The Role of Calcium in Neuronal Death and Regeneration after Neurite Transection in a Cell Culture Model

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
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Gordon Kwok Tung Chu, MD, M.Sc. Thesis Abstract, 1999

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Traumatic axonal injury in the central nervous system can result in neuronal death. While calcium entry following the trauma may be responsible for this cell death, calcium may be required for regeneration of the neurites.

The present study examines the role of calcium in neuronal death and in regeneration of neurites transected in a cell culture model using superior cervical ganglion neurons. To assess the relationship between calcium entry and cell death, the neurites were transected while immersed in media of different calcium concentrations (50 nM, 1.8 mM, 5 mM). The percentage of neurons that died did not correlate with the extracellular calcium concentration 72 hours after transection. The majority of cell death occurred within 660 μm of the cell bodies and within the first 24 hours after transection. To assess the relationship between calcium and regeneration, the neurites were transected while preloaded with the calcium chelator 1,2 bis-(2-aminophenoxy)ethane-N,N,N′, N′ - tetraacetic acid (BAPTA-AM) or immersed in a zero calcium medium. Neurons loaded with BAPTA-AM exhibited a delay in the initiation of regeneration after transection and the amount of branching per regenerating neurite. Neurons whose neurites were transected in zero calcium did not initiate regeneration until the normal calcium medium was restored 6 hours post transection. Furthermore, if the neuronal cell bodies were immersed in zero calcium medium while the neurites were immersed in normal calcium as the neurites were transected then there was a delay in the initiation of regeneration.

The results of these studies suggest that calcium entry after neurite transection is not important for neuronal death but is necessary for neurite regeneration in this cell culture model.

Acknowledgements

This thesis work is the result of contributions from many individuals who have aided me with the underlying hypotheses, conduction of the experiments, and the critical analysis of the data.

I would like to thank Dr. Tator for providing me with the opportunity to pursue this scientific work. His feedback on my work was always stimulating and enlightening. He has been an inspirational role model during my time in his laboratory. He has always been supportive, and his words of encouragement just when things seemed bleakest were much appreciated. It has been an interesting and exciting journey.

I would also like to thank the members of my program committee: Dr. Mills, Dr. Lozano, Dr. Charlton, and Dr. Eubanks. They have taught me valuable lessons concerning how to approach and answer a scientific question. I only hope that I can come close to their level of excellence in my future scientific endeavors. I would also like to thank them for their kindness and patience.

The technical assistance that I have received from Linda Lee, Rita van Bendegem, and Jim Loukides has been unparalleled. I wish them all the best. I would like to thank the summer students: Patrick Tawadros and Lauren Mendelson for their assistance. Thanks also to Maria Vespa, Sandy Amaral, and Frank Vidic for their aid.

I would like to thank my parents and my grandmother for their love and support without which all of this would not have been possible.

Finally, I would like to thank my girlfriend, Sandra for her support and for putting up with me through these many years.

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AM	acetoxymethyl ester
ANOVA	analysis of variance
BAPTA-AM	1,2 bis-(2-aminophenoxy)ethane-N,N,N′,N′ - tetraacetic acid
Ca^{2+}	calcium
$[Ca^{2+}]_i$	free intracellular calcium concentration
$[Ca^{2+}]_e$	extracellular calcium concentration
CICR	calcium induced calcium release
Cl^-	chloride
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N, N′,N′-tetraacetic acid
$\Delta f/f_0$	fractional change in fluorescence
Hepes	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
K^+	potassium
L-15	Leibovitz-15
Na^+	sodium
NMDA	N-methyl-D-aspartate
SCG	Superior Cervical Ganglion
SEM	standard error of the mean
TPEN	NNN'N'-tetrakis(2-pyridylmethyl)ethylene diamine

Introduction

Brain and spinal cord trauma can produce both immediate and delayed neuronal death. The cause of the immediate neuronal death is thought to be due to mechanical impact with irreparable disruption of cellular membrane, but delayed neuronal damage is believed to be due to a number of factors including ischemia and excitotoxicity secondary to decreased blood flow, hemorrhage, and edema (Tator, 1991). The delayed neuronal death is thought to involve calcium (Ca^{2+}) entry into neurons, but it is not known if Ca^{2+} entry from the initial trauma is sufficient to cause delayed neuronal death or whether Ca^{2+} entry from the resultant secondary injury (excitotoxicity, ischemia) alone is responsible for neuronal death. However, Ca^{2+} entry may also be beneficial to the neuron because it might be necessary for regeneration after transection. The following studies will examine the relationship of Ca^{2+} to both neuronal death and neurite regeneration.

Calcium Homeostasis in the Neuron

Calcium ions are ubiquitous intracellular second messengers which regulate numerous cellular functions. Neurons must tightly control the free cytosolic Ca^{2-} ion concentration ($[Ca^{2+}]_i$) to allow efficient Ca^{2+}-dependent signaling to occur. For relatively small or localized increases in $[Ca^{2+}]_i$ to trigger physiologic effects, resting $[Ca^{2+}]_i$ must remain at very low levels (around 100 nM, or 10^5 times lower than extracellular Ca^{2+} concentration $[Ca^{2+}]_e$). Because Ca^{2+} is necessary for cell survival, various mechanisms have evolved to maintain Ca^{2+} homeostasis. These mechanisms can be divided into four categories: Ca^{2+} influx, Ca^{2+} buffering, internal Ca^{2+} storage, and Ca^{2+} efflux.

Ca^{2+} Influx

Ca^{2+} influx occurs primarily through ion channels. These specialized pores in cell membranes are classified physiologically by their specific selectivities for certain ions (Ca^{2+}, K^+, Na^+, or Cl^-) and by their gating mechanism. Some ion channels respond to membrane voltage while others are associated with specific ligands. Voltage gated ion channels may contain binding sites for certain ligands, and ligand-gated channels may exhibit certain forms of voltage dependence. Voltage gated Ca^{2+} channels are numerous and include N, L, P, Q, T- types (Miller, 1992, Nooney et al, 1997). Ligand gated channels which permit Ca^{2+} entry include NMDA and certain AMPA/Kainate receptors. After trauma, Ca^{2+} influx may also occur through disruption of the cellular membrane.

Ca^{2+} Buffering

Calcium buffering allows the neuron to control the spread of Ca^{2+} ions within the cell. As Ca^{2+} ions enter into the cell, they are rapidly buffered by a number of cytoplasmic proteins, such as calmodulin, calbindin, and parvalbumin (Bainbridge et al, 1992). Approximately 95 to 99 % of Ca^{2+} ions entering the cell under physiological conditions are buffered in this fashion (Neher and Augustine, 1992, Zhou and Neher, 1993). The precise role of Ca^{2+} buffering molecules remains poorly understood, however, they may act to keep $[Ca^{2+}]_i$ at high levels in localized areas within cells, to limit those high $[Ca^{2+}]_i$ levels to those specific areas, and to rapidly dissipate the Ca^{2+} gradients, and thus limit the time course of activation of Ca^{2+}-dependent processes (Chard et al, 1993, Kasai and Peterson, 1994, Nowycky and Pinter, 1993, Roberts, 1994). Ca^{2+} buffers may also act as Ca^{2+} shuttles, carrying Ca^{2+} ions from their site of influx to and away from their site of action (Neher, 1986, Roberts, 1994, Sala and Hernandez-Cruz, 1990, Speksnijder

et al, 1989, Stern, 1992). These effects are dependent on the distribution, type, and concentration of the Ca^{2+} buffers within the cell.

Ca^{2+} Storage

When Ca^{2+} loads exceed the buffering capacity of the neuron, Ca^{2+} ions may be sequestered into organelles such as the smooth endoplasmic reticulum, mitochondria, and synaptic vesicles (Blaustein, 1988, Clapham, 1995). These organelles can store large quantities of Ca^{2+} under a variety of conditions, using active and passive Ca^{2+} transport mechanisms similar to those found in the plasma membrane (see below). Although Ca^{2+} storage in organelles is an efficient mechanism for controlling free cytosolic $[Ca^{2+}]_i$, this Ca^{2+} "lowering" system operates much more slowly than Ca^{2+} -binding proteins. Therefore, it is not capable of modulating rapidly changing or highly localized changes in $[Ca^{2+}]_i$ (Werth and Thayer, 1994).

Ca^{2+} Efflux

Due to the large extracellular-to-intracellular Ca^{2+} ion concentration gradient and the electrical driving force propelling the positively charged Ca^{2+} ions toward the negatively charged inner plasma membrane, Ca^{2+} efflux is an energy dependent event in the cell. Neurons have at least two Ca^{2+} extrusion mechanisms, adenosine triphosphate (ATP)-driven Ca^{2+} pumps (Ca^{2+} -ATPases) and an Na^+/Ca^{2+} exchange transport mechanism (Blaustein, 1988, Carafoli, 1992, Lwe et al, 1982, Miller, 1992, Naschshen, 1992, Stys et al, 1990). Ca^{2+} ATPases in the plasma membrane are modulated by calmodulin, a number of fatty acids, and protein kinases (protein kinases A and C). One ATP molecule is expended for each Ca^{2+} ion extruded. Ca^{2+} ATPases also exist in the

membranes of the smooth endoplasmic reticulum, acting as a mechanism of intracellular Ca^{2+} sequestration. These are calmodulin independent and sequester two Ca^{2+} ions for each ATP molecule. The Na^{+}/Ca^{2+} exchanger is triggered by a rise in $[Ca^{2+}]_i$, removing one Ca^{2+} ion for two to three Na^{+} ions that enter. This process is dependent on the Na^{+} gradient.

Calcium Ions and Cell Death

One of the first observations that Ca^{2+} ions were linked to cytotoxicity was the finding that livers damaged by toxins accumulated Ca^{2+} which suggested that Ca^{2+} entry may be responsible for tissue damage (McLean et al, 1965). Later experiments by Schanne et al (1979) revealed that adult hepatocytes in primary cultures were killed when exposed to various toxins (believed to affect plasma membrane integrity) in the presence, but not the absence, of extracellular Ca^{2+}. The authors thought that Ca^{2+} influx into cells was an absolute requirement for the expression of toxicity, and termed this process the "final common pathway of cell death." Even before this, Zimmerman and colleagues (1967) established a link between Ca^{2+} and cytotoxicity; they observed in isolated heart preparations that perfusion with Ca^{2+}-deficient solutions, followed by reperfusion with solutions containing Ca^{2+}, resulted in rapid cessation of contractility followed by massive, widespread cell death (the 'calcium paradox').

However, the hypothesis that cell death is dependent on Ca^{2+} ions was challenged by others (Cheung et al, 1986). For example, several reports indicated that cytotoxicity in hepatocyte preparations can be produced in the absence of Ca^{2+} (Fariss et al, 1985, Smith et al, 1981). More recently, the loss of hepatocyte viability during chemical hypoxia was

shown to occur prior to any measured rise in intracellular Ca^{2+} concentration (Lemasters et al, 1987), confirming that mechanisms other than those triggered by Ca^{2+} excess can also be cytotoxic. Similarly, cell death in the heart under some conditions can be triggered independently of variations in extracellular Ca^{2+} (Chizzonite and Zak, 1981), suggesting that in cardiac muscle, mechanisms other than those responsible for the "calcium paradox" may be operative.

While all forms of cell death may not involve Ca^{2+} influx, there is substantial evidence that cellular Ca^{2+} overload is associated with traumatic/ischemic neuronal death in the adult mammalian nervous system. Early studies in tissue cultures showed that amputated axons degenerated only if Ca^{2+} ions were present in the culture medium (Schlaepfer and Bunge, 1973). In recent experiments where Ca^{2+} accumulation was examined directly, neurodegeneration induced by neurotoxins such as capsaicin and glutamate was shown to be associated with increases in tissue Ca^{2+} (Jansco et al, 1984). Further investigations on the toxicity of excitatory amino acids (EAAs) in cultured neurons and brain slices confirmed an association between the observed toxicity and the presence of Ca^{2+} in the extracellular medium (Choi, 1985, Garthwaite and Garthwaite, 1986, Garthwaite et al, 1986, Hori et al, 1985). However, even this association was not absolute, as some subsequent studies produced evidence that was seemingly contradictory to the Ca^{2+} hypothesis (Collins et al, 1991, Price et al, 1985). Such contradictions illustrate that the rules governing the association between Ca^{2+} overload and neurotoxicity were poorly understood.

Animal studies also support the association between Ca^{2+} influx and damage to neural tissues. Within seconds to minutes after trauma to the rat spinal cord, $[Ca^{2+}]_e$

decreases to <0.01 mM (Stokes et al 1983, Young and Flamm, 1982, Young et al, 1982) and it was suggested that this decrease in $[Ca^{2+}]_e$ is due to cellular influx of Ca^{2+} with resultant cell death. Experimental spinal cord injury also produces significant Ca^{2+} accumulation in white matter axons (Balentine, 1988, Balentine et al, 1982, Balentine et al, 1984), possibly as a consequence of white matter anoxia/ischemia (Stys et al, 1990, Tator and Fehlings, 1991). Cerebral ischemia and epileptic seizures also appear to accelerate intracellular Ca^{2+} accumulation. This was demonstrated by studies using extracellular ion-selective microelectrodes, which showed a marked decrease in extracellular Ca^{2+} concentration following the induction of experimental cerebral ischemia (Gilbertson et al, 1991, Harris et al, 1981, Nicholson et al, 1977). Numerous later studies, using electron microscopy, autoradiographic techniques, atomic absorption spectroscopy, and free Ca^{2+} measurements with fluorescent Ca^{2+}-sensitive dyes, confirmed an increase in tissue and particularly cellular Ca^{2+} concentration following cerebral ischemia or seizures (Benveniste et al, 1988, Chen et al, 1987, Deshpande et al, 1987, Meyer, 1989, Silver and Erecinska, 1990, Simon et al, 1984, Uematsu et al, 1990, Uematsu et al, 1989, Uematsu et al, 1988).

The studies described above provide a foundation for what has been termed the "calcium hypothesis," which states that neuronal Ca^{2+} overload leads to subsequent neurodegeneration. For example, neurotoxic actions of Ca^{2+} overload have been ascribed to the over stimulation of enzymes such as phospholipases, plasmalogenase, calpains and other proteases, protein kinases, guanylate cyclase, nitric oxide synthetase, calcineurins, and endonucleases. Presumably, this leads to an overproduction of toxic reaction products such as free radicals, lethal alterations in cytoskeletal organization, or activation

of genetic signals leading to cell death such as apoptosis (Faroqui and Horrocks, 1991, Tominaga et al 1993). Many of the above noted studies demonstrated the neurotoxicity of Ca^{2+} in relation to EAA's and ischemia. Further evidence of Ca^{2+}'s role in neurotoxicity was demonstrated by studies revealing the neuroprotective effect of Ca^{2+} chelators such as BAPTA-AM after ischemia (Tymianski et al, 1993, Tymianski et al, 1994). In contrast, the relation between Ca^{2+} entry, neuronal death, and mechanical trauma is less well studied. Lucas et al (1990) used a laser to transect dendrites from embryonic murine spinal neurons in culture and demonstrated that cell death was reduced at 2 hours after injury when neurons were injured in low Ca^{2+} (30 μM) extracellular medium compared to cell death of neurons injured in normal (1.8mM) Ca^{2+}. However, by 6 hours there was no significant difference in cell death between the two groups. This study raises doubts as to whether Ca^{2+} influx secondary to trauma is ultimately responsible for neuronal death. However, at 30 μM, the $[Ca^{2+}]_e$ was still considerably greater than the $[Ca^{2+}]_i$. Therefore, 30 μM may not be a low enough Ca^{2+} concentration to rule out the deleterious effects of Ca^{2+} influx.

Regeneration

Return of function after neurite transection in vivo requires both a regrowth of injured neurites and reconnection with former targets. This is a complex process requiring a host of changes both intrinsic and extrinsic to the neuron. After transection in the peripheral nervous system (PNS), the cut axon forms multiple short sprouts and growth cones at the tips by several hours, the majority of the sprouts are later pruned as the axon elongates,

and eventually the axon reaches its target where it must subsequently form a new synapse (Bisby, 1995). In the central nervous system (CNS) of most mammals, spontaneous regeneration has yet to be demonstrated after neurite transection. It is unlikely that CNS neurons do not have the capability to regrow after injury because the experiments of several groups have shown that CNS neurons can regrow if presented with the right environment and trophic factors. Furthermore, these neurons can not only regrow but can also make functional connections (Aguayo et al, 1990, Cheng et al, 1996). These experiments demonstrate that at least some neurons in the CNS do have the capacity to regrow, but the environment may either have factors which inhibit regeneration or may lack factors which enhance regeneration. The reasons for this lack of success in the CNS most likely involves both an inhibitory environment and a weak intrinsic regenerative response to axotomy exhibited by CNS neurons (Caroni, 1997, Bahr and Bonhoeffer, 1994, Chen et al, 1995, Fawcett, 1992, Davies, 1994). After transection in the CNS, reactive astrocytes, oligodendrocytes, and myelin debris form a barrier to regeneration (Bahr and Bonhoeffer, 1994, Schwab and Bartholdi, 1996). Reactive astrocytes lack cell-surface and adhesion molecules that are necessary for axonal growth and transection induces the appearance of proteoglycans which are not permissive for regeneration. Oligodendrocytes and myelin contain growth inhibitory molecules (Schwab and Bartholdi, 1996, McKerracher et al, 1994) the neutralization of which results in limited axonal regeneration. An antibody directed to one such molecule has allowed some regeneration after spinal cord hemisection (Schwab and Bartholdi, 1996). Further evidence of the inhibitory nature of the environment of CNS neurons is demonstrated by the grafting experiments which have shown that allowing CNS axons to regrow through

PNS nerve grafts can improve the regenerative ability of these axons (Aguayo et al, 1990, Cheng et al, 1996). In the PNS, neurite transection causes "activation" of Schwann cells leading to proliferation and reexpression of cell surface adhesion molecules and extracellular matrix components (including collagen, fibronectin, laminin) which are favourable for regeneration (Bahr and Bonhoeffer, 1994, Schwab and Bartholdi, 1996). The myelin debris is also cleared out quickly by blood derived macrophages and monocytes. Therefore, it is clear that the PNS environment is far more conducive to regeneration compared to the CNS, and that successful regeneration must depend on a noninhibitory environment.

However, merely having a noninhibitory environment may be insufficient for regeneration. Equally important for regeneration is an intrinsic change in the neuron after neurite transection (Caroni, 1997, Ambron and Walters, 1996). It is believed that this intrinsic change involves the commencement of a growth program involving a redirection of gene transcription and protein synthesis. Evidence to support this change includes studies which have shown that there is an upregulation of GAP-43 after transection along with an increased synthesis of tubulins and actins and a decreased synthesis of neurofilament. There is also a reinduction of some genes including Tα1 tubulin and type IIβ tubulin (both embryonic forms of tubulin),(Miller et al, 1989, Hoffman and Cleveland, 1988). The transcription factors such as c-jun have also been shown to be increased with nerve transection (Herdegen et al, 1997). Further evidence of this change to a growth program was shown by Smith and Skene (1997). They showed that adult DRG neurons newly isolated for culture extended compact, highly branched neurites ("arborizing" growth) within the first 24 hours of plating, but by 48 hours, the neurites

were long and sparsely branched and showed "elongating" growth. However, if transcription was inhibited within the first 24 hours then this change from arborizing to elongating growth would be delayed. What causes the neuron to undergo such changes are unknown but it is believed that extracellular factors play a role in this change. Growth factors such as neurotrophic factors are thought to play a role in enhancing regeneration of a neuron after neurite transection, and it is possible that a lack of growth factor support in the CNS after injury can account for the weak regeneration response of the neuron (Schwab and Bartholdi, 1996, Kobayashi et al, 1997). In the PNS, Schwann cells increase their production of neurotrophic factors such as NGF and BDNF after nerve injury (Heuman et al, 1987, Meyer et al, 1992, Taniuchi et al, 1988) which is believed to enhance regeneration. However, growth factors are not the sole factors necessary for regeneration. For example, it is believed that Ca^{2+} entry after neurite transection may be necessary for growth cone formation and neuritogenesis (Ziv and Spira, 1997, Rehder et al, 1992, Kocsis et al, 1994).

Calcium entry after transection

After neurite transection, it has been shown that intracellular Ca^{2+} increases (Strautman et al, 1990, Ziv and Spira, 1993, Ziv and Spira, 1995, Sattler et al, 1996). The rise in Ca^{2+} reaches concentrations of up to 1 mM Ca^{2+} at the tip of the cut neurite (Ziv and Spira, 1995). Calcium levels in the soma were also noted to rise, but not to the same magnitude (Ziv and Spira, 1993). Calcium entry can occur via two routes. First, the transection causes depolarization of the membrane which activates voltage sensitive Ca^{2+} channels. Blocking of N-type and L-type channels attenuates the rise in intracellular

Ca^{2+} (Sattler et al, 1996) but the rise is not completely blocked. The second route is through the membrane disruption caused by the transection which results in a propagating wave of Ca^{2+} moving from the cut end towards the soma (Strautman et al, 1990, Ziv and Spira, 1993, Ziv and Spira, 1995). Though the initial intracellular Ca^{2+} rise may be due to Ca^{2+} entry from outside the neuron, this does not preclude contribution to the total Ca^{2+} increase from release from internal Ca^{2+} stores secondary to Ca^{2+} entry through the mechanism of Ca^{2+} induced Ca^{2+} release (CICR). Ziv and Spira (1993) concluded that there was very little contribution from internal stores in their system of injury because injection of Ca^{2+} into uninjured neurites did not trigger a propagating wave of elevated intracellular Ca^{2+} concentration, but they did not specifically block release from internal stores.

Calcium and Regeneration

The role of Ca^{2+} in regeneration is not well defined. Some groups have minimized the role of Ca^{2+} in regeneration (Campenot and Draker, 1989, Tolkovsky et al, 1990). Others however have suggested that Ca^{2+} is important for regeneration (Rehder et al, 1992, Kocsis et al, 1994, Lankford et al, 1995, Ziv and Spira, 1997). As noted above, Ca^{2+} performs a multitude of functions in the normal neuron from induction of gene expression to neurotransmitter release, and because of its ubiquitous action, it has been difficult to show its specific role in signaling regeneration after axonal transection. Furthermore, the exact localization of the Ca^{2+} signal for regeneration within the neuron has also proven difficult. Recently, it has been shown that a localized elevation of $[Ca^{2+}]_i$ at the cut end of the neurite in Helisoma and Aplysia neurons may be important for initiating

regeneration of neurites through growth cone formation (Rehder et al., 1992, Ziv and Spira, 1997). Also, it has been hypothesized that there is an optimal level of $[Ca^{2+}]_i$ in the neurite for growth cone formation and maintenance (Kater and Mills, 1991). At very low Ca^{2+} levels, the neuron dies, at high levels the growth cone collapses, and at very high levels the neuron dies. These studies have associated a Ca^{2+} rise in the neurites with the formation of growth cones and have implied that a localized Ca^{2+} rise at the cut tip of the neurite alone is sufficient for growth cone formation, but the importance of concomitant Ca^{2+} entry at the cell body was not addressed. However, several other studies have shown that decreasing the rise of $[Ca^{2+}]_i$ at the cell body can inhibit neurite initiation in freshly plated rat dorsal root ganglion neurons (Kocsis et al., 1994, Lankford et al., 1995) possibly by inhibiting a CICR phenomenon. Since in vitro transection of neurites may increase the $[Ca^{2+}]_i$ level of both the neurites and soma (Strautman et al., 1990, Ziv and Spira, 1993, Ziv and Spira, 1995, Sattler et al., 1996), it is possible that $[Ca^{2+}]_i$ at the cell body after transection may also be needed for initiation of neurite regeneration.

Objectives and hypotheses of present work

As indicated by the above, knowledge of the mechanisms concerning neuronal death and Ca^{2+} following neurite transection is incomplete. For example, the low extracellular Ca^{2+} concentration (30 μM) used by Lucas et al (1990) was not low enough to prevent Ca^{2+} influx after transection. Although, these investigators added ethylene glycol-bis(β-aminoethyl ether)-N,N, N′,N′-tetraacetic acid (EGTA) to lower the extracellular Ca^{2+} concentration, the study was unsuccessful because of a loss of cellular

adhesion. Therefore, they could not determine whether Ca^{2+} was involved in neuronal death after neurite transection.

Also, there is still uncertainty about the mechanism of involvement of Ca^{2+} in regeneration of neurites after transection. The studies of Redher et al (1992) and Ziv and Spira (1997) described above showed that increased Ca^{2+} at the tip of the transected neurite is needed for growth cone formation. However, they used Helisoma and Aplysia neurons for their experiments both of which are non-mammalian. It is possible that mammalian and nonmammalian neurons may regulate Ca^{2+} quite differently, and therefore, mammalian neurons may not require Ca^{2+} for regeneration. Furthermore, neither study examined the role of Ca^{2+} entry in the cell body and regeneration. Two studies have examined Ca^{2+} entry at the cell body and neurite initiation in mammalian neurons (Kocsis et al., 1994, Lankford et al., 1995) but these involved neurite initiation from the cell body rather than from a transected neurite. Lucas et al (1985) could not assess regeneration because it was rarely seen after neurite transection with the laser.

Therefore, the objectives of the present work are: 1) to assess the effect of Ca^{2+} entry after neurite transection on neuronal death; 2) to assess the effect of Ca^{2+} entry into mammalian neurons on regeneration after neurite transection; and 3) to assess the effect of Ca^{2+} entry at the cell body on regeneration after neurite transection.

The hypotheses formulated were: 1) Ca^{2+} entry after neurite transection is necessary for neuronal death; 2) Ca^{2+} entry after neurite transection is necessary for neurite regeneration in mammalian neurons; and 3) Ca^{2+} entry at the cell body after neurite transection is necessary for regeneration.

Materials and Methods

Calcium and Cell Death Experiments

Cell Culture

Superior cervical ganglia were isolated from newborn Sprague-Dawley rats (Charles River, Quebec) within 24 to 48 hours following birth and were dissociated both enzymatically with trypsin and mechanically with fire polished pipettes in a manner similar to that described by Campenot and others (Campenot, 1977). The cells were then cultured in collagen coated 35mm plastic tissue culture dishes in Leibovitz-15 (L-15) feeding medium (Gibco Laboratories, Grand Island, NY) supplemented with 2.5% rat serum, 200ng/ml of 2.5S nerve growth factor (Harlan Bioproducts, Madison, Wisconsin), sodium bicarbonate, a stable vitamin mix (containing L-aspartic acid, L-glutamic acid, L-proline, L-cystine, p-aminobenzoic acid, B-alanine, vitamin B12, myo-inositol, choline chloride, fumaric acid, coenzyme A, d-biotin, DL 6,8 thioctic acid), 2% glucose, 1% glutamine, 1% penicillin/streptomycin, 1% vitamin C, and 1% fresh vitamin mix (containing 6,7-dimethyl-5,6,7,8-tetra-hydropterine and glutathione). Cytosine arabinoside at a 1% concentration was also added to eliminate any dividing cells. These chemicals, unless otherwise specified, were all from Sigma Chemicals, Oakville, Ontario. Prior to plating the cells, the bottom of each culture dish was "scratched" using a pin-rake yielding 20 parallel tracks. The cells were plated in the center of the dish which allowed the neurites to extend laterally along the tracks during incubation under fully humidified conditions and 5% CO_2 for 14 days. At this stage, neurons had neuritic extensions measuring up to 10 mm in length.

Protocol and Injury Procedure

Each dish was subjected to a temporary change of medium from the L-15 feeding medium containing 1.8mM Ca^{2+} to one of the following $[Ca^{2+}]_e$ in a salt solution for a duration of 30min: 50 nM, 1.8 mM, or 5 mM. The salt solution consisted of double distilled water with 135 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 5.6 mM D-Glucose, 2 mM $NaHCO_3$, 10 mM Hepes (all materials from Sigma Chemicals, Oakville, Ontario). A duration of 30min was chosen because prior experiments in our laboratory have shown that 30min is sufficient for $[Ca^{2+}]_i$ levels to return to preinjury levels after neurite transection in SCG neurons (Chu et al, 1997). Furthermore, other studies have suggested that 30min is sufficient to allow post-transection membrane sealing (Xie and Barrett, 1991, Yawo and Kuno, 1985). In addition, in other forms of neurotoxic injury secondary to Ca^{2+} influx, it is the initial Ca^{2+} entry at the time of the injury, rather than any secondary Ca^{2+} rises afterwards, which contributes to neuronal death (Tymianski, 1996). For the 50nM Ca^{2+} medium, EGTA and a Ca^{2+} buffer software (CABUFFER, 1989, written by Jochen Kleinschmidt, NYU Medical Center) was used to calculate the exact amount of chelator required to achieve the desired free Ca^{2+} concentration. The pH of the solutions were maintained at 7.4.

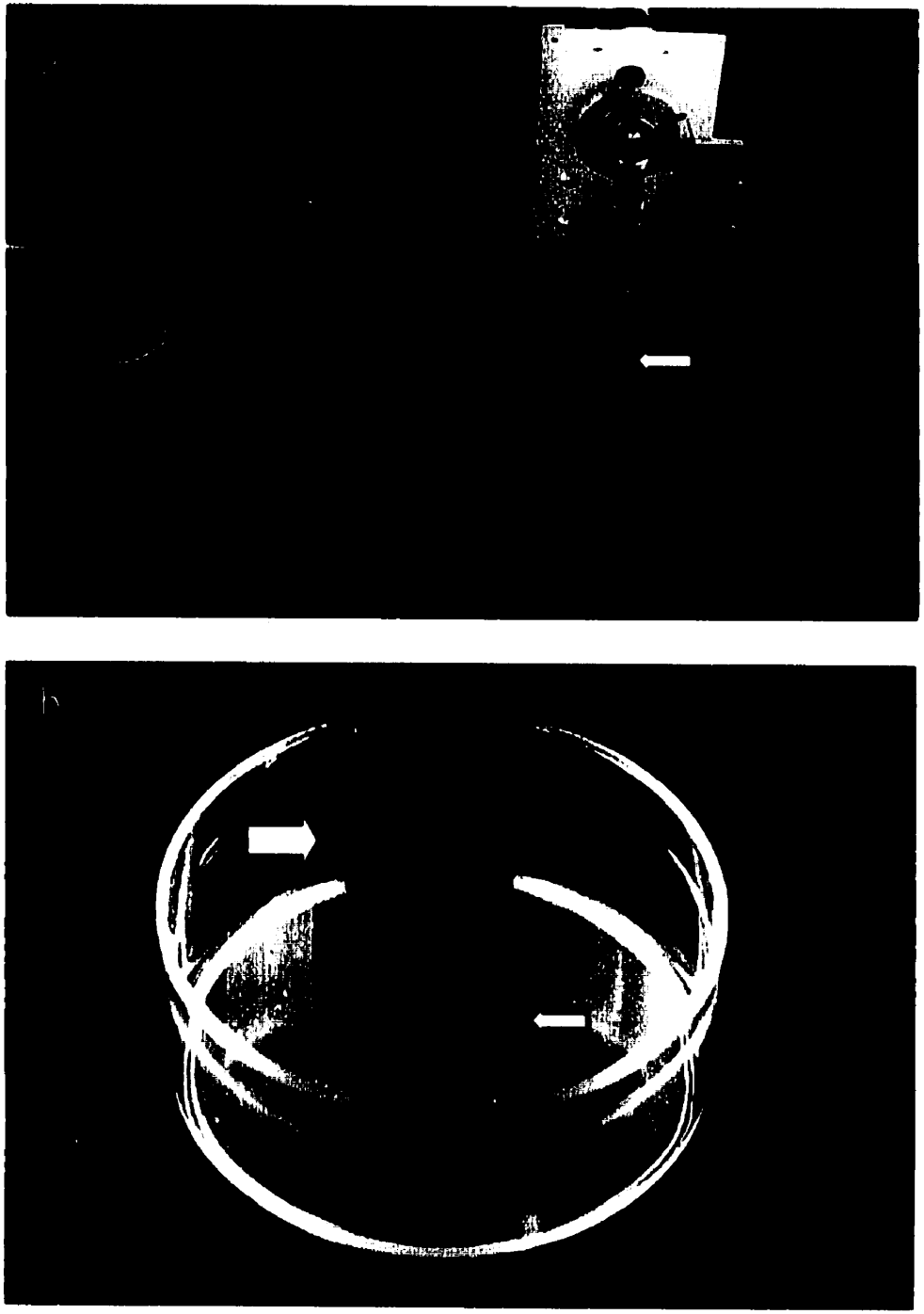
One minute after the change in solution, the cells were subjected to neurite transection using a custom made rubber-impactor transection device built by the University of Toronto Biomedical Engineering Department which produces a reliable and reproducible transection. The device consisted of an electrically driven vertical shaft to which was affixed a sharp edged (approximately 500 μm) rubber impactor (Fig. 1). The

Figure 1

a) The transection device with rubber impactor (arrow) attached to electric motor.

b) Culture dish with tracks (small arrow) and rubber impactor (large arrow) used to transect neurites.

Fig. 1



width of the rubber-impactor at its tip was 6.25 mm. The starting height of the rubber-impactor, as well as its velocity, could be varied independently of one another. However, for all experiments both the starting height and the velocity of the rubber impactor were held constant. During and after transection, the cells were exposed to one of the above-mentioned Ca^{2+} media for a total of 30 min. At the end of 30 min, the media was changed to the normal feeding medium containing 1.8 mM Ca^{2+}. The injury site was located by observing the cells at 20x under light microscopy.

The relationship between cell death and distance from the cell soma to the transection site was assessed by separating each track into 4 consecutive and adjacent "fields". Each field corresponded to one microscopic field of view at 20x such that field 1 extended from the injury site to 0.66mm away from the injury site, field 2 from 0.66mm to 1.32mm, field 3 from 1.32mm to 1.98mm, and field 4 from 1.98mm to 2.64mm (Fig. 2). These values were determined by measuring the length of one microscopic field of view at 20x with a stage micrometer. Thus, a cell body located in field 3, for example, would be 1.32mm to 1.98mm away from the transection site. This method is based on the assumption that all cells present in any of the fields extended neurites across the injury site. However, to avoid any uncertainty regarding whether the neurites of far cell bodies (those located in field 4 for example) actually crossed the injury site and were therefore transected by the injury device, only neurons in field 1 were examined to demonstrate the cell death versus $[Ca^{2+}]_e$ and the cell death versus post-transection time relationships.

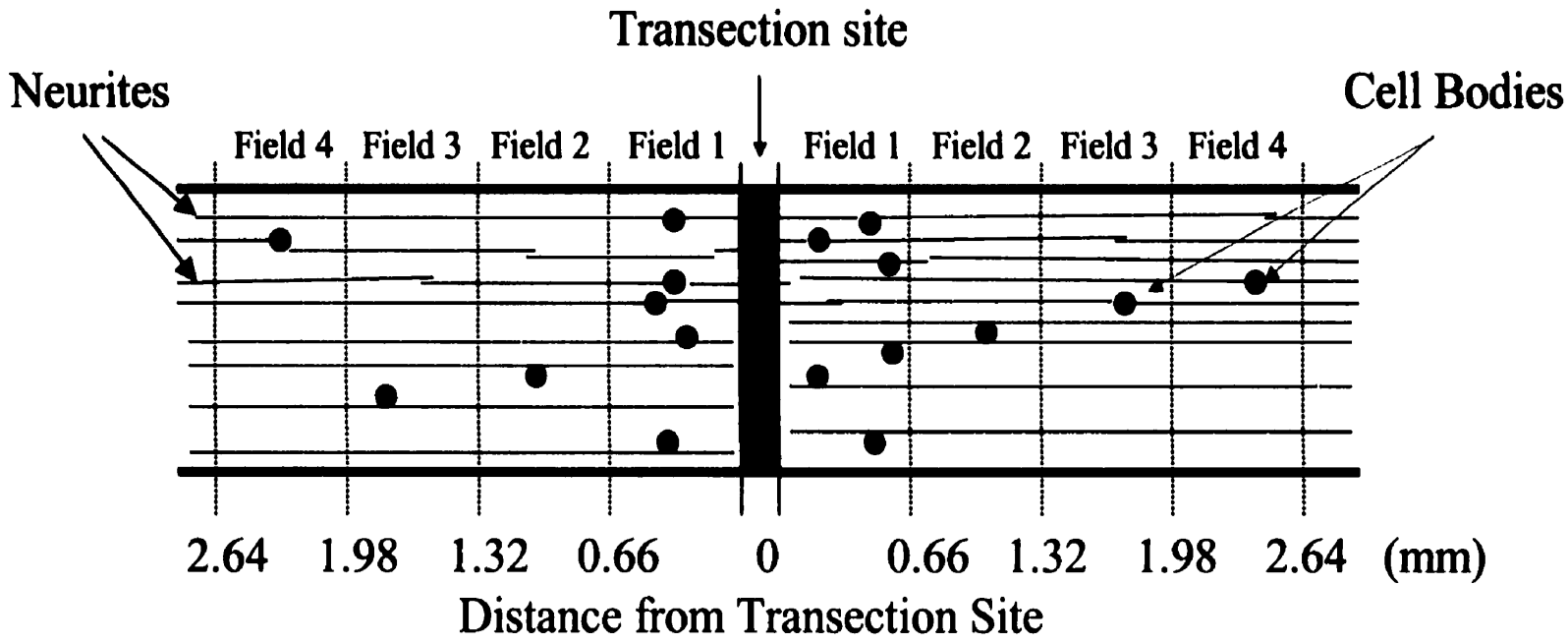
Figure 2

Schematic Representation of One Track

The thick horizontal lines were scratched onto the 35mm collagen coated dish prior to plating and served as tracks to direct neurite growth. The vertical hatched lines mark the microscopic fields at 20x and provide a scale for distance from the injury site. The neuronal cell bodies shown as circles have neurites (thin horizontal lines), which extend across the injury site. The cell bodies located at the injury site were not counted.

Fig. 2

One Track with Neuronal Cell Bodies and Neurites



20

Cell Death Analysis

Cell death was assessed by counting fluorescent cells at 20x under fluorescence microscopy using a mercury lamp and a rhodamine filter, after having added 5uL of propidium iodide (Molecular Probes) in 2 ml of media (0.25% concentration). Propidium iodide was added immediately after transection and at 30 minutes when the solutions were changed. Propidium iodide fluoresces when bound to the nucleic acids of dead cells (Ankarcrona et al, 1995). The percentage of dead cells was calculated at 30min, 2hrs, 24hrs, 48hrs and 72hrs post-injury by dividing the number of fluorescent nuclei by the total number of cells which were counted under phase contrast. Five tissue culture dishes were used for each of the three $[Ca^{2+}]_e$ groups and examined 5 tracks per dish totaling 25 tracks per group (n=25). For the uninjured controls, which were subjected to the same procedure except for the neurite transection, 2 dishes were examined and 9 tracks per group (n=9) were included. Cell death was expressed as a percentage (i.e. dead cells / total cells x 100) and statistical analysis was achieved by one way analysis of variance (ANOVA) with the Student-Newman-Keuls method for multiple pair-wise comparisons using statistical analysis software (SigmaStat, 1994, Jandel Scientific).

Calcium and Regeneration Experiments

Cell culture protocol

SCG neurons were cultured as previously described. Cultures were also grown in compartmented chambers as described by Campenot (1987) with the cell bodies plated in the middle compartment and the subsequent neurite growth extending into the side compartments. Briefly, these teflon chambers are divided into three compartments with

the side compartments larger in area than the middle compartment. Silicone grease (Dow Corning, Mississauga, Canada) is applied to the bottom of the chamber which allows adherence of the teflon chamber to the petri dish. The grease isolates the medium of each side compartment from the rest of the chamber and the petri dish. The medium of the middle compartment is open to the medium from the rest of the petri dish, but is isolated from the medium of the side compartments.

Loading protocol of SCG neurons

The SCG neurons were loaded with Fluo-3 AM (Molecular Probes, Eugene, Oregon) at 37°C and 5 % CO_2 for 45 minutes. The Fluo-3 AM was dissolved in dimethyl sulfoxide and then added to a loading solution to a final concentration of 5 μM. The loading solution consisted of the feeding medium as described above except that the rat serum was omitted and pluronic acid (0.01 %) was added. The rat serum was omitted because it decreased the loading of the Fluo-3 AM into the cells. After the loading time, the cells were washed in an indicator free solution to remove any extracellular indicator. To assess the ability of 1,2 bis-(2-aminophenoxy)ethane-N,N,N′, N′ - tetraacetic acid (BAPTA-AM) to chelate Ca^{2+} ions after neurite transection, SCG neurons were loaded with 5 μM of the Ca^{2+} indicator dye Fluo-3 AM (Molecular Probes, Eugene, Oregon) and BAPTA-AM (Molecular Probes, Eugene, Oregon). The concentrations of BAPTA-AM that were tested were 1 μM, 10 μM, or 100 μM. For the regeneration experiments, described below, only the chelator BAPTA-AM was loaded using the same procedure and at the same concentrations. A control group of SCG neurons were treated in the same manner except that BAPTA-AM was omitted from the loading solution.

Neurite transection

Neurons were allowed to grow for 10 to 14 days at which time their neurites extended more than 10 mm from the cell bodies (Fig. 3). The neurites were injured with a motor driven rubber impactor which descended vertically downwards perpendicular to the surface of the culture dish (Fig. 1). This device was designed and constructed by the Biomedical Engineering department at the University of Toronto and consists of an electrically driven vertical shaft to which was affixed a sharp edged rubber impactor. Both the starting height and the rate at which the impactor descended could be adjusted independently, but for these experiments they were held constant. This method of injury produced complete simultaneous transection of all the neurites in the path of the impactor with minimal stretching of the cell bodies or neurites. The neurites were transected at least 2 mm from the cell bodies. The experimental conditions under which the neurites were transected included: 1) entire neurons (cell bodies and neurites) preloaded with BAPTA-AM; 2) either the cell bodies alone or the entire cell were immersed in zero Ca^{2+} medium (growth medium supplemented with 5 mM EGTA and 20 mM Hepes); 3) entire neurons immersed in normal Ca^{2+} growth medium (1.8 mM Ca^{2+}) and then changed to zero Ca^{2+} medium 1 minute after transection; 4) entire neurons immersed in normal Ca^{2+} medium.

Analysis of $[Ca^{2+}]_i$ in neuronal somata

The cultures were viewed with a 20 X water immersion lens (Nikon, NA 0.4) and the Ca^{2+} levels in the soma of the same neurons prior to and after neurite transection were imaged with an upright Bio-Rad MRC 600 laser scanning confocal microscope (Bio-Rad,

Figure 3

Uninjured neurites from 10 day old SCG neurons in culture growing along a single track between two scratched lines in the collagen substrate.

Fig. 3

Hertfordshire, England). The images were acquired at a resolution of 384 X 256 pixels at 8 bits/pixel, with 488 nm excitation, 515 nm emission, 90 % neutral density filter to decrease the intensity of the laser, and confocal pinhole at 100 %. Pre and post transection Ca^{2+} images of the cell somata were acquired for both cells loaded with BAPTA-AM at the various concentrations or nonloaded cells. Due to the nature of the injury device the injuries could not be made directly on the microscope stage, and therefore, it was not possible to begin to record the Ca^{2+} images until 3 minutes after injury. However, previous experiments in our laboratory (Sattler et al., 1996, Chu et al., 1997) demonstrated that the Ca^{2+} rise in the somata of SCG neurons was maximal by 3 minutes and that the rise was maintained up to 30 minutes. The fractional change ($\Delta f/f_0$) in Fluo-3 fluorescence in the cell bodies between pre and post injury states was calculated. Similarly, the fractional change in fluorescence was also obtained for cells injured in zero Ca^{2+}.

Analysis of regeneration behavior

The cultures were viewed with a 20 X phase contrast lens (Olympus). Pre and post injury images of the injury site were recorded with a SIT video camera (C2400 model 8, Hamamatsu Photonics, Japan) or with a 35 mm camera (Nikon) attached to an inverted microscope (Nikon Diaphot-TMD). The video camera was controlled by software (Image-1, Universal Imaging, West Chester, PA) running on an 80386 microprocessor-based personal computer. Images were recorded immediately after injury, and then at 2, 6, 24, and 48 hours after injury. Regeneration was assessed by three methods: 1) initiation of regeneration defined as the percentage of tracks in each culture dish which had either growth cone formation or short sprouts emerging from the cut end

of the neurites at 2 or 6 hours after transection; 2) the number of branches per regrowing neurite at 2 and 6 hours after transection; and, 3) the average hourly rate of elongation in μm/hr from 6-48 hours after transection. Measurements were made using image analysis software (Sigmascan, Jandel) on a pentium based personal computer. The average hourly rate of elongation was based on the total distance regenerated and the time to regrow that distance. The total distance regenerated was determined by measuring the distance with the aid of fiduciary markers, between the lead neurite and the site of transection at 2, 6, 24, and 48 hours. In order to compare the rate of elongation between different groups, it was necessary to begin calculating the rate at 6 hours because at earlier times the 100 μM BAPTA-AM group had no initiation of regeneration. An earlier calculation would not differentiate the delay in initiating regeneration from the actual rate of elongation after regeneration had been initiated.

Statistical analysis of data

The percentage of tracks with regeneration were calculated by dividing the number of tracks with regeneration by the total number of tracks counted per dish, and then calculating the average of the averages of all the dishes. The values for $\Delta f/f_0$, branches per regrowing neurite, and the average hourly rate of elongation were analyzed with a one-way ANOVA followed by post-hoc analysis between groups by the Sudent-Neuman-Keuls Method on an IBM compatible computer (Pentium class) with statistical software (Sigmastat, Jandel Scientific).

Results

Calcium and Cell Death Experiments

Cell death versus $[Ca^{2+}]_e$

Figure 4 illustrates that at 50nM, cell death increased from 2.3% in the uninjured controls to 30.5% in the injured group at 72 hrs; at 1.8mM, cell death increased from 0.9% to 25.2% at 72 hrs; and at 5mM, it increased from 10.4% to 35.2% at 72 hrs. Thus, cell death due to neurite transection at 50nM was 13.3x greater than in the corresponding uninjured controls, at 1.8mM it was 28x greater and at 5mM it was 3.4x greater at 72 hrs. A comparison of the uninjured controls to the injured cells from field 1 at 72hrs post-injury in the same $[Ca^{2+}]_e$ group (i.e. a comparison of adjacent pair-wise columns in Fig. 4) revealed a statistically significant increase in cell death due to neurite transection in each case ($p<0.05$). Furthermore, when comparing the uninjured control groups, a statistically significant increase in cell death was found in the 5mM $[Ca^{2+}]_e$ group ($p<0.05$) compared with normal $[Ca^{2+}]_e$ (1.8mM). However, there was no significant difference in cell death between the injured groups at any of the three $[Ca^{2+}]_e$ studied ($p=0.278$).

Cell death versus post-transection time

The only statistically significant increase in cell death over time in field 1 (the first 0.66mm from the injury site) occurred between 2hrs and 24hrs post-injury in all three $[Ca^{2+}]_e$ groups (Fig. 5). Thus, even though the percentage cell death calculations consistently increased with time in all three $[Ca^{2+}]_e$ groups studied, the increments from 30min to 2hrs, 24 to 48hrs, and 48 to 72hrs were not statistically significant. However,

Figure 4

% Death in Field 1 versus $[Ca^{2+}]_e$ in Injured Cells and Uninjured Controls at 72hrs Post-Injury

The % death values from left to right are respectively: 2.3%, 30.5%, 0.9%, 25.2%, 10.4% and 35.2%. * indicates a statistically significant difference ($p<0.05$) in the 5mM uninjured control % death compared to the two other uninjured control groups. ** indicates a statistically significant difference ($p<0.05$) between % death in the injured groups and their respective uninjured control groups. There was no statistically significant difference ($p>0.05$) between % death in the three injured groups. (n=25 tracks per injured group and n=9 tracks per uninjured control group.)

Fig. 4

% Death in Field 1 vs. $[Ca^{2+}]_e$ at 72 hrs Post Transection

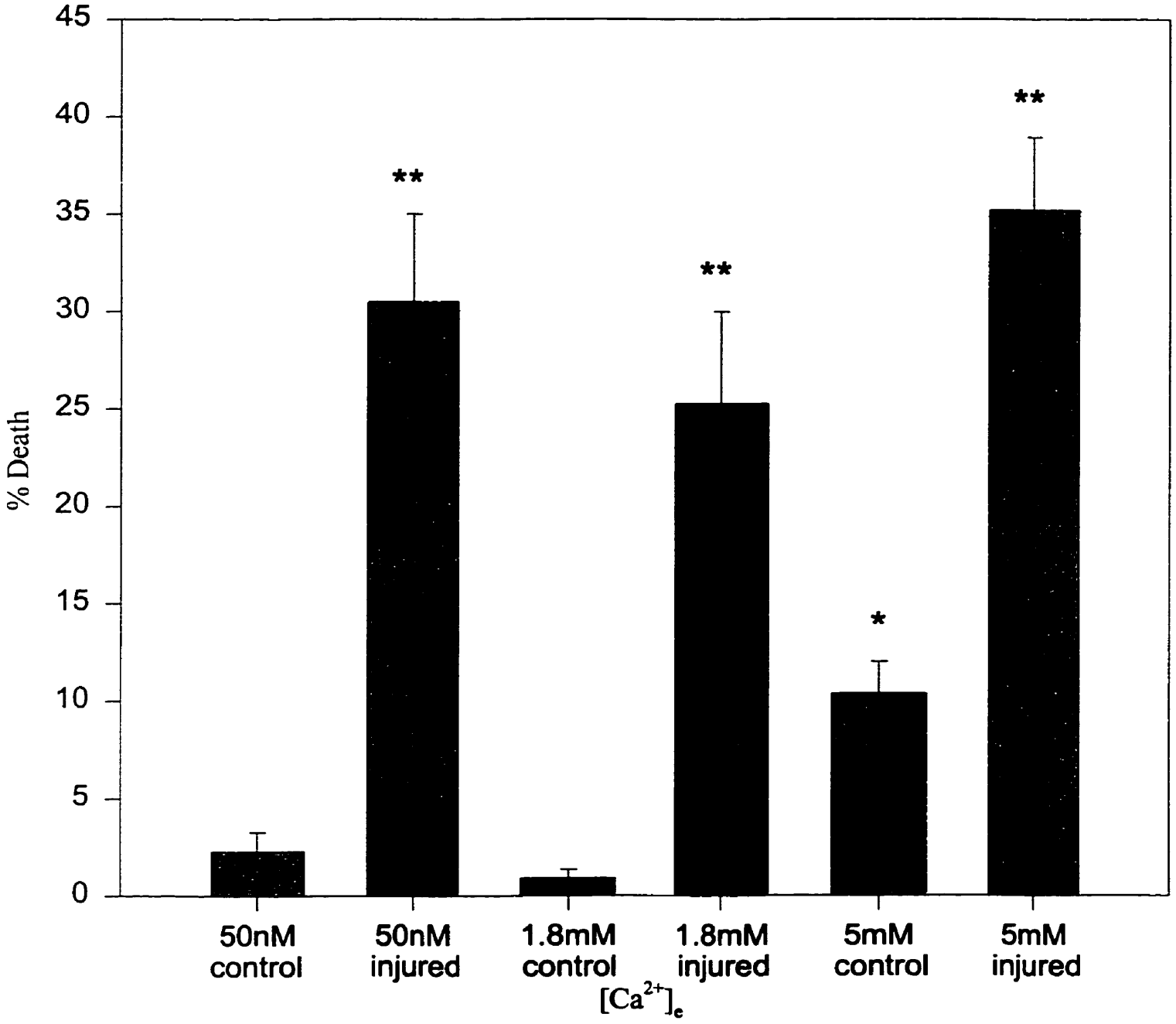


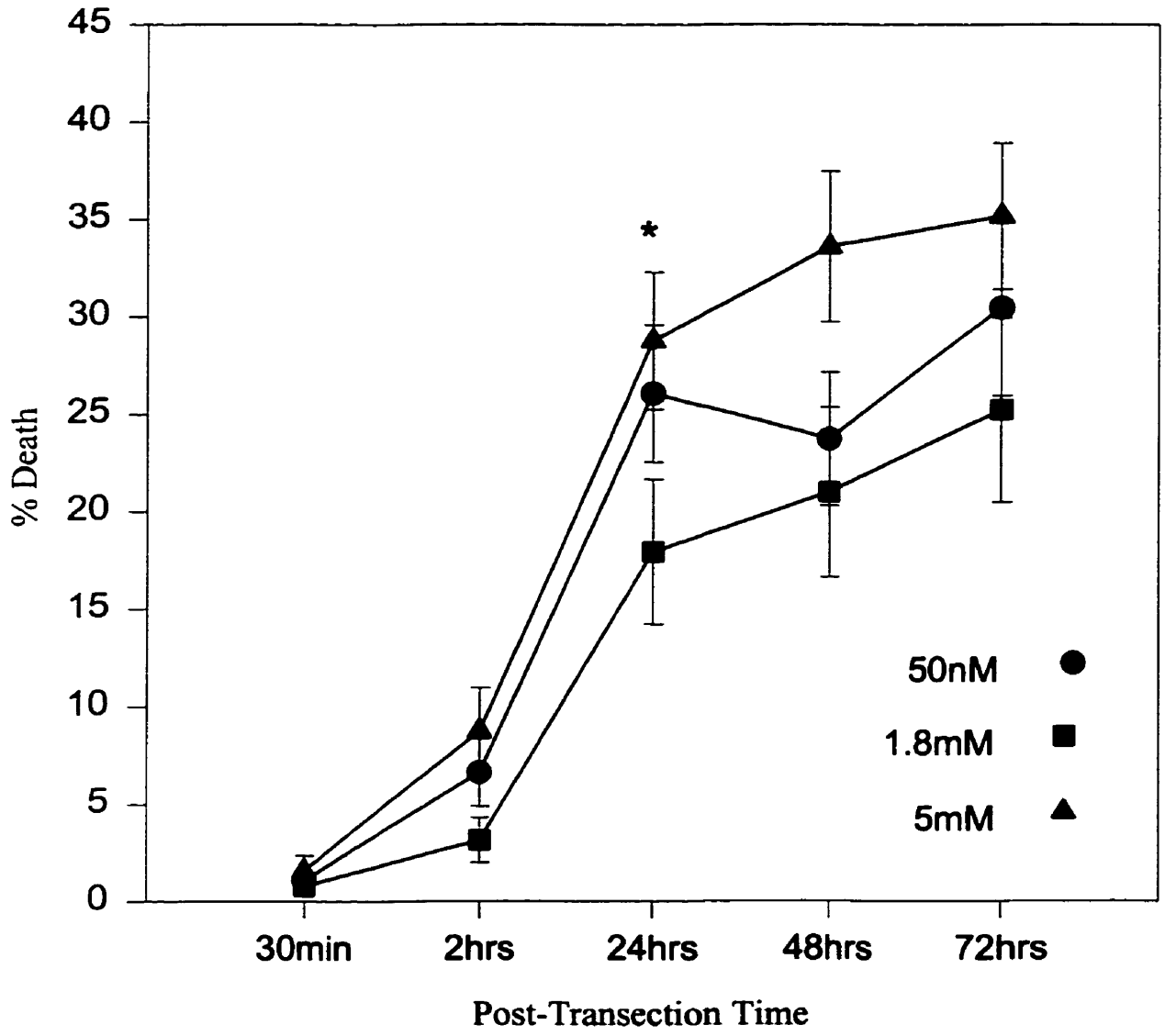
Figure 5

% Death in Field 1 versus Post-Transection Time in Injured Cells at 50nM, 1.8mM and 5mM $[Ca^{2+}]_e$, respectively.

* indicates a statistically significant increase ($p<0.05$) in cell death from 2hrs to 24hrs post- in all three $[Ca^{2+}]_e$.

Fig. 5

% Death in Field 1 vs. Post Transection Time of Transected Cells at 50 nM, 1.8 mM, and 5 mM $[Ca^{2+}]_e$



the 5mM uninjured control neurons showed a statistically significant increase in cell death ($p<0.05$) between 30min (where the death rate was at 0.55%) and 2hrs (where the death rate reached 7.23%) following the initial medium change (Fig. 4).

Cell death versus distance between the cell body and the transection site

At 72hrs post-injury, the percentage death increased as the distance between the cell body and the injury site declined (Fig. 6). Note, however, that the only statistically significant increase in cell death occurred between field 1 and field 2. Cell death beyond the second field were not significantly different from each other for each $[Ca^{2+}]_e$. Hence, cell death was significantly greater when the injury was within 0.66mm from the cell body than when it was more than 0.66mm away from the cell body.

Calcium and Regeneration Experiments

Initiation of regeneration is delayed by BAPTA-AM

By attenuating the rise of $[Ca^{2+}]_i$ after neurite transection with BAPTA-AM, it was established that a rise in $[Ca^{2+}]_i$ was needed to initiate regeneration. Neurons were loaded with BAPTA-AM at the concentrations described in the methods, and then their neurites were transected. Two hours after transection, each track was examined for initiation of regeneration from the site of transection (Fig. 7). For nonloaded neurons at 2 hours, 68.1% of the tracks had initiation of regeneration, whereas cells loaded with 1, 10, or 100 μM of BAPTA-AM had regeneration at 2 hours in 75 %, 41.7 % of tracks, and 0 %, respectively. However, by 6 hours (Fig. 8), 100 % of the tracks of nonloaded cells and 100 % the cells loaded with 1μM BAPTA-AM or 10 μM BAPTA-AM had regeneration, compared to 81.9 % of the tracks of cells loaded with 100 μM BAPTA-AM. At 24

Figure 6

% Death versus Distance from the Injury Site at 72hrs Post-Injury at 50nM, 1.8mM and 5mM $[Ca^{2+}]_e$, respectively.

Cell bodies in field 1 were within 0.66mm from the injury site, those in field 2 were 0.66mm to 1.32mm away from the injury site, those in field 3 were 1.32mm to 1.98mm away and those in field 4 were 1.98mm to 2.64mm away. * indicates a statistically significant difference ($p<0.05$) in % death between the cells in field 1 and those in field 2 at all three $[Ca^{2+}]_e$.

Fig. 6

% Death vs. Distance from the Transection Site at 72 hrs Post Transection at 50 nM, 1.8 mM, 5 mM $[Ca^{2+}]_e$

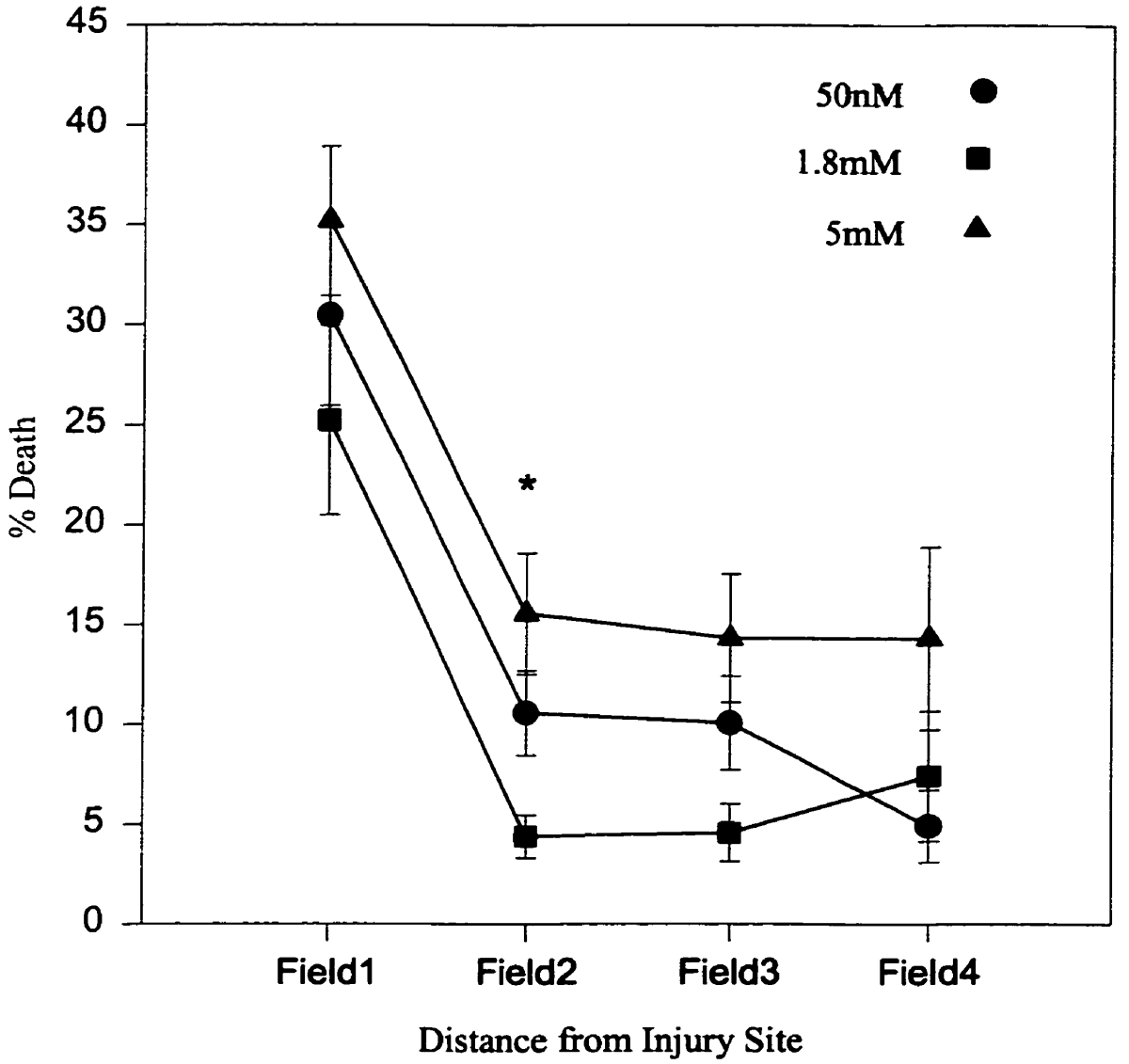


Figure 7

Transected neurites after 10 days in culture. The large arrow demarcates the transection site. Images a and c obtained at 0 and 2 hrs, respectively, after neurite transection of neurons not loaded with BAPTA-AM. Note appearance of growth cones and short sprouts emanating from the edge of the transection site (small arrows) 2 hrs after transection in c. Images b and d are at 0 and 2 hrs, respectively, after neurite transection of neurons loaded with 100 μM BAPTA-AM. At 2 hrs, there are no growth cones or short sprouts (d). Scale bar is 50 μm.

Fig. 7

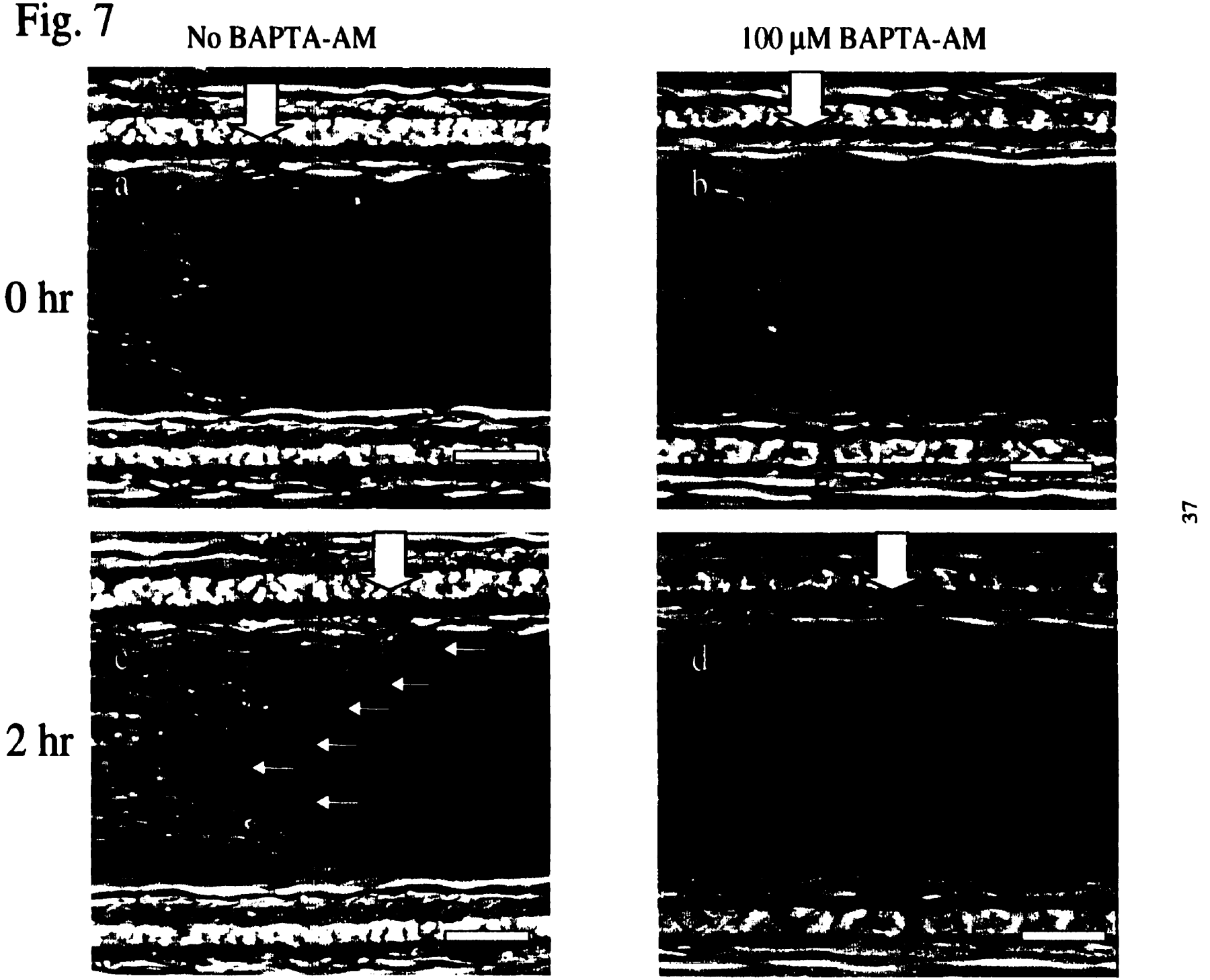
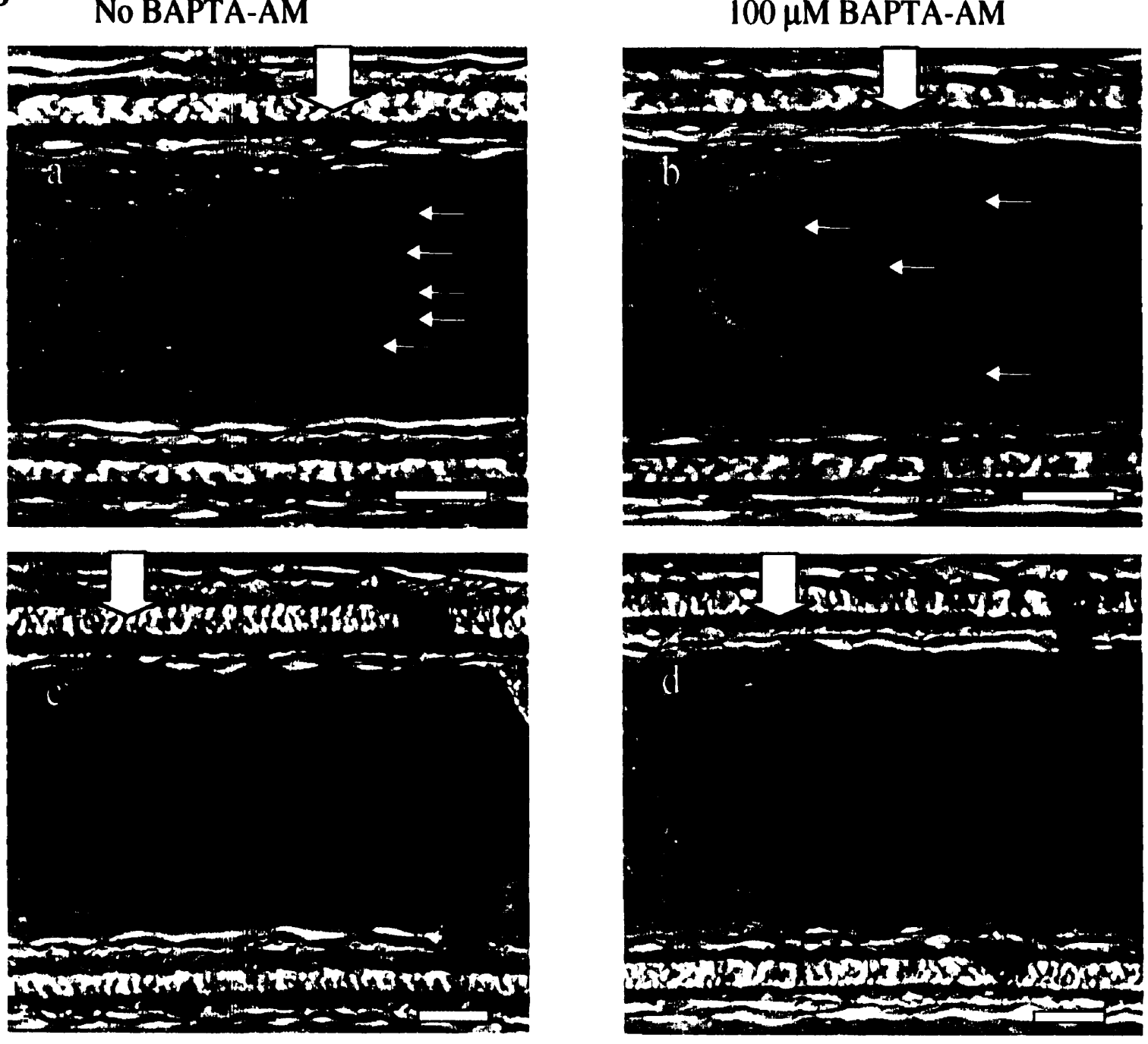


Figure 8

The same neurites as in Fig. 7 at 6 and 24 hours after transection. The large arrow demarcates the transection site. In the nonloaded neurons, note the addition of more growth cones and sprouts, and the elongation of the preexisting sprouts and branching (small arrows) at 6 hrs in a, and the entire transection area covered with regrowing neurites at 24 hrs in c. In the BAPTA-AM loaded neurons note that at 6 hrs there is some regrowth with minimal branching (small red arrows) in b, although at 24 hrs in d, the transection area is covered with regrowing neurites similar to c. Scale bar is 50 μm.

Fig. 8



hours, all tracks containing nonloaded or loaded cells at all 3 BAPTA-AM concentrations had regeneration. These results are summarized in Table 1.

BAPTA-AM prevents the intracellular Ca^{2+} rise at the soma after transection

After neurite transection, the fractional change in fluorescence of SCG neurons not loaded with BAPTA-AM was $\Delta f/f_0 = 0.06$ (n = 482 cells). For cells loaded with 1, 10, and 100 μM BAPTA-AM, the $\Delta f/f_0$ was 0.04 (n = 295 cells), -0.02 (n = 673 cells), and -0.04 (n = 170 cells), respectively. One way ANOVA revealed a statistically significant difference between these groups ($p < 0.0001$, Fig. 9). Post hoc analysis demonstrated a significant difference in $\Delta f/f_0$ between the nonloaded neurons and neurons loaded with either 10 or 100 μM BAPTA-AM, but not with 1 μM BAPTA-AM.

Branching after injury is reduced by BAPTA-AM

As noted above, two hours after transection, cells loaded with 1 or 10 μM BAPTA-AM and nonloaded cells had regeneration of neurites (Fig. 10). When the number of branches from these regrowing neurites were counted, it was found that the nonloaded cells had an average of 1.1 branches per new neurite (n = 180 neurites), whereas cells loaded with 1 or 10 μM of the chelator had 0.97 (n = 114 neurites), and 0.93 (n = 27 neurites) branches per regrowing neurite, respectively. These groups were not significantly different from each other ($p = 0.692$). However, at this time there were no new neurites in the cells loaded with 100 μM BAPTA-AM, and therefore, it was not possible to count the number of branches per new neurite. Six hours after injury (Fig. 10), the nonloaded cells had an average of 1.7 branches per neurite (n = 769 neurites). Cells loaded with 1 μM BAPTA-AM had an average of 1.8 branches per neurite (n = 259 neurites), whereas cells loaded

Table 1 **Percentage of Tracks with Regeneration of Neurons Preloaded with BAPTA-AM**

Group	2 hours %	6 hours %	24 hours %
1) 100 μM BAPTA-AM	0 *	81.9 ± 7 **	100
2) 10 μM BAPTA-AM	42	100	100
3) 1 μM BAPTA-AM	75	100	100
4) Nonloaded cells	68.1 ± 14	100	100

Table showing the percentage of tracks with regrowth at 2, 6, and 24 hours after neurite transection. Mean percentage ± SEM. 3 sets of cultures were analyzed at 2 hours for the nonloaded and 100 μM group, 4 sets of cultures were analyzed at 6 hours for the nonloaded and 100 μM group, and 1 set of cultures were analyzed for the 1 and 10 μM group. * and ** indicates $p < 0.05$ when compared to Group 4 at 2 or 6 hours post transection, respectively.

Figure 9

Histogram showing the effect of calcium chelation by BAPTA-AM on the rise of $[Ca^{2+}]_i$ in the soma after neurite transection. One way ANOVA comparison of $\Delta f/f_0$ between nonloaded, 1, 10, and 100 μM BAPTA-AM loaded neurons. The error bars are SEM. The 10 μM group is significantly different from all other groups (**, $p < 0.0001$) and the 100 μM group is significantly different from all other groups (*, $p < 0.0001$). n = number of cell bodies analyzed.

Fig. 9

The Effect of BAPTA-AM on Somal Calcium Rise after Neurite Transection

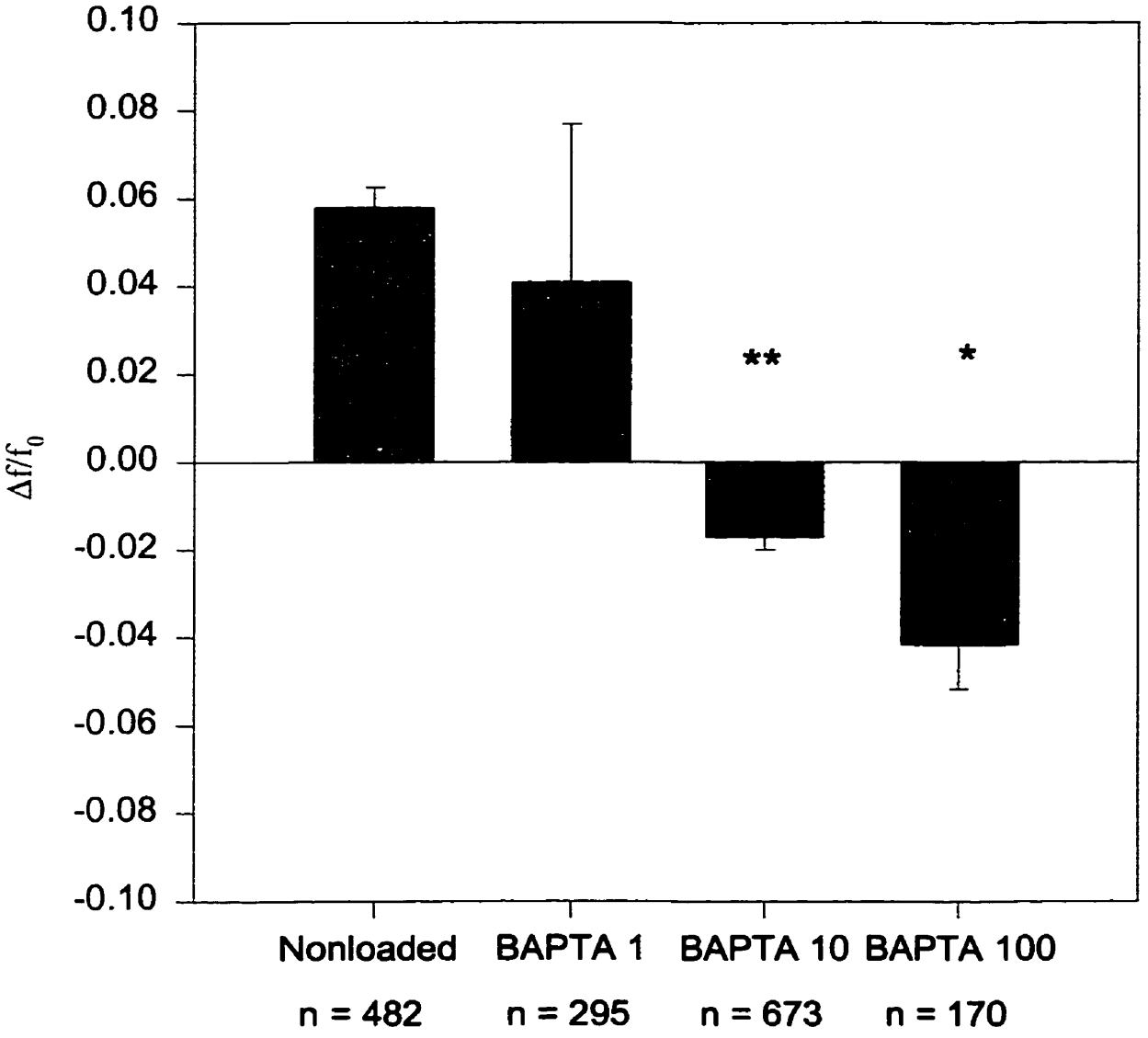
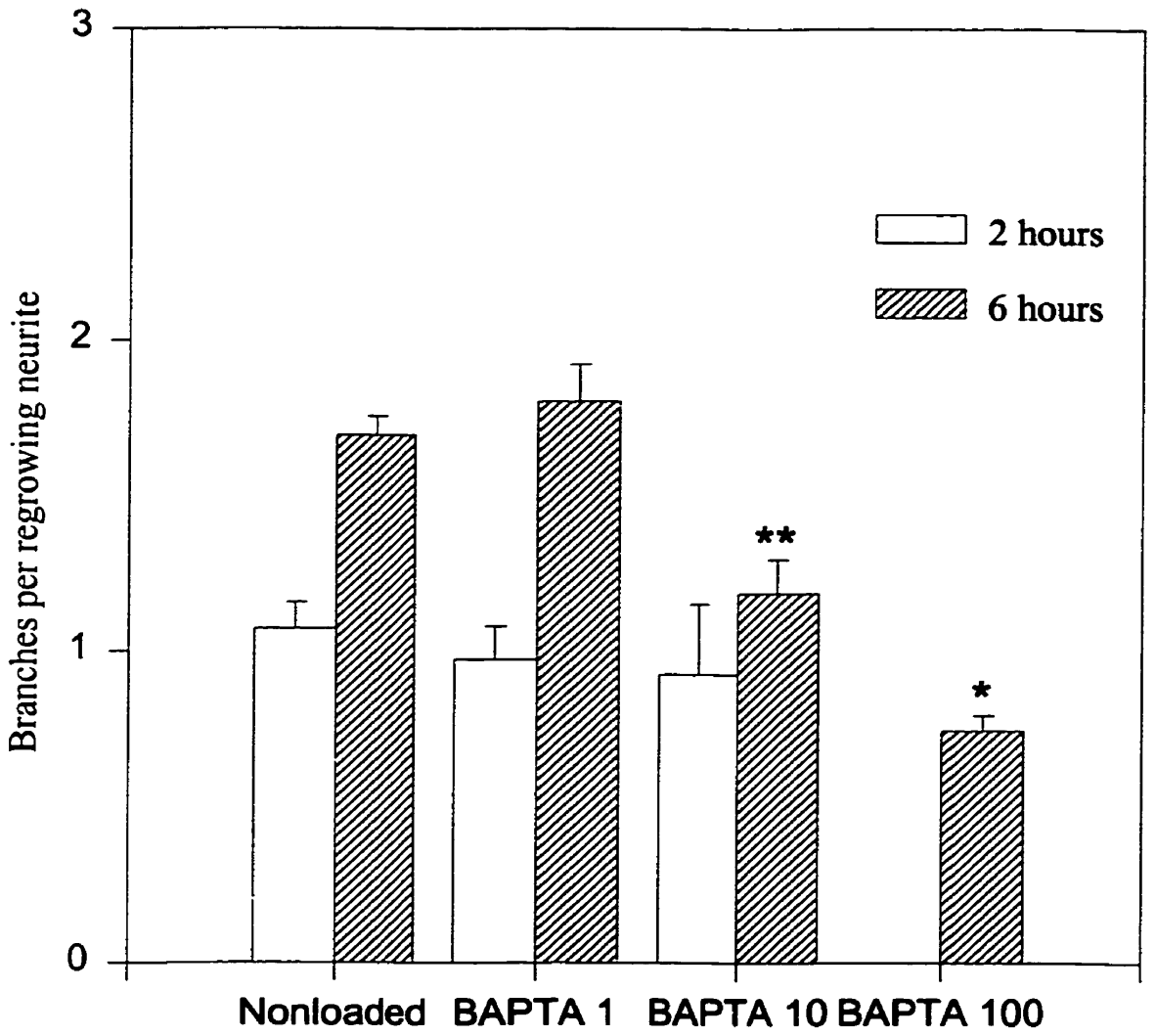


Figure 10

Histogram showing the number of branches per regrowing neurite 2 and 6 hours after neurite transection for cells loaded with 1, 10, or 100 μM BAPTA-AM loaded, and nonloaded neurons. Since there were no regrowing neurites in the 100 μM BAPTA-AM group, that group was not included in the calculations. The error bars are SEM. n = number of neurites analyzed. The groups were not significantly different ($p=0.692$) at 2 hours. The 10 μM group and the 100 μM group are significantly different from each other and all other groups at 6 hours (**,*, $p<0.0001$). The number of neurites analyzed at 2 hours were 180, 114, and 27 for nonloaded, 1, and 10 μM BAPTA-AM groups, respectively. The number of neurites analyzed at 6 hours were 769, 259, 155, 417 for nonloaded, 1, 10, and 100 μM BAPTA-AM groups, respectively.

Fig. 10

Branching at 2 and 6 hours after Neurite Transection



with 10 or 100 μM had 1.2 (n = 155 neurites) and 0.7 branches per new neurite (n = 417 neurites), respectively (Fig. 10). One way ANOVA of the four groups showed a significant difference ($p < 0.0001$), with the average number of branches per new neurite for the nonloaded group significantly greater than the 10 and 100 μM BAPTA-AM group, but not the 1 μM group. Both the 10 and 100 μM groups had significantly lower values than the 1 μM group, and the 100 μM group also had a significantly lower value than the 10 μM group. At 24 hours, branching in both loaded and nonloaded cultures was too numerous to count accurately (Fig. 8).

Average rate of regeneration is minimally affected by BAPTA-AM from 6-24 hours after injury, and from 24-48 hours

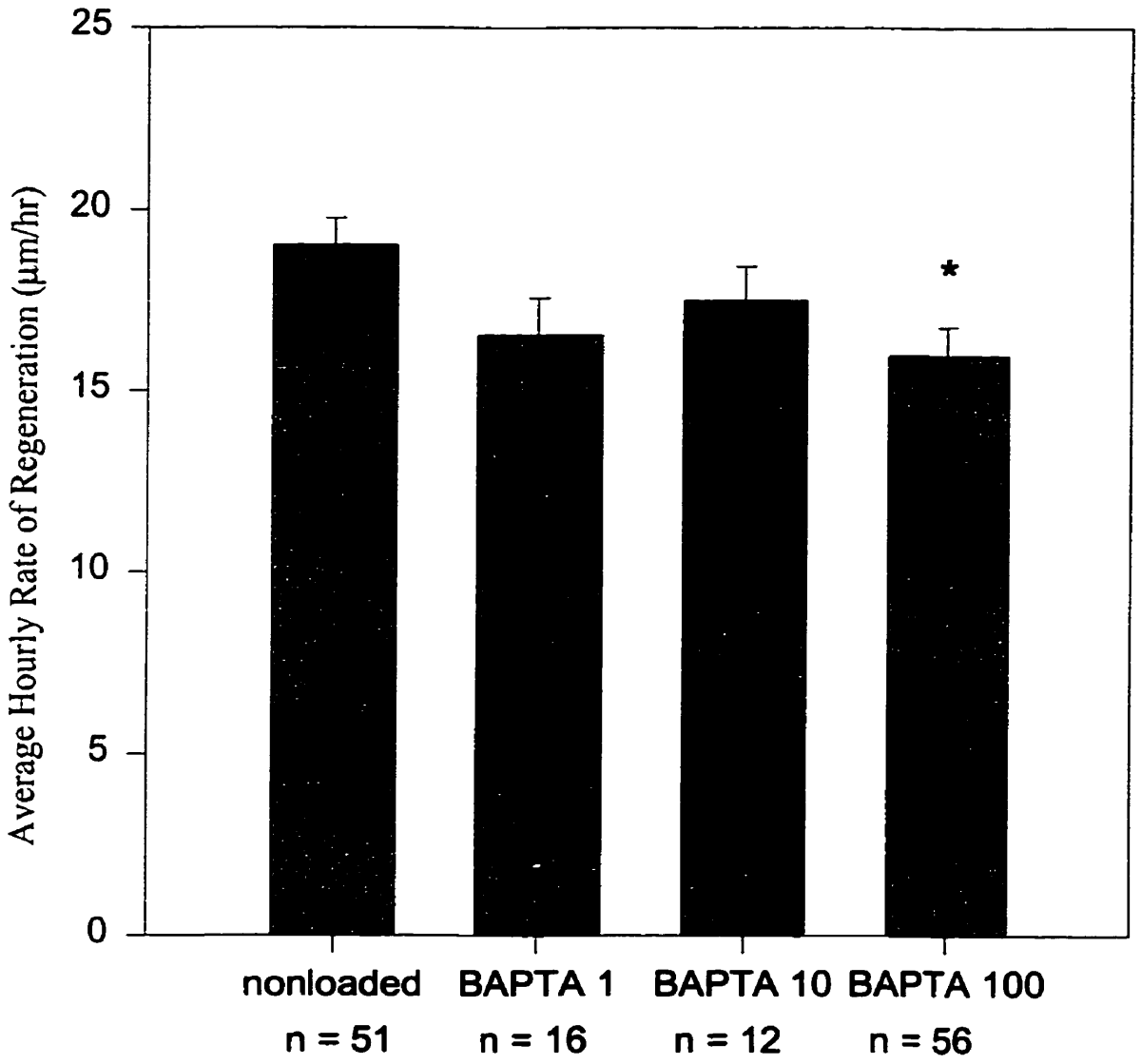
Between 6 and 24 hours, the average hourly rate of regeneration per track was 16.0 μm/hr and 19.0 μm/hr for cells loaded with 100 μM BAPTA-AM and nonloaded neurons, respectively. The average rate during the same period for the 1 μM was 16.5 μm/hr and for the 10 μM group, it was 17.5 μm/hr. One way ANOVA revealed a significant difference between groups ($p = 0.0294$, Fig. 11). However, multiple comparisons between the groups showed that the only groups to differ significantly were the nonloaded cells and the cells loaded with 100 μM BAPTA-AM. Between 24 to 48 hours the rate for nonloaded neurons was 20.9 μm/hr and for neurons loaded with 100 μM BAPTA-AM, it was 21.0 μm/hr. For the 1 and 10 μM BAPTA-AM loaded groups, the rate was 25.4 μM/hr and 21.3 μM/hr, respectively. Statistical analysis revealed only a significant difference in the rate of the 1 μM BAPTA-AM group compared to all the other groups ($p = 0.008$).

Figure 11

Histogram showing the average hourly rate of elongation (μm/hr) from 6 to 24 hours after neurite transection for 1, 10, or 100 μM BAPTA-AM loaded and nonloaded neurons. The error bars are SEM. The 100 μM BAPTA-AM group is significantly different from only the nonloaded neurons (* = $p<0.0294$). n = number of tracks analyzed.

Fig. 11

Rate of Regeneration from 6-24 hours after Neurite Transection



The initial rise in $[Ca^{2+}]_i$ at the cell body requires extracellular Ca^{2+}

There was minimal change in fluorescence in uninjured cells bathed in either zero Ca^{2+} ($\Delta f/f_0$=-0.01, n=116 cells) or normal Ca^{2+} solutions ($\Delta f/f_0$=0.007, n=112 cells, Fig. 12). After injury, the cell bodies of neurites injured in zero Ca^{2+} solution also showed minimal change in fluorescence ($\Delta f/f_0$=0.01, n=208 cells). In contrast, as previously noted, the cell bodies of neurites injured in normal Ca^{2+} solution showed a significantly larger $\Delta f/f_0$ ($\Delta f/f_0$=0.06, n=482 cells). The change in Ca^{2+} fluorescence was significantly different ($p < 0.0001$, Fig. 12) between cells injured in the zero Ca^{2+} solution compared to those injured in the normal Ca^{2+} solution and between injured cells and uninjured cells in normal Ca^{2+}, but there was no significant difference between injured and uninjured cells in zero Ca^{2+}.

Extracellular Ca^{2+} is required for initiation of regeneration (defined as the formation of growth cones or short sprouts emerging from the cut end of neurites)

Neurites transected in zero Ca^{2+} medium had no initiation of regeneration in any track for at least six hours after transection compared to 100 % of the tracks for neurites transected in normal Ca^{2+} (Fig. 13, 14). At six hours after transection (Fig. 14), the zero Ca^{2+} medium was replaced with normal Ca^{2+} and subsequently, two hours after this change in medium (8 hours after transection) 1.5 % of the tracks had initiated regeneration. At 12 hours after transection (6 hours after the change) 20.1% of the tracks had regeneration and by 24 hours after transection (14 hours after the change) all tracks had regeneration. To ensure that the lack of regeneration was not simply due to lack of membrane sealing which also requires Ca^{2+} influx (Yawo and Kuno, 1985, Xie and

Figure 12

Histogram showing the relationship between $[Ca^{2+}]_e$, neurite transection and somal calcium rise after neurite transection. One way ANOVA comparison of $\Delta f/f_0$ between uninjured and injured neurons in either normal or zero calcium solutions (* = $p < 0.0001$). n = number of cell bodies analyzed. The error bars are SEM.

Fig. 12

Relationship between somal calcium rises, transection, and extracellular calcium

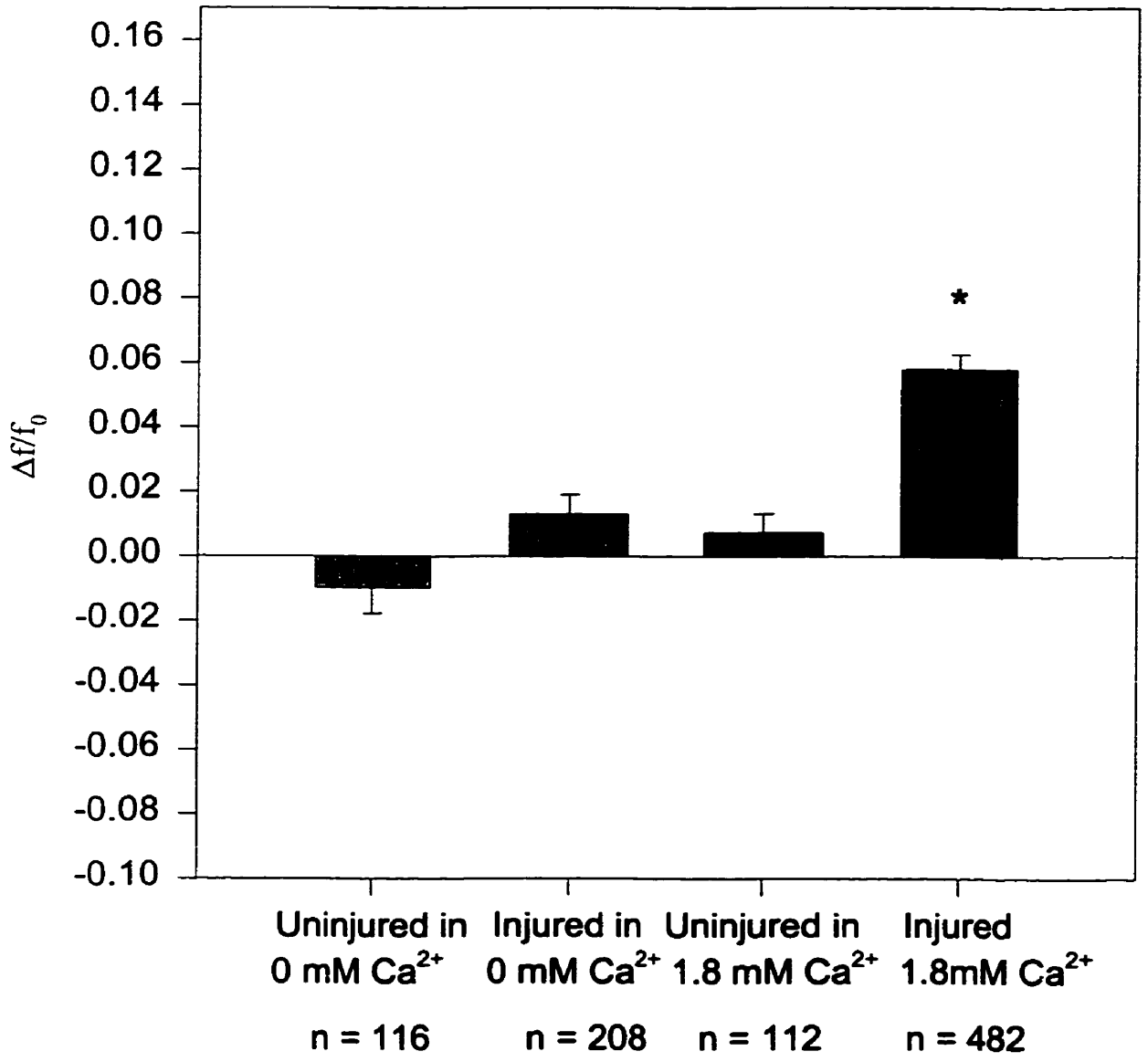


Figure 13

Neurites transected after 10 days in culture. The large arrow demarcates the transection site. Images obtained after neurite transection in 1.8 mM calcium medium (a, c) at 0 and 2 hrs, respectively. Note growth cones and short sprouts emanating from the edge of the transection site (small arrows) 2 hrs after transection in c. b and d show neurites transected in 0 mM calcium at 0 and 2 hrs, respectively. At 2 hrs, there is debris in the transection area in d, but no appearance of growth cones or short sprouts. Scale bar is 50 μm.

Fig. 13

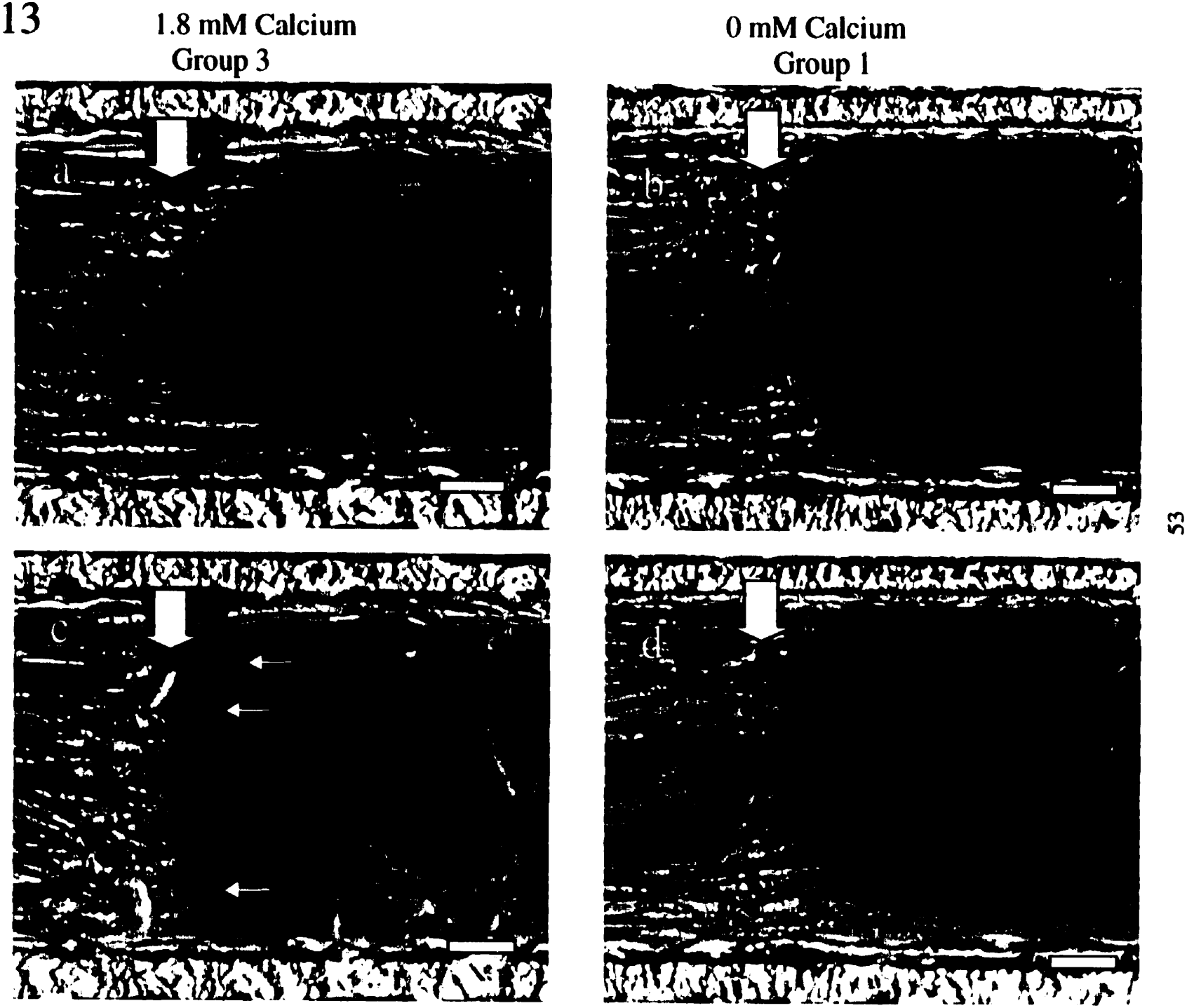


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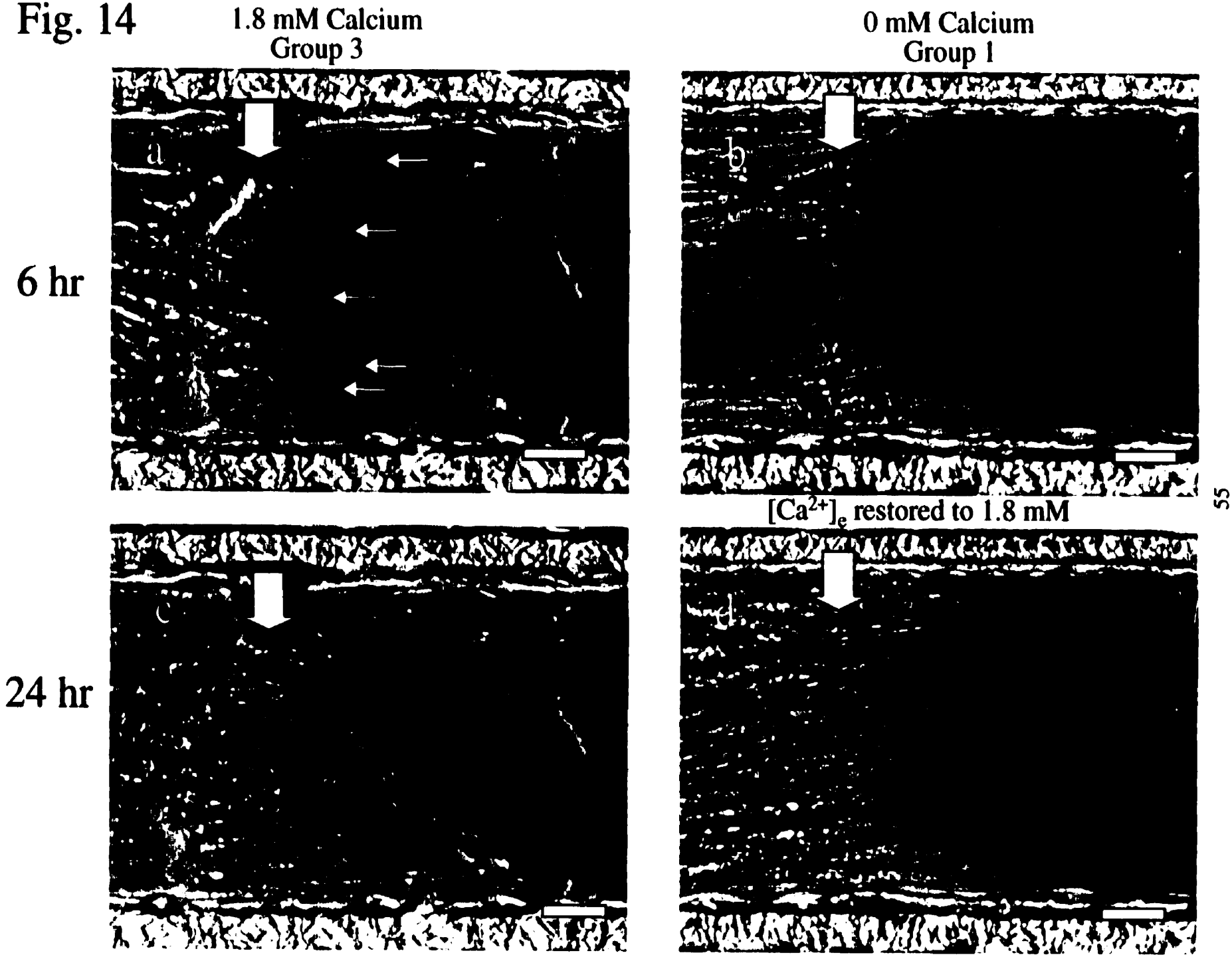
Figure 14

Images showing the same neurites as Fig. 13 at 6 and 24 hours after transection. The large arrow demarcates the transection site. In neurites transected in 1.8 mM calcium, note the addition of more growth cones and sprouts, and the elongation of the preexisting sprouts (small arrows) at 6 hrs in a while at 24 hrs, the entire transection area is covered with regrowing neurites in c. In neurites transected in 0 mM calcium note that at 6 hrs there are still no growth cones or short sprouts in b which contrasts markedly with a. The medium was changed to 1.8 mM calcium at 6 hrs, and at 24 hrs in d, the transection area is covered with regrowing neurites similar to (c). Scale bar is 50 μm.

Fig. 14


1.8 mM Calcium
Group 3
0 mM Calcium
Group 1
6 hr
24 hr
a
b
c
d
[Ca2+]e restored to 1.8 mM

55

Barrett, 1991), the neurites were transected in a normal Ca^{2+} medium which was then changed to zero Ca^{2+}. At six hours after transection, there was still no initiation of regeneration. Then, the medium was changed to normal Ca^{2+}, and at 8 hours after transection (2 hours after the change) 4.2 % of the tracks had regeneration. At 12 hours after transection (6 hours after the change) 70.8% of the tracks had regeneration, and by 24 hours, all tracks had regeneration (Fig. 14). These results are summarized in Table 2. To ensure that the lack of regeneration was not due to cell death, the viability stain propidium iodide was added to the media and the percentage of labeled cells was counted at 24 hours after transection. The percentage of cell death was 0 %, 0.29%, and 0.45% for neurites transected in normal Ca^{2+}, zero Ca^{2+}, and normal Ca^{2+} changed to zero Ca^{2-}, respectively (Table 3).

Calcium entry at the cell body after neurite transection is needed for optimal initiation of regeneration

To establish that Ca^{2+} entry at the cell body is required for initiation of regeneration, SCG neurons were grown in compartmented chambers. The 3 compartments in these chambers allow different Ca^{2+} concentrations in each compartment. The cell bodies were all isolated in the center compartment while the neurites grew into the side compartments. Prior to neurite transection, the media surrounding the cell bodies was exchanged for zero Ca^{2+} media while the neurites were immersed in normal Ca^{2+} medium. At 2 hours after transection, only 17.8 % of the tracks had initiated regeneration compared to 89.6 % for injured cultures in which both the cell body and neurites were in normal Ca^{2+} medium. At 6 hours, 86.2% of the tracks in the zero Ca^{2+} group had regeneration compared to 100% in the normal Ca^{2+} group. At 6

Table 2 **Percentage of Tracks with Regeneration of Neurons**

Injured in 0 mM $[Ca^{2+}]_e$

Group	Conditions During Transection	2 hours %	↓ 6 hours %	8 hours %	12 hours %	24 hours %
1.	0 mM $[Ca^{2+}]_e$	0 *	0 *	1.5 ± 1.5 *	20.1 ± 5.1 **	100
2.	1.8 → 0 mM $[Ca^{2+}]_e$	0 *	0 *	4.2 ± 4.2 *	70.8 ± 12.5 *	100
3.	1.8 mM $[Ca^{2+}]_e$	70.0 ± 6.9	100	100	100	100

Table showing the percentage of tracks which had initiation of regrowth at 2, 6, 8, 12, and 24 hours after transection. Mean percentage ± SEM. Group 1 (n = 5 culture dishes) were neurites which were transected in zero calcium. Group 2 (n = 2 culture dishes) were neurites transected in 1.8 mM calcium and then the solution was changed immediately to zero calcium. Group 3 (n = 3 culture dishes) were neurites transected in 1.8 mM calcium. The arrows indicate when the solutions were changed. * indicates $p < 0.05$ when compared to Group 3. ** indicates $p < 0.05$ when compared to Groups 2 and 3.

Table 3 **Percentage of Cell Death per Track at 24 hours after Transection**

Group	24 hours %
0 mM $[Ca^{2+}]_e$ (n = 7 tracks, 801 cells)	0.29 ± 0.14
1.8 mM $[Ca^{2+}]_e$ (n = 7 tracks, 1072 cells)	0
1.8 → 0 mM $[Ca^{2+}]_e$ (n = 7 tracks, 874 cells)	0.45 ± 0.28

Table showing the percentage of cell death per track 24 hours after transection for the three groups as defined in the text and Table 2. Mean percentage ± SEM

hours, the medium in the zero Ca^{2+} group was changed to normal Ca^{2+}, and by 24 hours 100 % of the tracks in both groups had regeneration. The results are summarized in Table 4.

Discussion

Calcium And Cell Death Experiments

These results indicate that the percentage cell death increases to 25.2-35.2% by 72 hours after neurite transection, but the majority of cell death occurs with the first 24 hours. The percent cell death is the same irrespective of whether the $[Ca^{2+}]_e$ was above or below normal levels of intracellular Ca^{2+}. There are three possible explanations for these results; 1) cell death is not dependent on the total amount of Ca^{2+} entry after transection; 2) cell death from increased Ca^{2+} entry is dependent on a specific route of Ca^{2+} entry or; 3) Ca^{2+} entry does not play an important role in neuronal death after neurite transection. The post-injury rise in $[Ca^{2+}]_i$ has been hypothesized to be responsible for initiating various cell death mechanisms (Balentine, 1983, Balentine et al, 1984, Choi, 1988, Emery et al, 1987, LoPachin et al, 1997, Tymianski et al, 1993, Tymianski, 1996, Young, 1992, Sattler et al, 1999). Calcium entry following neurite transection has been widely documented (Sattler et al, 1996, Strautman et al, 1990, Ziv and Spira, 1995), but whether this Ca^{2+} entry after neurite transection leads to cell death is not clearly established. Post-injury Ca^{2+} entry may directly or indirectly activate phospholipases (e.g. A_2 and C), arrest mitochondrial electron transport, depress cellular metabolism, lead to a loss of cytoskeletal integrity, possibly trigger the expression of several immediate-early genes such as c-fos, c-jun, c-myc, and lead to the formation and release of free radicals -

Table 4 **Percentage of Tracks with Regeneration of Neurons Injured with the Cell Bodies Immersed in 0 mM $[Ca^{2+}]_e$**

Group	2 hours	↓ 6 hours	12 hours	24 hours
	%	%	%	%
1) 0 mM $[Ca^{2+}]_e$ cell bodies	17.9 ± 11.3 *	86.2 ± 7.0	94.3 ± 3.5	100
2) 1.8 mM $[Ca^{2+}]_e$ cell bodies	89.6 ± 4.7	100	100	100

Table showing the percentage of tracks which had initiation of regrowth at 2, 6, 12, and 24 hours after transection. Mean percentage ± SEM. 6 sets of cultures were analyzed for Group 1, and 5 sets of cultures were analyzed for Group 2. Group 1 were neurites transected with the cell bodies immersed in zero calcium. Group 2 were neurites transected with the cell bodies immersed in normal calcium. The arrow indicates when the solutions at the cell bodies were changed. * indicates $p < 0.05$ when compared to Group 2.

all of which may potentially contribute to the ultimate destruction of injured cells (Smeyne et al, 1993, Trump and Berezesky, 1995, Tymianski, 1996, Young, 1992, Zhong et al, 1993). In addition, although much less is known about intranuclear Ca^{2+} regulation, there are reports that nuclei can accumulate Ca^{2+} in an ATP-dependent fashion (Nicotera et al, 1992), possibly leading to chromatin unfolding, and thus, rendering DNA vulnerable to endonuclease attack leading to apoptotic death (Evans, 1993, Trump and Berezesky, 1995). Furthermore, Ca^{2+} is also known to activate certain endonucleases which can modulate DNA strand breaks and alter gene expression (Arends et al, 1990, Trump and Berezesky, 1995). However, whether these Ca^{2+} activated mechanisms of cell death are dependent on the amount of Ca^{2+} entering the cell after transection is not known. Neurons injured in the higher Ca^{2+} environment should have had a higher amount of Ca^{2+} entry, and if the amount of Ca^{2+} entering the cell was important for cell death then the neurons in the present experiment in the higher Ca^{2+} environment should have shown a higher percentage of cell death than those transected in a normal or low Ca^{2+} environment. However, this did not occur. Although, the percentage cell death at 72 hrs was highest for the 5 mM $[Ca^{2+}]_e$ group, this percentage was still not significantly greater than the cell death percentages at the other concentrations. If one were to subtract 10% from the percent cell death because 10% of the neurons which died in 5 mM $[Ca^{2+}]_e$ were uninjured neurons, then the percent cell death due to transection alone would be 25% which is still similar to the other concentrations. Although no other experiments have correlated higher Ca^{2+} entry after transection with cell death, there have been several studies that have implicated total Ca^{2+} entry as important for glutamate toxicity (Kurth et al, 1989, Schramm and Eimerl, 1993). However, this correlation with glutamate toxicity

is disputed (Tymianski et al, 1993) and our results imply that the total amount of Ca^{2+} entry may not be important for neuronal death after neurite transection. For example, there could be a threshold level of $[Ca^{2+}]_i$ at which neurodegenerative processes are set in motion.

Alternatively, the route of Ca^{2+} entry may be the more important factor. In excitotoxic neuronal death, Ca^{2+} entry through NMDA receptors causes cell death whereas Ca^{2+} entry through other routes such as L-type voltage gated Ca^{2+} channels does not (Tymianski et al, 1993). After neurite transection in vitro Ca^{2+} entry is believed to be through both voltage gated Ca^{2+} channels and the disruption in the cell membrane (Sattler et al, 1996, Ziv and Spira, 1993). It is possible that Ca^{2+} entry through these routes will cause only 25-35% of the neurons to die but not more because these routes may limit the amount of Ca^{2+} that enters the cells regardless of the $[Ca^{2+}]_e$. Therefore, similar $[Ca^{2+}]_i$ rises would have occurred independent of $[Ca^{2+}]_e$, especially since non-specific Ca^{2+} entry through the cut end would have little effect on the cell body Ca^{2+} concentration, because of the limited diffusion of Ca^{2+} through the axoplasm (Strautman et al, 1990). It is also possible that the neuronal death observed in the present experiments was the result of rise in $[Ca^{2+}]_i$ from the release of Ca^{2+} from intracellular Ca^{2+} stores. It is known that Ca^{2+} may be stored within the neuron in the endoplasmic reticulum and the mitochondria (Clapham, 1995) ,and that after neurite transection Ca^{2+} is released into the cytoplasm. Although, it has been suggested that release from intracellular stores after neurite transection does not occur in Aplysia neurons (Ziv and Spira, 1993), this does not rule out such an event in rat sympathetic neurons.

Another possibility is that Ca^{2+} may not be involved in cell death in this model of

neurite transection. Schlaepfer and Bunge (1973) noted that amputated neurites of cultured rat sensory ganglia underwent granular disintegration of their axoplasm between 6 and 24 hours after transection. However, if the neurites were immersed in a low Ca^{2+} medium (25-50 μM) the neurites retained their longitudinal continuity and retained their neurofilaments although the microtubules were disrupted. If the transected neurites were immersed in a medium containing 6 mM EGTA then both neurofilaments and microtubules were preserved. Similarly, George et al (1995) found that Ca^{2+} influx through specific ion channels was responsible for the degeneration of transected neurites. The above studies concentrated on the degeneration of neurites distal to the transection (Wallerian degeneration), but the survival of the proximal neurite and cell body was not studied. In 1990, Lucas et al examined neuronal survival and low $[Ca^{2+}]_e$ after dendrotomy of embryonic mouse spinal neurons in culture. Using a laser microbeam system, they transected the primary dendrite 100 μm from the cell body and then assessed cell survival of neurons injured in either 1.8 mM Ca^{2+} or 30 μM Ca^{2+}. As noted previously, they could not reduce the Ca^{2+} concentration further in their studies due to loss of cellular adhesion if the $[Ca^{2+}]_e$ was below 15 μM. They found that transection in the 30 μM Ca^{2+} medium decreased the percentage cell death at 2 hours after injury, but at 6 hours after transection there was no difference in cell death between the two groups. However, 30 μM Ca^{2+} is greater than the $[Ca^{2+}]_i$ so it is likely there was still Ca^{2+} influx into the neuron after transection. Therefore Ca^{2+} influx could still play a role in neuronal death. However, in the present studies neurons injured in 50 nM Ca^{2+} medium did not show a lower death rate compared to controls. Furthermore, although data for figure 12

was collected under different conditions than the cell death experiments with respect to amount of EGTA used and distance from the soma to the transection site, the figure nevertheless demonstrates that when neurites are transected in low calcium media there is no rise in $[Ca^{2+}]_i$ at the soma. Thus, these results support the hypothesis that Ca^{2+} entry does not play a significant role in neuronal death after neurite transection, at least not for the first 30 minutes after transection.

Other ions may enter the neuron after neurite transection. Sodium will enter neurons after neurite transection both through the cut end and through voltage gated sodium channels secondary to the depolarization of neurons after transection. The rise in intracellular sodium after transection would increase the demand on energy resources within the cell as its Na^+/K^+ ATPase pumps respond to the increased sodium load. This extra energy demand caused by the trauma could cause some neurons with lower energy reserves to die from energy depletion. The sodium/calcium exchanger in the mitochondrial membrane may also play a role in cell death. It may also be that mechanical disruption of the cytoskeleton triggers a certain pattern of cell signaling that leads to cell death (perhaps via apoptosis) (Trump and Berezesky, 1995).

With respect to cell death versus post-transection time, it was found the majority of cell of death had occurred between 2 and 24hrs. Other studies have shown that detectable ultrastructural changes in transected mouse spinal neurons indicating whether the cells were going to live or die could be observed as early as 2hrs post-transection and definitively by 24hrs post-transection (Emery et al, 1987, Lucas et al, 1985). These results are consistent with the present findings.

The uninjured control neurons showed a percentage cell death of approximately 10% when exposed to 5mM $[Ca^{2+}]_e$ for 30min, as opposed to a percentage cell death of about 1% in normal physiological conditions. Normally, neurons maintain internal Ca^{2+} homeostasis by constantly extruding excess intracellular Ca^{2+} by active transport across the plasma membrane using Ca^{2+} ATPases (Carafoli, 1992, Young, 1992). However, this mechanism of intracellular Ca^{2+} regulation is slow and energy demanding (Young, 1992). Thus, it is possible that the uninjured controls in the 5mM $[Ca^{2+}]_e$ group experienced a period of elevated $[Ca^{2+}]_i$ that contributed to their higher death rate. In 1982 Balentine et al., for example, found that non-traumatized spinal cord tissue died of necrosis following exposure to high $[Ca^{2+}]_e$, leaving traces of calcification or hydoxyapatite deposits within axons. It is also possible that the cells' attempt to reestablish normal $[Ca^{2+}]_i$ may have been exhaustive and lead to ATP starvation and death by metabolic collapse. Calcium ions can exacerbate ATP deficiencies by interfering with mitochondrial electron transport which leads to both a blockage of ATP production (Carafoli and Lehninger, 1971, Clenendon and Allan, 1979, Ito et al, 1978, Vink et al, 1990, Young, 1992) and the emergence of free radicals from the resulting inappropriate oxygen reduction (Young, 1992). Moreover, there are Ca^{2+} activated proteases that appear capable of disturbing Ca^{2+} ATPases in the plasma membrane, thus compounding the problem (Trump and Berezesky, 1995). There are two alternative explanations; elevated $[Ca^{2+}]_e$ may have affected certain cell surface receptors and prompted internal cascades leading to cell death; or, high $[Ca^{2+}]_i$, may have affected the permeability of some chloride channels (Trump and Berezesky, 1995), and may have altered cell volume through electrolyte imbalance and thus contributed to cell death.

The relationship between cell death and distance from the cell body to the transection site found in the present studies was similar to the results obtained by Lucas et al. They found a 30% cell death rate when the dendrite transection was 0.15mm from the cell body of mouse cultured spinal neurons (Lucas et al, 1985). The present study showed similar results, with a death rate of approximately 30% when the neurite transection was within 0.66mm from the cell body. Cell death after transection beyond that distance was significantly less. It is possible that the cell bodies in fields 2-4 had a progressively lower percentage of neurites which extended across the site of injury, and therefore, the decrease in cell death may have been due to the fact that fewer neurons had neurite transection. However, if this were the case then the decrease in death rate should have decreased as a linear function of distance from the injury site. Also, it has been shown in other studies that cell death after axotomy in rat retinal ganglion cells depends on the distance between the lesion and the cell body (Berkelaar et al, 1994). Similarly, expression of GAP-43 (a growth associated protein), for example, is altered depending on the distance of the axonal injury from the rat retinal ganglion cell body (Doster et al, 1991), indicating that the distance from the axonal injury site to the cell body has an effect on various cellular events. Alternatively, the relationship between death and distance from the cell body may be explained by the extent of the ionic currents across the lesion site: the farther the injury from the cell body, the smaller the number of ions which enter the cell body and contribute to cell death (Lucas et al, 1985). Furthermore, neurite transection results in many neurodegenerative events in the neurites such as microtubule disassembly and neurofilament disaggregation (Lucas et al, 1985), and the farther the lesion from the cell body, the less this damage can affect the cell body and

contribute to cell death.

Calcium And Regeneration Experiments

These results demonstrate that Ca^{2+} entry into sympathetic neurons in culture after neurite transection do not necessarily play a role in neuronal death. However, Ca^{2+} entry into neurons may still signal other cellular events after neurite transection, such as the regeneration of neurites after transection. The present results suggest: 1) initiation of regeneration and branching after neurite transection require intracellular Ca^{2+}; 2) Ca^{2+} influx is necessary for this initiation of regeneration; and 3) Ca^{2+} influx at the cell body accelerates the initiation of regeneration after transection.

Cells loaded with the Ca^{2+} chelator BAPTA-AM showed a delay in the onset of regeneration although the effects of BAPTA-AM were temporary, possibly due to the extrusion of BAPTA from the cell after 6 hours or saturation of the BAPTA-AM molecule. An alternate explanation for the temporary effect is that there may be a Ca^{2+} independent mechanism involved in regeneration, though this is less likely as shown by the experiments conducted in the zero Ca^{2+} medium discussed below.

The effect of Ca^{2+} chelation on branching was quite dramatic, demonstrating Ca^{2+}'s importance to the branching of neurites. These findings are similar to two other recent studies which also examined the relationship between Ca^{2+} and branching (Reitstetter and Yool, 1998, Ramakers et al, 1998). Reitstetter and Yool (1998) showed that rat cerebellar purkinje neurons depolarized by high potassium showed increased dendritic outgrowth and branching. This response could then be inhibited by the addition of $NiCl_2$ (an R-type and T-type Ca^{2+} channel antagonist). Furthermore, thapsigargin, a

Ca^{2+}-ATPase inhibitor which depletes internal Ca^{2+} stores, could also decrease dendritic branching. Ramakers et al (1998) used cultured embryonic rat cortical neurons to demonstrate that depolarization of these neurons induced an increase in the size of the lamellipodia of all growth cones. Prolonged depolarization for 24 hours caused an increase in axonal branching but not dendritic branching. These two studies suggest that Ca^{2+} is needed for the branching of neurites. However, Wakade et al (1995) grew chick sympathetic neurons in a low Ca^{2+} medium and found that the cells grew as single cells with thin branching neurites in contrast to neurons grown in higher Ca^{2+} concentrations which caused cell aggregation and thick neurites. This study suggests that Ca^{2+} may cause neurite fasciculation rather than branching. However, this study was conducted with chick neurons while the other studies were conducted with rat neurons, and there could have been a difference due to the different species involved.

There was less effect of BAPTA-AM on the average hourly rate of elongation than the extent of branching. From 6-24 hours after transection, the rate of elongation was only significantly different between the group with the highest loading concentration of BAPTA-AM (100 μM) and the nonloaded cells. From 24-48 hours after transection, the rate of regeneration was not significantly different between the nonloaded, 10, and 100 μM BAPTA-AM loaded groups, though the average rate was significantly greater for the 1 μM group. There are several possible explanations as to why the rate of elongation did not appear to be as affected as branching. It is possible that the BAPTA-AM was extruded after 6 hours, and thus the effect of low $[Ca^{2+}]_i$ lasted for a shorter time than was necessary to measure the rate of elongation. For example, if the neurons had been

reloaded with BAPTA-AM, there may have been a significant decrease in the rate of elongation. However, several reports have shown that Ca^{2+} is not required for neurite elongation (Campenot and Draker, 1989, Tolkovsky et al., 1990). Campenot and Draker (1989) demonstrated with compartmented chambers that transected SCG neurites could grow into the side compartments containing zero Ca^{2+} medium as long as the cell bodies in the middle compartment remained immersed in a normal Ca^{2+} medium. It must be noted that the neurites in that study were transected in a normal Ca^{2+} medium. Thus, these studies suggest it is possible that neurite elongation and branching are two independent phenomena with branching being dependent on Ca^{2+}, whereas elongation is largely Ca^{2+} independent. Indeed, some authors have identified differences between factors required for branching and those required for neurite elongation (Yasuda et al., 1990, Caroni, 1997, Smith and Skene, 1997). For example, certain growth associated proteins such as GAP-43 have been shown to promote branching (Caroni, 1997), but not elongation (Aigner et al., 1995, Strittmatter et al., 1995). Others have noted that neurites formed in culture may be either short in length and highly branched or long but with far fewer branches (Yasuda et al., 1990, Smith and Skene, 1997). Our results suggest that Ca^{2+} may be a signal for branching after transection but not for neurite elongation.

In the present study it was shown that the source of the rise in $[Ca^{2+}]_i$ at the cell body must initially be from the extracellular medium. This confirms the results of previous studies (Borgens et al., 1980, Happel et al., 1981, Mata et al., 1986, Strautman AF et al., 1990, Ziv and Spira, 1993, Ziv and Spira, 1995, Sattler et al., 1996) which have suggested that Ca^{2+} enters the cell through voltage gated Ca^{2+} channels as well as through the disruption in the cellular membrane due to the transection. In Sattler et al (1996), it

was suggested that stretch activated ion channels may also play a role in Ca^{2+} entry although, the neurites in that experiment were transected close to the cell bodies (3 cell body lengths away), and the neurites and cell bodies were stretched prior to neurite transection. In contrast, in the present study, the neurites were transected with a crush injury at a much greater distance from the cell body so it is unlikely that either the cell bodies or neurites were stretched significantly prior to transection, and therefore it is unlikely that Ca^{2+} entered through stretch receptors. The role of CICR is not ruled out by the present study, and it is possible that Ca^{2+} entry through neurite transection causes release from stores.

It is unknown by what mechanism the somatic $[Ca^{2+}]_i$ increased from a transection of distal neurites in this cell culture system. It is possible that depolarization at the transection site caused an action potential to be propagated retrogradely and subsequently voltage gated calcium channels were opened. It is also possible that only passive spread or electrotonic conduction occurred along the neurite to the soma leading to opening of the voltage gated calcium channels if the length constant for the neurites in this culture system was large enough. Neither of these possibilities can be eliminated by these experiments.

Elimination of this rise by transecting the neurites in zero Ca^{2+} will delay the initiation of regeneration and that regeneration only occurred after the normal Ca^{2+} medium was restored. This result is in agreement with previous studies of non-mammalian neurons which implicated a transient rise in $[Ca^{2+}]_i$ at the time of injury as sufficient for growth cone formation (Rehder et al., 1992, Ziv and Spira, 1997). However, since Ca^{2+} is essential for many cellular functions, it has been suggested that

the spatiotemporal aspects of Ca^{2+} signaling are just as important as the actual increase in Ca^{2+} concentration (for review see Tymianski and Tator, 1996, Berridge, 1998). Rehder et al (1992) suggested that the Ca^{2+} transient at the site of injury was needed to reseal the membrane of the cut neurite, and that once the membrane was sealed a growth cone could then form. They demonstrated this by transecting neurites in a normal Ca^{2+} solution and then changing the solution within 1 minute to one which had no Ca^{2+}: the growth cones still formed at the usual time after transection. A similar experiment was performed in this culture system, but no formation of growth cones was found up to six hours after the transection. Since Rehder et al (1992) used neurons from Helisoma while rat neurons were used in this study, these conflicting results could imply that mammalian neurons require more than a transient influx of extracellular Ca^{2+} after injury for initiation of regeneration, and that the Ca^{2+} influx is required for more than membrane resealing. Rehder et al further noted that the neurons injured in low Ca^{2+} died after 1-3 hours in that solution due to leaking of the cytoplasm through the unsealed membranes. However, in the present studies there was very little neuronal death even after six hours in the zero Ca^{2+} medium suggesting that some membrane sealing may have occurred although no regeneration had taken place. Ziv and Spira (1997) also suggested that the Ca^{2+} rise after transection was needed for more than resealing. They showed that raising the $[Ca^{2+}]_i$ in the Aplysia neurite by local application of a Ca^{2+} ionophore to a concentration similar to that achieved by cutting a neurite, allowed growth cone formation from the intact neurite. They further demonstrated that if they maintained the $[Ca^{2+}]_i$ level of the cut neurite below the level needed to initiate growth cones, but high enough for membrane sealing then no growth cone would form. From these results, they hypothesized that localized

and transient elevation of $[Ca^{2+}]_i$ in neurites may be sufficient for growth cone formation. However, other reports have demonstrated that a Ca^{2+} rise in the rat neuronal cell body may be necessary for initiation of regeneration (Kocsis et al., 1994, Lankford et al., 1995). Whether Ca^{2+} entry at the cell body during and after transection was important for initiation of regeneration was tested in the present experiments and it was found that initiation of regeneration was severely delayed in the majority of neurites, but not stopped entirely as it was when the entire neuron was immersed in zero Ca^{2+}. These results imply that Ca^{2+} entry into the cell body after neurite transection is required for optimal regeneration, and acts to decrease the delay between the transection and the initiation of neurite regeneration. However, Ca^{2+} entry at the cell body is not an absolute requirement for regeneration because some initiation still occurred in some tracks (17 %) in those experiments. One possible explanation why initiation of regeneration did not completely stop could be that neurites contain all the machinery necessary to remodel the cytoskeletal proteins at the site of injury to form growth cones and initiate regeneration. This machinery is Ca^{2+} activated, and recently it has been shown that calpains may be part of this Ca^{2+} activated machinery (Gitler and Spira, 1998). However, Ca^{2+} entry at the cell body may aid in this process by activating calpains at the cell body which may then increase the efficiency with which the cut end is transformed into a growth cone. It is possible that Ca^{2+} entry at the cell body may also activate different molecular processes than at the cut end, such as gene transcription. For example, it is known that Ca^{2+} entry through voltage gated Ca^{2+} channels can lead to gene transcription in depolarized, undamaged neurons (Bading et al., 1993), and a similar mechanism may occur in transected neurons. Since the neurons in these cultures were all transected during the

initial dissection, the in vitro injury was the second neurite transection experienced by these neurons. It is not known whether regeneration associated genes were activated by the somal Ca^{2+} rise initiated by the second injury. However, it is known that a first injury (the conditioning lesion) can increase the regenerative potential of the neuron after a second injury (Richardson and Issa, 1984). Therefore, it is possible that the Ca^{2+} entry after the second transection may reactivate or enhance transcription of genes initially transcribed during the first transection. It is also possible that the Ca^{2+} entry may have caused activation of other genes not activated by the first injury which can then enhance regeneration.

Campenot and Draker (1989) had previously shown neuronal death when the SCG cell bodies were immersed in zero Ca^{2+} medium for 3 consecutive days, whereas in the present studies the cell bodies remained in zero Ca^{2+} for only 6 hours with little neuronal death (Table 3).

A specific model of regeneration is suggested by the observation that the regeneration of neurites was abolished by a global lack of extracellular Ca^{2+} surrounding the cell bodies and transected neurons as opposed to being delayed when only the cell body was deprived of extracellular Ca^{2+}. The model indicates that Ca^{2+} entry at the site of transection activates mechanisms essential for regeneration. Whether these mechanisms are merely needed to reseal the ruptured membrane or are also needed for growth cone formation is not completely understood. Calcium entry at the cell body optimizes the regeneration processes either by enhancing the mechanisms initiated at the site of injury or by activating new mechanisms that enhance regeneration. The finding that zero Ca^{2+} at the cell body delayed the regeneration, but did not inhibit it completely, implies that there

may be non Ca^{2+} dependent mechanisms which are activated or that Ca^{2+} from internal stores may be released in lieu of Ca^{2+} entry.

Though BAPTA and EGTA are chelators with high affinity for Ca^{2+}, they also chelate other divalent ions such as zinc (Zn^{2+}). Zinc is increasingly being recognized as playing an important role in CNS injury (Choi and Koh, 1998). The role that zinc plays in axonal regeneration has not been fully explored. It is possible that the results observed with the present studies may be due in part to chelation of Zn^{2+} as well as Ca^{2+}. This issue needs to be explored further.

Conclusion

It must be emphasized that this in vitro study of neurite transection may differ greatly from in vivo axonal injury. In the in vivo situation, CNS axonal transection may be associated with excitotoxicity, ischemia, and deprivation of trophic factors, none of which is present in this model. Nevertheless, this study reinforces the finding that most neurons survive axonal transections which are more than 1 mm distance from the cell bodies such as in spinal cord injury (Jenkins et al, 1993, Theriault and Tator, 1994). Therefore after spinal cord transection, the neurons closest to the transection site such as anterior horn cells and dorsal root ganglion (DRG) neurons will die while the more distal DRG, brainstem, and cortical neurons will survive. Furthermore, this study indicates that the majority of early cell death, if it occurs, takes place between 2hrs and 24hrs post-injury. Finally, these results suggest that early post-transection cell death is independent of extracellular Ca^{2+} influx, leaving in question the role of the post-transection $[Ca^{2+}]_i$ rise secondary to this influx. The present study also showed that post-transection Ca^{2+} influx

was needed for the initiation of regeneration in mammalian neurons, and that Ca^{2+} entry at the cell body after transection is necessary for optimal neurite initiation. What mechanisms are activated by the Ca^{2+} entry are unknown and need to be elucidated. Whether Ca^{2+} entry at the cell body or site of injury after axonal transection is important for in vivo regeneration remains to be tested. If Ca^{2+} entry is important than rapid restoration of normal $[Ca^{2+}]_e$ after spinal cord injury may be beneficial since it is known that $[Ca^{2+}]_e$ decreases precipitously after spinal cord injury. The in vivo situation may be more complicated due to the influence of additional factors such as non-neuronal cells, and the longer distances from the axonal injury site to the somata, in injuries such as spinal cord injury. Discovering which Ca^{2+} activated mechanisms are involved in regeneration in culture may lead to an understanding of which ones are involved in vivo. Furthermore, the effects of Zn^{2+} chelation on regeneration have not been ruled out.

Future Directions

Although early Ca^{2+} entry after neurite transection did not cause neuronal death, Ca^{2+} may still be involved. Ca^{2+} released from internal stores alone may be sufficient for neuronal death after neurite transection. Ca^{2+} may have been a contributing factor to cell death if the cells had been maintained at the higher concentration (5 mM) for a longer time period. Therefore, it may not have just been a question of "how much Ca^{2+} for neuronal death", but rather "how much, for how long, and from where". Furthermore, if Ca^{2+} does not play a prominent role in cell death after neurite transection then what other factors are involved ? It is possible that sodium influx is important in neuronal death after neurite transection (Agrawal and Fehlings, 1996). It is also possible that the

disruption of cytoskeletal components such as neurofilaments may cause neuronal degeneration (Julien and Mushynski, 1998). The type of neuronal death after neurite transection that occurs in this model needs to be explored further. It is not known whether these neurons died through necrotic or apoptotic mechanisms.

With regards to Ca^{2+} and regeneration, the present studies demonstrated the importance of Ca^{2+} influx after neurite transection at the soma and the whole cell proper. However, the role of Ca^{2+} from internal stores remains to be explored. Furthermore, the issue of what time period is long enough for Ca^{2+} to enter the cell after neurite transection for initiation of regeneration needs to be examined further. For example, in these studies it was shown that maintenance of the normal Ca^{2+} medium for 1 minute after transection was insufficient for initiation, therefore it is not known how long after neurite transection do these cells need to be immersed in normal Ca^{2+} for initiation to occur. What Ca^{2+} activated mechanisms are required for initiation of regeneration remains unknown. It has been suggested that calpain and other proteases are necessary for growth cone formation in Aplysia neurons (Ziv and Spira, 1998, Gitler and Spira, 1998). However, calpain activation after traumatic injury in mammalian neurons has been associated with neuronal degeneration instead of regeneration (Buki et al, 1999). What Ca^{2+} activated mechanisms are required for branching of neurites are unknown. Whether specific routes of Ca^{2+} entry are needed for initiation of regeneration are unknown. For example, does initiation require Ca^{2+} entry through a specific Ca^{2+} channel ? Also, it is not known whether other factors such as neurotrophins which may stimulate regeneration in neurons act through Ca^{2+}. It is possible that the NGF induced branching of SCG neurons is mediated through

Ca^{2+} by activation of the NGF receptors. Finally, to assess the effects of Zn^{2+} on regeneration, a more specific Zn^{2+} chelator such as TPEN could be used.

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