NEURONAL VIABILITY OR DEATH AFTER DENDRITE AMPUTATION WITHIN 200 μm OF THE PERIKARYON: CORRELATION WITH ELECTROPHYSIOLOGIC AND MORPHOLOGICAL CHANGES AND THE PRESENCE OR ABSENCE OF EXTRACELLULAR CALCIUM

A DISSERTATION

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Date

To the Provost of the Graduate School:

I am submitting herewith a dissertation written by Jen Hill Lucas entitled "Neuronal Viability or Death After Dendrite Amputation Within 200 μm of the Perikaryon: Correlation With Electrophysiologic and Morphological Changes, And the Presence or Absence of Extracellular Calcium". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Molecular Biology.

Michael H Droge
Michael Droge, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted:

Leslie M. Thompson
Provost of the Graduate School

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ABSTRACT

The responses of cultured mammalian spinal neurons to physical trauma were observed following UV laser amputation of primary dendrites within 400 µm of perikarya. The following observations were made. First, neuronal survival is a function of lesion distance from the perikaryon and of process diameter at the lesion site. For an average lesion diameter of 3.2 µm, dendrite transections at 50 µm, 100 µm, and 150 µm were associated with survival probabilities of 31%, 54% and 70%, respectively. Second, the fate of the injured cells was definitely established 24 hours after injury and very likely was determined as early as two hours. Third, phase microscopic observation revealed that the early stages of deterioration leading to cell death were associated with increased cytoplasmic phase brightness; this optical effect correlated ultrastructurally with the appearance of numerous, small, electron-lucent vacuoles and swollen mitochondria. Fourth, the magnitude and time course of injury potentials recorded at the somata were a function of the lesion distance and did not return to prelesion levels within 30 minutes after transection. Fifth, at 24 hours after surgery, the average membrane potential of lesioned neurons was 8% below that of control

neurons. Sixth, at a lesion distance of approximately 300 μm both anticipated injury potentials and the probability of cell death approach zero. The influence of lesion physical parameters upon neuronal survival after dendrite amputation suggests that neuronal deterioration and death after dendrite amputation trauma depend upon the magnitude of the injury currents reaching the soma. Removal of calcium from the culture medium, however, does not protect lesioned neurons and actually decreases the probability of survival. It is, therefore, hypothesized that the influx of external sodium after physical trauma may mediate the death of injured neurons both indirectly by triggering the release of endogenous mitochondrial calcium stores, and directly by causing osmotic swelling and irreversible damage to mitochondria.

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CHAPTER I

INTRODUCTION

I. Rationale for the Study

Systematic investigation of the nervous system responses to trauma and mechanisms of repair date from early in the century (Schmaus, 1980; Allen, 1911, Allen, 1914; Young, 1949; Cajal, 1928). Such studies drew much of their original impetus from interest in the treatment of the unparalleled injuries received in the course of modern warfare (Young, 1949). At present, in our highly mobile society, the epidemic of moving vehicle accidents has transformed such injuries into a fact of modern living.

Most investigations of CNS trauma have utilized experimental models which focused on damage at the tissue level (Goodkin, 1973; Yeo, 1976; Puchala and Windel, 1977; Balentine, 1978; Molt et al., 1979; Balentine et al., 1982). A limitation of such models has been the difficulty of discriminating between the purely neuronal responses to injury and the secondary effects of other cells and tissues.

Using various types of "micro-knives" several investigators have studied the reactions of isolated single neurons to perforation or transection injuries (Peterfi and Kapel, 1928; Levi and Meyer, 1945; Shaw and Bray, 1977;

Yawo and Kuno, 1983, 1985). Two experimental systems were utilized which allowed surgery to be performed on individual neurons with minimal involvement of other cells and tissues. One approach was to isolate single invertebrate axons within an excised nerve cord so that separation from surrounding cells was achieved while attachment to the cell body and synaptic contacts were preserved (Yawo and Kuno, 1983, 1985). An alternative was to operate upon neurons growing in monolayer cultures of nervous tissue (Peterfi and Kapel, 1928; Levi and Meyer, 1945; Shaw and Bray, 1977). The latter approach also made possible examination of the responses of individual vertebrate neurons to axotomy.

Despite these advances, mechanical methods of cell surgery do not offer the control necessary for creating experimental lesions without concomitant distortion and even tearing of other areas of an operated cell. This limitation is particularly disadvantageous to studies of the responses of relatively small mammalian neurons. Such distortion has precluded concurrent electrical recording close to the lesion site during cell surgery. Most importantly, without the capability of creating highly localized injuries the physical parameters of the lesions could not be determined and the responses of neurons to physical trauma, including the probability of cell survival, could not be assessed quantitatively.

To resolve these difficulties, techniques of laser microbeam cell surgery have been developed which permit creation of lesions as small as one micrometer (μm) in diameter at precise locations along target neurites with minimal disturbance of other portions of target cells, surrounding structures and intracellular electrodes (Higgins et al., 1980; Gross et al., 1983; Kirkpatrick et al., 1985). This precision, in conjunction with the accessibility of individual neurons in monolayer cultures, has permitted the first observations of the effects of transection injuries specifically to dendrites (ibid.). Lesions at dendritic loci undoubtedly play an important role in CNS injury. Dendrites within the CNS may ramify over distances as great as 1000 μm from the soma (Shepherd, 1979). In addition, a dendritic lesion may represent a simpler form of neuronal injury than axotomy where myelination could introduce additional physical and chemical complicating factors (Lubinska, 1956; Kao et al., 1977).

II. Purpose of the Study

This study represents the first systematic examination of the early morphological and electrophysiologic responses of individual mammalian CNS neurons to dentrite amputations performed at precise locations within 400 μm of perikarya. The probability of neuronal survival following transection

trauma has been evaluated as a function of the physical parameters of a transection lesion and correlated with these early responses. The purpose of these investigations is to provide baseline data for future studies of physical, chemical and pharmacological interventions which may increase the probability of neuronal survival after physical trauma or accelerate the rate of recovery of surviving cells.

III, Definitions

Axon A type of neurite which is capable of conducting action potentials away from the parikaryon. Action potentials which reach the axon terminum cause the release of chemical transmitters which then carry the signal to a target cell (neuronal, muscle or glandular tissue). Such a point of contact is known as a chemical synapse. Each neuronal cell possesses only one axon which may extend for very long distances. However, axons may sprout profusely at their termini to generate more than one synaptic ending. Axons generally lack ribosomes and must depend upon the cell body for structural and other proteins.

Axon Reaction Expression coined by Lieberman (1971) to designate the responses commonly observed within the cell bodies of neurons after axotomy. These responses include dispersal of the Nissl substance (rough endoplasmic reticulum), swelling of the perikaryon, nuclear

eccentricity, and a general large increase in the synthesis of DNA, RNA and proteins. Lieberman intended this expression to replace "chromatolysis" which had been traditionally used as a collective term for this set of reactions but whose literal meaning refers only to the disperal of the Nissl substance.

Axotomy Transection of severing of an axon.

Chromatolysis The dispersal of the Nissl substance which occurs soon after axotomy.

Central Nervous System (CNS) That portion of the nervous system which includes the brain and spinal cord. All neurons whose cell bodies are located within either the spinal cord or the brain are considered to be part of the CNS. Thus, motoneurons are considered part of the CNS even though their axons project to targets in the periphery.

Dendrite A type of neurite which provides the major portion of the receptive membrane for synaptic contacts. Depending on its type, a neuron may possess one, many or no dendrites. Dendrites are not usually as long as axons but in the spinal cord some may extend up to 1000 μm from the neuronal perkaryon. Unlike axons, dendrites do contain rough endoplasmic reticulum and are not dependent upon protein synthesis in the soma.

Membrane Potential (MP) The voltage or potential created by an unequal distribution of charged molecules

across cell membranes. This polarity makes the inside of a neuron negative relative to the extracellular environment. In neuronal cells the average membrane potential is approximately -60 millivolts (mV). The membrane potential of excitable cells is often referred to as the resting potential (RP) to distinguish it from transient membrane voltage variations such as action potentials.

Necrosis A process of cellular deterioration culminating in cell death which is the result of a lethal environmental insult. Necrosis should not be confused with apoptosis which is the programmed cell death that occurs during development.

Neurite A general term for the stable cytoplasmic processes which extend from the somata of neuronal cells. The two types of neurites are axons and dendrites.

Nissl Substance A term used to denote the rough endoplasmic reticulum of neurons. Named for the man who developed a stain for the rough endoplasmic reticulum.

Perikaryon Term for the soma or cell body of a neuron.

Peripheral Nervous System (PNS) Includes portions of the nervous system outside the brain and spinal cord. More precisely, neurons whose cell bodies are located outside the brain and spinal cord such as those of the dorsal root ganglia belong to the peripheral nervous system.

Synapse An area of contact between a neuron and a target cell (neuronal, muscle or glandular) which is specialized for the transfer of chemical or electrical signals. Synapses are of two general types: chemical or electrical.

IV. Research Hypotheses

A. That the probability of neuronal survival after dendrite amputation within 150 μm of the perikaryon is a function of the lesion distance.

B. That the probability of neuronal survival after dendrite amputation at a fixed lesion distance from the perikaryon is a function of the diameter of the dendrite at the point of transection.

C. That early morphological and optical changes detectable with phase contrast microscopy can be used to determine neuronal viability after dendrite amputation within 150 μm of the perikaryon.

D. That the magnitude of early (within 30 minutes) injury potentials (percent loss of resting potential) recorded at the perikaryon after dendrite amputation is a function of lesion distance from the perikaryon.

E. That the magnitude of early (within 30 minutes) injury potentials may be correlated with the probability of neuronal survival.

F. That the average membrane potentials of neurons which survive dendrite amputation close to the perikaryon usually remain below the average potentials of unoperated control cells within the same culture for at least 24 hours.

G. That neuronal death after dendrite amputation is mediated by massive influxes of extracellular sodium and/or calcium into the cell across the lesion.

V. Limitations of Working in Culture

Primary monolayer cultures of spinal cord cells represent a drastically simplified two-dimensional tissue in which the individual neurons can be visualized and accessed to a much greater degree than they can in explants or the intact cord. Cultured neurons have been used for years to study the electrophysiologic and morphological properties of these cells (Nelson and Peacock, 1973; Peacock et al., 1973; Ransom et al., 1977a,b,c,; Nelson et al. 1981).

The culture model, however, despite its welcome simplicity, is not without limitations. Among these are: a) the physical trauma to which the neurons are subjected during the process of tissue dissociation, b) the loss of pheripheral components of the spinal cord circuitry upon removal of the cord from the animal, c) variability of the strength of neuronal adhesion to the glial layer, and d) variability in the contents of the serum component of the culture medium.

A. Tissue Dissociation

Mechanical dissociation of CNS tissues by trituration subjects the neuronal cells to substantial shear stress which can--and usually does--remove many neurites. Some neurons may survive dissociation better than others. Thus, it is possible that methods of tissue dissociation introduce a selection bias for those neuronal types which are more likely to survive a physical trauma. This may simple by the result of different physical parameters (i.e. shearing of the smaller diameter neurites on a smaller class of neurons is less of a trauma than shearing of larger diameter neurites on a larger neuronal cell type). Alternatively, this selection bias may represent more serious differences involving membrane properties and cytoskeletal components.

B. Loss of Peripheral Circuit Components

A simple spinal cord circuit consists of a receptor cell, an afferent neuron (dorsal root ganglion cell), one or more interneurons, an efferent neuron (motoneuron) and a peripheral target cell (muscle or gland)(Figure 1). Removal of the cord for culture eliminates peripheral components of this circuit (dorsal root ganglia and target cells).

Studies of severed peripheral nerves have shown that they undergo a well-defined series of changes known collectively as the "axon reaction" which includes:

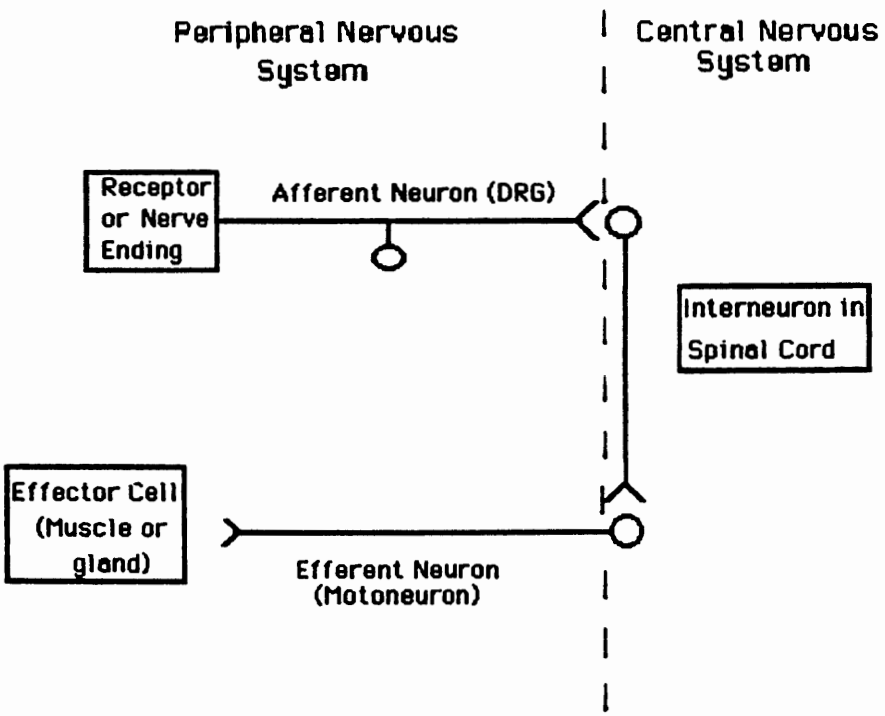


Figure 1. A simple spinal cord circuit.

a) chromatolysis, b) nuclear eccentricity, c) perikaryal swelling, d) deafferentation and e) a massive increase in RNA, DNA and protein synthesis. If axotomized neurons fail to reestablish normal synaptic contacts within a few months they will die.

Although interneurons in our cultures may have been able to reestablish synaptic contacts with their usual targets, efferent motoneurons would not have been able to do so. Thus, it is likely that some of the cells on which we operated three to five weeks after seeding had not recovered from the physical trauma of dissociation and loss of synaptic contact with their targets. Indeed, electron microscopic (EM) analysis of the ultrastructure of unoperated, control neurons revealed that some have plentiful rough endoplasmic reticulum while others had virtually none thereby indicating a persistent chromatolytic state. In addition, decreased electrical stimulation of neurons which results from the loss of afferent input due either to removal of the cord from the animal or to the deafferentation which accompanies axotomy has also been suggested to cause eventual deterioration (Kelly, 1981). The fact that some of the neurons in our cultures were still recovering from dissociation, deafferentation and loss of targets undoubtedly contributed to the variability of some of the results in the present study.

C. Variability in the Strength of Neuronal Adhesion

The substrate to which neurons in our cultures adhere is really a sandwich consisting of (from bottom to top): 1) the charged glass surface of the coverslips which are glued to the culture dish, 2) a (presumably) positively charged layer of combustion products which results from flaming of the glass with a propane torch (Lucas et al., 1986a), 3) a positively charged layer of polylysine which is applied after flaming, 4) various serum proteins, secreted proteins and divalent cations which are adsorbed onto this surface from the medium, and, finally, 5) a cellular layer composed of a mixture of glia, fibroblasts and some epithelial cells. The neurons stratify and attach to the upper surface of the support cell layer.

There is some preliminary evidence that the extent to which a dendrite can retract after severing may dictate the rapidity and efficiency of resealing and the amount of damage done by penetrating injury currents (Appendix A). Retraction, in turn, depends not only upon direct adhesion of a neuron to the support cells but also on the strength of attachment of each element of the substrate to the others around and below. The combined influence of these various elements may significantly influence the probability of neuronal survival after dendrite transection, the rate of deterioration of critically injured neurons, the amount

of residual damage in surviving neurons and the rate of recovery of surviving neurons after the injury. We have attempted to reduce the variations in adhesion parameters by the use of standardized procedures for substrate preparation and by limiting the choice of cells for surgery to those neurons growing on a confluent layer of support cells. Nevertheless, the large number of elements which compose the substrate in our cultures means that there is a great potential for variation in the overall strength of the adhesive forces opposing retraction of the proximal segment of an amputated dendrite.

D. Variations in the Contents of the Serum Component of Culture Media

One of the generally acknowledged biggest problems in tissue culture is the batch variability of serum due to age, sex, and health of the animal donors. Surveys of the components of sera from a number of commercial sources revealed that overall osmolarity varied from 328 to 390 mOsm/l (Hohn, 1975) and total protein varied from 30 to 90 mg/l (Olmsted, 1967; Hohn, 1975). There was also a wide range in the content of electrolytes, simple sugars, phospholipids, cholesterol and hormones (Olmsted, 1967; Hohn, 1975). These variables can have profound and differing effects upon the adhesion, development and general "health" of the various cell types growing in primary culture. For

this reason, attempts have been made to develop "defined media" in which the concentrations of all components are known (Hayashi and Sato, 1976; Mather and Sato, 1979; Barnes and Sata, 1980).

The development of defined media for growth of primary neuronal cultures, however, has had a mixed record of success (Bottenstein and Sato, 1980; Bottenstein et al., 1980; Honnegger et al., 1979; Messer et al., 1980; Messer et al., 1981). Even in these studies a "pre-incubation" period in medium with serum was required for initial cell adhesion and neuronal survival (ibid.). Furthermore, the necessity of seeding cells at high densities in defined media has been suggested to cause a conditioning effect which may negate the designation of these media as "defined" (Messer et al., 1981). Thus, most techniques of neuronal culture still require culture medium containing at least a small amount of serum.

In our laboratory a number of samples of fetal bovine and horse serum from several commercial sources were evaluated prior to purchase for their effects on cell adhesion, culture development and long-term maintenance. Once a decision was made a six-month supply of the selected lot of serum was purchased. Although this has reduced the variability from culture to culture somewhat, a number of different serum lots have been utilized over a period of

several years to seed and maintain the cultures for the studies of neuronal trauma. It is, therefore, possible that varying amounts of some serum factors have not only contributed to variations in the adhesion and general health of the neurons selected for experimental trauma, but may also have played a direct role in the ability of these cells to survive a transection lesion. Certainly, studies of trauma conducted in serum-free medium have revealed an increase in the ratio of surviving control neurons to surviving lesioned neurons (see Results section).

CHAPTER II

MATERIALS AND METHODS

I. Animals Used

BALB/C mice were obtained from Charles River, Wilmington, Massachusetts. The animals were maintained in the animal facility of the Department of Biology at Texas Woman's University.

II. Cell Culture

A. Preparation of Culture Substrate

For most experiments cells were seeded on flamed, polylysine-coated (MW = 70-150 kD, 25 µg/ml, Sigma) glass coverslips affixed to the bottom of the 60 mm culture dishes (Corning) with Eukitt (F.O. Kindler, Freiburg, Germany). Flaming has been demonstrated to increase the wettability of many surfaces thereby enhancing cell adhesion as well as the application of traditional ahdesion-promoting compounds (Lucas et al., 1986a). The procedure for flaming requires three one-second exposures of the glass surfaces to a propane flame held at a distance of 1 centimeter (cm) (ibid.).

B. Culture Procedure and Maintenance

The procedure for culture of dissociated spinal tissue is based on the method of Ransom et al. (1977a). Pregnant female mice (13 to 14 days gestation) were anesthetized using CO_2 narcosis and killed by cervical dislocation. The pregnant uterus from each mouse was removed and placed into two changes of ice-cold DlSGH. DlSGH is a balanced salt solution containing 8 gm NaCl, 0.4 gm KCl, 0.045 gm $Na_2HPO_4 \cdot 7H_2O$, 0.03 gm KH_2PO_4 and 0.0012 gm phenol red per liter. To each liter of salt solution is also added 7.5 gm sucrose and 3 gm D-glucose. Hepes buffer (3 gm/L) is added to the salt solution and the pH is adjusted to 7.3-7.4 by the addition of 1 N NaOH or 1 N HCl. The osmolarity of DlSGH is brought up to 330 milliosmols (mOmols) by the addition of dextrose. The salt solution is then sterilized by filtration. Embryos were removed from the uterus and also placed in ice-cold DlSGH. The spinal cords were dissected out of the embryos using sterile technique.

Seven to ten cords were placed into a petri dish and minced finely. Three milliliters of DlSGH containing 0.25% trypsin and 0.05% DNase (Sigma) were added to the cords and they were placed in a 10% CO_2 incubator at 37°C for 15 minutes. At the end of this period enzymatic activity was stopped by the addition of 10 ml of culture

medium (Minimum Essential Medium, GIBCO) containing 10% horse serum and 10% fetal bovine serum (MEM10/10). The tissue was then pelleted by centrifugation at 200 g for one minute.

After centrifugation the supernatant was removed and discarded and 1.5 ml of fresh MEM10/10 was added to the tissue. The pellet was then triturated by drawing it back and forth ten times through the tip of a sterile Pasteur pipette. Remaining tissue fragments were allowed to settle for one minute. The supernatant containing suspended single cells was then removed and placed into a second tube. One milliliter of MEM10/10 was then added to the fragments. Trituration of the remaining fragments was repeated a second and a third time using Pasteur pipettes with flame-narrowed tips. At the end of the entire dissociation procedure there were 3.5 ml of cell suspension for every seven to ten spinal cords. The separate suspensions were then pooled.

The number of cells per milliliter of suspension was calculated using hemacytometers. Sufficient MEM10/10 was added to the suspension to bring the total number of cells (neuronal and nonneuronal) per milliliter to 1×10^6. One half milliliter of suspension was placed on prepared glass coverslips affixed to the bottom of 60 mm plastic tissue culture dishes (see above). The cultures were then placed into a 10% CO_2 incubator at $37^{\circ}C$.

After 24 hours medium on the coverslips containing non-adhered cells was removed and three milliliters of MEM10/10 was added to the dishes. A second complete medium change with MEM10/10 was performed three days after seeding. Five-to-seven days after seeding when the cultures were confluent a complete medium change with Minimum Essential Medium containing 10% horse serum (MEM10) and fluoro-deoxyuridine (FdU) plus uridine (Sigma, 5.4×10^{-5} M and 1.3×10^{-4} M) was performed. Fluorodeoxyuridine retards the proliferation of dividing cells. After 24 hours the medium containing FdU was removed and replaced with MEM10. Thereafter, partial medium changes with MEM10 were performed every three days. Spinal cord cultures were utilized for laser cell surgery three-to-five weeks after seeding when they are generally considered to have stabilized (Peacock et al., 1973; Ransom et al., 1977a).

III. Target Cell and Target Neurite Identification and Selection

A. Identification for Neuronal Cells

Experimental and control cells were identified as spinal cord neurons based upon the following morphological criteria: 1) presence in the uppermost stratum of the culture, 2) multiple long, branched cell processes, 3) large, bulging, phase-bright cell bodies and

4) well-defined nuclei and nucleoli (Peacock et al., 1973; Fischbach and Nelson, 1977; Ransom et al., 1977a). Electrophysiologic studies have confirmed these criteria in at least 78% of the selected cells.

B. Identification of Dendritic Processes

Most of the cell processes selected for laser surgery were identified as dendrites (or multiple dendrite bundles) on the basis of light microscopic (LM) examination. Dendrites were distinguished from axons based on the following morphological criteria: 1) they arise directly from the soma, 2) they are thick and tapering, 3) they branch at an acute angle to the parent process, 4) they display irregular contours and 5) they lack the characteristic axon hillock and initial segment (Peters, Palay and Webster, 1976). All target processes were transected proximal to the first major branch point.

Ultrastructural analyses have shown that dendrites are rarely isolated and are often accompanied by other, smaller fibers. Dendritic fascicles are also frequently encountered. In either situation the neurites of a target cell represent the largest component of a bundle. Neurite diameters at the target sites ranged from 1 to 5 μm with an average process diameter of 3.2 + 0.1 μm.

IV. Laser Cell Surgery

The laser microbeam system is depicted in Figure 2. Laser cell surgery was performed with a pulsed nitrogen (UV) laser operating at 337 nm and a maximum output of 14 kW. A helium-neon (HeNe) laser (630 nm, 1 mW), colinearly aligned with the nitrogen work laser, provided a visible target beam.

The HeNe and UV laser beams were reflected into a Leitz Orthoplan microscope by a beam aligner and focused through Zeiss Ultrafluar quartz objectives onto target neurons. A 32X phase objective (2.3 μm minimum laser focus diameter) was used to effect process transections in the studies of cell survival. The Ultrafluar objectives are heat sensitive and cannot be exposed to 37^{o}C medium. Therefore, a plastic sleeve sealed at one end with a matched (350 μm thick) quartz coverslip was used to protect the 32X objective from heat and immersion damage (Figure 3B). With the recommended glycerin (index of refraction = 1.454) between the objective and the coverslip (inside the sleeve) optical distortion was kept to a minimum. Sleeve misalignment could be discerned from asymmetries in the reflected HeNe diffraction pattern. Intracellular recording arrangements (Figure 3A) necessitated the use of a 10X Zeiss Ultrafluar objective (4.5 mm minimum laser focus diameter) with a longer 7.4 mm) working distance. Because the 10X

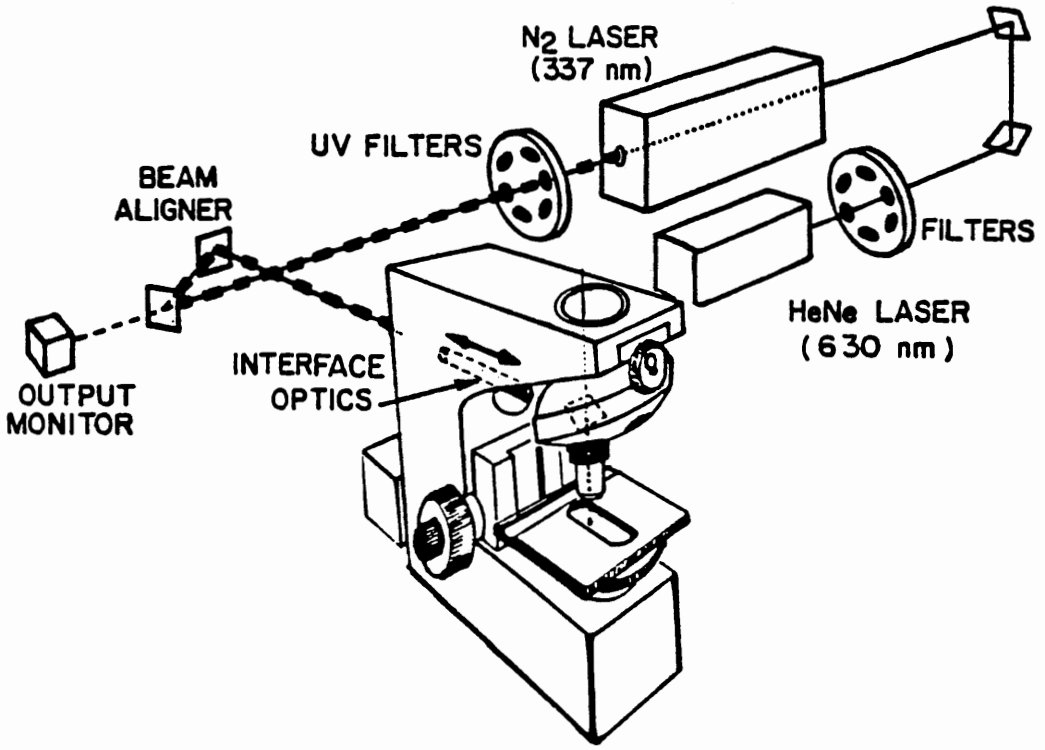


Figure 2. The laser microbeam system. The UV (337nm) and HeNe laser beams are aligned colinearly, reflected into a Leitz Orthoplan microscope, and focused onto a target on the microscope stage.

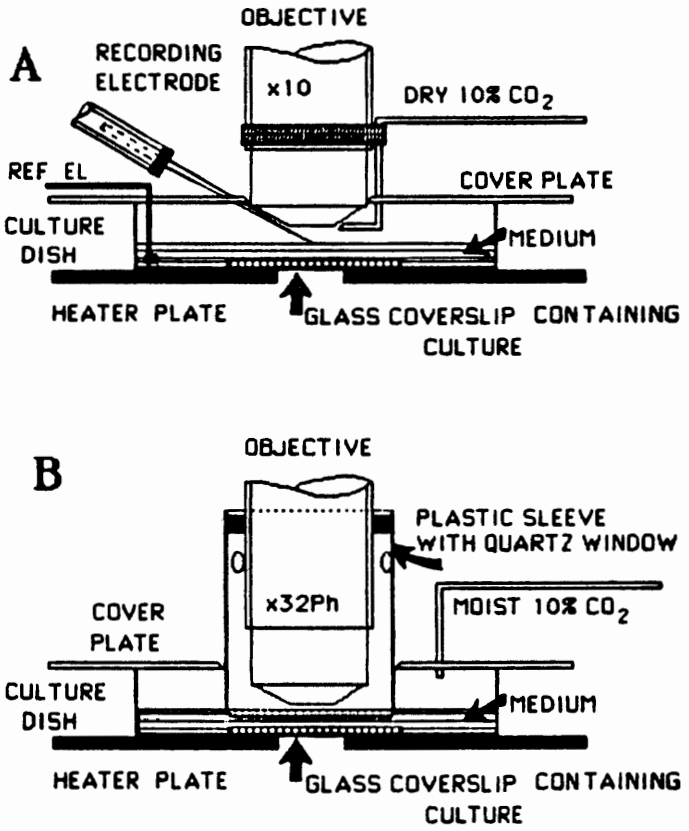


Figure 3. A. Arrangements for laser cell surgery and concurrent electrophysiologic recording using a 10X quartz objective (Zeiss Ultrafluar, working distance 7mm). A dry stream of 10% CO_2 in air controls medium pH and is directed at the lens to prevent condensation. B. Laser cell surgery arrangements used for neuronal survival experiments. The 32X phase Zeiss quartz objective is protected from the warm culture medium by a plastic sleeve sealed with a matched quart coverslip.

objective was positioned above the surface of the medium, there was no need for a protective sleeve.

All laser surgery was performed using the multiple shot, low energy method described by Gross et al. (1983). Laser pulses (12 nanoseconds/pulse) were delivered at a firing frequency of 20 Hz in all experiments. This method of laser surgery permits creation of precise transections without concurrent pressure wave effects (ibid). Target neurites were irradiated until LM examination showed complete separation of proximal and distal segments (usually 10-15 seconds) with no remaining interconnections.

V. Electrophysiology: Injury Potential Studies

Figure 3A shows the arrangements for the intracellular recording. The 10X objective was introduced into the culture dish through an opening in the cap. A stream of 30% dry CO_2 in air at a flow rate of 10 to 15 ml/min was directed at the lens of the objective to prevent condensation and to maintain pH of the medium. The high CO_2 concentration was required to achieve a 10% CO_2 atmosphere and compensate for the mixing with room air through the microelectrode access port. Distilled water (250 µl/0.5 h) was added to the medium to maintain proper osmolarity. Temperature of the medium was controlled between 35°C and 37°C by a DC-heated copper plate below the dish. All

recordings in this series of experiments were made within three hours of removal of a culture from the incubator.

Prepulled glass recording electrodes (100 to 200 megohms) were obtained from Frederick Haer, Co. and filled with 3 M filtered potassium acetate. A silver/silver chloride wire in the medium served as a reference electrode. Signals were fed into a Grass P-16 or a WPI KS-700 amplifier and displayed on an analog storage oscilloscope (Tektronix model 5311). Permanent records of injury potentials were made on a chart recorder.

VI. Cell Survival Studies

Arrangements of culture dishes during cell survival studies were similar to those for electrophysiology (Figure 3B). The 32X objective within its plastic sleeve was introduced into the dishes through a specially constructed cap and immersed in the medium. The protective sleeve was UV irradiated for 30 minutes prior to each experiment. The cap and CO_2 line were rinsed with 70% ethanol and also UV irradiated for 30 minutes. A 20 to 25 ml/min flow of filtered (Gelman, 0.2 µm) 10% CO_2 in moist air was used to maintain pH at 7.4 and create a positive pressure within the culture dish. Two hundred microliters of triple distilled water were added at 30 minute intervals to compensate for evaporation and

maintain osmolarity. Temperature of the dishes was kept between 35°C and 37°C.

All surgery was performed in the absence of antibiotics or antimycotics. Precautions such as UV irradiation, soaking in ethanol and filtration of the CO_2 as well as the positive pressure minimized the chance of bacterial or fungal contamination during an experiment. Cultures remained free of contamination for at least three days after cell surgery.

Laser marks were made below the surface of the glass coverslip to assist relocation of both experimental and control cells. Such relocation marks were made without disturbing either target neurons or the glial layer (see, for example, Figure 6). Photographs of individual cells were taken to assist relocation. All experimental cells in a culture dish were operated at the same distance (50, 100, or 150 μm). Experiments generally lasted two to two-and-a-half hours, after which cells were returned to the incubator. A 50% medium change with Minimum Essential Medium (MEM, GIBCO) and 10% horse serum was performed 24 hours after surgery.

VII. Data Collection and Analysis

A. Selection of Target and Control Neurons

Care was exercised to select control and target neurons which were free of obvious signs of deterioration such as the presence of vacuoles, cytoplasmic granularity, a plasma membrance with a "ragged" appearance, or the absence of clearly visible somal features (nucleus and nucleoli). Neurons were chosen from areas of a culture in which the neuronal elements were relatively dispersed; this low density allowed light microscopic visualization of the perikarya and most of the major neurites or neurite bundles. Individual cells were also selected from widely separated areas of the culture to minimize the possibility of inadvertently lesioning either the control neurons or previously operated cells due to fasciculation of neurites. For both the electrophysiologic and cell survival studies only neurons on areas of confluent glial carpet were chosen to insure greater uniformity of adhesion parameters.

B. Measurement of Lesion Physical Parameters

Measurements of lesion distance and lesion diameter (diameter of the dendrite at the target site prior to transection) were made from light micrographs of target neurons taken prior to dendrite amputation.

C. Cell Survival Studies

1. Determination of Cell Viability

Certain morphological changes observed with phase contrast microscopy have been shown to correlate strongly with cell survival or cell death after transection trauma. Cells which were viable 24 hours after surgery usually continued to have clearly visible nuclei and nucleoli. Dead cells resembled highly vacuolated bags of debris which might be either swollen or shrivelled depending on the stage of deterioration. Intense vacuolization is one of the few morphological criteria which has been generally accepted as indicative of cell death (see Lieberman, 1971 for review). Remnants of dead cells normally disappeared within a few days. EM analysis of lesioned neurons has provided ultrastructural confirmation of these judgements of cell viability or death (see Appendix B). By 24 hours after surgery virtually all experimental cells could be categorized as either viable or dead.

2. Cell Survival Calculations

During each cell survival experiment six to eight experimental neurons and the same number of control neurons were selected. The low number of cells per experiment represented a compromise between statistical requirements and the need to minimize shear stress on the cells resulting

from movement of the culture below the quartz window of the plastic sleeve protecting the 32X objective (less than 100 µm clearance; Figure 2B).

Survival of experimental and control cells in normal medium (MEM10) was checked at 24, 48 and 72 hours after neurite amputation. In a few experiments surviving cells were followed for longer periods. Ten experiments (a total of 60 experimental and 60 control neurons) were performed at each lesion distance (50 µm, 100 µm and 150 µm). Percent cell survival was calculated for the cells in each experiment at each time after surgery. After dropping the high and low survival values in each set of experiments (all cells operated at the same lesion distance), the mean percent survival and the standard deviation was calculated (N_{final} = 8 experiments). Results of these studies were compared graphically.

Data from the group of experiments at the lesion distance for which there was the greatest statistical variation (100 µm) were used to determine the effect of lesion diameter upon neuronal survival. To these data were added data from a separate set of experiments (61 operated neurons) in which the cells were also operated at a lesion distance of 100 µm. The data were separated into groups based upon lesion diameter. Percent cell survival for each lesion diameter was calculated and the results were represented graphically.

D. Injury Potential Studies

1. Criteria of Recording Stability

In the studies of changes in membrane potentials during and immediately after surgery, cells were held for three to five minutes prior to process amputation. Data presented in this paper were taken from records in which there was no evidence of system or electrode instability (excessive drift, noisy baseline, rapid unexplained potential changes, etc.) either before or after laser cell surgery.

Resting potentials of surviving neurons were measured at intervals up to 24 hours after dendrite amputation. Recordings were considered acceptable only if the cells were held stably for a minimum of 30 seconds after penetration. The maximum potential recorded during this period (usually the value recorded on the oscilloscope upon penetration) was taken as representative of the membrane potential.

2. Measurement of Injury Potentials

Changes in membrane potentials were measured for 30 to 45 minutes after dendrite amputation. An injury potential was calculated as the percent loss of the pre-surgery membrane potential. Injury potentials were plotted as a function of lesion distance.

Membrane potentials of recovering operated neurons at four to 24 hours were ascertained by averaging the

potentials of the surviving cells (a minimum of five measurements per experiment). These average potentials of surviving cells were then compared to the average potentials of unoperated control neurons in the same culture (based upon a minimum of ten measurements).

E. Early Morphological Change Studies

Sixty-one neurons were subjected to dendrite amputation at a distance of 100 μm. Morphological and optical changes in the lesioned neurons were noted at 30 minutes and two hours after surgery. Viability of these cells at 24 hours was also determined. Attempts were made to correlate the observed early morphological and optical changes with eventual cell survival or death.

F. Studies of Neuronal Survival in Calcium-Free Medium

To study the effect of low calcium on cell survival after dendrite amputation at 100 μm, the MEM10 in which neuronal cultures were normally maintained was replaced one hour prior to surgery by calcium-free MEM (GIBCO) without horse serum. A control series of experiments was performed in MEM containing the normal concentration of calcium but without serum (MEMO). Survival was also observed for neurons operated in MEM containing 6mM EGTA (Sigma). The percent cell survival was noted at two hours after surgery in each

experiment and the average was calculated for each series. Using the average percent survival values, the ratio of surviving control (unoperated) neurons to surviving operated neurons was calculated for each protocol. The results of these studies were compared to one another and to the results of the dendrite amputation studies in MEM containing normal calcium and 10% horse serum.

CHAPTER III

RESULTS

I. Neuronal Survival as a Function of Lesion Diameter and Lesion Distance from the Perikaryon

Figure 4 shows clearly that cell survival was a function of the distance of the lesion from the perikaryon. Neuronal survival was observed for three days after dendrite amputation at distances of 50, 100 and 150 µm. The plateau values indicate that whereas only 31% of cells operated at 50 µm were alive at 24 hours, 54% of cells operated at 100 µm and 71% of cells operated at 150 µm survived. In almost every case cell death occurred within the first 24 hours after surgery. Cells which survived this period remained viable at 48 and 72 hours. Survival among control cells was 100% in almost every experiment.

Dendrites taper with distance from the perikaryon, therefore, the increased survival of operated cells at greater lesion distances was probably also a function of smaller lesion diameters. Determination of the diameter of a lesion is difficult because the process of resealing can begin almost immediately. However, lesion diameter at the moment of transection may be estimated by measuring the diameter of a neurite at the target site. The percent

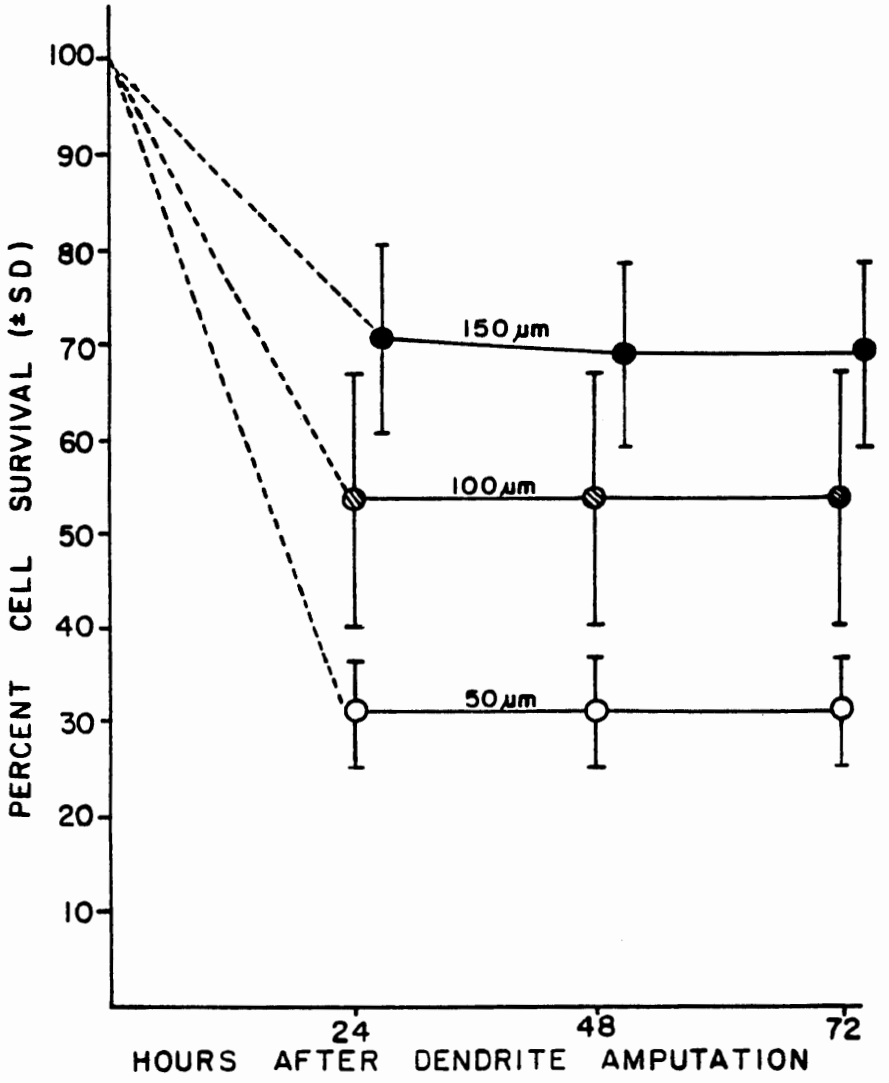


Figure 4. Cell survival as a function of time after dendrite transection trauma. For an average process diameter of 3 µm, dendrite transections of 50 µm, 100 µm, and 150 µm were associated with percent cell survival values of 31%, 54% and 71% respectively. Each point on the graph represents the mean percent survival $\pm$ standard deviation calculated from the results of 10 separate experiments (a total of 60 cells). In general, operated cells that were alive at 24 hours were still viable at 48 and 72 hours.

survival of cells operated at the same distance (100 μm) was plotted as a function of the diameter of the target process at the lesion site. Figure 5 shows that the diameter of a target neurite at the lesion was a factor in cell survival. Therefore, lesion diameter and lesion distance from the perikaryon were both determinants of neuronal viability after transection.

We have observed that both antimycotic and antibiotic agents (Funizone, 2.5 μg/ml MEM, penicillin/streptomycin, 100 U/ml MEM and 100 μg/ml MEN, GIBCO) increase the probability of neuronal death after transection trauma. For example, approximately half of the experimental cells survived dendrite amputation at 100 μm in medium without Fungizone whereas by three days after surgery only 20% survived in medium with Fungizone. Survival of control neurons was also lower in medium containing Fungizone. Thus, these agents were eliminated from our cultures. The resulting increased likelihood of contamination forced us to restrict most of our observations of cell survival after neurite amputation to a three-day period. In a few experiments, however, it was possible to monitor cell survival for longer periods. In these cultures operated cells which survived the first 24 hours were still alive at seven days. Figure 6 depicts a neuron during a six-day period after neurite transection. Somal swelling and

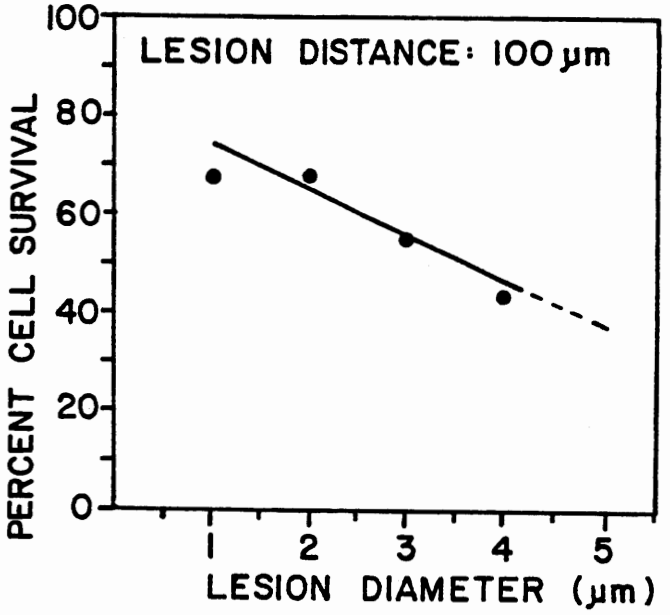


Figure 5. Percent cell survival as a function of lesion diameter. At a lesion disctance of 100 µm the probability of survival decreases as lesion diameter increases. Each point represents between 13 and 48 observations made 24 hours after surgery.

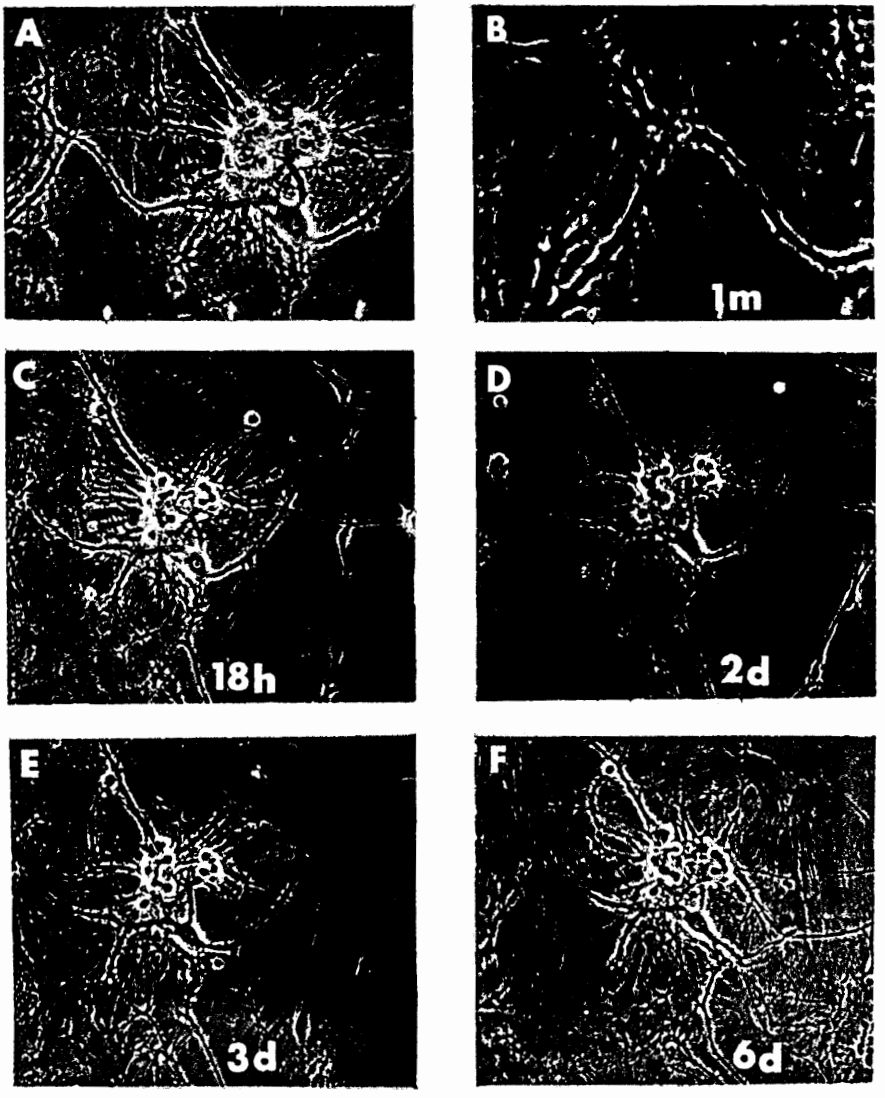


Figure 6. Survival of CNS neuron over a six-day period after dendrite transection 100 m from the soma. A. Cell prior to surgery. Arrow indicates target area. Mag. x 320 B. Higher magnification of target area one minute after surgery (arrow). Mag. x 550 C-F. Cell at 18 hours, two days, three days, and six days after surgery. Note laser relocation marks on the right side of the photographs. Arrows point to the laser impact area. Mag. x 320.

cytoplasmic granularity (C-E) were not uncommon in cells which survived dendrite amputation. On this occasion, the neuron recovered its normal appearance within six days after surgery (F).

Reformation of an amptuated process was not observed in the early post-traumatic period (three days). Even sprouting at the cut end of the proximal segment was rarely seen. However, some regrowth of transected neurites has been seen in approximately one-third of the surviving neurons observed for more than six days (preliminary data). It is apparent, therefore, that longer observation periods are necessary for the investigation of possible regenerative responses of cultured mammalian spinal neurons following dendrite amputation.

II. Early Morphological Changes and the Probability of Neuronal Survival

The viability of neurons after dendrite amputation close to the perikaryon may be assessed within a few hours of transection (Table I). Observations of 61 neurons operated at a lesion distance of 100 µm demonstrated that cells which would survive had normal somal features (distinct nucleoli and nuclear region) two hours after transection trauma. Fatally injured cells could usually be recognized on the basis of distinct morphological

TABLE I

EARLY MORPHOLOGICAL INDICATORS OF CELL VIABILITY AFTER AMPUTATION TRAUMA*

CATEGORY**	MORPHOLOGICAL CHARACTERISTICS AT 2 HOURS+		
	PHASE-BRIGHT CYTOPLASM	DISTINCT NUCLEUS	DISTINCT NUCLEOLUS
Viable Cells a-30 (49%)	10%	43%	90%
Moribund a-21 (34%)	81%	0%	14%
Dead Cells++ a-10 (16%)	0%	100%	100%

* data from 61 neurons operated at 100 µm from the cell body

** categories established by observation at 2 hours with viability checks at 24 hours; all cells designated "moribund" at 2 hours were dead at 24 hours; viable cells at 2 hours remained viable at 24 hours.

\+ characteristics are not mutually exclusive.

++ dead cells were very deteriorated but disappearance of the phase brightness permitted visualization of somal features

changes. Of these, one-third displayed at two hours the large vesicles and obvious deterioration associated with cell death. The remaining neurons (designated in Table I as "moribund") were usually characterized by intensely phase-bright cytoplasm and an absence of distinct nuclear features. Figure 7 shows the changes in a moribund cell during the three hour period immediately after dendrite transection.

EM analyses of the ultrastructural changes in neurons fixed two hours after dendrite transection have confirmed LM evaluations of viability or death (see Appendix B) and provided some insight into the process of deterioration after transection trauma. The characteristic phase brightness of moribund neurons was shown to result from the formation of large numbers of small membrane-bound vesicles which diffract light and obscure the nuclear region. These vesicles were derived from swollen, fragmented endoplasmic reticulum and irreversibly damaged mitochondria. In dead neurons the large vacuoles visible with LM were probably the result of coalescing of these smaller vesicles. Consequently, as cells deteriorated further, the phase-brightness disappeared and the nuclear features were again discernable amid the large vacuoles and cystoplasmic debris.

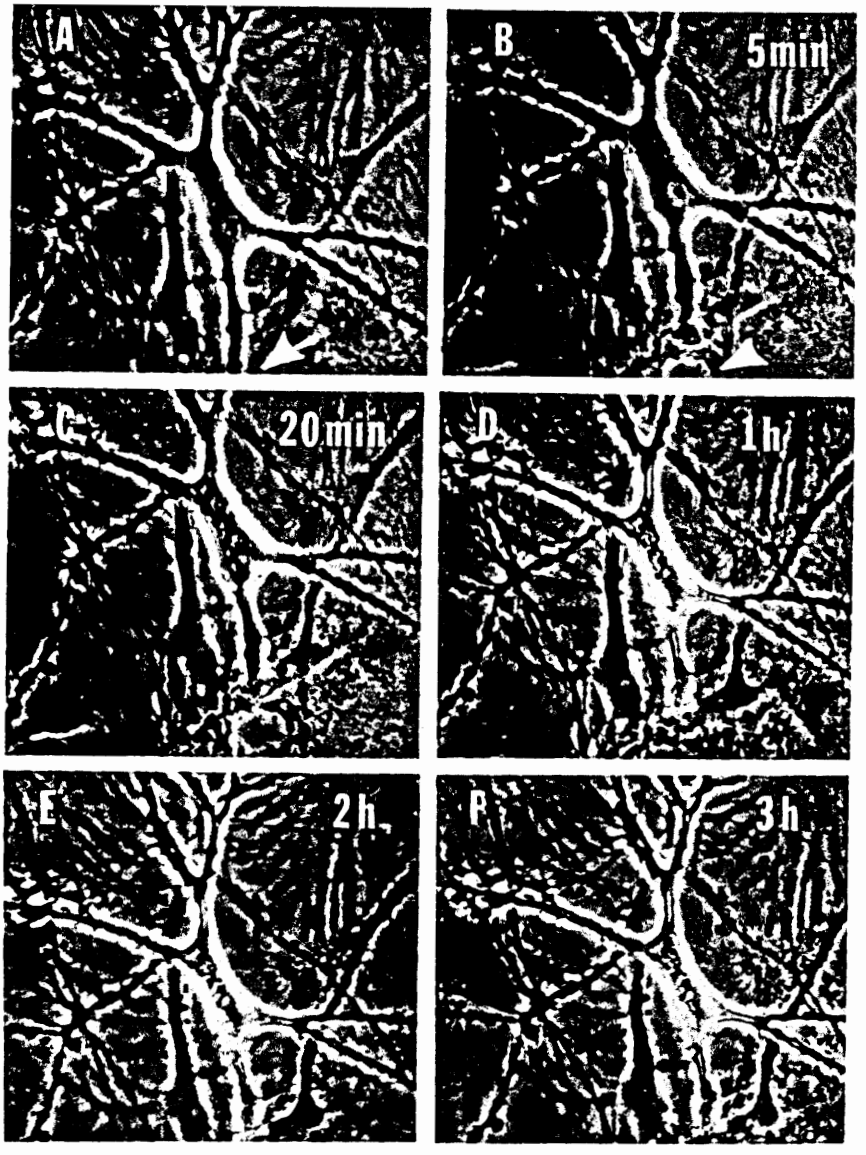


Figure 7. Early changes observed with phase contrast light microscopy in moribund neuron after dendrite amputation. A. Neuron prior to surgery. Target area (arrow) was approximately 60 μm from the edge of the soma. B. Cell five minutes after surgery. Note swelling of the proximal segment of the cut process. C. Cell at 20 minutes after transection. Pale patches encircling the nucleus first appeared in the somal cytoplasm nine minutes after surgery. D. At one hour after transection phase-brightness has spread into processes, and the nucleus and nucleolus are no longer visible. E-F. Neuron at two hours and three hours. Note the apparent decrease in somal volume during the three-hour interval. Cells displaying this combination of features (lack of distinct nucleus or nucleolus and phase-bright cytoplasm) are reduced to debris by 24 hours.

The percentage of viable cells (49%) and nonviable (moribund plus dead) cells (51%) at two hours agrees closely with the results of the separate three-day cell survival study (Fig. 4) of neurons operated at 100 µm. These data indicate that the fate of a neuron injured close to the perikaryon is decided as early as two hours after lesioning. Although it is possible that the fate of lesioned neurons may be determined even earlier than two hours, morphological and optical changes observed 30 minutes after dendrite amputation were not sufficiently consistent to serve as indicators of eventual cell survival or death.

III. Injury Potential Profiles and Magnitudes as Functions of Lesion Distance from the Perikaryon

Over 100 recordings were made from neurons before, during and after process amputation at distances ranging from 10 to 400 µm from the edge of the soma. Changes in membrane potential following transection were monitored for periods up to 45 minutes. Fifty of the most stable records were selected for analysis of injury potentials. No neurons with RP's less than 40 mV were used in this series of experiments. The average RP value was 56 $\pm$ 5.5 mV. Seventy-eight percent of these cells demonstrated spike activity during recording confirming their identification as neurons. This figure probably underestimates the real

percentage as not all neurons in culture are spontaneously active (Peacock et al., 1973). Even electrode penetration or neurite transection (where depolarization is relatively slow) may not always elicit spike activity.

Figure 8 shows representative recordings from five different neurons before, during and after neurite transection at distances ranging from 50 µm to 375 µm. Such recordings were characterized by an initial RP loss (peak) within the first five minutes after transection followed by establishment of a new membrane potential (plateau phase) which was less negative than the original RP.

Figure 9 illustrates the fact that different injury potential patterns were characteristic of different lesion distances. As indicated by Figure 8, most cells operated at distances greater than 50 µm displayed peak and subsequent plateau phases. The percent RP loss of the plateau phase generally exceeded that of the initial peak when a lesion was made close to the soma (Figure 9B, 9C) but fell below the latter at greater lesion distances (Figure 9D). The peak-to-plateau transition represented an increase in resistance probably resulting from several factors including partial membrane resealing, reduction in the lesion diameter and accumulation of organelles in the cytoplasm adjacent to the lesion. A similar pattern was often seen immediately following electrode penetration of a cell and

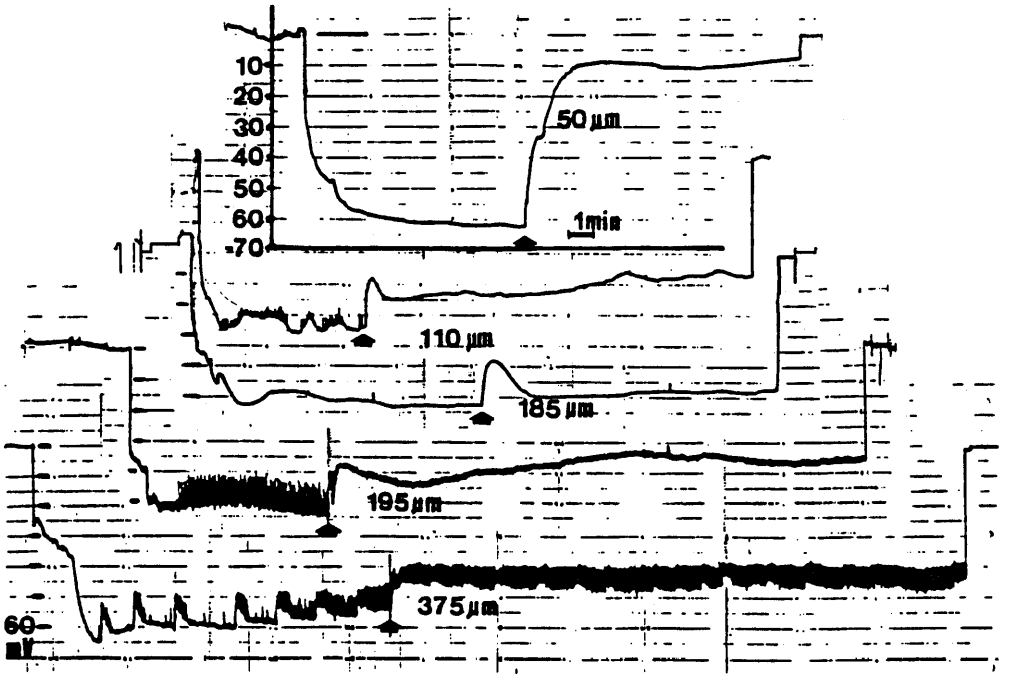


Figure 8. Representative recordings of injury potentials after neurite transection. Lesion distances ranged from 50 to 375 µm from the cell body. Arrows indicate time at which transections occurred. Note the characteristic peak and plateau phases of the injury potential profiles after transections at lesion distances greater than 50 µm. Although two of the target neurons were not spontaneously active, fast-sweep oscilloscope observations revealed spike activity during surgery. 10 mV/larger vertical division. 1 minute/large horizontal division.

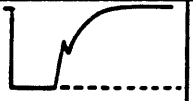
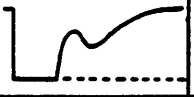
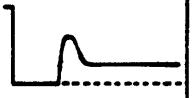
CHARACTERISTIC INJURY POTENTIAL PROFILES	LESION DISTANCE (μm)					
	0-30 (N=5)	31-60 (N=14)	61-100 (N=9)	101-150 (N=8)	151-200 (N=9)	> 200 (N=5)
A 	80%	—	11%	—	—	—
B 	20%	79%	11%	—	—	—
C 	—	14%	11%	25%	—	—
D 	—	—	44%	38%	89%	20%
E 	—	7%	22%	38%	11%	80%
F 	—	—	—	—	—	—

Figure 9. Characteristic patterns of injury potentials vary with distance of the lesion from the cell body. An initial peak followed by a stable plateau potential is usually seen within five minutes after transection at distances greater than 50 μm (C-F). The downward curve of the peak phase of the injury potential represetns an increasing resistance possibly due to partial resealing of the membrane at the lesion. The plateau phase represents an equilibrium between residual injury currents and outward pumping of ions. Complete recovery of presurgery potentials (F) has not been observed within a 30 minute timeframe.

probably represented closing of the plasma membrane around the electrode tip. The plateau phase of the injury potential represented an equilibrium condition during which inflowing (injury) currents were in balance with outflowing or sequestering currents (membrane pumps). At no time has complete recovery of pre-transection potentials in neurons operated within 400 µm of their perikarya been observed during the 30 to 45 minute period after dendrite amputation (Figure 9F).

The correlation between distance of the lesion from the perikaryon (i.e. the recording electrode and the percent RP losses during the plateau phase is depicted in Figure 10. It is immediately apparent that the magnitude of the RP reduction after neurite transection decreased with distance from the soma. Fifty-to-one hundred micrometers seemd to represent a pivotal rnage of lesion distances. At lesion distances less than 50 µm, the magnitude of the injury potentials was greater than 80%. However, at lesion distances greater than 100 µm, injury potentials fell below 30%. A similar correlation was obtained when the percent potential loss during the peak phase (peak apex) was plotted against lesion distance (data not included). Interestingly, the variability of the response at lesion distances between 50 µm and 100 µm echos the greaer statistical variation observed in the set of cell survival experiments perofrmed at 100 µm (Figure 4).

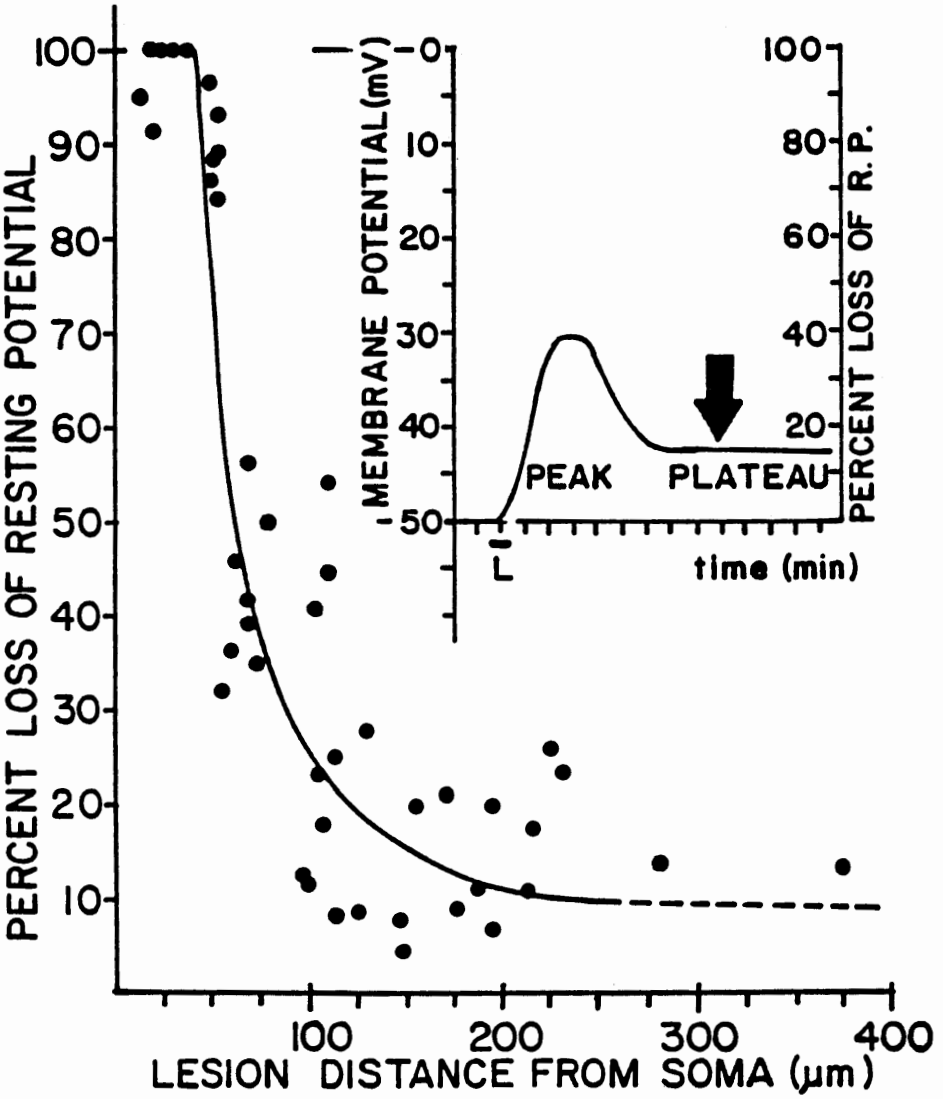


Figure 10. Effect of lesion distance on plateau phase potentials (indicated by arrow in inset) expressed as percent loss of resting potential. Plateau phase potentials are remarkably stable and show little variation between ten and 30 minutes after surgery.

IV. Correlation of Early Injury Potentials and Cell Survival

Injury potentials reflect the magnitude of the ion fluxes across the lesion. Therefore, it is of interest to determine whether changes in potential recorded at the perikarya of physically injured neurons can be correlated with mortality.

Electrode withdrawal from small mammalian neurons represents a second injury which invalidates any subsequent cell survival observations. Therefore, a correlation of early injury potentials recorded at the somata of neurons after process transection with the probability of survival of those same cells is not easy to demonstrate experimentally. However, an attempt may be made to correlate the results of the separate cell survival and injury potential studies.

It has been demonstrated that neuronal death after dendrite amputation is a function of distance of the lesion from the perikaryon (see Figure 4). By plotting cell survival as a function of lesion distance (data from Figure 4) one can predict that at 250-300 µm no further cell death is anticipated (Figure 11A). It has also been shown that plateau phase potentials are a function of lesion distances from the perikarya (see Figure 10). This relationship may also be represented by plotting the

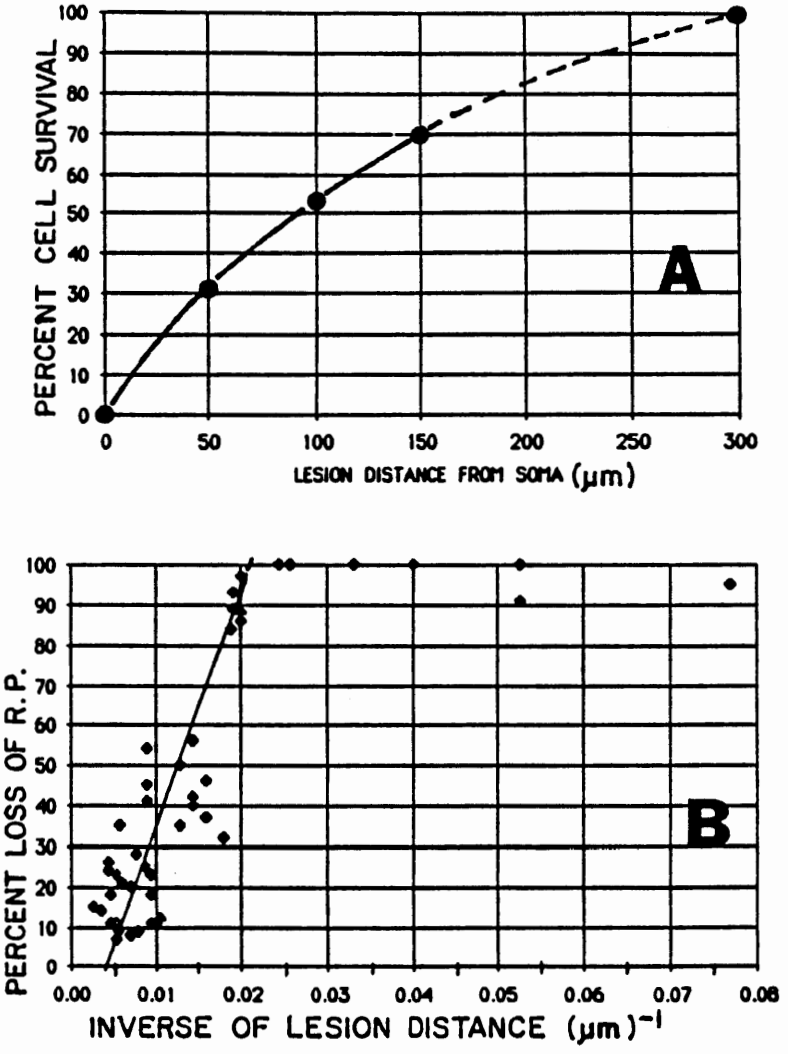


Figure 11. Correlation of neuronal survival with injury potentials recorded at the soma. A. By plotting percent cell survival as a function of lesion distance (data from Figure 4) one may predict 100% cell survival for neurons lesioned beyond 250-300 µm. B. Similarly, when injury potentials (data from Figure 10) are plotted as a function of the reciprocal of lesion distance, the x intercept of approximately 0.0037 suggests that there will be only very small changes in the potential at the soma for lesions beyond 250-300 µm.

the values from Figure 10 against the reciprocal of the distance (Figure 11B). From the linear approximation one can infer that at 250-300 μm (i.e. 0.0037 μm^{-1} at the x intercept) injury potential changes will be small or imperceptible at the soma, and that at 45 μm (0.022 intercept) 100% RP loss may be expected. Thus, cell survival and electrophysiologic data obtained from separate sets of experiments indicate that at transection distances sufficiently removed to cause only very small potential changes at the perikaryon the probability of cell death is also reduced to zero (100% survival).

V. Membrane potentials of Surviving Neurons

To ascertain statistically whether surviving cells recovered their original membrane potentials, intracellular recordings were made at four and 24 hours after dendrite amputation at a lesion distance of 100 μm (Figure 12). At both times the membrane potentials of neurons which survived transection trauma averaged 8% below the membrane potentials of control cells from the same cultures. These findings suggest that in most cases reestablishment of normal ion gradients was incomplete 24 hours after dendrite amputation.

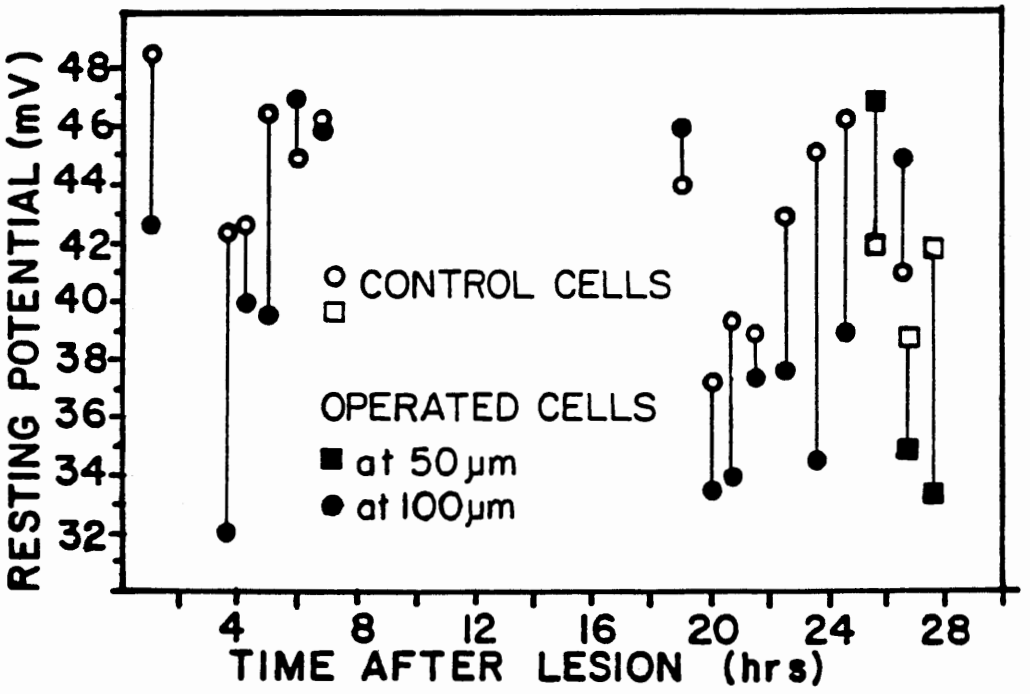


Figure 12. Prolonged depression (up to 24 hours) of resting potentials of surviving neurons after dendrite amputation indicates incomplete recovery of normal membrane permeability and selectivity characteristics. Each pair of data points represents the average of the membrane potentials of 5-9 lesioned cells (closed symbols) and the average of the membrane potentials of 10-20 unoperated control cells from the same culture (open symbols). In 76% of these experiments (13 of 17) the average potential of viable operated cells was below that of the unoperated controls. The slight decrease in the membrane potentials of control cells over time is probably the result of general culture stress due to repeated manipulation and observation. □ , neurons operated 50 µm from the perikaryon; ○ neurons operated 100 µm from the perikaryon.

VI. Neuronal survival after dendrite amputation in low calcium

To determine the effect of extracellular calcium upon cell survival neurons were operated at a distance of 100 μm from their perikarya in calcium-free MEM or MEM containing 6 mM EGTA. The results of these studies are shown in Table II.

Within three hours after replacement of normal MEM10 with MEM containing 6 mM EGTA (two hours after dendrite amputation) the cultures showed great deterioration. The support cell layer had become non-confluent and some areas of the substrate were completely bare of cells. Few cells had a normal appearance at two hours. A large percentage of the control neurons appeared dead or moribund. By 24 hours cultures in EGTA were reduced to debris.

The average survival at two hours of neurons operated in calcium-free medium was substantially reduced from the anticipated survival of neurons in complete medium (Table II, see also Figure 4). However, the survival of control neurons under these conditions was also slightly reduced. To achieve a calcium-free environment serum also had to be eliminated from the culture medium in these experiments; consequently, a set of control experiments in serum-free MEM with calcium was also performed for

TABLE II

NEURONAL SURVIVAL AFTER DENDRITE AMPUTATION:

Manipulation of Ca^{++} and Serum Levels

DATE	PROTOCOL	2hr VIABILITY Control	Operated	RATIO C/O
1985(n=10)	MEM, normal Ca^{++}, 10% serum	100%	54%	1.85
	MEM without Serum			
3/11/86		70%	22%	
3/12/86		100%	13%	
3/13/86		87.5%	37.5%	
3/16/86		85.7%	25%	
	Average	85.8%	24.4%	3.5
	MEM without Ca^{++} & Serum			
1/25/86		100%	0%	
1/26/86		87.5%	25%	
1/27/86		87.5%	25%	
1/28/86		87.5%	12.5%	
1/29/86		100%	0%	
	Average	92.5%	12.5%	7.4
	MEM with EGTA (6mM)			
3/15/86	no serum	71%	57%	
3/15/86		50%	25%	

comparison. To correct for the general contribution of serum to maintenance of neuronal viability, the results of these separate sets of experiments has been expressed as a ratio of the average percent survival of control cells to the average percent survival of operated cells (C/O ratio). In medium with serum and a normal calcium concentration the C/o ratio was 1.85. Removal of serum increased the ratio of surviving control cells to surviving operated cells to 3.5. When both serum and extracellular calcium were removed the C/o ratio increased to 7.4. These results seem to indicate that, contrary to most current opinion, removal of extracellular calcium does not enhance cell survival. Indeed, the greatly increased mortality of lesioned neurons in calcium-free medium indicates that external calcium may be critical to their survival after physical trauma. Data on survival of lesioned and control neurons 24 hours after addition of serum-free medium or serum-free medium without calcium was not included as cultures showed extensive deterioration.

DISCUSSION

I. Injury Currents and Neuronal Injury: Historical Background

Historically, measurements of ionic fluxes and electrical changes after axotomy have been used to: A) ascertain the degree to which neuronal membranes are capable of resealing and reestablishing molecular selectivity after lesioning, and B) explain the mechanisms underlying the phenomena of axonal degeneration and drying-back.

A. Recovery of Membrane Competence in Neuronal Cells after Transection Injury

Electrophysiologic investigation into the capacity of excitable cell membranes to reseal after physical injury began before the turn of the century (Engelmann, 1877) and has continued to the present (Lorente de No, 1947; Rothschuh, 150, 1951; Eccles et al., 1958; Ueda, 1959; Easton, 1960, 1965a, 1965b; DeMello et al., 1969; Deleze, 1970; Borgens et al., 1980; Meiri et al., 1981; Yawo and Kuno, 1983, 1985). Table III summarizes the major findings of those studies which were performed on neuronal cells. While these investigations have produced some important

TABLE III

MEMBRANE RESEALING IN NEURONS FOLLOWING NEURITE TRANSECTION

Study	Model	Electrode Distance From Lesion	Conclusions
Engelmann (1877)	frog sciatic nerve	0-4000µm	EMF* at lesion reduced 53% 1-2 hrs after axotomy, 7% residual EMG at 24 hrs; normal EMF 500µm or more from lesion
Eccles (1958)	cat ventral roots	---	most neurons had lower RP and AP 14-44 days post axotomy**
Easton (1960, 1965)	frog sciatic nerve or spinal root	approx. 1 cm	cut nerve ends "heal" but exact time course not clear
Borgens et al. (1980)	larval lamprey	at transection site	persistant injury currents up to 6 days after transection
Meiri et al. (1981)	cockroach giant axon	200-300µm and 5000 ± 500µm	recovery of RP, AP and input resistance required 8-10 days
Yawo and Kuno (1983, 1985)	cockroach giant axon	500-1000µm	complete recovery of RP magnitude and input resistance in 5-30 min

*EMF: electromotive force

**Eccles attributed this finding to secondary electrode injury of chromatolyzed neurons and only used those cells with normal spike potentials for his measurements of the electrical properties of neurons in chromatolysis.

insights into the mechanisms of membrane resealing after physical rupture, Table III demonstrates that even after 100 years the question of the completeness of that resealing is still under debate (Borgens et al., 1980; Meiri et al, 1981; Yawo and Kuno, 1983, 1985).

In the present study I recorded continuously for periods up to 45 minutes from over 50 mammalian CNS neurons during and after dendrite amputation at lesion distances within 400 µm of the perikarya. Not once have I observed total recovery of a presurgery resting potential. The data show, in fact, that average potentials in surviving neurons remained approximately 8% below those of controls even 24 hours after neurite transection. It is interesting to note that a small residual "electromotive force" (7% of the maximum) was measured by Engelmann (1877) at transection sites along frog sciatic nerves 24 hours after severing (50 observations). Meiri et al. (1981) found that membrane potentials 200 µm to 300 µm from the lesion in single cut cockroach axons were profundly reduced for as long as ten days (5-12 observations at each time). Undoubtedly a major factor in the reported differences in the degree and duration of these potential changes after transection is the order of magnitude difference in the diameters of vertebrate and invertebrate neurites (a few micrometers vs 40-50 µm).

Reduced MP values in lesioned neurons have usually been attributed to incomplete recovery of normal membrane selectivity characteristics. Both Meiri et al. (1981) and Borgens et al. (1980) reported that small injury currents continued to leak across the lesion for days. However, impairment of membrane pump function after injury may also account for the lower average MP's in operated neurons. Experimental spinal cord trauma has been reported to inhibit activity of the Na+/K+ ATPase (Clenendon et al., 1978). Inhibition of pump function could be caused by an increase in plasma membrane fluidity due to the activation of phospholipase A_2 by calcium entering the cell across the lesion (Sun et al., 1983). Alternatively, depressed pump function could be caused by reduced ATP production resulting from mitochondrial damage and/or a loss of adenosine from the injured cell. Preliminary electron microscopic (EM) of surviving neurons 24 hours after dendrite amputation reveals that in most cases there is residual damage in the form of mitochondrial swelling and cytoskeletal disorganization (see Appendix A).

Injury currents have only recently been analyzed quantitatively in terms of their ionic components (Borgens et al., 1980; Meiri et al., 1981). Using techniques of ion replacement, Borgens et al. (1980) have shown that the injury currents entering the cut faces of severed lamprey

spinal cord axons consist primarily of massive influxes of sodium (45-47% of the toal ion flux in μAmps/cm^2) and calcium ions (32%). Similarly, in transected cockroach giant axons Meiri et al. (1981) meausred a four-fold increase in sodium conductance and a twenty-fold increase in calcium conductance at thelesion; gradual restoration in normal ion conductances took place over several days. Low-level ion leakage across the cut face of a transected neurite may actually be desireable. Meiri et al. (1981) demonstrated that the electrical properties at the transection site are similar to those of embryonic tissues and that the persistent calcium current at the lesion may stimulate regeneration of a cut axon much as calcium fluxes at a growth cone are necessary to elongation.

B. Degenerative Changes after Neurite Transection

The major changes in the cytoplasmic ultrastructure which have been reported to occur in neurons following axotomy include mitrochondrial swelling and disruption of cristae, vacuolization, microtubule disassembly and neurofilament disaggregation (Mire et al., 1970; Schlaepfer and Bunge, 1973; Schlaepfer, 1974; Bird, 1978; Sole, 1980). Most of these changes are consistent with the known effects of elevated levels of free calcium in the cytoplasm (Rossi

and Lehninger, 1964; Schlaepfer, 1971; Gilbert et al., 1975; Hunter et al., 1976; Mohri, 1976; Dustin, 1978; Coelho and Vercesi, 1980; Pant and Gainer, 1980), and, indeed, Schlaepfer and coworkers have reported a direct relationship between calcium influx into severed axons and the cytoskeletal deterioration (Schlaepfer and Bunge, 1973; Schlaepfer, 1974). While calcium-induced microtuble disassembly is reversible and neurofilaments may be replaced (Schlaepfer, 1971; Gilbert et al, 1975; Mohri, 1976; Dustin, 1978; Pant and Gainer, 1980), the severe mitochondrial damage characterized by high amplitude swelling is generally considered irreversible (Rossi and Lehninger, 1964; Hunter et al., 1976; Coelho and Vercesi, 1980). Situations in which increases in free calcium are prolonged may virtually shut down mitochondrial production of energy (ibid.).

Not surprisingly, observations of the cytoplasmic changes following dendrite amputation are very similar to descriptions of changes in axonal structures near a transection lesion (Lucas et al., 1986b; Emery et al., 1986; Gross and Higgins, 1986; see also Appendix B). Electron micrographs of severed neurites fixed within minutes after surgery reveal that ultrastructural damage forms a gradient between the lesion and soma with the greatest deterioration close to the transection site and less severe changes at the

leading edge of the damage front (Emery et al., 1986; Gross and Higgins; see also Appendix C). These findings also support the hypothesis that the ionic currents across the lesion mediate the cytoplasmic damage typical of transection trauma. However, in the present study attempts to protect lesioned neurons by removing extracellular calcium following the protocols used by Schlaepfer and Bunge (1973) were unsuccessful and mortality among both lesioned and control neurons was actually increased. Furthermore, although ultrastructural analyses have not yet been performed, LM observations have confirmed the development of the phase bright moribund stage in neurons lesioned in calcium-free medium indicating that these cells are still undergoing the same process of deterioration observed in complete medium containing normal levels of calcium. Thus, although external calcium may normally play some role in deterioration and death of neurons after transection trauma, it is apparently not necessary for either phenomenon. Possible explanations for this surprising effect are discussed in Sections III and IV.

II. Neuronal Survival after Transection Injury

Phenomena such as membrane resealing and the degenerative changes which result from influxes of extracellular cations undoubtedly have important

implications for neuronal viability after transection trauma. However, previous investigations of these events in axotomized neurons have not correlated them specifically with the probability of cell survival.

A. Injury Potentials and Neuronal Survival

Injury potentials represented as percent RP loss reflect the magnitude of ion fluxes at a lesion and may indicate the extent to which extracellular cations penetrate into a lesioned cell. In separate experiments both the injury potentials and cell survival were determined to be functions of lesion distance from the perikaryon. Comparison of the effective range of lesion distances in each study predicts that the incidence of cell death and the magnitude of the injury potentials (plateau phase) will fall to zero at lesion distances between 250-300 μm.

B. Morphological Indicators of Damage Spread and Cell Survival

Neuronal viability at 24 hours after dendrite amputation close to the perikaryon is easily determined on the basis of LM observations. However, shortly after lesioning, fatally injured neurons do not necessarily display obvious deterioration except for a slowly developing cytoplasmic phase brightness (see Figure 7). From EM

observations (lucas et al., 1986b; Emery et al., 1986; see also Appendix B) it is now clear that the intense phase brightness which pervades a moribund cell within two hours after transection trauma corresponds to the spread of numerous small vesicles and swollen nitochondria throughout the cytoplasm. Thus, the probability of neuronal survival may be correlated with the extent of this damage which is indicated by the spread of the optical effect.

It is interesting to note that formation of the myriad of small vesicles in moribund neurons would probably not be detectable with differential interference contrast microscopy. Phase contrast microscopy may be considered an imperfect form of interference microscopy due to incomplete separation of the interfering light beams. As a result, images are surrounded by halos of opposite contrast (Barer, 1965). Thick cells and processes show this phenomenon most clearly at their peripheries. The appearance of phase brightness in the center of somata and dendrites is a separate phenomenon and is probably associated with internal structural changes. It is likely that increased scattering and the summation of the halo effect around the numerous, small hydrated vacuoles in moribund neurons generated the observed phase brightness. This interpretation also explains the loss of cytoplasmic phase brightness in dead cells in which the small vesicles have coalesced into much larger structures.

III. Injury Currents and Cell Death

The ultrastructural damage in dead and moribund neurons after dendrite amputation (Appendix B) confirms closely to descriptions of necrosis (Wyllie, 1981; Bowen, 1984; Wyllie et al., 1984). The necrotic form of cell death typically orcurs in cells exposed to lethal environmental insults (ibid.). Many investigations have suggested that, regardless of the nature of the lethal agent, cell death is ultimately mediated by influxes of extracellular calcium (Schanne et al., 1979; Trump et al., 1978, 1979; Kane et al., 1980a, 1980b). Likewise neuronal death after CNS injury has been linked to elevated intracellular calcium (Balentine and Spector, 1977; Happel et al., 1981; Young et al., 1982; Stokes et al., 1983). In the present study, however, neurons lesioned in the absence of extracellular calcium following the protocols described by Schalepfer and Bunge (1973) continued to die (Table II). In fact, percent cell death of neurons after amputation of dendrites at 100 µm from their perikarya actually increased from 46% to 88%.

There is an important difference in the model of neuronal trauma employed by Schlaepfer and Bunge (1973) and that utilized in the present study which may account for the apparent contradiction in the results of investigations performed in calcium-free medium. In the earlier study experimental trauma to neurons was achieved by severing

bundles of axons growing from cultured DRG explants. In explants neurites within fascicles are protected from environmental perturbations by a number of surrounding structures including myelin, a three-dimensional extracellular matrix, and sheaths of connective tissue. In cultures of dissociated nervous tissue, however, the neuronal cells are relatively isolated and, therefore, more exposed to any environmental perturbations. This may explain whi in the present study even removal of serum by itself resulted in a reduced proportion of surviving operated neurons, while in the 1973 study it was reported that the DRG explant cultures could be maintained in serum-free medium for as long as one week with no obvious deterioration (Schlaepfer and Bunge, 1973). Likewise, extracellular calcium has been demonstrated to stabilize cell membranes (Manery, 1966). As a result, its removal may cause an increase in cell membrane permeability which develops more rapidly in neurons in monolayer cultures than in the relatively protected neurons within explants. In the present study lesioned neurons were particularly vulnerable to the removal of calcium; the C/O ratio (percent surviving control neurons to percent surviving lesioned neurons) in serum-free medium without calcium was roughly twice that in serum-free medium with calcium and almost four times the ratio in MEM with both serum and calcium (Table II).

Thus, rather than having desired prophylactic effect upon lesioned neurons the absence of calcium probably constituted a second insult. The critical question, however, still remains: in the absence of extracellular calcium, what is responsible for the increase in neuronal mortality in general and among lesioned neurons in particular?

IV. Expansion of the Calcium Hypothesis of Cell Death

Any hypothesis which explains the continued death of physically injured neurons in the absence of extracellular calcium must also account for the ultrastructural damage commonly observed in neurons after transection trauma which is typical of an increase in the concentration of free calcium in the cytoplasm. The most likely explanation is that transection trauma causes the release of intracellular stores of bound calcium. Although free cytoplasmic calcium is normally only 10^{-7} M, the concentration of total calcium in both neuronal and other types of cells is 0.4 to 2.0 mM (Baker, 1976; Albers et al., 1983). Sequestered calcium is stored primarily in the endoplasmic reticulum and in mitochondria (ibid.). If released, the concentration of free calcium in the cytoplasm would be more than sufficient to account for the loss of microtubules and disaggregation of neurofilaments (Mohri, 1976; Dustin, 1978; Pant and Gainer, 1980).

The most likely trigger for release of intracellular stores of bound calcium is the massive influx of sodium which has been shown to be the other major component of the injury current (Borgens et al., 1980). Sodium is known to trigger a two-for-one exchange with calcium across mitrochondrial membranes (Carafoli et al., 1974; Crompton et al., 1986; Crompton et al., 1977; Crompton and Heid, 1978; Nedergaard, 1984; Carafoli and Penniston, 1985). Excitable cells are normally equipped to handle small, transient influxes of sodium. However, if the concentration of sodium is high, it can cause the release of significant amounts of mitochondrial calcium (ibid.) which then can dismantle the cytoskeleton.

The possibility that sodium may be directly responsible for the irreversible high amplitude mitochondrial swelling which is considered to be the lethal lesion after physical trauma has not been addressed in the literature. Despite the two-for-one exchange of sodium for calcium, there is general agreement that, at concentrations of sodium chloride less than 50 mM, mitochondria do not swell (Brierley, G.P. et al., 1978; Jung, D.W. and Brierly, G.P., 1978). Carofoli, however, does allude to swelling of mitochondria at pathological concentrations of sodium chloride (Carafoli, et al., 1984). If mitochondrial swelling is extreme, irreversible damage will occur (Trump et al., 1974).

Thus, the sodium component of the injury current may play both indirect and direct roles in the ultrastructural deterioration and death of neurons after physical injury. Indeed, it is probable that conflicting reports of the protective effects of calcium removal from the medium of cells exposed to a variety of toxic insults (Schanee et al., 1979; Kane et al., 1980 a,b; Stacey and Klaassen, 1982; Fariss et al., 1985) are the result of penetration of the cells by varying amounts of extracellular sodium. A diagram of the various internal and plasma membrane pumps for calcium is given in Figure 13. A diagram of the hypothesized events following a large influx of sodium is presented in Figure 14.

The finding that removal of extracellular calcium not only does not protect lesioned neurons but actually increases the probability of cell death can also be explained. Yawo and Kuno (1983, 1985) have demonstrated that membrane resealing at the transection site of a severed axon is the result of an increase in membrane fluidity due to calcium activation of phospholipase A_2. In the absence of extracellular calcium, resealing of the reputured membrane is probably delayed allowing a greater influx of sodium which mediates more widespread ultrastructural damage and increases the probability of cell death.

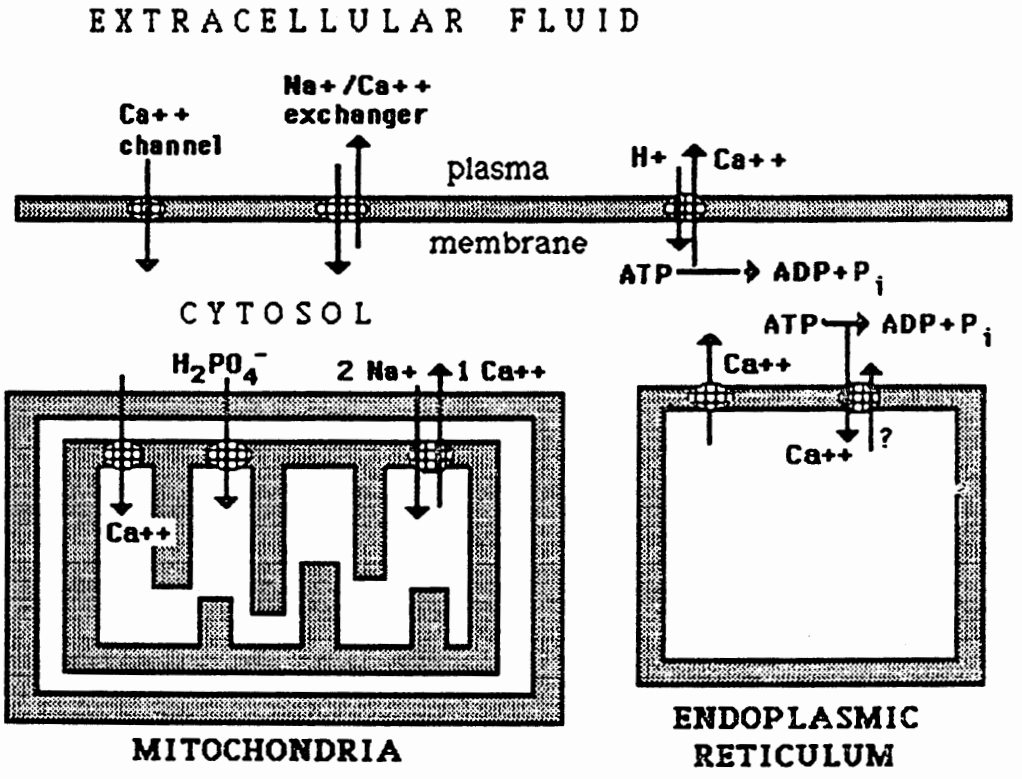


Figure 13. Summary of Na+ and Ca++ transport mechanisms identified for mitochondria; smooth endoplasmic reticulum, and plasma membrane in excitable cells.

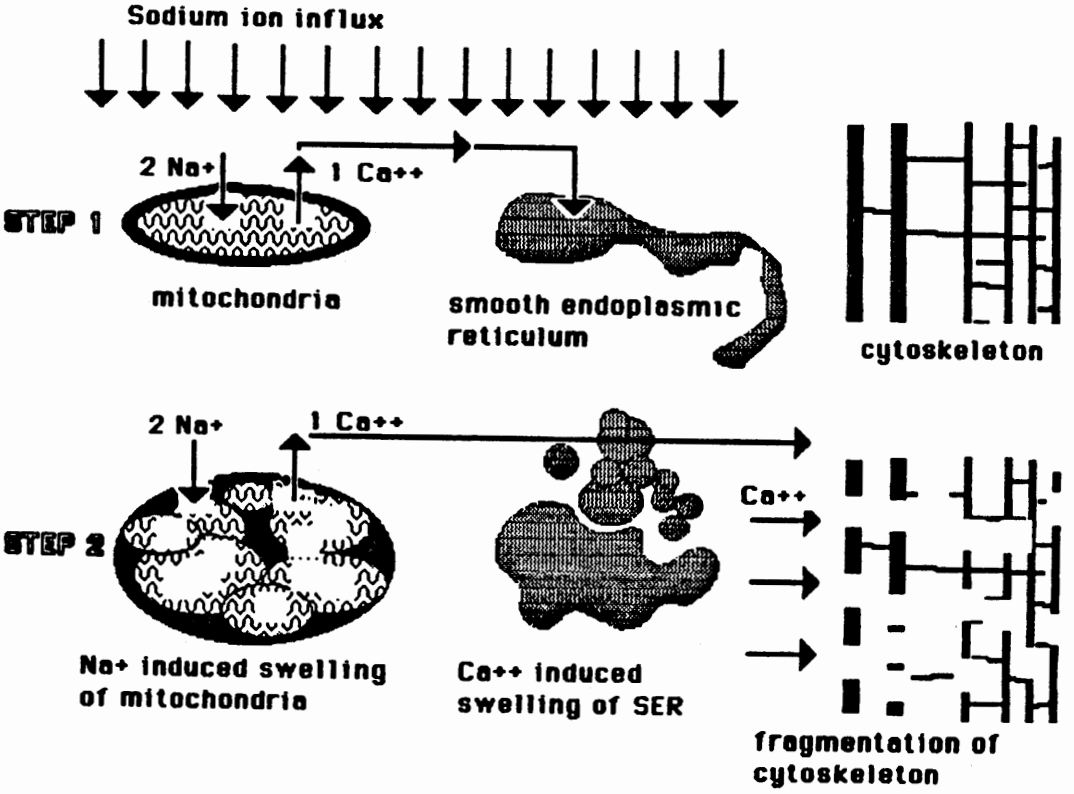


Figure 14. The sodium/endogenous calcium hypothesis of cell death. Sodium ion influx accelerates an ATP-independent 2Na+ for 1Ca++ exchange across the inner mitochondrial membrane. The released endogenous Ca++ is initially sequestered rapidly by the SER, keeping the free calcium concentration below 1 µm. As more sodium enters the cell and more Ca++ is released from mitochondria, two pathological reactions are triggered: (a) sodium-induced osmotic swelling of mitochondria and (b) calcium-induced swelling and fragmentation of the SER as Ca++ saturation is approached. Failure of further SER sequestering causes the free calcium concentration in the cytosol to rise and initiates fragmentation of the cytoskeleton.

V. Recovery of Surviving Neurons after Dendrite Amputation

Neurons which survive dendrite amputation probably do so as a result of their successful efforts to restore the concentration of intracellular cations to normal levels. Recovery of membrane potentials of surviving neurons to approximately 90% of control cell potentials within 24 hours after lesioning implies restoration of near normal ionic gradients. Electron microscopy has provided some preliminary visual evidence of this recovery process. All cells fixed within the first few minutes of dendrite amputation show reduced numbers of microtubules in the target neurite (Gross et al., 1983; Kirkpatrick et al., 1985; Sole, 1980; Emery et al., 1986; Gross and Higgins, 1986). In surviving neurons at two and 24 hours after dendrite amputation microtubules are present, even in the cytoplasm adjacent to the lesion, but lack their usual longitudinal orientation (see Appendices A and B, Figures A2 and A3). It is therefore, probable that the microtubules seen in viable neurons within 24 hours after laser surgery have actually been reassembled as a result of reestablishment of physiological levels of free calcium (10^{-7} M).

VI. Neuronal Survival and the Injury Potential Profile

The injury potential profile probably constitutes a very accurate record of the stages in the survival responses of neurons after transection. Injury potential profiles have not previously been recognized as characteristic of the early reactions to lesioning and have not been described as a function of lesion distance from the soma. Yawo and Kuno (1983, 1985) do, however, show such profiles with the peak and plateau phases among their figures.

The peak phase of the typical injury potential probably represetns the actual membrane resealing at the lesion. Yawo and Kuno (1983, 1985) have demonstrated that membrane resealing is dependent upon calcium activation of phospholipase A_2 which then casues an increase in membrane fluidity. Preliminary attempts to correlate physical parameters of the lesion with characteristic intervals during the peak phase (see inset Figure 4) indicate a direct relationship between time to the inflection point on the rising slope and lesion diameter. However, confirmation of this trend as well as analysis of other portions of this phase will require additional observations.

Given that rapid membrane resealing is a prerequisite for cell survival, it probably is not, in itself,

sufficient to insure the viability of neurons after transection trauma. Restoration of normal ion concentrations undoubtedly constitutes an important aspect of the cell survival response. Studies have demonstrated that cells have a remarkable ability to prevent large concentrations of injected calcium from spreading within the cytoplasm (Baker, 1976; Rose and Loewenstein, 1975). Some of the means by which the free calcium is controlled include calcium binding proteins such as calmodulin and even tubulin, sequestering by mitochondria and the endoplasmic reticulum and removal to the extracellular space by various plasma membrane pumps (Baker, 1976; Levine and Williams, 1982; Moore and Dedman, 1982; Penniston, 1983). Removal of sodium is largely dependent upon the Na+/K+ pump.

The plateau phase of injury potentials represents an equilibrium established after the initial resealing between the infolowing (injury) and outflowing ionic currents. The outflowing current must reflect in part removal of calcium from an injured neuron by pumping across the plasma membrane. The magnitude of this plateau phase relative to the peak phase indicates how successfully the cell is coping with both the initial ionic influx across the lesion as well as leakage from resealing. For lesions beyond 100 µm from the perikaryon (the pivotal distance for cell

survival) most plateau phase potentials are not larger than their peak phases.

VII. Physical Parameters of the Lesion: Deciding Factors in Cell Survival?

It has been determined that the probability of neuronal survival after neurite transection increases with increased distance from the perikaryon (Figure 4). The diameter of the cut neurite at the lesion has also been demonstrated to influence survival and probably accounts for much of the statistical variability at each lesion distance. These findings show that the dimensions of the "conduit" between the soma and lesion are major factors in cell survival after dendrite transection. It is this physical structure which determines: 1) the magnitude of the injury currents across the lesion and 2) the degree to which they penetrate into the perikaryon and other processes. Influence upon the latter is a matter of both the total number of pumping, binding and sequestering entities between the lesion site and the soma as well as passive cable properties of the proximal segment.

VIII. The Point of Irreversibility after Dendrite Amputation

It is tempting to interpret the finding that cell death is a function of transection lesion distance from the soma to mean that damage directly to the nucleus and impairment of nuclear functions constitute the critical insult to the injuryed cell. However, this is not necessarily the case. Even in dead cells ultrastructural changes within the nucleus do not appear to be as profound as those of the cytoplasm (Appendix B); indeed, the most obvious alteration, which is clumping of the chromatin, is generally considered to be reversible (Wyllie, 1981; Bowen, 1984; Wyllie et al., 1984). Rather, spread of irreversible damage to mitochondria into the perikaryon may simple create a situation in which the nucleus is surrounded by a sea of "dead" cytoplasm in which two few high energy molecules are produced. Nuclear energy starvation may be the final stage of a lethal physical insult to a cell and deleterious direct effects of calcium or sodium upon the nucleus may only serve to increase the rapidity of cell death. This interpretation would also be consistent with the results of the studies of the effects of ischemia which have identified the stage of widespread irreversible mitochondrial damage as the "point of no return" (Trump et al., 1974).

IX. Significance to Studies in Neurobiology

There are a number of practical implications of these findings for studies in neurobiology. Techniques of laser microbeam surgery were developed to be used in culture to simplify the circuitry of neuronal networks (Higgins et al., 1980; Gross et al., 1983). The present findings indicate that under normal culture conditions laser cell surgery is not feasible at distances less than 100 µm if the majority of the cells are to live. In addition, laser microsurgical changes of neuronal geometry for the purpose of investigation of altered calbe properties cannot be recommended within this critical 100 µm distance. Manipulations of culture media which increase the likelihood of survival of neurons operated *in vitro* will be necessary if surgical modification of neurons close to their perikarya is required.

Our findings regarding the probability of neuronal survial after lesioning close to the soma may also be relevant to some of the more traditional methodologies in neurobiology. For instance, data acquired using the conventional tissue slice of only a few hundred micrometers thickness may have to be reinterpreted as this preparation could cause death of many circuit components. The survival studies may also explain the difficulty of culturing larger

neurons from adult tissue with larger diameter neurites; the bigger cells probably sustain more serious damage during the process of dissociation (Scott, 1977). This same argument might also explain why the use of dissociated adult nervous tissues has been less successful in intracerebral grafting experiments (Gage et al., 1983/84).

These studies also indicate that 24 hours after dendrite amputation the membrane potentials of small mammalian neurons remain slightly below those of control cells (present upper limit of observations). Meriri et al. (1981) reported an even more significant depression in potentials of cockroach neurons as well as membrane impedence changes which persisted for as long as ten days. The evidence that neuronal membranes of surviving cells do not fully recovery their normal electrical properties for days after neurite transection should be taken into account by those studying the effects of altered dendritic geometry (Miller and Jacobs, 1984).

We have stated that the fate of neurons operated within 200 μm of their perikarya is probably decided within two hours of the injury. The opportunities for clinical intervention to save these cells are, therefore, very limited. In the surviving cells however, depressed membrane potentials reflect an abnormal state of increased membrane permeability and/or depressed ion pump function

which persists for at least 24 hour. The question arises whether a neuron in this final recovery phase is more vulnerable to further trauma as well as to a variety of secondary physical and chemical perturbations (ischemia, free radical formation, etc.) common to CNS trauma (Balentine, 1985; Young, 1985). Having established the baseline data for neuronal recovery under normal culture conditions, the next logical step would be to investigate neuronal vulnerability under the less than optimal conditions which are found in CNS tissue after trauma. It is not unreasonable to speculate that increased vulnerability during the long plateau phase of recovery may be a contributing factor to observed post-traumatic complications.

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APPENDIX A

RESIDUAL DAMAGE IN SURVIVING NEURONS

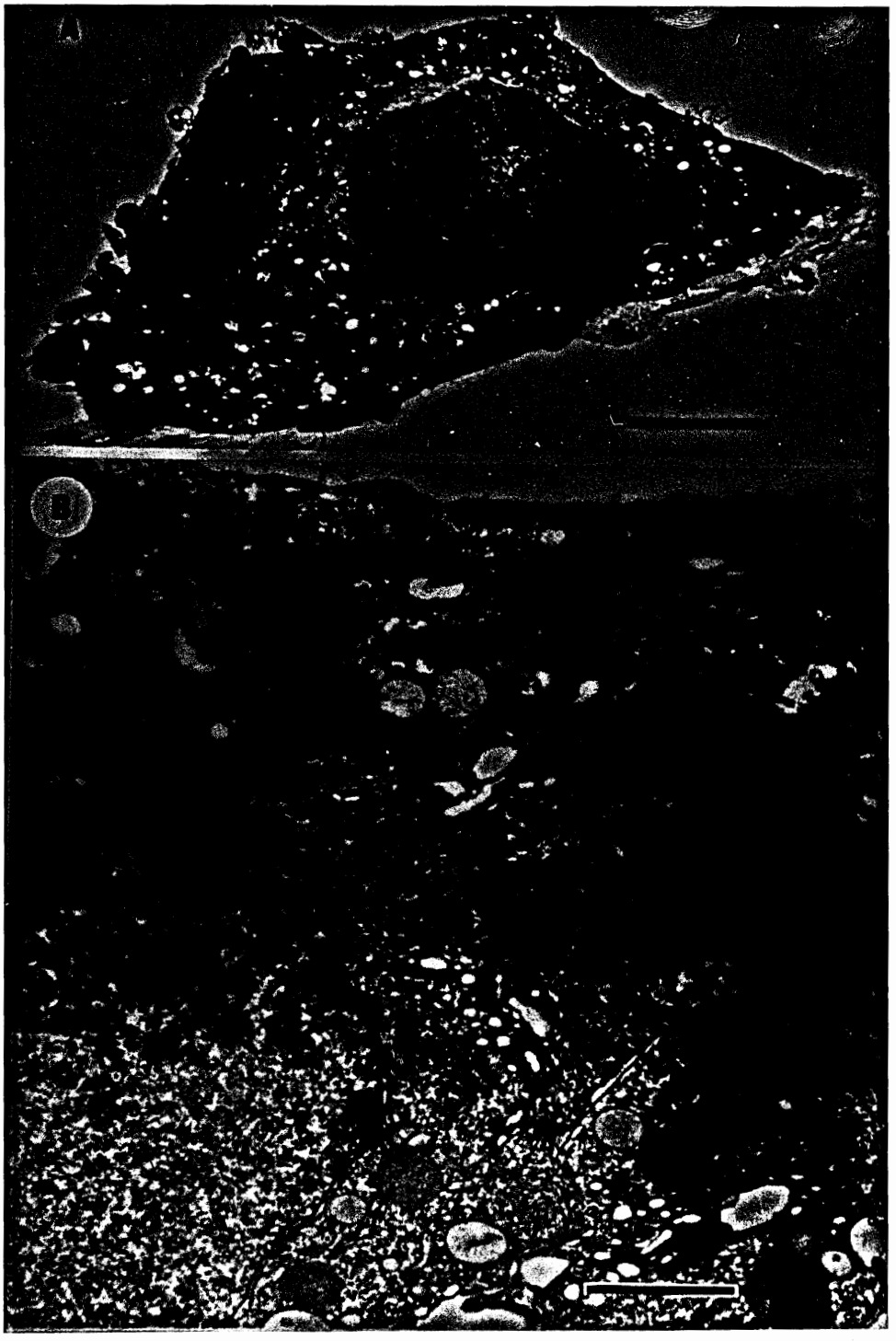

Figure A1. Spinal neuron 24 hours after transection of a dendrite 100 µm from the perikaryon. A. While the cell is viable, mitochondria are swollen and show internal disruption. Scale bar = 5 µm. B. Some swollen golgi/ER cisternae are present, but the extensive vesiculation characteristic of moribund cells is absent and undilated ER is common. The nucleus appears unaffected and microtubules are still present in the cytoplasm. Scale bar = 1 µm. Electron micrograph courtesy of Dr. Dennis Emergy.

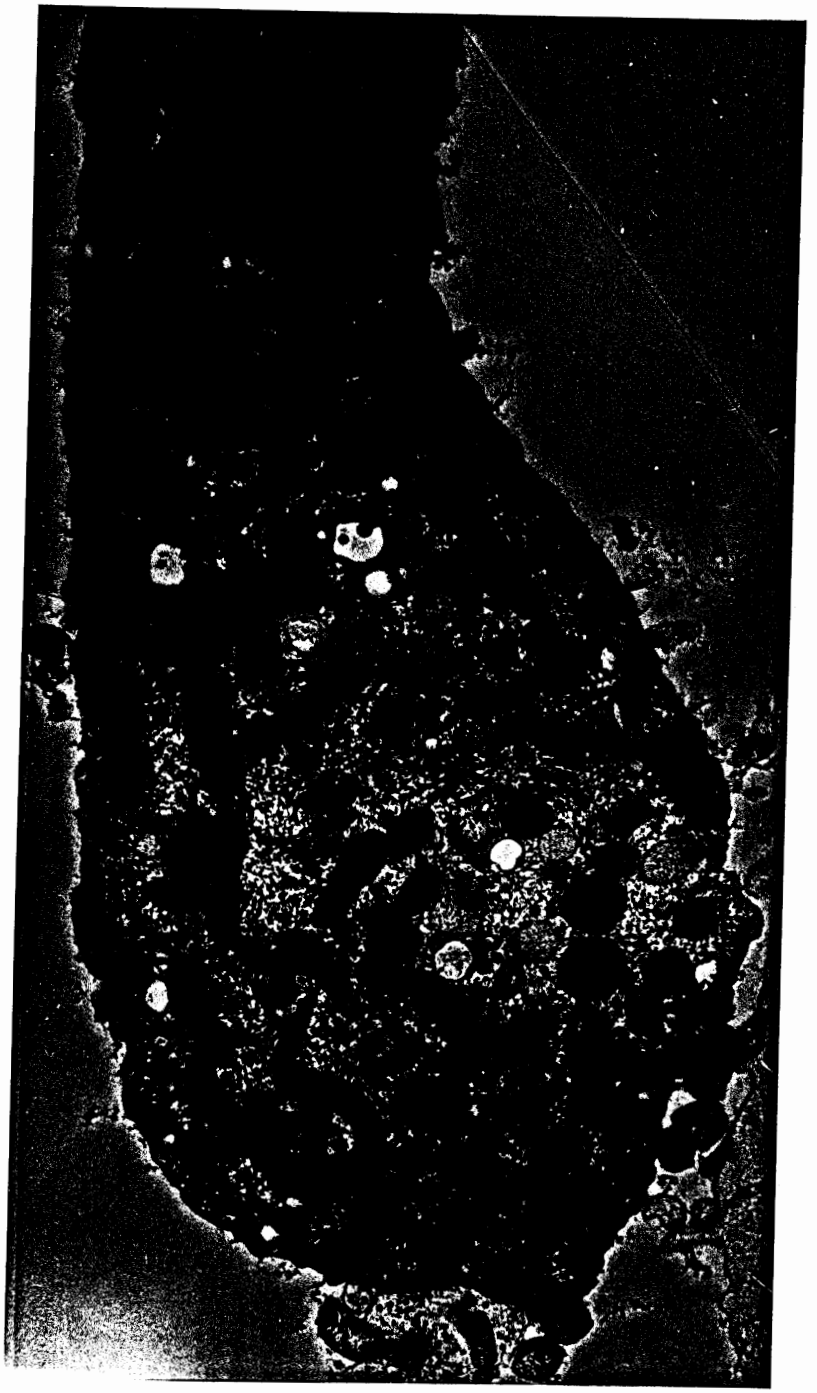

Figure 2A. "Retraction ball" at end of the proximal segment of a transected dendrite of a surviving neuron. Although ER derived membrane-bound vesicles are present, mitochondria appear completely normal displaying neither electron lucent areas nor swelling. Behind the swollen terminus microtubules are longitudinally oriented. Microtubules are also present within the terminal swelling although reduced in number and disoriented. A neighboring neurite with intact synaptic contact which was apparently attached along the dendrite prior to transection has been shifted to the center of the terminal swelling by retraction of the cut process. The lesser degree of ultrastructural disruption in this surviving cell at two hours suggests that retraction of the cut process may accelerate resealing of the severed process. Lesion distance = 100 μm. Fixation time = 2 hours. Electron micrograph courtesy of Dr. Dennis Emery.

APPENDIX B

CORRELATION OF MORPHOLOGICAL AND OPTICAL CHANGES IN VIABLE, MORIBUND AND DEAD NEURONS AFTER DENDRITE AMPUTATION WITH ULTRASTRUCTURAL CHANGES

APPENDIX B

LM ASSESSMENT OF NEURONAL VIABILITY AFTER DENDRITE AMPUTATION: CORRELATION OF MORPHOLOGICAL AND OPTICAL CHANGES WITH ULTRASTRUCTURAL CHANGES

Cells that were judged viable on the basis of their appearance in the light microscope two hours after dendritic transection (100 m from the perikaryon) varied somewhat in ultrastructure. Some were virtually indistinguishable from control cells. Others showed some vacuolization of the cytoplasm and swelling of the mitochondria (Figure A4). Enlargement of cut processes proximal to the lesion site, accompanied by secondary narrowing proximal to such swellings (Figure A4 A) was seen in some transected dendrites. Microtubules appeared disorganized and lacked longitudinal orientation within cut processes (Figure A4 B), but closer to the perikaryon the microtubules were normally oriented.

Contacts between the transected dendrites and other neurites were not affected by dendritic transection. Adhesions between the transected dendrite and other processes can be seen in Figure A4 B, and some of these have the characteristic appearance of chemical synapses.

Cells which were judged to be moribund two hours after dendritic transection based on light microscopic changes

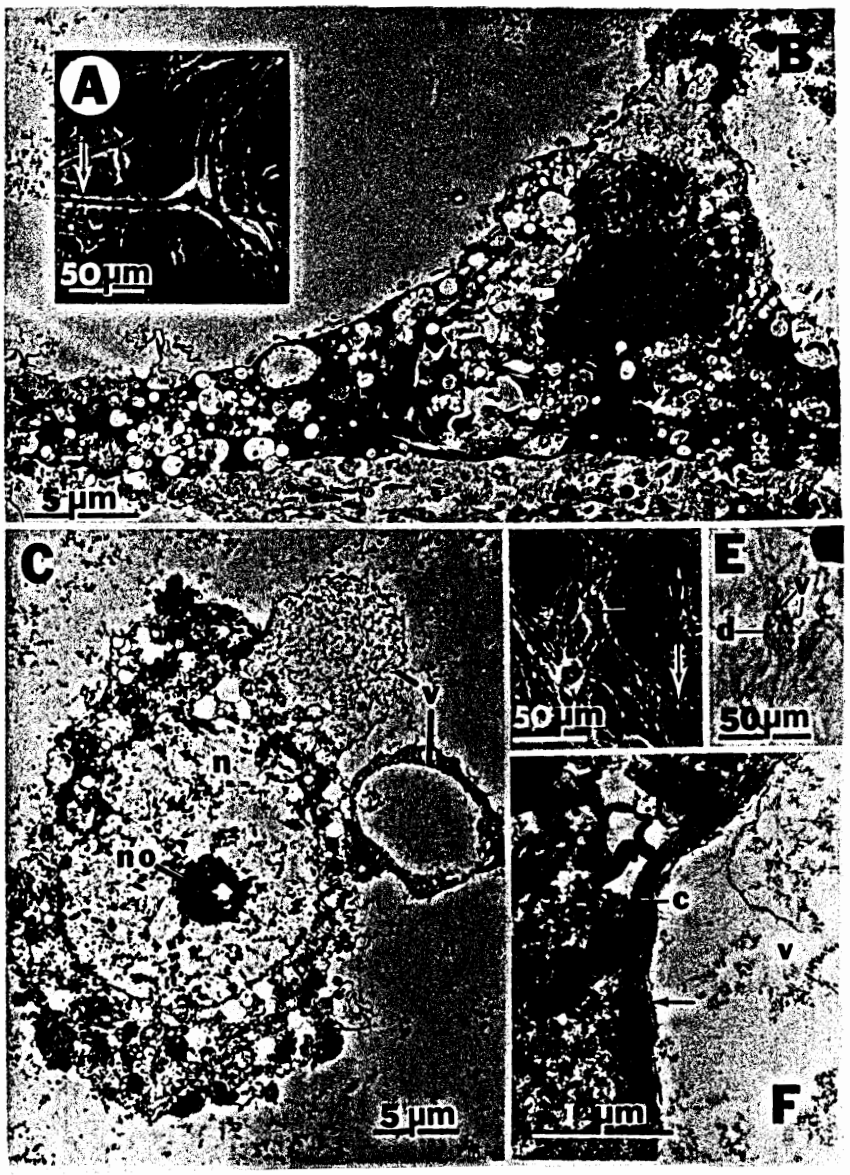
A
50 µm
B
5 µm
C
v
n
no
5 µm
50 µm
E
d
50 µm
c
v
1 µm
F

Figure A3. Neurons that appear moribund (upper) and dead (lower) two hours after laser transection of a dendrite. A. Light micrograph of the moribund neuron showing the site of laser surgery (arrow). B. Electron micrograph of the moribund neuron shown in A. Note the extensive vacuolization of the cytoplasm. C. Electron micrograph of a dead neuron two hours after dendrite transection. Both the nucleu (n) and nucleolus (no) are still visible. Two large putative vesicles (v) or blebs are present near the cell. D.E. Light micrographs of the neuron in C before (D) and after (E) laser cell surgery (arrow). The vesicles (v) are visible in E. F. Electron micrograph of part of the lower vesicle shown in C. A double membrane (arrow) separates the flocculent contents of the vesicle (v) from a cytoplasmic cortex (c). Electron micrograph courtesy of Dr. Dennis Emery.

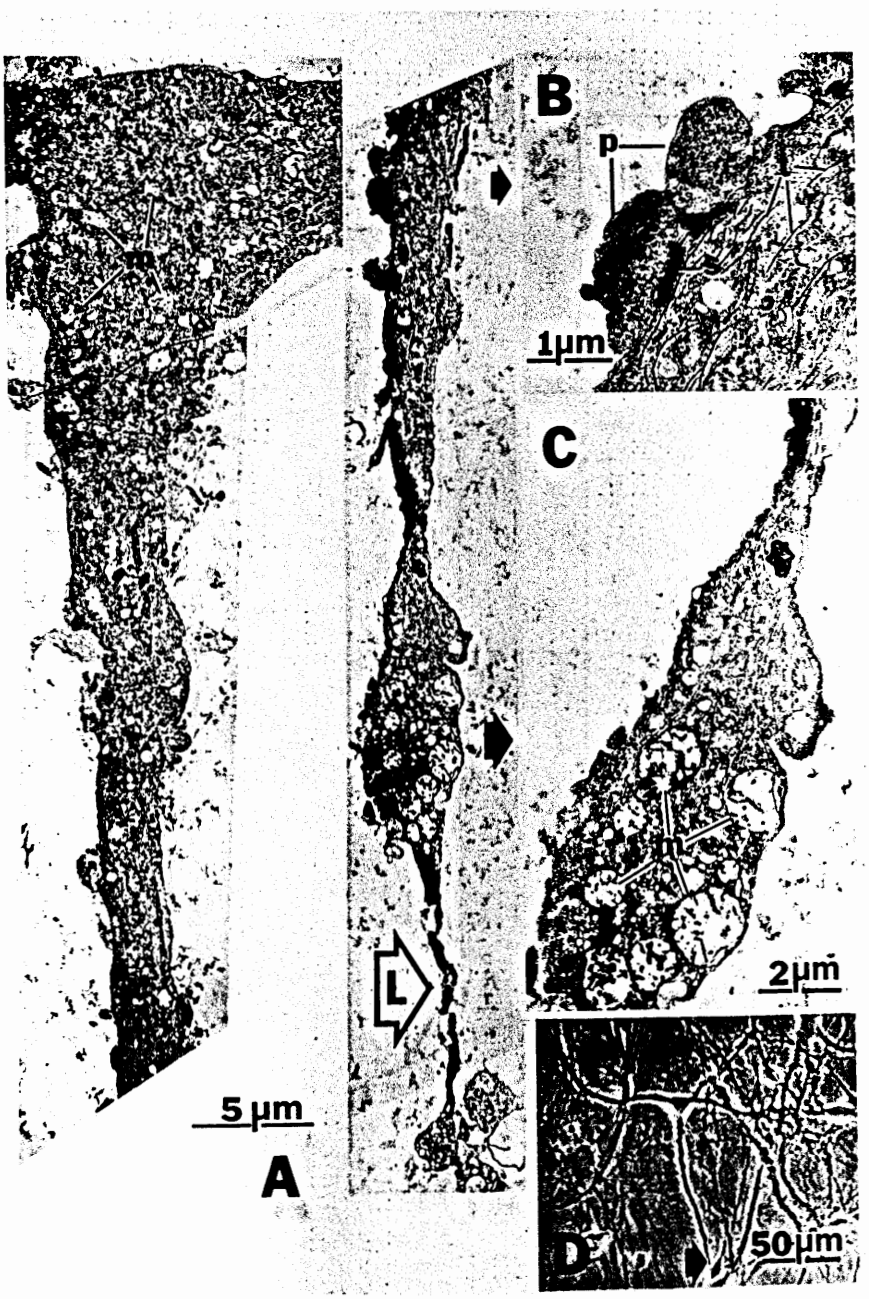
B
p
1µm
C
m
2µm
L
5µm
A
D
50µm

Figure A4. A neuron two hours after dendrite transection that appears viable on the basis of light microscopy. A. Electron photomontage of the neuron showing the cell body (left panel) and the transected process to the site of laser transection (L, right panel). Some swollen mitochondria (m) are visible in the soma. B. Enlargement of a portion of the dendrite in A showing two processes from other cells (p) contacting the dendrite. One process has formed a chemical synapse (s) onto the transected dendrite. Short segments of microtubules (t) are visible in the dendrite C. Enlarged dendritic segment proximal to the site of the site of transection showing swollen mitochondria (m). D. Light micrograph showing the neuron before laser surgery. The site of transection is indicated by the arrow. Electron micrograph courtesy of Dr. Dennis Emery.

such as increased internal phase brightness, manifested a highly consistent constellation of ultrastructural changes. As shown in Figure A3 A-B, the cytoplasm of these cells was generally filled with vesicles and the cytoplasmic ground substance very electron dense. Microtubules were totally absent from the cytoplasm of the moribund neurons. Such cells had an overall electron dense when viewed with EM. Close inspection revealed that the vesicles derived from swollen fragmented endoplasmic reticulum and from grossly swollen mitochondria. However, the nuclei and nucleoli of these cells were usually visible in sections, and the nuclear membranes appeared intact for the most part. Clumping of the chromatin in moribund cells gave the nuclei a mottled appearance at this stage. The presence of the many clear vesicles around the nuclei of moribund neurons which would probably have obscured these organelles from view with the light microscope. Synapse-like contacts between moribund cells and other neurons remained intact.

Cells judged on the basis of light microscopy to be dead two hours after dendrite transection showed characteristic and extensive ultrastructural disruption (Figure A3 C-F). Most mitochrondria were very condensed and electron dense in appearance. There were still some membranous systems with an electron dense matrix which appeared to be remnants of the endoplasmic reticulum.

and large vacuoles might also be present. The cytoplasmic ground substance was usually ver clear at this stage. There was no evidence of ribosomes, microtubules or fibrils in the cytoplasm of dead cells. The nuclei of dead cells were still visible, both ultrastructurally and with LM, and the nuclear membranes were mostly intact. The nuclei were very granular internally. In some cases, nucleoli in dead neurons appeared dispersed. Synapse-like contacts were still present on the surface of the dead cells.

With the light microscope large vesicles or blebs were often seen near the dead cells (Figure A3 E). Ultrastructurally these appeared to have a flocculent matrix surrounded by a membrane and/or a thin cytoplasm-like layer (Figure A3 F). These structures were assumed to have been extruded from the injured cells.

APPENDIX C

GRADIENT OF ULTRASTRUCTURAL CHANGES WITHIN A TRANSECTED DENDRITE

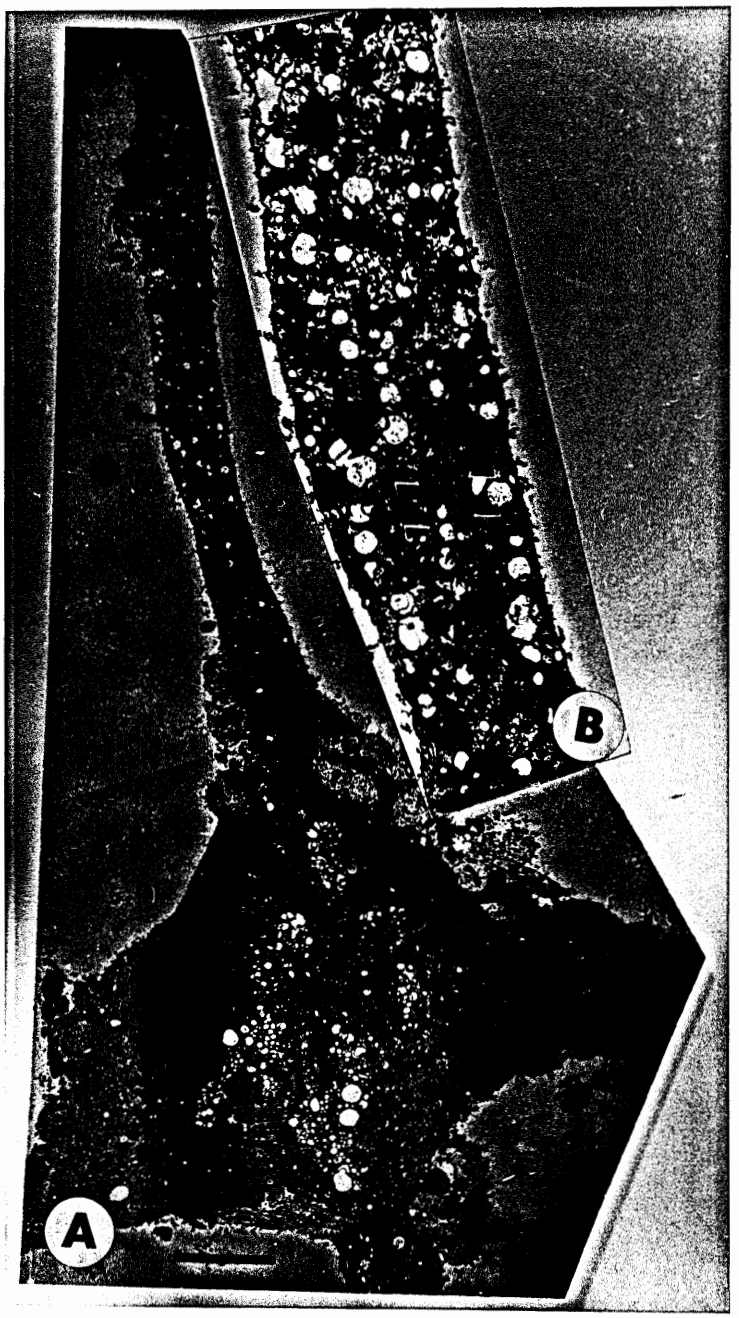
B
A

Figure A5. Spinal neuron fixed seven minutes after dentrite amputation 50 m from the edge of the perikaryon. A. The target process (P) shows a gradient of degenerative changes spreading from the transection site (top) towards the soma. Clear areas are apparent in the mitochondria of the soma as well as the dendrite (arrowheads). Note the clusters of clear vesicles (V) in the central cytoplasm of the soma. The nucleus (N) is eccentrically located. Scale bar = 5 µm.
B. Enlarged portion of transected process of same neuron. The middle of this segment is 20 µm from the soma (below) and 30 µm from the lesion (above). This segment seems to represent a transition region along the damage gradient. The cytoplasm in the half of the segment which is closer to the lesion has swollen mitochondria (M) and very few microtubules. The cytoplasm of the half of the segment which is closer to the soma still contains intact microtubules and mitochondria without obvious swelling. Degeneration in the form of swollen vesicles derived from cisternae of the ER and clear areas within mitochondria is apparent both above and below the region of transition.