Chapter 14

THE EFFECTS OF DIRECT AND INDUCTIVELY COUPLED CURRENT, AND NERVE GROWTH FACTOR, ON NERVE GENERATION IN VITRO*

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Introduction

For the last twenty years, there has been increasing interest in the effects of electrical stimulation on various tissues and organ systems. Most of this interest has stemmed from the basic studies of Fukada (1957), Yasuda (1953), Becker (1960), Bassett and Becker (1962) and has dealt with the piezoelectricity of bone and fracture healing. More recent work on electrical stimulation covers the range from observing the effects on amputated mammalian limbs (Becker, 1972; Sisken et al., 1979) to determining the effects on macromolecular synthesis in cell cultures (Norton et al., 1979).

Our interest has been primarily in the area of nerve regeneration in vitro (Sisken and Smith, 1975; Sisken and Lafferty, 1979; Sisken and Sisken, 1979). We have used nerve growth factor (NGF) as a standard in our experiments since nerve growth factor is the best known neurotrophic substance available at present. Its primary actions are (1) to increase cell survival, (2) to increase cell size, and (3) to increase neurite outgrowth in specific neuronal populations of the embryonic and adult peripheral nervous system. We have found that direct current (Sisken and Lafferty, 1979), nicotine (Sisken and Sisken, 1979), and dibutryl cyclic AMP (Roisen et al., 1972) also stimulate neurite outgrowth from these same neuronal populations.

A different mode of delivering electric current, pulsed electromagnetically induced current (Pilla, 1974) provides another kind of electrical environment; it is noninvasive and can be modified in its waveform configuration. This type of treatment has been reported to affect bone cells (Shteyer et al., 1979), limb regeneration in salamanders (Smith and Pilla, 1979), and calcification in cases of nonunions in humans (Bassett et al., 1979).

It was decided, therefore, to test this alternate type of electric current in our culture system. We will present the results of our experiments compar-

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ing the effects of two different types of electrical stimulation, direct and induced, with each other and with that of nerve growth factor.

MATERIALS AND METHODS

Eight day chick embryos obtained from eggs incubated at 39°C were placed into dissecting dishes and, under sterile conditions, trigeminal, dorsal root, and lumbar sympathetic chain ganglia were dissected from these embryos and placed into 60 mm Falcon culture dishes (3-4 per dish). The dishes were not coated with collagen nor were plasma clots used since either situation might have impeded current flow in dishes used for electrical experiments. The ganglia were covered with 8 ml of complete medium containing 85% Dulbecco® MEM, 10% fetal calf serum, glucose at final concentration of 600 mg%, plus penicillin (1250 units/ml) and streptomycin (12 mg/ml). All culture dishes, treated and untreated, were incubated at 39°C in a water-jacketed incubator containing 95% air, 5% CO_2.

The dishes were distributed into four groups: a control group, a group (used as a standard) treated with 2.5s nerve growth factor (10^{-8}M), a group treated with direct current, and a group treated with pulsed electromagnetically induced current via coils. In the direct current treated group, the tops of the culture dishes were exchanged for tops modified so that they contained tantalum electrodes (Fig. 14.1). The circular geometry of the electrodes were chosen in order to avoid electric field "edge effects." Four such dishes were loaded onto a plastic bus, which was placed in a 39°C incubator containing 95% air, 5% Co_2. The leads from the bus were fed out of the incubator to connect to a .65 volt battery and picoammeter.

The electric field ($\bar{E}$) in the dish was determined by solving the boundary value problem with the following assumptions:

1. The charge distributions are uniform over the electrode surfaces. This assumption is made so that the computations are manageable. The assumption is reasonable since the diameter of the wire electrodes is small with respect to the distance between them, so a nonuniform charge distribution would only cause a significant perturbation of the field near the electrode surface.
2. The bottom of the dish is a semi-infinite slab. The dish will sit on a thick Plexiglas® slab.
3. The medium has infinite depth. Since the field strength will diminish rapidly as the liquid-air interface is approached, the perturbation due to the interface is negligible.
4. There are no supports for the electrodes. This is the weakest assumption. The influence of the dielectric supports is being investigated.
5. The dielectric constant for the medium is the same as that for water, namely 80. (Personal communication, R. Buvet, Paris, France).

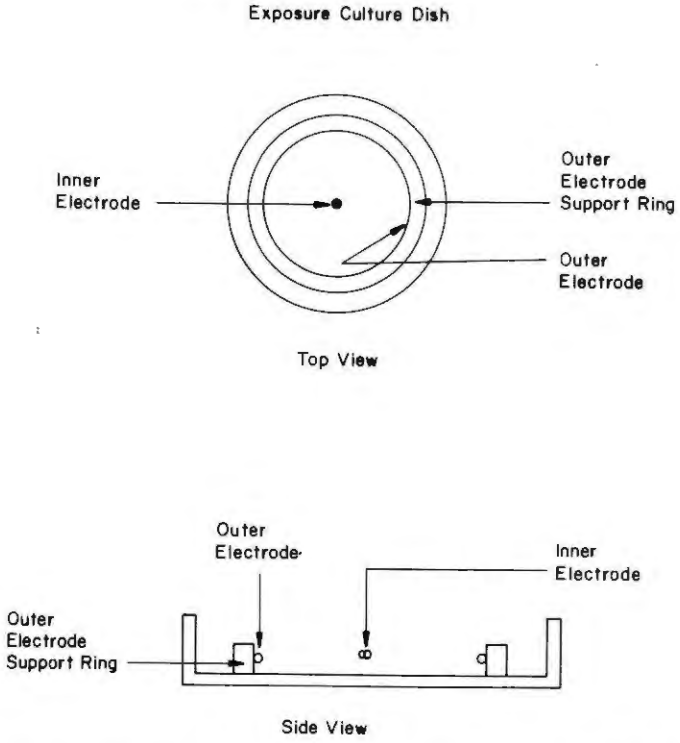


Figure 14-1. Top and side view of culture chamber containing two electrodes. Explants of nervous tissue are placed approximately half-way between the inner and outer electrodes.

6. There are no lead-in wires connected to the electrodes. The small diameter of the wire should produce a negligibly small perturbation in the field away from the wire surface.

The electric field distribution in the dish was computed from the electrostatic scalar potential (Φ):

$$\nabla^2\Phi = -\frac{\rho_s}{\epsilon}$$

where

∇^2 is the Laplacian operator
Φ is the solution to Poisson's equation
ρ_s is the surface densities on the electrodes
ϵ is the permittivity.

The boundary conditions are that (1) the potential difference between the two electrodes is known and (2) the same total charge exists on each electrode.

The electric field $\bar{E}$ is given by

$$\bar{E} = - \text{grad } \Phi$$

where grad is the gradient operator.

The current $\bar{J}$ density is given by:

$$\bar{J} = \sigma \bar{E}$$

where σ is the conductivity of the medium.

This model has been solved directly for meaningful parameter values. The resulting electric field lines are shown in Figure 14-3A. Figure 14-3B gives the field strength/current density as a function of radial distance along the bottom of the dish where the tissue explants are located.

The model discussed in No. 4 neglects the potentially significant influence of the dielectric support ring. The approximate configuration shown in Figure 14-2B represents an improved model. The influences of the supports may be accounted for by using an equivalent charge representa-

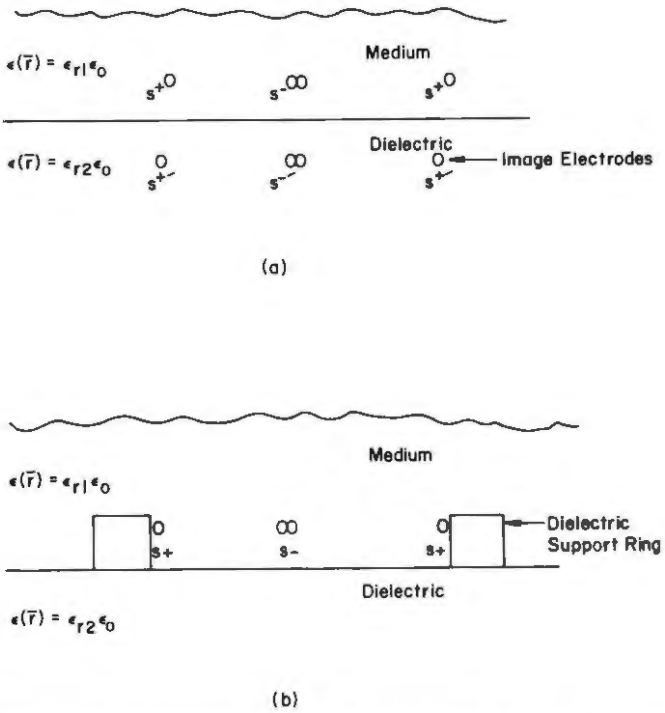


Figure 14-2. (a) Schematic representation of two electrodes (s^+ and $s^{-\prime}$) in a conducting medium of infinite extent above image electrodes ($s^{+\prime}$ and s') in a dielectric slab of infinite extent. The permitivity of the environment is denoted by $\epsilon(\bar{r})$. The symbols ϵ_{r1} and ϵ_{r2} are the dielectric constants of the medium and the dielectric material respectively. ϵ_0 is the free-space permitivity.

(b) Same as (a) except the dielectric support rings are shown.

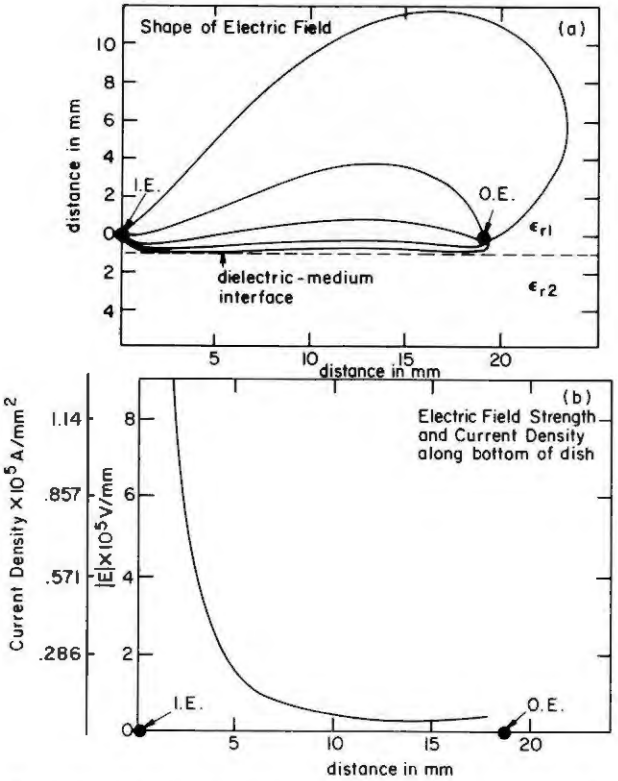


Figure 14-3. The shape of the electric field (a) and the electric field strength and current density (b) contained in the culture chamber. I.E. = inner electrode, O.E. = outer electrode.

ϵ_{r1} = the dielectric constant of the medium.

ϵ_{r2} = the dielectric constant of the plastic dish bottom.

tion. There are two alternative methods for including this charge in the field in Figure 14-2B:

(a) volume polarization charge in dielectrics [2]
(b) surface equivalent charge imposed on the dielectric surfaces [3]

Either of these methods require numerical solutions to the integral equations arising from the formulations. In general, (a) is simple conceptually and costly computationally and (b) is conceptionally involved — it requires significant analytical preprocessing — but it is computationally efficient and is the method we have chosen to pursue. Using the theoretical calculations presented above together with the total current obtained per dish (5-20 ηA) in our laboratory experiments, explants located at 10 mm from the center electrode are presumed to be exposed to current densities in the range of 0.5-0.05 ηA/mm^2.

The culture dishes to be treated with electromagnetically induced cur-

rent were placed in the air gap between the two facing coils set in the incubator, as illustrated in Figure 14-4A. Both sets of coils were connected to a 110 V line-operated power source and housed in the same incubator used for the other treated and untreated culture groups but set at least twelve inches from each other and six inches above the other shelves. Two different current waveforms (Fig. 14-4B) configured for electrochemical information (Pilla, 1974, 1979) were tested on these explants. Both single signals are kinetically unipolar, i.e. they possess an opposite polarity too short in duration to affect the slow specific absorption and transport processes in cells. The culture dishes containing explants were removed from the coils after forty-eight hours of continuous treatment and left in the incubator for an additional forty-eight hours.

Neurite outgrowth was determined after four days of incubation according to a modification (Fenton et al., 1970) of the original semiquantitative technique devised by Cohen et al. (1964). It assigns a score to each ganglion based on the number, length, and degree of branching of the neurites emanating from the original explant. The scoring system assigns "zero" to a ganglion with no outgrowtb and +5 to a ganglion with maximal outgrowth. Treatment with $10^{-8}M$ 2.5s nerve growth factor routinely yielded a +5 score. To compare treatments, scores for all ganglia in their respective groups were added together, a mean and standard error of the mean were obtained. A Student's t-test was used to determine significance.

Additional morphological evidence of stimulation by direct current was obtained by fixing trigeminal ganglion cultures from the various treatment groups for two and one-half hours in 3.5% glutaraldehyde in 0.1*M* cacodylate buffer containing $CaCl_2$ followed by two hours in 1% $O_5U_4$0.1*M* cacodylate buffer and subsequent dehydration and imbedding in Epon®. Sections 1μ thick were mounted on microscope slides and stained with 0.1% toluidine blue.

To ascertain the effect of these treatments on calcium efflux patterns, trigeminal ganglia were dissected out and treated immediately, electrically or with NGF, for twenty hours; then ^{45}Ca was added to obtain a final concentration of 1μc/ml of tissue culture medium. While still under treatment, the tissue explants were exposed to the ^{45}Ca for ninety minutes to maximally load the tissues with radioactive calcium. At the end of that time, the cultured explants were carefully taken off the culture dish, placed on a polycarbonate filter, and washed with 2 ml aliquots of Dulbecco® phosphate-buffered saline *minus* $CaCl_2$ at one minute, two minutes, five minutes, and ten minutes. Each wash was collected in a separate vial and saved for counting. At the end of the wash period, the tissue was placed in 10% sodium dodecyl sulphate for two days to be solubilized. Aliquots of the solubilized tissue samples were used to determine both the protein content (Lowry method, 1951) and the content of

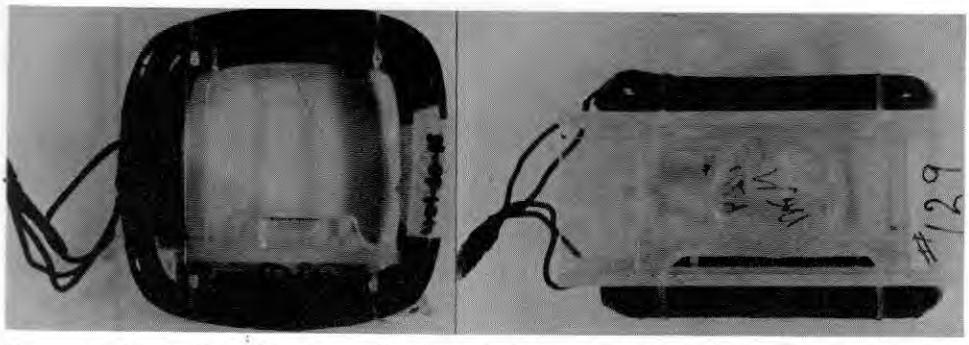


Figure 14-4A: Coils with culture dish for administration of electromagnetically induced current.

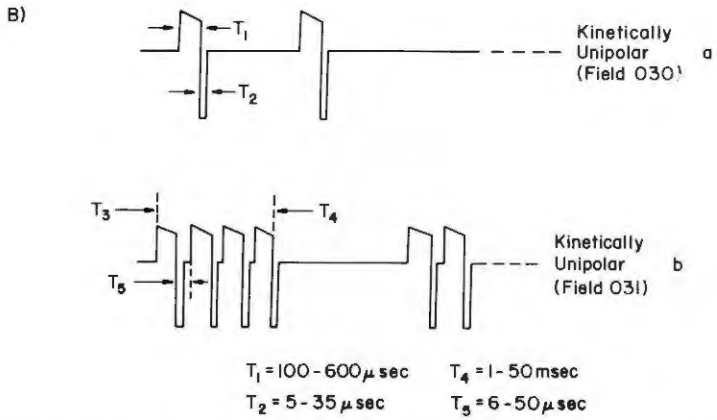


Figure 14-4B. Waveform configuration for field #030 and #031.

isotope using a liquid scintillation system. All counts were normalized to courts per minute per μg tissue protein. Counts for washes and tissue were added together to give total ^{45}Ca uptake as CPM/μg protein. Thereafter, the percentages remaining in the tissue after each wash period were calculated.

Results

Only minimal neurite formation was obtained in untreated control cultures of trigeminal and sympathetic ganglia; those from dorsal root ganglia explants gave variable results. Table 14-I presents the data obtained from both sensory (trigeminal, dorsal root) and sympathetic (lumbar chain) ganglia after either direct or induced current. In comparison to control cultures, both types of electrical treatment significantly stimulated neurite outgrowth from both types of sensory ganglia. However, sympathetic ganglia were not affected by the induced waveform signal but *did* respond to direct current. In comparison to the score obtained in

TABLE 14-1
EFFECT OF DIRECT OR INDUCED CURRENT ON NEURITE OUTGROWTH OF SENSORY AND SYMPATHETIC GANGLIA IN VITRO

		Induced Current		
	Control	*#030 Unit*	*#031 Unit*	*Direct Current*
Trigeminal Ganglion				
Number of Ganglia	46	53	11	45
Mean ± S.E.M.	0.93 ± 0.16	2.51 ± 0.21	1.09 ± 0.47	2.15 ± 0.11
p =		.001	not significant	.001
Dorsal Root Ganglion				
Number of Ganglia	34	17		38
Mean ± S.E.M.	1.5 ± 0.28	3.06 ± 0.36	2.71 ± 0.13	
p =		.001		.01
Sympathetic Ganglion				
Number of Ganglia	18	10		18
Mean ± S.E.M.	0.39 ± 0.29	0		2.27 ± 0.36
p =				.001

cultures treated with nerve growth factor, both types of electrical stimulation produced about a half-maximal response. Of the two different induced waveforms shown in Table 14-I, only the single pulse configuration (#030 unit) consistently produced positive effects.

Examples of typical cultures of ganglia treated either chemically (NGF) or electrically (D.C. or I.C.) are presented in Figures 14-5 through 14-7. Scanning electron micrographs of trigeminal ganglia (Fig. 14-5) at three different magnifications demonstrate that remarkable neurite outgrowth was obtained after exposure to low levels of direct current (Fig. 14-5D). A profuse growth of fibers have emanated from the original explant in the direct current (Fig. 14-5D) and nerve growth factor-treated cultures (Fig. 14-5G). Higher magnification (1200×) of these fibers reveals a more complicated picture; the outgrowth fibers appear as multi-stranded, intertwining cables (Fig. 14-5E). NGF cultures (Fig. 14-5H) contain, in addition, many more glial and nonneuronal cells that obscure the complex cable arrangement of nerve fibers. Under our culture conditions, the distal ends of the neuronal processes always appear to make contact with migrated nonneuronal cells; fibers ending on the surface of the dish were rarely seen. The termination of these fibers was, in many cases, quite complex, producing extensive growth cones that expanded into a variable number of microspikes. This can be seen in the direct current group (Fig. 14-5F), with maximal growth cone expansion occurring after NGF treatment (Fig. 14-5I)

Representative phase contrast and dark field micrographs of cultures after the three types of treatment are presented in Figure 14-6. Although either type of electrical treatment produces extensive outgrowth of nerve fibers (Fig. 14-6B, C, D, F), a bushy profusion of fibers is seen only after

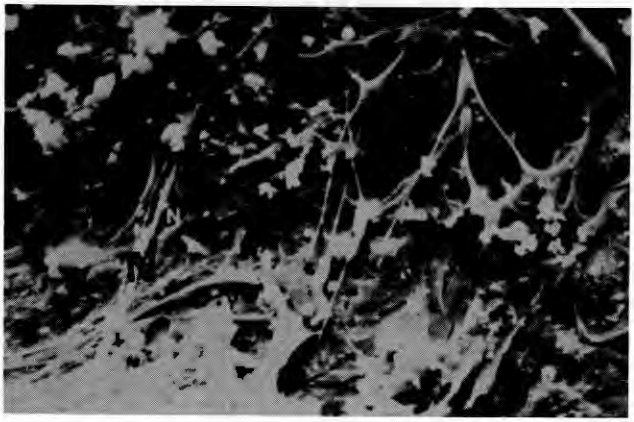
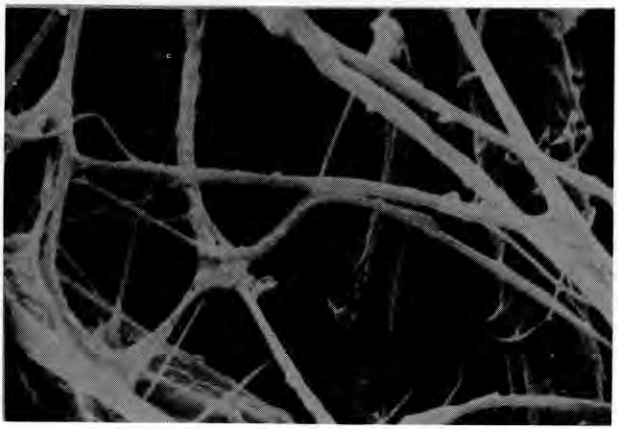
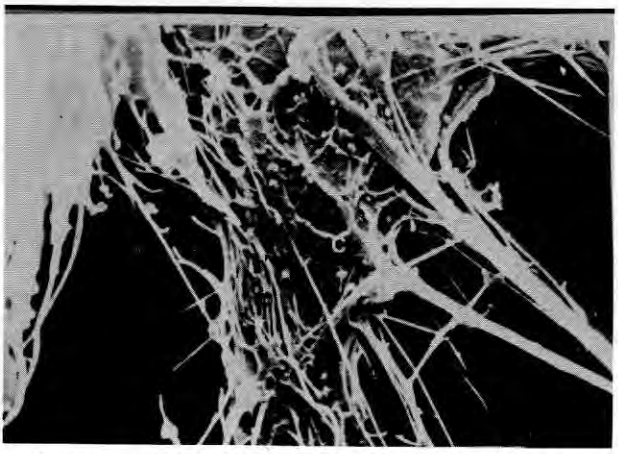


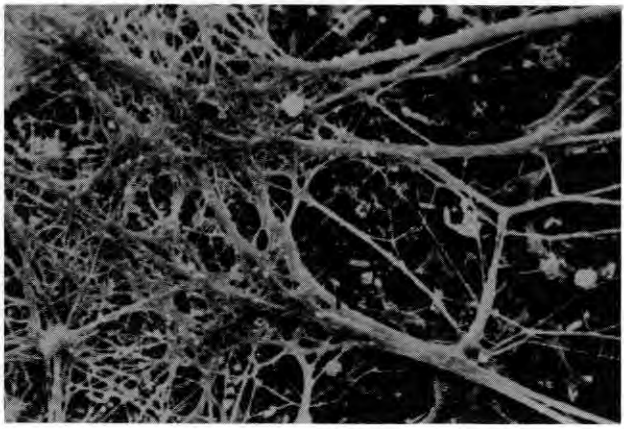
Figure 14-5: Scanning electron micrographs of trigeminal ganglia. Cultures after four days of continuous treatment. *A*. Control, ×120. N = neurites.



B. Control, ×1,200.

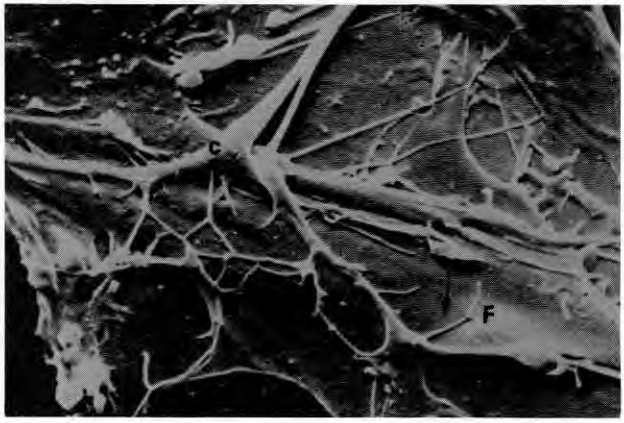


C. Control, ×3,000. C = growth cone; F = fibroblast.

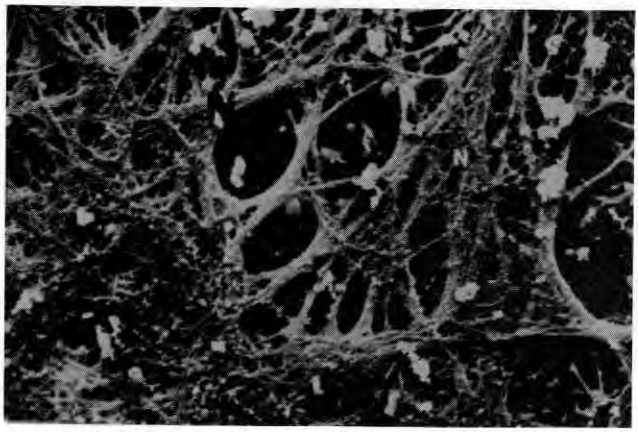


D. Direct current, ×120. N = neurites.

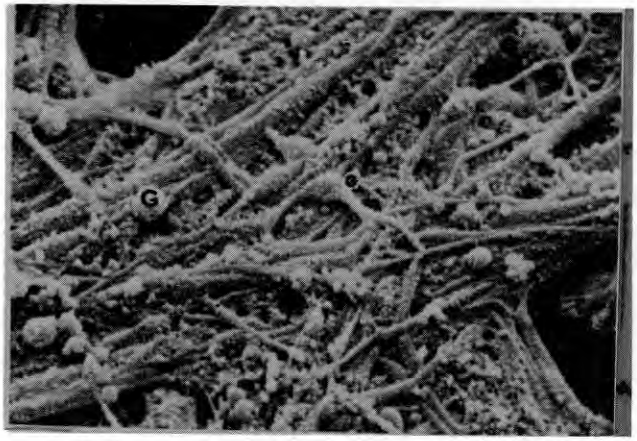
E. Direct current, ×1,200. G = glia.



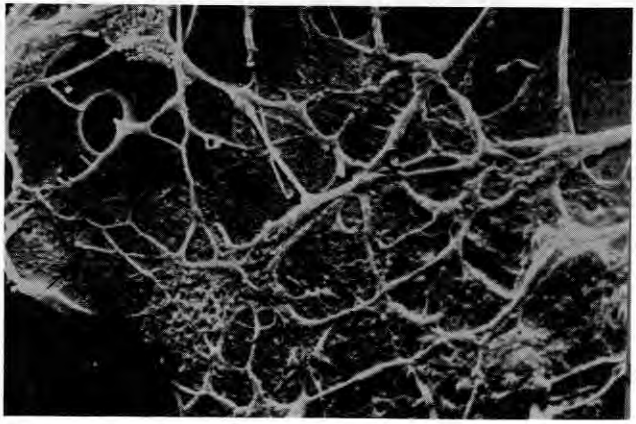
F. Direct current, ×3,000. C = growth cone; F = fibroblast; arrow = microspikes from growth cone.



G. Nerve growth factor, ×120. N = neurites.



H. Nerve growth factor, ×1,200. G = glia.



I. Nerve growth factor, ×3,000. C = growth cone; F = fibroblast; arrow = microspikes from growth cone.

maximal doses of NGF (Fig. 14-6A, E). Direct current most often elicits very long fragile processes (especially in dorsal root ganglia) (Fig. 14-6D) while the induced current waveform produces shorter processes that are not as dense as those seen after treatment with NGF (Fig. 14-6B, C).

Examples of neurons residing in the original explant (trigeminal ganglia) are presented in Figure 14-7 which shows 1 micron Epon sections of explants fixed after four days of continuous treatment (control, direct current, NGF) (Fig. 14-7B, E, F, C) or two days of single pulse induced current followed by two days of normal incubation (Fig. 14-7D). Unincubated eight day ganglia are also included (Fig. 14-7A) to compare the relative size of the neurons after four days in vitro. Most eight day neurons contain proportionally smaller amounts of cytoplasm relative to the nucleus. After four days of incubation with no treatment (Fig. 14-6B), most neurons have disintegrated and the culture contains mostly nonneuronal cells. The neurons that have survived are somewhat larger. The neuronal population after continuous direct current has largely survived and many of the neurons have not only increased in size beyond that of the control group but have also undergone differentiation (Fig. 14-7E, F). Neurons treated with single pulse induced current (Fig. 14-7D), which provoked neurite outgrowth from them, demonstrate a different series of events that will be explored in future studies. A large proportion of the neurons are filled with vacuoles and appear to be undergoing degeneration. It seems possible that initially they were stimulated to grow and send out processes and then underwent metabolic decline. Explants of trigeminal ganglia treated with NGF demonstrate the maximum degree of survival, hypertrophy, and differentiation obtained in our treatment series (Fig. 14-7C).

Representative sections of these trigeminal cultures were examined for relative increases in neuronal size as compared to unincubated ganglia. The maximum diameter of the neuronal population was determined for each group and the data are presented in Table 14-II. These data confirm the subjective evaluation that we made by scanning the sections.

To test the effect of these experimental procedures on Ca^{++} efflux

TABLE 14-II
PERCENT OF MAXIMUM DIAMETER OF EIGHT DAY UNINCUBATED TRIGEMINAL GANGLIA NEURONS

Treatment	*# Cells Measured*	*%*
Control	10	123
IC	20	123
DC	32	141
NGF	26	153

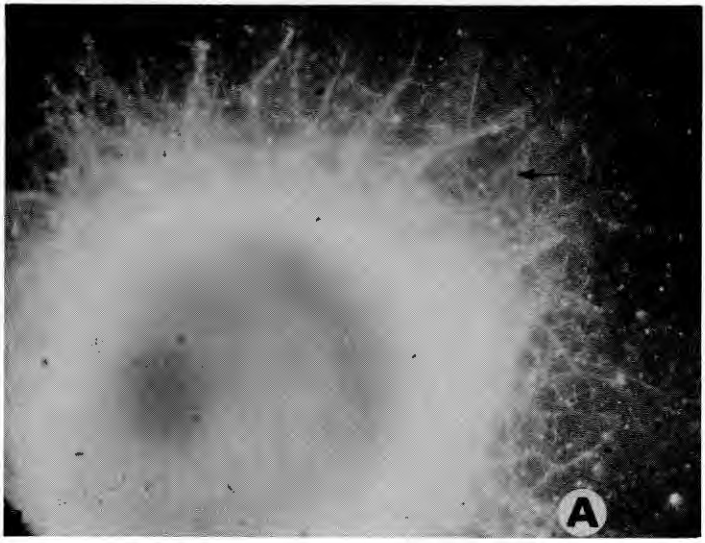
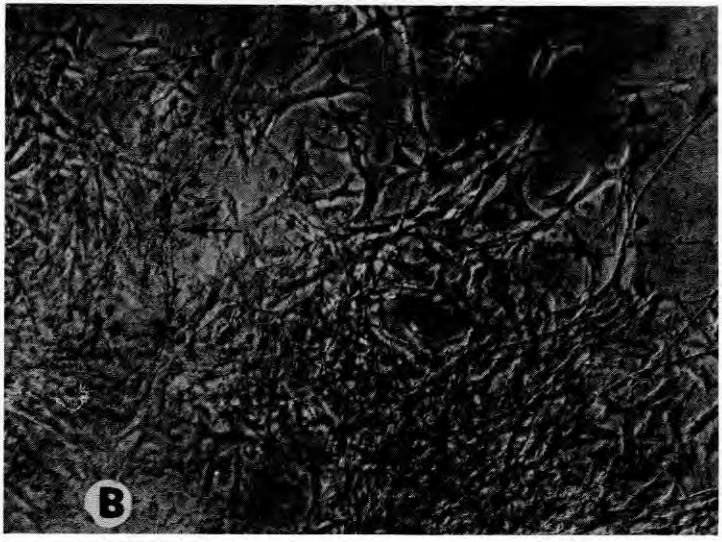
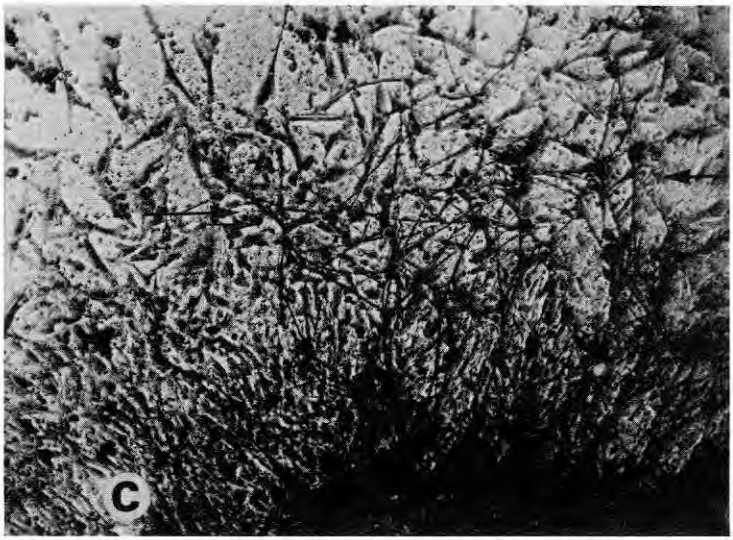


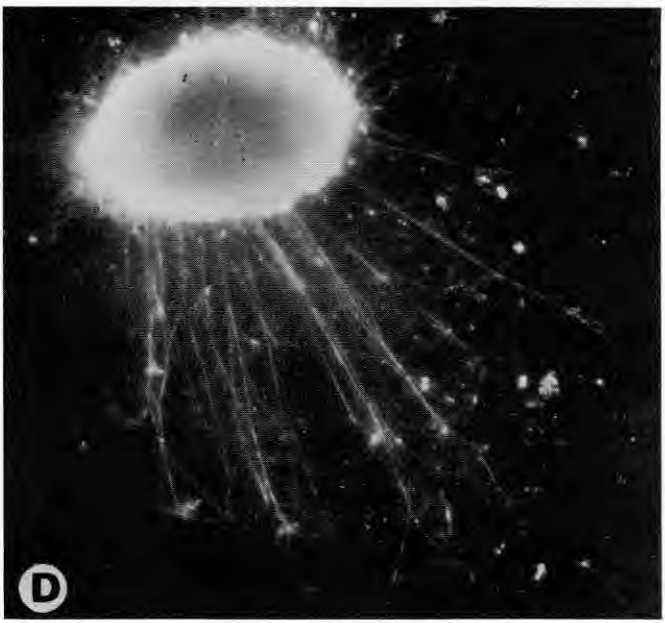
Figure 14-6. Phase and darkfield photographs of different treatments on different ganglia. Note the density of fibers in ganglia treated with NGF (A, E) as opposed to the long, delicate strands produced by direct current (D, F). Induced current (B, C) produces an intermediate type of outgrowth. Arrows indicate neurites. *A*. Trigeminal, NGF, darkfield, ×60.



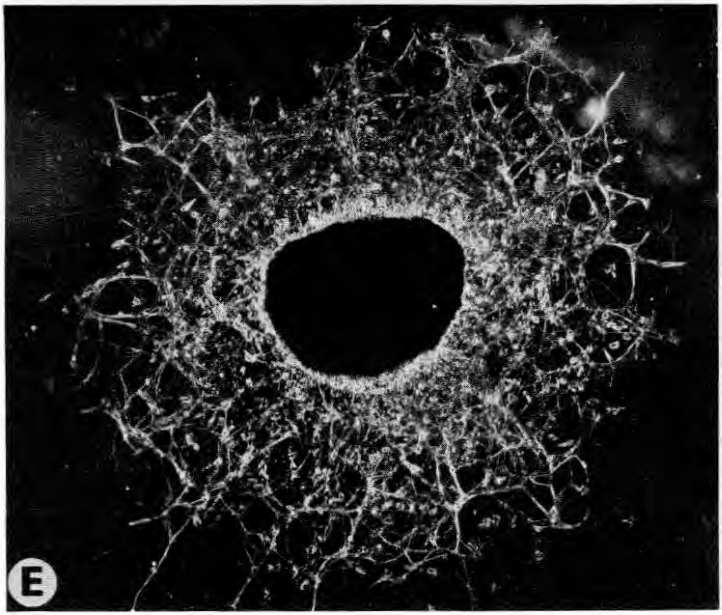
B. Trigeminal, induced current, phase, × 186.



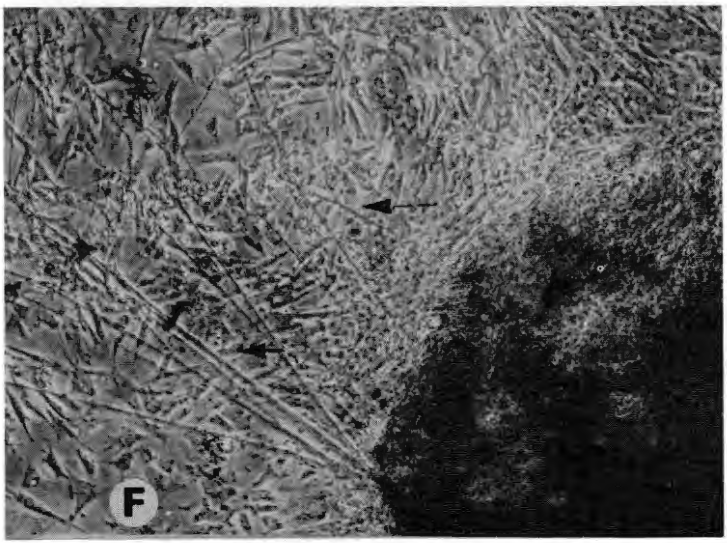
C. Dorsal root, induced current, phase, ×186.



D. Dorsal root, direct current, darkfield, ×50.



E. Sympathetic, NGF, darkfield, ×50.



F. Sympathetic, direct current, phase, ×200.

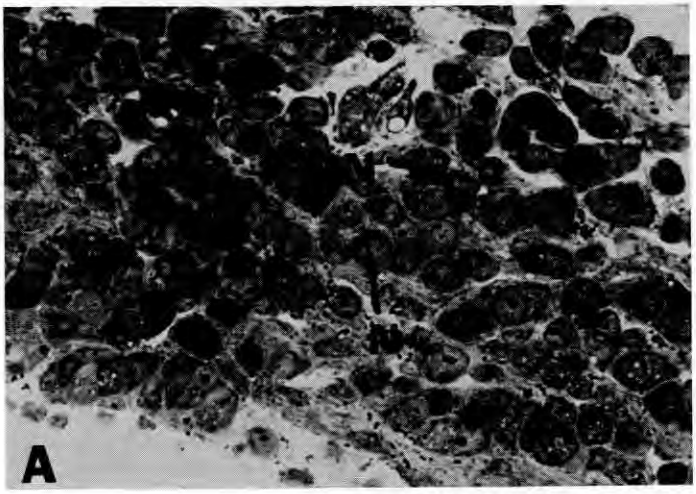
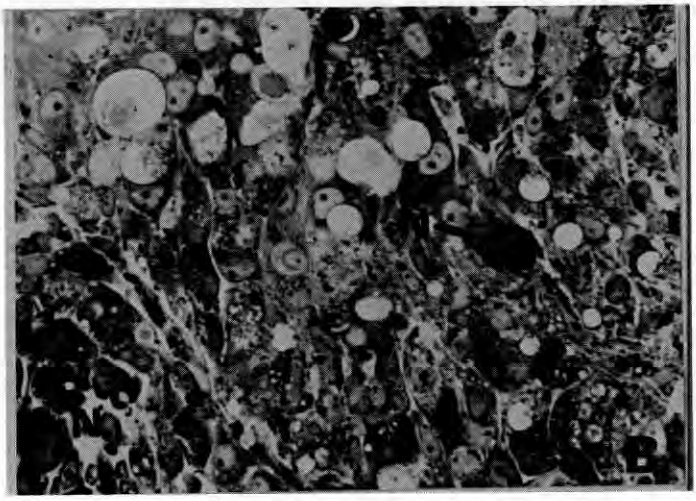
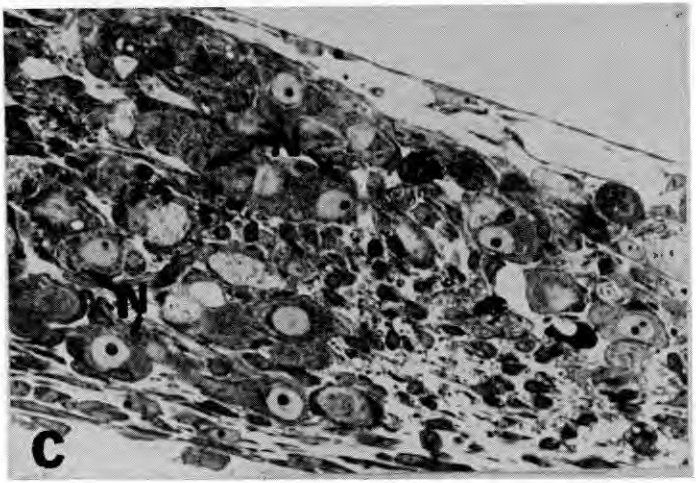


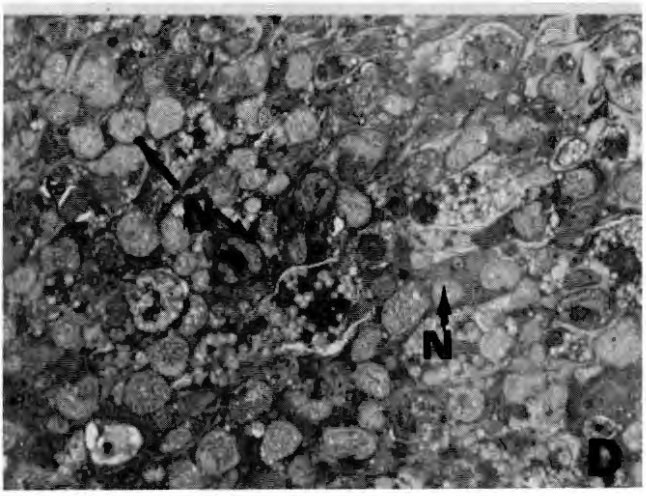
Figure 14-7. One micron Epon sections stained with Toluidine blue of the original explant of trigeminal ganglia. Four days after treatment in vitro. × 1,250. *A*. Unincubated ganglia contain both "light" and "dark" neurons.



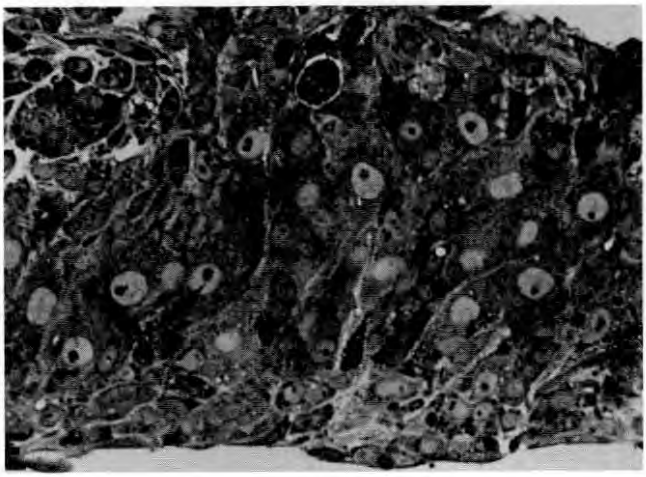
B. Untreated (control) cultures show many signs of degeneration including large vacuoles in cells, myelin figures, and distorted cell morphology.



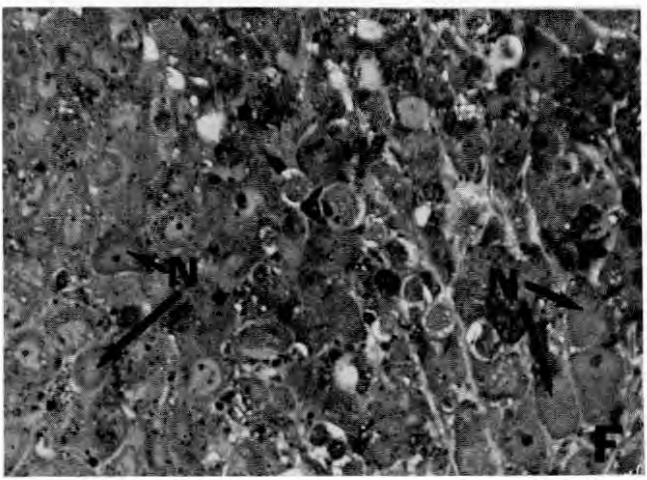
C. NGF cultures demonstrate good neuronal survival and differentiation.



D. Induced current appears to cause stimulation and then destruction of the neuronal population.



E. Direct current cultures demonstrate good neuronal survival and differentiation.



F. Direct current cultures demonstrate good neuronal survival and differentiation

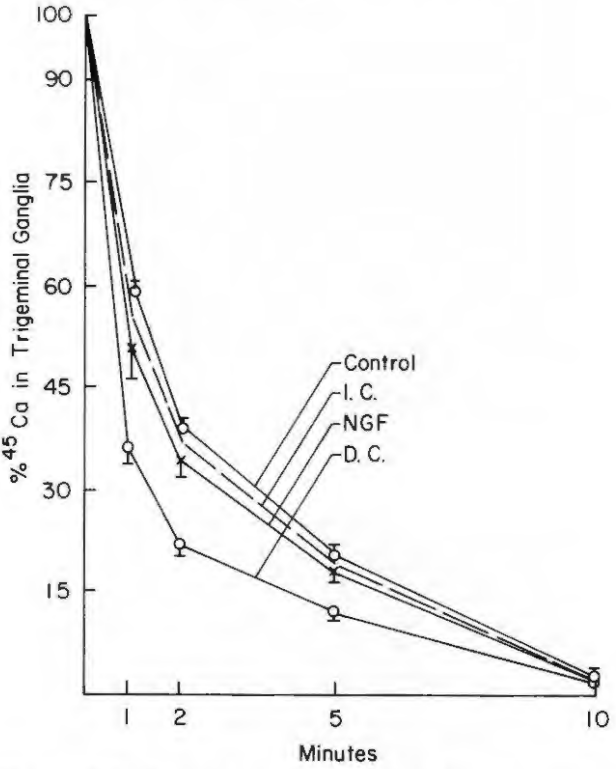


Figure 14-8. Efflux of ^{45}Ca from trigeminal ganglia. Successive washes with phosphate buffered saline minus calcium. Percentage remaining calculated on a basis of CPM/μg protein; Standard Error of the Mean (S.E.M.) are presented at each point.

patterns, treated ganglia were incubated for twenty hours, loaded with ^{45}Ca while under treatment for ninety minutes, and washed successively with calcium-free phosphate buffered saline. Samples of both tissue and washes were counted in a liquid scintillation counter. All counts for each sample of ganglia were added together to obtain total counts per minute per μg protein and the percent counts remaining in the tissue at each time period was plotted. The efflux curve generated is presented in Figure 14-8.

None of the treatments appear to have significantly affected the total uptake of Ca^{++} (Duncan's Multiple Range Test; Table 14-III). However, a plot of the percent of ^{45}Ca remaining at each time period (Figure 14-8) shows that the direct current treatment did significantly affect the wash-out kinetics of ^{45}Ca from the tissue. Within the first two minutes, approximately 61 percent of the total ^{45}Ca was removed from control, induced current, or NGF cultures; at this same time period, approximately 78 percent of the ^{45}Ca was removed from direct current treated cultures.

TABLE 14-III
TOTAL UPTAKE OF ^{45}CA AFTER NINETY MINUTES INCUBATION

Treatment	*Total CPM/μg Protein*
Control	165.08 ± 38.87
IC	204.94 ± 38.3
DC	177.84 ± 20.64
NGF	116.9 ± 17.28

Discussion

The purpose of these experiments was to compare the effects of different types of electrical stimulation on explanted sensory and sympathetic ganglia, and in turn to compare these to the well-known effects of nerve growth factor. In all cases, the final references were the control condition (no treatment) and a standard treatment, nerve growth factor (at a maximal concentration). We have also extended our previous endeavors to test low levels of direct current on different types of nervous tissue. Thus, we can now include another sensory ganglion (dorsal root) and the sympathetic chain ganglia as constituting discrete groups of neurons that respond to direct current. In addition, administration of a different mode of electrical stimulation, electromagnetic induced current, was also observed to stimulate neurite extension from sensory ganglia. However, only the single pulse waveform, as opposed to a train of pulses, has been shown to be effective; sympathetic ganglia were not stimulated by either type waveform.

Using all of the criteria that we have examined including the results from previous publications (Sisken and Smith, 1975; Sisken and Lafferty, 1979) we can make the following conclusions:

1. Direct current (in the range of 5-20 ηA/dish, current density range of 0.5-0.05 ηA/mm^2) applied in a nonuniform field in the absence of nerve growth factor produces a luxuriant outgrowth of neurites from explants of trigeminal, dorsal root, and sympathetic chain ganglia. The outgrowth obtained was about twice that found in untreated ganglia, but only one-half of that produced by maximal doses of NGF (Table 14-I). The processes produced by direct current stimulation tend to be longer and thinner, sparsely enveloped by glial cells, and grow towards the cathode.
2. Observation of the neurons residing within the original explant of cultures treated with direct current has revealed a pattern of increased survival of the neuronal population. The microscopic appearance of the cell bodies substantiates the relative gross differences seen in whole mounts, i.e. in cultures stimulated with direct current, the neuronal cells were larger and more differentiated than control neurons but

were not as large nor as differentiated as NGF-treated neurons (Table 14-II).

3. Uptake and incorporation of ^{3}H-leucine into eight day trigeminal ganglia appear to parallel the results mentioned above, i.e. direct current doubled the uptake and incorporation into ganglia relative to untreated ganglia, but was not as effective as the NGF treatment (Table 14-IV).
4. Efflux of ^{45}calcium was significantly affected *only* in direct-current-treated cultures. In this respect, NGF-treated ganglia responded in a manner similar to control cultures (Fig. 14-8).
5. The experiments concerning pulsed electromagnetically induced current, i.e. single pulse (+15 mV, 0.325 msec, 72 Hz), are too recent to evaluate in a general way. The pronounced cell degeneration that we found after two days of incubation and treatment followed by two days of incubation with no treatment must be explored.

In reference to points 1 through 3, the current densities (0.5-0.05 $\eta A/mm^2$ for 96 hours) that we have obtained in our culture system (nonuniform field) differ markedly from those used by other investigators. Marsh and Beams (1946) used a wedge-chamber with an agar-salt bridge to produce a nonuniform field and obtained a range of current densities; an optimum density of 120 $\mu A/mm^2$ stimulated neurites to grow to the cathode. The maximum incubation period was 28.5 hours, and no current effect was observed below 80 $\mu A/mm^2$. Jaffee and Poo (1979) cultured ganglia in nerve growth factor, then exposed them to an electric field in a rectangular chamber with an agar-salt bridge in a uniform field obtaining current densities of 1.92-192 $\mu A/mm^2$ to determine cathodal orientation. The maximum incubation period in the electric field was eight hours. They determined that the neurite growth rate to the cathode for seven to nine day dorsal root ganglia ranged from 11.8 (0.01 poise) $\mu m/hr$ to a maximum of 65 $\mu m/hr$ (1.0 poise). We reported a rate of 100 $\mu m/hr$ (.01 poise) for twelve day trigeminal ganglia (Sisken and Smith, 1975). It is difficult to compare the results in these two papers to ours since their current densities used were so much greater and were used for shorter time periods than in our experiments. Additionally, agar salt bridges were

TABLE 14-IV
UPTAKE AND INCORPORATION OF ^{3}H-LEUCINE INTO EIGHT DAY TRIGEMINAL GANGLION WITHIN THE FIRST TWENTY HOURS OF INCUBATION

	# Ganglia	*% of Ganglia*
Control	17	100
Direct Current	20.5	190
Nerve Growth Factor	14	210

employed to administer current, while we have used either platinum or tantalum wires, a system used by many clinicians to treat bone fractures. They have pursued primarily the question of growth orientation of nerve processes to the cathode, while we feel that our morphological and biochemical evidence has demonstrated a true *growth-stimulation* of the neuronal cell and its processes.

The question of electrode products due to the insertion of tantalum wire in the incubation medium has been addressed in the following ways. (1) Tantalum is a highly stable metal; examination of the media obtained at the end of our experiments using x-ray fluorescence spectroscopy (University of Kentucky Institute Mining and Minerals Research) has revealed no tantalum present (sensitivity $10^{-8}M$). (2) Preincubating media for three days with the electrodes and battery in place and "on" and then adding freshly dissected explants and turning battery "off" followed by observation of their growth after four days demonstrated no stimulation, i.e. they were comparable to control cultures. (3) Addition of Ta_2O_5 (10^{-6}-$10^{-8}M$) to the media had no deleterious or stimulatory effects on growth of ganglionic explants. (4) Since the possibility exists that the concentration of H_2O_2 is increased at the cathode, histochemical tests for peroxidase and catalase (Herzog and Fahimi, 1976) were performed on control ganglia and in ganglia treated with direct current. No differences in the concentration of either enzyme were noted.

In defining the binding characteristics of calcium ions, both Borle (1969) and Weiss (1978) refer to that portion of the calcium pool that is "readily depleted" (half-time of 1.58 min in HeLa cells, under 5 min in vascular smooth muscle) as a "low affinity Ca^{++}"; this Ca^{++} is bound to the extracellular surface glycoprotein of the plasma membrane. High affinity Ca^{++} is more tightly bound, located internal (perhaps intracellularly) to the low affinity component, and is removed more slowly. Since the effect of direct current on the efflux rate of ^{45}Ca occurs within the first two minutes (a 28% increase over control), we feel that the electrical current is acting primarily on the low-affinity, readily depleted Ca^{++} pool. A similar type of result was obtained by Bawin, Sheppard, and Adey (1976, 1978) who described the effects of weak electromagnetic fields on calcium efflux from chick cerebral tissue. A specific window of electromagnetic radiation (147 MHz amplitude modulated field) caused an increase of 10 percent or more in the rate of ^{45}Ca efflux.

In our experiments, twenty hour incubation with NGF ($10^{-8}M$) had no significant effect on ^{45}Ca efflux from trigeminal ganglia. Using rat phaeochromocytoma cells (PC 12), Landreth, Cohen, and Shooter (1980) demonstrated that NGF, which causes these cells to cease division and extend neurites, had no significant influence on either ^{45}Ca uptake or efflux rates. Their efflux studies consisted of preincubation with high

(300η*M*) concentrations of NGF for twenty-five hours or low concentrations of NGF (3η*M*) for sixty minutes. They conclude that the calcium ion may not be involved directly with the mechanism of action of NGF in these cells. However, an absolute requirement for calcium has been demonstrated for neurite outgrowth in the presence of NGF (7 ng/ml) in dorsal root ganglia (West and Stach, 1979).

Varon and Skaper (1980) and Varon and Adler (1980) have implicated the sodium ion rather than the calcium ion as having a primary role in the mechanism of action of NGF, especially as it relates to the NGF-receptor complex. Borle (1967) has reviewed the literature concerning calcium in the nervous system. He cites a number of theories and proposes that the calcium ion, which stabilizes the membrane, is associated with decreased permeability for sodium and potassium. Upon electrical stimulation, calcium ions are removed from some surface membrane sites, which then allow for increased *sodium* transport. Since many of the attributes of NGF are mimicked by direct current stimulation, and sodium ions may play a major role in NGF-action, the mechanism of action of direct current may also be ultimately to affect sodium transport.

SUMMARY

1. Both nAmp levels of direct current and pulsed electromagnetically induced current have been tested for stimulatory effects on neurite outgrowth in cultures of sensory and sympathetic ganglia of the eight day chick embryo.
2. Significant increases in fiber index over control values have been attained in cultures of sensory ganglia exposed to either type of electric current. Sympathetic ganglia were stimulated by direct current only. In all cases, the fiber index attained with either type of current was not equivalent to that produced by a standard preparation, $10^{-8}M$ 2.5s NGF.
3. Cultures of trigeminal ganglia treated with direct current not only produced long, complex fibers with expanded growth cones but also demonstrated an increase in neuron size and preservation; again, equivalence to the standard NGF effects were not observed.
4. Uptake and incorporation of ^{3}H-leucine into trigeminal ganglia increased twofold over control values after exposure to either direct current or NGF.
5. An increase in ^{45}Ca efflux within the first two minutes occurred in cultures exposed to direct current and did not occur in NGF or induced current treated cultures. This readily depleted calcium probably represents that portion of the calcium pool that is bound to the external surface of the cell membrane.

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