981a). Pathway selection by chick lumbosacral motoneurons during normal development. *Proc. R. Soc. Lond [Biol.]*, 214, 1-18.

LANCE-JONES, C. & LANDMESSER, L. (1981b). Pathway selection by embryonic chick motoneurons in a experimentally altered environment. *Proc R. Soc. Lond. [Biol.]*, 214, 19-52.

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

LANDER, A.D., FUJII, D.K., & REICHARDT, L.F. (1985). Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned media. *Proc. Natl. Acad. Sci. USA*, 82, 2183-2187.

LANDER, A.D. (1990). Mechanisms by which molecules guide axons. *Curr. Op. Cell Biol.*, 2, 907-913.

LANDMESSER, L. (1978). The development of motor projection patterns in the chick hindlimb. *J. Physiol.*, 284, 391-414.

LANGMAN, J. & HADEN, C. (1970). Formation and migration of neuroblast in the spinal cord of the chick embryo. *J. Comp. Neurol.*, 138, 419-432.

LANSER, M.E. & FALLON, J.F. (1984). Development of the lateral motor column in the limbless mutant chick embryo. *J. Neurosci.*, 4, 2043-2050.

LANSER, M.E., CARRINGTON, J.L., & FALLON, J.F. (1986). Survival of motoneurons in the brachial lateral motor column of limbless mutant chick embryos depends on the periphery. *J. Neurosci.*, 6, 2551-2557.

LANSER, M.E. & FALLON, J.F. (1987). Development of the brachial lateral motor column in the wingless mutant chick embryo: motoneuron survival under varying degree of peripheral load. *J. Comp. Neurol.*, 261, 423-434.

LEBER, S.M., BREEDLOVE, S.M., & SANES, J.R. (1990). Lineage, arrangement, and death of clonally related motoneurons in chick spinal cord. *J. Neurosci.*, 10, 2451-2462.

LE DOUARIN, N.M. (1980). The ontogeny of the neural crest in avian chimaeras. *Nature*, 286, 633-669.

LETOURNEAU, P.C. (1975). Possible roles for cell-to-substratum adhesion of axonal elongation. *Dev. Biol.*, 44, 92-102.

LEVI-MONTALCINI, R. (1950). The origin and development of the visceral system in the spinal cord of chick embryo. *J. Morphol.*, 86, 253-283.

LEVITT, P.R., COOPER, M.L., & RAKIC, P. (1981). Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: An ultrastructural immunoperoxidase analysis. *J. Neurosci.*, 1, 27-39.

LEVITT P.R., COOPER, M.L., & RAKIC, P. (1983). Early divergence and changing proportion of neuronal and glial precursor cells in the primate cerebral ventricular zone. *Dev. Biol.*, 96, 427-484.

LIESI, P., DAHL, D., & VAHERI, A. (1983). Laminin is produced by early rat astrocytes in primary cultures. *J. Cell Biol.*, 96, 920-924.

LINDER, E., VAHERI, A., RUOSLAHTI, E., & WARTIOVARA, J. (1975). Distribution of fibroblast surface antigen in the developing chick embryo. *J. Exp. Med.*, 142, 41-49.

LYSER, K.M. (1968). An electron-microscope study of centrioles in differentiating motor neuroblasts. *J. Embryol. Exp. Morphol.*, 20, 343-354.

MacDERMOTT, A.B. & WESTBROOK, G.L. (1986). Early development of voltage-dependent sodium currents in cultured moused spinal cord neurones. *Dev. Biol.*, 113, 317-326.

MacHANWELL, S. & BISCOE, T.J. (1981). The sizes of motoneurons supplying hindlimb muscles in the mouse. *Proc. R. Soc. Lond.B*, 213, 201-216.

MacLEAN, N. & HALL, B.K. (1987). Cell commitment and differentiation. Cambridge University Press. Cambridge, Great Britain.

MARTIN, A.H. & LANGMAN, J. (1965). The development of the spinal cord examined by autoradiography. *J. Embryol. Exp. Morphol.*, 14, 23-25.

MARTINOU, J.C., LE VAN THAI, A., CASSAR, G., ROUBINET, F., & WEBER, M.J. (1989a). Characterization of two factors enhancing Choline Acetyltransferase activity in cultures of purified rat motoneurones. *J. Neurosci.*, 9, 3645-3656.

MARTINOU, J.C., BIERER, F., LE VAN THAI, A., & WEBER, M.J. (1989b). Influence of the culture substratum on the expression of choline acetyltransferase activity in purified motoneurons from rat embryos. *Dev. Brain Res.*, 47, 251-262.

MASUKO, S., KUROMI, H., & SHIMADA, Y. (1979). Isolation and culture of motoneurones from embryonic chicken spinal cords. *Proc. Natl. Acad. Sci. USA*, 76, 3537-3541.

McCARTHY, K.D. & DE VELLIS, J. (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat central tissue. *J. Cell Biol.*, 85, 890-899.

- McCOBB, D.P., BEST, P.M., & BEAM, K.G. (1989). Development alters the expression of calcium currents in chick limb motoneurons. *Neuron*, 2, 1633-1643.
- McCOBB, D.P., BEST, P.M., & BEAM, K.G. (1990). The differentiation of excitability in embryonic chick limb motoneurons. *J. Neurosci.*, 10, 2974-2984.
- McLENNAN, I.S. (1982). Size of motoneuron pool may be related to number of myotubes in developing muscle. *Dev. Biol.*, 92, 263-265.
- McMANAMAN, J.L., SMITH, R.G., & APPEL, S.H. (1985). Low molecular-weight peptide stimulates cholinergic development in ventral spinal cord cultures. *Dev. Biol.*, 112, 248-252.
- McMANAMAN, J.L., CRAWFORD, F.G., STEWART, S.S., & APPEL, S.H. (1988). Purification of a skeletal muscle polypeptide which stimulates choline acetyltransferase activity in cultured spinal cord neurons. *J. Biol. Chem.*, 263, 5890-5897.
- McMANAMAN, J.L., CRAWFORD, F.G., CLARK, R., RICHTER, J., & FULLER, F. (1989). Multiple neurotrophic factors from skeletal muscle: demonstration of effects of bFGF and comparison with the 22 kd ChAT development factor. *J. Neurochem.*, 53, 1763-1771.
- McMANAMAN, J.L., OPPENHEIM, R.W., PREVETTE, D. & MARCHETTI, D. (1990). Rescue of motoneurons from cell death by a purified skeletal muscle polipeptide: Effects of the ChAT developmental factor, CDF. *Neuron*, 4, 891-898.
- MILLARUELO, A.I., NIETO-SAMPEDRO, M., & COTMAN, C.W. (1988). Cooperation between nerve growth factor and laminin or fibronectin in promoting sensory neuron survival and neurite outgrowth. *Dev. Brain Res.*, 38, 219-238.
- MOODY, S.A. & HEATON, M.B. (1983). Ultrastructural observations of the migration and early development of trigeminal motoneurons in chick embryos. *J. Comp. Neurol.*, 216, 20-35.
- MORRIS, D.G. (1978). Development of functional motor innervation in supernumerary hindlimbs of the chick embryo. *J. Neurophysiol.*, 41, 1450-1465.
- MUDGE, A.W. (1984). Schwann cells induce morphological transformation of sensory neurones in vitro. *Nature*, 309, 367-369.
- MULLER, H.W., BECKH, S., & SEIFERT, W. (1984). Neurotrophic factor for central neurones. *Proc. Natl. Acad. Sci. USA*, 81, 1248-1252.
- NARAYANAN, C.H. & HAMBURGER, V. (1971). Motility in chick embryos with substitution of lumbosacral by brachial and brachial by lumbosacral spinal cord segments. *J. Exp. Zool.*, 178, 415-432.
- NEUGEBAUER, K.M., TOMASELLI, K.S., LILIEN, J., & REICHARDT, L.F. (1988). N-cadherin, NCAM, and integrins promote retinal neurite outgrowth on astrocytes in vitro. *J. Cell Biol.*, 107, 1177-1188.

NEWGREEN, D. & THIERY, J.P. (1980). Fibronectin in early avian embryos: synthesis and distribution along migratory pathways of neural crest cells. *Cell Tissue Res.*, 211, 269-291.

NOBLE, M., FOK-SEANG, J., & COHEN, J. (1984). Glia are a unique substrate for the *in vitro* growth of central nervous system neurons. *J. Neurosci.*, 4, 1892-1903.

NORNES, H.O. & DAS, G.D. (1974). Temporal pattern of neurogenesis in spinal cord of rat. I. An autoradiographic study-time and sites of origin and migration and settling patterns of neuroblasts. *Brain Res.*, 73, 121-138.

NURCOMBE, V., TOUT, S., & BENNETT, M.R. (1985). Motoneurone survival and neuritic outgrowth promoted by different cell types in embryonic muscle. *Dev. Brain Res.*, 21, 49-60.

O'BRIEN, R.J. & FISCHBACH, G.D. (1986). Isolation of embryonic motoneurons and their survival *in vitro*. *J. Neurosci.*, 6, 3265-3274.

OKADO, N. & OPPENHEIM, R.W. (1984). Cell death of motoneurons in the chick embryo spinal cord. IX. The loss of motoneurons following removal of afferents inputs. *J. Neurosci.*, 4, 1639-1652.

OKUN, L.M. (1981). Identification and isolation *in vitro* of neurones marked *in situ* by retrograde transport. Short course Syllabus "New Approaches in Developmental Biology". Soc. for Neurosci., Los Angeles, CA, Oct. 17-18, 1981.

OPPENHEIM, R.W. & CHU-WANG, I-W. (1977). Spontaneous cell death of motoneurons following peripheral innervation in the chick embryo. *Brain Res.*, 125, 154-160.

OPPENHEIM, R.W., CHU-WANG, I-W., & MADERDRUT, J.L. (1978). Cell death of motoneurons in the chick embryo spinal cord. III. The differentiation of motoneurons prior to their induced degeneration following limb-bud removal. *J. Comp. Neurol.*, 177, 87-112.

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

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

OPPENHEIM, R.W. (1986). The absence of significant postnatal motoneuron death in the brachial and lumbar spinal cord of the rat. *J. Comp. Neurol.*, 246, 281-286.

OPPENHEIM, R.W. (1987). Muscle activity and motor neuron death in the spinal cord of the chick embryo. In: *Selective neuronal death*. Wiley, Chichester (Ciba Foundation Symposium 126), pp 96-112.

OPPENHEIM, R.W., HAVERKAMP, L.J., PREVETTE, D., McMANAMAN, J.L., & APPEL, S.H. (1988). Reduction of naturally occurring motoneuron death *in vivo* by target-derived neurotrophic factor. *Science*, 240, 919-922.

- OPPENHEIM, R.W. (1990). Identification of early developing projections from spinal interneurons in the chick embryo with a neuron specific β -tubulin antibody: evidence for a new 'pioneer' pathway in the spinal cord. *Development*, 108, 705-716.
- PATTERSON, P.H. (1978). Environmental determination of autonomic neurotransmitter functions. *Ann. Rev. Neurosci.*, 1, 1-17.
- PATTERSON, M.K. Jr. (1979). Measurement of growth and viability of cells in culture. In: *Cell Culture*. W.B. Jakoby and I.H. Pastan, ed.. *Methods in Enzymology*, vol. LVIII; pp. 141-152. Academic Press Inc., London.
- PHELPS, P.E., BARBER, R.P., BRENNAN, L.A., MAINES, V.M., SALVATERRA, P.M., & VAUGHN, J.E. (1990). Embryonic development of four different subsets of cholinergic neurons in rat cervical spinal cord. *J. Comp. Neurol.*, 291, 9-26.
- PILAR, G. & LANDMESSER, L. (1976). Ultrastructural differences during embryonic cell death in normal and peripherally deprived ciliary ganglia. *J. Cell Biol.*, 68, 339-356.
- PITTMAN, R. & OPPENHEIM, R. (1978). Neuromuscular blockage increases motoneurone survival during normal cell death in the chick embryo. *Nature*, 271, 364-366.
- PITTMAN, R. & OPPENHEIM, R.W. (1979). Cell death of motoneurons in the chick spinal cord. IV. Evidence that a functional neuromuscular interaction is involved in the regulation of naturally occurring cell death and the stabilization of synapses. *J. Comp. Neurol.*, 187, 425-446.
- PIXLEY, S.K.R. & DE VELLIS, J. (1984). Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. *Dev. Brain Res.*, 15, 201-209.
- PRESTIGE, M.C. (1967). The control of cell number in the lumbar ventral horns during the development of *Xenopus laevis* tadpoles. *J. Embryol. Exp. Morphol.*, 18, 359-387.
- PRESTIGE, M.C. (1970). Differentiation, degeneration and the role of the periphery: quantitative considerations. In: *The Neurosciences. Second Study Program*. F.O.Schmitt (ed.); pp 73-82. The Rockefeller University Press. New York.
- PRICE, J. & HYNES, R.O. (1985). Astrocytes in culture synthesize and secrete a variant form of fibronectin. *J. Neurosci.*, 5, 2205-2211.
- PRICE, J., TURNER, D., & CEPKO, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA*, 84, 156-160.
- PURVES, D. & LICHTMAN, J.W. (1985). *Principles of Neural Development*. Sinauer Associates Inc. Publishers. Sunderland, Massachusetts.

QUINN, L.S., HOLTZER, H., & NAMEROFF, M. (1985). Generation of chick skeletal muscle cells in groups of 16 from stem cells. *Nature*, 313, 692-694.

RAKIC, P. (1972). Extrinsic cytological determinants of basket and stellate cell dendritic pattern in the cerebellar molecular layer. *J. Comp. Neurol.*, 146, 335-354.

RAKIC, P. (1981). Neuronal-glia interaction during brain development. *Trends Neurosci.*, 4, 184-187.

RAKIC, P. (1984). Emergence of neuronal and glial cell lineages in primate brain. In: *Cellular and Molecular Biology of Neuronal Development*. Ira B. Black (ed.); pp. 29-50. Plenum Press, New York and London.

RANSOM, B.R., NEALE, E., HENKART, M., BULLOCK, P.N., & NELSON, P.G. (1977). Mouse spinal cord in cell culture. I. Morphology and intrinsic neuronal electrophysiological properties. *J. Neurophysiol.*, 40, 1132-1150.

RIOPELLE, R.J. & CAMERON, D.A. (1984). Neurite-promoting factors from embryonic neurons. *Dev. Brain Res.*, 15, 265-274.

ROGERS, S.L., LETOURNEAU, P.C., PALM, S.L., McCARTHY, J., & FURCHT, L.T. (1975). Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. *Dev. Biol.*, 98, 212-220.

ROGERS, S.L., McCARTHY, J.B., PALM, S.L., FURCHT, L.F., & LETOURNEAU, P.C. (1985). Neuron-specific interactions with two neurite-promoting fragments of fibronectin. *J. Neurosci.*, 5, 369-378.

ROGERS, S.L., EDSON, K.J., LETOURNEAU, P.C., & McLOON, S.C. (1986). Distribution of laminin in the developing peripheral nervous system of the chick. *Dev. Biol.*, 113, 429-435.

ROGERS, S.L., LETOURNEAU, P.C., PETERSON, B.A., FURCHT, L.T., & MCCARTHY, J.B. (1987). Selective interaction of peripheral and central nervous system cells with two distinct cell-binding domains of fibronectin. *J. Cell Biol.*, 105, 1435-1442.

ROLE, L.W., MATOSSIAN, U.R., O'BRIEN, R.J., & FISCHBACH, G.D. (1985). On the mechanism of ACh receptor accumulation at newly formed synapses on chick myotubes. *J. Neurosci.*, 5, 2197-2204.

ROLE, L.W. & FISCHBACH, G.D. (1987). Changes in the number of chick ciliary ganglion neuron processes with time in cell culture. *J. Cell Biol.*, 104, 363-370.

ROSS, J.J., DUXSON, M.J., & HARRIS, A.J. (1987). Formation of primary and secondary myotubes in rat lumbrical muscles. *Development*, 100, 383-94.

RUTISHAUSER, U., GRUMET, M., & EDELMAN, G.M. (1983). Neural cell adhesion molecule mediates initial interactions between spinal cord neurons and muscle cells in culture. *J. Cell Biol.*, 98, 145-152.

- SAITO, K. (1979). Development of spinal reflexes in the rat fetus studied *in vitro*. *J. Physiol.*, 294, 581-594.
- SAKLA, F.B. (1959). Postnatal growth of the cervical spinal cord of the albino mouse and the dendritic organization of its ventral horn cells. *J. Comp. Neurol.*, 113, 491-505.
- SANES, J.R. (1982). Laminin, fibronectin and collagen in synaptic and extrasynaptic portion of muscle fiber basement membrane. *J. Cell Biol.*, 93, 442-451.
- SCHAFFNER, A.E. & OLEK, A.J. (1986). The developmental appearance of alfa-bungarotoxin binding sites on rodent spinalcord neurons in cell culture. *Dev. Brain Res.*, 25, 239-247.
- SCHAFFNER, A.E., ST. JOHN, P.A., & BARKER, J.L. (1987). Fluorescence-activated cell sorting of embryonic mouse and rat motoneurons and their long-term survival *in vitro*. *J. Neurosci.*, 7, 3088-3104.
- SCHMECHEL, D.E. & RAKIC, P. (1979). A golgi study os radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes. *Anat. Embryol.*, 156, 115-152.
- SCHNAAR, R.L. & SCHAFFNER, A.E. (1981). Separation of cells types from embryonic chicken and rat spinal cord: characterization of motoneuron enriched fractions. *J. Neurosc.*, 1, 204-217.
- SCHOLL, D.A. (1953). Dendritic organization in the neurons of the visual and motor cortices of the cat. *J. Anat.*, 87, 387-407.
- SEILHEIMER, B. & SCHACHNER, M. (1988). Studies of adhesion molecules mediating interactions between cells of peripheral nervous system indicate a major rol for L1 in mediating sensory neuron growth on Schwann cells in culture. *J. Cell Biol.*, 107, 341-351.
- SHOUKINAS, G.M. & Hinds, J.W. (1978). The development of the cerebral cortex in the embryonic mouse: an electron microscopy serial section analysis. *J. Comp. Neurol.*, 179, 795-830.
- SIDMAN, R.L. (1970). Cell proliferation, migration and interaction in the developing mammalian CNS. In: *The Neuroscience 2nd Study program*. F.O. Schmitt (ed.), pp 100-. The Rockefeller Univ. Press, New York.
- SIDMAN, R.L. & RAKIC, P. (1973). Neuronal migration, with special reference to developing human brain: A review. *Brain Res.*, 62, 1-35.
- SIKDAR, S.K., WARING, D.W., & MASON, W.T. (1986). Voltage activated ionic current in gonadotrophs of the ovine pars tuberalis. *Neurosci. Lett.*, 71, 95-100.
- SLACK, J.R. & POCKETT, S. (1982). Motor neurotrophic factor in denervated adult skeletal muscle. *Brain Res.*, 247, 138-140.

- SMITH, C.L. & HOLLYDAY, M. (1983). The development and postnatal organization of motor nuclei in the rat thoracic spinal cord. *J. Comp. Neurol.*, 220, 16-28.
- SMITH, R.G., McMANAMAN, J., & APPEL, S.H. (1985). Trophic effects of skeletal muscle extracts on ventral spinal cord neurones in vitro: separation of a protein with morphologic activity from proteins with cholinergic activity. *J. Cell Biol.*, 101, 1608-1621.
- SMITH, R.G., VACA, K., McMANAMAN, J., & Appel, S.H. (1986). Selective effects of skeletal muscle extract fractions on motoneuron development in vitro. *J. Neurosci.*, 6, 439-447.
- SOLOMON, F. (1979). Detailed neurite morphogenesis in sister neuroblastoma cells are related. *Cell*, 16, 165-169.
- SOLOMON, F. (1981). Specification of cell morphology by endogenous determinants. *J. Cell Biol.*, 90, 547-553.
- SPERRY, D.G. & GROBSTEIN, P. (1985). Regulation of neuron numbers in *Xenopus Laevis*: effects of hormonal manipulation altering size at metamorphosis. *J. Comp. Neurol.*, 232, 287-298.
- STANDLER, N.A. & BERSTEIN, J.J. (1982). Degeneration and regeneration of motoneuron dendrites after ventral root crush: computer reconstruction of dendritic fields. *Exp. Neurol.*, 75, 600-615.
- STANDLER, N.A. & BERSTEIN, J.J. (1984). Dendritic alteration of spinal motoneurons after ablation of somatomotor cortex. *Exp. Neurol.*, 83, 264-273.
- STERN, C.D., FRASER, S.E., KEYNES, R.J., & PRIMMETT, D.R.N. (1989). A cell lineage analysis of segmentation in the chick embryo. *Development*, 104, 1-21.
- ST.JOHN, P.A., KELL, W.M., MAZETTA, J.S., LANGE, G.D., & BARKER, J.L. (1986). Analysis and isolation of embryonic mammalian neurons by fluorescence-activated cell sorting.
- STRAZNICKY, K. (1963). Function of heterotopic spinal cord segments investigated in the chick. *Acta Biol. Hung.*, 14, 145-155.
- TANAKA, H. & OBATA, K. (1982). Survival and neurite growth of chick embryo spinal cord cells in serum-free culture. *Dev. Brain Res.*, 4, 313-321.
- TANAKA, H., SAKAI, M., & OBATA, K. (1982). Effects of serum, tissue extract, conditioned medium, and culture substrata on neurite appearance from spinal cord explants of chick embryo. *Dev. Brain Res.*, 4, 303-312.
- TANAKA, H. & OBATA, K. (1983). Survival of HRP-labeled spinal motoneurons of chick embryo in tissue and cell cultures. *Dev. Brain Res.*, 9, 390-395.

- TANAKA, H. & LANDMESSER, L.T. (1986). Cell death of lumbosacral motoneurons in chick, quail, and chick-quail chimera embryos: a test of the quantitative matching hypothesis of neuronal cell death. *J. Neurosci.*, 6, 2889-2899.
- TAPSCOTT, S.J., BENNETT, G.S., & HOLTZER, H. (1981a). Neuronal precursor cells in the chick neural tube express neurofilament proteins. *Nature*, 292, 836-838.
- TAPSCOTT, S.J., BENNETT, G.S., TOYAMA, Y., KLEINBART, F., & HOLTZER, H. (1981b). Intermediate filament proteins in the developing chick spinal cord. *Dev. Biol.*, 86, 40-54.
- TEMPLE, S. & RAFF, M.C. (1986). Clonal analysis of oligodendrocytes development in culture: evidence for a developmental clock that counts cell divisions. *Cell*, 44, 773-779.
- TENNYSON, V.M. (1970). The fine structure of the developing nervous system. In: *Developmental Neurobiology*; W. Himwich (ed.); pp 47-114. Thomas, Springfield, Illinois.
- TESSIER-LAVIGNE, M. & MUDGE, A.W. (1987). Distribution of calcitonin gene-related peptide in chick motoneuron pools. *Soc. Neurosci. Abstr.*, 13, 1609.
- THANOS, S. & BONHOEFFER, F. (1983). Investigation on the development and topographic order of retinotectal axons: anterograde and retrograde staining of axons and perikarya with Rhodamine *in vivo*. *J. Comp. Neurol.*, 219, 420-430.
- TIMPL, R., ROHDE, H., ROBEY, D.G., RENNARD, S.I., FOIDART, J.-M., & MARTIN, G. (1979). Laminin- a glycoprotein from basement membranes. *J. Biol. Chem.*, 254, 9933-9937.
- TOMASELLI, K.J., NEUGEBAUER, K.M., BIXBY, J.C., LILIEN, J., & REICHARDT, L.F. (1988). N-cadherin and integrins: two receptor systems that mediate neuronal process outgrowth on astrocytes surfaces. *Neuron*, 1, 33-43.
- TOSNEY, K.W. & LANDMESSER, L. (1985). Specificity of motoneuron growth cone outgrowth in the chick hindlimb. *J. Neurosci.*, 5, 2336-2344.
- TOSNEY, K.W., WATANABE, M., LANDMESSER, L., & RUTISHAUSER, U. (1986). The distribution of NCAM in the chick hindlimb during axon outgrowth and synaptogenesis. *Dev. Biol.*, 114, 437-452.
- TROPEA, M., JOHNSON, M.I., & HIGGINS, D. (1988). Glial cells promote dendritic development in rat sympathetic neurons *in vitro*. *Glia*, 1, 380-392.
- VAN STRAATEN, H.W.M., HEKKING, J.W.M., BEURSGENS, J.P.W.M., TERWINDT-LOUWENHORST, E., & DRUKKER, J. (1989). Effect of the notochord on proliferation and differentiation in the neural tube of the chick embryo. *Development*, 107, 793-803.
- VARON, S.S. & RAIBORN, C.W. (1969). Dissociation, fractionation and culture of embryonic brain cells. *Brain Res.*, 12, 180-199.

VARON, S.S. & BUNGE, R.P. (1978). Trophic mechanisms in the peripheral nervous system. *Ann. Rev. Neurosci.*, 1, 327-361.

VOYVODIC, J.T. (1987). Development and regulation of dendrites in the rat superior cervical ganglion. *J. Neurosci.*, 7, 904-912.

WALTON, K. & FULTON, B. (1983). Hydrogen peroxide as a source of molecular oxygen for in vitro mammalian CNS preparations. *Brain Res.*, 278, 387-393.

WENGER, B.S. (1951). Determination of structural patterns in the spinal cord of the chick embryo studied by transplantation between brachial and adjacent levels. *J. Exp. Zool.*, 116, 123-149.

WENTWORTH, L.E. & HINDS, J.W. (1978). Early motoneuron formation in the cervical spinal cord of the mouse: An electron microscopic serial analysis. *J. Comp. Neurol.*, 177, 611-634.

WENTWORTH, L.E. (1984a). The development of the cervical spinal cord of the mouse embryo. I. A Golgi analysis of ventral root neuron differentiation. *J. Comp. Neurol.*, 222, 81-95.

WENTWORTH, L.E. (1984b). The development of the cervical spinal cord of the mouse embryo. II. A Golgi analysis of sensory, commissural and association cell differentiation. *J. Comp. Neurol.*, 222, 96-115.

WILKIN, G.P., MARRIOTT, D.R., & CHOLEWINSKI, A.J. (1990). Astrocyte heterogeneity. *Trends Neurosci.*, 13, 43-46.

WILLIAMS, R.W. & HERRUP, K. (1988). The control of neuron number. *Ann. Rev. Neurosci.*, 11, 423-453.

WINDLE, W.F. & BAXTER, R.E. (1936). Development of reflex mechanisms in the spinal cord of albino rat embryos. Correlation between structure and function and comparison with the cat and the chick. *J. Comp. Neurol.*, 63, 189-209.

WISNIEWSKI, H.M. (1987). Introduction. In: *Selective Neuronal Death*. Ciba Foundation Symposium 126. John Wiley & Sons; Sussex, U.K.

YAGINUMA, H., SHIGA, T., HOMMA, S., ISHIHARA, R., & OPPENHEIM, R.W. (1990). Identification of early developing projections from spinal interneurons in the chick embryo with a neuron specific β -tubulin antibody: evidence for a new 'pioneer' pathway in the spinal cord. *Development*, 108, 705-716.

YIP, J.W. & YIP, Y.P.L. (1990). Changes in fibronectin distribution in the developing peripheral nervous system of the chick. *Dev. Brain Res.*, 51, 11-18.

Identification and electrical properties of rat spinal motoneurones in culture

BY M. FRUNS, C. KRIEGER and T. A. SEARS. *Sobell Department of Neurophysiology, Institute of Neurology, Queen Square, London WC1N 3BG*

The evidence that the survival and growth of motoneurones is dependent on trophic factors is partly based on tissue culture studies in non-mammalian species. In such studies the *in vitro* identification of motoneurones is a major problem. To overcome this for studies in the mammal we have labelled motoneurones by the retrograde transport of fluoro-chromes from muscle prior to cell dissociation (Okun, 1981) and subsequently identified them using an image-intensification video camera and video recorder.

Pregnant rats were anaesthetized with Pentobarbital (40 mg/kg) and embryos were exteriorized and 1–3 μ l of fluoro-chrome (Lucifer Yellow–wheat-germ agglutinin, rhodamine isothiocyanate or sulphorhodamine) were injected into the hindlimb muscles using a glass micropipette. Embryos of 13.5–15 days gestation were removed from the mother, decapitated, eviscerated and incubated in a mixture of oxygenated Ham's F12 and Dulbecco's Modified Eagle's media at 30 °C. Embryos between 15 and 18 days were kept *in utero* in the anaesthetized mother. After 8–16 h *in utero* or *in vitro* the spinal cord was removed, sectioned and the ventral portion enzymically dissociated and plated on to glass coverslips in Petri dishes.

Image intensification greatly increased the ability to detect labelled motoneurones and by reducing the exposure time minimized light-induced damage.

Motoneurones comprised 0.5–2% of the total number of plated cells and were seen for up to 3–4 days in culture. They had cell body diameters between 15 and 25 μ m and had up to four processes. These techniques enabled whole-cell patch-clamp recording to be applied to identified motoneurones as early as 6–12 h in culture. Such recordings demonstrated input resistances of between 150 and 950 M Ω which are similar to those observed in cultured chick motoneurones (O'Brien & Fischbach, 1986). Spontaneously occurring action potentials were not observed, although spikes could be evoked by stimulation and these were sometimes followed by after hyperpolarizations.

Thus retrograde labelling with fluoro-chromes in conjunction with image intensification allows the identification of embryonic mammalian motoneurones in heterogenous culture. Identified motoneurones can survive in culture for a limited time and have some of the properties of motoneurones *in vivo*. Further use of these methods should facilitate studies of the mechanisms by which muscle-derived factors influence the development and maintenance of motoneurone structure and function.

Supported by the International Spinal Research Trust, the MRC (Canada) and the Motoneurone Disease Association.

REFERENCES

- OKUN, L. M. (1981). In *New Approaches in Developmental Neurobiology*, ed. GOTTLIEB, D., pp. 109–121. Washington: Society of Neuroscience.
O'BRIEN, R. J. & FISCHBACH, G. D. [(1986). *J. Neurosci.* 6, 3265–3274.

NSL 05010

Identification and electrophysiological investigations of embryonic mammalian motoneurons in culture

Manuel Fruns, Charles Krieger and Thomas Anthony Sears

Sobell Department of Neurophysiology, Institute of Neurology, London (U.K.)

(Received 13 July 1987; Revised version received 20 August 1987; Accepted 1 September 1987)

Key words: Rat; Motoneuron; Cell culture; Ionic current; Fluorescent retrograde labelling

The retrograde transport of fluorochromes from muscle was used to label embryonic rat motoneurons *in vivo*. The fluorescent motoneurons were subsequently detected in cultures of dissociated spinal cord neurones using an image-intensification video camera, the images from which allowed identification of the motoneurons under phase-contrast microscopy. Such motoneurons were mostly phase dark, often with a multipolar appearance and were detectable for up to 4 days in culture. Whole-cell patch-clamp recordings from these motoneurons demonstrated mean input resistances of 635 M Ω and resting membrane potentials of -65 mV; action potentials could be evoked and, under voltage-clamp, inward and outward currents were present.

Cultures of dissociated cells from mammalian spinal cord have been frequently used for the study of neural development and for investigations of basic mechanisms relevant to diseases affecting motoneurons [13]. In such cultures it has not been possible to distinguish motoneurons from other neurones. In this report we describe the labelling of rat motoneurons by the retrograde axonal transport of fluorochromes from muscle [10] and their subsequent detection in cultures of dissociated spinal neurones using an image-intensification video camera. The labelled cells have been studied electrophysiologically using the whole-cell patch-clamp method.

Timed sperm-positive Sprague–Dawley rats of 13.5–15 and 17–19 days gestation (E) were used. Rats were anaesthetized with pentobarbital (30–40 mg/kg) and E13.5–15 embryos were aseptically removed, decapitated, eviscerated and placed in Hank's balanced salt solution (HBSS, Flow, U.K.) with 0.6% glucose and 0.003% H₂O₂ at room temperature. Either Lucifer yellow conjugated with wheat germ agglutinin, 5% sulphorhodamine, or 0.4% rhodamine ITC (R-ITC) (all from Sigma, U.K.) were pressure injected (1–3 μ l) into the hindlimb and thoracic muscles. Embryos were incubated in DMEM:F12 (1:1, Flow, U.K.) bubbled with 95% O₂–5% CO₂ at 30°C for 4–6 h. E17–E19 embryos were injected *in utero* into the same sites and with the same

Correspondence: T.A. Sears, Sobell Department of Neurophysiology, Institute of Neurology, Queen Square, London WC1N 3BG, U.K.

fluorescent labels as above and the embryos were maintained in the anaesthetized mother for a further 8–15 h. Subsequently spinal cords were removed from injected embryos and freed from meninges and dorsal root ganglia. The ventral spinal cord was sectioned, fragmented and incubated in 0.25% trypsin in Ca^{2+} , Mg^{2+} -free HBSS for 20 min at 37°C. Spinal cord fragments were washed twice in DMEM:F12 containing DNase (40 $\mu\text{g}/\text{ml}$, Sigma), dissociated and centrifuged in 3.5% bovine serum albumin. Cells were resuspended and $3\text{--}5 \times 10^4$ cells were plated onto poly-D-lysine and collagen-coated glass coverslips glued in 35 mm tissue culture dishes.

Dissociated cultures were grown in a mixture of DMEM:F12 (1:1) supplemented with 10% heat-inactivated horse serum, glutamine (2.5 mM), glucose (0.6%), insulin (10 U/ml), HEPES (10 mM), penicillin (100 U/ml) and streptomycin (50 $\mu\text{g}/\text{ml}$) at 37°C in 10% CO_2 -90% air. These heterogeneous cultures had a seeding efficiency of 70–75% with a viability of 85–90% by Trypan blue. After 6–12 h, labelled cells were detected under fluorescence microscopy with the aid of an image-intensification video camera (Panasonic, model 1900) attached to an inverted Zeiss microscope (ICM 405). The video camera was connected to a video tape recorder (VTR, Sony, U-matic, 5080) and an image analysis system (Sight Systems, U.K.). Each field, viewed through a $\times 40$ Neofluar objective (N.A. 0.75) was exposed to the exciting light for less than 3 s and the image captured in the VTR and image processor. Fluorescence and phase-contrast images were compared thus allowing the labelled motoneurons to be identified in phase-contrast. Whole-cell patch-clamp recordings [4] were made from these identified motoneurons using a List EPC-7 amplifier. Fire polished Sylgard-coated microelectrodes (1.5 mm o.d.) with tip diameters of 1–2 μm and resistances of 1–10 $\text{M}\Omega$ were advanced onto the cell under phase-contrast microscopy. The seal resistance was greater than 5 $\text{G}\Omega$ before breaking the patch. Recordings were corrected for the fast capacitative transients of the electrode. The data was filtered (Bessel, 2.5 kHz, –3 dB), recorded on an FM tape recorder and the signals were displayed on a digitizing oscilloscope and plotted on an x-y recorder.

Retrograde labelling of lumbosacral and thoracic motoneurons was clearly observed under the fluorescence microscope in fixed sections of spinal cord from intact injected embryos (Fig. 1A). In cultures of dissociated spinal cord neurones, individual motoneurons could occasionally be strongly labelled particularly within a few hours of dissociation (Fig. 1B,C). However, as previously noted by Okun [10], the majority of motoneurons were difficult to identify directly through the microscope because of their faint fluorescence. The identification was greatly improved with the use of the image-intensification video camera as illustrated in Fig. 1D,E for a 4-day-old labelled cell whose fluorescence was not detectable when viewed directly under the microscope. The number of labelled cells at 12 h in culture was 1–1.5% of the total cell number in E13.5–15 cultures and 0.3–0.5% of E17–19 cultures. These observations were independent of the fluorochrome used but more strongly labelled cells were seen using rhodamine labels. The number of labelled motoneurons in culture progressively decreased with time, but some motoneurons were seen for up to 4 days, especially in cultures from younger embryos. The fluorescent motoneurons had cell body diameters of 15–25 μm , were phase dark and had up to 4 processes

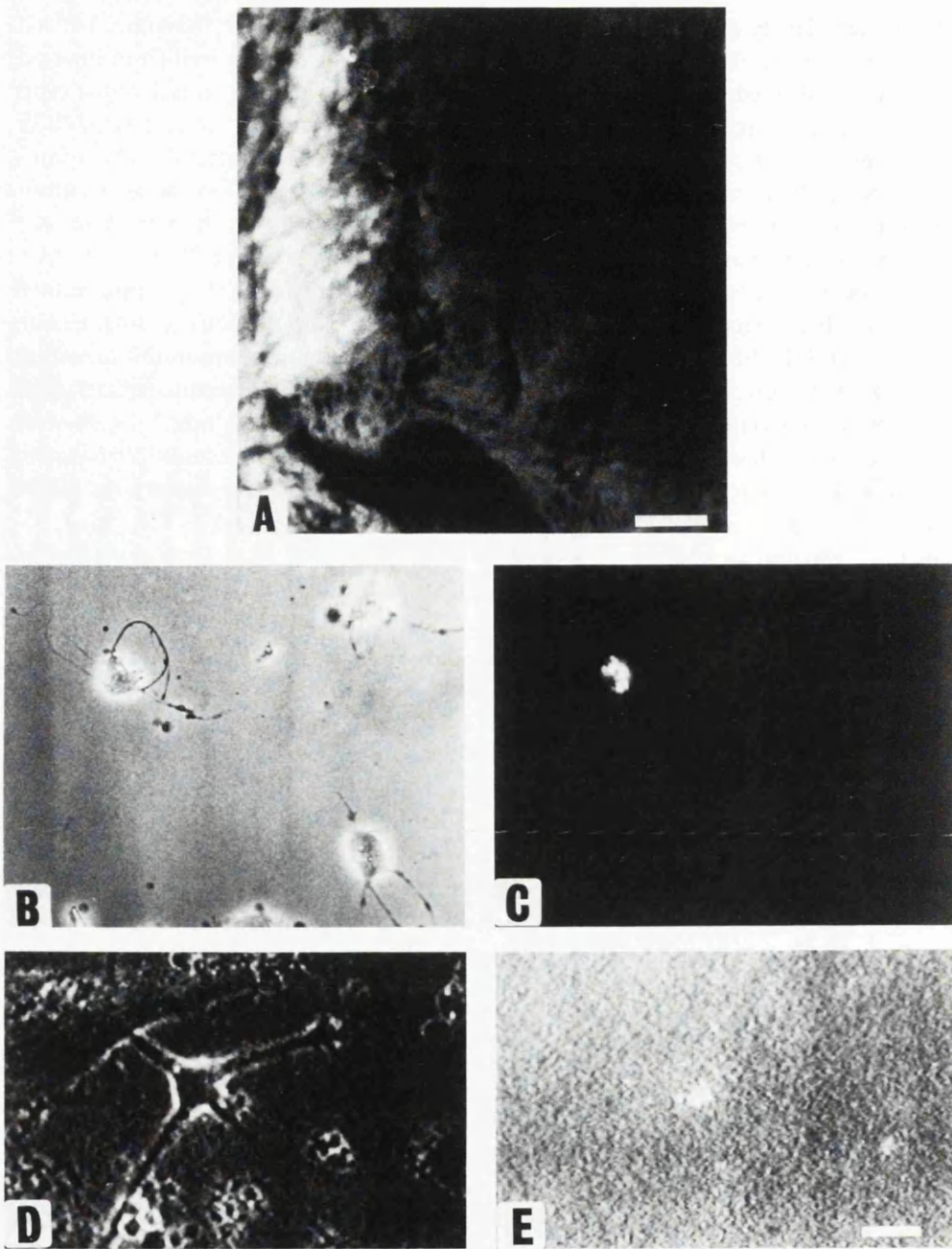


Fig. 1. A: retrograde labelling of thoracic motoneurons. A: 10 μm paraffin section of a 4% paraformaldehyde fixed thoracic spinal cord from an E14 embryo injected 6 h previously with rhodamine B in the right intercostal muscles. Fluorescence microscopy. Bar = 50 μm . B: phase contrast and C: fluorescence micrographs of an identified dissociated motoneurone from an E14 embryo after 6 h in culture. D: phase contrast and E: fluorescence micrographs of a dissociated motoneurone taken from the video monitor. Bar = 20 μm .

(Fig. 1D,E). There was also evidence for motoneurone death as suggested by the appearance of foamy, vacuolated, labelled motoneurones with short processes which were not electrically excitable; their number increased with the age of the embryo and also with their duration in culture.

The mean input impedance of identified motoneurones was $635 \pm 369 \text{ M}\Omega$ (\pm S.D., $n=10$) and the mean resting membrane potential was $-65.6 \pm 15.1 \text{ mV}$ ($n=10$). The capacitance of 10 motoneurones was calculated by determining the area of the capacitive current under voltage-clamp and the mean value was $19.1 \pm 9.6 \text{ pF}$. Plots of the rate of change of membrane potential were made from 4 motoneurones and they demonstrated curves fit by a single exponential corresponding to a mean membrane time constant of $18.6 \pm 7.1 \text{ ms}$. Some motoneurones had spontaneous, slow irregular fluctuations of membrane potential but these fluctuations did not generate action potentials (Fig. 2A). Action potentials were produced in response to depolarizing currents (Fig. 2B) or anode-break stimulation (Fig. 2C) in 7 of these 10 motoneurones; these had a mean amplitude of $59.1 \pm 14.9 \text{ mV}$ with a mean overshoot of

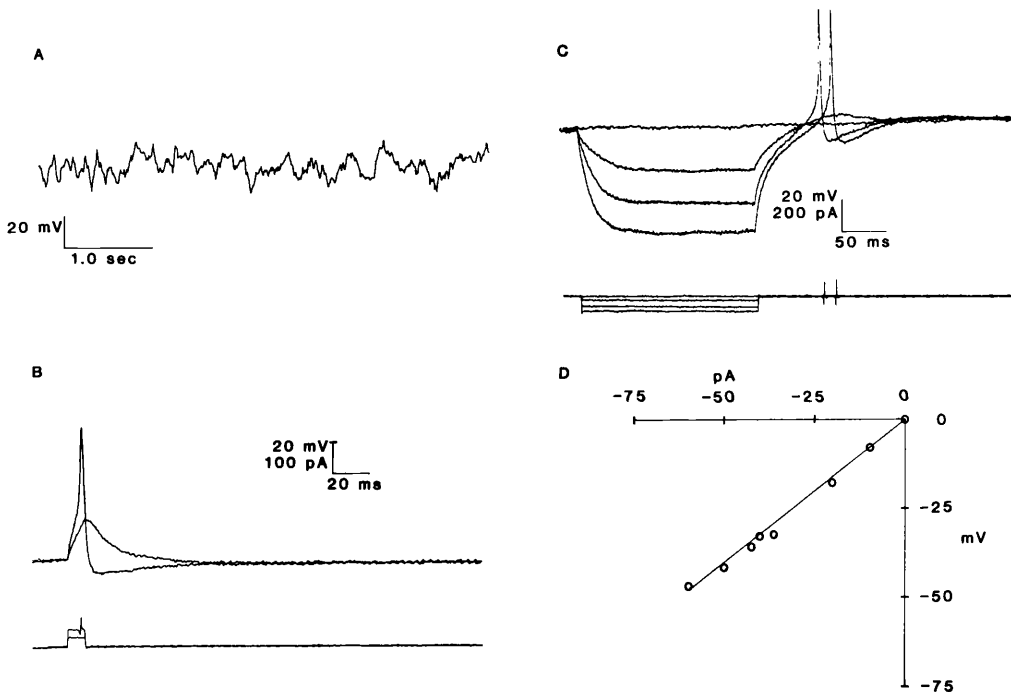


Fig. 2. Voltage recordings from identified motoneurones. A: membrane potential recordings demonstrating spontaneous voltage fluctuations. B: membrane potential changes to depolarizing current pulses of 10 ms duration. An evoked action potential is followed by a hyperpolarization. C: membrane potential changes (upper panel) to hyperpolarizing current steps (lower panel). D: $I-V$ plot obtained from records shown in C. Voltage scale is with respect to resting membrane potential (-55 mV). B-D are from same cell. The pipette solution was (in mM): KCl 140.0, MgCl_2 2.0, CaCl_2 1.0, HEPES 10.0, EGTA 10.0 adjusted to pH 7.2. The bath solution was composed of (mM): NaCl 140.0, KCl 5.0, MgCl_2 1.0, CaCl_2 1.8, HEPES 10.0, glucose 10.0, pH 7.3.

7.2 ± 12.3 mV and a mean duration at half-maximum amplitude of 2.4 ± 0.7 ms. In 3 of these motoneurons, action potentials were followed by afterhyperpolarizations having a mean duration of 57.3 ± 3.7 ms (Fig. 2B,C). The resistance of these motoneurons remained constant during hyperpolarizing current pulses indicating the absence of inward rectification (Fig. 2D).

When motoneurons were voltage-clamped in an external medium containing K^+ , Na^+ and Ca^{2+} , using a K^+ concentration in the pipette similar to that of the intracellular concentration, depolarization from a holding potential of -75 mV evoked both inward and outward currents in 7 of 10 motoneurons (Fig. 3A). The remaining 3

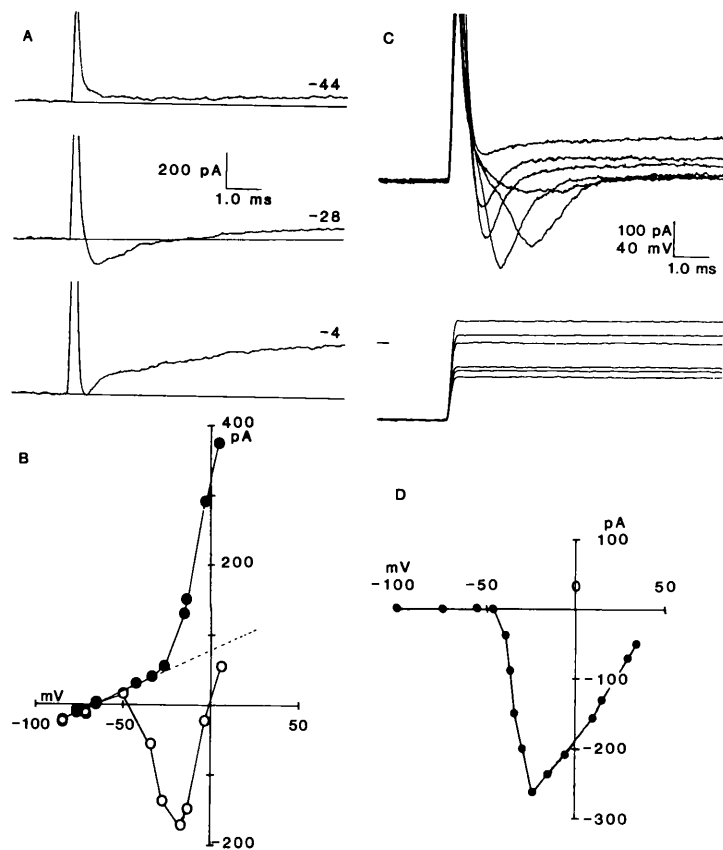


Fig. 3. Membrane current records from identified motoneurons. A: representative current recordings evoked by depolarizations from a holding potential of -75 mV to values indicated to the upper right of each trace. B: $I-V$ plot of the data shown in A. Open circles indicate the minimum values of the currents within 2 ms of the stimulus and filled circles indicate current values at 20 ms after the stimulus. Points are uncorrected for leak resistance. Extrapolated leak conductance is shown by interrupted lines. C: current records (upper panel) produced by depolarizing voltage commands from a holding potential of -90 mV (lower panel). D: $I-V$ relation of the peak inward current for the data in C. Data points are represented by filled circles. Data were not corrected for leak currents. Pipette solution in A, and bath solution in A and C as in Fig. 2. Pipette solution in C was (in mM): Cs acetate 140.0, $MgCl_2$ 2.0, $CaCl_2$ 1.0, HEPES 10.0, EGTA 10.0 adjusted to pH 7.2.

motoneurons demonstrated outward current only. The current-voltage relation uncorrected for leak resistance is shown in Fig. 3B. The inward current (open circles) deviated from the leak current at about -50 mV, it peaked at around -15 mV and had an extrapolated reversal potential of about $+25$ mV. A late, outward current was also present, having a threshold of -30 mV and this increased in magnitude with larger command voltages (filled circles). In two motoneurons examined, potassium currents were abolished by inclusion of Cs^+ in the pipette solution allowing the inward current to be observed in the absence of an outward current as illustrated in Fig. 3C. The current-voltage relationship for this inward current (Fig. 3D) showed that the current had a threshold of about -50 mV, it peaked at about -25 mV and had an extrapolated reversal potential of about $+50$ mV.

These results demonstrate that electrically excitable embryonic mammalian motoneurons labelled by the retrograde transport of fluorochromes from muscle can be detected in cultures of dissociated spinal cord neurones. The use of an image intensification video-camera with an image store system, not only facilitates the detection of the labelled cells, but also reduces the exposure to exciting light and consequently the risk of damage. Cultures of fluorescent labelled motoneurons from avian embryos enriched by cell sorting [1,2,9,10] and cultures of labelled motoneurons from rat embryos enriched by density gradient techniques [12] have been described showing morphological features similar to the present results. Such features include most commonly a phase dark, multipolar cell body of $15-25$ μm diameter.

These electrical recordings appear to be the first from identified embryonic mammalian motoneurons in culture. The motoneurons have input impedances of the same order of magnitude as observed in avian motoneurons in culture [9] but are much higher than those observed by conventional intracellular recording from *in vitro* preparations of isolated hemisectioned neonatal rat spinal cord [3]. These higher impedances can be attributed to the smaller size of cells and the less damaging effects of patch-clamp recording. Resting membrane potentials were similar to those observed in motoneurons in preparations of hemisectioned neonatal rat spinal cord [3,5]. Some motoneurons showed spontaneous fluctuations of the membrane potential in the absence of morphological or physiological evidence of synaptic contacts. Such fluctuations are reminiscent of those seen in cultured gonadotrophic cells of the ovine pituitary [11] but in both cases their origin and nature is unknown. Depolarizing and anode-break stimuli produced action potentials similar to those observed *in vivo* [8] or in isolated hemisectioned spinal cords from neonatal rats [3,5]. After-hyperpolarizations were present in some motoneurons. Voltage-clamp data show that cultured motoneurons possess an inward current and a late, potassium-dependent outward current. Although the ionic dependence of this inward current was not investigated with tetrodotoxin, it is likely to be sodium-dependent as it has a reversal potential of about $+50$ mV and has similar kinetics to the sodium-dependent inward current seen in cultured mouse spinal cord neurones [6,7].

In previous work on the avian spinal cord, fluorescent cell sorting following retrograde labelling of motoneurons has been used to obtain motoneurone-enriched cultures of dissociated spinal cord neurones thus facilitating the study of identified

motoneurons in culture. In the present work the use of a video intensification camera has enabled fluorescent labelled motoneurons to be identified in heterogeneous non-enriched cultures of dissociated mammalian spinal cord neurones and thus allowed their morphological and electrophysiological study.

Supported by the International Spinal Research Trust, MRC (Canada) and the Motoneurone Disease Association.

- 1 Calof, A.L. and Reichardt, L.F., Motoneurons purified by cell sorting respond to two distinct activities in myotube-conditioned medium, *Dev. Biol.*, 106 (1984) 194–210.
- 2 Eagleson, K.L. and Bennett, M.R., Motoneurone survival requirements during development: the change from immature astrocyte dependence to myotube dependence, *Dev. Brain Res.*, 29 (1986) 161–172.
- 3 Fulton, B.P. and Walton, K., Electrophysiological properties of neonatal rat motoneurons studied in vitro, *J. Physiol. (Lond.)*, 370 (1986) 651–678.
- 4 Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J., Improved patch clamp techniques for high resolution current recording from cells and cell-free membrane patches, *Pflugers Arch.*, 391 (1981) 85–100.
- 5 Harada, Y. and Takahashi, T., The calcium component of the action potential in spinal motoneurons of the rat, *J. Physiol. (Lond.)*, 335 (1983) 89–100.
- 6 Krieger, C. and Sears, T.A., The development of voltage-dependent conductances in murine spinal cord neurones in culture, *J. Physiol. (Lond.)*, 381 (1986) 70P.
- 7 MacDermott, A.B. and Westbrook, G.L., Early development of voltage-dependent sodium currents in cultured mouse spinal cord neurones, *Dev. Biol.*, 113 (1986) 317–326.
- 8 Naka, K.-I., Electrophysiology of the fetal spinal cord. I. Action potentials of the motoneuron, *J. Gen. Physiol.*, 47 (1964) 1003–1022.
- 9 O'Brien, R.J. and Fischbach, G.D., Isolation of embryonic chick motoneurons and their survival in vitro, *J. Neurosci.*, 6 (1986) 3265–3274.
- 10 Okun, L.M., Identification and isolation in vitro of neurons marked in situ by retrograde transport. In *New Approaches in Developmental Neurobiology* (short course syllabus), Society for Neuroscience, 1981, pp. 109–121.
- 11 Sikdar, S.K., Waring, D.W. and Mason, W.T., Voltage activated ionic currents in gonadotrophs of the ovine pars tuberalis, *Neurosci. Lett.*, 71 (1986) 95–100.
- 12 Smith, R.G., Vaca, K., McManaman, J. and Appel, S.H., Selective effects of skeletal muscle extract fractions on motoneurone development in vitro, *J. Neurosci.*, 6 (1986) 439–447.
- 13 Varon, S., Manthorpe, M. and Longo, F.M., Growth factors and motor neurones, In L.P. Rowland (Ed.), *Human Motor Neurone Disease*, *Advances in Neurology*, Vol. 36, Raven, New York, 1982, pp. 453–471.