NEURONAL SURVIVAL AFTER DENDRITE AMPUTATION: INVESTIGATION OF INJURY CURRENT BLOCKAGE

THESIS

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After dendrite transection, two primary injury current pathways may acount for cell death: (1) the lesion current at the site of injury and (2) the voltage sensitive calcium channels along the dendrite. Lesions were made with a laser microbeam in mouse spinal monolayer cell cultures. Polylysine was tried as a positively charged "molecular bandage" to block the lesion current. The calcium channel blockers, verapamil and nifedipine, were used to reduce the calcium channel current. Control toxicity curves were obtained for all three compounds. The results show that neither verapamil, nifedipine, nor polylysine (MW: 3,300) protect nerve cells after dendrite amputation 100 μm from the soma. The data also indicate that these compounds do not slow the process of cell death after such physical trauma.

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CHAPTER I

INTRODUCTION

Background and Rationale for this Study

Modern systematic scientific investigations of the responses of the nervous system to varying kinds of trauma and mechanisms of repair date from the middle of the last century (Brown-Sequard, 1849, 1850; Engelmann, 1877; Schamus, 1890; Allen, 1911; Cajal, 1928). Interest in the treatment of the unparalleled injuries received during modern warfare gave added impetus to these investigations (Young, 1949). At present, in our highly mobile society, the high rate of moving vehicle accidents has transformed such injuries into a fact of modern living.

In spite of the sophisticated advances in modern surgical therapy, the present day managment of central nervous system (CNS) trauma remains unsatisfactory. In the case of spinal cord trauma, one of the most common CNS injuries, current treatment consists of immobilization with skeletal traction, surgical decompression with or without incision of dural coverings, and immediate or delayed bone fusion to provide stabilization (Collins, 1983; Dean, 1984). Unfortunately, even with these treatments, a very high

percentage of such patients eventually develop varying degrees of paralysis. The weakness of clinical treatments and the poor understanding of the events of CNS trauma have forced scientists to re-examine the injury and recovery mechanisms of CNS trauma in more detail.

Many investigators have utilized experimental models which focus on damage at the tissue level (Balentine, 1978, 1982; Bresnahan, 1978; Molt et al., 1979; Feringa et al., 1980; Krikorian et al., 1981). A major disadvantage of such models has been the difficulty of discriminating between the pure neuronal responses to injury and the reactions of other cells and tissues. With the advent of reliable culture methods, it has now become possible to observe the primary responses of cells to mechanical injury in isolation from the homeostatic influences of the intact animal (Schwertschlag, 1986). Additionally, many researchers have developed different methods for creation of experimental physical lesions in single cells. Most of these models have utilized various types of "microknives" for cell surgery (Levi and Meyer, 1945; Mire et al., 1970; Schlaepfer and Bunge, 1973; Shaw and Bray, 1977; Bird, 1978; Sole, 1980; Yawo and Kuno, 1983, 1985). However, mechanical methods of cell surgery do not offer the control necessary for creating experimental lesions. These methods have disadvantages such as involvement of neighboring structures and target cell process

stretching. Such distortion also precludes concurrent electrical recording close to the lesion site during cell surgery. Consequently, the responses of neurons to physical trauma, including the probability of cell survival or death, cannot be assessed quantitatively as functions of injury severity.

In order to resolve these difficulties, Gross, Lucas and coworkers have developed techniques of laser microbeam cell surgery (Gross et al., 1983). Laser cell surgery methods confer the capability of creating highly localized cellular injuries as small as one micrometer in diameter at precise locations along target neurites. Laser cell surgery causes minimal disturbance of other portions of the target cell, surrounding structures, and intracelluar electrodes. This precision, together with the accessibility of individual neurons in monolayer cultures, has permitted the first observation of the effects of defined neurite transection injuries on nerve cell electrical properties, on ultrastructure, and on the likelihood of neuronal survival (Gross et al.,1983; Kirkpatrick et al., 1985; Lucas et al., 1985; Gross and Higgins,1987; Emery et al., 1987; Lucas, 1987).

Neurons have a unique cellular morphology. Nerve cell processes or "neurites" extend long distances from the soma. This geometry permits creation of experimental transection lesions at varying distances from perikarya and allows

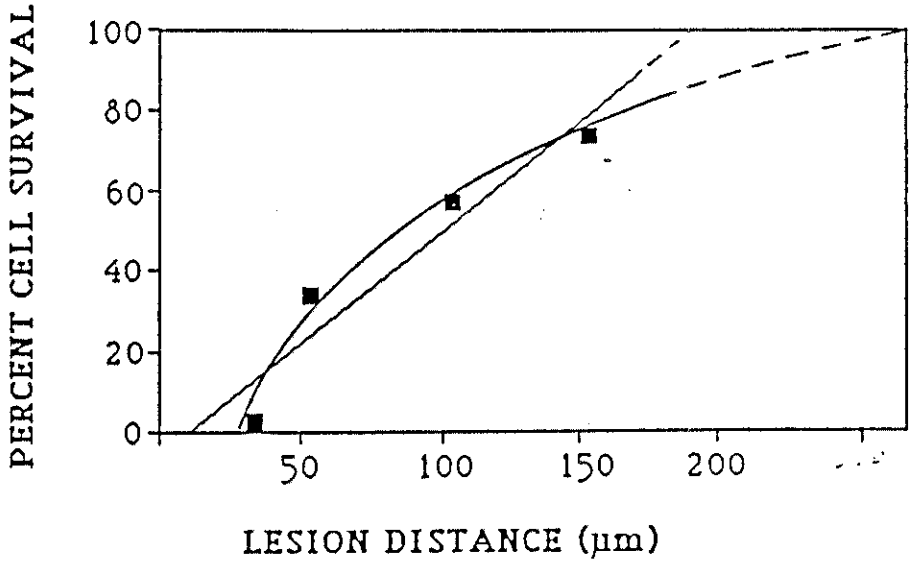
temporal separation of the local injury events and eventual cell death. This separation of injury and death is impossible in most other cell types.

Based upon previous studies performed in this laboratory, we know that nerve cell survival or death after neurite transection is a function of three lesion physical parameters. They are lesion distance from the soma, lesion diameter, and the extent of proximal segment retraction (Lucas et al., 1985; Lucas, 1987; Fig. 1, 2). According to Lucas and coworkers (1985), 24 hours cell survival after dendrite amputation at lesion distances of 50 μm, 100 μm, and 150 μm is 30%, 53%, and 70% with respect to each. These data indicate that the greater the lesion distance, the higher the probability of cell survival. Increased survival of operated cells has also been found to be inversely proportional to lesion diameter (Lucas et al., 1985). Finally, the probability of nerve cell survival after dendrite transection has been shown to increase as a function of proximal segment retraction (Lucas, 1987).

Laser surgery methods also permit expression of the effect of environmental manipulations for treatment of physically injured neurons in terms of absolute numbers of surviving cells. These findings have established baseline data for neuronal recovery under normal culture conditions. They serve as reference data for future studies in which the

Figure 1. Effect of lesion distance and diameter on cell survival. *A*, The probability of nerve cell survival 24 hours after amputation of a primary neurite increases with distances of the lesion from the perikaryon. This graph represents 58 experiments (580 lesioned cells). The viability of 330 of the lesioned neurons was also evaluated at 48 hours and 72 hours. No further cell death was observed at these longer post-injury intervals. Survival of 580 unoperated control cells was almost 100%. The data predict that 100 % survival will occur at approximately 200 µm (linear fit) or 300 µm (hyperbolic fit) (From Lucas et al., 1987). *B*, The probability of nerve cell survival after amputation of a primary dendrite is inversly proportional to lesion diameter. This graph represents data from 35 experiments (350 lesioned cells). Lesion distance was 100 µm in every case (From Lucas et al., 1987).

A.


PERCENT CELL SURVIVAL
100
80
60
40
20
0
50
100
150
200
LESION DISTANCE (µm)

B.

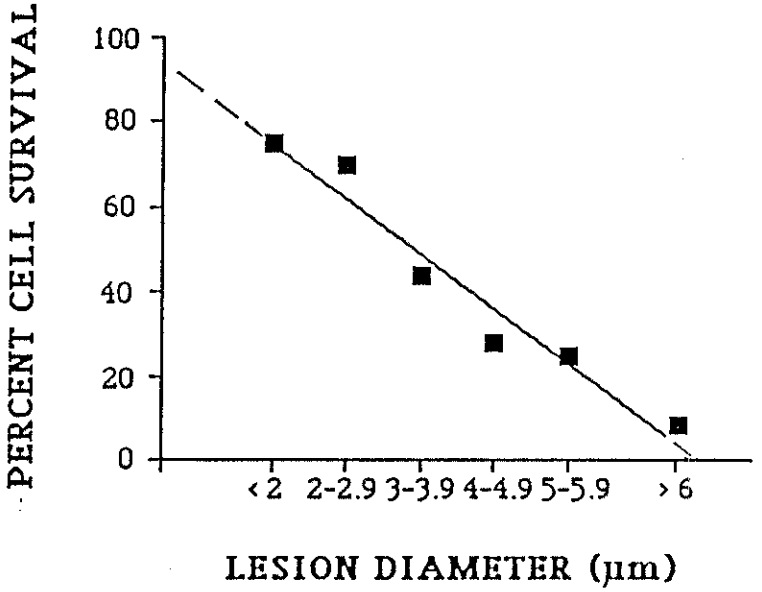
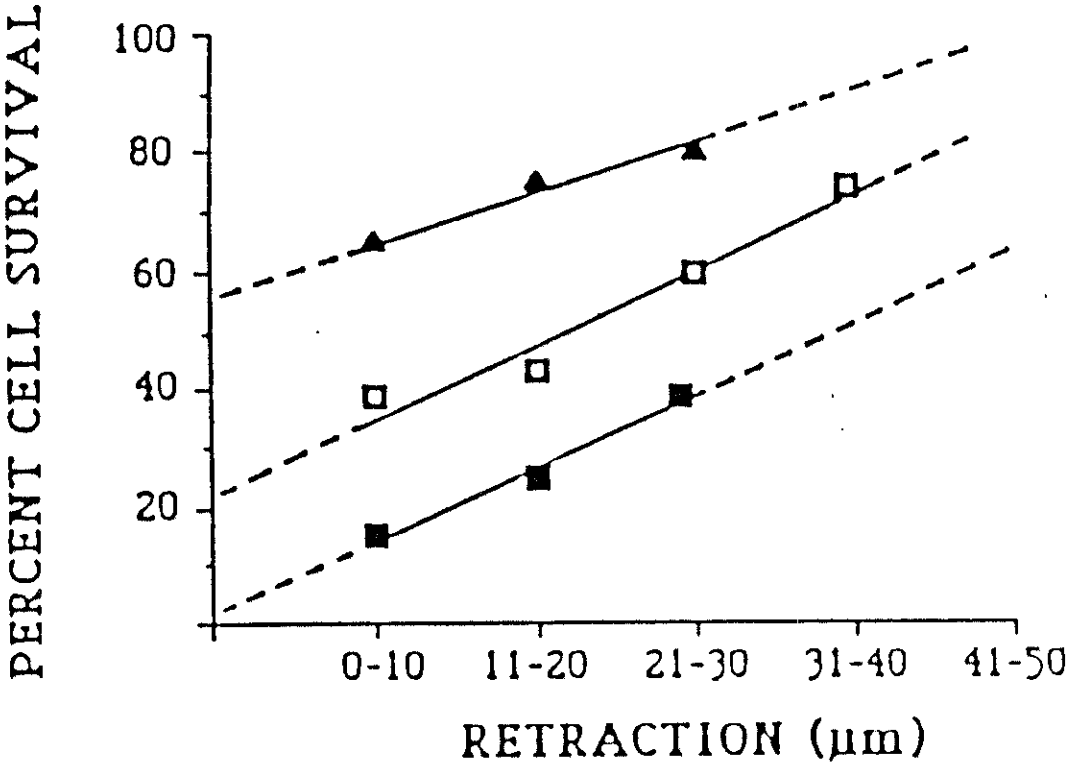

PERCENT CELL SURVIVAL
100
80
60
40
20
0
< 2
2-2.9
3-3.9
4-4.9
5-5.9
> 6
LESION DIAMETER (µm)

Figure 2. Effect of dendrite retraction from point of transection on cell survival. Lesion distance was 100 μm in all cases. The three functions represent survival data for 206 cells within three ranges of lesion diameter: 2.0-2.9 μm (triangles), 3.0-3.9 μm (open squares) and 4.0-4.9 μm (closed squares) (From Lucas, 1987).


PERCENT CELL SURVIVAL
100
80
60
40
20
0-10
11-20
21-30
31-40
41-50
RETRACTION (µm)

effects of various manipulations of the external environment (i.e., the culture medium) on cell survival after neurite transection injuries are evaluated.

Many types of injury including physical trauma are followed by large amounts of calcium crossing the membrane (Schlaepfer and Bunge, 1973; Schlaepfer, 1974; Schanne et al., 1979; Kane et al., 1980; Trump and Berezesky, 1985; Young, 1985). According to Trump (1985) and Morgan (1986), calcium will probably cause irreversible changes in the cell by activation of phospholipases, proteases and endonucleases; by disruption of organelles; and by inhibition of key metabolic enzymes. As a consequence, this influx of calcium has been called "the final common pathway" of cell death (Schanne et al., 1979).

Calcium has been determined to be a major component of injury currents after axotomy (Borgens et al., 1980). Almost one-third of the injury current at the cut end of a severed axon consists of calcium ions (Borgens et al., 1980), while calcium conductance at an axon transection lesion has been demonstrated to increase as high as twenty-fold (Meiri et al., 1981). The major changes in the cytoplasmic ultrastructure that have been reported to occur in neurons following axotomy include mitochondrial swelling and disruption of cristae, vesiculation, primary of the smooth endoplasmic reticulum (SER), microtuble 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 (Schlaepfer and Bunge, 1973; Durham, 1974; Schlaepfer, 1974; Dustin, 1978; Pant and Gainer, 1980; Trump et al.,1985). Indeed, Schlaepfer and coworkers (1973, 1974) have demonstrated a direct relationship between calcium influx into severed axons and this deterioration. All of these studies support the hypothesis that calcium is the agent which causes pathological changes leading ultimately to the death of neurons after physical injury.

I postulate that there are at least two possible avenues by which calcium may enter a cell after physical injury: 1) through the physical lesion itself, and/or 2) through calcium channels in the plasma membrane.

Calcium Entry Through the Lesion

As reported by Borgens et al. (1980), ionic currents enter the injured cell directly through the cut face of a severed axon. Meiri and coworkers (1981) also measured changes in ionic conductances at the tip of cockroach giant axons before and after transection injury, and showed that a large injury current entered through the axonal tips. As already described, previous research done in this laboratory shows that the fate of a lesioned cell is determined by

events occurring at the lesion (Lucas et al., 1985; Lucas, 1987). In addition to viability studies, electron microscope analyses have shown that a sequence of ultrastructural changes spreads from the lesion (Emery et al.,1987; Gross and Higgins, 1987). Quantative analysis of damage after dendrite amputation has shown that the number and the density of cell organelles, especially neurofilaments and microtubles, decrease as one approaches the site of transection (Emery et al.,1987; Gross and Higgins, 1987). Mitochondrial swelling is also a function of distance from the lesion (Emery et al.,1987; Gross and Higgins, 1987). These studies support the hypothesis that the physical lesion is probably a major pathway through which calcium currents enter an injured cell.

Calcium Entry Through Calcium Channels

Cytosolic calcium levels are maintained at a level of 10^{-8} to 10^{-7}M against a steep gradient of extracellular calcium (10^{-3}M). A transient increase in intracellular calcium, which acts as an important intracellular signal, can be initiated in two major ways. First, calcium can be released from intracellular storage sites associated with the endoplasmic reticulum and mitochondria. Secondly, the intracellular calcium may also rise due to an increase in the calcium permeability of the plasma membrane, which is normally virtually impermeable to calcium. This is achieved

by the opening of specific channels through which calcium can pass into the cytoplasm down its electrochemical gradient.

Like muscle and endocrine cells, nerve cells have voltage dependent calcium channels (Nowycky et al., 1985; Miller, 1987). The function of these calcium channels are not fully understood. However, Nowycky et al.(1985) postulated that calcium channels within neurons might contribute to threshold behavior or rhythmic activity, and may also be important for dendritic spike formation and neurotransmitter release.

Information about the total number of functional calcium channels in a membrane patch or whole cell comes from estimates of the unitary current. Estimates of channel density range from ~1 per μm^2 in GH_3 pituitary tumor cells (Hagiwara, 1982) and 5-15 per μm^2 in chromaffin cells (Fenwick et al., 1982), up to 30-60 per μm^2 in snail neurons (Krishtal et al., 1981). By using patch clamp techniques, Krishtal et al. (1981) measured calcium current through small electrically isolated patches of the membrane of snail neurons. Their results showed that the maximum calcium inward current which flows through a single calcium channels was about 0.1 pA. As a single channel current of 1 pA corresponds to a flux of three million divalent cations per second (Tsien, 1983), an open calcium channel can probably pass a maximum of $3x10^5$ calcium ions per second. Therefore, based upon the chromaffin cell and snail neuron data, when

calcium channels are fully open, one could anticipate that there are from $4.5x10^6$ to $1.8x10^7$ calcium ions entering mammalian chromaffin cells and snail neurons per cubic micrometer of membrane area each second.

According to recent studies (Miller, 1987; Kamp and Miller, 1987), there are at least three kinds of calcium channels within neurons. They are: 1) L-type, which opens in response to strong depolarization and generates long-lasting currents. 2) T-type, which opens in response to weak depolarizations and generates a relatively tiny conductance change that underlies a transient current. and 3) N-type, which is neither T nor L (Nowycky et al., 1985; Miller, 1987). These channels have been named "voltage-sensitive calcium channels" (VSCC) because their activities and pharmacological effects are highly dependent upon membrane potential (Kamp and Miller, 1987).

Recent studies have shown that the L-type calcium channel is the main site of action of calcium antagonist drugs (Kamp and Miller, 1987). Electrophysiological studies have revealed that L type channels are mainly localized in dendrites (Llinas and Yarom, 1981). The L-type channels in chick dorsal root ganglion neurons are activated by large depolarization (-10mV to positive) (Miller, 1987).

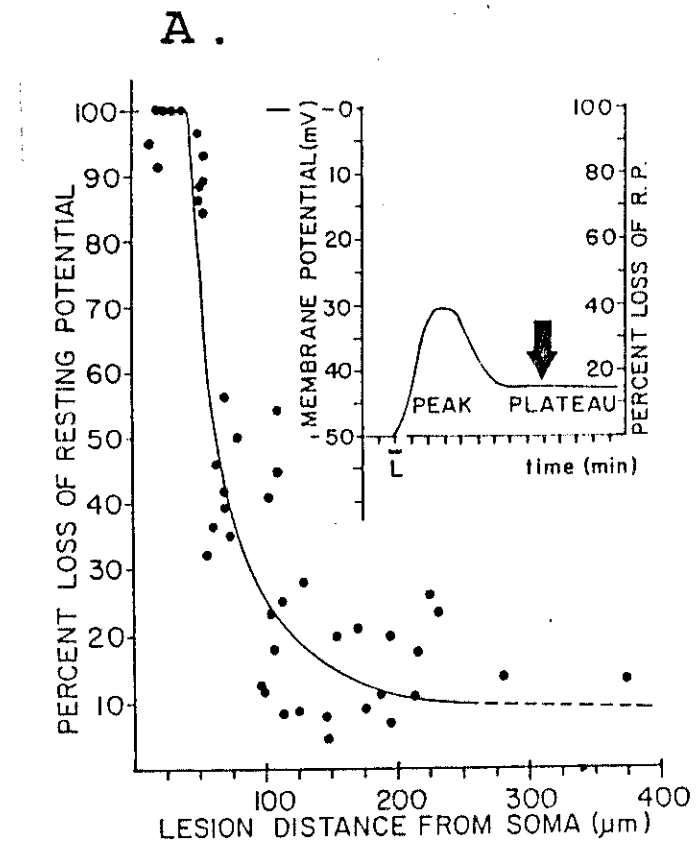
In our cultures, the resting potentials (RP) of spinal cord neurons average 56 $\pm$6 mV (Lucas et al., 1985).

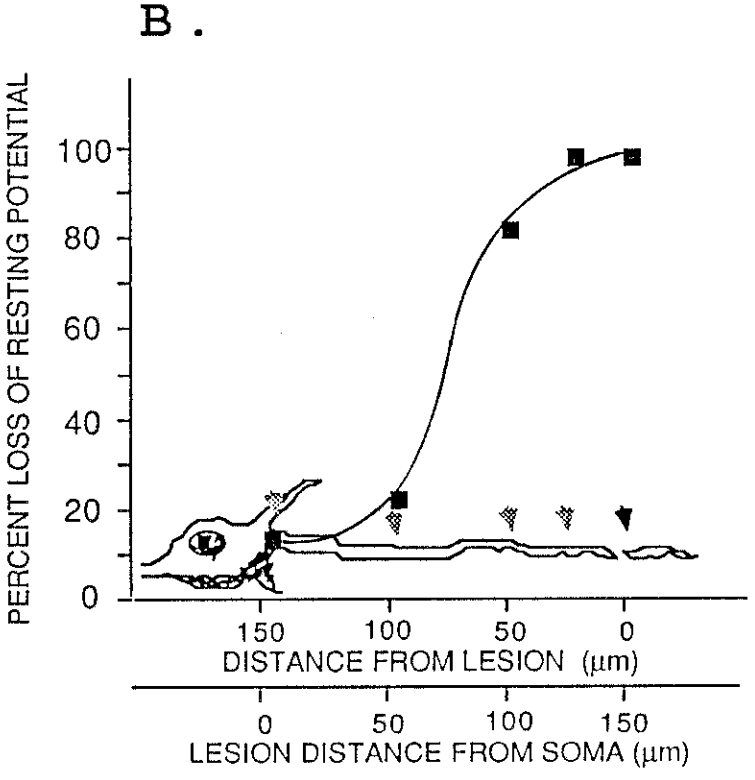
Transection of a primary dendrite is followed by a loss of RP which decreases with distance from the lesion (Lucas et al., 1985; Fig. 3 *A, B*). Electrophysiological studies have shown that the RP losses at 25 μm and 50 μm from the transection point were approximately 100% and 85% respectively (Lucas et al., 1985). Therefore, within 50 μm of the lesion I anticipate a potential loss to -10mV or less. This would probably be strong enough to open the L-type calcium channels along a lesioned neurite. As a consequence, calcium ions should enter the lesioned cells through these channels after dendrite amputation.

In addition to the obvious loss of membrane potential caused by a lesion, the injury may disturb the ordering of the lipids in the membrane sufficiently to cause non-specific calcium leakage. Based upon previous studies (Blaustein and Weismann, 1970, Blaustein et al., 1972; Dipolo, 1979; Hirata and Axelrod, 1980), White and coworkers (1983) proposed several mechanisms of calcium influx including: 1) a depolarization-dependent entry which is through the voltage-sensitive calcium channels, 2) a depolarization-independent steady state entry system which is possibly ATP-dependent and Na-dependent (Dipolo, 1979), and 3) an entry related to changes in membrane fluidity (Hirata and Axelrod., 1980)

Among these mechanisms, the third calcium entry pathway suggests a relation between the plasma membrane fluidity and

Figure 3. *A*, The effect of lesion distance on plateau phase potentials (indicated by arrow in inset) expressed as percent loss of resting potential measured in the cell body. Plateau phase potential are remarkably stable and show little variation between 10 and 30 minutes after surgery (From Lucas et al., 1985). *B*, Simulation of the expected effect of lesion distance on membrane potential in dendrites. These values were taken from figure 3 A and assume that the mesurement in dendrites will be essentially the same as the mesurement in the cell body. The lesion distance from soma corresponds to figure 3 A. However, the data points have been taken to represent membrane potential in dendrites at 0, 25, 50, and 100 μm from the lesion. For example, the data point at 50 μm from the lesion in B was taken from the 50 μm lesion distance in A.


A.
PERCENT LOSS OF RESTING POTENTIAL
100
90
80
70
60
50
40
30
20
10
MEMBRANE POTENTIAL (mV)
-0
10
20
30
40
50
PERCENT LOSS OF R.P.
100
80
60
40
20
PEAK
PLATEAU
L
time (min)
100
200
300
400
LESION DISTANCE FROM SOMA (μm)


B.
PERCENT LOSS OF RESTING POTENTIAL
100
80
60
40
20
0
150
100
50
0
DISTANCE FROM LESION (μm)
0
50
100
150
LESION DISTANCE FROM SOMA (μm)

non-specific calcium leakage after physical trauma. In addition to this, Yawo and Kuno (1983, 1985) have shown that after physical trauma, phospholipase A_2, which can be activated by calcium ion, enhanced cut end membrane resealing following transection of a giant axon. This was probably achieved through the cleavage of phospholipids which is thought to enhance membrane fluidity (Poole et al., 1970; Yawo and Kuno, 1983, 1985). Furthermore, the membrane resealing was found to be highly dependent on temperature. In their studies, every axon examined above 22^0 C showed resealing following transection, no resealing occurred below 13^0 C which was thought to be below the phase transition temperature of membrane lipids (Yawo and Kuno, 1983, 1985). The temperature-dependent phenomena showed again that enhancement of resealing is due to the changes of membrane fluidity mediated by calcium ion and phospholipase A_2 after transection injury. Besides that, Kimelberg and Papahadjopoulos (1974) demonstrated a relationship between membrane fluidity and the function of membrane-bound enzymes. According to their studies, inhibition of $Na^+ + K^+$ - ATP ase activity may be due to decreased molecular motion or conformational freedom mediated by cholesterol. Therefore, it is not unreasonable to speculate that a dendrite transection lesion may change lipid composition in the

affected process. The resulting change in membrane fluidity might also affect calcium channel function.

Objective

The purpose of these studies was to determine whether calcium entry through the physical lesion or through voltage-sensitive calcium channels causes the death of nerve cells after transection of primary dendrites 100 μm from the edge of their perikarya. I tried to determine the relative importance of these two means of calcium ion entry by attempting to block each one and observing changes in the probability of cell survival or death.

Spcific Aims

In order to prevent calcium influx after physical trauma, two different agents, calcium channel antagonists and positively-charged molecules ("molecular bandages"), were used in my neuronal survival studies to block membrane and lesion currents respectively.

1) Calcium channel antagonists were utilized to block calcium channels in order to prevent calcium influx across the plasma membrane after neurite transection. The following calcium antagonists were tested:

   a. Verapamil, a papaverine

   b. Nifedipine, a dihydropyridine

2) I attempted to increase the resistance to injury

currents at the lesion by adding positively-charged macromolecules which could serve as "molecular bandages". As the concept of "molecular bandages" is highly speculative, these experiments constituted pilot studies. Different sizes of molecules as well as a series of different concentrations were selected for testing.

a. Low molecular weight amino acid polymers: polylysine (MW: 3300)

b. Medium molecular weight amino acid polymers: polylysine (MW: 14,100 and 57,000)

Significance of Specific Aims

Calcium Channel Blockers

Calcium entry through voltage dependent calcium channels is the main physiological mechanism of calcium transport across the intact plasma membrane. Many researchers have used calcium channel antagonists or blockers to prevent the entrance of calcium into cells injured by anoxia, ischemia and physical trauma, and to increase the likelihood of cell survival (Faden et al., 1984; Ford and Malm, 1985; Wauquier et al., 1985; Schwertschlag et al., 1986; Faden, 1987). Although calcium channel blockers have been used to treat a variety of cell and tissue types exposed to different insults, their major clinical benefit has been in the treatment of cardiovascular disorders. Calcium channel

blockers, verapamil and nifedipine, have found therapeutic application in cardiac arrhythmias, myocardial ischemia syndromes, and systemic hypertension (see Urthaler, 1986 for review). Verapamil and nifedipine have been demonstrated to be effective for minimization of the area necrosis after myocardial infarction (Downey, 1983; Reimer, 1977; Henry, 1978). Calcium blockers or antagonists have also been shown to protect brain against hypoxia and ischemia (Wauquier et al., 1985).

There is some controversy about whether calcium channel blockers have direct beneficial effects on injured cells. This is because in the intact animal or in isolated organ preparations the protective effects of the calcium channel blockers are mediated by an antagonism of vascular constriction. Using the isolated perfused rat kidney preparation, Malis and coworkers (1983) demonstrated protection from anoxic injury by norepinepherine only when verapamil was given before the insult. In contrast to this finding, verapamil and nifedipine have been shown to be effective in culture for reduction of renal cell death after anoxia (Schwertschlag et al., 1986). This demonstrated that at least some of the salutary effects of these two calcium blockers are independent of vasodilation. One of my objectives of the present study was to eliminate vascular

effects and homeostatic influences from single cell events after physical trauma.

Although calcium channel blockers have shown protection for many kinds of tissue and cells after different traumas, attempts to protect damaged cells and spare function after experimental spinal cord physical injury *in vivo* with calcium antagonists have so far not been successful (Faden et al. 1984; Ford and Malm, 1985). Faden (1987) has stated that one problem with these *in vivo* studies is that no dose-response curves have been established for maximum nontoxic levels of calcium channel antagonists. By using an *in vitro* culture system for the study of the cellular mechanisms involved in physical injury, I have been able to establish the first toxicity curve for uninjured spinal cord neurons exposed to verapamil or nifedipine. This information has permitted me to study the effects of these compounds (at their maximum nontoxic concentrations) on spinal neuron survival after a defined dendrite amputation injury. By working in culture all indirect vascular or homeostatic effects of these agents on injured nerve cell survival have been eliminated.

"Molecular Bandages" to Increase Resistance to Calcium Current at the Lesion

An important study was done by Roederer et al. (1983) which followed up the findings of Borgens et al. (1980) on

ion currents at transection lesion. In their study, prevention of axonal necrosis in lampreys after spinal cord transection and enhanced regeneration were achieved by application of a DC current across the lesion which apparently conteracted the injury currents. Similar injury currents are most probably responsible for the deterioration of mammalian neurons after transection-type injuries. Borgens (1988) stated recently even more emphatically that, after axotomy, the great intermingling of ions at the tip is not just a passive process but is related to active charge movement driven by the membrane voltage in the more proximal, healthy portion of the axon. Thus, the Roederer and Borgens studies suggest a new approch to the development of therapies which address the ionic insult to the cell.

Lucas and coworkers have stated that the reduction of injury currents at the lesion is probably central to any future therapy (Lucas et al., 1985; Gross and Higgins, 1987; Emery et al., 1987; Lucas, 1987). This might be achieved through enhancement of membrane resealing by the addition of structural proteins or lipids. This approach would essentially use the injury current to reduce itself by allowing it to draw positively charged macromolecules into the lesion. While the charges of such molecules would fix them against the breach in the membrane or underlying cytoskeleton, their size could prevent entry in to the

injured cell's interior. The exact size such molecule should have in order to be effective would have to be determined. We have dubbed such molecules "molecular bandages".

The reason that I selected polylysine as molecular bandage candidate is that it is positively charged, it is used routinely with cultured cell to enhance adhesion, and it is available in a wide range of molecular weights.

CHAPTER II

MATERIALS AND METHODS

Animals Used

BALB/C mice were obtained from Charles River, Wilmington, Massachusetts. The animals were maintained in the animal facility of the Center for Network Neuroscience and Department of Biological Sciences at the University of North Texas.

Cell culture

Preparation of Culture Substrate

Routine culture support for this research was provided by the laboratory culture staff (L. Czisny and J. Douglas). For completeness and the information to reader, the entire culture procedure is included in this section.

All the cells were grown on the glass coverslip which were affixed to the bottom of 60 mm culture dishes (Corning). The glass coverslip were flamed and polysine-coated (25 µg/ml, Sigma) before the cells were seeded. Some coverslip were laminin-coated (8 µg/cover slip) to enhance cell growth in addition to the above procedure. Cell

viability test with laminin were conducted to make sure that cell survival with laminin was the same as that with previous culture procedure. The reason for flaming the coverslips is that it has been demonstrated to enhance cell adhesion and also the application of the traditional adhesion-promoting compounds (Lucas et al., 1986).

Culture Procedure and Maintenance

The procedure for culture of dissociated spinal tissue was based on the method of Ransom and coworkers (1977). Thirteen to fourteen days old pregnant mice were killed by cervical dislocation and an incision was made through the skin of the abdomen. The skin was reflected on either side of the abdomen, the peritoneum was opened, and the uterus from each mouse was removed and rinsed twice with cold sterile Hepes-buffered D_1SGH (NaCl 0.8%, KCl 0.04%, $Na_2H_2PO_4$-$7H_2O$ 0.009%, KH_2PO_4 0.003%, HEPES 0.188%, glucose 0.3%, and sucrose 0.75% or enough sucrose to bring the final osmolarity to 320 milliosmoles). Embryos were removed from the uterus and transfered to a 60 mm petri dish containing cold D_1SGH. A second transfer of the embryos to a dish with fresh cold D_1SGH was performed. The remaining steps were performed using sterile techniques. With the aid of a stereomicroscope, the spinal cords were dissected out of the embryos using very fine forceps. The cords were placed into

a cold D_1SGH solution. All cords were removed in this manner and pooled in one dish.

Seven-to-eight cords were transferred into a dry, 60 mm tissue culture dish. The tissue was minced to a gelatinous mass by using two scalpel blades. One milliliter of cold D_1SGH was added to the minced cords and both tissue and D_1SGH were then transfered to a sterile 15 ml centrifuge tube using a siliconized Pasteur pipette. Pasteur pippettes were siliconized to prevent the tissue and cells from sticking to the glass surfaces. Another milliliter of D_1SGH was added to the culture dish as a rinse and the remaining pieces of cord were pipetted into a centrifuge tube. The tissue fragments were spun down for two minutes at approximately 800 RPM. The supernatant was discarded after centrifugation.

A solution of 0.25% trypsin and 0.5 mg/ml DNase (Sigma) was added to the dissociated tissue and incubated in a 10% CO_2 atmosphere at 37°C for 20 min. At the end of this period the enzymatic activity was stopped by addition of six ml of culture medium (Minimum Essential Medium, GIBCO) containing 10% horse serum and 10% fetal bovine serum (MEM10/10). The tissue was pelleted again by centrifugation at 700 RPM for one-to-two minutes.

After centrifugation the supernatant was removed and discarded and two ml of fresh MEM10/10 was added to the tissue. The pellet was then triturated fifteen times through

the tip of a sterile Pasteur pipette. Remaining tissue fragments were allowed to settle to the bottom of the tube. The supernatant containing suspended single cells was then removed and placed into a second tube. Trituration of the remaining fragments was repeated a second and third time by adding one-to-two milliliters of MEM10/10 and using a Pasteur pipette with a narrowed opening. The top layer of single cells was drawn off and pooled with previous single cells. At the end of the entire dissociation procedure, the cell pool was gently triturated to obtain a uniform suspension.

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 $1x10^{6}$. Eight tenths of a milliliter of suspension was placed on prepared glass coverslip in each 60 mm dish. The cultures were then placed into a 10% CO_2 incubator at 37°C.

After 24 hours the medium on the coverslips (containing non-adhered cells) was removed and three milliliters of fresh MEM10/10 was added to the dishes. A second complete medium change with MEM10 was performed three days after seeding. Seven-to-eight days after seeding when the carpet of nonneuronal cells was usually confluent, a complete medium change with MEM containing 10% horse serum MEM10 and fluoro-deoxyuridine (FdU) plus uridine (U) (Sigma) was performed. The final concentration of FdU and U were $1.3x10^{-3}$% and

$3.3x10^{-3}$% (W/V) respectively. Fluorodeoxyuridine retards the proliferation of dividing cells. After 48 hours the medium containing FdU + U was removed and replaced with MEM10. Therafter, all the cells were fed every three-to-four days with usually one milliliter medium changes (remove one milliliter old medium and replace with one milliliter fresh MEM10). Spinal cord cultures were utilized for laser cell surgery three-to-four weeks after seeding when they were generally considered be stable cultures (Ransom et al. 1977).

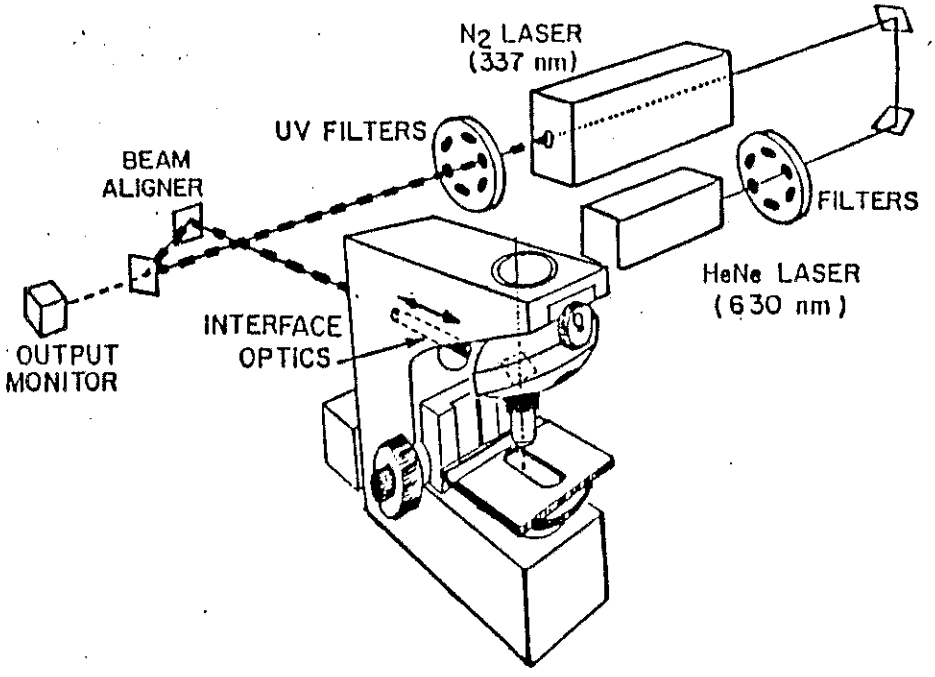
Laser Cell Surgery

Laser cell surgery was performed with a pulsed nitrogen laser which was collinearly aligned with a helium-neon (HeNe) (Gross et al., 1983; Lucas et al., 1985) (Fig. 4, *A*, *B*). The pulsed nitrogen instrument which performed the actual cell surgery has a fixed wavelength of 337 nm. Maximum power output was 14 kW. The HeNe laser (630 nm) provided a visible target beam (1 mW). The HeNe and laser beams were reflected into a Leitz Orthoplan microscope by a beam aligner and focused through Zeiss Ultrafluar quartz objectives onto target neurons.

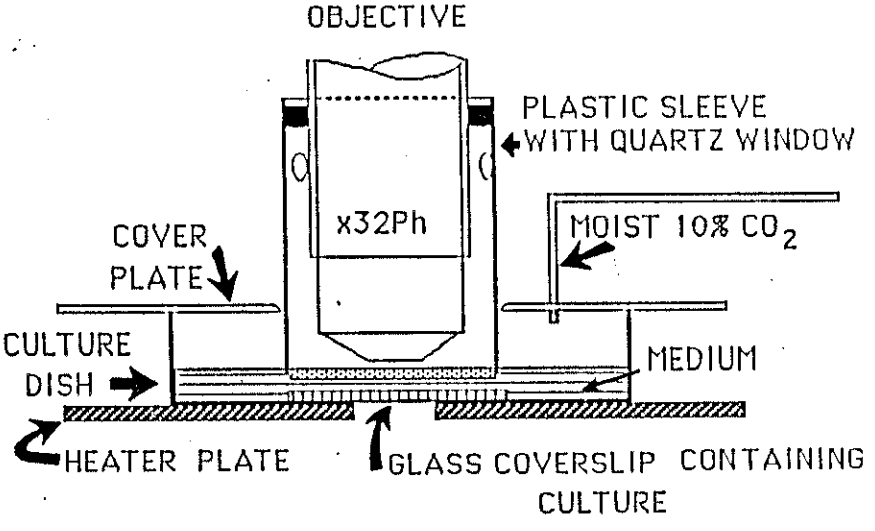
The cultures were maintained at 37°C by a copper DC heating plate on the microscope stage. A moist stream of 10% CO_2 in air was directed over the medium in the dish to

Figure 4. The laser cell surgery system. *A.* The laser microbeam system. The UV (337 nm) and HeNe (630 nm) laser beams are aligned colinearly, reflected into a Leitz Orthoplan microscope, and focused onto a target on the microscope stage (Lucas et al., 1985). *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 platic sleeve sealed with a matched quartz coverslip. The cell were maintained between 35^0 and 37^0 C during surgery. 200 µL of distilled water was added to the culture dish every 30 min. to compensate for evaporation and maintain normal osmolarity of the medium (Lucas et al., 1985).

A.


N_2 LASER
(337 nm)
UV FILTERS
BEAM
ALIGNER
FILTERS
HeNe LASER
(630 nm)
INTERFACE
OPTICS
OUTPUT
MONITOR

B.


OBJECTIVE
PLASTIC SLEEVE
WITH QUARTZ WINDOW
x32Ph
MOIST 10% CO_2
COVER
PLATE
CULTURE
DISH
MEDIUM
HEATER PLATE
GLASS COVERSLIP CONTAINING
CULTURE

maintain the the pH at approximately 7.4. Two hundred microliters of ultrapure water were added at 30 minute intervals to compensate for evaporation and maintain osmolarity. A 32x phase objective (2.3 μm minimum laser focus diameter and 4.2 μm^2 minimum laser focus area) was used to effect process transections in the studies of cell survival. Ultrafluar objectives are temperature sensitive and could be damaged if exposed to medium at 37°C. Therefore, a ventilated plastic sleeve containing a matched (350 μm thick) quartz coverslip at one end was used to protect the 32X objective. With the recommended glycerin between the objective and coverslip (inside the sleeve), optical distortion was kept to a minimum. Sleeve misalignment could be discerned from asymmetries in the reflected HeNe diffraction pattern.

All transections were effected by firing multiple laser pulses at a firing frequency of 20 Hz and an energy density of about 1~3 $\mu J/\mu m^2$. The firing interval usually varied from 10~50 sec (200~1000 shots total). This method of laser surgery permitted creation of precise transections without concurrent pressure wave effects (Gross et al., 1983). Laser marks were made below the surface of the glass coverslip to assist relocation of both experimental and control cells. Such sub-surface relocation marks were made without disturbing either target neurons or the glial layer.

Photographs of individual cells were taken to assist relocation. Target neurites were irradiated until microscope examination showed complete separation of proximal and distal segments with no remaining interconnections. All experimental cells were operated at same distance (100 μm). Experiments generally lasted 1-1.5 hours for marking and 30-45 min for transection, after which cells were returned to the incubator. A total medium change with condition medium was performed just after marking to prevent culture contamination.

Drug Toxicity Testing

Drug Preparation

Verapamil (MW: 491.1), nifedipine (MW: 346.3) and polylysine (MW: 3,300, 14,100, 57,000) were all obtained from the Sigma Company. Verapamil were dissolved in 0.9 % saline, nifedipine were dissolved in 100 % ethanol, and polylysine were dissolved in MEM. Because of light sensitivity, verapamil and nifedipine were prepared in the dark. All the drug solutions were made from powder no longer than two weeks prior to use and were stored as instructions indicated.

Drug Toxicity Studies

Toxicity tests were conducted for all compounds. These experiments were performed on unoperated nerve cells exposed

to different concentrations of these agents for 24 hours. Cell viability (see below) was checked at two hours and 24 hours. For cell surgery experiments, we used the highest concentration at which no cell death occurred in the toxicity studies.

Cell Survival Studies

Target Cell and Target Neurite Identification and Selection

Experimental and control cells were identified as spinal cord neurons based upon the following morphologic criteria: a) presence in the uppermost stratum of the culture, b) multiple long, branched cell processes, c) large, bulging, phase-bright cell bodies and d) well-defined nuclei and nucleoli (Ransom et al., 1977). Previous studies have shown that at least 80% of the selected cells have the potential of being neuronal membrane property (Lucas et al., 1985).

Cells 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 posibility of inadvertently lesioning either the control neurons or previously operated cells. For the cell survival studies, only neurons on areas of confluent

glial carpet were chosen to insure great uniformity of adhesion parameters.

Although a great deal of work has been done on axon transection (Levi and Meyer, 1945; Mire et al., 1970; Schlaepfer and Bunge, 1973; Shaw and Bray, 1977; Bird, 1978; Sole, 1980; Yawo and Kuno, 1983, 1985), so far no studies of dendrite amputation have been published in the literature by other laboratories. Dendrites within the CNS may ramify over distances as great as 1000 micrometers from the soma (Shepherd, 1979) and, lesions at dendrite loci undoubtedly play an important role in CNS injury. In addition, a dendrite 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; Lucas et al., 1985). Finally, with phase contrast (Phc) light microscopy, it is often difficulty to locate the point of origin of the axons in the neurons in our mouse spinal cord monolayer culture. This is very important in order to measure distance of a lesion from the perikaryon.

All the cell processes selected for laser surgery were identified as dendrites (or multiple dendrite bundles) by light microscopic examination. Dendrites were distinguished from axons based on the following criteria: a) they arise directly from the soma, b) they are thick and tapering, c) they branch at acute angles, d) they display irregular

contours and e) they lack the characteristic axon hillock and initial segment (Peters et al., 1976). All target dendrites 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 (Gross and Higgins, 1987). Dendritic fascicles were also frequently encountered. In either situation electron microscopy studies of cross section of these processes have shown that the dendrite of a target cell represents the largest component of a multiple neurite bundle (Gross and Higgins, 1987). Neurite diameters at the target ranged from two to five μm.

Care was exercised to select control and target neurons which were free of any obvious signs of deterioration such as presences of vacuoles, cytoplasmic granularity, a plasma membrane with a "ragged" appearance, or the absence of clearly visible somal freatures (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 nerite bundles. Individual cells were also selected from widely separated areas of the culture to minimize the possibility of inadertently lesioning either the control neurons or previously operated cells due to fasciculation of neurites.

Measurement of Lesion Physical Parameters

Measurement of lesion distance was made before cell surgery with a ruler within the ocular. Lesion diameter (diameter of the dendrite at the target site prior to transection) and retraction of proximal segment after dendrite transection were measured from light micrographs of target neurons taken prior to dendrite amputation (for lesion diameter) and five minutes after transection (for retraction study).

Prevention of Culture Contamination

All surgery was performed in the absence of antibiotics. Sterilization precautions, such as UV irradiation of the objective, soaking in ethanol, and filtration of the air/mixture CO_2, as well as the positive chamber pressure from the gas flow, minimized the chances of bacterial or fungal contamination during an experiment. Cultures remained free of contamination for at least two days after cell surgery in all my toxicity and cell survival studies except four cases (less than 4% of total experiments had contamination).

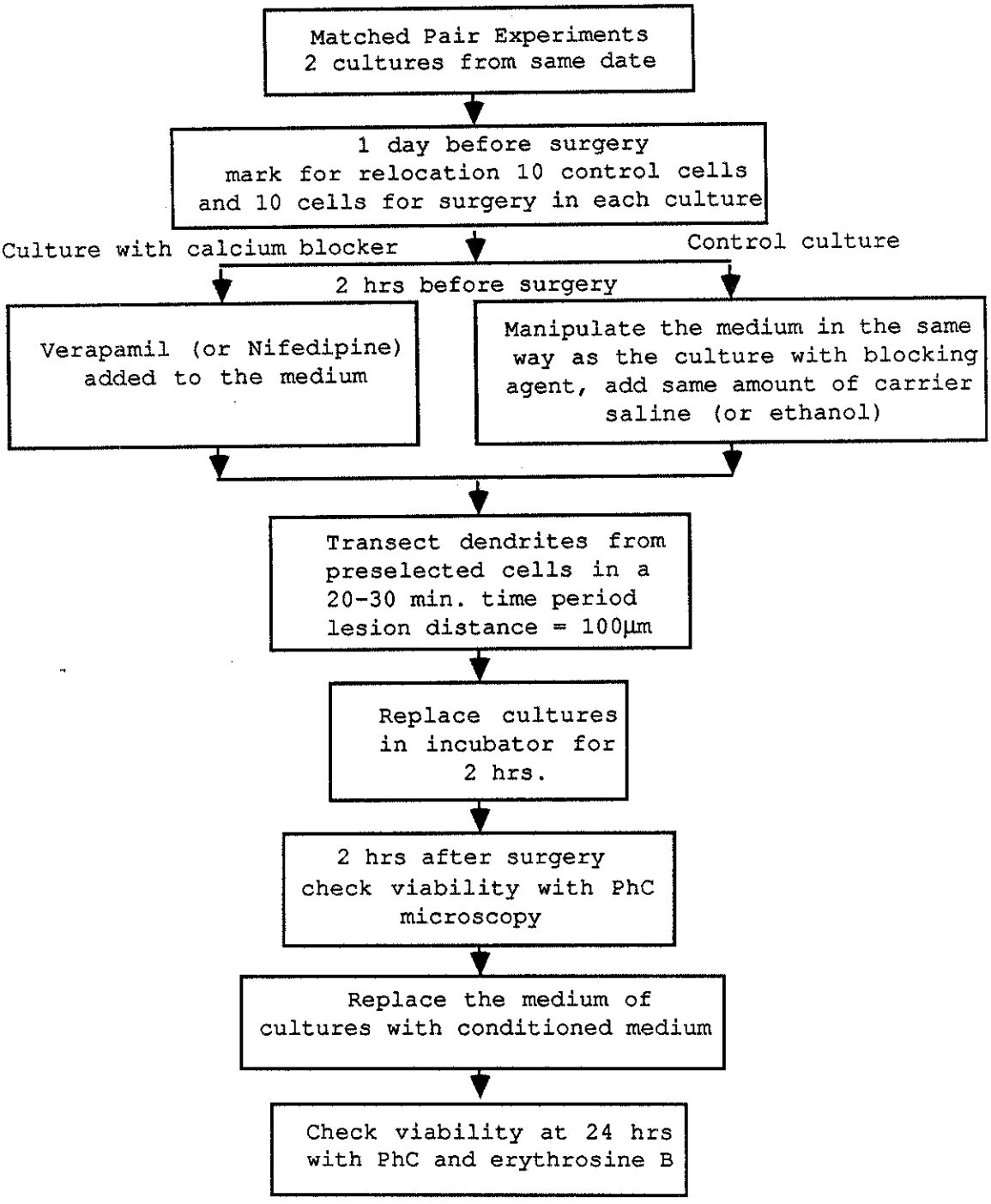
Experimental Protocol

Calcium Channel Antagonists

Matched pair experiments were performed using two cultures from the same seeding date. Previous studies have shown that cell survival after neurite amputation was a function of lesion distance from the perikaryon, and was inversely proportional to lesion diameter (Lucas et al., 1985). For the calcium antagonist studies I operated at a constant distance. Thus, in each culture a primary dendrite was transected from each on ten experimental cells at a lesion distance of 100 µm from the edge of the perikaryon. This was determined prior to laser cell surgery by using a ruler within the ocular. Ten other cells served as unoperated control cells. Lesion diameter of the neurite at the target point was allowed to vary between two µm to five µm. Verapamil or nifedipine was added to the conditioned medium on the cells two hours prior to laser cell surgery. The control culture received an equal volume (10 µl/ml) of either saline (for verapamil) or 100% ethanol (for nifedipine). Control and operated cell viability was evaluated two hours after surgery using PhC observation and 24 hours using a combination of PhC observation and erythrosine B dye exclusion. After the two hours observation all of the medium from each culture was removed and replaced

Figure 5. Experimental protocol for studies of calcium channel blocking effects.

EXPERIMENTAL PROTOCOL



with fresh conditioned medium (no drug) (Fig. 5). Therefore, total exposure time to calcium channel blocker was four hours.

Molecular Bandage

Matched pair experiments were performed using two cultures from the same date. As in the calcium antagonist survival studies, a primary dendrite was transected from each of ten experimental cells in a culture at a lesion distance of 100 µm from the edge of the prikaryon. Ten other cells served as unoperated control cells. Polylysine at molecular weights of approximately 3,300, 14,100 or 57,000 were used for these studies, with MEM as the carrier. Because these molecules must be mobile to act as molecular bandages, polysine was added to lesioned cells very shortly (10-25 min) after dendrite amputation. The control culture (with the same number of dendrite amputation) received an equal volume of MEM. Control and operated cell viability were evaluated two hours after surgery using phase contrast (PhC) observation and 24 hours using a combination of PhC and erythrosine B dye exclusion.

Dertermination of Cell Viability

Previous work from this laboratary has shown that the viability of nerve cells subjected to dendrite amputation

within 200 μm of the perikaryon can be determined using PhC light microscopy two hours after lesioning (Lucas et al., 1987). Viable lesioned nerve cells have distinct nucleoli and nuclear regions (Lucas et al., 1985). Dead cells usually show obvious deterioration which includes formation of large cytoplasmic vacuoles and membrane blebs (Lucas et al., 1985). Moribund (i.e., dying) neurons are usually characterized by an intensely phase-bright cytoplasm and an absence of distinct nuclear features (Lucas et al., 1985). Almost all the cells which appear moribund at two hours will be dead at 24 hours. Electron microscopy has shown that the phase-brightness characteristic of this kind of cell is probably due to the formation of a large numbers of cytoplasmic vesicles derived from the swollen mitochondria and endoplasmic reticulum. These vecicles diffract light and obscure the nuclear region (Lucas et al., 1985). Grossly dilated mitochondria are indicative of irreversible injury (Trump et al., 1974; Wyllie et al., 1980). The large vesicles associated with neuronal death were probably the result of coalescing of these small vesicles (Lucas et al., 1985).

At 24 hours after surgery viability was determined by a combination PhC light microscope and application of a vital stain. Erythrosine B dye was used according to the method of Phillips with the modifications described by Emery et al.

(1987) to confirm cell viability or death. A stock solution of erythrosine B was prepared at a concentration of 0.4%, and 0.4 ml of the stock was added to each milliliter of culture medium and placed on the operated culture. As soon as the culture medium is mixed with erythrosine B, the culture was maintained at the proper pH by gassing it with 10% CO_2 in air. After four minutes, the culture was rinsed with D_1SGH and immediately observed with PhC microscopy to assess the uptake of the dye. Dead cells took up erythrosine B and appeared pink. By contrast, viable cells with intact plasma membranes did not stain. The majority of moribund cells also failed to stain with erythrosine B (Lucas et al., 1985).

CHARPTER III

RESULTS

Drug Toxicity

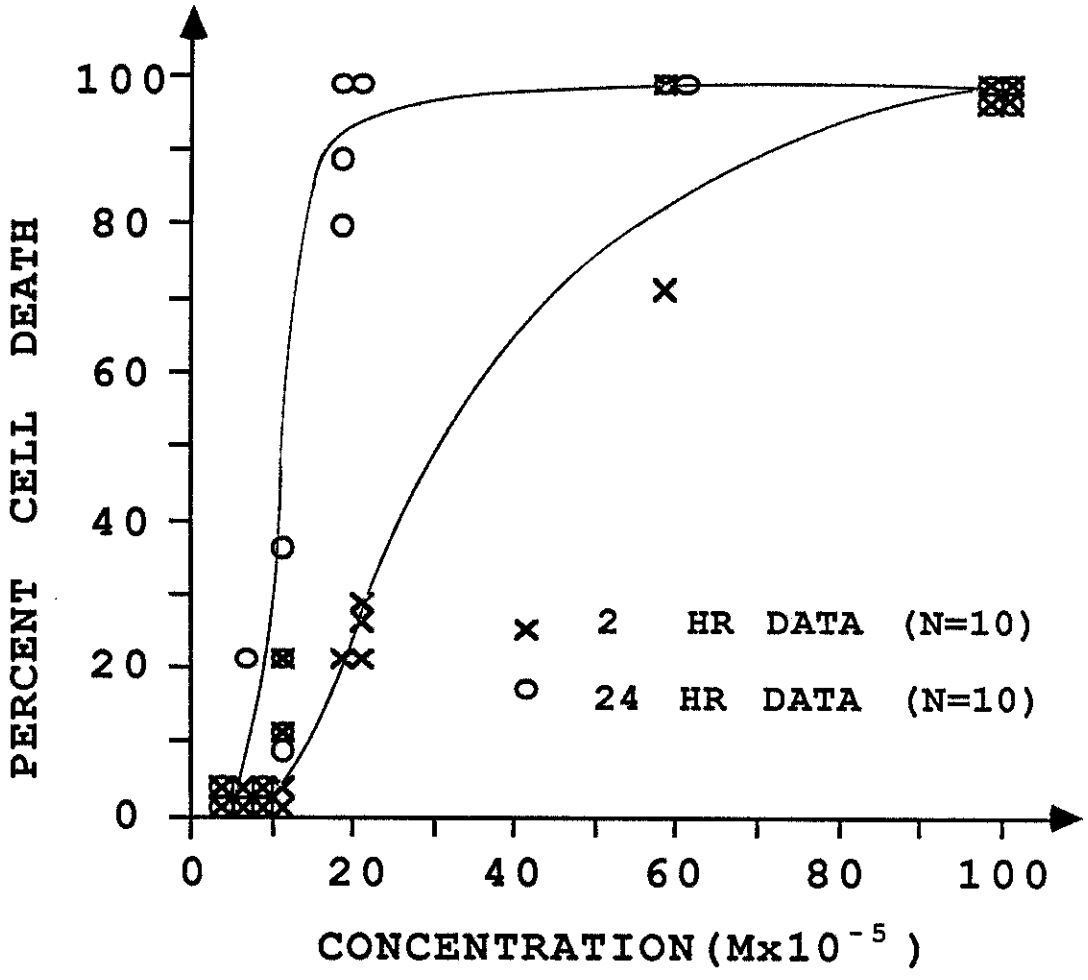
Toxicity of Verapamil

I have performed 20 experiments (10 matched pairs) using different concentrations of verapamil ($100x10^{-5}$ M, $60x10^{-5}$ M, $20x10^{-5}$ M, $10x10^{-5}$ M, $8x10^{-5}$ M) dissolved in 0.9% saline. The results are shown in Figure 6. The highest concentration which did not cause unoperated cell death was $8x10^{-5}$ M. With this concentration, the cells appeared healthy at two hours but did develop large, dark "granules" at 24 hours when observed with phase contrast (PhC) light microscpy although no cell death could be detected with erythrosine B. According to Boll and Lux (1985), with the minimum concentration of $5x10^{-5}$ M, verapamil completely blocked calcium channels in avian dorsal root ganglion cells. Therefore, our concentration is close to the effective range reported in the literature for neuronal cells.

Toxicity of Nifedipine

Six cultures were treated with two different concentrations of nifedipine. Three of them received

Figure 6. Toxicity of verapamil as a function of concentration. Toxicity studies of verapamil were performed on unoperated nerve cells exposed to five different concentrations. Cell viability was checked at two hours (x) and 24 hours (o) after addition of verapamil. Each cross or circle represents survival percentage calculated from ten unoperated cells. A total of 200 cells were selected for the verapamil toxicity studies.


PERCENT CELL DEATH
100
80
60
40
20
0
0
20
40
60
80
100
CONCENTRATION(Mx10^{-5})
2 HR DATA (N=10)
24 HR DATA (N=10)

10^{-6} M of nifedipine and the others received 10^{-4} M. At these two concentrations of nifedipine, there was 100% unoperated cell survival in the toxicity tests. Because nifedipine failed to dissolve in 100% ethanol (solvent) when the concentration of stock solution was above 10^{-2} M, I chose 10^{-2} M as highest stock solution which yielded final concentration of nifedipine of 10^{-4} M. (10 μL ethanol stock/1mL culture)

Toxicity of Polylysine

Toxicity as a Function of Molecular Weight

Three different molecular weights of polylysine, 3,300, 14,100, and 57,000, were tested. Seventy unoperated cells (in seven cultures) were selected for toxicity tests of polylysine at a concentration of $5x10^{-7}$ M. The results are shown in Figure 7. At the molecular weight of 3,300, no cell death could be detected two hours and 24 hours after unoperated cell exposure to polylysine. By contrast, at the molecular weight of 57,000, all of the neurons were dead at two hours after unoperated cell exposure to polylysine. At a molecular weight of 14,100, the mean two hours death was 35±5% and the mean 24 hours death was 75±15%. These data indicate that the higher the molecular weight, the great the toxicity.

Figure 7. Toxicity as a function of molecular weight of polylysine. Three different molecular weights of polylysine, 3,300, 14,100, and 57,000 were tested at a concentration of $5x10^{-7}$ M. Each point represents ten unoperated cells. A total of 70 cells were selected for toxicity studies.

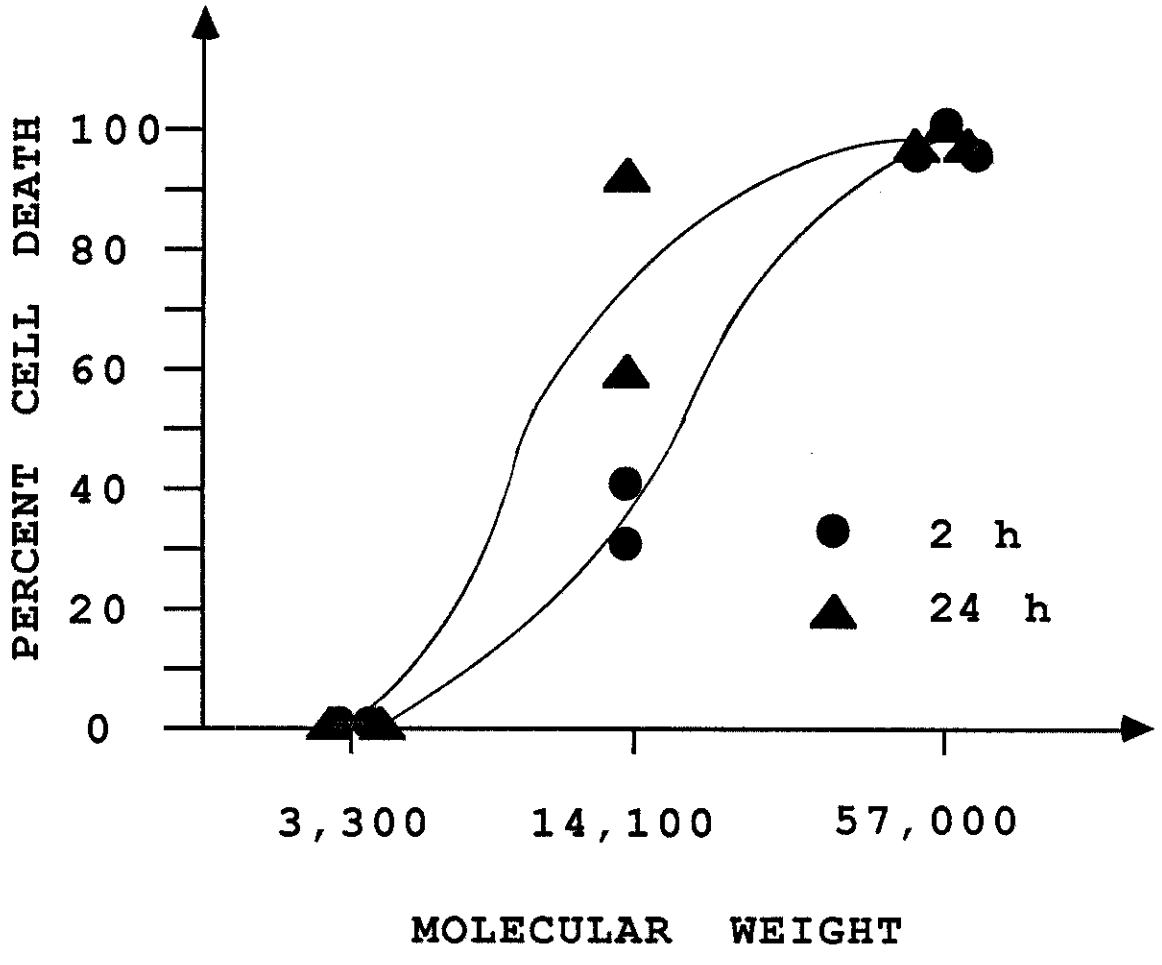
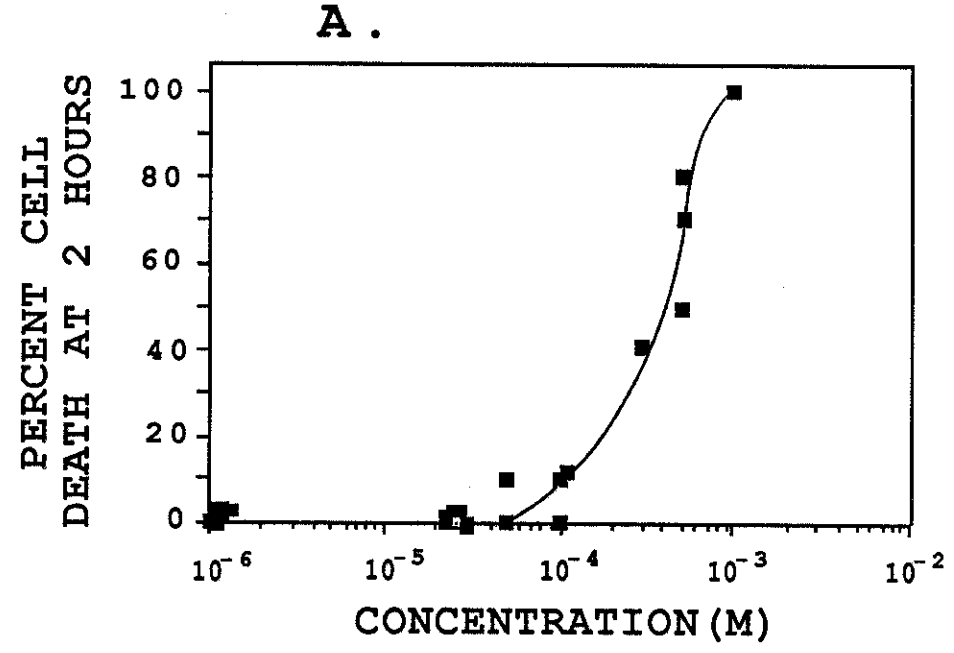
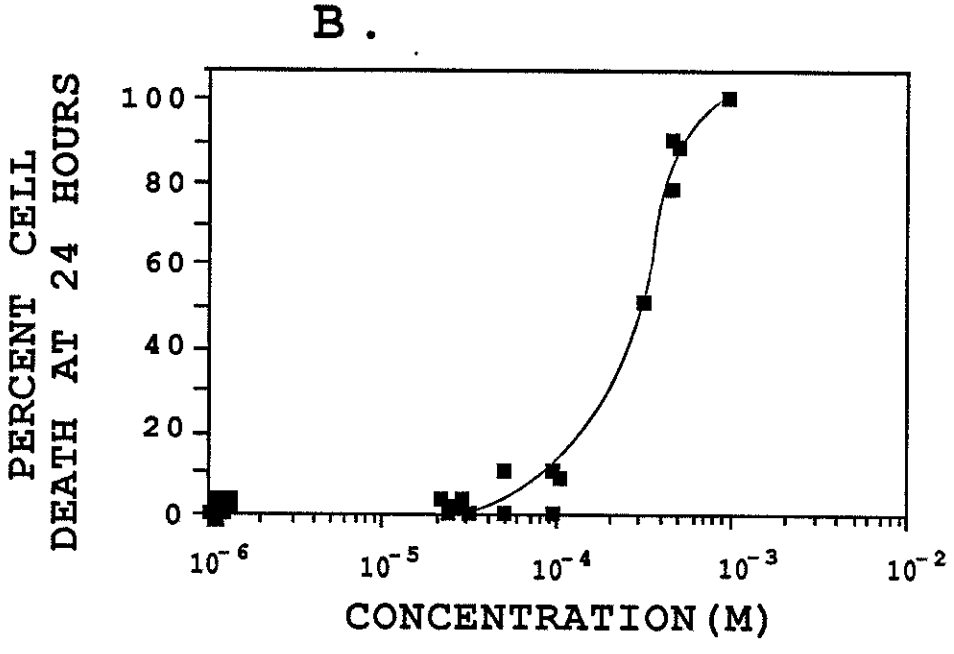

PERCENT CELL DEATH
100
80
60
40
20
0
2 h
24 h
3,300
14,100
57,000
MOLECULAR WEIGHT

Figure 8. Toxicity of polylysine (MW: 3,300) as a function of concentration. This toxicity study was performed on unoperated cells exposed to seven different concentrations. *A*, Viability of nerve cells two hours after exposure to polylysine. Each square represents ten unoperated cells. *B*, Viability of nerve cells 24 hours after exposure to polylysine. Each square represents ten unoperated cells. A total of 210 cells was selected for this toxicity study.


A.
PERCENT CELL DEATH AT 2 HOURS
100
80
60
40
20
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
CONCENTRATION (M)


B.
PERCENT CELL DEATH AT 24 HOURS
100
80
60
40
20
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
CONCENTRATION (M)

Toxicity as a Function of Concentration

Molecular Weight of 3,300

I have performed 21 experiments (210 cells) using seven different concentrations of polylysine: 10^{-3} M, $5x10^{-4}$ M, $3x10^{-4}$ M, 10^{-4} M, $5x10^{-5}$ M, $2.5x10^{-5}$ M, 10^{-6} M (Fig. 8). The toxicity studies of polylysine were conducted on unoperated cells exposed to polylysine for at least 24 h. Viability of the nerve cells was checked at both two hours and 24 hours after exposure to polylysine. As shown in figure 8, the highest concentration which did not cause unoperated cell death two hours after exposure to polylysine was $2.5x10^{-5}$ M. There was no additional cell death at 24 hours.

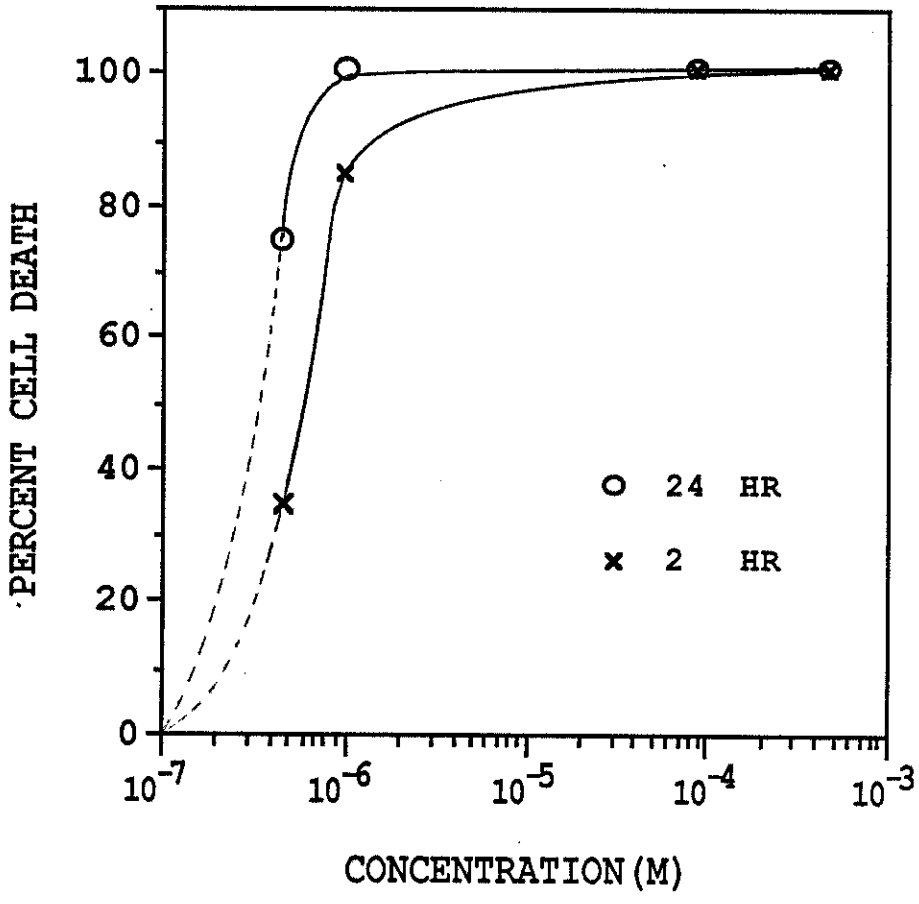
Molecular Weight of 14,100

Eight experiments (80 unoperated cells) were performed using four different concentrations: $500x10^{-6}$ M, $100x10^{-6}$ M, $1x10^{-6}$ M, $0.5x10^{-6}$ M (Fig. 9). At the lowest concentration which I tested, $5x10^{-7}$ M, the mean cell death two hours after exposure to polylysine was 35±5%, and the mean cell survival 24 hours after exposure to polylysine was 75±15%.

Molecular Weight of 57,000

Two concentrations of polylysine, 10^{-6} M, and $0.5x10^{-6}$ M, were tested in six experiments (60 unoperated cells). At

Figure 9. Toxicity study of polylysine (MW: 14,100) was performed on unoperated cells exposed at four different concentrations. Cell viability was checked at two hours and 24 hours after addition of polylysine. Each point represents 20 unoperated cells. A total of 80 cells was selected for these toxicity studies.

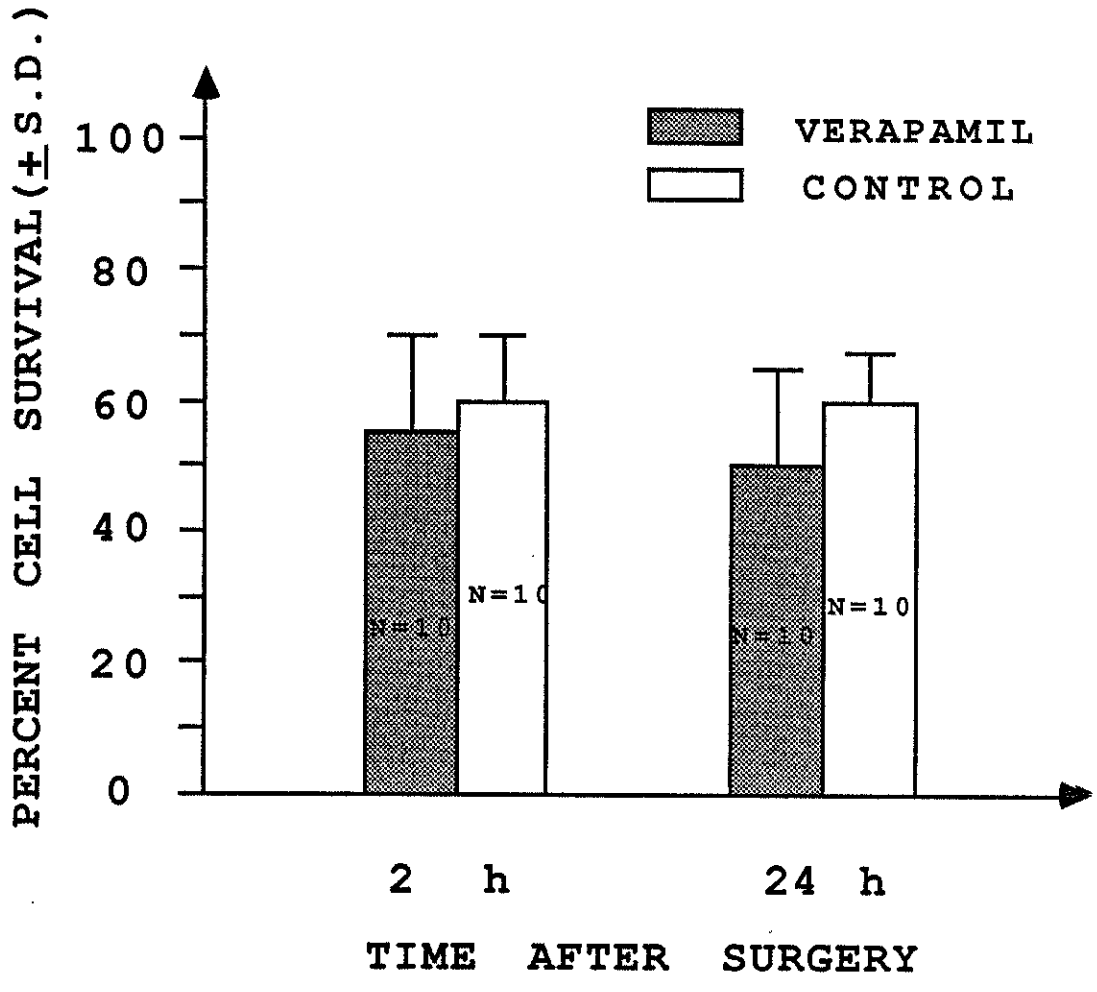

PERCENT CELL DEATH
100
80
60
40
20
0
24 HR
2 HR
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
CONCENTRATION (M)

these concentrations of polylysine, all the unoperated cells were dead by two hours after exposure to polylysine.

Calculation of Number of Molecules of Polylysine per Unit Volume of Medium

Using the concentration and molecular weight of polylysine, I calculated the number of molecules per unit volume. One mole of a substance contains $6x10^{23}$ molecules. Therefore, at a concentration of $25x10^{-6}$ M, there are $25x10^{-6}$ x $6x10^{23}$ or $1.5x10^{19}$ molecules in every liter. As 1 ml equals 1 cm^3 and 1 mm^3 equals 10^9 μm^3, there are $1.5x10^4$ molecules within every μm^3 (1 μm^3 =10^{-12} cm^3, 1 ml = 10^{-3} L, 1 L = 10^{15} μm^3). Consequently, at a molecular weight of 3,300 and concentrations of $25x10^{-6}$ M or 10^{-6} M, there are $1.5x10^4$ or $6x10^2$ molecules of polylysine in every cubic micrometer of medium. At a molecular weight of 14,100 and a concentration of $5x10^{-7}$ M (the lowest concentration I used at this molecular weight), there are only $3x10^2$ molecules per μm^3. At a molecular weight of 57,000, one of the concentrations I tested was also $5x10^{-7}$ M. At a molecular weight of polylysine of 3,300 which consists about 22 lysine, the length of this polyamine is about 220 A (each lysine is about 10 A long). As it will form a loose random coil in solution, the diameter of polylysine at molecular weight of 3,300 will probably less than 220 A. In order to test the efficacy of

Figure 10. Lesioned nerve cell survival in the presence of $8x10^{-5}$ M verapamil. Cell viability was checked at two hours and 24 hours after dendrite amputation. Shadowed bars represent the experiments with verapamil and open bars refer to control experiments without verapamil. Twenty experiments (200 cells) were performed.


PERCENT CELL SURVIVAL (± S.D.)
100
80
60
40
20
0
VERAPAMIL
CONTROL
N=10
N=10
N=10
N=10
2 h
24 h
TIME AFTER SURGERY

polylysine as a molecular bandage, I wanted to make available a high density of molecules of polylysine to the lesioned cell. Therefore, I chose polylysine at a molecular weight of 3,300 and a concentration of 25×10^{-6} M to test as a molecular bandage.

Studies of Cell Survival after Dendrite Amputation

Neuronal Cell Survival in the Presence of Verapamil

For the verapamil studies, 20 experments (ten pairs) were performed using concentration at 8×10^{-5} M. At two hours after surgery the mean operated cell survival in the presence of verapamil was 56±15% (S.D.). Two hour survival of operated cells in medium without verapamil was 61±9%. Mean survival of the two groups at 24 hours was 49±16% and 59±7% respectively (Fig. 10). Unoperated cell survival in each group was 100% at two hours and 24 hours. At two hours, the moribund cell population of the verapamil-treated group was 4±7%. Moribund cells accounted for 8±7% of the control group (Fig. 11). These data indicate that calcium antagonist, verapamil, fails to protect neurons after dendrite amputation. Mortality rates between control cells and verapamil-treated cells were not significantly different.

Figure 11. Viability of nerve cells two hours after lesioning in the presence of $8x10^{-5}$ M verapamil (ten experiments with verapamil and ten control experiments without verapamil). The total number of viable, moribund, or dead cells was 100 in each group. (ten operated cell in each experiment)

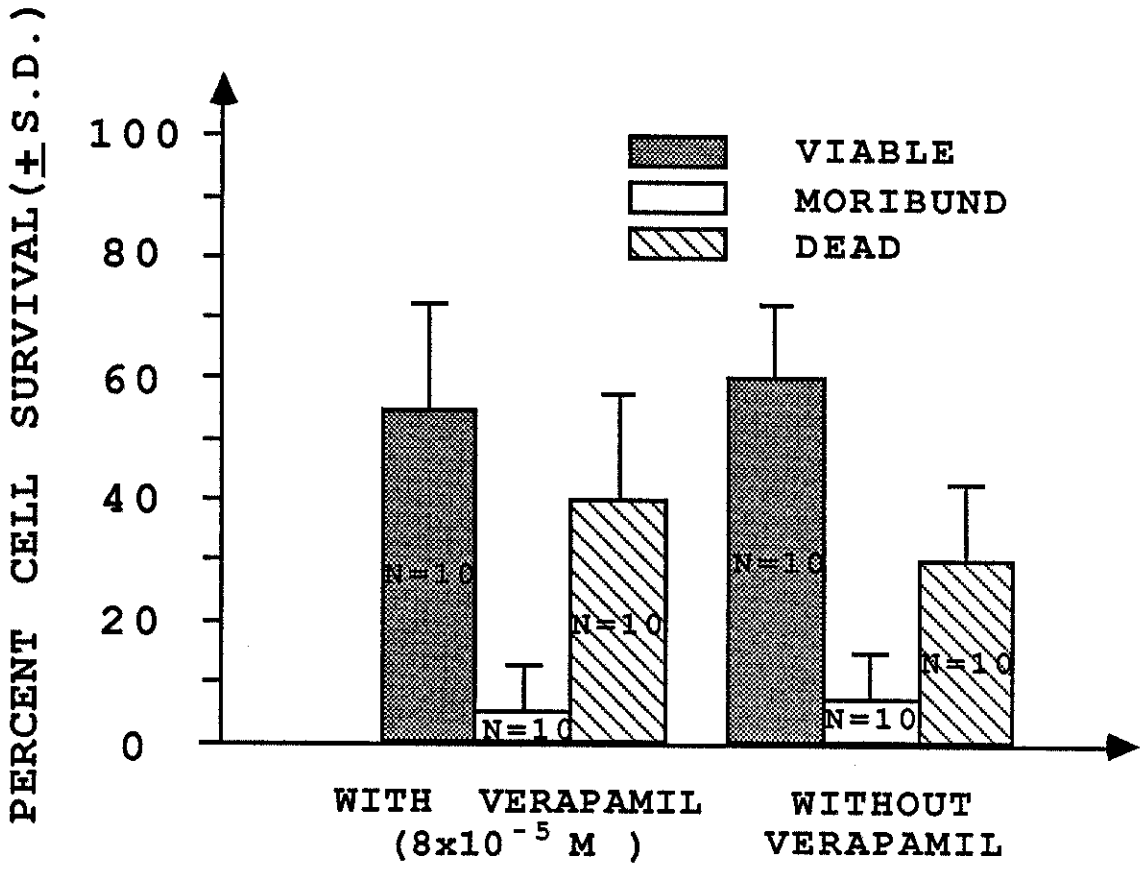
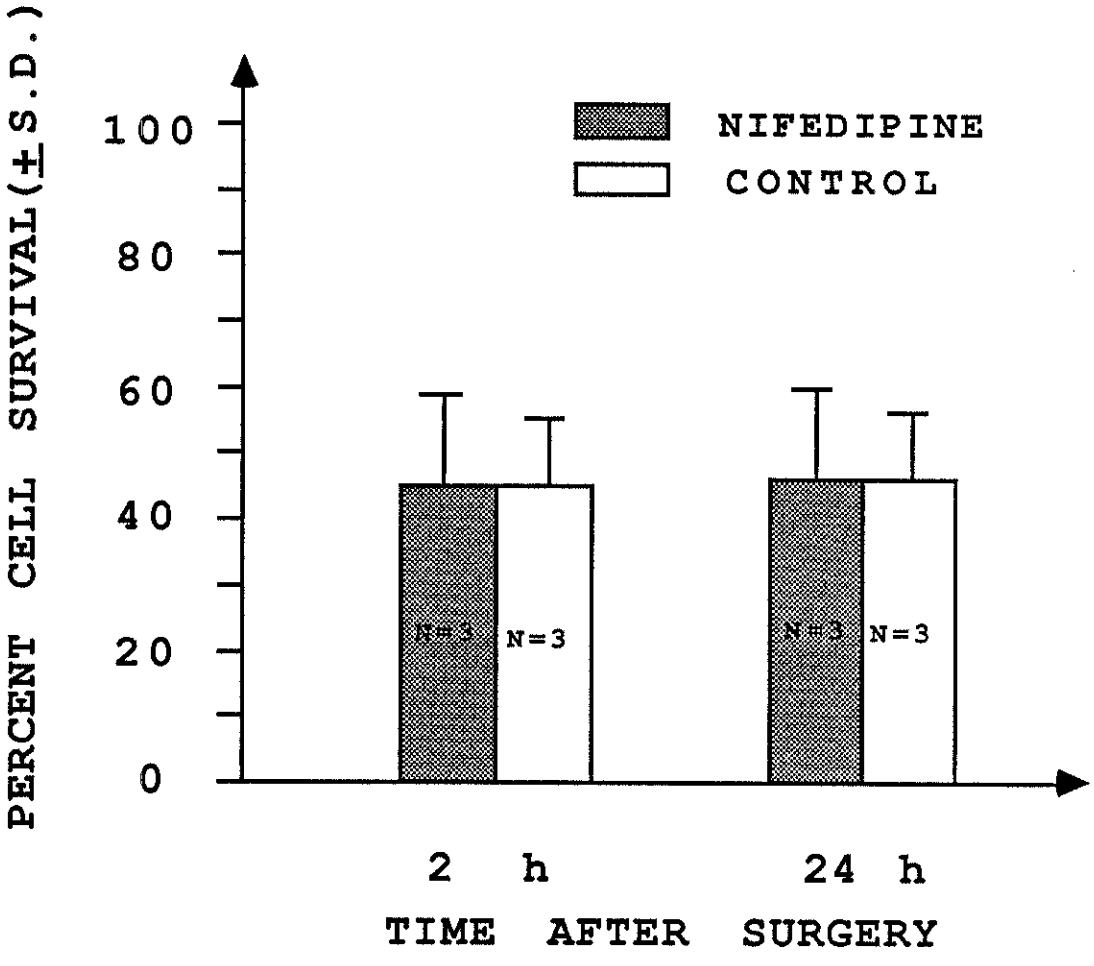

PERCENT CELL SURVIVAL(±S.D.)
100
80
60
40
20
0
VIABLE
MORIBUND
DEAD
N=10
N=10
N=10
N=10
N=10
N=10
WITH VERAPAMIL
(8x10⁻⁵ M)
WITHOUT
VERAPAMIL

Figure 12. Lesioned nerve cell survival in the presence of 10^{-4} M nifedipine. Cell viability was checked at two hours and 24 hours after dendrite amputation. Shadowed bars represent the experiments with nifedipine and open bars refer to control experiments without nifedipine. Six experiments (60 cells) were performed as a cell viability study using nifedipine. Thirty neurons in three experiments served as a control group.


PERCENT CELL SURVIVAL (± S.D.)
100
80
60
40
20
0
NIFEDIPINE
CONTROL
N=3
N=3
N=3
N=3
2 h
24 h
TIME AFTER SURGERY

(Contingency chi-square with continuity correction, $X^2 = 0.27$, $0.5 < P < 0.75$). The two hour viability shows that the proportion of moribund cells was not significantly different when calcium antagonist were present. These data indicate that verapamil does not slow the process of deterioration leading to cell death after physical injury.

Neuronal Cell Survival in the Presence of Nifedipine

Three pairs of experiments were performed using nifedipine at a concentration of 10^{-4} M. The mean of the two hours operated cell survival for these experiments with and without nifedipine was 47±16% and 47±10% respectively. The mean of the 24 hour operated cell survival with and without nifedipine was also 47±16% and 47±10% (Fig. 12). Unoperated cell survival in each group was 100% at both two hours and 24 hours. These data also indicate that nifedipine fails to protect neurons after dendrite transection close to the perikaryon.

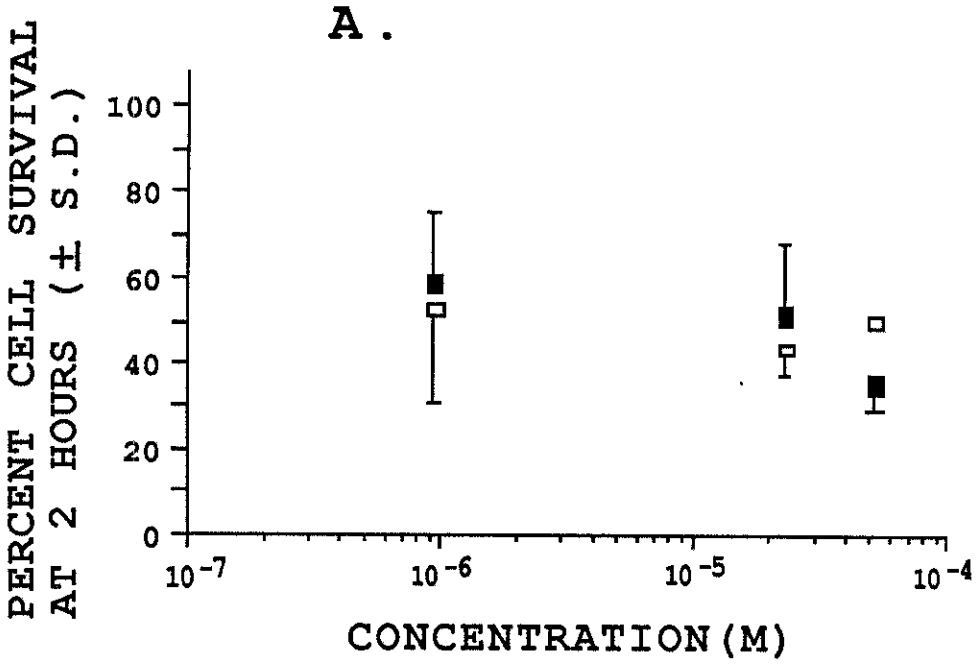
These results agree with *in vivo* studies which also showed that calcium channel antagonists were unable to reduce tissue damage and spare function after central nervous system physical trauma (Faden et al., 1984; Ford and Malm, 1985; Faden, 1987).

Neuronal Cell Survival in the Presence of Polylysine

For these studies polylysine at a molecular weight of 3,300 and at three concentrations, $50x10^{-6}$, $25x10^{-6}$, and $1x10^{-6}$ M was tested as a molecular bandage (Fig. 13). Twelve experiments (six pairs) were performed at a concentration of 10^{-6} M. At two hours after surgery the mean operated cell survival in the presence of polylysine was 60±16%. Two hour survival of operated cells in the medium without polylysine were 52±20%. Mean survival of the two groups at 24 h was 55±19% and 43±9% respectively. Unoperated cell survival in each group was 100% at two hours and 24 hours. At two hours, the moribund cell population of the polylysine-treated group was 13±12%. Moribund cells accounted for 8±7% of the control group (Fig. 14).

At a concentration of $2.5x10^{-5}$ M, I performed five experiments with five additional control experiments. The means of the two hours operated cell survival with and without polylysine were 52±16% and 43±5% respectively. The mean of the 24 hours operated cell survival with and without polylysine were also 52±16% and 43±5% (Fig. 13). Unoperated cell survival in each group was 100% at two hours and 24 hours. At two hours after surgery, the moribund cells of the polylysine-treated group and control group were 12±10% and 10±8% repectively.

Figure 13. Lesioned nerve cell survival in the presence of $1x10^{-6}$ M, $25x10^{-6}$ M, $50x10^{-6}$ M polylysine. Dark squares represent cells operated in the presence of polylysine. Open squares represent cells operated in control group without polylysine. *A*, Lesioned nerve cell survival two hours after dendrite amputation. Twenty-six cultures (260 cells) were selected for cell viability study using polylysine. *B*, Lesioned nerve cell survival 24 hours after dendrite amputation. Twenty-six cultures (260 cells) were selected for cell viability tests of polylysine as a molecular bandage.


A.
PERCENT CELL SURVIVAL
AT 2 HOURS (± S.D.)
100
80
60
40
20
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
CONCENTRATION (M)

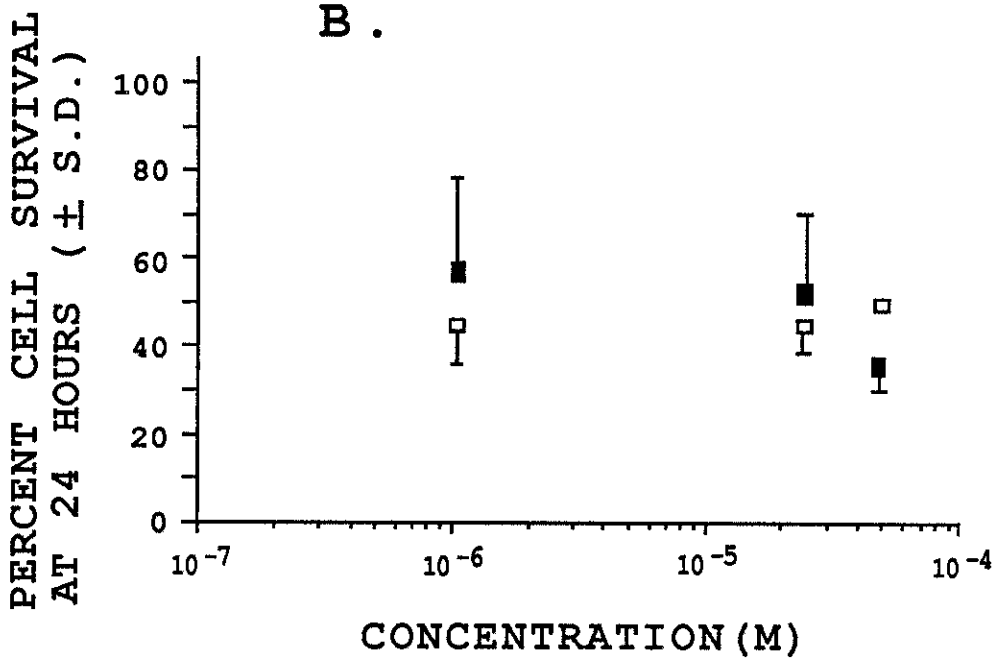
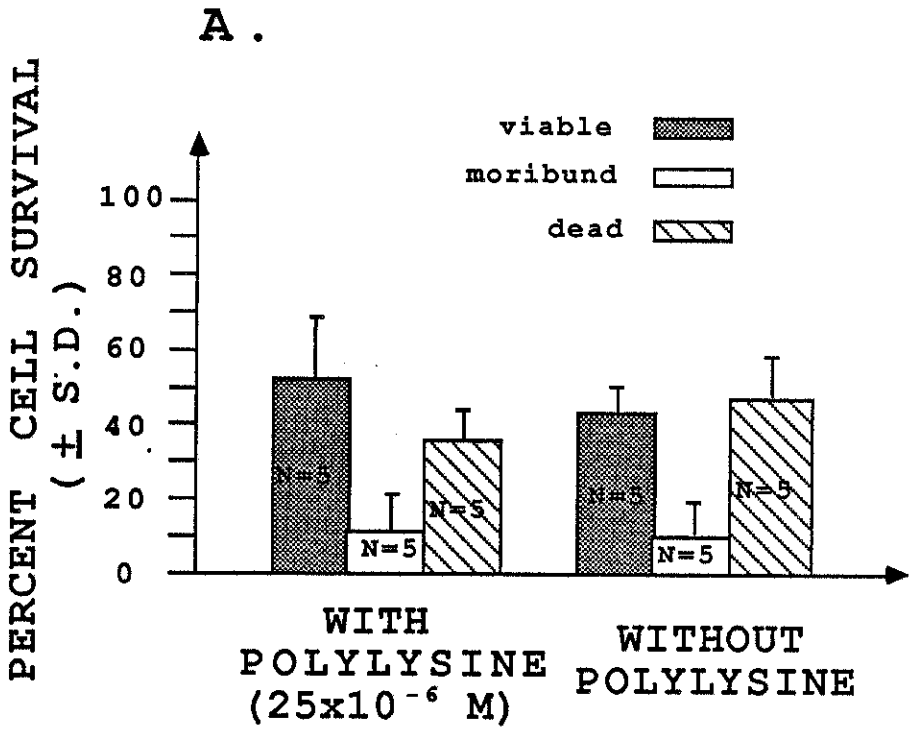
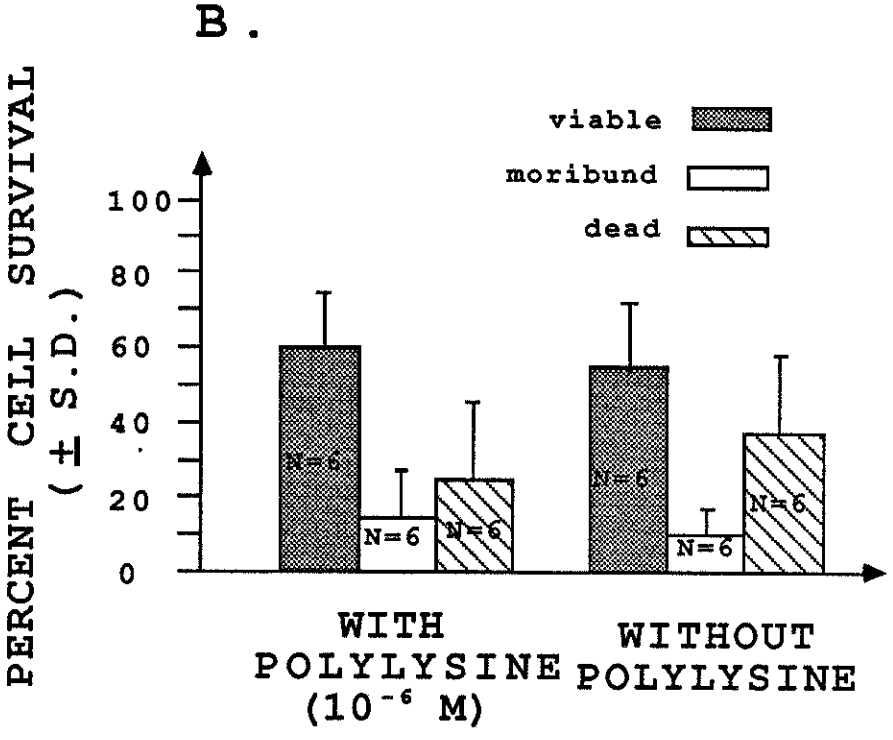

B.
PERCENT CELL SURVIVAL
AT 24 HOURS (± S.D.)
100
80
60
40
20
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
CONCENTRATION (M)

Figure 14. Lesioned nerve cell survival two hours after dendrite amputation in the presence of polylysine. Darked bars represent viable cells, shadowed bars represent dead cells, and open bars represent moribund cells. *A*, Two hours viability in the presence of 25×10^{-6} M polylysine. A total of ten experiments (five with polylysine and five controls) were performed (100 operated cells). *B*, Two hours viability in the presence of 10^{-6} M polylysine. A total of twelve experiments (120 cells) were performed. Six of these served as a control group (no polylysine).


A.
PERCENT CELL SURVIVAL (± S.D.)
viable
moribund
dead
100
80
60
40
20
0
N=5
N=5
N=5
N=5
N=5
N=5
WITH POLYLYSINE (25x10⁻⁶ M)
WITHOUT POLYLYSINE


B.
PERCENT CELL SURVIVAL (± S.D.)
viable
moribund
dead
100
80
60
40
20
0
N=6
N=6
N=6
N=6
N=6
N=6
WITH POLYLYSINE (10⁻⁶ M)
WITHOUT POLYLYSINE

Finally, at a concentration of $5x10^{-5}$ M, four experiments (two pairs) were performed (Fig. 13). The means of the two hours operated cell survival with and without polylysine were 35±5% and 50±0% respectively. The mean of the 24 h operated cell survival with and without polylysine were also 35±5% and 50±0%.

These data indicate that, at the maximum nontoxic concentration, polylysine fails to increase the likelihood of cell survival or to slow the process of cell deterioration after physical injury close to the neuronal perikaryon.

Neuronal Survival with the Measurement of Retraction and Lesion Diameter

In order to determine the effect on survival of these various agents more precisly, I performed 20 experiments (ten matched pairs) in which the extent of proximal segment retraction and lesion diameter were also measured. These lesion parameters have been shown to influence the probability of survival after dendrite transection (Lucas, 1987). Ten of these experiments were performed using polylysine and the other ten experiments were conducted using verapamil. The molecular weight and concentration of polylysine was 3,300 and $2.5x10^{-5}$ M. The concentration of verapamil was $8x10^{-5}$ M. After measuring lesion diameter and the extent of retraction, operated cells were divided into

four groups based on their calculated R/D (retraction /diameter) values. As is shown in Figures 15 and 16, neither verapamil nor polylysine significantly increased cell survival even when the factors of lesion diameter and proximal segment retraction were taken into account.

Figure 15. Lesioned nerve cell survival 24 hours after dendrite amputation in the presence of $8x10^{-5}$ M verapamil. A total of 100 cells (in ten experiments) were divided into four groups according to their R/D values (retraction of proximal segment/lesion diameter). Dark bars represent cells operated in the presence of verapamil. Open bars represent survival in the control group.

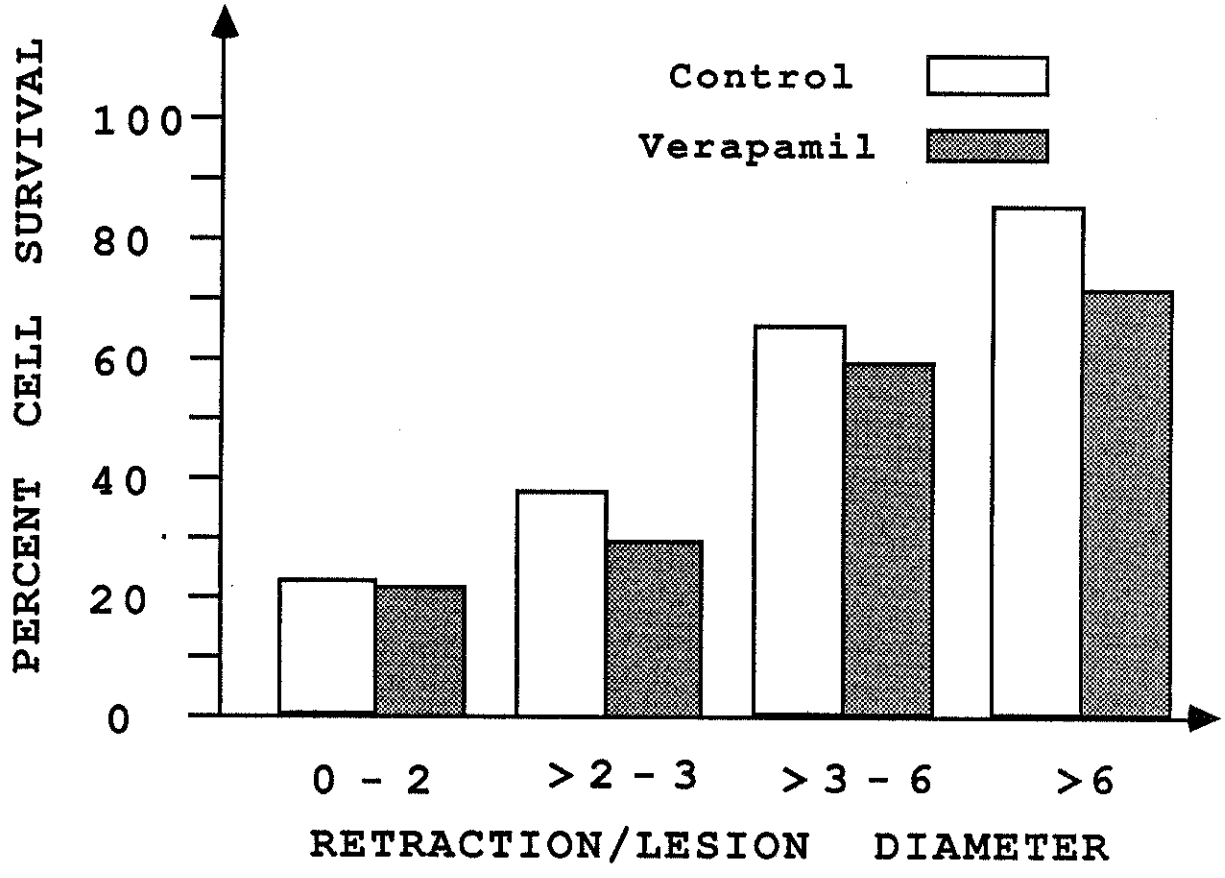
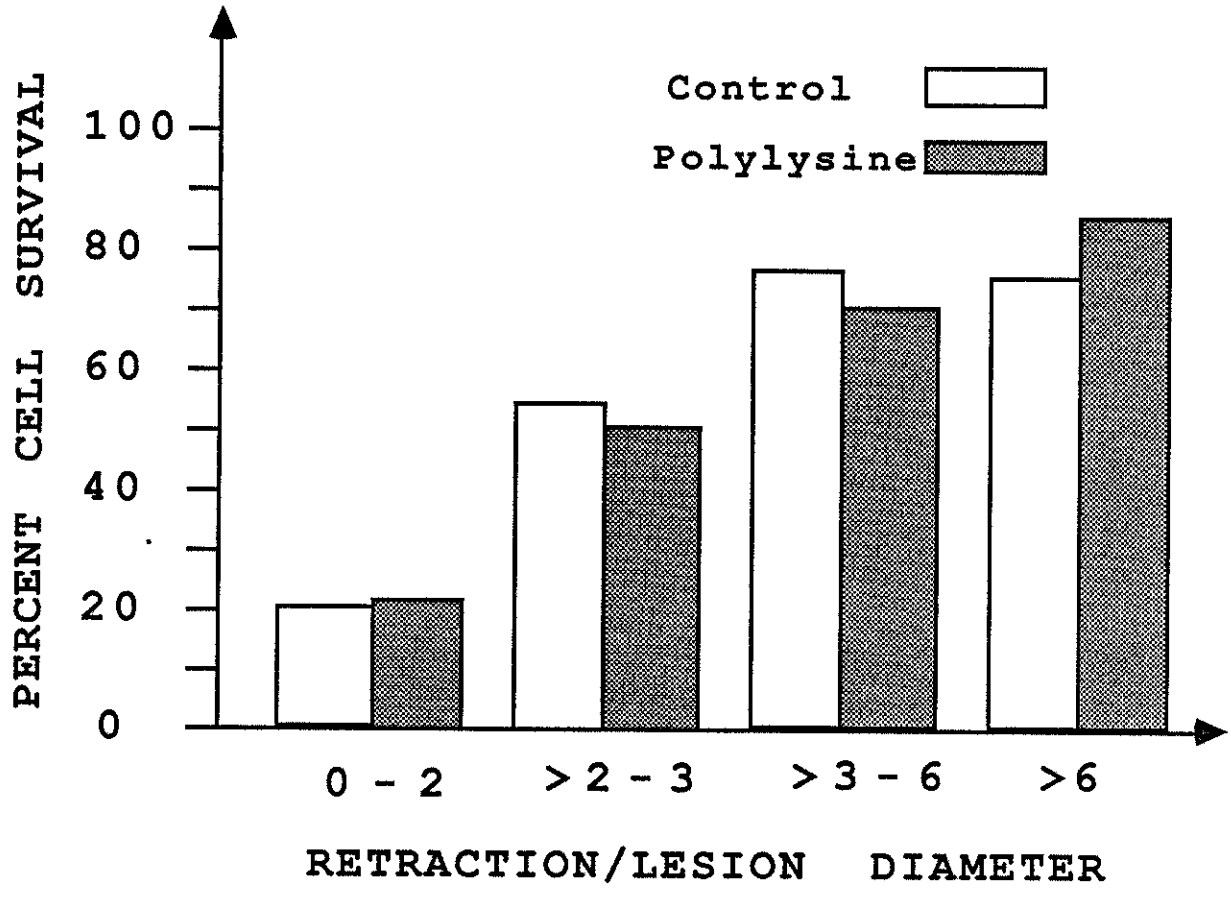

PERCENT CELL SURVIVAL
100
80
60
40
20
0
Control
Verapamil
0 - 2
> 2 - 3
> 3 - 6
> 6
RETRACTION/LESION DIAMETER

Figure 16. Lesioned nerve cell survival 24 hours after dendrite amputation in the presence of 25×10^{-6} M polylysine (MW: 3,300). A total of 100 operated cells (ten experiments) were divided into four groups according to their R/D values (retraction of proximal segment/lesion diameter). Dark bars represent cells operated in the presence of polylysine and open bars represent the control group of cells (no polylysine).


PERCENT CELL SURVIVAL
100
80
60
40
20
0
Control
Polylysine
0 - 2
> 2 - 3
> 3 - 6
> 6
RETRACTION/LESION DIAMETER

CHAPTER IV

DISCUSSION

Injury Currents After Neuronal Physical Trauma

Historical Background and New Thoughts

While electrophysiologic investigation into the capacity of excitable cell membrane to reseal after physical injury began before the turn of the century (Engelmann, 1877) and continued to the present (Lorente de No, 1947; Rothschuh, 1950, 1951; Easton, 1960; Deleze, 1970; Borgens et al., 1980; Meiri et al., 1981; Yawo and Kuno, 1983, 1985; Lucas et al., 1985), injury currents through the lesion after neuronal physical trauma have only recently been analyzed quantitatively in terms of their ionic components (Borgens et al., 1980; Meiri et al., 1981, Borgens, 1988). Using techniques of ion replacement, Borgens and co-workers (1980, 1988) have shown that the injury currents entering the cut faces of severed lamprey spinal cord axons consist roughly of 50% sodium while most of the balance is carried by calcium. Similarly, increased conductances for these ions have been measured in the transected giant fibers of the cockroach nerve cord. Using conventional eletrophysiological

techniques, Meiri and co-workers (1981) measured a four-fold increase in sodium conductance and twenty-fold increase in calcium conductance at the lesion; gradual restoration in normal ion conductances took place over several days. A similar situation was observed in mammalian central nerve system after physical injury. In addition, following experimental spinal cord injury in animals, there appears to be a rapid and profound decrease in extracellular calcium, presumably resulting from the movement of calcium into cells (Young and Flamm, 1982; Young, 1985, 1986, 1987). By using two methods, the electron probe and atomic absorption spectroscopy, Young and co-workers found that in cat thoracic spinal cords injured by a standardized weight-drop contusion, extracellular calcium decreased from 1.2 mM before injury to <0.01 mM within minutes (Young, 1985). Similar extracellular changes have been reported in a dog spinal injury model by Stokes et al. (1983, 1984). One likely possibility is that the calcium entered cells through the disrupted plasma membrane (Young, 1985). In addition, both extracellular and tissue measurements of sodium and potassium have indicated that, after spinal cord injury, these ion also undergo massive shifts down their respective gradients across cell membranes (Young, 1985).

Although a direct cause-effect relationship between calcium and neuronal death has been difficult to establish, a strong, persistent injury current consisting mainly of

sodium and calcium has been demonstrated repeatedly after neuronal physical injury (Borgens et al., 1980; Meiri et al., 1981; Borgens, 1988). The relationship between the concentration of intracellular free calcium and cell deterioration has also been studied extensively (Schlaepfer and Bunge, 1973; Schlaepfer, 1974; Durham, 1974; Dustin, 1978; Pant and Gainer, 1980; Trump et al., 1985). Calcium entry into cells has been reported to activate degradative enzymes, to initiate and aggravate the cascade of arachidonic acid metabolites, to stimulate aerobic glycolysis, to increase membrane permeability and, to degrade cytoskeleton such as microtubules and neurofilaments (see Young, 1985 for review). Consequently, many investigators have hypothesized that calcium entry causes the widespread cell death and loss of function which occurs after severe CNS injury (Schlaepfer, 1977; Banik et al., 1980; Young and Flamm, 1982; Balentine, 1982, 1983; Hsu et al., 1984; Trump and Berezesky, 1985).

By contrast, although injury currents through the lesion site after neuronal physical injury are well documented (Borgens et al., 1980; Meiri et al., 1981; Borgens, 1988), injury currents through ion channels in the plasma membrane of injured cells have not been given much attention. Although many studies have suggested that the fate of a lesioned cell is determined by events occurring at the the lesion (Lucas et al., 1985; Lucas et al; 1987; Emery et al.,

1987; Gross and Higgins, 1987; Borgens, 1988), the injury current through ion channels in the membrane cannot be ignored. The existance of voltage-sensitive calcium channels (VSCC) has been clearly demonstrated (Nowycky et al., 1985; Miller, 1987; Kamp and Miller, 1987; Tsien et al., 1987), and neurite transection injury has been shown to cause a depolarization which is probably strong enough to open the calcium channels within 50-100 μm of the lesion site (Lucas et al., 1985; Miller, 1987, Tsien et al., 1987).

Based upon previous studies on plasma membrane calcium channel function (Hagiwara, 1982; Fenwick et al., 1987; Krishtal et al., 1979; Tsien, 1983), one can estimate the total number of calcium ions which cross a given area of the plasma membrane after injury. Studies of snail neuron and mammalian chromaffin cells have shown that, within 50 μm of the lesion site, approximately 4.5×10^6 to 1.8×10^8 calcium ions will enter an injured cell through VSCC each second for every square micrometer of membrane area (Hagiwara, 1982; Fenwick et al., 1987; Krishtal et al., 1979; Tsien, 1983; Lucas et al., 1985). In addition, ischemia, which is part of the secondary injury phase after physical trauma, can also produce calcium-related cellular deterioration (Siesjo, 1981; also see Young, 1985, 1986, 1987 for reviews).

In the present series of experiments, I have attempted to determine whether the calcium currents through the VSCC

contribute to the development of the sequence of pathological changes which occurs after physical trauma.

Expansion of the Concept of Injury Currents

In addition to massive shifts of different ions down their respective electrochemical gradients across a physical lesion, ionic currents which cross the membrane through the VSCC should also be considered as part of injury currents after trauma. Current theories on the mechanisms of calcium influx include: (1) depolarization-dependent entry (Nowycky et al., 1985; Tsien et al., 1987; Miller, 1987), (2) depolarization-independent steady state entry system (Dippolo, 1979), and (3) receptor-mediated calcium entry. Receptor mediated entry can be caused by: *a.* the changes in membrane fluidity related to shifts in membrane phospholipid content induced by adrenergic agonists (Hirata and Axelrod, 1980), *b.* N-methyl-D-aspartate (NMDA) receptor activation (MacDermott et al., 1986), and *c.* nicotinic cholinergic agonists (Adams et al., 1980). Approximately, 50-60% of the total calcium influx through plasma membrane ion channels has been found to be insensitive to ATP, is sensitive to the membrane potential, and is partially inhibited by external cobalt (Dipolo, 1979).

Although ATP is necessary for calcium to enter via the depolarization-independent pathway, elevation of intracellular sodium and calcium will probably activate this

calcium influx (Dipolo, 1979). Interestingly, Dipolo (1979) suggested that in the presence of ATP and intracellular sodium, intracellular calcium probaly will activate the calcium influx along a sigmoid curve without saturation up to a concentration of 1 μM. Intracellular sodium and calcium probably will increase in neuronal tissue after physical injury (Young, 1985). Intracellular calcium activity exceeding 1 μM will activate proteinases (Schlaepfer, 1979; Schaffer et al., 1983), and phospholipases (Hass, 1981; Banik et al., 1984) that degrade cell structures and release damaging metabolites (Demopoulos et al., 1980) similar to those observed in mechanical or ischemic injury. Thus, it is likely that the depolarization-independent calcium entry system is also partially responsible for the nerve cell deterioration after physical injury.

At present there is no direct evidence that changes in membrane fluidity can increase calcium leakage across cell membrane. However, Yawo and Kuno (1983, 1985) have shown that after physical trauma, phospholipase A_2, which is activated by the calcium influx, is instrumental in membrane resealing. Hirata and Axelrod (1980) have suggested that an increase in membrane fluidity may open calcium ion channels and activate the calcium-requiring phospholipase A_2. In addition, several investigators found that the changes in membrane fluidity caused by physical injury (Yawo and Kuno,

1983, 1985) will affect the function of membrane bound permease such as the Na^+/K^+-ATPase (Kimelberg and Papahadjopoulos, 1974; Clendenon et al., 1978; Faden a et al., 1987). Therefore, a physical lesion may itself trigger a change in membrane fluidity in the affected process and, consequently, enhance the calcium influx.

In summary, physical injury may open many pathways for injury currents to penetrate the injured cells in addition to the one at lesion site. Immediately after trauma, ionic currents will enter a cell through the lesion as well as through VSCC which are activated by membrane depolarization. Shortly after that, the increase of intracellular sodium and calcium will activate calcium influx through the depolarization-independent calcium influx system (Dipolo, 1979). When intracellular calcium concentration exceeds 1 μM, calcium influx via non-specific openings in the injured membrane will become saturated. However, when the concentration rises above 1 μM, calcium will activate proteinases and phospholipases that degrade cell structures. Activation of phospholipase A_2 will also increase membrane fluidity which will augment the calcium influx into the cell. All of these events are presently thought to cause a series of pathological changes leading to cell death. It is of great importance that we understand the relative importance of each of these calcium injury currents in order to develop

effective treatments which will save injured nerve cells after physical trauma.

Neuronal Survival After Physical Injury in the Presence of Calcium Channel Blockers

Neuronal Cell Survival in the Presence of Verapamil and Nifedipine

Based upon previous studies, I anticipated that verapamil and nifedipine would block the L-type calcium channels in our mouse spinal neurons, and increase nerve cell survival after physical injury. However, my results show that neither verapamil nor nifedipine significantly increased the numbers of nerve cells which survived dendrite amputation. This is surprising because verapamil and nifedipine significantly increased the likelihood of survival of cultured renal cells after anoxia (Schwertschlag et al., 1986).

There are some reasons which may explain this difference. The biggest difference between these two models is the type of injury. Physical injury differs from anoxia in the size of the area of membrane disruption. Large calcium and sodium currents will enter a cell at the lesion site (Borgens et al., 1980; Meiri et al., 1981; Young, 1985; Borgens, 1988). The magnitude of these currents will probably determine the fate of a lesioned cell (Lucas et al.,

1985; Emery et al., 1987; Gross and Higgins, 1987; Lucas, 1987). Therefore, although I blocked calcium channels along the neurite after physical injury with these two agents, the injured cells still had a large opening which allowed massive amount of calcium and sodium to enter and, probably, kill them.

The VSCC are not the only channels by which calcium can enter injured cells. According to Dipolo (1879), only 50-60% of calcium entry is depolarization-dependent. In addition, current evidence suggests that the available calcium antagonists block only the L-type VSCC (Kamp and Miller, 1987). Apparently, the calcium channel blockers presently available are not by themselves capable of protecting nerve cells after neurite transection injuries as they do not address the major component of injury currents.

Voltage-sensitive Calcium Channels

The existence of calcium permeability in excitable cells has been appreciated for 30 years (Fatt et al., 1953) -- almost as long as we have known about voltage-dependent membrane permeabilities to sodium and potassium (Hodgkin and Katz, 1949; Hodgkin and Huxley, 1952). Yet, to this day, our understanding of calcium channels has lagged far behind our knowledge of sodium or potassium channels. This situation is ironic because calcium channels are widely distributed and rival their better-characterized counterparts in diversity of

biological function (Hagiwara and Byerly, 1981).

For many years, biological or biochemical analysis of calcium channels has been seriously hampered by experimental problems (see review Tsien, 1987). But the state-of-the-art has improved recently with the development of powerful methods for recording calcium activity, and of blocking drugs that may interact with calcium in a potent and specific manner. Using voltage clamp techniques and dihydropyridine derivatives, Nowycky and co-workers (1985) demonstrated three types of neuronal calcium channels, L-type, N-type and T-type, with different calcium agonist sensitivity. Among them, the L-type channel has been attracted most attention in terms of electrophysiological properties and pharmacological characteristics (see Kamp and Miller, 1987 for review).

L-type channels are thought to be located in dendrites (Linas and Yarom, 1981) and are activated by large depolarizations (positive to -10 mV) (Nowycky et al., 1985). L-type channels can generate large calcium conductances that cause long-lasting currents which can be blocked by inorganic and organic calcium channel blockers, and toxins (See Miller, 1987 for review). Unlike N-type channels, which are thought to play a dominant role in the depolarization-evoked release of neurotransmitters (Hirning et al., 1988), the function of L-type channel are still not clear. However, quantitative analysis has so far largely been restricted to

dihydropyridine-sensitive (DHP-sensitive) L-type calcium channels (See review Tsien et al., 1987). Radiolabeled calcium antagonists (^{3}H-DHPs) have been used biochemically to isolate one type of VSCC and identify it as a multisubunit glycoprotein complex consisting of at least two subunits with molecular weights of 130-145 kD and 32 kD (Curtis et al., 1984; Borsotto et al., 1984; Flockerzi et al., 1986).

Among several classes of compounds which have been shown to interact with VSCC, the most important from the theoretical point of view has been the dihydropyridines. Nifedipine is a dihydropyridine (See Miller, 1987 for review). Others like verapamil also show potent antagonistic effects on the VSCC in neurons (Boll and Lux, 1985; Kamp and Miller, 1987). In NCB-20 (neuroblastoma x Chinese hamster brain hybrid), for instance, the concentration of nifedipine required to inhibit the depolarization-sensitive component of $^{45}Ca^{2+}$ uptake by 50% is 9.2 nM and verapamil's is 1.8 μM (Freedman et al., 1984). With the concentration from 20 to $100x10^{-6}$ M, verapamil can strongly depress the slow inactivation (L-type channel) calcium conductance in avian dorsal root ganglion (DRG) cells (Boll and Lux, 1985). Current evidence suggests that the function of the calcium antagonists is voltage-dependent as well as the frequency of opening and closing of the calcium channel (see Kamp and Miller, 1987 for review). It has been shown that DHP calcium

antagonists can be up to 1000-fold more potent at blocking VSCC at depolarized potentials compared with hyperpolarized potentials (Sanguinetti and Kass, 1984).

Until recently it was not known whether mammalian CNS neurons contain the DHP-sensitive VSCC. By using microspectrofluorimetry and the calcium-sensitive dye fura-2, Thayer and co-workers (1987) found that virtually all mouse CNS neurons posses functional DHP-sensitive VSCCs.

Extracellular Calcium and Cell Death

Cell deterioration after injury has been linked to elevated intracellular calcium (Schlaepfer and Bunge, 1973; Durham, 1974; Schlaepfer, 1974; Dustin, 1978; Pant and Gainer, 1980; Young et al., 1982; Trump et al., 1985). Consequently, some investigators have called the calcium influx the "final common pathway" of cell death (Schanne et al., 1979). The degenerative changes which result from influxes of extracellular cations undoubtedly have important implications for neuronal viability after transection trauma.

However, a significant lowering of external calcium after injury has not conferred the protection anticipated. For instance, *in vitro* neuronal trauma studies in this laboratory showed that the percent cell survival in the low calcium medium and normal calcium medium after amputation of dendrite at 100 μm from their perikarya was not significantly different (30 ± 7 % and 23 ± 8 % respectively) (Lucas et al., In

press). In addition, Smith and co-workers (1981) reported that toxic injury to isolated rat hepatocytes was not dependent on extracellular calcium. Therefore, it seems that calcium influx is not the final common pathway in every injury situation. The critical question then is: when external calcium is greatly reduced, what is responsible for the death of neurons after transection trauma?

As I mentioned in the first chapter, calcium can be released from intracellular storage sites such as the smooth endoplasmic reticulum and mitochodria (Spat et al., 1986; Berridge and Irvine, 1984; Carafoli, 1974). Therefore, the most likely explanation of cell death without extracellular calcium is that physical 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 0.2 to 0.4 mM (Baker, 1976; Albers et al., 1983). Sequestered calcium is stored primarily in the endoplasmic reticulum and in mitochondria (Baker, 1976; Albers et al., 1983). 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. Sodium has been determined as a major component of the injury current (Borgens et al., 1981; Meiri et al.,1981; Borgens, 1988). Sodium is known to trigger a two-for-one exchange with calcium across the mitochondrial inner membrane (Carafoli et al., 1974; Crompton et al., 1976; 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 might cause the release of significant amounts of mitochondrial calcium (Carafoli et al., 1974; Crompton et al., 1976; Crompton et al., 1977; Crompton and Heid, 1978; Nedergaard, 1984; Carafoli and Penniston, 1985) which then could dismantle the cytoskeleton.

Sodium may have a second portal of entry into cells injured in a low calcium environment. Calcium ions are believed to be firmly bound to the exterior surface of the plasma membrane of cells and to stabilize the plasma membrane (Manery, 1966). Reduction of extracellular calcium will result in an increase of cell permeability to sodium (Hays et al., 1965) and potassium (Conn et al., 1957). In a situation of low extracellular calcium, the membrane will probably become more "leaky" and allow sodium to rush into the injured

cell. Once again, the increased intracellular sodium will probably trigger the release of calcium from mitochondria. Such scenario has been reported to occur in the so called "calcium paradox" (Zimmerman and Hulsmann, 1966). Thus, theoretically, protection probably will not be effective unless both extracellular calcium and sodium are lowered after injury. Goldberg and co-workers (1986) have already reported that protection of cultured neurons after ischemic injury was only achieved when levels of both calcium and sodium were lowered. In this study, when calcium was buffered to a very low level (1 μM), no increase in neuronal survival was observed as long as sodium and potassium were maintained at normal concentration (140 mM and 5 mM respectively). A significant sparing of neurons, however, was observed when the decrease in calcium was combined with lowered sodium (45 mM) (Goldberg et al., 1986). This is consistent with Rothman's (1984) observation that TTX, a drug that blocks the sodium-dependent action potential, also protects hippocampal cell from anoxic damage. Therefore, these results support the idea that an sodium influx can also contribute to neuronal damage after injury.

Neuronal Survival After Dendrite Amputation in the Presence of a "Molecular Bandage"

Dynamic Resealing After Transection Injury

The electrophysiological properties of nerve cells have been used as indicators of membrane resealing after transection injury (Borgens et al., 1980; Meiri et al., 1981; Yawo and Kuno, 1983, 1985; lucas et al., 1985; Borgens, 1988). However, because of a lack of appropriate techniques, the acute morphological reactions of individual neurons in response to transection injury have received less attention. Using the squid giant axon, Gallant (1988) observed a initial partial constriction of the cut end approximately five minutes after axotomy. Similar results were reported by Lucas (1987) who found that the extent of proximal segment retraction within five minutes after injury is a factor in cell survival after dendrite amputation. This morphological phenomenon is in agreement with the electrophysiological studies done earlier by Lucas and co-workers (1985) which showed that the membrane potential partially recovered around five minutes after dendrite transection injury, and also with separate observation by the same group which showed that the percent RP loss after transection is less when retraction is greater (unpublished data). Therefore, although it is difficult to determine the exact size of the cut face after

transection injury, local changes near the lesion during the acute phase probably influence the fate of the injured neuron after transection trauma.

Neuronal Cell Survival in the Presence of Molecular Bandage

Prevention of axonal necrosis in lampreys after spinal cord transection has been achieved by application of a DC current across the lesion which apparently conteracted the damaging injury currents (Roederer et al., 1983). This points the way to the development of the therapies which address the ionic insult to an injured cell.

The interior of cells is negative compared to the extracellular environment. This electric field will cause movement of positive ions (Borgens et al., 1980; Borgens, 1988). It should be possible to enhance resealing by allowing the electric field to draw positively charged large molecules into the lesion which can then serve as "molecular bandage". Based on this hypothesis, I transected 180 neurons in medium containing polylysine, a positively charged polyamine. The results of these experiments show, however, that there was no significant difference in the likelihood of survival between the group of neurons injured with polylysine in the medium and the group of neurons injured in control medium without polylysine.

In the molecular bandage experiments, I controlled for a number of factors such as exposure time of injured neurons to polylysine, numbers of molecules of polylysine present in the medium per unit volume and the toxicity of polylysine. In order to make a positive molecule to serve as a molecular bandage, however, there are probably several other factors which need to be considered. First, while the charge of the large cations would in theory fix them against the membrane breach or underlying cytoskeleton, the size of the molecules must be great enough to prevent their penetrating deep in to the injured cell's interior. Therefore, the size of the molecular bandage is important if it is to "plug" the hole in the injured cell.

Some degrees of initial constriction of the lesion site have been observed in giant squid axons (Gallant, 1988). In addition, by injecting cobalt intracellularly and observing the precipitate as an indication of leakage of cobalt through the open cut end of a transected axon, Meiri and coworkers (1981) postulated a formation of a barrier to cobalt and possibly to large molecule start at four hours and an efficient barrier to cobalt was formed at 20 to 24 hours after axon transection. Nevertheless, statistic data from direct measurement on the size of the cut face of injured cell after transection trauma has not been seen in the literature yet. In order to determine the lesion size, more

morphological study on CNS trauma in vitro system needs to be done.

The mobility of the positively charged molecule is also critical for its function. In order to retain maximum mobility in the electric field, a molecular bandage should remain positively charged until it binds to the membrane breach. In my experiments, however, it is possible that associated anions masked the positive charges on the polylysine molecules. The polylysine molecules then would lose their mobility and consequently could not be drawn by the field across the disrupted cell membrane. In order to avoid this, the ions in the culture medium may have to be replaced by a neutral solute such as sucrose. In my opinion, this would be a logical next step in the molecular bandage studies.

Others like the toxicity of counter ion Br^- of polylysine may also affect the function of the molecular bandage by causing more cell death. In order to eliminate this possibility, polylysine with different counter ion (Br^- and Cl^-) were tested (unpublished data). The results showed that there was no significant difference between the polylysine with bromine and with chlorid in terms of numbers of surviving cell. Therefore, the toxicity of counter ion of bromine associated with polylysine has no more effects than chloride based upon my toxicity studies.

Further Comments and Suggestion on Neuronal Trauma Study

The laser microbeam/culture model clearly does not provide an exact replication of all the conditions found in a situation of physical injury *in vivo*, yet it can serve as a model system for studying the mechanisms of neuronal damage in isolation from some of the complexities of the *in vivo* situation. Furthermore, when compared with mechanical methods of surgery, the laser microbeam does provide a more precise experimental injury (Gross et al., 1983; Lucas et al., 1885).

In the present trauma model, the laser microbeam was utilized to transect primary dendrites. Physical injury differs from anoxia and ischemic injury as it causes a major local membrane disruption. Therefore, the pathways of the damaging calcium and sodium currents are quite different in the two types of injury and will probably require different avenues of treatment. I believe that in order to protect nerve cells after transection injury, agents such as calcium channel blockers which leave the cut face open will not be effective.

Because of the massive injury current which penetrates an injured neuron at the lesion, I think that development of an effective molecular bandage should be given priority in the effort to find new treatments for CNS physical trauma. I

also believe, however, that as there are two avenues by which calcium and sodium can enter injured cells, channel blockers still may be beneficial if used to assist the molecular bandage. Finally, reducing the concentrations of calcium and sodium could also help to protect physically injured nerve cells.

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