ELECTROPHYSIOLOGICAL AND MORPHOLOGICAL ANALYSES OF MOUSE SPINAL CORD MINI-CULTURES GROWN ON MULTIMICROELECTRODE PLATES

DISSERTATION

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By

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The electrophysiological and morphological properties of small networks of mammalian neurons were investigated with mouse spinal cord monolayer cultures of 2 mm diameter grown on multimicroelectrode plates (MMEPs). Such cultures were viewed microscopically and their activity simultaneously recorded from any 2 of 36 fixed recording sites. The specific aims achieved were: 1. development of techniques for production of functional MMEPs and maintenance of mini-cultures; 2. characterization of the spontaneous activity of mini-cultures; 3. application of inhibitory and disinhibitory agents; 4. development of staining methods for cultured neurons; and 5. initial light microscopic analysis with correlation of electrophysiological and morphological characteristics.

Mini-cultures represented a vastly simplified model of neuronal networks *in situ*. Cultures exhibited vigorous, spontaneous, coordinated activity, primarily in the form of bursts. Activity levels increased with culture age up to 4 weeks and also varied with neuronal density. Responses to

disinhibition (bicuculline, strychnine) and inhibition (glycine, GABA) were organotypic. After disinhibition, cultures of less than 500 neurons displayed epileptiform activity similar to that observed *in situ*, with increases in burst frequency, regularity, and stereotyping of burst envelope form.

The Bodian-Nissl stain was developed from section staining methods, allowing light microscopic analysis of mini-cultures and correlation of morphology with activity. Cultures exhibited such great complexity that correlation of a spike with its source could not often be achieved. Morphological and electrophysiological variability between cultures was not eliminated by control of culture variables.

Further investigations will include: 1. culture improvements and culturing of other nervous system regions; 2. selection of mini-cultures for experiments, reducing experimental variability; 3. expanded electrophysiological data collection systems with computer-assisted analysis; 4. expanded pharmacological testing; and 5. development of a histological battery for comprehensive morphological analysis. This research exposed both the advantages and difficulties of studying neuronal networks grown on MMEPs and serves as a springboard for planning of future experiments.

ACKNOWLEDGMENTS

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Rita and I are pleased to announce that "our" dissertation is complete. Mato and Cali greatly assisted me in setting appropriate priorities. I am very grateful to Carol Gardner, the Gathings family, and the Cloutier clan, who commiserated and listened, and to Sallie, who mainly just listened. Finally, I remember with special fondness the various sessions with Cindy, Essie, Becky, and Gay, when we explored pendulum theory, astronomy, and botany and engaged in affective prose.

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CHAPTER 1

PROJECT INTRODUCTION, SPECIFIC AIMS, AND RATIONALE

Nervous System Performance as a Network Phenomenon

The complex signal transmission, processing, and storage tasks which the mammalian central nervous system routinely performs depend on the networking of millions of neurons. It is becoming increasingly clear, however, that basic computational phenomena, such as simple pattern generation, response reliability, and fault tolerance, arise from the properties of small networks of 30-100 neurons (Mountcastle, 1978; Delcomyn, 1980; Shaw et al., 1982). The interactions of neurons forming functional circuits in the vertebrate central nervous system (CNS) remain undefined, although some theoretical progress is being made (Grossberg, 1971; Hopfield, 1982; Cohen and Grossberg, 1983; Hopfield and Tank, 1986; Linsker, 1986; Cooper and Scofield, 1988; Selverston, 1988). An extreme paucity of biological data exists in this important area of neuroscience, and the chemical and structural complexity of CNS tissue necessitates the development of simplified experimental model systems for the study of neuronal networks.

Review of Preparations for Electrophysiological Studies

Invertebrate Preparations

In order to approach the problem of correlating nervous system structure with its physiological function, a number of workers have performed a variety of elegant experiments using the simpler neuronal circuits of invertebrates (Stent, 1983; Abrams, 1985; Getting, 1985; Miller and Selverston, 1985; Getting, 1988; Harris-Warrick and Flamm, 1987). Invertebrate nervous systems are generally composed of only a few thousand cells arranged in easily accessible ganglia (Miller and Selverston, 1985; Benjamin et al., 1985) Invertebrate neurons are also typically much larger than those of vertebrates, allowing easy impalement of cells for electrophysiological monitoring. The ganglia of invertebrates are composed of rigidly defined neuronal populations. This stereotyping allows the characterization and experimental manipulation of specific identified neurons (Marder and Hooper, 1985; Ross et al., 1987; Chiel et al., 1988).

Although research with invertebrate preparations has resulted in circuit diagrams for specific networks and useful generalizations concerning nervous system physiology, the results achieved with invertebrates do not necessarily represent the morphological and physiological characteristics of higher animals. For example, electrotonic synapses (gap junctions) occur frequently in

invertebrate neuronal networks and have major impact on activity patterns (Eccles, 1964; Miller and Selverston, 1982), but such synapses are less common in vertebrate systems (Fischbach and Dichter, 1974; Fulton, 1986). Invertebrate neurons also exhibit a high degree of process arborization (Hildebrande, 1985). Thus, even though invertebrate nervous systems are considered simpler than those of vertebrates based on neuronal populations, their extensive dendritic tree formation results in extraordinarily complex cell-to-cell interconnections.

Vertebrate Preparations

In Situ Studies

Although the study of vertebrate nervous systems presents some technical problems, researchers have developed several strategies to study the complex vertebrate neuronal circuitry. Implantation of probes into CNS regions allows the monitoring of nervous systems *in situ* (Mountcastle, 1978; McClellan and Farel, 1985; Freeman and Baird, 1987). Often, these experiments can provide long-term electrophysiological data from behaving animals, and whole system responses to pharmacological agents and other stimuli can be tested. Despite these advantages, work with vertebrate nervous systems *in situ* poses some limitations. Surgical procedures and probe insertion can result in cellular damage, so that monitored activity only

approximates that of intact systems. The investigation of small groups of neurons in these preparations is difficult due to the influence of other interconnected neural systems. This problem can be only partially mitigated by surgical or pharmacological simplification (Delcomyn, 1980; Grillner and Wallen, 1985; Alonso et al., 1987). Finally, probes must generally be positioned using stereotactic coordinates, and the cells being monitored cannot be visualized during the assay period.

Studies With Isolated CNS Regions

The relative inaccessibility of the intact vertebrate CNS has provided an incentive for the study of dissected nervous system pieces or tissue slices (Crain, 1976; Gahwiler, 1981; Droge and Leonard, 1983; Thomson et al., 1984; Dekin et al., 1985; McClellan and Farel, 1985; Horne et al., 1986; Smith and Feldman, 1987). These preparations approximate to varying degrees the physiological behavior of their counterparts *in situ*. The behavior of an isolated subregion can be studied in the absence of influence from other CNS areas. Pharmacological agents may be applied directly to the tissue, with more clearly defined effects.

Nevertheless, the use of isolated CNS regions poses certain limitations. Long-term experiments cannot be performed because the preparations are generally not viable for more than 24 hr. In addition, even the simplest of

these preparations consists of several layers of cells and retains enormous physiological and morphological complexity. Only a few of the neurons participating in the functional circuit can be visualized during the assay period or identified after intracellular dye injection. Thus, determination of all neuronal interconnections in even these simplified systems is exceedingly difficult. Tissue dissection or sectioning without allowing a lengthy recovery period introduces reaction to trauma as an experimental variable. The passage of electrodes through layers of tissue can also cause additional damage to the network being monitored. The maintenance of small CNS fragments or explants in culture (Gahwiler et al., 1973; Gahwiler, 1981) allows the design of long-term experiments and recovery from dissection reactions, but the other limitations inherent to the study of multi-layered tissue remain.

Studies With Dissociated Monolayer Cultures

In recent years, culture methods have been perfected which allow cellular monolayers of dissociated subregions of the CNS to be maintained and studied for periods of up to several months (Ransom et al., 1977; Jackson et al., 1982; MacDonald et al., 1986; Mattson et al., 1988). The cell density of these cultures is greatly reduced relative to that of the intact nervous system, and the effects of variables such as myelination and vascularization are

absent. Although these cultures may not exhibit all of the characteristics of the corresponding CNS regions *in vivo*, they do serve as greatly simplified cellular models of neuronal network behavior.

Neurons in culture form an essentially two-dimensional monolayer network atop a glial cell carpet. Even though the tissue is dissociated and cells are seeded randomly, the neurons appear to interact in a specific manner (Fischbach and Dichter, 1974; Camardo et al., 1983). They form synapses and generate spontaneous, coordinated bursting patterns that are frequently rhythmic (Nelson and Peacock, 1973; Gross and Lucas, 1982; Thomson et al., 1984; Droge et al., 1986). Responses to pharmacological agents often mimic those observed in the intact animal (Crain and Bornstein, 1972; Droge et al., 1986). Exposure of cell monolayers to applied pharmacological agents is achieved even more rapidly and with greater uniformity than in tissue slices. Finally, under optimal conditions, each neuronal cell body and its major processes which contribute to the monitored activity can be visualized during the electrophysiological assay, a major advantage that studies with intact animals, tissue slices, or even explant cultures cannot consistently provide (Gahwiler, 1981).

Review of Conventional Electrophysiological Monitoring Methods

Intracellular Recording

Monitoring of the electrical activity of neurons in culture may be accomplished using either intracellular or extracellular monitoring methods. Intracellular recording requires the impalement of neurons and is used to measure membrane potentials of individual neurons and to chart small, low-frequency changes such as subthreshold potentials (Snodderly, 1973). However, mechanical disturbances arising from external vibrations and temperature fluctuations can result in either tissue or electrode movement with consequent loss of resting potentials. Therefore, long-term intracellular monitoring is impractical, and reimpalement of small vertebrate neurons for developmental studies is generally not feasible.

Dye Monitoring of Activity

A recent technique involves electrical recording using activity-sensitive dyes which are applied to and taken up by neurons. When combined with a photo-detection system, the activity of all of the neurons in a microscopic field may be monitored simultaneously (Grinvald and Segal, 1984; London et al., 1987). Unfortunately, the dyes developed so far are toxic, and recording times for cells in culture are

limited to less than 1 min. This extreme limitation of assay time precludes the use of dye monitoring as the sole means of obtaining electrophysiological data.

Extracellular Recording

For conventional extracellular analyses, an electrode is placed in close proximity to the cells to be monitored, and the difference in potential between it and a reference electrode elsewhere in the preparation is measured. Extracellular electrodes cannot yet be used to chart small, subthreshold potential changes because signal strength decreases as a function of the electrode distance squared. However, high magnitude, transient events such as action potentials are of sufficient signal strength to be detected above noise levels (Buchwald et al., 1973). Because extracellular analysis of cultured monolayers is noninvasive, a culture may theoretically be monitored periodically for several weeks or longer without mechanical damage to the target cell or to other network components.

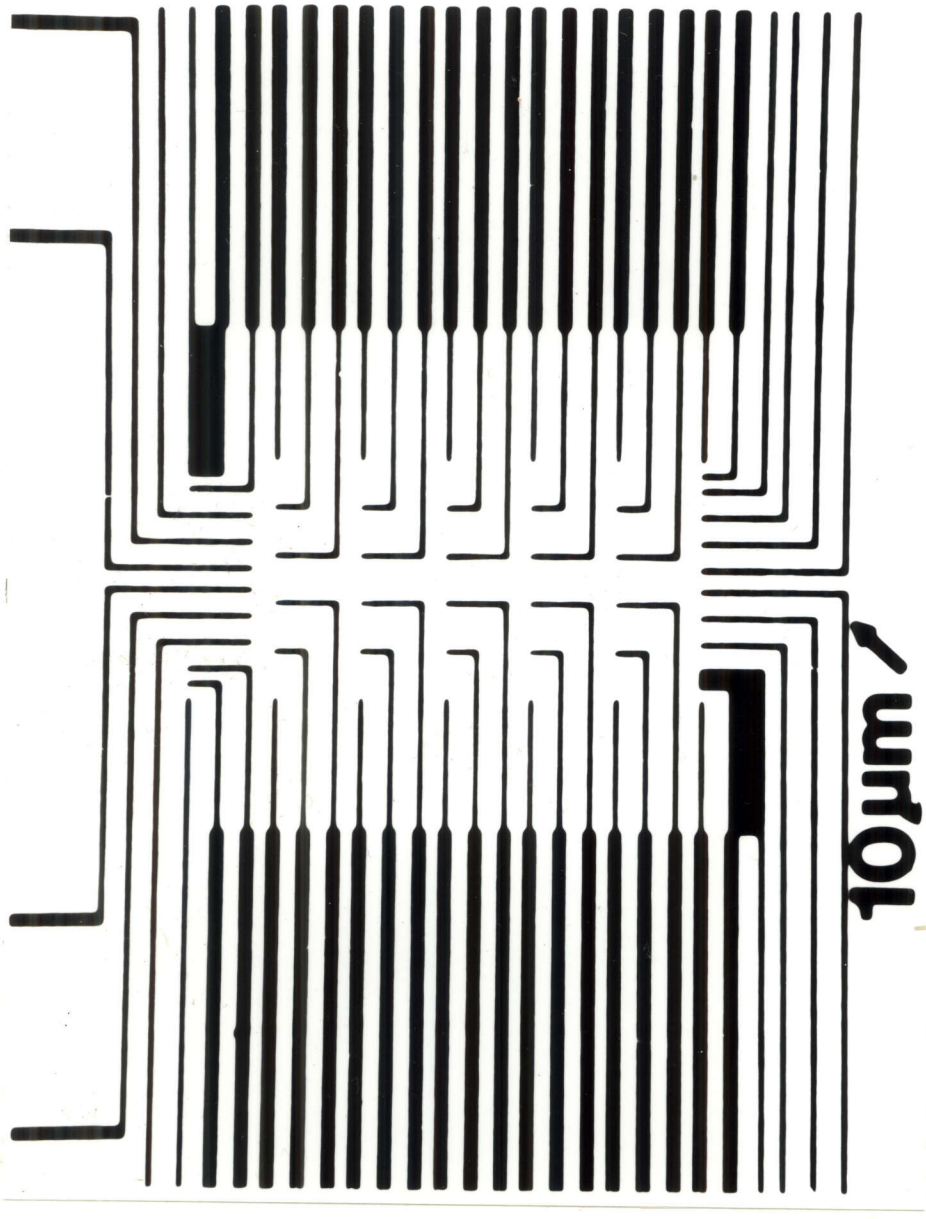
Several investigators have studied the electrophysiological properties of neurons in culture utilizing intra- or extracellular electrodes brought into a culture chamber (Crain, 1976; Bergey et al., 1987). Although these methods have allowed the initial characterization of neuronal network properties *in vitro*, their use is cumbersome. The number of electrodes which

can be employed simultaneously, and thus the number of cells which can be monitored, is constrained by space limitations and the need for microscopic visualization of electrode placement. The characteristics of network behavior have therefore been deduced from the sequential monitoring of a limited number of observation sites. In addition, these methods require open chambers, making pH and osmolarity variations difficult to control. These variations result in excessive culture stress, so that long-term monitoring of the same neuronal circuit during its formation and development is difficult or impossible.

The Multimicroelectrode Plate (MMEP) as a Tool for Extracellular Recording

Some researchers have devised alternatives to bringing the electrodes to the cell culture by seeding neurons upon electrode matrices (Thomas et al., 1972; Gross et al., 1977; Pine, 1980). One such electrode matrix design is called the multimicroelectrode plate (MMEP), a glass plate with a fixed pattern of metal, thin-film conductors photoetched upon it (Gross et al., 1977; Gross, 1979; Gross and Lucas, 1982; Gross et al., 1985). The center electrode array is continuous with photoetched leads extending to the edge of the plate to serve as contact strips for amplifier probes. The MMEP allows simultaneous, multi-site, extracellular monitoring of neurons cultured upon it (Fig. 1).

Figure 1. Schematic diagram of a 38-electrode MMEP showing the central photoetched conductor pattern.


10µm

The fixed MMEP electrode matrix allows the monitoring of only those neuronal network components adhered on or near the conductors. While random cell seeding and adhesion make selection of the cells to be monitored a function of their proximity to recording craters, the multiple conductors permit a much more thorough, multi-site sampling and characterization of neuronal circuit activity than is attainable with external electrode systems. Because MMEP monitoring is noninvasive and can be used in conjunction with closed chambers and perfusion systems, the functional development of a neuronal circuit can be studied over periods of up to several months. In addition, visualization of network components during electrophysiological recording is possible.

Characterization of Neuronal Mini-Cultures on MMEPS: Specific Aims

The MMEP promises to be a useful tool for the analysis of network properties of neurons in culture, but it is a recent innovation whose potential as well as technical and neurobiological limitations must be systematically characterized. The objective of this project was to apply the MMEP to the study of small neuronal monolayer cultures. In order to achieve this objective, the specific aims of the project were as follows: 1. modification of existing preparative protocols to produce optimally functional MMEPs

and development of techniques to produce and maintain cultures of only 300-2000 neurons growing in a restricted region over the MMEP recording matrix; 2. systematic characterization of the spontaneous electrical activity produced by these simplified neuronal monolayer networks; 3. application of inhibitory and disinhibitory pharmacological agents in order to explore the development of "organotypic" behavior in such cultures; 4. development of a new histological processing method for use with neuronal monolayers; 5. initial light microscopic morphological analysis; and 6. correlation of electrophysiological and morphological variables (Fig. 2).

Rationale for Specific Aims

MMEP Preparation and Mini-Culture Production

Insulation Clarity and Electrode Impedance Stability

One of the first project aims was to address the technical problems associated with the reliable production of MMEPs. First, the conductor insulation material exhibited optical deterioration upon exposure to warm saline, resulting in an inability to visualize cells growing on the insulation surface. Refinements of the insulation procedure were required to enhance insulation bonding to the MMEP plates, minimizing optical distortion and allowing morphological observation. Second, a difficulty arose in the use of MMEPs with transparent

Fig. 2. The specific aims of the project were addressed sequentially, as portrayed in this project flow diagram.

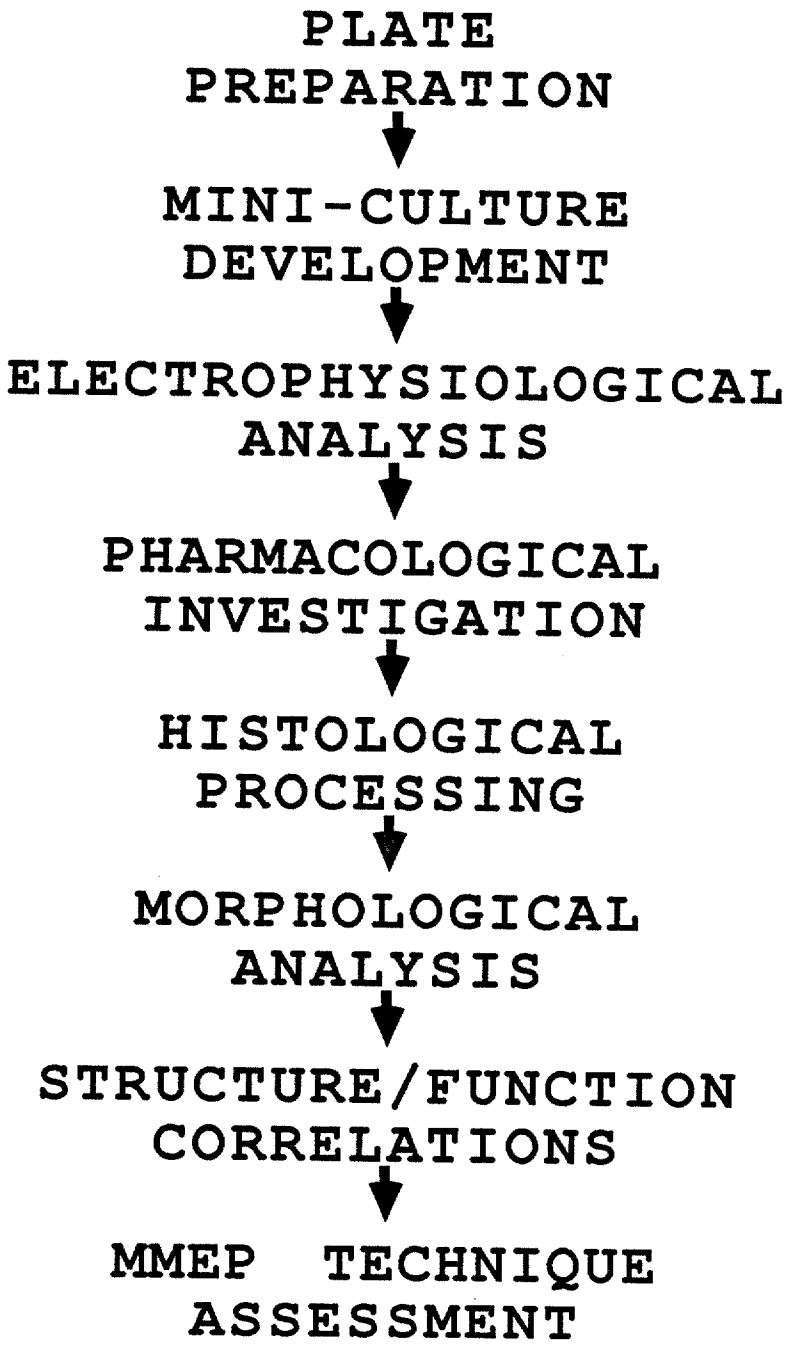


PLATE
PREPARATION
MINI-CULTURE
DEVELOPMENT
ELECTROPHYSIOLOGICAL
ANALYSIS
PHARMACOLOGICAL
INVESTIGATION
HISTOLOGICAL
PROCESSING
MORPHOLOGICAL
ANALYSIS
STRUCTURE/FUNCTION
CORRELATIONS
MMEP TECHNIQUE
ASSESSMENT

indium-tin oxide (ITO) conductors. The tips of such conductors had to be electroplated with gold to produce satisfactory electrode impedances. The investigation of various electroplating reagents and protocols was required for the production of stable impedance values.

Preparation and Maintenance of Spinal Cord Mini-Cultures

Spinal cord tissue was chosen as the first CNS subregion to be cultured on the MMEP (Gross and Lucas, 1982) because it represents a large, relatively homogeneous CNS fraction which is easily dissected from fetal animals. Initial electrophysiological studies were conducted with neuronal monolayers which covered the entire MMEP surface (Gross and Lucas, 1982; Gross et al., 1982; Gross et al., 1985). Because neurite outgrowth was extensive, activity monitored at the electrode sites could have been produced by a cell located >1 cm away from the recording matrix. Restriction of the culturing area to a 1-3 mm diameter region centered over the recording matrix was envisioned as a method to reduce network complexity and thereby to simplify morphological and electrophysiological correlations. However, modification of the established culturing protocol and investigation of the special maintenance requirements of these mini-cultures would be required.

Electrophysiological Characterization

Production of spatially restricted spinal cord "mini-cultures" consisting of only 300-2000 neurons was expected to result in the formation of neuronal networks of sufficient complexity to display spontaneous electrical activity, while still retaining the simplicity necessary for morphological characterization. It was not known, however, whether such small neuronal networks would survive in culture or whether they would generate the spontaneous, coordinated activity observed in larger, spatially unrestricted networks. If such activity was observed, an investigation of mini-culture spontaneous electrophysiological behavior would then follow. Activity characteristics such as the concentration of spikes into bursts of activity, the development of patterned activity, and the extent of activity coordination between electrode sites would be qualitatively compared with those which had previously been observed with larger networks.

Pharmacological Studies

Investigation of neuronal mini-culture responses to inhibitory and disinhibitory pharmacological agents was planned to follow the characterization of spontaneous activity. Investigation of mini-culture response to the application of glycine and gamma aminobutyric acid (GABA) and their antagonists would help to establish the influence

of these inhibitory neurotransmitters on network activity. In addition, mini-culture reactions would be compared with those observed by other researchers working with more complex preparations (tissue slices, intact animals, etc.). This comparison would be used to determine whether neuronal mini-cultures exhibited "organotypic" behavior. The display of such activity would help to establish the use of monolayer cultures as model systems for the study of neuronal network behavior.

Histological Processing

While phase contrast microscopy is valuable for visualization of living cells, it is inadequate for extensive morphological analysis. A routine histological protocol was required to: 1. allow the distinguishing of neurons from glial and other supporting cells present in dissociated spinal cord cultures; 2. stain fine neuronal processes to allow tracing of neuronal network components; and 3. highlight somal and dendritic morphology. In addition, any histological procedure developed for use with mini-cultures on MMEPs would have to be compatible with the organic insulation surface. In an attempt to develop a histological protocol which would meet these criteria, classical methods for the staining of sectioned neuronal tissue would be combined and modified for use with cell cultures.

Light Microscopic Morphological Analysis

After electrophysiological monitoring and histological processing, spinal cord mini-cultures would be characterized morphologically. The neuronal population would be counted and typed according to size and form. In addition, the documentation of the local network of fibers and somata surrounding each electrode might be possible. The mini-culture neuronal population would also be compared with that of less spatially restricted neuronal cultures.

Correlation of Electrophysiological and Morphological Variables

The acquisition of a body of data describing the structure and function of small monolayer networks would permit initial correlations between the morphological and electrophysiological characteristics of neuronal mini-cultures. The variability between cultures would be assessed, and minimization of this variability might be possible. The following relationships would be investigated: 1. neuronal density as a function of the number of cells seeded and culture age; 2. activity levels as a function of neuronal density and culture age; 3. the percentages of neuronal cell types as a function of neuronal density; and 4. the effect of differences in neuronal populations on observed activity levels.

MMEP Technique Assessment

Because this work represented the first systematic characterization of neuronal cultures using the multimicroelectrode plate, an assessment of the advantages and current limitations of the MMEP for the investigation of small neuronal networks would result. Many of the studies, such as initial pharmacological testing, would provide a base of data to justify the design of more extensive experiments requiring more sophisticated equipment and experimental protocols. The achievement of the listed specific aims would make culturing of neuronal mini-networks and histological processing routine procedures, provide basic information concerning the structural and functional characteristics of neuronal mini-cultures, and guide the development of future experiments.

CHAPTER 2

PRODUCTION OF SPINAL CORD MINI-CULTURES ON MMEPS: MMEP PREPARATION AND CULTURE MODIFICATIONS

Introduction

Initial electrophysiological experiments using tissue fragments and dissociated spinal cord cells cultured on MMEPs had been performed successfully prior to the planning of this study (Gross et al., 1977; Gross, 1979; Gross and Lucas, 1982; Gross et al., 1982). The first MMEPs were designed and manufactured by Siemens Corporation (Munich) and were fabricated with gold microelectrodes. After repeated use, these MMEPs became non-functional, and additional plates were not available from Siemens.

Polytronix, Incorporated (Richardson, TX) produced second-generation MMEPs, substituting indium tin oxide (ITO) electrodes for gold (Gross et al., 1985). These new MMEPs could potentially function as well as the original ones for electrical monitoring. In addition, while the opaque gold conductors had obscured portions of the culture, the ITO electrodes were transparent and allowed visualization of the neuronal network growing over the MMEP conductors. Thus, MMEPs with transparent electrodes promised to facilitate morphological analysis of cultured neuronal monolayers.

Despite the anticipated advantages of the new MMEP design, several technical problems were encountered in preparing the plates for culture. First, insulating the ITO MMEPs according to the procedure developed for gold MMEPs resulted in progressive optical deterioration of the insulation layer when it was incubated under warm saline during the culture maintenance period. This deterioration was often so severe that the neuronal monolayers could not be observed microscopically after only 2 weeks in culture. Clear cell visualization was essential for morphological analysis of neuronal networks, and modification of the insulating protocol was required to improve the optical stability of the MMEP insulation layer.

In addition to the insulation problem encountered with the new MMEPs, it had previously been determined that the ITO conductors required the electroplating of a layer of gold onto the conductor tips to reduce the electrode impedances (Gross et al., 1985). However, the plating procedure in use did not produce stable impedances. It was therefore essential to develop a method for reliable electrode plating to allow monitoring of spike activity on ITO MMEPs.

Modifications of the culturing protocol were also necessary in order to reduce neuronal network complexity. The MMEP insulation layer (DC 648, Dow Corning, Midland, MI) also served as the surface upon which cells were seeded, but

this polysiloxane resin was extremely hydrophobic and would not support the attachment and growth of cultured cells. However, Lucas and co-workers (1986) determined that brief, direct exposure of the DC 648 to a propane flame (1 sec) produced a hydrophilic surface which supported cell adhesion.

In initial experiments, the entire MMEP insulation layer was flamed, allowing the growth of a large, complex neuronal monolayer network over the expanse of the MMEP surface (Gross and Lucas, 1982, Gross et al., 1985). Neurons adhered on opposite sides of the plate (up to 3-4 cm apart) could conceivably interconnect via their neurites and could contribute to the activity monitored from the central recording matrix. Flaming of selected regions of the insulation through a metal mask was proposed as a method to produce a greatly restricted cell adhesive region (Lucas et al., 1986). Since cells would adhere and grow only on the areas of the MMEP contacted by the flame, it would be possible to create "recording island cultures" of 1-2 mm in diameter centered over the MMEP electrode matrix.

The production of recording island cultures would simplify network analysis by confining cells to the recording matrix region, limiting the neuronal population to be monitored, and restricting process extension. However, it was not known whether such small networks could be maintained in long-term culture, and extensive modifications

of the culturing methods might be required to enhance the survival of neurons in mini-culture. For example, the larger cultures probably produced sufficient medium conditioning factors necessary for neuronal survival and development, whereas restricted cultures might require treatment with conditioned medium (Banker, 1980; Sakellaridis et al., 1986; Unsicker et al., 1987).

As an alternative to the exogenous application of conditioned medium, the requirement for trophic factor enrichment might be met by co-culturing larger neuronal networks on the MMEP which were spatially separated from the mini-culture. Such conditioning cultures would secrete the medium conditioning factors required for the maintenance of the small central culture but would not be a part of the recording island network. The conditioning areas could also serve as internal controls for comparison of mini-culture morphology with that of less restricted cultures.

Materials and Methods

MMEP Preparation

MMEP Insulation

MMEPs with 38 photoetched indium-tin oxide conductors were custom manufactured by Polytronix, Inc. (Richardson, TX). New MMEPs were cleaned thoroughly prior to insulation by soaking them in a detergent solution (Sunlight, Lever Brothers, New York, NY, 8 hr), followed by boiling in 3%

hydrogen peroxide (20 min), thorough rinsing with deionized water, and air drying. Previously used MMEPs could be recycled by removal of the insulation layer with a 6% KOH/82% ethanol/12% water solution (8-12 hr), followed by the standard cleaning procedure.

After cleaning, MMEPs were insulated by application of polysiloxane resin (DC 648 Semiconductor Coating, Dow Corning) according to the method developed for gold MMEPs by Gross (1979). While the MMEP was spinning at approximately 4000 rpm, a 100-µl drop of the resin solution was applied at a viscosity of approximately 100 centipoise. This procedure created an insulation layer of 1.5-2.0 µm thickness in the center of the electrode plate. Such a thin insulation layer in the plate center was required to allow laser deinsulation of electrode tips.

In order to raise the shunt impedance of individual conductors from 5 Mohm to more than 25 Mohm, a 10-15 µm-thick insulation layer was hand painted to either side of the recording matrix to within 2 mm from the edge of the recording area, covering all conductors leading to the amplifier contact strips. After a period of 1 hr at room temperature, the insulated plates were oven-cured according to the following schedule: 4 hr at 150°C; 16 hr at 200°C; 4-6 hr at 250°C.

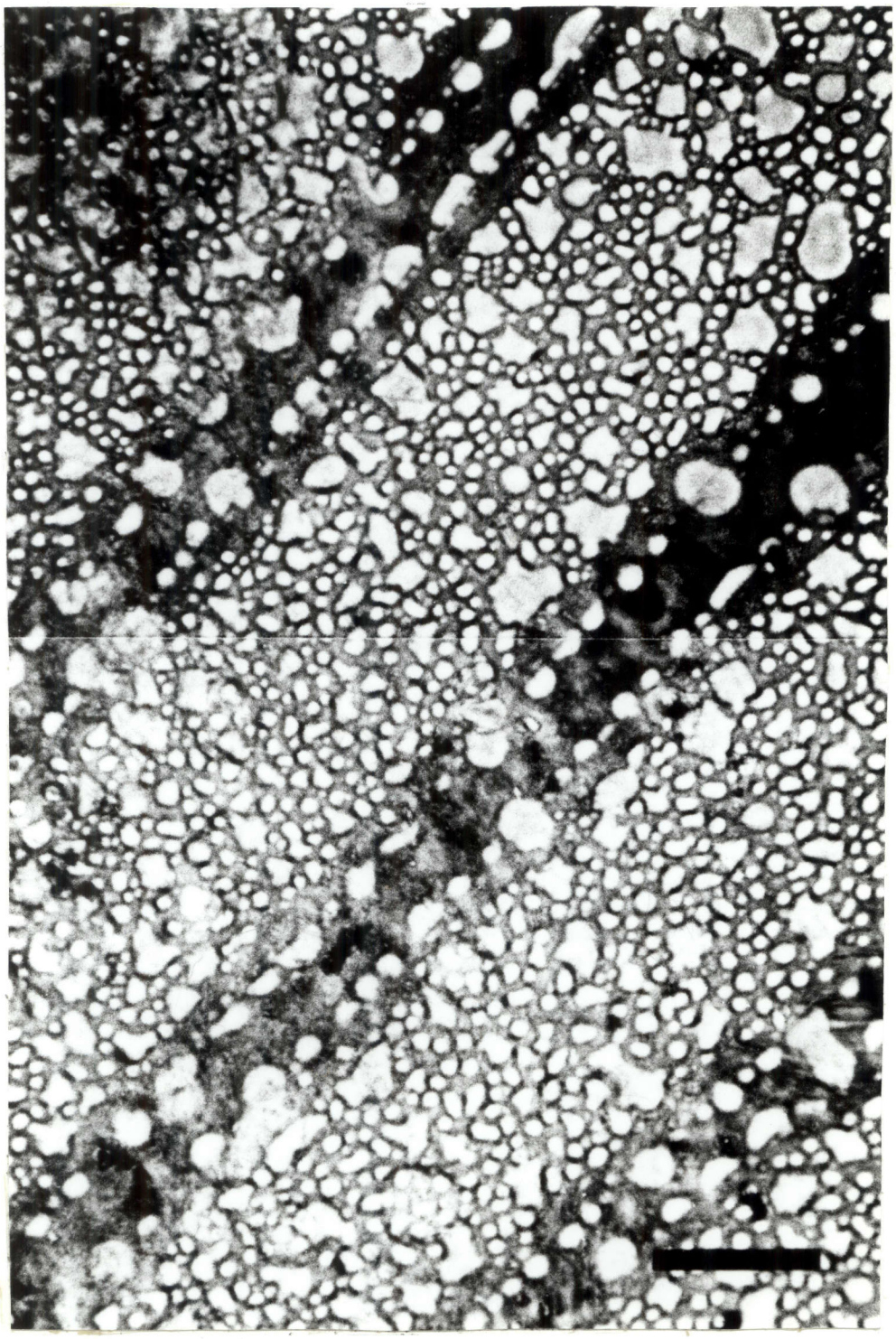
The DC 648 polysiloxane resin material was electrically stable under the culturing conditions. It was capable of

withstanding the autoclaving and flaming procedures and maintained high shunt impedances even after 3 months under warm saline. Unfortunately, although its insulating properties were maintained, the resin deteriorated optically. This deterioration, which appeared as a "bubbling" of the insulation layer in the plane of focus of the cells, had been observed occasionally on the original gold MMEPs, but was much more pronounced on the ITO MMEPs.

With prolonged incubation, the bubble formation worsened, often completely obscuring the neuronal culture (Fig 3). Deterioration was sometimes only slight or moderate on new MMEPs insulated for the first time, but MMEPs cleaned and reinsulated often exhibited extreme deterioration. Steps in the histological procedure reversed this deterioration (to be discussed in Chapter 5), but microscopic visualization of living cells during electrophysiological monitoring was impossible.

Various insulation materials had previously been tested (Gross, 1979), and DC 648 had exhibited the best combination of features (nontoxic, electrically stable under warm saline, capable of being deinsulated by laser). Therefore, rather than testing other possible insulation materials, an effort was made to improve the optical stability of DC 648 by systematically modifying the cleaning, insulation, and culture preparation procedures. First, each step of the standard MMEP deinsulation and cleaning procedure was

Figure 3. Optical deterioration of the MMEP insulation surface was manifested as a "bubbled" appearance. Although its electrical properties were not adversely affected, insulation deterioration made visualization of cells cultured on the insulation surface impossible. Diagonal lines represented the ITO electrodes. The insulation layer over conductors frequently exhibited less deterioration. Scale bar, 100 μm.



prolonged to ensure an optimal glass surface for insulation application. In an effort to further optimize insulation-to-glass bonding, surface moisture was reduced by heating the MMEPs at various temperatures up to 150°C and insulating them under a nitrogen stream immediately after removal from the oven. The oven curing temperature and schedule were varied as well, and substitution of UV sterilization for autoclaving was also tested as a means to produce improved insulation stability.

Laser Deinsulation of Conductors

Deinsulation of conductor tips was performed according to the method developed by Gross (1979) using single 12 ns laser pulses at a wavelength of 337 nm and an energy density of approximately 2 $\mu J/\mu m^2$. A small amount of the electrode tip in the laser focus was vaporized, resulting in gas pressure-induced removal of the insulation layer. "Laser cratering" of the resin produced deinsulation of a 10 μm X 10 μm area at each conductor tip. These deinsulated regions served as the electrophysiological monitoring sites.

Electrode Impedance Measurement and Optimization

Electrode impedances were measured according to the method of Gross (1979), with an AC circuit similar to that described by Robinson (1968). A 1 kHz, 50 mV peak-to-peak signal from an oscillator was passed through a 100 Mohm resistor, and subsequently through the electrode to ground.

The voltage drop across the electrode was amplified and displayed on an oscilloscope and on a chart recorder. Two conductors were left insulated for long-term monitoring of shunt impedance. Electrode tip impedance was determined graphically from the equivalent impedances measured after deinsulation (shunt and tip in parallel).

ITO conductors possessed higher interface impedances than the previously employed gold electrodes, resulting in significant signal loss. This loss of signal was eliminated by light gold plating of the laser-exposed electrode tips (1% gold chloride at 1 V for 5-10 sec, Gross et al., 1985). This plating procedure reduced the electrode impedances to $\leq$3 Mohms, similar to values obtained with pure gold electrodes. However, the gold-ITO bond was not always stable, and the impedances of some electrodes often rose to >10 Mohms following preparation for culture (surface cleaning, autoclaving or UV sterilization, and flaming). Additional conductors developed high impedances following cell seeding and incubation under medium for several weeks. Some of the electrodes with increased impedances displayed visible loss of the plated gold, while others appeared to retain the plating but exhibited unacceptable impedance values. Plating instability on some MMEPs was so severe that over 50% of the electrodes displayed unacceptable impedance values after 4 weeks in culture.

In order to improve conductor electroplating, the concentration of the gold chloride solution, the plating voltage, and plating time were systematically varied. In addition, other gold plating solutions were tested for production of acceptable and stable electrode impedances, including chloroauric acid (0.25-1.0%, Sigma, St. Louis, MO), commercial gold plating alloys (Hunter Mini-Plating System, Bridgewater, NJ), and a commercial pure gold plating solution (SG-10, cyanide-based, Transene, Rowley, MA). Since any electrode plating procedure must not result in toxicity to neuronal cultures, MMEPs from each electroplating test group were seeded with spinal cord cells and observed for signs of toxicity after 1 and 3 weeks in culture.

Production of Dissociated Spinal Cord Mini-Cultures

Formation of Recording Island Regions

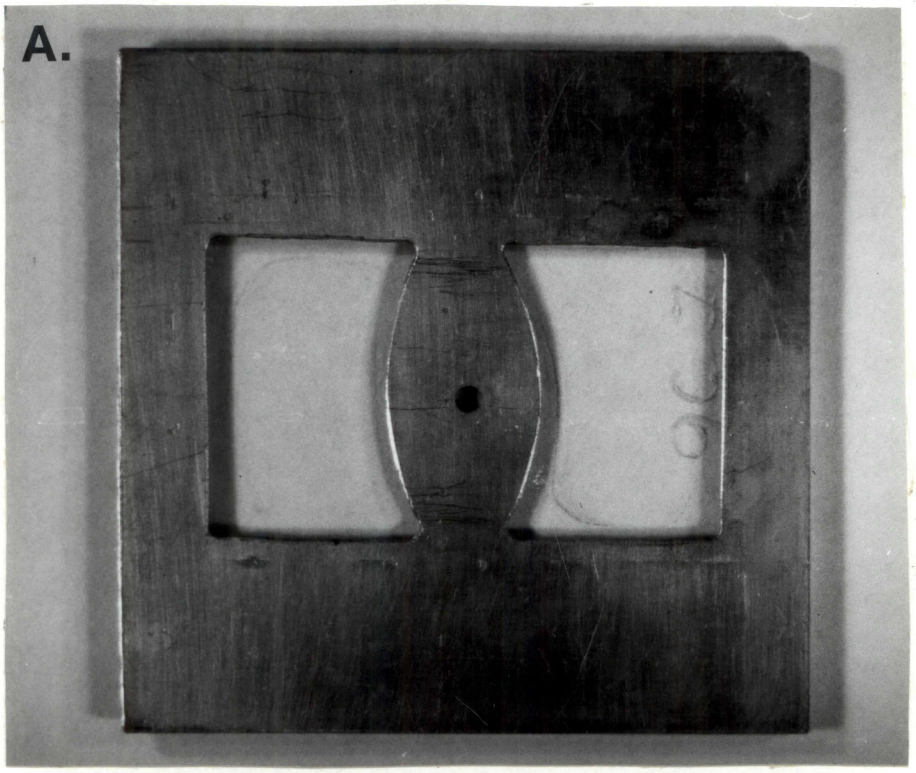
After conductor deinsulation and gold plating, MMEPs were soaked in a detergent solution (10% Linbro, Flow Laboratories, McLean, VA), brushed lightly, and rinsed thoroughly with ultrapure water. The plates were then sterilized by autoclaving (132°C, 10-15 min). Exposing the MMEP insulation to a 1-2 sec propane or butane flame changed the originally hydrophobic insulation layer to a hydrophilic surface which would support cell adhesion and growth (Lucas et al., 1986). This effect was thought to result

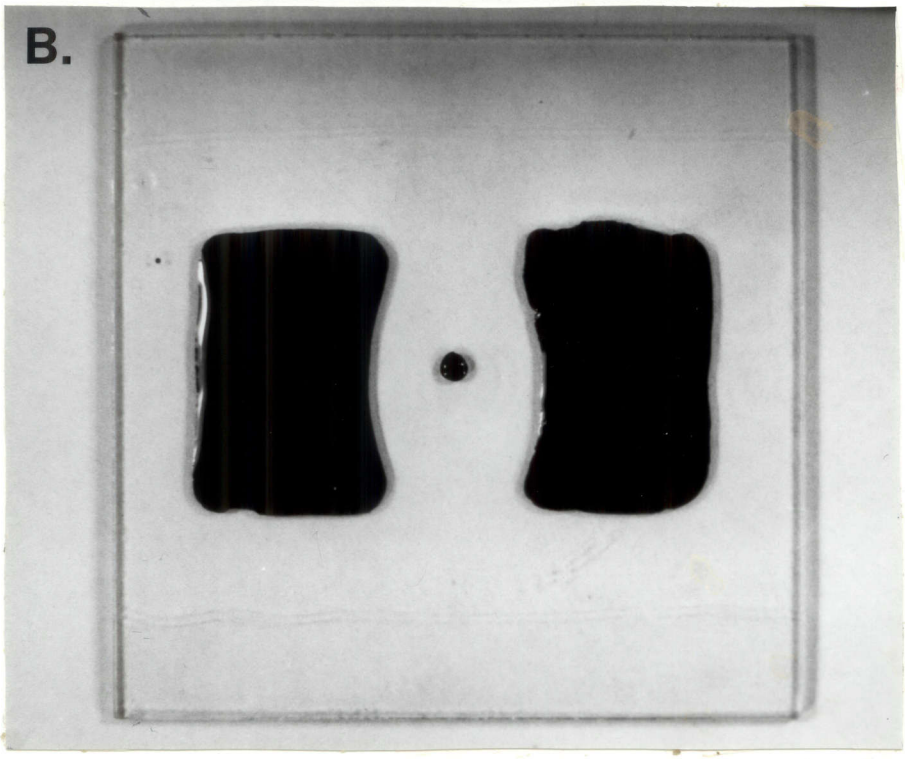
from the deposition of oxidation products onto the insulation surface.

Flaming the entire MMEP surface resulted in the formation of a large adhesive area measuring approximately 3 cm X 4 cm (1200 mm^2). In an attempt to restrict the cellular adhesion region, an aluminum mask was fabricated having a 1 mm central aperture (approximately 3 mm^2 in area) and two larger open areas of approximately 300 mm^2 each, spaced 0.5 cm from the mask center. This mask was sterilized and placed over the MMEP, aligning the center mask aperture with central MMEP conductor matrix using a stereomicroscope. Flaming of the MMEP was then performed with the mask in place, producing a central 1-3 mm diameter hydrophilic "recording island" separated from two spatially more unrestricted "conditioning areas" (Fig. 4). Total surface adhesive area was approximately 600 mm^2.

After flaming, a solution of poly-L-lysine (1-5 X 10^5 M_r, 25 mg/ml, Sigma) was applied to the adhesive areas to further promote single cell adhesion and to minimize cell clustering (Lucas et al., 1986). In order to minimize the size of the central adhesive surface, a droplet of polylysine was placed on the MMEP and immediately drawn off, leaving a film of solution on the recording island. The conditioning areas each received several drops of the polylysine solution, which was also drawn off after 1 hr. The residual fluid was allowed to dry on the MMEP surface

Figure 4. *A*, An aluminum mask was placed over the MMEP prior to flaming in order to produce restricted hydrophilic areas. *B*, The areas of the MMEP surface exposed to flame became hydrophilic, accepting the deposition of water, whereas adjacent areas were water-repellent. A dye solution (1% thionin) was employed to display this hydrophilic effect.


A.


B.

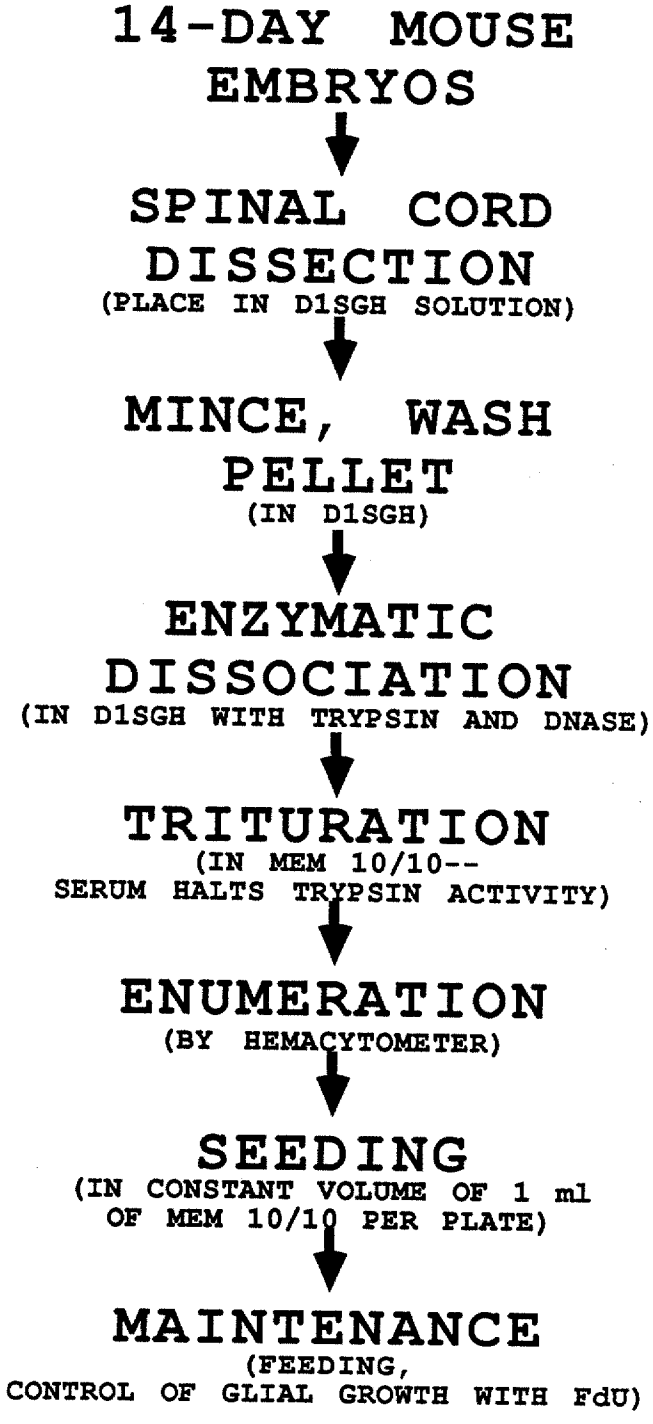
overnight. A sterile silicone gasket spread with a thin layer of stopcock grease was then placed firmly onto the MMEP surface. This gasket formed a reservoir for the 1-2 ml of culture medium which would bathe the neuronal culture.

Dissociation and Culture of Spinal Cord Tissue

Spinal cord tissue was cultured according to the method of Ransom and coworkers (1977) as modified by Gross and Lucas (1982). The culturing procedure is reviewed schematically in Figure 5. Pregnant female mice (13-14 d gestation) were anesthetized by CO_2 narcosis and killed by cervical dislocation. The gravid uteri were removed and rinsed twice with ice-cold HEPES-buffered D1SGH (a balanced salt solution containing sucrose and glucose, GIBCO, Grand Island, NY). Embryos were removed from the uteri aseptically and placed in cold D1SGH. Spinal cords were removed from the embryos using sterile technique and placed in cold D1SGH until all dissections were complete.

The spinal cords (in groups of 7-10) were placed in a disposable petri dishes and minced finely. After rinsing with D1SGH and collection by centrifugal pelleting (200 X g, 1 min), each group of minced spinal cords was added to a D1SGH solution containing 0.25% trypsin and 0.05% DNase (Sigma). The tissue was then placed in a 10% CO_2 incubator at 37°C for 15 min. At the end of this incubation period, trypsinization was halted by the addition of 6 ml of culture

Figure 5. Flow diagram of spinal cord culturing procedure.


14-DAY MOUSE EMBRYOS
SPINAL CORD DISSECTION
(PLACE IN D1SGH SOLUTION)
MINCE, WASH PELLET
(IN D1SGH)
ENZYMATIC DISSOCIATION
(IN D1SGH WITH TRYPSIN AND DNASE)
TRITURATION
(IN MEM 10/10--
SERUM HALTS TRYPSIN ACTIVITY)
ENUMERATION
(BY HEMACYTOMETER)
SEEDING
(IN CONSTANT VOLUME OF 1 ml
OF MEM 10/10 PER PLATE)
MAINTENANCE
(FEEDING,
CONTROL OF GLIAL GROWTH WITH FdU)

medium (Minimum Essential Medium, GIBCO) containing 10% horse serum and 10% fetal bovine serum (MEM 10/10). The tissue fragments were then pelleted by centrifugation (200 X g, 1 min).

After centrifugation, the pellet was removed and suspended in 1.5 ml of fresh MEM 10/10. The tissue was then triturated 10 times using a siliconized Pasteur pipette. Tissue fragments were allowed to settle for 2 min. The supernatant fluid containing suspended dissociated cells was collected, and 1 ml of MEM 10/10 was added to the fragments. Trituration was repeated a second and third time on the remaining tissue using flame-narrowed, siliconized pipettes. All supernatants were then pooled.

The number of cells per milliliter of the pooled cell suspension was calculated from hemacytometer cell counts, and sufficient MEM 10/10 was added to dilute the cell density to 8 X 10^5/ml. Each MMEP was then seeded with 1 ml of the cell suspension; this volume was required for the medium to spreading cover the MMEP surface. Because the recording island and conditioning region surfaces totalled approximately 600 mm^2 (550-700 mm^2), this seeding corresponded to a density of approximately 1300 cells/mm^2 of adhesive surface. Seeding was expressed as the total cells plated rather than the total neurons seeded because the dissociated spinal cord suspension was composed of neuronal and non-neuronal cells, and neurons could not be

distinguished from other cell types. After plating, the MMEPs were then placed into a 10% CO_2 incubator at 37°C.

Culture Maintenance

After 24 hr, the culture medium containing any non-adhered cells and cellular debris was removed, and 1.5 ml of MEM 10/10 was added to the plates. A second complete medium change with MEM 10/10 was performed 3 d after seeding. When the glial carpet became confluent (5-7 d in culture), a complete medium change with MEM 10 (10% horse serum, no fetal bovine serum) containing fluoro-deoxyuridine (FdU) plus uridine (Sigma, at concentrations of 5.4×10^{-5} M and 1.3×10^{-4} M, respectively) was performed. FdU retards the proliferation of dividing cells, controlling the growth of glial cells and allowing the survival of the non-dividing neuronal population (Ransom et al., 1977). The medium containing FdU was removed after 48 hr and replaced with MEM 10. Until the time of electrophysiological assay, cultures were maintained with half medium changes (MEM 10) performed every 3 d.

Results

Optimization of MMEP Preparation

Maintenance of Insulation Clarity

None of the attempts to improve the optical stability

of the DC 648 insulation layer by modification of the insulating procedure were effective. Although proper deinsulation and cleaning of MMEPs was required, prolongation of the cleaning steps did not improve insulation clarity. Likewise, reduction of surface moisture by oven drying, immediate insulation of warm plates, and insulation under a nitrogen stream appeared to only slightly improve insulation stability. Neither modification of the oven curing temperature and schedule nor the substitution of UV sterilization for autoclaving resulted in reduced deterioration.

Optical deterioration of the insulation was minimal over the metal conductors and was most severe on the glass surfaces, suggesting that the soda lime glass bases of the ITO MMEPs might not support proper bonding of organic resins. Therefore, plates of other types of glass were insulated and examined for signs of deterioration. All insulated soda lime glass samples exhibited moderate to severe deterioration, while Pyrex and other borosilicate glass samples displayed only slight optical deterioration. Although information regarding the type of glass used by Siemens to produce the original MMEPs was unavailable, it was likely of a borosilicate rather than a soda lime formulation.

No deterioration occurred on pieces of insulated quartz. Barrier glass, consisting of a soda lime glass base

with a thin coating of quartz, was obtained for use as a photoetching substrate (Donnelly Corporation, Midland, MI). Insulated barrier glass exhibited optical stability similar to that of quartz at a greatly reduced cost. Therefore, barrier glass became the substrate of choice for MMEP manufacture.

Gold Plating and Optimization of Electrode Impedance

Gold plating of the ITO conductors with gold chloride solutions in any of the concentrations, plating voltages, and plating times tested did not result in reproducibly stable electrode impedances. Substitution of 1% chloroauric acid for the 1% gold chloride solution produced slightly more consistent results, but impedance increases were still frequent. The Hunter Mini-Plating System produced acceptable and stable electrode impedances, but this alloy was toxic; no cells remained near the conductor matrix after 1 week in culture.

Plating with the cyanide-based Transene pure gold plating solution SG-10 (2-3 min, 500 mV) resulted in stable electrode impedances with no visible loss of gold plating following plate preparation for culture. Furthermore, the plating solution did not appear to deposit cytotoxic residues. The Transene solution was thus routinely employed for the gold plating of ITO MMEPs. A plating protocol employing low voltages over an extended time period

(400-500 mV, 2-3 min) resulted in optimal electrode impedance stability.

Production of Dissociated Spinal Cord Mini-Cultures

Flaming of the MMEP surface through a mask produced a spatially isolated recording island culture of 1-3 mm in diameter centered on the MMEP conductor matrix and two larger conditioning regions on either side of the plate. Cells adhered and grew in these regions, and there was no adhesion or process extension between the separate areas (Fig. 6). The boundaries between the adhesive and nonadhesive regions were sharp (Fig. 7), and neurons forming the network were easily visualized on the ITO MMEPs (Fig. 8).

Conditioning Areas and Mini-Culture Maintenance

No modifications of the culturing procedure for coverslips were required to promote the growth of spinal cord mini-cultures on MMEPs, aside from adjustment of cell seeding. Approximately 5-10 X 10^5 cells suspended in 1.0 ml of medium were required for production of MMEP cultures of moderate cell density (corresponding to 800-1700 cells seeded/mm^2 of the total cell adhesive area provided by the recording island and the conditioning regions). Seeding of 8 X 10^5 cells/plate (approximately 1300 cells/mm^2 of adhesive surface) became the standard plating procedure for MMEPs.

Figure 6. Photograph of an entire MMEP culture showing the central recording island culture (actual size, 2 mm diameter) and the conditioning regions. The adhesion boundaries were distinct and sharp. This culture was stained by the Nissl method to enhance contrast.

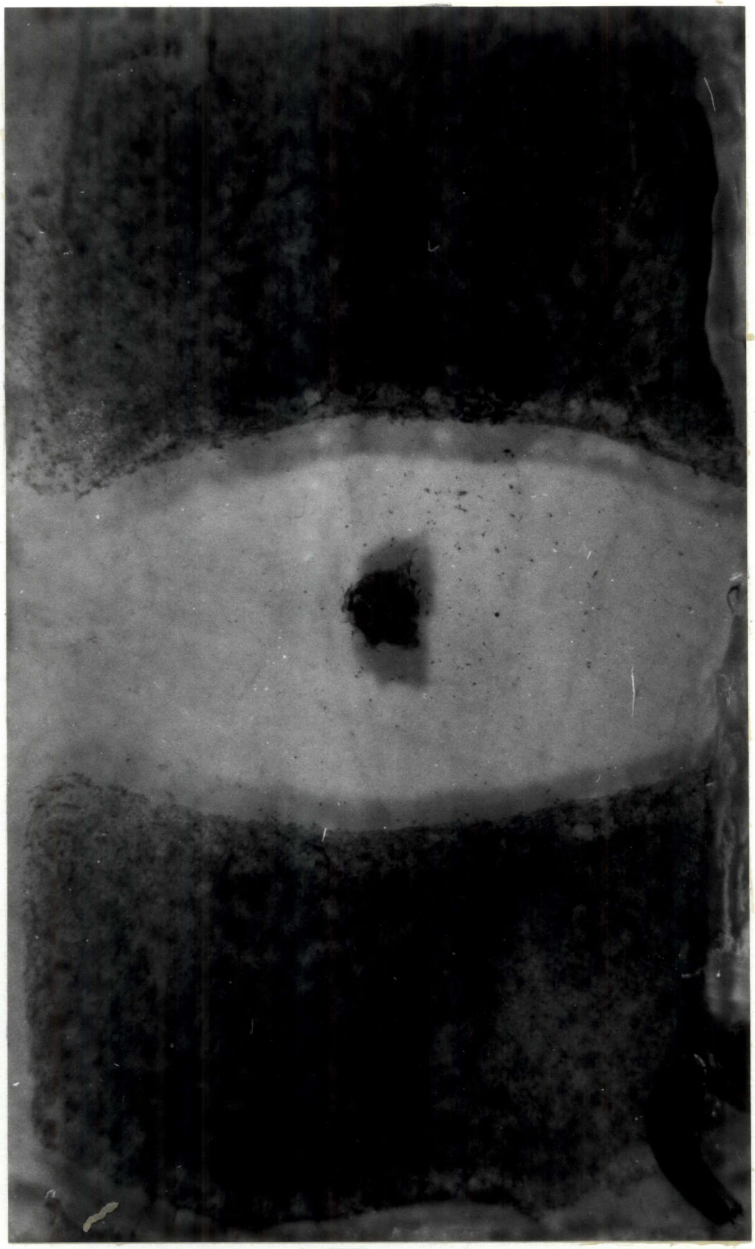


Figure 7. *A*, Photomicrograph of a recording island culture. Somal adhesion and process extension did not occur outside of the island boundaries. Approximately 600 neurons were contained within this 2 mm diameter recording island culture. This MMEP was one of the original MMEPs manufactured by Siemens Corporation (Munich) which possessed opaque gold conductors. The culture was stained by a combined Bodian/Nissl procedure in order to highlight the neuronal elements (see Chapter 5). Scale Bar, 250 μm. *B*, Enlargement of the region circled in *A*, showing details of cells and their processes. Scale Bar, 100 μm.

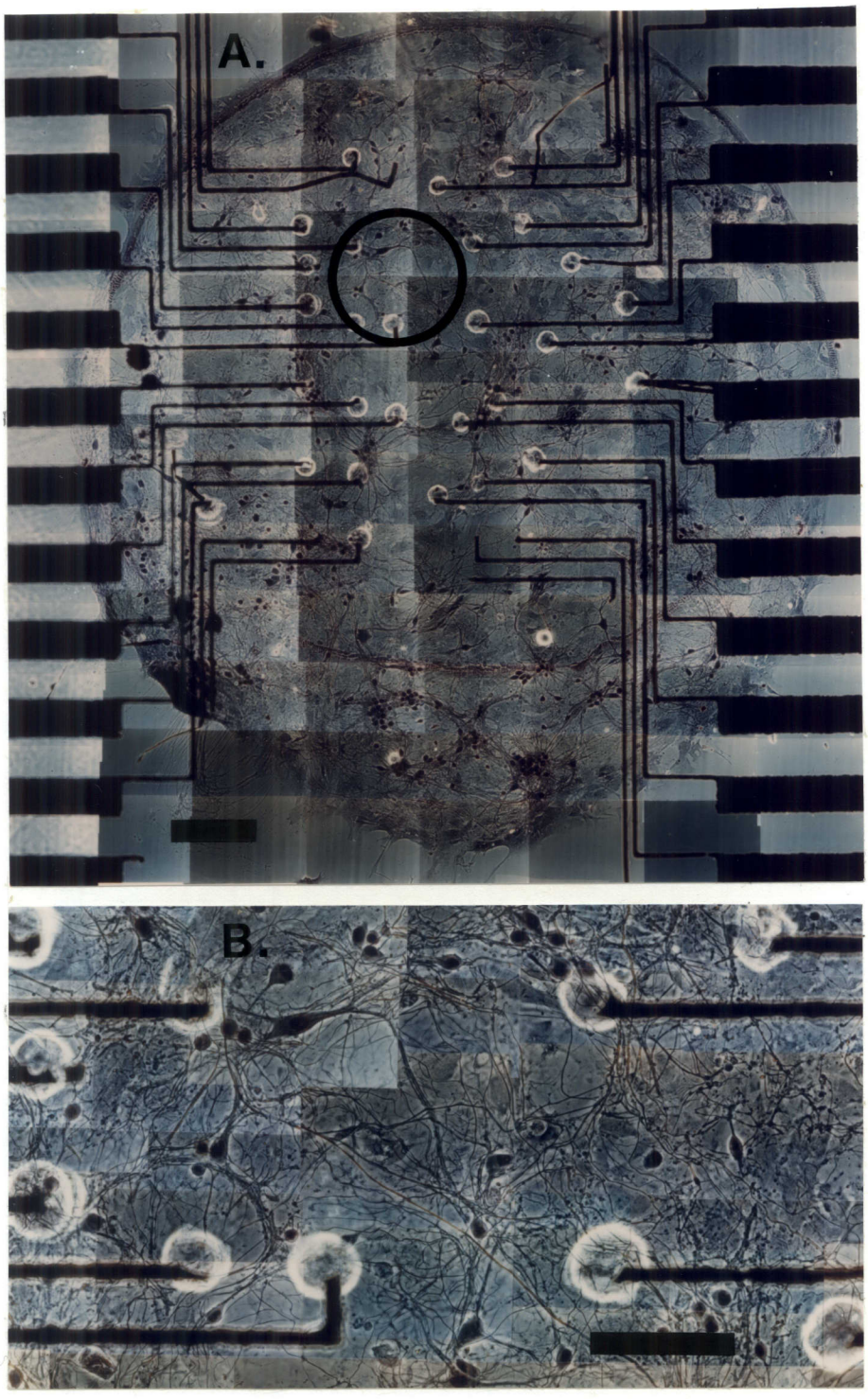
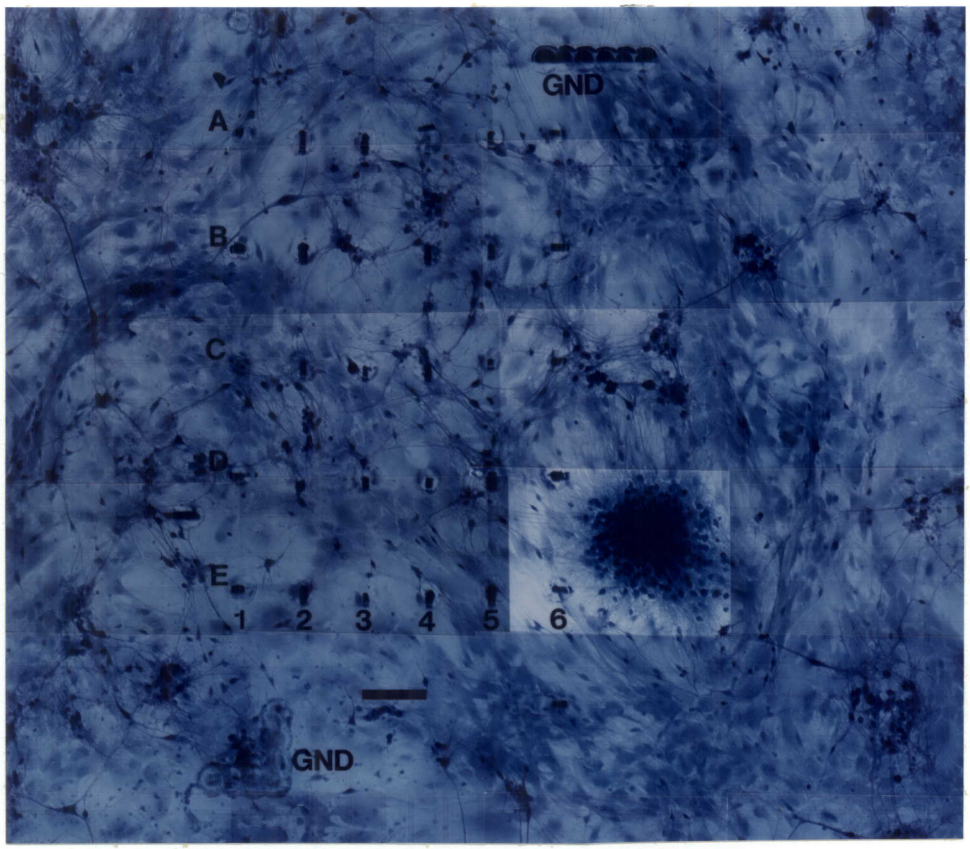

A.
B.

Figure 8. Photomicrograph of the central region of a recording island culture grown on an ITO MMEP. Letters and numbers indicate columns of the conductor array. The transparent ITO electrodes were barely visible, allowing much better visualization of neuronal network components than was achieved with gold MMEPs. However, the deinsulation craters and gold plating of electrode tips partially obscured the recording sites. This culture was stained by a combined Bodian/Nissl procedure in order to highlight the neuronal elements (see Chapter 5). Scale bar, 100 μm.


GND
A
B
C
D
E
1
2
3
4
5
6
GND

The large conditioning areas were essential for the production and maintenance of recording island cultures. Seeding of 5-10 X 10^5 cells on MMEPs with no conditioning regions (only the central area flamed) resulted in recording island cultures of extremely high cell density. Such cultures were often several cell layers thick (precluding morphological analysis) and also exhibited poor health by week 2 in culture, as evidenced by the presence of excessive cellular debris and the granular appearance of neurons.

Seeding fewer cells (2-4 X 10^5) on such plates initially resulted in recording island cultures of moderate density, but these mini-cultures did not survive well. Cell deterioration was apparent within 1 week, and no neurons remained by week 4. Supplementation of the feeding medium with up to 50% conditioned medium helped to prevent neuronal deterioration but was not as effective as the presence of conditioning cultures. Thus, the conditioning areas first served as a cellular "sinks" to prevent the overpopulation of the recording island. These larger cultures then probably produced labile conditioning factors for cell survival within the small recording island.

Neuronal Reaggregation and Border Clustering

Cell adhesion was initially uniform throughout the adhesive area, and well separated, single neurons were observed at 24 hr. However, cell clustering and neuronal

aggregation was evident by day 7. By week 4, recording island cultures frequently exhibited a pronounced "edge effect," a clustering of cells at the edge of the island (Fig. 9). The neurons which aggregated at the border of the adhesive region contacted other neurons lying on the electrode matrix and extended processes over conductors. Therefore, they contributed to the monitored activity. Edge clustering was also observed at the periphery of the larger conditioning areas, but to a lesser extent. It is unlikely that the edge of a flamed region was inherently more adhesive; otherwise, cells would have initially adhered there preferentially. Such cell aggregation at the island periphery complicated morphological studies and remained a primary complicating factor for analysis of recording island cultures.

Discussion

MMEP Preparation

Although technical difficulties were encountered in the production of MMEPs, modifications of the existing preparation procedures were sufficient to eliminate most of these problems. In order to produce an optically clear insulation layer, substitution of barrier glass for soda lime glass was essential. Gold plating of the ITO conductor tips was required to produce proper electrode impedances. Of the four plating solutions tested, only the SG-10

Figure 9. Photograph of a portion of a recording island culture border showing extreme aggregation of cells at the island edge. The aggregates followed the curvature of the island border (arrow). Note the fiber cables extending toward the conductor matrix. Although aggregation to this extent was rare, many recording island cultures exhibited at least some degree of neuronal clustering at the island periphery. Such aggregation complicated morphological analysis. This culture was stained by the combined Bodian/Nissl procedure to highlight neuronal elements (see Chapter 5). Scale bar, 100 μm.

electroplating solution from Transene produced stable electrode impedance values without toxicity.

Spinal Cord Culture

Production of Recording Island Cultures

Flaming MMEPs through a mask produced recording island cell adhesive regions centered over the conductor matrix. Restriction of cell adhesion reduced electrophysiological and morphological complexity by decreasing the number of neurons participating in the monitored neuronal network and by decreasing the possible process extension distance. The recording island cultures possessed sharp boundaries and made no contact with adjacent culture regions.

Neurons appeared to grow as well in mini-culture as they did on more spatially unrestricted surfaces. However, larger cultures growing adjacent to the recording island were required for optimal mini-culture maintenance. The cells growing in these regions probably produced cell conditioning factors necessary for culture health. In addition, the conditioning regions served as additional hydrophilic surface which competed with the recording island area for the attachment of cells after seeding. This served to lower the neuronal density in the recording island region, thus contributing to reduction of network complexity.

Neuronal Reaggregation, Fasciculation and Clustering

Neurons were initially seeded in a suspension of single cells and adhered separately to the substrate at 24 hr. However, by 1 week in culture, neurons had typically reaggregated, forming tight cell clusters with their neurites arrayed in large cables extending from the aggregate. Neuronal clustering was noted in spatially unrestricted cultures grown on coverslips as well as on the MMEP recording island and conditioning areas. Other researchers have also reported the reaggregation phenomenon (Seeds and Haffke, 1978; Akagawa and Barnstable, 1986; Anderson et al., 1988), and it is generally considered to be a characteristic of neuronal monolayer cultures. Cell clustering and process fasciculation complicated network analysis, increasing the difficulty of unequivocally linking observed activity with the cell producing it.

In addition to the general neuronal reaggregation observed by many researchers, the recording island cultures also frequently displayed substantial aggregation at the island border. It is possible that the flaming procedure or the application of polylysine produced an especially cell-adhesive edge. However, neurons did not initially adhere to the island edge preferentially. Possibly, cells moving randomly might encounter the hydrophilic-hydrophobic junction at the island border, and the non-adhesive nature of the surrounding surface would impede their further

outward progress. Such cells would remain "stalled" at the island periphery, and the subsequent random movement of other cells from the island center would then result in aggregation at the island edge. The neurons themselves might be involved in active migration or might be passively moved by the shifting glial carpet. Regardless of its cause, however, neuronal aggregation, with process fasciculation and the clustering of cells at the recording island border, represented one of the most formidable problems to be solved in order to perform efficient morphological-electrophysiological correlations.

CHAPTER 3

ELECTROPHYSIOLOGICAL ANALYSES OF NEURONAL MINI-CULTURES

Introduction

Activity Characterization

The spontaneous electrophysiological activity of tissue fragments and dissociated spinal cord monolayer cultures grown on MMEPs had been successfully monitored prior to the initiation of this project (Gross and Lucas, 1982). However, no systematic analysis of the observed activity had been performed, and the effects of culture variables on the expression of electrophysiological activity had not been appraised. In addition, the preparation of mini-cultures was only in the planning stages, and it was not yet known whether such cultures could exhibit spontaneous activity similar to that observed in larger neuronal cultures. Therefore, the first priorities for this study were to produce neuronal mini-cultures and to determine whether they exhibited spontaneous electrical activity. Providing that such activity was observed, the following tasks would then be attempted: 1) qualitative comparison of activity produced by mini-cultures with that previously observed with larger cultures; 2) systematic characterization of activity; and 3) determination of

differences in activity levels or patterns resulting from changes in culture variables.

Culture Variability

MMEP mini-cultures were quickly shown to exhibit spontaneous activity, so that systematic electrophysiological analyses were attempted. However, only 2-5 MMEPs were cultured per week, and initial characterization of the activity monitored from MMEP mini-cultures therefore utilized pooled data from multiple culture dates. The extreme variability of MMEP cultures was not apparent prior to the semi-quantitative analysis of data from many experiments. Although some general activity characteristics of mini-cultures became evident, statistical treatment of the data was difficult.

Small variations in the cell seeding pool, in plate preparation and culturing procedures, or in culture age at the time of assay could have been responsible for the observed variability. Thus, experiments were planned which would utilize a larger number of MMEPs from the same culture date with several of the culture variables controlled. Histological processing and morphological analysis of these cultures would also be performed so that morphological and electrophysiological characteristics could be correlated (Chapter 6).

Optimization of Cell Seeding

Minimization of the culture adhesion area was expected to result in simplified neuronal networks. However, an accompanying minimization of neuronal density within the adhesion island was also desirable to further simplify network analysis and to facilitate morphological-electrophysiological correlations. Optimally simplified networks would consist of the minimum number of neurons required to exhibit spontaneous, coordinated activity on several electrodes. An experimental series was initiated to determine the cell seeding requirements for the routine production of active mini-cultures with low neuronal density.

Time Course of Activity Development

In addition to the establishment of the optimal cell seeding density, determination of the effect of culture age on electrophysiological activity was necessary. Other researchers (Jackson et al., 1982; Nelson and Brenneman, 1982; Van Huizen et al., 1987) determined with intracellular methods that vigorous spontaneous spike activity occurred after approximately 5-7 d in culture. It was necessary to determine whether activity monitored with the MMEP technique would occur with the same time course. It was hypothesized that network formation and the resultant development and coordination of activity would proceed in a stepwise manner.

Cultures seeded with the previously determined optimum number of cells and maintained under controlled culture conditions would be used to appraise the time course for activity development.

Materials and Methods

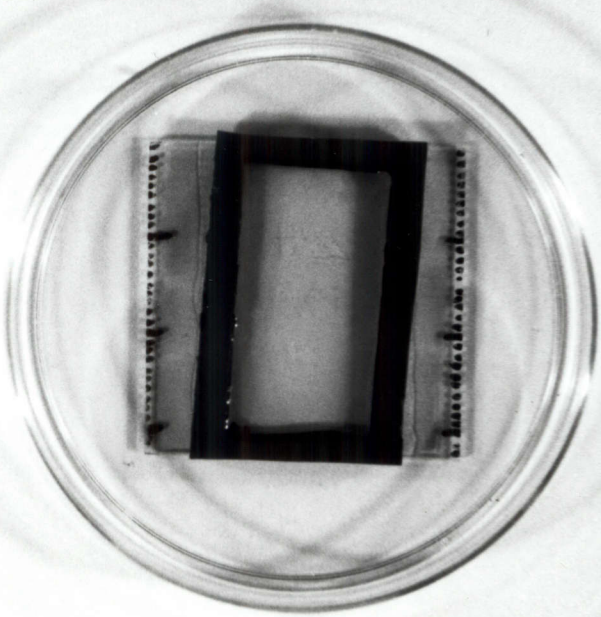
Chamber Assembly, Culture Maintenance

MMEP cultures were typically assayed after 4-6 weeks in culture. The MMEP was removed from the incubator, placed aseptically into a recording chamber (Fig. 10) and returned to the incubator for at least 1 hr. This waiting period after assembly of the chamber allowed the culture to recover from the physical manipulation. In order to minimize reactions to chemical stress, the culture medium bathing the cells was retained and used to fill the chamber reservoir, and MMEP cultures were not assayed within 24 h after a maintenance feeding.

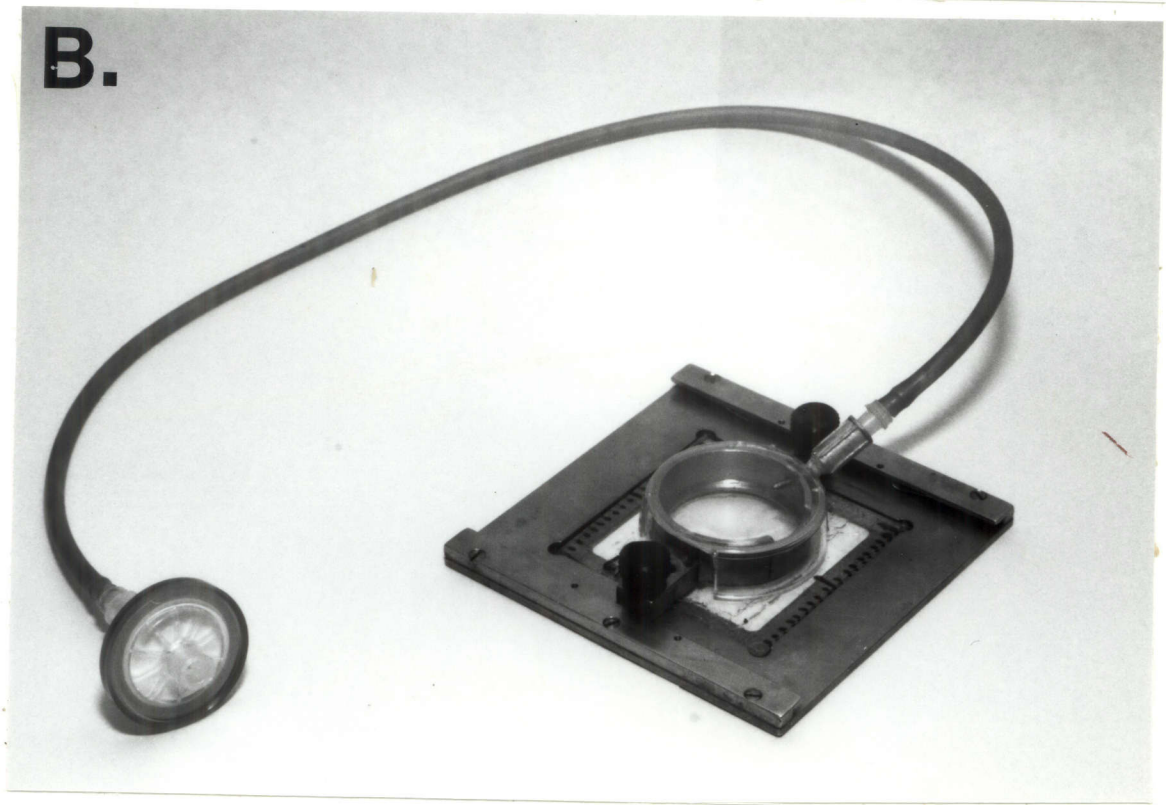
At the time of assay, the open chamber assembly was capped with a clear plastic cover to prevent contamination, to maintain pH, and to allow microscopic viewing during electrophysiological monitoring. Cultures were maintained at 34-37°C during recording with a heater plate which was attached to the microscope stage. A constant filtered stream of water-saturated 10% CO_2/90% air was applied over the medium surface through a chamber port at a rate of

Figure 10. MMEP chamber assembly. *A*, MMEP culture in petri dish with silicon gasket in place for medium retention. *B*, MMEP plate with attached open chamber system. The MMEP served as the chamber floor. Thumb screws joined the chamber base and reservoir and placed sufficient pressure on the rubber reservoir gasket to result in medium retention over the culture. Medium was pipetted off before gasket removal (*A*) and replaced on the culture after chamber assembly.

A.



B.



20-30 ml/min. This gas stream served to maintain the pH of the bicarbonate-buffered culture medium.

Monitoring System

The recording equipment consisted of two Neurolog systems (Digitimer Ltd., Welwyn Garden City, England), permitting simultaneous electrical monitoring from two electrodes. Each recording unit consisted of a high impedance head stage, two amplifier modules, a filter module, and an audio amplifier. Short probes connected the head stage with the conductor leads at the edge of the MMEP. The probe assemblies were mounted on micromanipulators to facilitate the sequential monitoring of conductors.

Action potentials were displayed on an analog storage oscilloscope (Tektronix, model 5113, Beaverton, Oregon) and photographed with a Polaroid camera (Cambridge, MA). After passing through a full-wave rectifier circuit, the activity was saved on a chart recorder (Linseis, Princeton-Jct., NJ or Soltec, Sun Valley, CA). The inertia of the pens resulted in a form of signal integration. Chart recorders were unable to reproduce waveforms of individual spikes but were a valuable tool for the recording of bursting patterns. A 14-channel tape recorder (Racal Store 14DS, Sarasota, FL) was acquired during the latter stages of this project, and some electrophysiological experiments were taped. A Masscomp 5700 mainframe computer (Westford, MA) was also

obtained. Software was developed for the acquisition and storage of taped data (Raymond et al., 1987), and the stored activity was printed in simulated chart record form.

Quantitation of Activity

Electrode impedances were tested just prior to electrophysiological assay, and only conductors with impedances $\leq$5.5 Mohm were considered functional. An electrode classified as nonfunctional may have been scratched during the cleaning or insulation procedures (creating an open circuit) or may have lost the gold plating at the conductor tip. No activity was ever recorded from electrodes with impedances higher than 5.5 Mohm; the noise levels associated with high impedance precluded signal detection. Overall MMEP activity was expressed as a percentage of the total functional electrodes active.

After a brief scan of all electrodes to identify conductors displaying spontaneous activity, strychnine was added to the culture bath at a final concentration of 7.5 μM. Strychnine competes with the inhibitory neurotransmitter glycine for receptor binding and thus produces one form of disinhibition (Simmonds, 1986; Aprison et al., 1987). Initially, this pharmacological agent was applied in order to maximize the number of active electrodes: Conductors which exhibited activity either before or after the addition of strychnine were considered active. Further investigations performed with strychnine

were directed toward detection of burst pattern changes after disinhibition and are discussed in Chapter 4.

Control of Culture Variables

Control of Cell Seeding

In order to systematically determine the cell seeding requirements for production of optimal electrophysiological activity levels, 20 MMEPs were cultured on each of 2 culture dates (40 plates total). It was anticipated that seeding a large number of plates during single culture periods might decrease the wide variations observed between MMEP cultures of multiple culture dates. Plates were flamed through the same mask to produce approximately 600 mm^2 of adhesive surface area (a 3-5 mm^2 recording island and 2 conditioning regions of approximately 300 mm^2 each).

These MMEPs were divided into 4 groups for each culture (5 replicates/test) and plated with variable numbers of cells. Seedings were varied over a wide range for the first series (2.5, 5, 7.5, and 10 X 10^5 cells/plate, corresponding to a range of 400-1700 cells/mm^2 of adhesive surface), and over a narrower, suspected critical range for the second series (4, 5, 6, and 7 X 10^5 cells/plate, or 700-1200 cells/mm^2 of adhesive surface). All cell seedings were plated in a constant volume of 1 ml of culture medium, the volume of fluid required to spread over the culture area. All cultures were assayed at week 4; it was not possible to

monitor all of the MMEPs of a group on the same day, but all plates were assayed at 28 d ± 2 d.

Control of Culture Age at Assay

Similarly, to investigate the effects of age on electrophysiological activity, 20 MMEPs were prepared identically and cultured on each of 2 culture dates, for a total of 40 plates. The same number of cells was seeded on each plate (8×10^5 cells/plate in 1 ml of culture medium, or 1300 cells/mm^2 of adhesive surface, within the seeding range suspected to produce maximal activity). Five plates from each culture date were monitored at weeks 1, 2, and 3 in culture, and a total of 8 plates were monitored at week 4 (2 plates lost to fungal contamination). It was not possible to assay all of the MMEPs of a group on the same day, but all of the MMEPs were monitored at the appropriate age (7, 14, 21, and 28 d) ±1 d.

Grouping of Data from Controlled Seeding and Age Experiments

The experiments to test seeding and age effects described above were performed on 4 successive culture dates. The same lots of all culture supplies (including sera) were used for the production and maintenance of all of the cultures, and the plate preparation, culturing and maintenance procedures were also identical for all plates. In order to maximize the replicates for statistical and graphical purposes, data from the experiments were pooled in

various patterns (Table 1). The "TOTAL" file included data from all of the MMEP experiments. The "SEED" file was composed of data from all MMEPs which were assayed at 4 weeks but which had variable seeding densities. The "AGE" file contained all the data from MMEPs seeded within the range of 6-8 X 10^5 cells and assayed at 1, 2, 3, or 4 weeks. Table 2 provides a summary of the number of replicates in each group within the SEED and AGE files.

Results

General Characteristics of Activity

Quantitation of Active Electrodes

A majority of MMEP mini-cultures exhibited some form of spontaneous electrical activity. Activity was displayed on as few as 1 or as many as 36 electrodes during any given experiment, but vigorous activity observed on 5-10 conductors was common. Since an average of 30 electrodes per MMEP displayed acceptable impedance values, activity was thus commonly monitored from at least 17-33% of all functional electrodes. This level of activity was similar to that which had been observed with spatially unrestricted cultures (Droge et al, 1986).

When MMEP mini-cultures which had exhibited few or no active electrodes were observed microscopically, excessive debris and an absence of a confluent glial carpet was often

Table 1. Summary of experiments to determine the effects of cell seeding concentration and culture age on the activity and morphology of mini-cultures

No. of MMEPS	Culture date	Cells seeded per plate[b] (x 10^{-3})	Assay age (weeks)	Data files[a] TOTAL	AGE	SEED
5	8-28-86	250	4	+	-	+
4	8-28-86	500	4	+	-	+
5	8-28-86	750	4	+	+	+
3	8-28-86	1000	4	+	-	+
5	9-04-86	800	1	+	+	-
5	9-04-86	800	2	+	+	-
5	9-04-86	800	3	+	+	-
4	9-04-86	800	4	+	+	+
5	9-11-86	800	1	+	+	-
5	9-11-86	800	2	+	+	-
5	9-11-86	800	3	+	+	-
5	9-11-86	800	4	+	+	+
5	9-18-86	400	4	+	-	+
5	9-18-86	500	4	+	-	+
5	9-18-86	600	4	+	+	+
5	9-18-86	700	4	+	+	+
Number of MMEPS analyzed				76	54	46

[a]The TOTAL file included electrophysiological and morphological data from all 76 MMEP experiments. The SEED file was composed of MMEP experiments which were seeded at variable cell concentrations and assayed at 4 weeks. The AGE file was composed of data from MMEPs which were seeded with 6-8 x 10^5 cells and assayed at varying ages

[b]Cells were seeded in a constant volume of 1 ml onto a constant cell adhesion area of 600 mm^2/plate.

Table 2. Summary of the number of replicates in the SEED and AGE files.

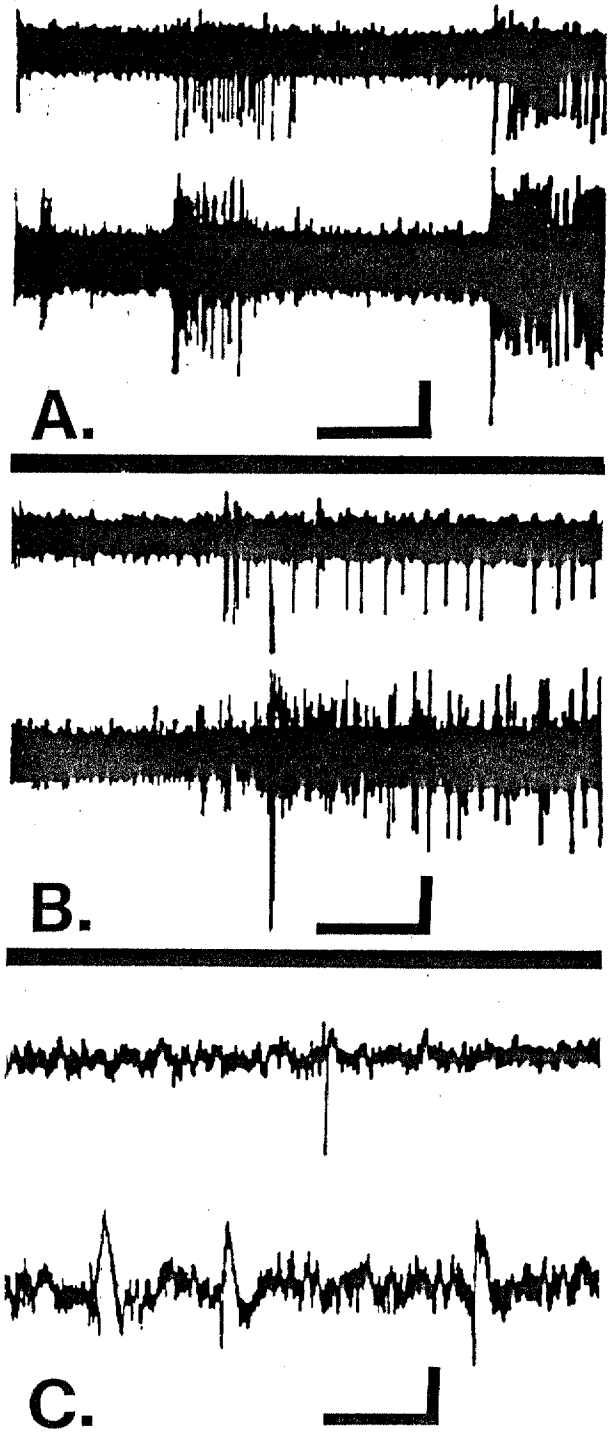
Cell Seeding Concentration ($X\ 10^{-3}$)	Culture age (weeks)	Number of replicates
SEED file		
250	4	5
400	4	5
500	4	9
600	4	5
700	4	5
750	4	5
800	4	9
1000	4	3
AGE file		
600-800	1	10
600-800	2	10
600-800	3	10
600-800	4	24

apparent. The neuronal cells in these cultures often appeared stressed, exhibiting vacuolization of somata and graininess of processes. In contrast, cultures which appeared healthy, with little debris or intracellular vacuolization, generally exhibited some spontaneous activity.

Spike and Burst Characteristics

The monitored activity was primarily in the form of bursts of action potentials rather than random single spiking (Fig. 11*A*). Observed burst frequencies ranged from <1 burst/min to >60 bursts/min, but 64 of 83 active cultures (approximately 80%) exhibited burst frequencies of between 3 and 20/min. Burst durations ranged from <50 msec to >20 sec, with a majority of burst durations between 200 msec and 3sec. Occasionally (in < 10% of active cultures), continuous tonic firing was observed on one or more electrodes, accompanied or unaccompanied by periodic bursting. The magnitude of the observed spikes ranged from 60-1000 μV, although 100-400 μV signals were most common. These typical signal voltages corresponded to oscilloscope tracings with signal-to-noise ratios of 1.5-10:1. In 70-80% of observed bursting activity, a higher amplitude spike would precede a burst of action potentials of smaller amplitude (Fig. 11*A, B*). Similar "initiating discharges"

Figure 11. Oscillographic records of spontaneous activity monitored from 2 electrodes simultaneously. All vertical scale bars, 40 μV. *A*, An example of bursting activity. Scale bar, 1 sec. Spikes were concentrated in intense "packets" (bursts), and little interburst spiking was apparent. *B*, The initiation of a burst recorded at a faster sweep from the same electrodes. Scale bar, 50 msec. A burst was often initiated by a spike of large amplitude which preceded spikes of smaller amplitude. *C*, A portion of a burst recorded from the same electrode at fast sweep. Scale bar, 2 msec. At this recording speed, individual action potential waveforms were distinguishable, and the number of active units could thus be estimated.


A.
B.
C.

were also routinely observed by Provine (1986), who worked with an *in situ* chick spinal cord preparation.

Distinguishing Units of Activity

The amplitude and form of the action potentials produced by a neuronal element monitored at the same location are considered nonvariable (Buchwald et al., 1973). Therefore, spikes recorded from different neurons on the same electrode were often distinguishable by differences in amplitude and duration. Occasionally, a conductor would be monitored which exhibited only one unit of activity (Fig. 12*A*). However, 2-4 distinguishable activity units from one electrode were common (Fig. 12*B*), and as many as 8 or more different units were sometimes observed (Fig. 12*C*).

The number of activity units determined by observed differences in spike form was probably systematically underestimated because spikes of similar amplitude and duration could not be easily distinguished. Generally, spikes of 500-1000 μsec are associated with axonal firing, whereas spikes of longer duration are attributable to cell body spiking (Towe, 1973). Since a majority of the monitored spikes were of $\leq$ 500 μsec and more axons than somata were generally observed near the recording craters, the vast majority of the observed activity was monitored from axons (Fig. 13).

Figure 12. Slow sweep oscillographic records of representative electrical activity from 3 different electrodes accompanied by corresponding fast sweep records. *A*, One large unit of activity was recorded from this electrode, as well as one much smaller unit which was almost indistinguishable from the noise line. *B*, Two or three different activity waveforms were discernable in this record. *C*, Frequently, many units participated in the activity recorded from a single electrode. When firing frequency was high, waveforms superimposed, and distinguishment of all activity units was difficult. Horizontal scale bars, 1 sec, slow sweeps; 5 msec, fast sweeps. Vertical scales for all traces, approximately 40 μV.

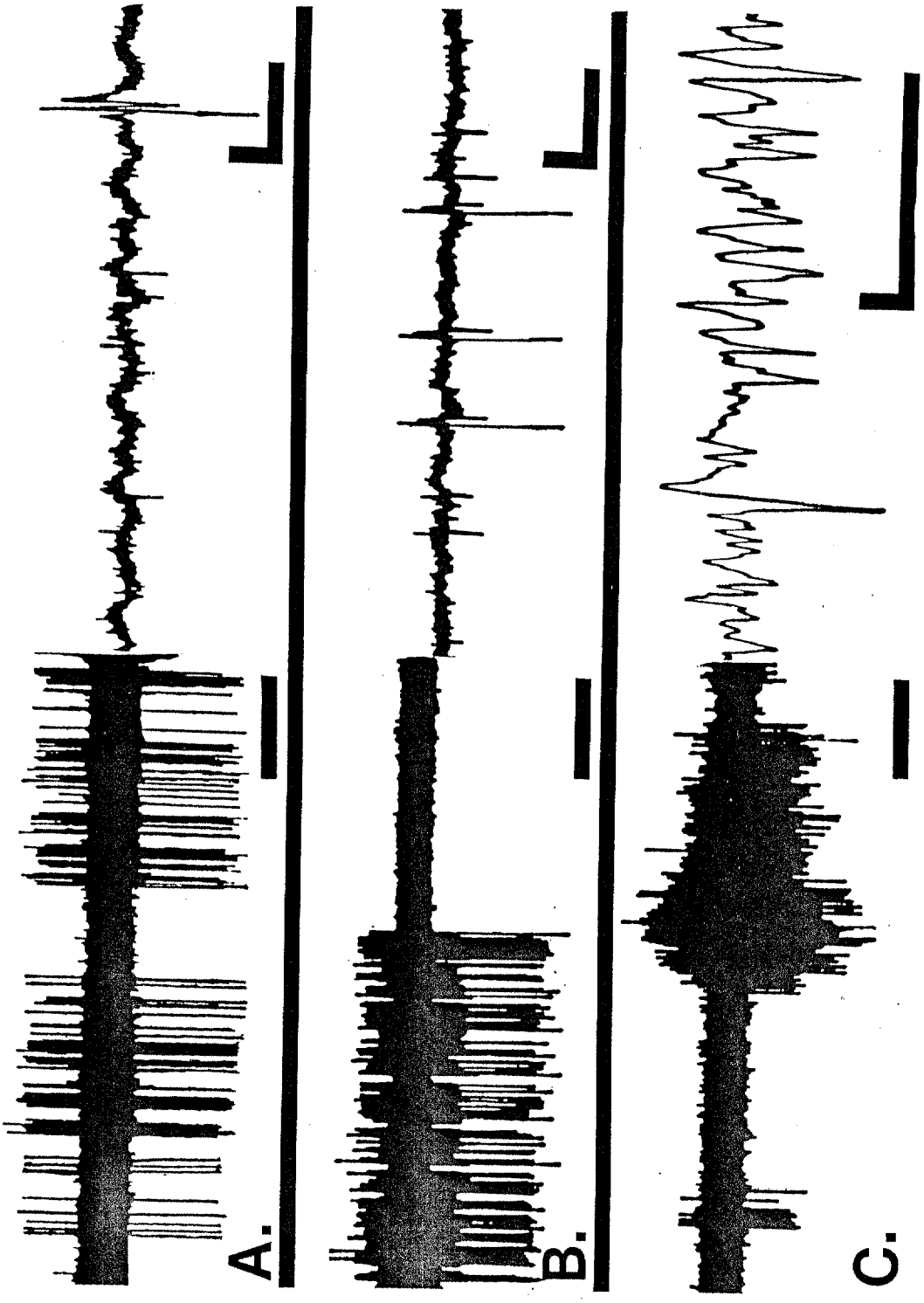
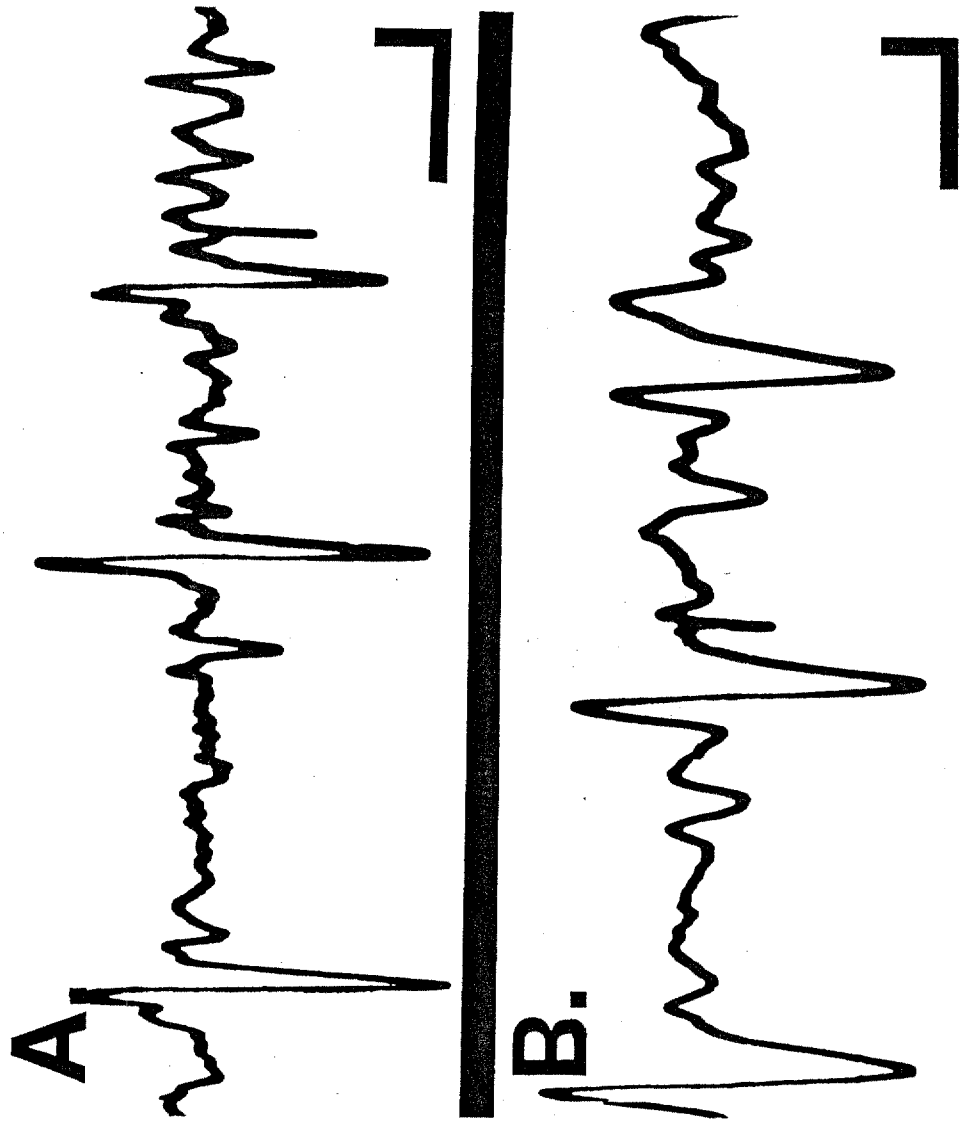

A.
B.
C.

Figure 13. High-speed oscillographic recordings of action potential waveforms. *A*, The short duration and smooth waveform of these signals suggested sodium spikes monitored from axons. *B*, A putative somal spike (typically 1-2 msec in duration). Horizontal scale bars, 1 msec. Vertical scales, approximately 40 μV.


A.
B.

Activity Coordination

Strict Coupling of Activity

Electrophysiological activity was monitored from two electrodes simultaneously. It was therefore possible to assess the coordination between any two electrode sites. By monitoring active electrodes in tandem, the degree of coordination between all active electrodes was determined. Activity observed on one electrode was almost always tightly coupled to that of all other electrodes, generally with no more than a 2-5 msec difference in onset time (Fig. 14). Occasionally, a spike or small burst at one electrode would precede the burst trains on other electrodes by more than 5 msec (Fig. 14*C, D*).

Incomplete Coupling of Activity

Some exceptions to the trend toward strict coordination between all electrode sites were observed, however. Single spikes occurring during the interburst interval at one electrode site were often not observed on other conductors (Fig. 15*A*). Although burst onsets were usually tightly coupled, burst terminations were sometimes not as rigidly coordinated (Fig 15*B*). Continuous spiking (i.e. tonic activity) was sometimes observed from one conductor which was absent on most or all other electrodes (Fig. 15*C*). However, when an electrode exhibited tonic

Figure 14. Coordination of activity. *A, B,* Slow-speed oscillographic records of bursting activity recorded simultaneously from 2 electrodes. *A,* Bursting activity was coupled between electrodes 1 an 2. Note, however, that the single spike activity exhibited by electrode 1 was not coupled to similar activity on electrode 2. *C,* Coupling was also observed between electrodes 3 and 2. Therefore, it was concluded that burst activity was coordinated on all 3 electrode sites. *B, D,* High-speed sweeps of bursts from the same electrodes. Initiation of bursts on all of the electrodes occurred within 5 msec. It should be noted that the number of units and their form varied between electrode sites, indicating minimal electrical cross-talk. Horizontal scales, 1 sec, *A, C,* 5 msec, *B, D.* All Vertical scales, 40 μV.

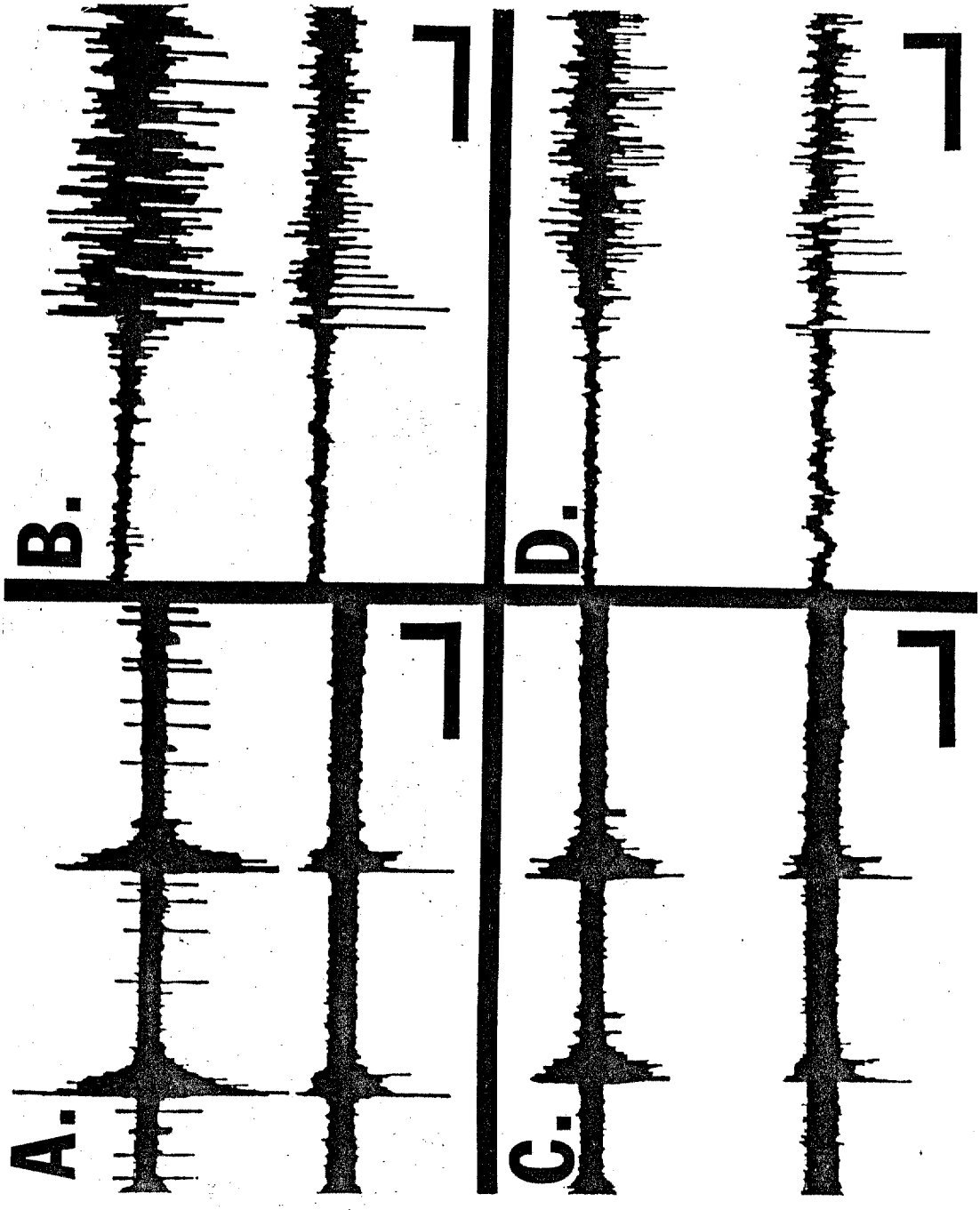
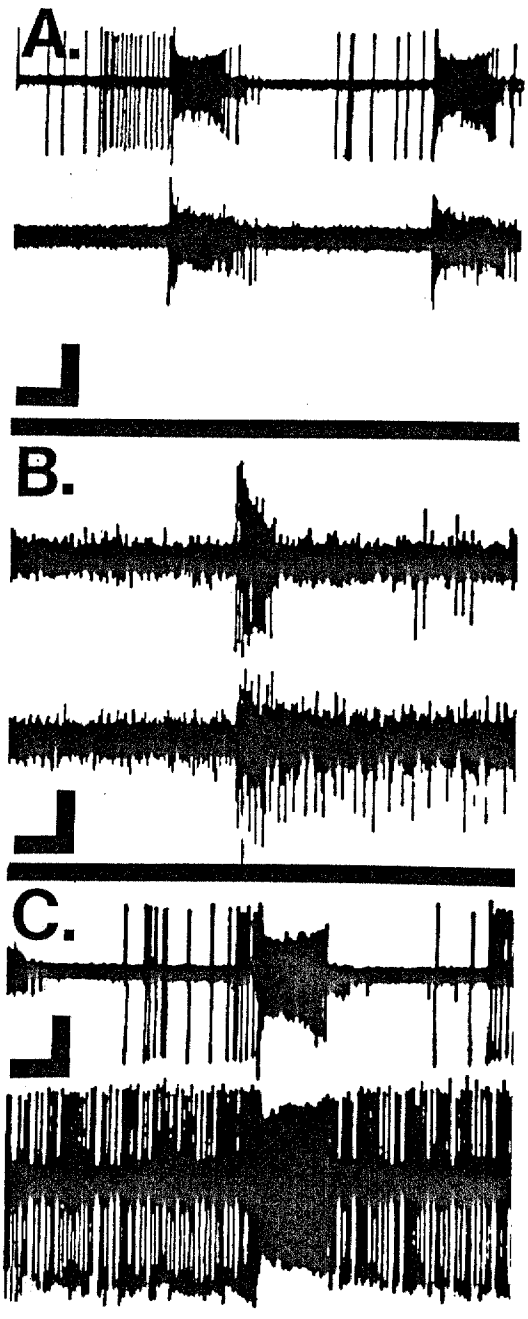

A.
B.
C.
D.

Figure 15. Oscillographic sweeps recorded simultaneously from 2 electrodes showing exceptions to the strict coordination of activity. *A*, Electrode 1 exhibited single spiking between bursts. Electrode 2 displayed bursting activity only. The periods of bursting were coordinated between the 2 electrodes. *B*, Onset of bursting between electrodes 1 and 2 was coordinated, but terminations were not coordinated. *C*, Electrode 1 exhibited nonregular single spiking, whereas electrode 2 displayed continuous single spike activity. Bursts, however, were coordinated between the electrode sites. Horizontal scale bars, 1 sec, vertical scales, approximately 60 μV.


A.
B.
C.

activity as well as bursting activity, the bursts tended to be coordinated with the bursting from other electrodes.

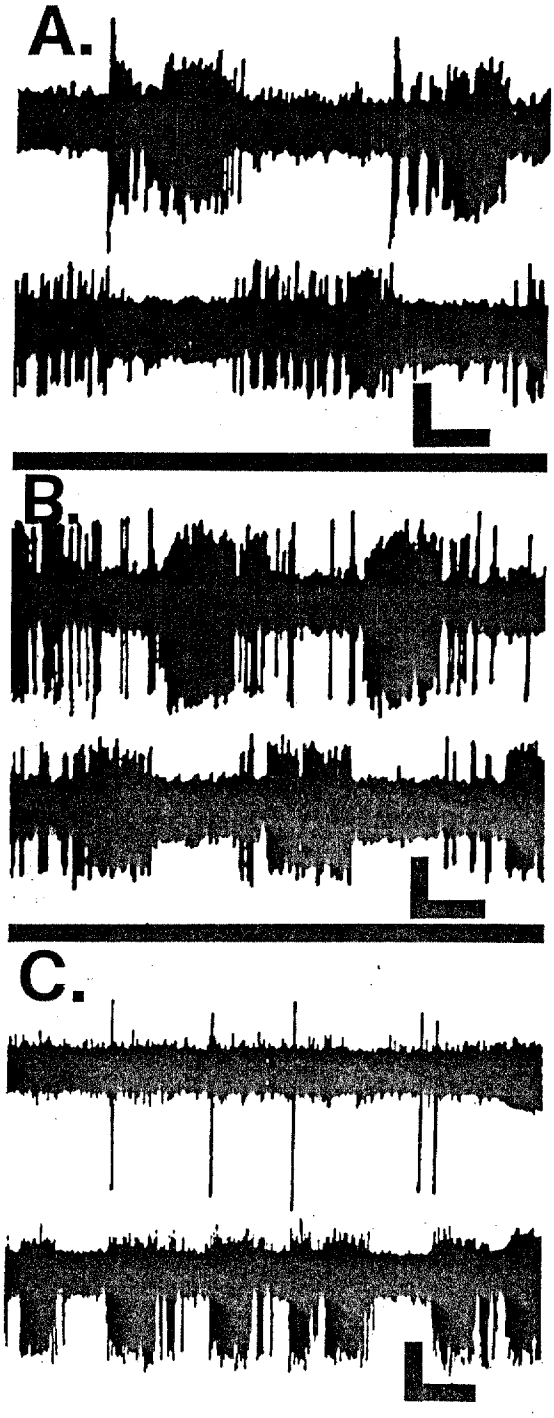
Alternating Activity

One interesting phenomenon infrequently observed was an alternating pattern of activity between two electrodes. Bursting at one electrode site was associated with a silent period at the other, and vice versa (Fig. 16). This form of activity was considered coordinated, but in an antagonistic rather than synergistic mode. Alternating activity was observed in only a small minority of MMEP experiments (approximately 1%), but its presence indicated that at least some of the mini-cultures were capable of complex electrical behavior.

Burst Analysis

For certain studies, such as determining the number of active units per electrode, distinguishing between axonal and somal spiking, and critically assessing coordination between two electrode sites, oscillographic records of individual action potentials were essential. However, such photographs could provide a detailed portrayal of only a few seconds of activity at most, necessitating simultaneous chart recording to provide a continuous record of general activity characteristics over periods of several minutes to hours. The recorder pen inertia produced a form of signal integration, and individual spikes, except those of

Figure 16. Oscilloscope records of simultaneous activity on 2 electrodes demonstrating alternating patterns of activity. *A*, Electrodes 1 and 2 exhibited almost 100% alternation of bursting activity. When one electrode displayed bursting, the other electrode was silent. *B*, Alternation of bursting with an overlapping "on" time. Units at electrode 1 initiated a burst. As this burst became less intense, units at electrode 2 began to burst. A portion of the interburst silent period was shared. *C*, A period of bursting at one electrode was preceded by single spike activity on another electrode site. Horizontal scale bars, 1 sec, vertical scales, approximately 40 μV.


A.
B.
C.

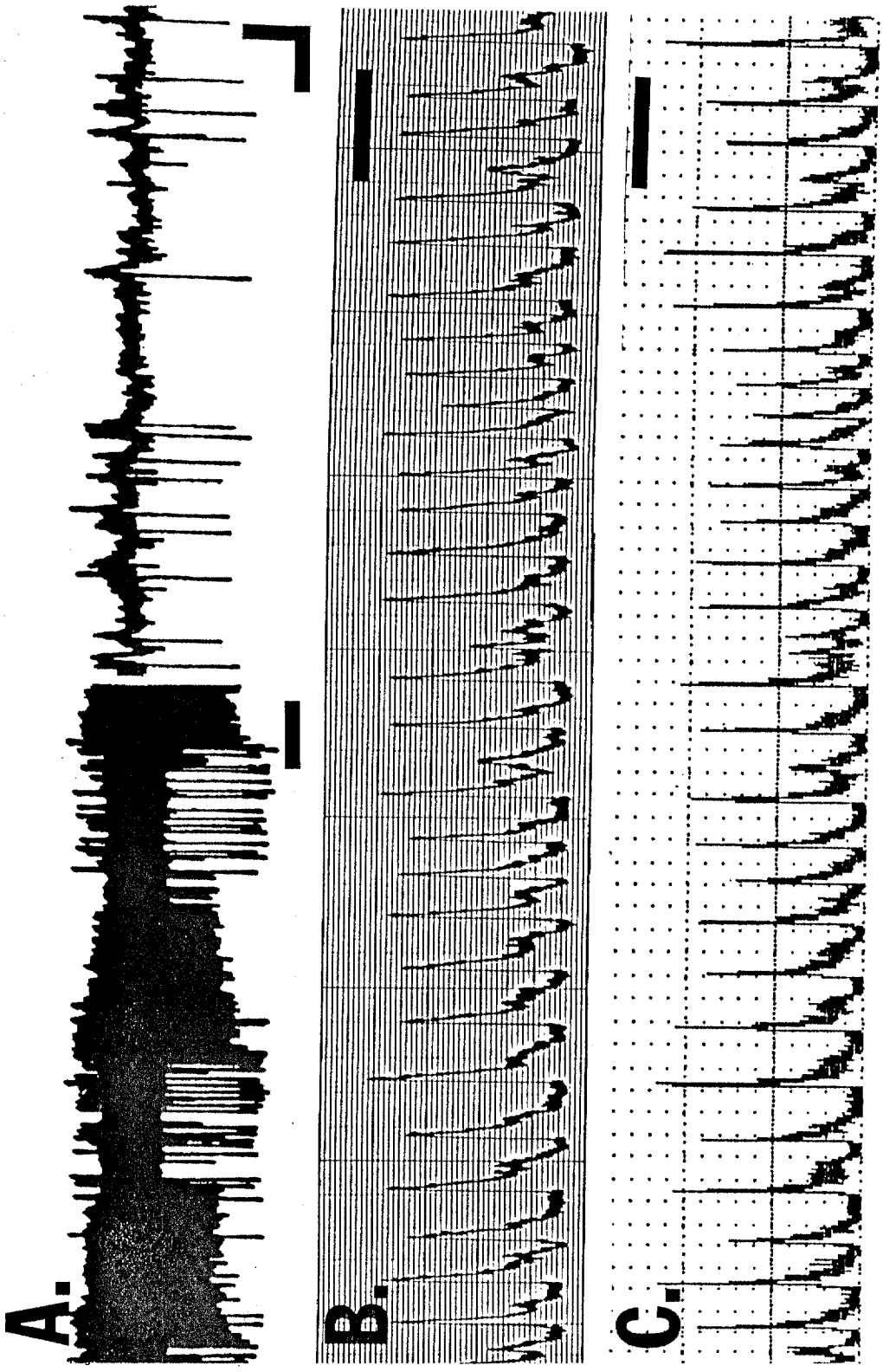
exceptional voltage, were not recorded. Thus, random and tonic single spiking activity was lost in background noise, but burst patterns were well displayed (Fig. 17). Changes in burst amplitude recorded from the same electrode during the course of an experiment were related to changes in spike amplitude and/or spike frequency.

Vigorous multi-unit activity was generally present on several electrodes during each experiment. A quantity of data was thus produced which was too large for prolonged periods of activity to be successfully analyzed at the single spike level. Analysis of chart records, with designation of the burst, rather than the action potential, as a single electrophysiological event represented a second, more global, level of electrophysiological analysis. Changes in burst characteristics and patterns would reflect overall network dynamics rather than the activity fluctuations of a single cell. Because burst analysis represented a simplified method for electrophysiological characterization of a neuronal population over a prolonged period, it was employed in investigations of the effects of pharmacological agents on MMEP mini-cultures (Chapter 4).

Activity as a Function of Cells Seeded

MMEPs in the SEED group (seeded with variable numbers of cells in a constant 1 ml seeding volume, assayed at 4 weeks in culture, see Tables 1 and 2) were used to

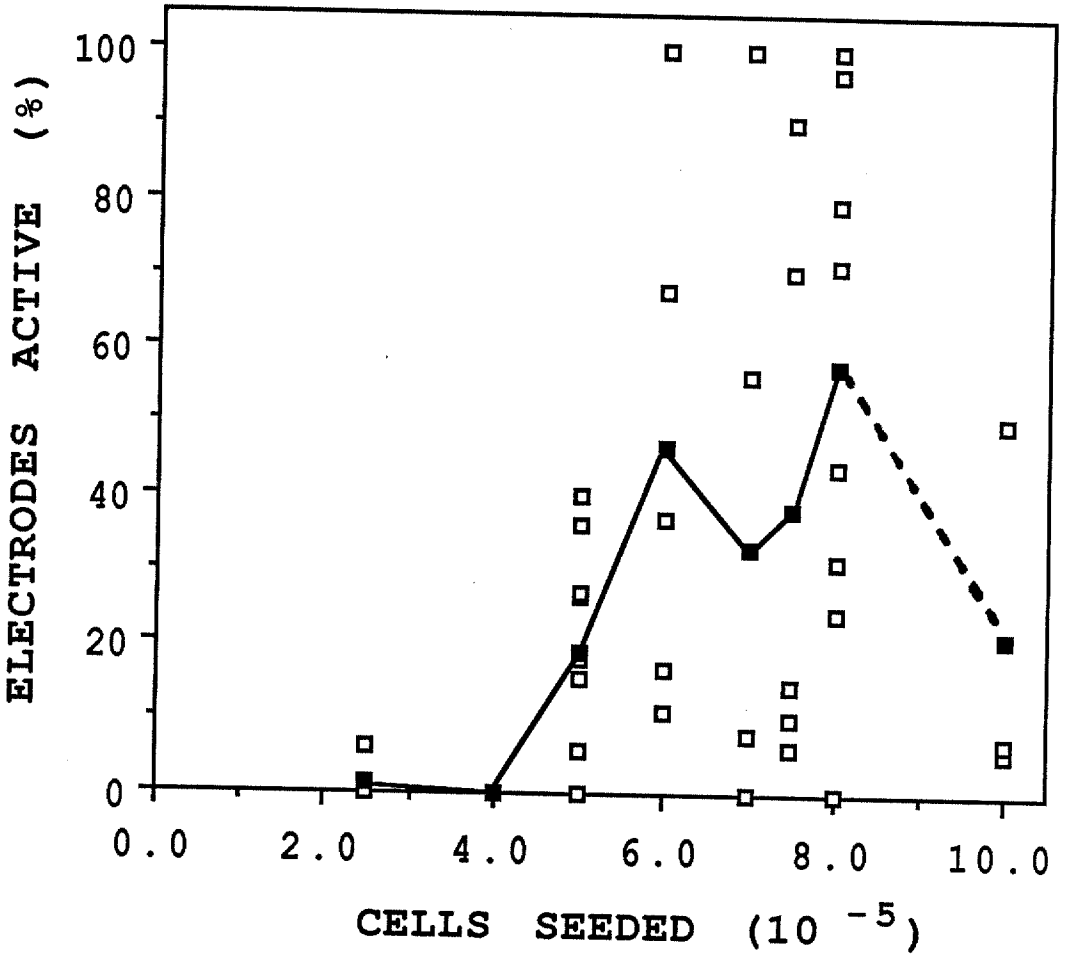
Figure 17. Comparison of an oscilloscope record and a chart recording of electrode activity. *A*, The oscilloscope record of electrode activity portrayed both single spikes and bursts of action potentials. However, these records were limited to only a few seconds of continuous activity. Horizontal scale bars, 1 sec, slow sweep, 5 msec, fast sweep. Vertical scale, approximately 40 μV. *B*, A chart record of activity from the same electrode depicted periods of bursting as an integrated envelope of activity. Single spikes were lost in the noise line. It was possible to continuously chart electrode activity over a period of several minutes. Scale bar, 10 sec. *C*, Computer-generated simulated chart record of same activity portrayed in *B*. Note the very similar burst envelopes. Scale bar, 10 msec.


A.
B.
C.

determine the effect of cell seeding on the quantity of observed electrical activity, measured as the percentage of active electrodes. MMEPs seeded with 2.5 or 4 X 10^5 cells exhibited very little activity at 4 weeks in culture (Fig. 18). Seeding 5 X 10^5 cells resulted in increases in the percentage of active electrodes, and the seeding of 6 X 10^5 cells produced a further increase in the mean percentage of active electrodes. Seedings above this level, however, resulted in no consistent increases in active electrodes; in fact, all seedings of between 6 and 8 X 10^5 cells resulted in similar mean levels of activity (40-50% of the functional electrodes active). Although the percentage of active electrodes was low at low seeding densities, the type of activity and degree of coordination between active electrodes were the same as those encountered with cultures of more densely seeded cultures.

It should be noted that while a general pattern emerged from the plot of mean percentages of electrodes active versus cells seeded, the levels of activity exhibited by individual cultures in any seeding group were extremely variable. For example, the percentage of active electrodes varied from 0-100% for MMEPs seeded at 8 X 10^5 cells. Because the activity range was so great, the differences between seeding groups were not statistically significant. However, the envelope formed by the maximum observed activity in each seeding group paralleled the

Figure 18. Scatterplot of the percentage of active electrodes at 4 weeks as a function of the number of cells seeded (SEED file data). Activity was maximized by counting as active all of those electrodes which exhibited activity either before or after the addition of 10 μM strychnine. Seedings of less than 6 X 10^5 cells resulted in low levels of activity. Both the envelope function of maximum activity levels attained in each seeding group (dotted curve) and the mean percentages of active electrodes (solid curve) reached a plateau value at seedings of 6-8 X 10^5 cells. Further increases in seeding densities did not produce increases in the percentage of active electrodes. Mean activity levels decreased at seedings of 1 X 10^6 cells. However, this value was obtained from only 3 experiments, and random data scatter could explain the decrease in activity.

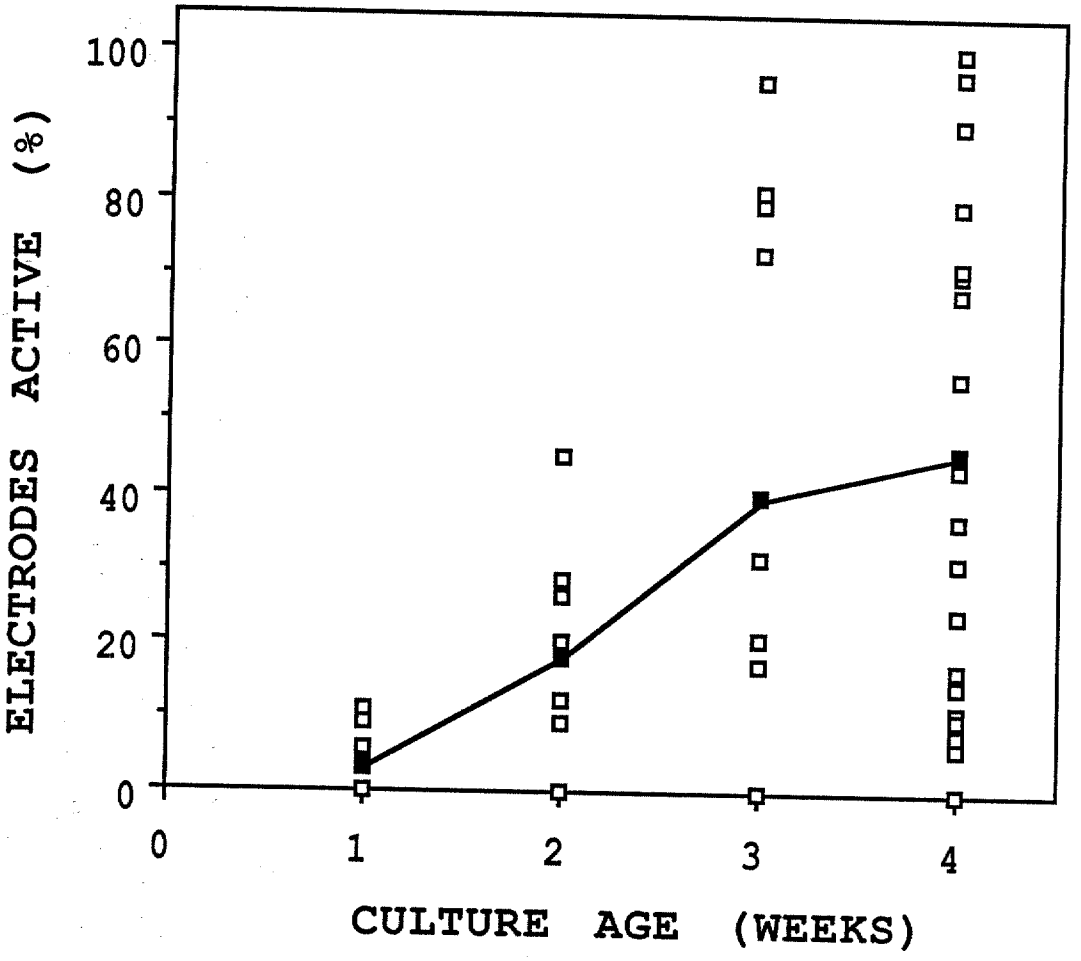

ELECTRODES ACTIVE (%)
100
80
60
40
20
0
0.0
2.0
4.0
6.0
8.0
10.0
CELLS SEEDED (10 -5)

trend suggested by the mean activity values, with the first observation of 100% electrodes active occurring at a seeding of 6 X 10^5 cells (Fig. 18). The mean percentage of active electrodes appeared to decline with seedings of 1 X 10^6 cells. However, this data point was probably invalid; two plates in this group were sacrificed to fungal contamination, and the poor performance of the 3 remaining cultures may have resulted from undetected contamination.

Activity as a Function of Culture Age

MMEPs of the AGE group (seeded with 6-8 X 10^5 cells in a constant 1-ml seeding volume and assayed at 1, 2, 3, or 4 weeks in culture, see Tables 1 and 2) were used to study the effect of culture age on the quantity of activity. The mean percentage of electrodes active on MMEPs after 1 week in culture was extremely low. This percentage rose steadily during weeks 2 and 3 and continued to rise slightly by the fourth week in culture, reaching a mean of 45% electrodes actives (Fig. 19). These data paralleled those of other laboratories (Jackson et al., 1982; Nelson and Brenneman, 1982; Van Huizen et al., 1987) and indicated that activity monitored by extracellular methods increases with age up to at least 4 weeks in culture. It must be noted, however, that despite controlled culture conditions, the activity variability in cultures assayed during weeks 3 and 4 was great, ranging from 0-100% electrodes active.

Figure 19. Scatterplot of the percentage of active electrodes as a function of culture age (AGE file data). Activity was maximized by counting as active all of those electrodes which were active either before or after the addition of 7 µM strychnine. Although considerable variability was exhibited by MMEPs experiments within each age group, the mean percentages of active electrodes increased between weeks 1 and 4 (lines).


ELECTRODES ACTIVE (%)
100
80
60
40
20
0
0
1
2
3
4
CULTURE AGE (WEEKS)

This may indicate that the neuronal networks formed in dissociated culture were variable or that degeneration resulting from inadequate culture maintenance (insufficient supplies of trophic factors, etc.) or undetected contamination in some cultures competed with the developmental trend toward increased activity levels. However, the curve formed by tracing the envelope of maximal activity levels showed the same trend toward increased activity with age that the mean activity levels indicated (Fig. 19).

When the activity characteristics of cultures of different ages were compared, a developmental progression emerged. One-week-old cultures displayed more random single spikes (Fig. 20). Bursting, if present, was less intense (indicative of lower spike density within the burst), and spike amplitude never exceeded 100 μV. Most of the activity monitored from 2-week-old cultures was in the form of intense bursts with spike amplitudes averaging 150 μV. Activity at 2 weeks was often indistinguishable from the activity of some 3- and 4-week-old cultures (Fig. 20). A scatterplot of the maximum spike amplitude monitored on each MMEP as a function of culture age (Fig. 21) showed a tendency toward increasing signal strength between weeks 1 and 4. The development of electrophysiological activity was observed in a single culture which was assayed during

Figure. 20. Oscillographic records depicting development of activity as a function of culture age. Each photograph was recorded from MMEP cultures from the same culture date assayed between weeks 1 and 4. *A*, One-week-old cultures exhibited low-frequency, low-amplitude spiking. Bursts, when present, were not intense. Coordination between electrodes was occasionally already present. *B*, Two-week-old cultures displayed spiking of higher amplitude and intense bursting. The activity of some 2-week-old cultures was indistinguishable from older cultures. *C*, *D*, During weeks 3 and 4, maximum spike amplitude tended to increase. The majority of monitored activity was in the form of coordinated bursts. Horizontal scale bars, 1 sec, Vertical scales, approximately 40 μV.

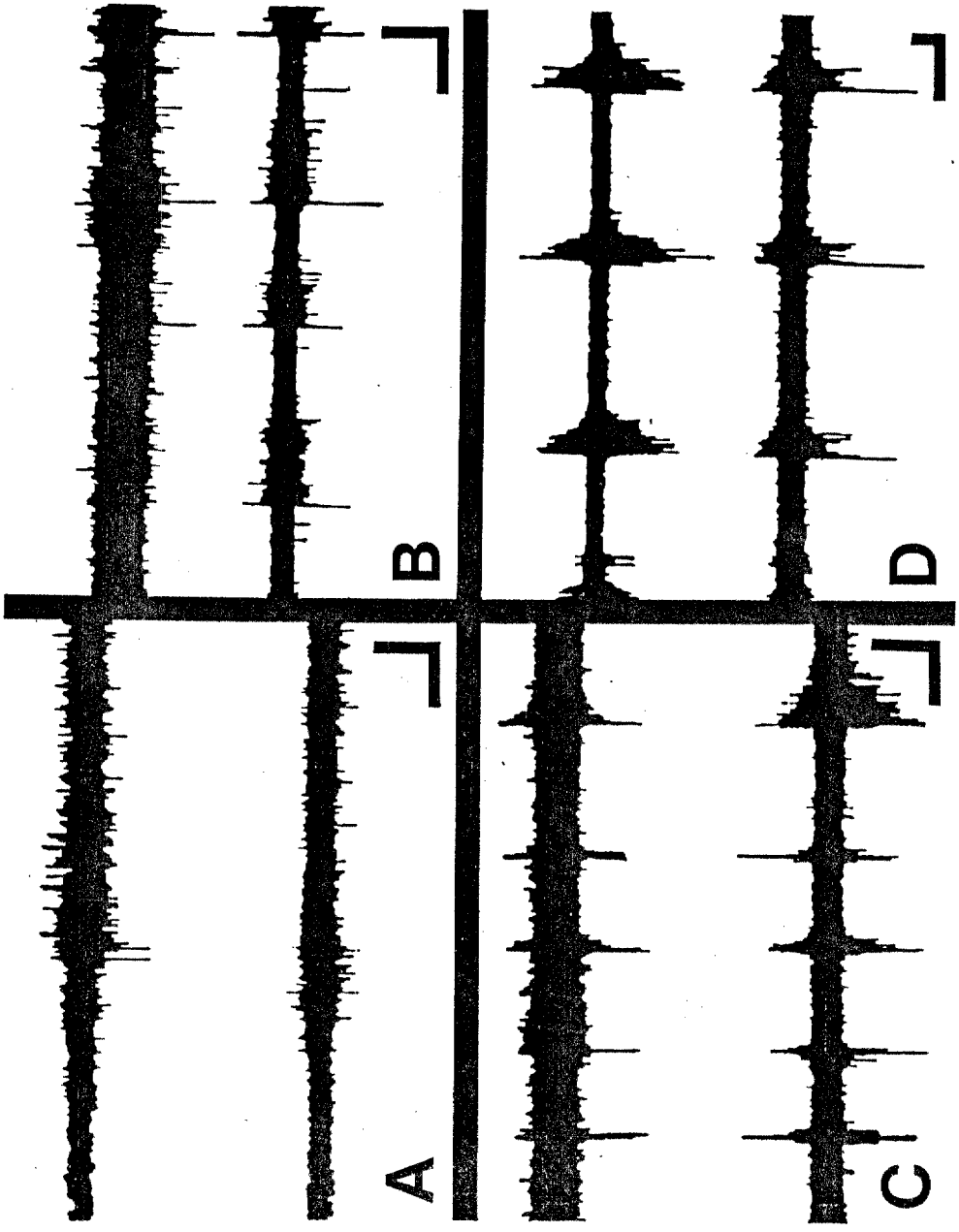
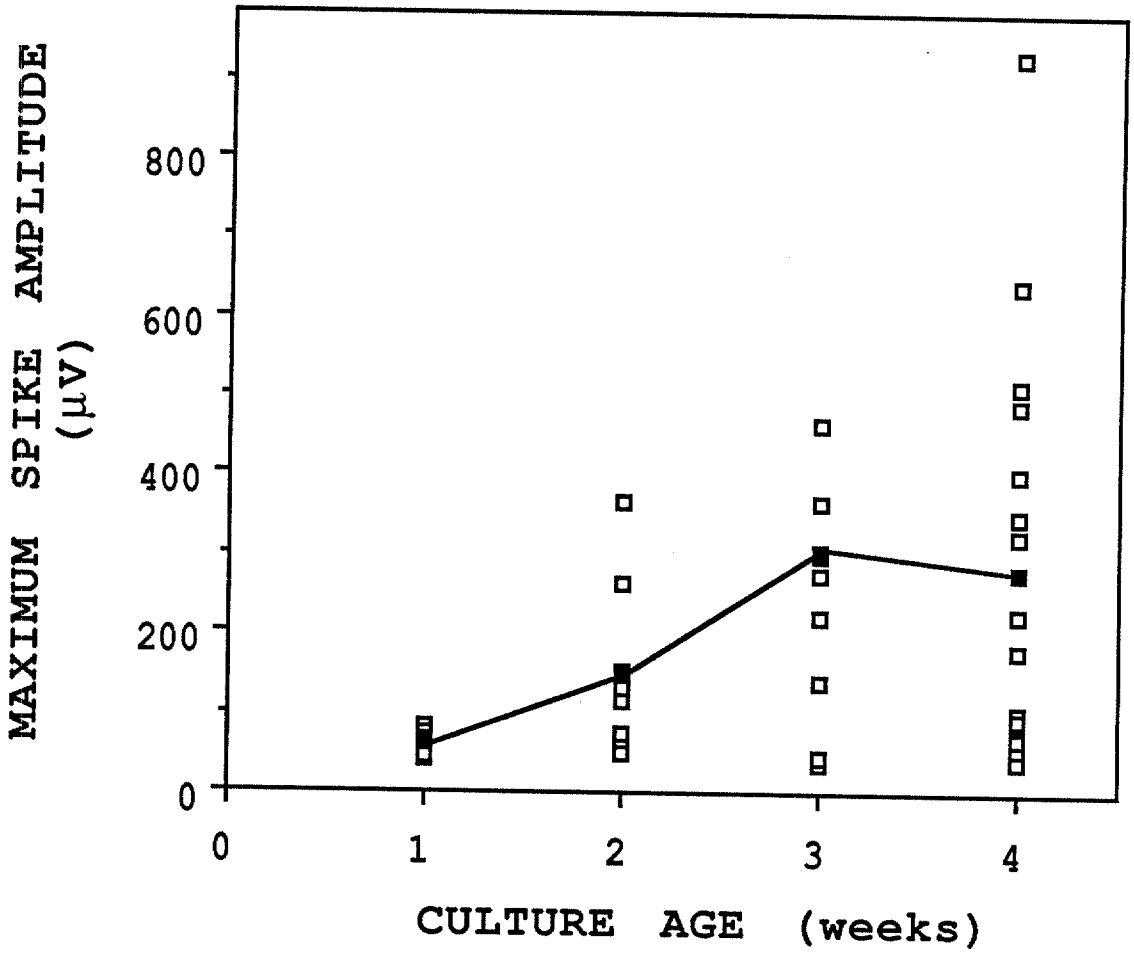

A
B
C
D

Figure 21. Scatterplot of the maximum action potential amplitude recorded on each MMEP versus culture age (AGE file data). The mean values for each age group are connected by lines. MMEPs demonstrating activity during week 1 exhibit very low amplitude spiking. Maximum spike voltage tended to increase during weeks 2 and 3, reaching a plateau between weeks 3 and 4.


MAXIMUM SPIKE AMPLITUDE (μV)
800
600
400
200
0
0
1
2
3
4
CULTURE AGE (weeks)

week 1 and again at week 3. The initially low amplitude spiking and low intensity bursting was replaced with vigorous bursting and greater spike amplitude (Fig. 22).

Discussion

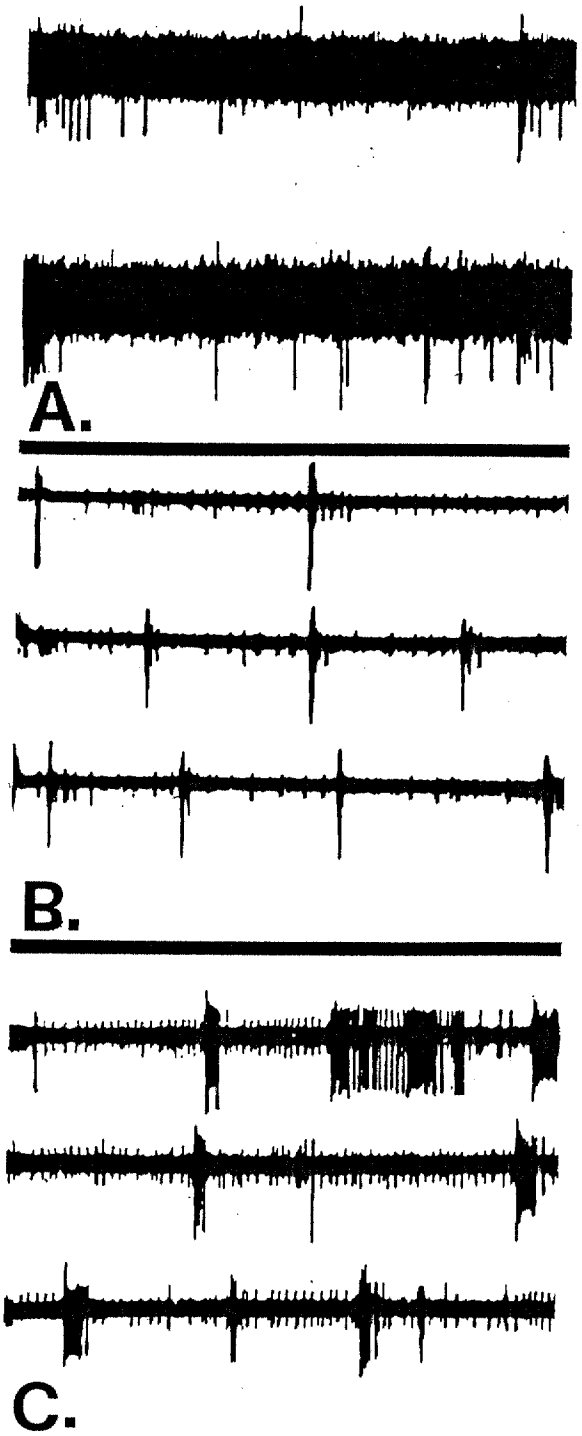
Comparison of Mini-culture and Larger Culture Activity

Activity recorded from mini-cultures paralleled that observed with spatially unrestricted MMEP cultures (Droge et al., 1986). As seen with the larger neuronal networks, spontaneous activity was frequently observed, primarily in the form of bursts of action potentials. The mini-culture spike amplitudes and burst frequencies did not differ from those of larger cultures. Activity coordination was similar to that of unrestricted cultures, and tonic continuous firing and alternating activity were observed with approximately the same frequency in large and mini-cultures. Thus, simplification of cultured neuronal networks by restriction of adhesion area did not result in noticeable differences in electrophysiological behavior.

Activity Coordination

The degree of activity coordination between electrodes on the mini-culture was striking. Although random, uncoordinated spiking predominated during week 1, activity was almost exclusively coordinated by 2 weeks in culture. Coordination was even displayed by cultures of low seeding

Figure 22. Oscillographic records showing the development of activity in a single culture assayed three times during its first 23 days in culture. *A*, Activity on day 7 was sparse and of low amplitude.*B*, Activity monitored from the same electrode on day 16 was of higher spike amplitude. Spikes were concentrated in clear bursts. *C*, Activity on day 23 was much more vigorous, with steady tonic activity as well as intense bursts. Scale bars, 1 sec. Noise levels for all traces were approximately 40 μV.


A.
B.
C.

density which exhibited a low percentage of active electrodes. This suggested that functional networks were formed in these cultures, regardless of the poor capacity for electrophysiological monitoring of cultures of such low neuronal density. Total activity coordination was not observed on all electrodes, however. In particular, the occasional presence of alternating activity indicated the capacity of the mini-culture to produce complex electrophysiological behavior and helped to further demonstrate that the coordination of activity between electrodes resulted from network formation rather than mere cross-talk phenomena or simple pacemaker activity.

Effects of Culture Variables, Data Scatter

Control of the culture variables of initial cell seeding and culture age at time of assay were required for systematic characterization of mini-culture activity. In addition to these controls, other variables, such as chemical lots, were kept constant for the control studies. Variable seeding studies indicated that 6-8 X 10^5 cells seeded per plate would result in optimally active cultures. Studies with cultures of different ages showed the development of increased activity levels between weeks 1 and 4, as evidenced by increases in the percentage of active electrodes and in spike amplitude. There was also an apparent tendency toward increased burst intensity and

coordination between electrodes as cultures aged. Nevertheless, substantial variability in activity levels was encountered, even though these cultures were controlled for seeding or culture age. This variability was one of the greatest problems remaining in the quantitative electrophysiological analysis of mini-cultures on MMEPs.

CHAPTER 4

ANALYSIS OF MMEP MINI-CULTURE ACTIVITY FOLLOWING PHARMACOLOGICAL TREATMENT WITH DISINHIBITORY AND INHIBITORY AGENTS

Introduction

Prior to this study, the effects of certain pharmacological agents on the electrophysiological activity of MMEP cultures had been investigated. Magnesium chloride, which competes with the calcium-mediated exocytotic event of synaptic transmission (Crain, 1976), had been applied to spontaneously active cultures and resulted in a cessation of activity (Gross and Lucas, 1982). This result supported, but did not confirm, the contention that the monitored activity was mediated by synaptic transmission. Strychnine, a receptor blocker of the inhibitory neurotransmitter glycine (Crain, 1976; Simmonds, 1986; Aprison et al., 1987), had also been applied to MMEP cultures, resulting in enhanced activity levels. Cursory experiments with other drugs, such as glutamate and aspartate, had met with less success, but it appeared that spinal cord monolayer cultures could respond to pharmacological manipulation in a manner similar to drug effects observed *in situ*.

Pharmacological studies with spinal cord mini-cultures had not been performed. However, when such cultures exhibited vigorous spontaneous activity similar to that of the larger monolayer cultures (MacDonald and Barker, 1981; Gross and Lucas, 1982), it was anticipated that their responses to drug application might prove similar as well. A systematic investigation of the effects of certain inhibitory and disinhibitory pharmacological agents on mini-culture activity was thus initiated. Two general types of drugs were employed for these studies: agents which compete with inhibitory neurotransmission (bicuculline, a GABA antagonist, and strychnine, a glycine antagonist, Simmonds, 1986) and the inhibitory neurotransmitters themselves (GABA and glycine). Both GABAergic and glycinergic neurons had been shown to be present in the spinal cord *in situ* (Cooper et al., 1982).

Subsequent to the qualitative observation of drug effects, the quantitation of certain features of mini-culture responses to the pharmacological agents was planned. While oscillographic records portrayed detailed electrophysiological activity at the resolution level of the individual action potential, the data were too complex for general analysis of activity changes. In addition, oscilloscope pictures could not provide an uninterrupted record of the activity on an electrode over a period of several minutes. Although chart recordings did not allow

resolution of the individual active units provided by oscillograph pictures, they did provide a continuous record of total electrode activity in a simpler, integrated form, the burst (see Fig. 17). Alterations in burst pattern or burst envelope characteristics were more easily quantified and could be used as a better indicator of changes in the activity of the neuronal population than detailed spike analysis. Thus, changes in mini-culture activity following drug application were studied by burst analysis using data obtained from chart recorders.

Materials and Methods

General Procedure for Drug Application

All pharmacological agents were applied directly to the 2 ml chamber bath in 100 µl aliquots. In order to determine the rate of drug mixing in the bath, a dye solution (1% thionin) was added to a mock chamber assembly containing 1.8 ml of culture medium. The assembly was mounted on the recording stage to simulate the conditions of electrophysiological assay and pharmacological additions. The test chamber received the standard flow of 10% CO_2 (25-30 ml/min), which caused fluid movement, resulting in enhanced mixing of added agents to the bath. Thirty replicates of this experiment indicated that dye mixing occurred within 5-10 sec. This required mixing time

correlated well with the observed onset of electrophysiological reactions to the applied agents.

All drugs, with the exception of bicuculline, were dissolved in MEM 10, the standard medium used for culture feedings. Bicuculline solubility characteristics required its suspension in a solution of MEM 10 with a final concentration of 1.0 mM HCl, which was further diluted by a factor of 20 upon its addition to the chamber reservoir. The methiodide salt of bicuculline was employed because it does not degrade to the inactive bicucucine in saline solutions (Olsen et al., 1975). At least one application of 100 µl of the appropriate drug solvent preceded addition of any drug to serve as a carrier control.

Addition of Disinhibitory Agents

Dosage Selection

For disinhibition studies, strychnine (100 µM) or bicuculline (500 µM) was added directly to the recording chamber bath (containing 1.5-2.0 ml of medium), resulting in a final drug concentration of 5-7.5 µM or 25-38 µM respectively. These concentrations were chosen for routine application because they were within the range recommended in the literature (Crain, 1976; Jahr and Nicoll, 1982; Cohen and Harris-Warrick, 1984; Ault et al., 1986; Horne et al., 1986), they produced demonstrable and relatively stable changes in bursting behavior in a majority of

cultures, and a 10-fold increase in this drug dosage did not result in toxicity-associated activity cessation within 30 min. In order to establish a dose-response curve, sample cultures were treated with sequential applications of drug solutions of increasing concentrations (range: 10^{-9}-10^{-3} M). Although the culture dose responses varied greatly, the disinhibitor concentrations chosen for routine investigation reproducibly promoted strong drug responses without producing electrophysiological or morphological deterioration of the cultures.

Dual Disinhibition Investigations

Dual disinhibition experiments with both bicuculline and strychnine were performed by applying the second agent (in the concentration stated above) after the culture had established a disinhibition-associated activity pattern and appeared to exhibit the full reaction to the first drug (application interval, approximately 15-45 min). Experiments were performed with each agent added first in order to test the effect of drug application order.

Application of Inhibitory Agents

The inhibitory neurotransmitters glycine and GABA (in 500 μM stock solutions) were added to the culture bath to result in final drug concentrations of 25-38 μM. Inhibitors were applied to a few spontaneously bursting cultures prior to the addition of other drugs. However, in

order to show the ability of the inhibitors to reverse disinhibition, the majority of inhibitory drug tests occurred after disinhibition with strychnine and/or bicuculline.

Results

Effects of Disinhibitory Agents on Activity

Initial Responses to Disinhibition

A prolonged, intense burst occurred within 1-5 sec after the application of bicuculline or strychnine (Figs. 23, 24*B*). A "silent period" of 5-10 sec was occasionally observed after this "paroxysmal" burst. A short period (<1 min) of irregular tonic activity then followed, which preceded a prolonged sequence of regular bursting activity characterized by stereotyping of the integrated burst envelope shape. The application of drug solvent produced a similar initial paroxysmal burst (23, 24*A*), but it was generally of slightly lower amplitude and of shorter duration than that produced by disinhibition. Also, in contrast to the long-term electrophysiological effects of the disinhibitory agents, the characteristic activity pattern prior to drug solvent addition was generally re-established within 5 min.

Figure 23. Initial electrophysiological responses to 7 μM strychnine application illustrated by representative computer-generated chart recordings. Addition of 100 μl of medium produced an initial prolonged burst and briefly increased activity. However, within 1 min, burst activity returned to control levels. Application of 7 μM strychnine resulted in a pronounced initial burst, followed by a period of intense tonic firing. Within 1 min, bursting activity returned, with increased frequency compared with pre-drug activity. Burst regularity was also increased, and the shapes of the burst envelopes were more highly stereotyped. Scale bar, 10 sec.

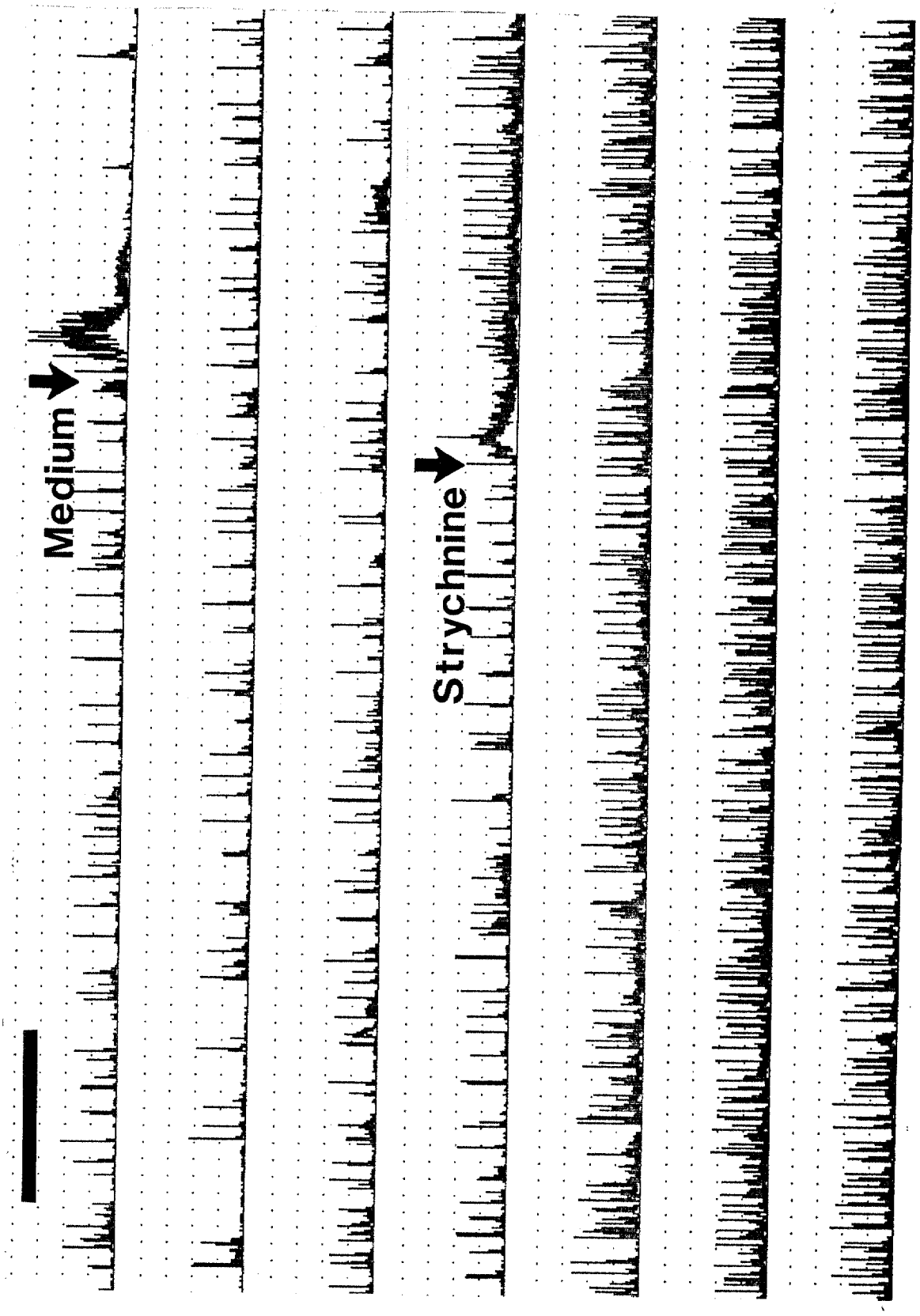
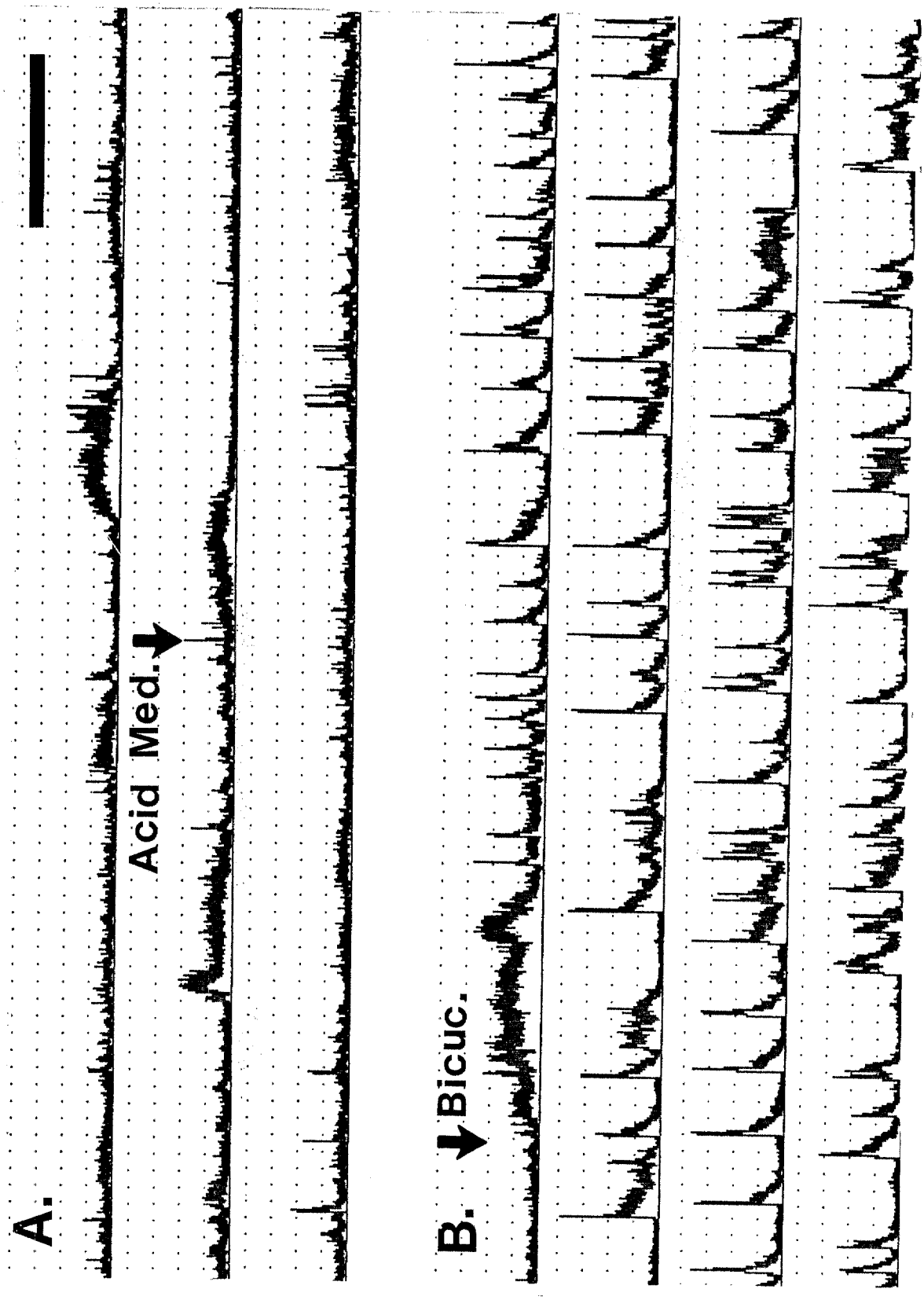

Medium
Strychnine

Figure 24. Electrophysiological response to the application of bicuculline. *A*, Addition of acid medium (drug solvent) had little effect on the quantity or form of activity. *B*, Addition of 35 μM bicuculline produced a pronounced initial burst, followed by an increase in burst frequency and intensity. Burst regularity was also increased, and the forms of the burst envelopes were stereotyped. Scale bar, 10 sec.


A.
Acid Med.
B.
Bicuc.

Changes in Burst Frequency after Disinhibition

Determination of burst frequency values for statistical comparison. Burst frequencies were determined by counting all bursts during 1-min increments at intervals of 3 min throughout each experiment. During periods of pronounced frequency changes, burst frequencies were determined for each 1-minute bucket. The frequency of spontaneous bursting prior to drug addition was generally <20/min. Although additions of the control drug carrier solutions produced immediate changes in burst frequency, effects of longer than 5 min occurred in only 6 of 38 experiments. In these cases, the frequency established by medium addition was used as the control frequency for evaluation of drug effects on burst frequency.

Burst frequency effects: single drug application. Following the application of 10 μM strychnine or 50 μM bicuculline, the bursting rate generally increased (Figs. 25 and 26), usually reaching a peak frequency within 3-10 min. Culture activity often remained at this peak level, forming a stable "plateau frequency." One culture was treated with strychnine, monitored, and returned to the incubator without a medium change. When this culture was reassayed after 12 hr, it exhibited vigorous activity with only a 20% decay in burst frequency. The activity characteristics exhibited by this culture also remained similar (Fig. 27). This activity stability was not

Figure 25. Plot of burst frequency during the course of an experiment in which strychnine was applied. Additions of MEM 10 (M), the standard culture feeding medium and drug solvent, had only small effects on burst frequency. Appplication of 7 μM strychnine (S) resulted in a large, rapid increase in burst frequency. A plateau frequency was achieved within 10-15 min.

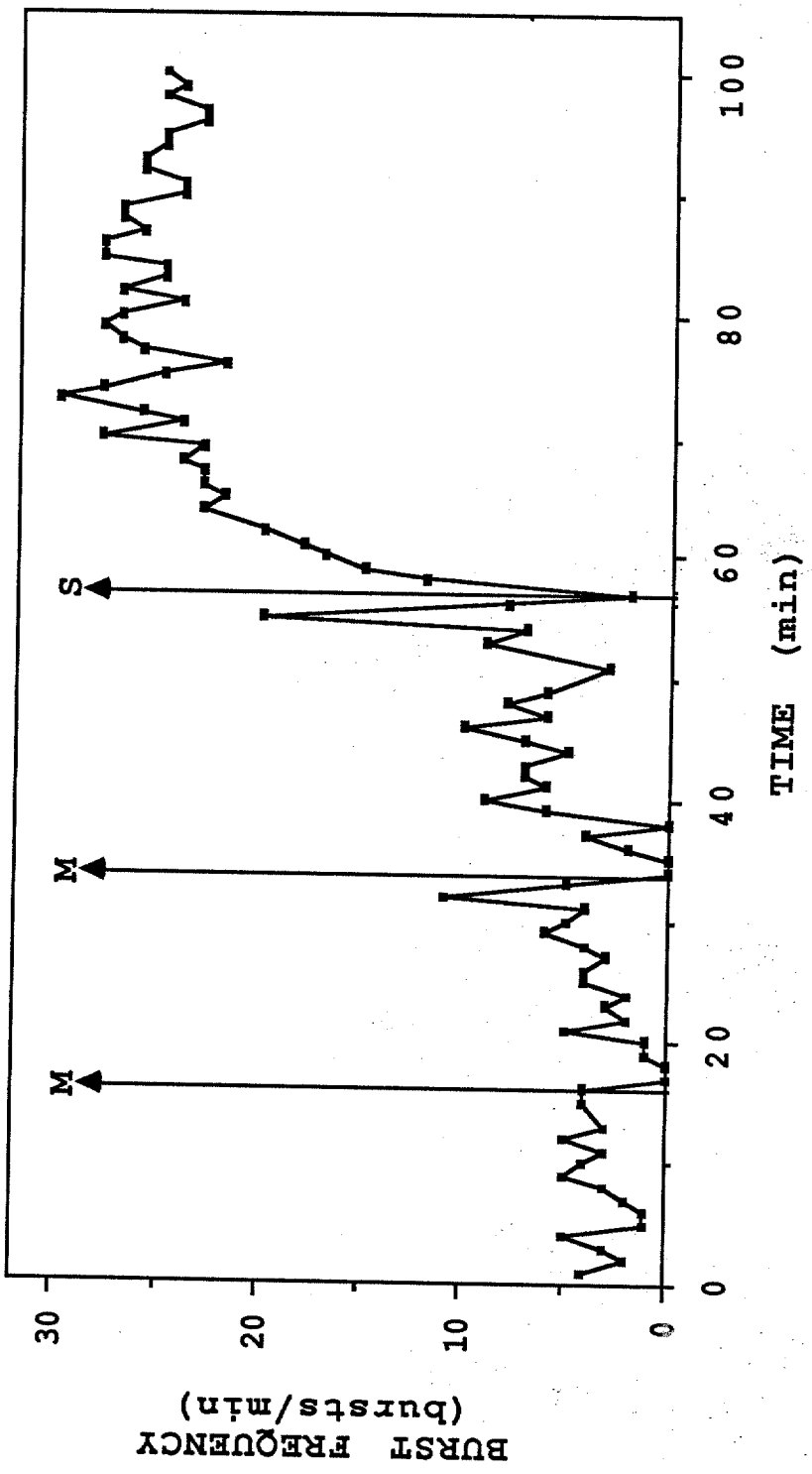

BURST FREQUENCY
(bursts/min)
30
20
10
0
0
20
40
60
80
100
TIME (min)
M
M
S

Figure 26. Plot of burst frequency during the course of an experiment in which bicuculline was applied. Additions of MEM 10 (M) and acidified MEM 10 (AM), the bicuculline solvent, had pronounced but short-term effects on burst frequency. Application of 35 μM bicuculline (B) resulted in a rapid and pronounced increase in burst frequency. Although burst frequency readings varied considerably both prior to and following bicuculline addition, the increase in burst frequency to at least 35-40 bursts/min was established within 5-10 min of drug application.

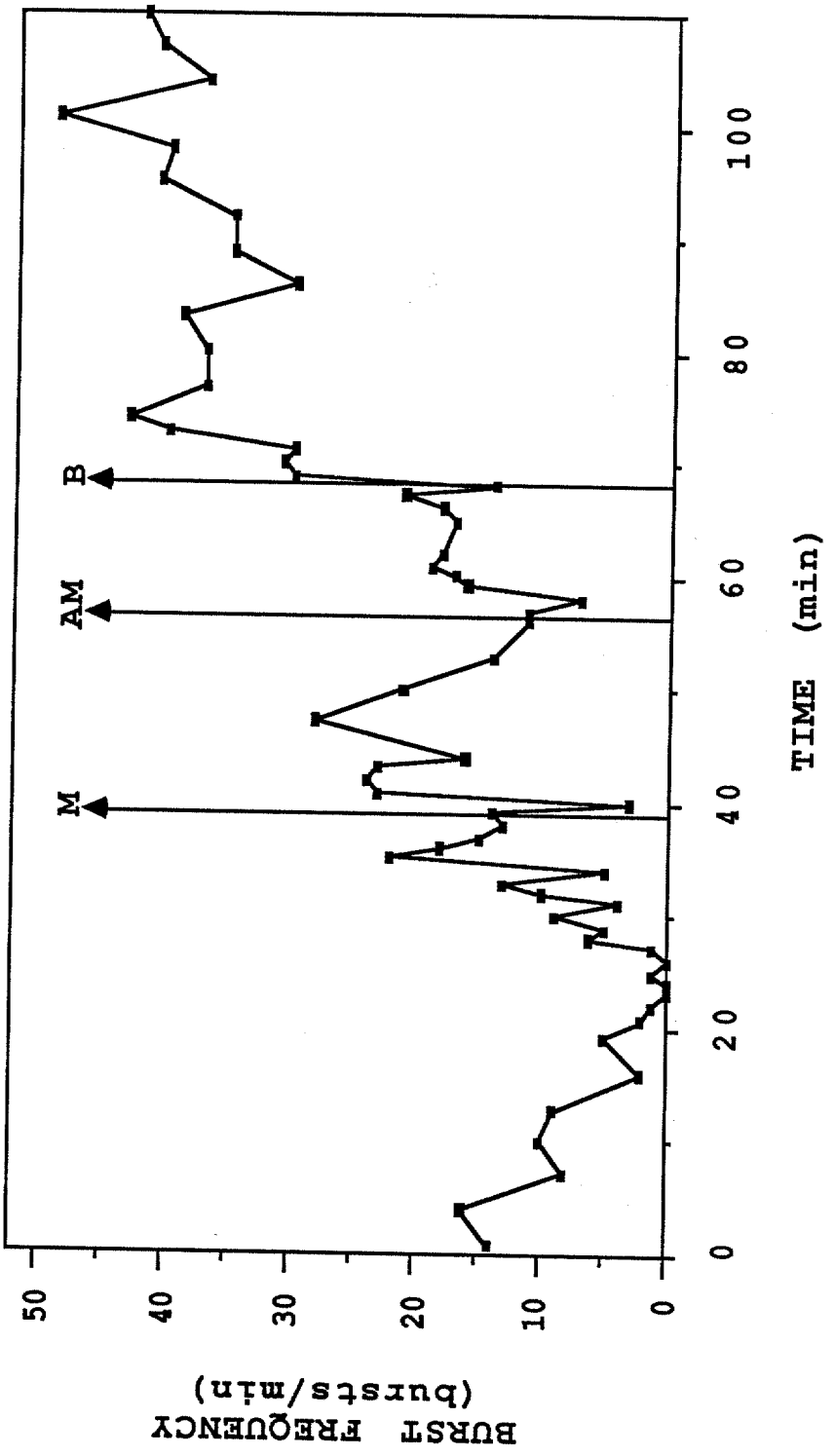
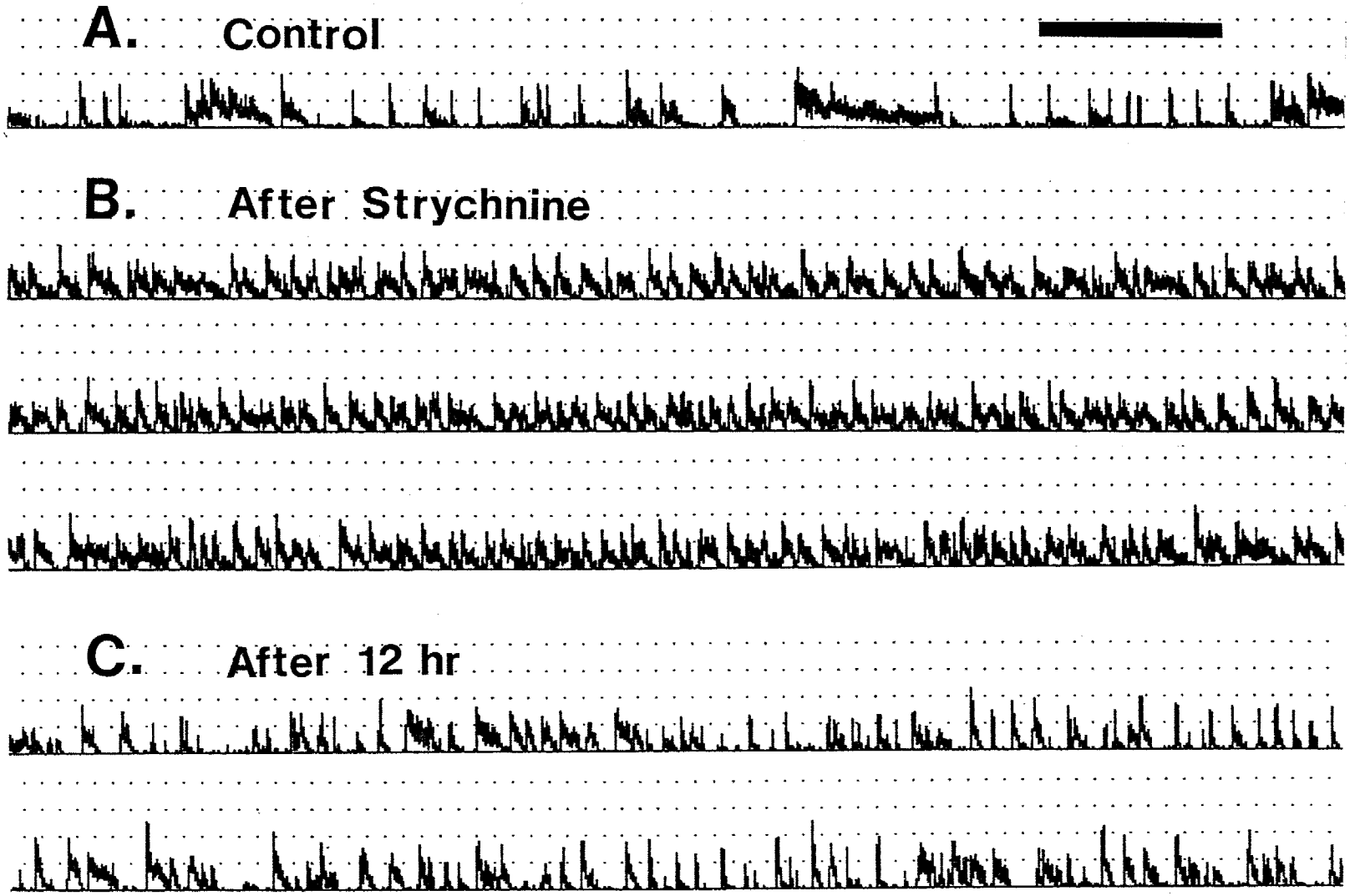

BURST FREQUENCY
(bursts/min)
50
40
30
20
10
0
0
20
40
60
80
100
TIME (min)
M
AM
B

Figure 27. Long-term effects of disinhibitor treatment illustrated by computer-generated chart recordings. This culture displayed vigorous spontaneous activity (*A*), received 7 μM strychnine, exhibited rhythmic bursting (*B*), and was returned to the incubator. After 9 hr, it continued to display disinhibited activity, although burst intensity, frequency, and regularity had somewhat decayed. Scale bar, 10 s.


A.
Control
B.
After Strychnine
C.
After 12 hr

representative of typical experiments, and some cultures displayed rapid burst frequency decay (Fig. 28). However, burst frequencies often plateaued at an increased frequency for up to 30 min (Figs. 25, 26).

For statistical comparison, predrug burst frequency was measured for 1 min 5 min prior to disinhibitor application. Post-disinhibition frequency was defined as the plateau burst frequency, or the frequency 5 min following disinhibitor application in those cultures which did not establish a plateau. Although a wide variation in response to the disinhibitors was observed, bicuculline produced a mean frequency increase of 59% above control levels (N = 15 experiments, Table 3). Strychnine was generally even more effective in producing an increased frequency than bicuculline (88% mean frequency increase, N = 10, Table 4). A few cultures, however, exhibited a decrease in burst frequency following disinhibition. In almost all cases of disinhibition-produced burst frequency decrement, the initial burst frequency had been high (Fig. 29). In 8 of the 11 experiments displaying initial burst frequencies of $\geq$32 bursts/min, disinhibitory drug application resulted in a decreased burst frequency (Table 5).

Burst frequency effects: dual disinhibition experiments. Dual drug effects were investigated by sequential application of both disinhibitors and

Figure 28. Plot of burst frequency changes during the course of a pharmacological experiment. Although a higher burst frequency was achieved after 7 µM strychnine treatment (S), this increased frequency decayed over a 10-min period. In fact,the effect of strychnine addition was almost as transient as that of MEM 10 addition (M). The addition of 35 µM bicuculline (B) increased burst rate beyond the levels attained with strychnine, but the effects also decayed over the 10-minute observation period. 35 µM glycine was added at minute 58, resulting in a cessation of burst activity (to be discussed in inhibitor results section).

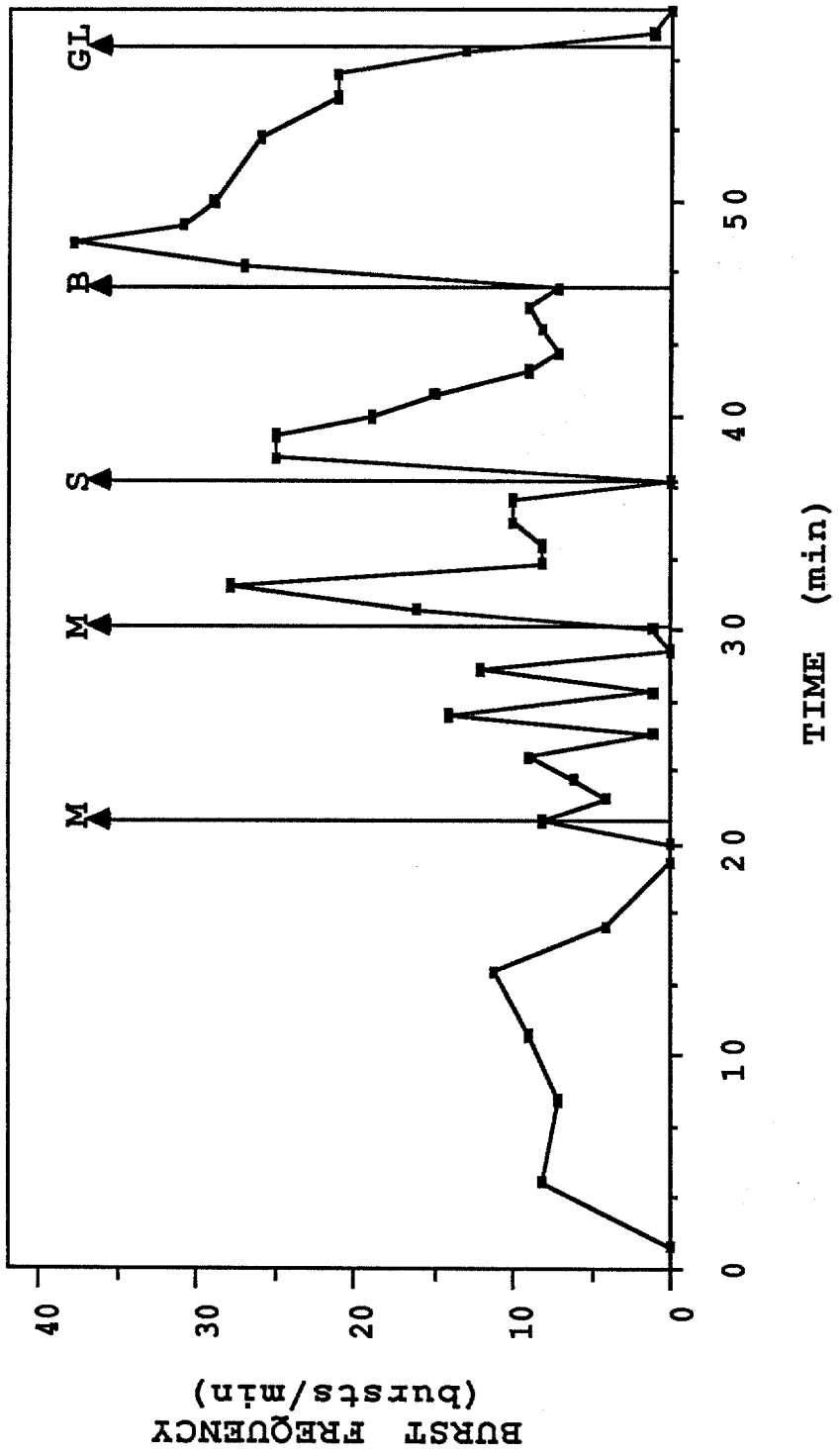

BURST FREQUENCY
(bursts/min)
0
10
20
30
40
0
10
20
30
40
50
TIME (min)
M
M
S
B
GL

Table 3. Summary of bicuculline effects on burst frequency

MMEP no.	Initial frequency[a] (bursts/min)	Frequency following bicuculline[b] (bursts/min)	Burst frequency difference (bursts/min)	Frequency change (%)
G2	5	23	+18	+360
H142	22	37	+15	+68
H5	10	15	+5	+50
H26	10	15	+5	+50
H33	15	20	+5	+33
H48	20	25	+5	+25
H49	9	14	+5	+56
H74	20	20	0	0
H78	22	13	-9	-41
H100	16	25	+9	+56
J35	5	11	+6	+120
J37	16	36	+20	+125
L2	16	21	+5	+31
AA22	16	14	-2	-13
AA25	38	20	-18	-48
Mean frequency change				+58

[a]Predrug burst frequency was defined as the burst frequency measured for 1 min 5 min prior to bicuculline application.

[b]Postdrug burst frequency was defined as the frequency plateau established after 35 μM bicuculline administration or the burst frequency determined 5 min following drug application in those cultures not exhibiting a plateau.

Table 4. Summary of strychnine effects on burst frequency

MMEP no.	Initial frequency[a] (bursts/min)	Frequency following strychnine[b] (bursts/min)	Burst frequency difference (bursts/min)	Frequency change (%)
G19	7	10	+3	+43
G26	12	18	+6	+50
H11	8	25	+17	+213
H35	13	20	+7	+54
H70	35	60	+25	+71
J5	23	45	+22	+96
J19	22	40	+18	+82
J24	35	53	+18	+51
K2	10	23	+13	+130
L5	10	18	+8	+80
Mean frequency change				+87

[a]Predrug burst frequency was defined as the burst frequency measured for 1 min 5 min prior to strychnine application.

[b]Postdrug burst frequency was defined as the frequency plateau established after 7 μM strychnine administration or the burst frequency determined 5 min following drug application in those cultures not exhibiting a plateau.

Figure 29. Burst frequency decrease in response to pharmacological disinhibition. Although most cultures responded to strychnine with increased burst frequency, this mini-culture exhibited decreased frequency of bursting after disinhibition with 7 μM strychnine (S). This MMEP culture and others which exhibited decreased burst frequency in response to disinhibitory agents often displayed high pre-drug burst frequencies. MEM 10 additions (M) did not affect burst frequency.

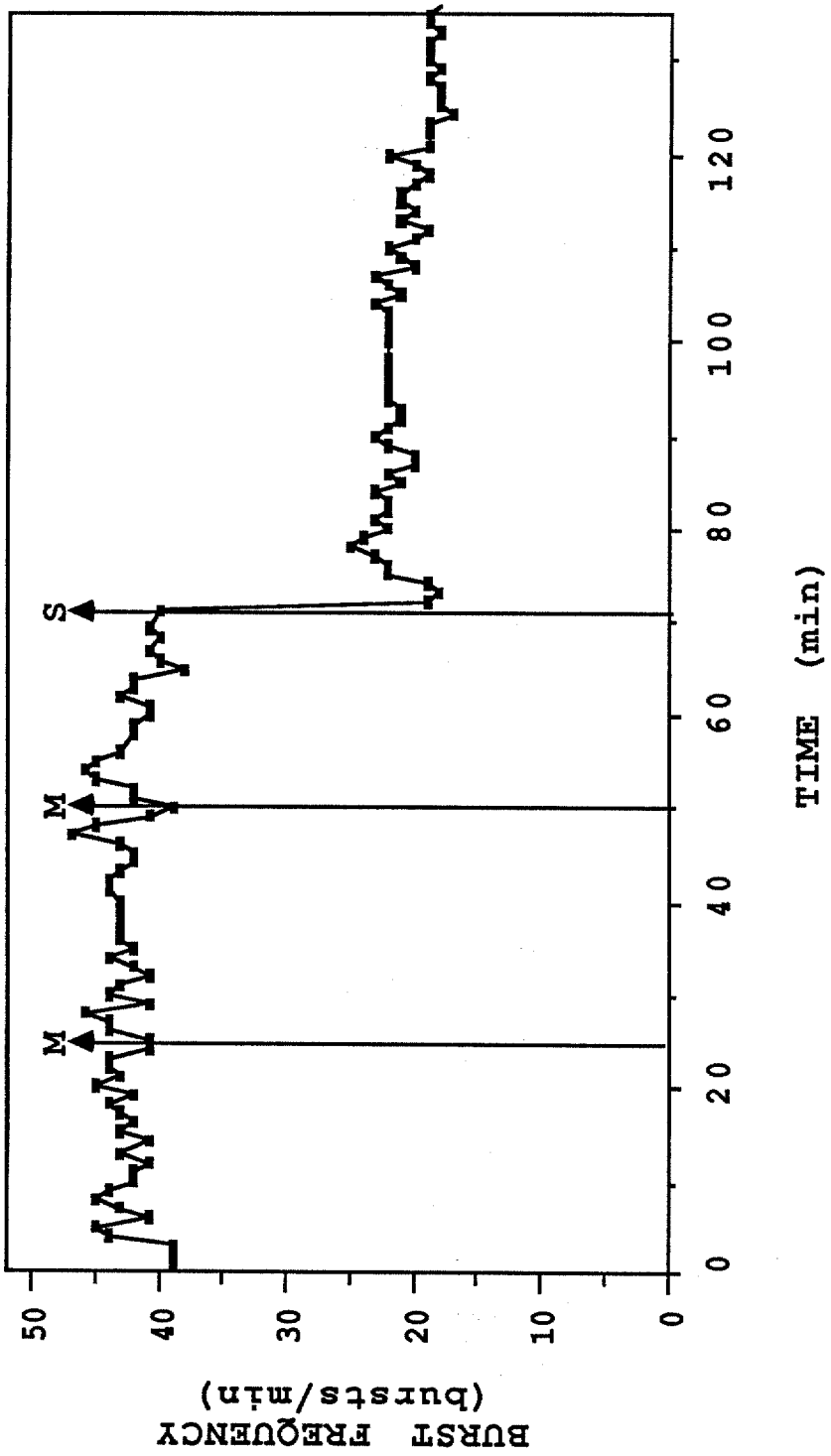

BURST FREQUENCY
(bursts/min)
50
40
30
20
10
0
0
20
40
60
80
100
120
TIME (min)
M
M
S

Table 5. Summary of the responses of cultures with high burst frequencies to disinhibition

MMEP number	Drug applied[a]	Initial burst frequency[b] (bursts/min)	Frequency following disinhibition[c] (bursts/min)	Burst frequency change (%)
G37	S	32	25	-28
H8	S	42	52	+24
H50	S	45	25	-44
H70	S	42	60	+42
H73	S	44	22	-50
H89	S	38	8	-79
H92	S	45	85	+89
H97	S	85	40	-53
H107	S	46	33	+28
H111	S	35	21	-40
AA25	B	35	19	-46

[a]S indicates application of 7 μM strychnine, and B indicates the administration of 35 μM bicuculline.

[b]Predrug burst frequency was defined as the burst frequency measured for 1 min 5 min prior to drug application.

[c]Postdrug burst frequency was defined as the frequency plateau established after disinhibitor administration.

measurement of a second post-drug frequency. Dual disinhibitor application resulted in a mean burst frequency increase of 123% above that of the original control frequency (N = 25, Table 6). Thus, the application of two disinhibitors generally increased burst frequency above levels achieved by one agent alone (Figs. 28, 30, and 31, and Table 6). Such an enhanced response was not observed when two aliquots of strychnine were applied sequentially (mean increase 75%, N = 10). Drug application order may have had an effect on maximum frequency increase (106% mean increase for experiments in which bicuculline followed strychnine, N = 10; 134% mean increase for experiments with strychnine following bicuculline, N = 15). Due to the wide variation in culture responses to pharmacological agents, however, this difference was not statistically significant.

Disinhibition: Burst Regularity

Measurement of burst regularity: burst period range decrement. The regularity of bursting monitored as integrated activity on the chart recorder increased after disinhibition with either bicuculline or strychnine (Figs. 32 and 33). This was exhibited both as a stereotyping of burst envelope form and as greater regularity of bursting pattern. In an attempt to quantify development of burst pattern regularity, the maximum and minimum burst periods were measured for 1-minute increments at 2-minute intervals

Table 6. Summary of the effects of dual disinhibitor application on burst frequency

MMEP no.	Drug application order[a]	Initial frequency[b] (bursts/min)	Frequency change, drug 1[c] (%)	Frequency change, dual drug[d] (%)
G19	S,B	7	+43	+57
G26	S,B	12	+50	+100
H11	S,B	8	+213	+250
H35	S,B	13	+54	+131
H70	S,B	35	+71	-3
J5	S,B	23	+96	+65
J19	S,B	22	+82	+164
J24	S,B	35	+51	+71
K2	S,B	10	+130	+130
L5	S,B	10	+80	+100
G2	B,S	5	+360	+560
H142	B,S	22	+68	+18
H5	B,S	10	+50	+100
H26	B,S	10	+50	+700
H33	B,S	15	+33	+33
H48	B,S	20	+25	+30
H49	B,S	9	+56	+56
H74	B,S	20	0	+40
H78	B,S	22	-41	-50
H100	B,S	16	+56	0
J35	B,S	5	+120	+320
J37	B,S	16	+125	+219
L2	B,S	16	+31	+50
AA22	B,S	16	-13	-13
AA25	B,S	38	-48	-53
Mean frequency change (%)			+70	+123

[a]S indicates application of 7 μM strychnine, and B indicates the administration of 35 μM bicuculline.

[b,c,d]See the methods section and Tables 4-1 and 4-2 for discussion of burst frequency determinations and calculations of postdrug effects.

Figure 30. Burst frequency plot of a sample experiment in which sequential dual disinhibition with bicuculline and strychnine was performed. A burst frequency plateau was reached with 35 µM bicuculline (B). Application of 7 µM strychnine (S) produced a second, higher plateau frequency. MEM 10 or acidified MEM 10 additions (M, AM) produced only transient effects.

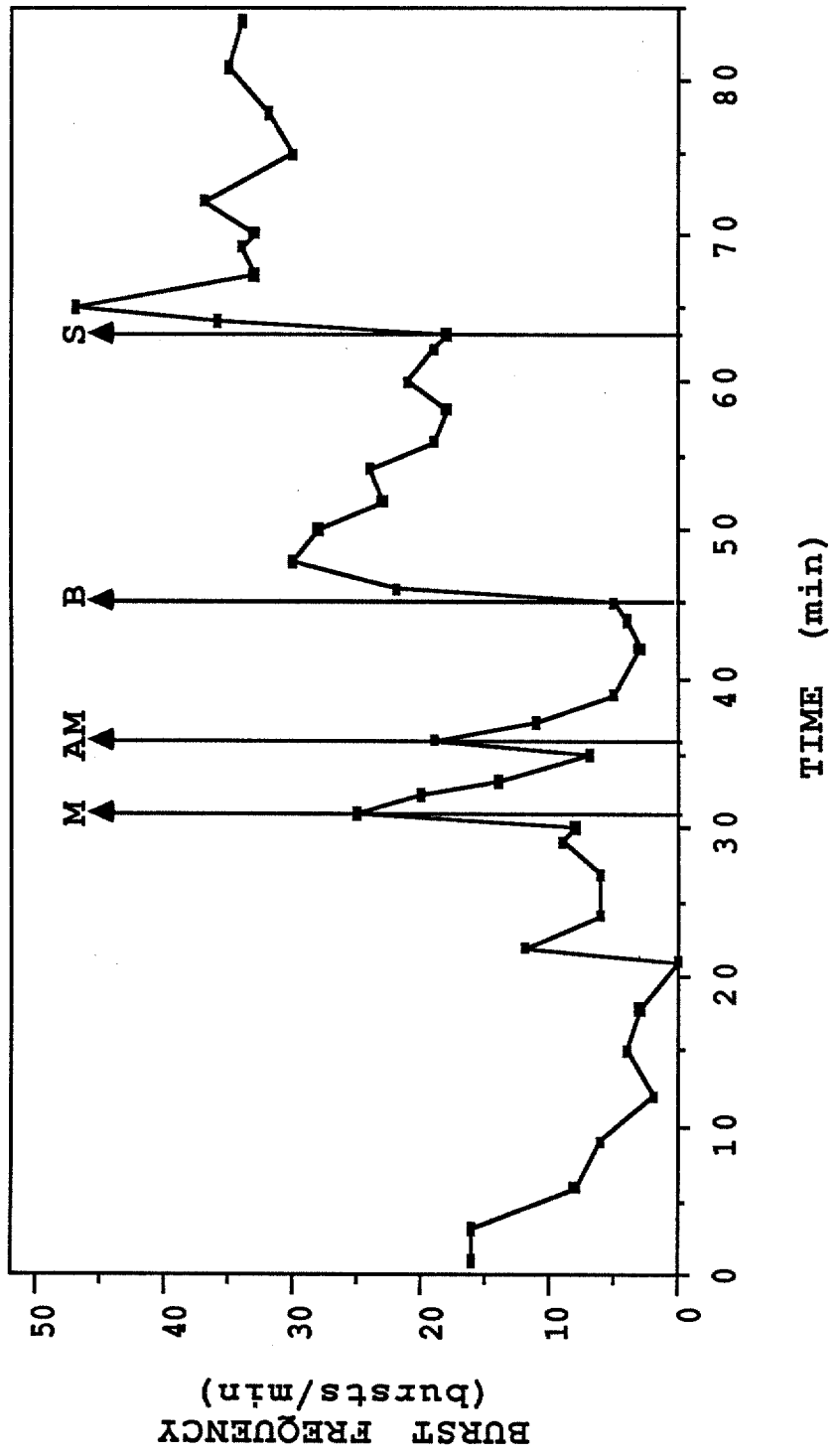

BURST FREQUENCY
(bursts/min)
0
10
20
30
40
50
0
10
20
30
40
50
60
70
80
TIME (min)
M
AM
B
S

Figure 31. Bar graph comparison of the mean frequency increases following the addition of bicuculline, strychnine, or both disinhibitors. Both bicuculline (35 μM) and strychnine (7 μM) increased burst frequency above control levels. Dual drug application resulted in frequency increases beyond that produced by either drug alone. N=15, bicuculline; 10, strychnine; 25, dual drug.

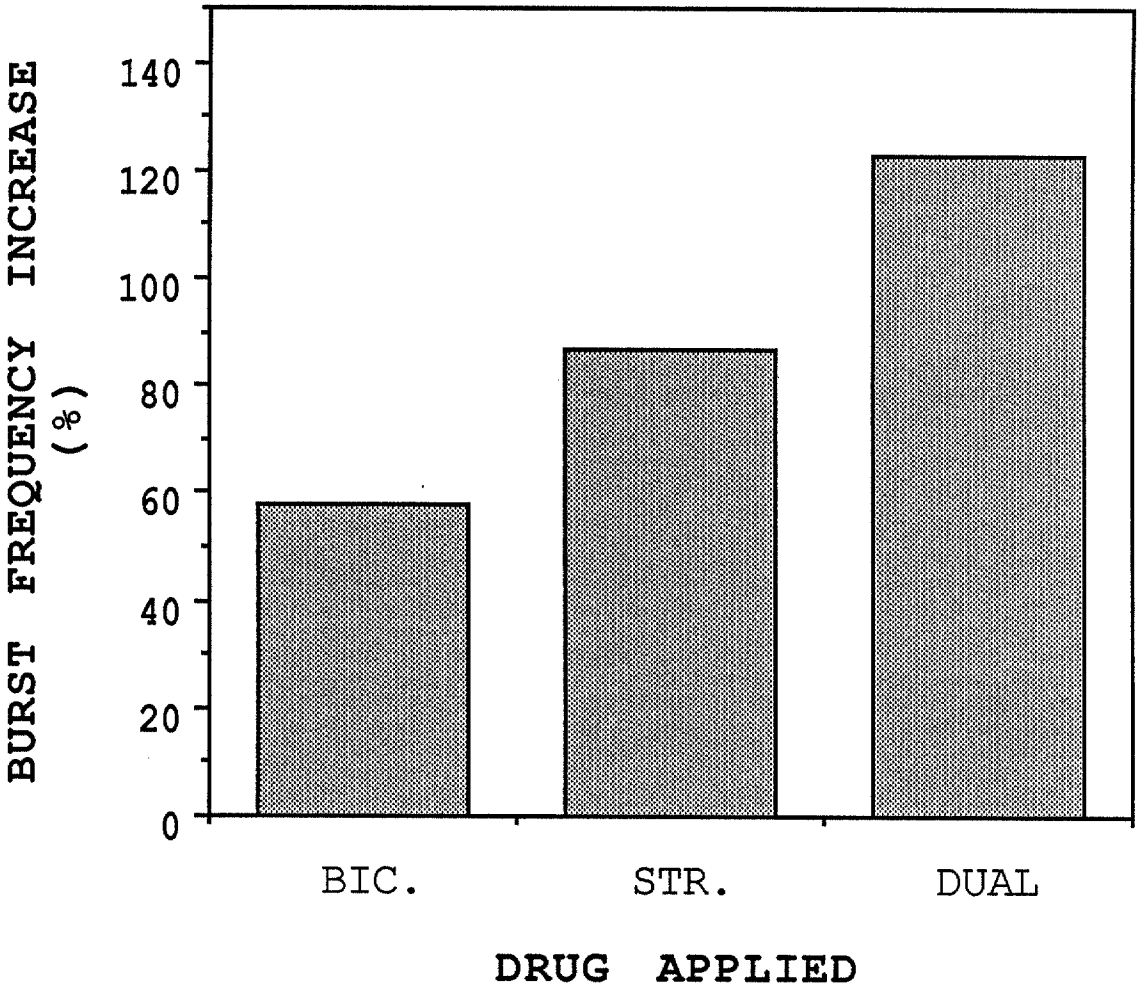

BURST FREQUENCY INCREASE (%)
140
120
100
80
60
40
20
0
BIC.
STR.
DUAL
DRUG APPLIED

Figure 32. Computer-generated chart record illustrating the production of regular bursting patterns after disinhibition with bicuculline (B, 35 μM). Not only was burst regularity increased, but the form of the burst envelopes became more similar. This suggested that the intraburst spike activity had become more stereotyped. Scale bar, 10 sec.

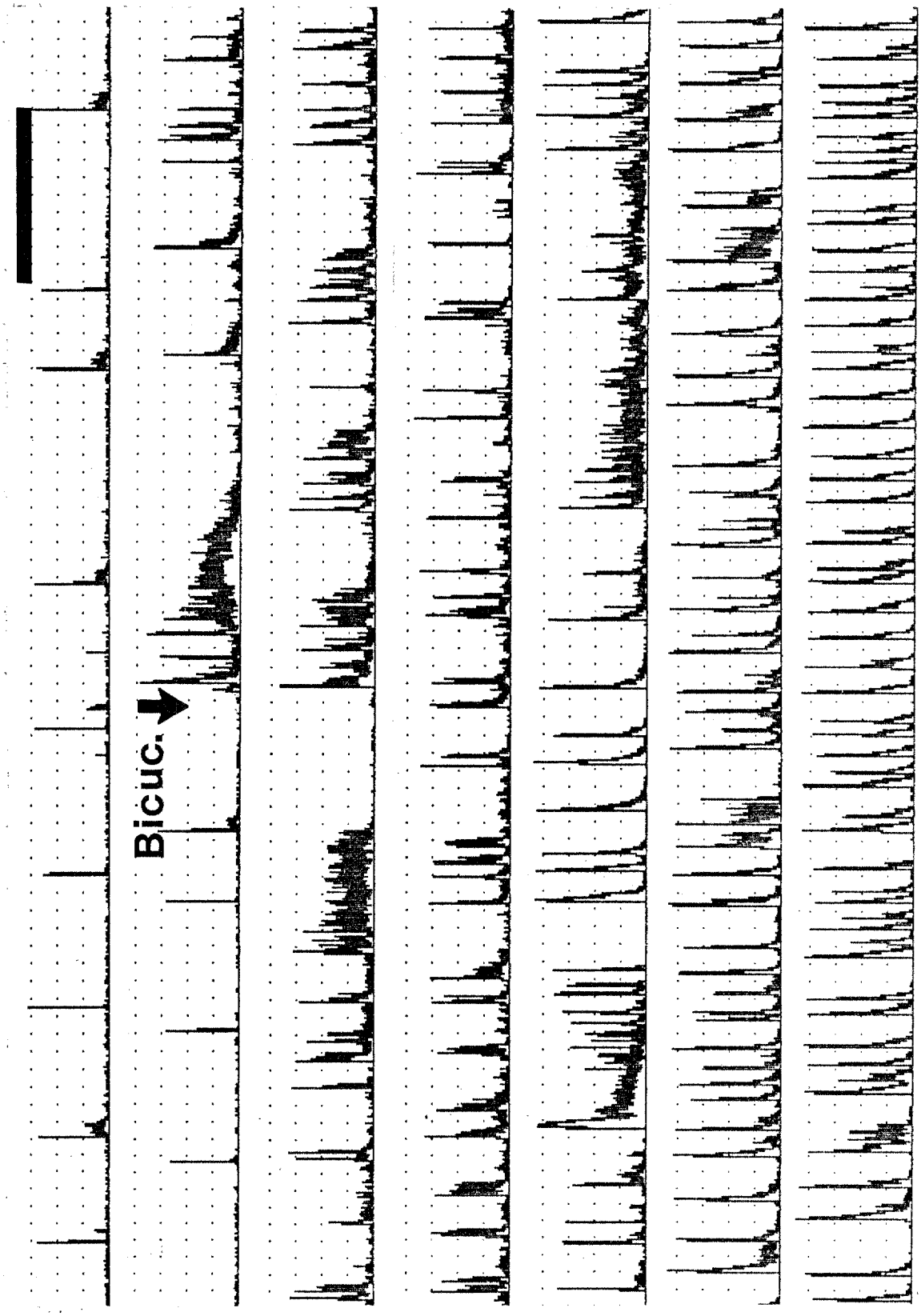
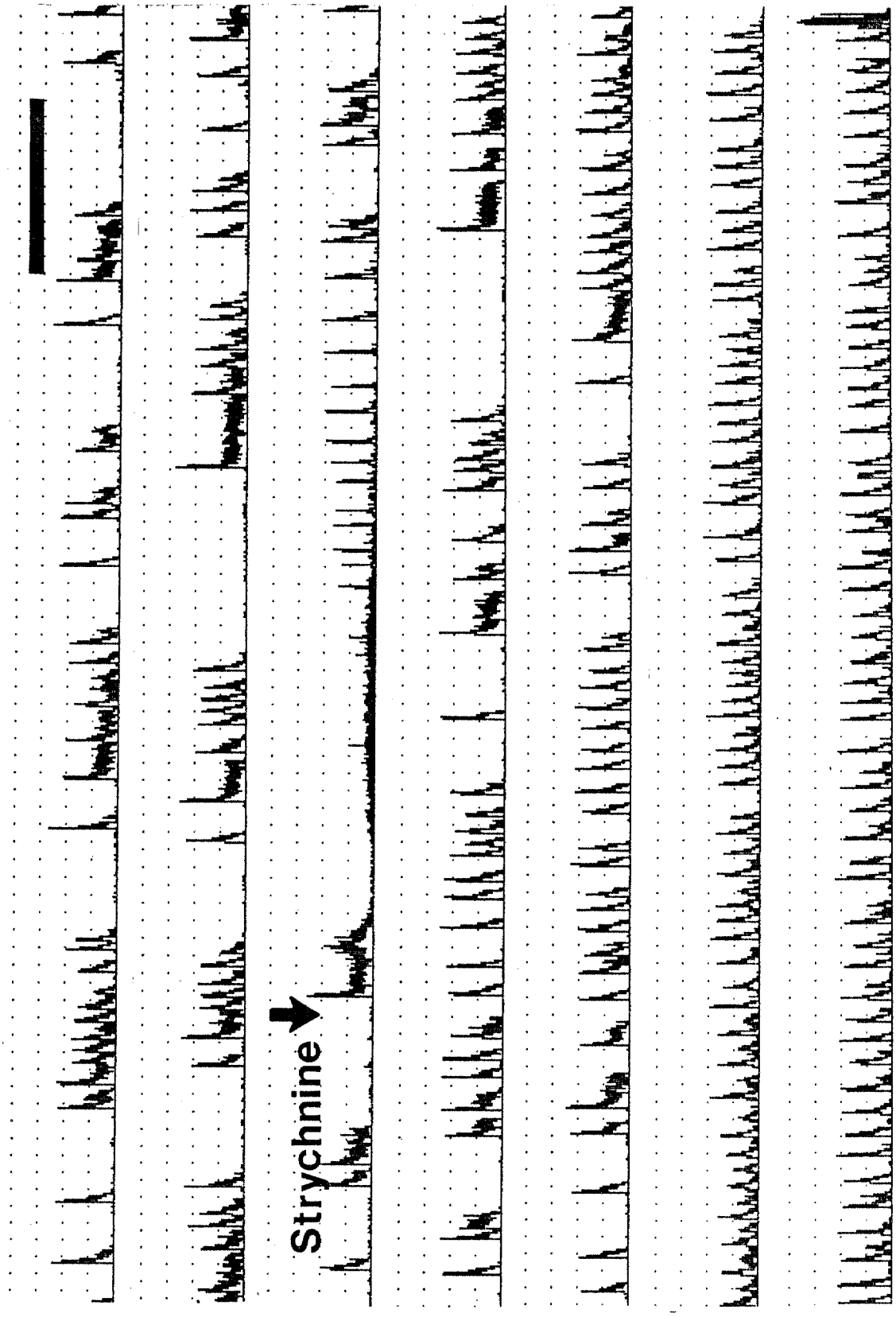

Bicuc.

Figure 33. Chart record showing the development of a regular bursting pattern following disinhibition with strychnine (S, 7 μM). Stereotyping of the burst envelopes also occurred.


Strychnine

throughout the course of selected experiments. Plots of these maximum and minimum periods (Figs. 34 and 35) provided a graphical representation of fluctuations in burst period range, a measurement of the regularity of burst activity. Both bicuculline and strychnine produced dramatic and stable reductions of the burst period range in comparison with that of control levels. Additions of control media did not produce such a period range decrease.

Measurement of burst regularity: coefficient of variation of burst period. Mean burst period, and therefore the burst period range, would be expected to vary inversely with the burst frequency. Both disinhibitors generally produced an increased burst frequency. Thus, a decrease in burst period range could occur independently of increased regularity. Therefore, the burst period variability was normalized to omit frequency effects by expressing variation as a percentage of mean period (coefficient of variation, C. V.). The periods of all bursts during a one-minute increment 5 min prior to and 5 min following each disinhibitor application were measured and used to calculate the control, single drug, and dual drug period C. V.

The cultures exhibited a high degree of variation, both in control period C. V. and in response to pharmacological treatment. However, application of the disinhibitors generally resulted in a decrease in burst

Figure. 34. Plot of maximum and minimum burst periods during a strychnine disinhibition study. The area between the maximum and minimum plots represents changes in burst period range throughout the experiment. Strychnine application (7 µM, S) reduced the burst period range, whereas application of MEM 10 (M) did not have this effect. Bicuculline addition followed strychnine treatment (35 µM, B), but in this experiment, it did not appear to produce an appreciable decrease in burst period range below that achieved by strychnine.

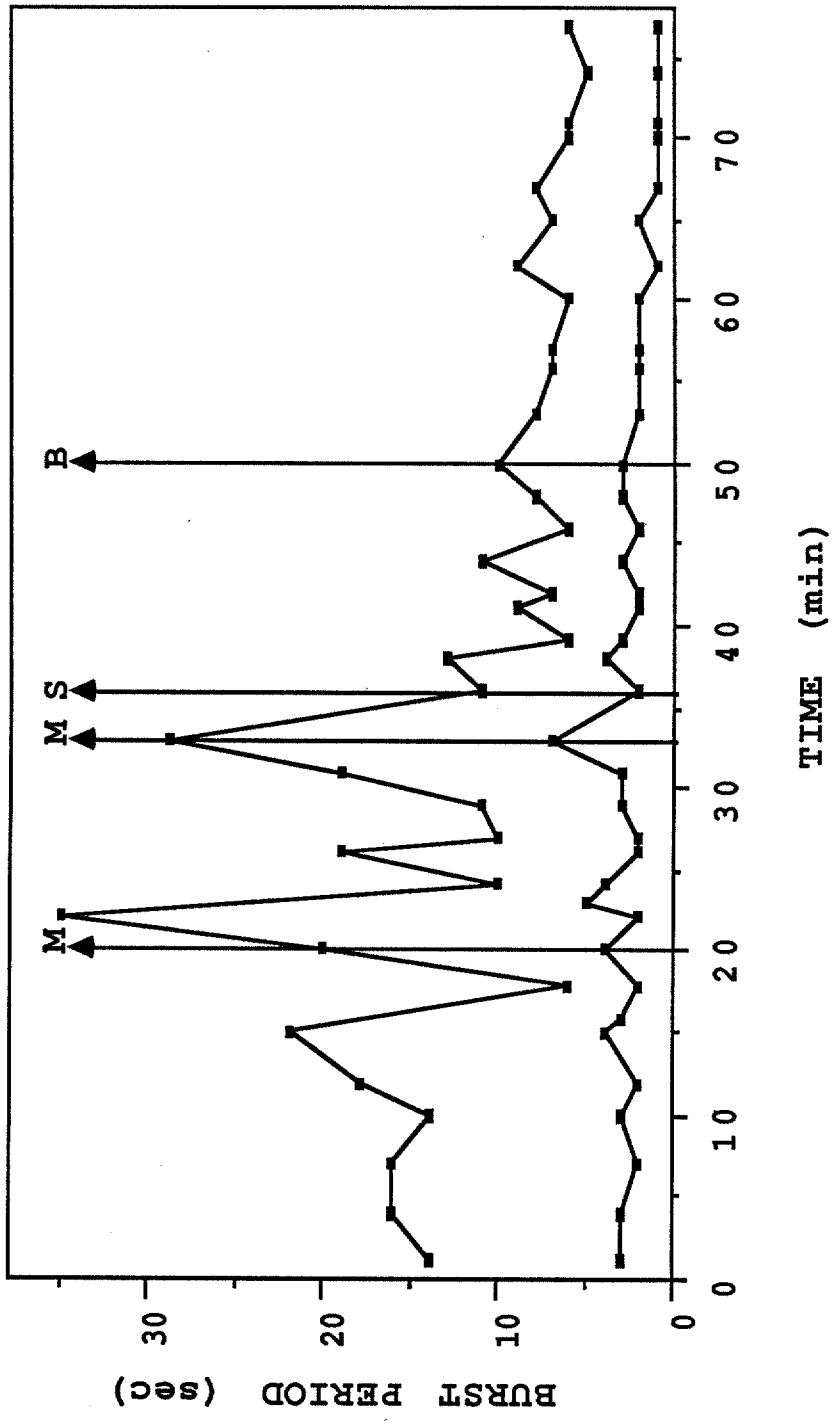
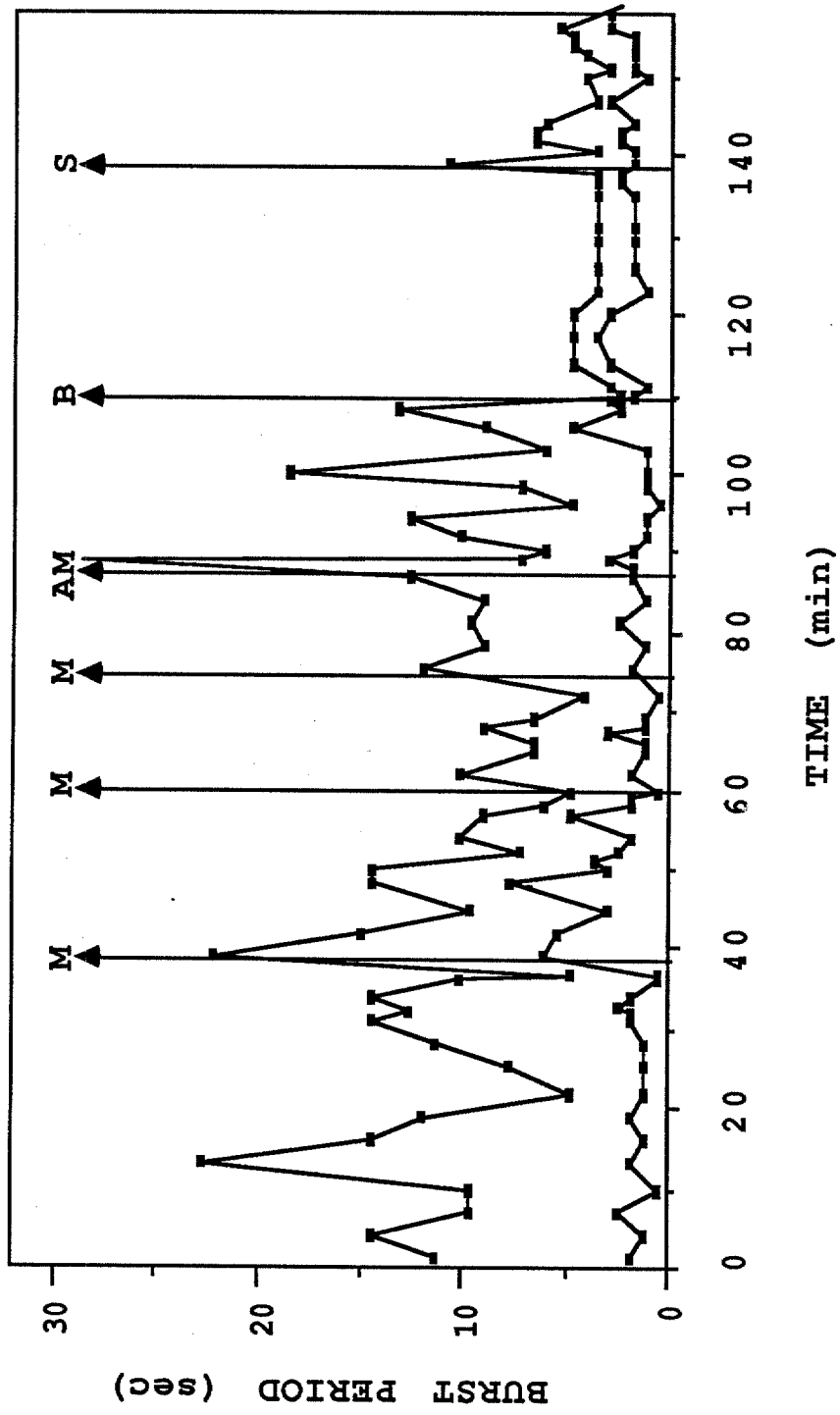

BURST PERIOD (sec)
0
10
20
30
M
M S
B
0
10
20
30
40
50
60
70
TIME (min)

Figure 35. Plot of maximum and minimum burst period ranges during a bicuculline disinhibition study. The area between the two lines represents burst period range. Bicuculline application (35 μM, B) reduced the period range, whereas application of MEM 10 or acidified MEM 10 (M, AM) did not have this effect. Strychnine was applied following bicuculline (7 μM, S), and the decreased period range was maintained but not further reduced.


BURST PERIOD (sec)
30
20
10
0
0
20
40
60
80
100
120
140
TIME (min)
M
M
M
AM
B
S

period C. V. in comparison with the mean control value, indicating a clear enhancement of burst regularity following disinhibition (Table 7, Fig.36). Cultures treated with strychnine displayed a mean period C. V. decrease of 35%, while those treated with bicuculline exhibited even greater regularity, with a C. V. decrement of 52%. Treatment with both drugs resulted in only slightly greater period C. V. decrement (58%) than application of bicuculline alone. The order of drug application did not appear to be critical for maximization of regularity (period C. V. decrement = 55% with strychnine followed by bicuculline; 60% with bicuculline followed by strychnine).

Effects of Inhibitory Agents on Activity

Effects on Spontaneous Activity

In only a few experiments was an inhibitor applied to a spontaneously bursting culture prior to the addition of disinhibitory agents (N = 4). In these cases, the application of 35 μM glycine or GABA resulted in the immediate cessation of burst activity. Single-spike activity occasionally occurred, but this was often also suppressed. Those cultures which exhibited recovery of bursting activity displayed marked attenuation of burst frequency. The subsequent addition of bicuculline or strychnine to the culture restored burst activity and

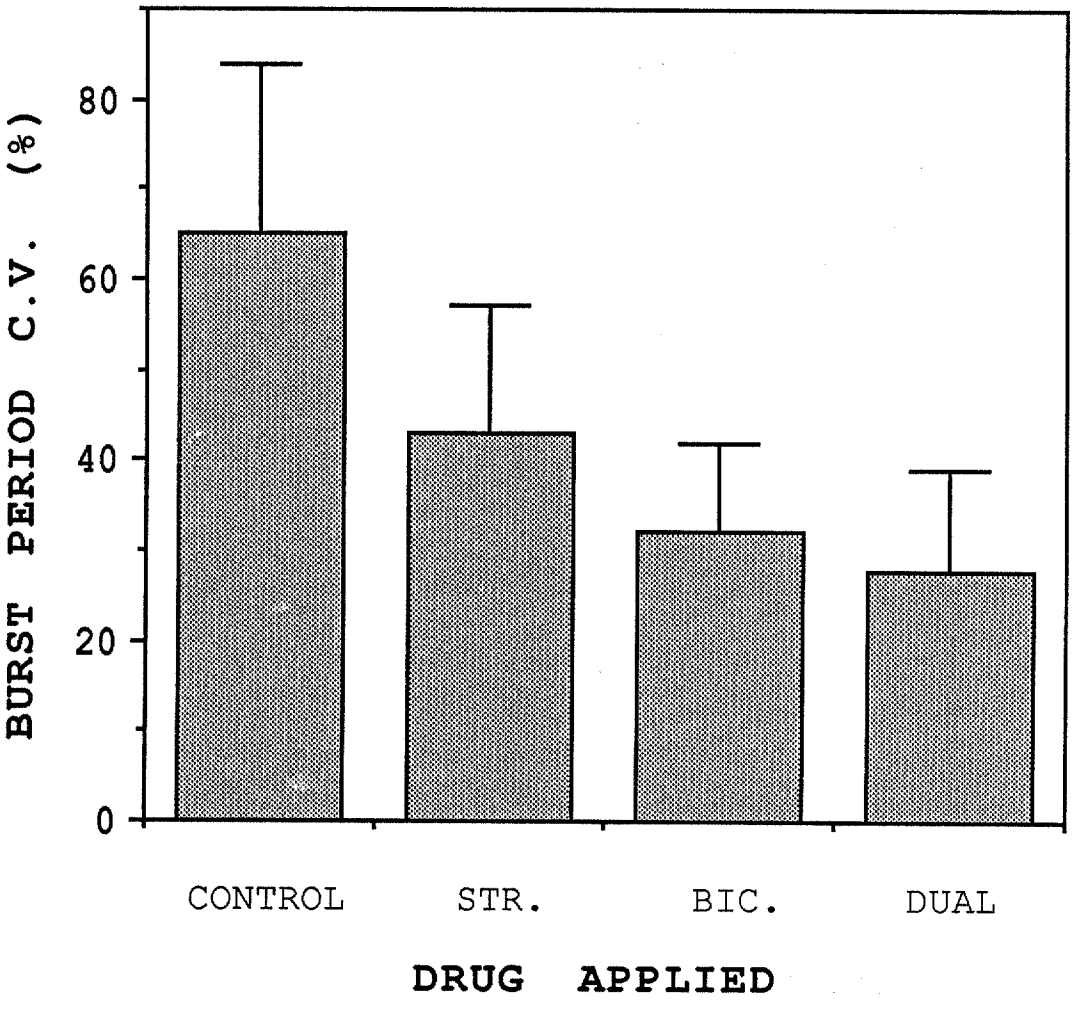
Table 7. Burst period coefficients of variation: effects of single and dual disinhibitor application

MMEP No.	Drug Application Order[a]	Predrug C.V. (%)[b]	First Drug C.V. (%)[c]	Dual Drug C.V. (%)[d]
G19	S,B	68	41	25
G26	S,B	75	30	30
H11	S,B	81	26	26
H35	S,B	67	39	33
H70	S,B	50	44	34
J5	S,B	33	45	27
J19	S,B	50	21	42
J24	S,B	62	65	15
K2	S,B	61	66	34
L5	S,B	90	52	34
G2	B,S	115	30	24
H142	B,S	60	22	18
H5	B,S	81	27	23
H26	B,S	72	34	18
H33	B,S	95	29	20
H48	B,S	88	24	17
H49	B,S	56	22	11
H74	B,S	47	39	40
H78	B,S	51	44	27
H100	B,S	43	14	20
J35	B,S	53	38	27
J37	B,S	57	40	55
L2	B,S	57	21	20
AA22	B,S	75	49	26
AA25	B,S	41	43	51
Mean C.V. %		66	36	28

[a]S indicates application of 7 μM strychnine, and B indicates the administration of 35 μM bicuculline.

[b,c,d]Calculations of the pre- and postdrug burst period coefficients of variation were made from measurements of all burst periods during 1-min increments 5 min prior to any drug application and 5 min following the administration of each drug. See the methods section for further detail.

Figure 36. Bar graph of burst period variability (coefficient of variation of mean burst period) as a function of disinhibitory drug treatment. Decreased burst period variability is representative of increased burst pattern regularity. Both bicuculline and strychnine decreased period variability. Dual application of disinhibitors resulted in a small further increase in regularity beyond that achieved by a single drug. Bars represent 1 SD.


BURST PERIOD C.V. (%)
80
60
40
20
0
CONTROL
STR.
BIC.
DUAL
DRUG APPLIED

resulted in burst frequencies greater than spontaneous, pre-drug levels.

Effects on Disinhibited Activity

Glycine and GABA were usually applied following the addition of disinhibitory agents. The bath fluid containing previously added drugs was not replaced because sudden medium changes produced profound changes in activity and visible morphological stress reactions. Therefore, the disinhibitory agents remained in the culture bath, and glycine or GABA (35 μM) only rarely produced complete and sustained burst cessation. Bursting generally resumed within 1-2 min, but with attenuated frequency (Fig. 37). Because the drug application protocol for each experiment varied, direct comparison between experiments was difficult (Tables 8 and 9). Nevertheless, regardless of the previous drug history, both glycine and GABA generally reduced burst frequency.

Successive Disinhibition-Inhibition Experiments

Several experiments were performed by applying a disinhibitory agent (to enhance burst activity), followed by addition of an inhibitory drug (to cause burst cessation or burst frequency attenuation), and finally a second application of disinhibitory agent (to restore burst activity). As anticipated, such alternation of disinhibitory and inhibitory treatment did enhance,

Figure 37. Chart records depicting the effects of inhibitors on burst activity subsequent to treatment with disinhibitors. *A*, Application of 35 μM glycine. *B*, Application of 35 μM GABA. After addition of either drug, burst cessation occurred, although some single spike activity remained. Within 1-2 min, burst activity returned, but burst frequency was attenuated. Scale bars, 10 sec.

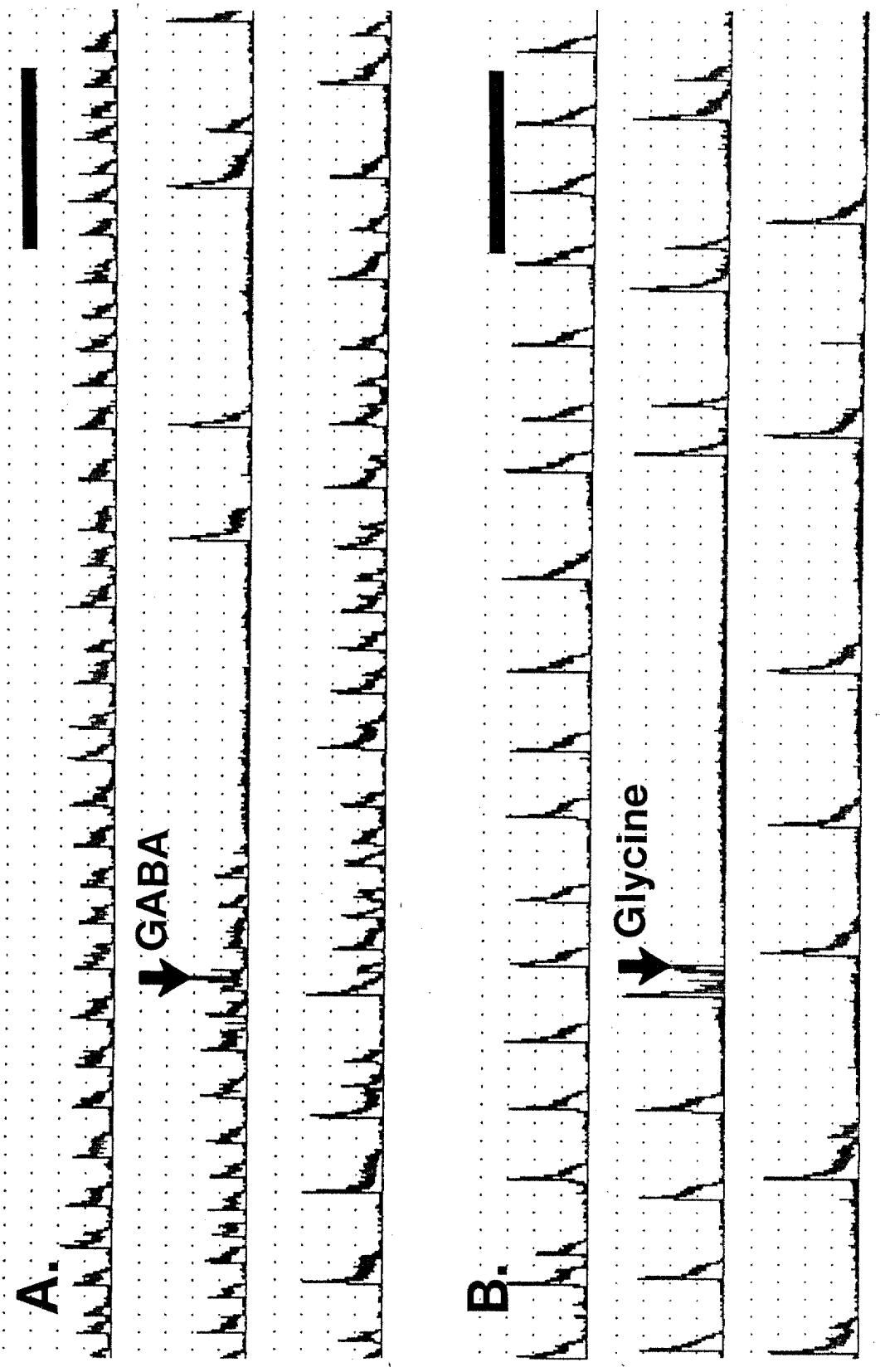

A.
GABA
B.
Glycine

Table 8. Summary of glycine effects on burst frequency

MMEP no.	Drugs applied before glycine*	Freq. before glycine (/min)	Freq. after glycine (/min)	Freq. change (%)
G1	S,S,PE	40	0	-100
G2	B,S+,GB	20	16	-20
G19	S,B	15	6	-60
G26	S,B,GB	8	0	-100
H142	GB,GB,B,S+	27	29	+7
H5	B,S,GB	6	0	-100
H8	None	35	0	-100
H11	S,B	27	0	-100
H26	B,S,GB	11	4	-64
H33	B,S,GB	6	2	-67
H35	S,B	25	16	-35
H70	S,S,B,S,B,GB	11	0	-100
H74	B,S,PI,GB	10	6	-40
H75	B,GB	4	4	0
H78	B,S,GB	11	10	-9
H97	PI,S,S+,B,GB	40	30	-25
H100	B,S,GB	18	15	-17
J4	PE,S,GB,PI,PI,S,B,GB	28	20	-29
J5	S,B	38	22	-42
J15	PE,PE,PE,S+,B,GB,GB	33	19	-42
J19	None	22	0	-100
J24	S,S+,B,PI	63	51	-19
J35	GB,B	6	0	-100
J37	B,S,GB	31	5	-84
L2	PE,B,S,GB	6	4	-33
L4	GB,B,B,B,B,S	8	6	-25
L5	S,B	8	8	0
AA22	B,S+,GB	8	8	0
Mean ±1 SD		20±14	10±12	-50±38

*Drug symbols represent final concentrations of the following agents: B, 35 μM bicuculline; GB, 35 μM GABA; PE, 10,000 units/ml penicillin; PI, 35 μM picrotoxin; S, 7 μM strychnine; S+, 75 μM Strychnine. All drugs were applied in MEM 10 except bicuculline, which was applied in acid MEM 10 (described in Methods, Chapter 4). Drugs were applied in the order listed.

Table 9. Summary of GABA effects on burst frequency

MMEP no.	Drugs applied before GABA*	Freq. before GABA (/min)	Freq. after GABA (/min)	Freq. change (%)
G1	S,S,PE,GL,B,PE,S	6	3	-50
G2	B,S+	35	20	-43
G19	S,B,GL	6	0	-100
G26	S,B	28	8	-71
H142	None	29	27	-7
H5	B,S	23	6	-74
H8	GL,S	57	0	-100
H11	S,B,GL,S+	34	34	0
H26	B,S	60	11	-82
H33	B,S	17	6	-65
H35	S,B,GL	16	3	-81
H70	S,S,B,S,B	31	11	-65
H74	B,S,PI	29	10	-66
H75	B	13	4	-69
H78	B,S	13	11	-15
H97	PI,S,S+,B	43	40	-7
H100	B,S	18	18	0
J4	PE,S	34	0	-100
J5	S,B,GL	22	12	-45
J19	GL,S	40	3	-93
J24	S,S+,B,PI,GL	51	60	+18
J35	None	11	0	-100
J37	B,S	53	31	-42
L2	PE,B,S	23	6	-74
L4	None	33	0	-100
L5	S,B,GL	8	0	-100
AA22	B,S+	10	8	-20
AA25	B,S	17	0	-100
Mean ±1 SD		27±15	12±14	58±36

*Drug symbols represent final concentrations of the following agents: B, 35 μM bicuculline; GL, 35 μM glycine; PE, 10,000 units/ml penicillin; PI, 35 μM picrotoxin; S, 7 μM strychnine; S+, 75 μM Strychnine. All drugs were applied in MEM 10 except bicuculline, which was applied in acidic MEM 10 (described in Methods, Chapter 4). Drugs were applied in the order listed.

abolish, and then restored bursting activity patterns in many cultures (Fig. 38). Unfortunately, the drug "stacking" effects produced by sequential application of multiple drugs was difficult to interpret quantitatively because cultures often became stressed during prolonged experiments with extensive pharmacological testing. This culture stress reaction was displayed morphologically as intracellular vacuolization of neurons and an increase in extracellular debris. Decreases in burst intensity and frequency and the incomplete restoration of activity patterns were the corresponding electrophysiological indicators of culture stress. Nevertheless, multiple drug application studies showed that the observed pharmacological effects could be reversed by their antagonists.

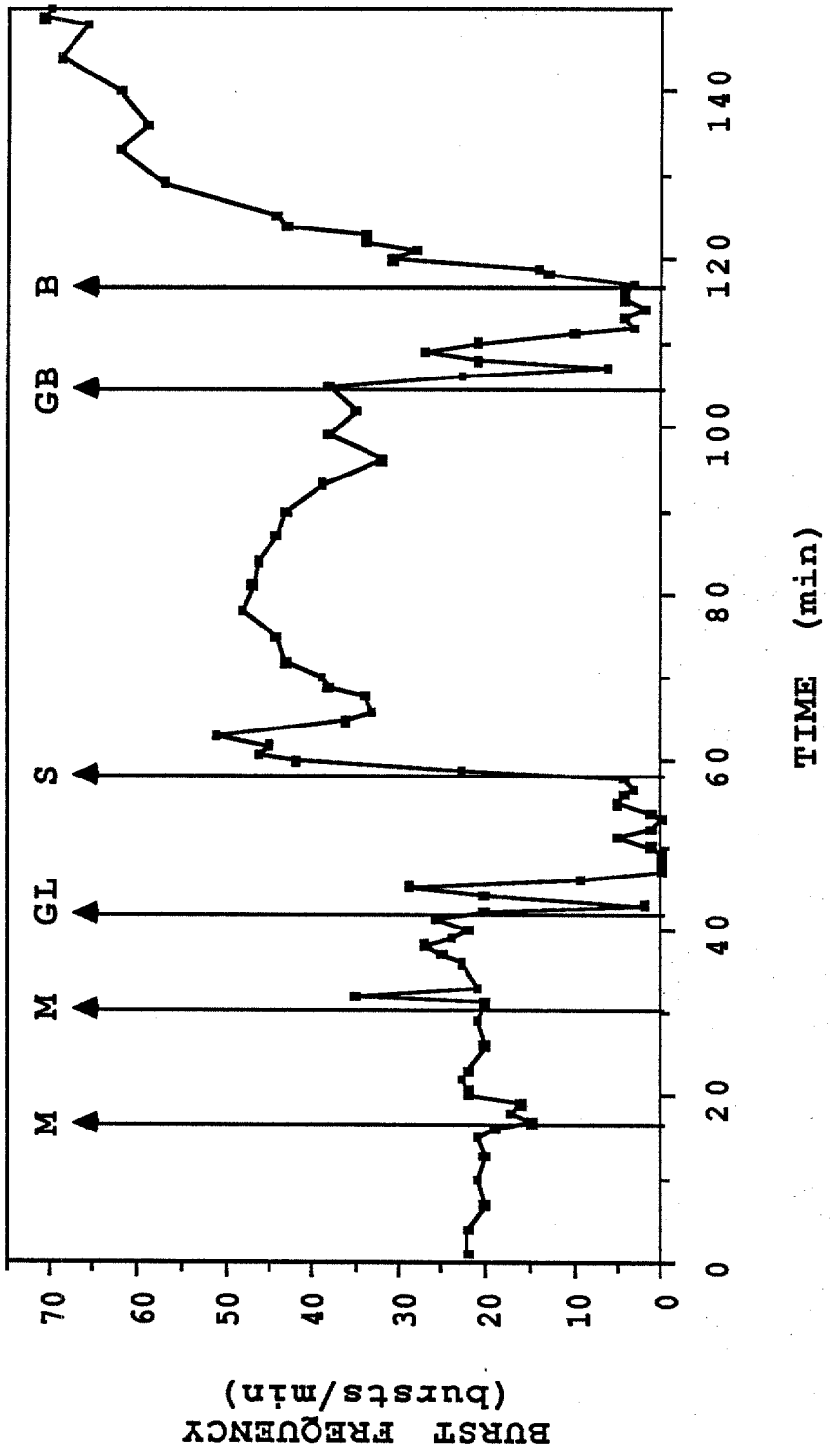
Discussion

Disinhibition Effects

Frequency Increases

The broad variation in burst frequency response to disinhibitory drug application made rigorous statistical evaluation of the pharmacological data impossible. Nevertheless, trends were apparent regarding frequency changes following the application of disinhibitory agents. Addition of either bicuculline or strychnine generally

Figure 38. Plot of fluctuations in burst frequency throughout the course of one complex pharmacological experiment. While medium additions (M) had little effect on burst frequency, glycine and GABA (GL, GB, 35 μM) markedly attenuated burst activity. Inhibitor effects were reversed by disinhibitor application (S, 7 μM strychnine; B, 35 μM bicuculline), and vice versa. Thus, activity could be successively inhibited and disinhibited.


BURST FREQUENCY
(bursts/min)
0
10
20
30
40
50
60
70
M
M
GL
S
GB
B
0
20
40
60
80
100
120
140
TIME (min)

produced increases in burst frequency in comparison to control levels. Strychnine was perhaps more effective in increasing burst frequency than bicuculline. Dual application of the agents generally produced a greater frequency increase than either drug applied singly or than a double application of strychnine. An unexpected frequency decrease after disinhibition was observed in a few cultures which displayed a high spontaneous burst frequency prior to drug application. These cultures may have represented a different metabolic or synaptic state, resulting in their non-typical reaction to the addition of pharmacological agents.

Decay of Disinhibition-Induced Frequency Increases

The often observed decay in an established plateau burst frequency could have resulted from culture stress and consequent metabolic changes, or perhaps from inactivation of the pharmacological agents. Data from other researchers concerning the metabolic breakdown of bicuculline or strychnine were not available. However, MMEP cultures monitored in open chambers for several hours without the addition of pharmacological agents also exhibited a slow decrease in bursting activity. Bath osmolarity during a 4-hr assay typically rose from 330 mOsM to 390 mOsM. These changes were gradual and could have produced the observed gradual changes in activity. Assay times for controlled

drug testing in the open chamber system was therefore limited to 2-3 hr. Osmolarity changes were not corrected by the addition of distilled water because trial applications of hypotonic solutions to the culture bath often resulted in the cessation or drastic alteration of activity for prolonged periods.

Disinhibition-Induced Regularity

Application of either bicuculline or strychnine resulted in an increase in burst regularity as measured by the reduction of the burst period C. V. Bicuculline was possibly a more potent promotor of burst regularity than strychnine in the concentrations employed, even though it did not generally increase burst frequency as greatly as strychnine. Therefore, the potency of a drug to promote regularity did not necessarily predict its effectiveness for increasing burst frequency. Dual application of both inhibitors tended to produce slightly greater regularity than the addition of either drug singly.

Disinhibition-produced "Epileptiform Activity"

The electrophysiological sequelae observed following strychnine or bicuculline administration strongly paralleled the EEG tracings monitored during an epileptic seizure (Ayala, 1973; Kandel and Schwartz, 1985). Seizure onset is associated with a long, intense burst ("interictal paroxysm"). This is followed by a silent period, then a

short phase of tonic activity ("tonic seizure"). Finally, a period of regular, coordinated, rhythmic bursting develops ("clonic seizure").

This sequence of events was frequently observed in disinhibited spinal cord cultures. The only major difference between an epileptic seizure *in situ* and disinhibition-evoked epileptiform activity produced by the mini-culture was in the duration of the response. While a seizure event is complete within a few minutes and is followed by a postictal depression phase, the epileptiform activity produced by disinhibition of spinal cord cultures remained in the clonic bursting phase for prolonged periods. Prolongation of the clonic bursting phase probably resulted from the continued presence of the disinhibitor in the culture bath.

Disinhibition Studies: Significance

"Organotypic" response to bicuculline and strychnine. Bicuculline and strychnine block two of the major inhibitory receptor classes known to be operative *in situ* in the spinal cord, the $GABA_A$ and the glycine receptors, respectively (Crain, 1976; Barker, 1985; Simmonds, 1986;). Their application to spinal cord cultures resulted in a disinhibitory response similar to that observed in CNS systems *in situ* and in slice preparations and explants (Crain, 1976; Grillner and Wallen, 1980; Alger, 1984; Cohen

and Harris-Warrick, 1984). Thus, networks of only a few hundred spinal neurons reacted in an *organotypic* fashion to pharmacological manipulation.

Both bicuculline and strychnine are considered relatively specific in their action, producing only minimal interference with other ion channel systems (Simmonds, 1986; Aprison et al., 1987). Other possible disinhibitory agents considered for pharmacological study with mini-cultures, such as penicillin (a $GABA_A$ antagonist), and picrotoxin (a $GABA_B$ antagonist, Van Huizen et al., 1987) are known to have other effects, such as direct excitation, in addition to their inhibitory transmitter system antagonism (Davenport et al., 1979; Simmonds, 1986). Trial applications of these two agents to spinal cord cultures resulted in highly variable responses, with a general increase in activity levels predominating. These responses were consistent with a disinhibition model of action, but the necessary intracellular testing could not be performed, and their reported nonselectivity discouraged the use of penicillin or picrotoxin for systematic disinhibition studies.

Dual disinhibitor application: a model of "total disinhibition"? Cultures treated with strychnine and bicuculline could not be considered totally disinhibited for the following reasons: 1) $GABA_B$ receptors remained unantagonized; and 2) the possible influence of a variety

of neuromodulators remained uninvestigated. Nevertheless, dual disinhibition of spinal cultures with strychnine and bicuculline represented the functional elimination of two of the major inhibitory influences known to be present in the spinal cord.

Differential responsiveness to disinhibitors. A majority of the cultures displayed a reaction to each disinhibitor, indicating the presence of receptors for both glycine and GABA. However, some cultures appeared to be more responsive to strychnine while others were influenced more by bicuculline. This might indicate variability in the populations of neurons growing on each recording island mini-culture. Strong strychnine sensitivity could reflect an inhibitory circuit dominated by glycinergic cells, whereas amplified response to bicuculline could indicate an inhibitory network made up primarily of GABAergic neurons.

Activity analysis: complexity and variability. Electrophysiological complexity and culture-to-culture variability critically limited the usefulness of spinal cord monolayer cultures as a model system for the investigation of neurobiological problems. Although many neurobiologists consider the action potential as the basic electrophysiological unit, activity has actually been studied in a hierarchy of detail, ranging from the analysis of single ion channels to the recording of field potentials and EEGs (Ayala et al., 1973; Armstrong et al., 1987; Korn

et al., 1987; Vibert et al., 1987). The investigation of burst characteristics rather than the analysis of individual action potentials was not unprecedented (Ault et al., 1986; Korn et al., 1987). It provided a good overview of the activity patterns of the mini-culture neuronal population and allowed the initial quantitation of the effects of pharmacological agents. Burst characteristics, such as frequency and envelope form, were highly variable prior to drug application. However, treatment with disinhibitory agents resulted in greater burst pattern regularity and burst envelope stereotyping; these changes indicate that disinhibition produced a decrease in activity variability. Thus, disinhibition might represent a form of functional network simplification.

CHAPTER 5

HISTOLOGICAL PROCESSING OF MMEP MINI-CULTURES: DEVELOPMENT AND EVALUATION OF THE BODIAN-NISSL STAIN

Introduction

Morphological analysis was planned to follow the successful electrophysiological characterization and pharmacological manipulation of MMEP mini-cultures. Previous observation of neuronal monolayers had been performed with living or fixed cultures using only phase contrast microscopy. This method had revealed general morphological features and allowed the production of photographic montages showing major network components. However, it was anticipated that proper histological processing would enhance the contrast of neuronal somata and processes, allowing more detailed morphological analysis and fiber tracing. A routine histological procedure was required to achieve the following objectives: 1. stain specificity for distinguishing of neurons from glial and supporting cells; 2. definition of somal and dendritic morphology; 3. dark staining of fine processes to allow network tracing; and 4. compatibility with the

MMEP insulation surface. The features of existing section staining methods were researched in an attempt to select one and adapt it for use with neuronal cultures (Table 10).

The Bodian procedure was chosen as a promising neuronal fiber staining method (Bodian, 1936). It was expected to selectively stain the neurons present in spinal cord mini-cultures because Protargol-S, the Bodian silver albumose reagent (Polysciences, Warrington, PA), was shown to bind preferentially to neurofilament protein (Gambetti and Autilio-Gambetti, 1981). The Bodian method produces the dark staining of neuronal fibers associated with standard Golgi methods, but it is considerably less capricious: the Bodian procedure generally stains a majority of the neurons, whereas Golgi methods typically stain only 5% of the neurons in a particular section (Cox, 1982). Nissl procedures were investigated for use as a Bodian counterstain to highlight perikaryal domains. Nissl methods, with specificity for rough endoplasmic reticulum, stain the reticulum-rich neuronal somata preferentially (Sheehan and Hrapchak, 1980).

Materials and Methods

Fixation

The combined Bodian/Nissl staining protocol (Fig. 39) was derived from standard paraffin section methods and was

Table 10. Comparison of primary features of classical neuronal staining methods

Stain	Specificity	Consistency	Reference
Holzer	Glial fibers	Good	Holzer, 1921 McManus, 1960
Silver carbonate	Glia	Fair	Globus, 1927
Cajal	Neurons	Fair	Bolles, 1946
Golgi	Neurons	Poor	Golgi, 1873 Bielschowsky, 1902
Rapid Golgi	Neurons	Poor	Cox, 1891 Moliner, 1958
Nauta	Neurons, Myelin	Fair	Nauta and Gygax, 1951
Luxol fast blue	Myelin	Good	Kluver and Berrera, 1953
Nissl	Neurons (soma)	Good	Nissl, 1894 Fletcher, 1947
Bodian	Neurons	Good	Bodian, 1936

Figure 39. Flowchart of the basic steps of the combined Bodian-Nissl procedure with accompaning primary reagents.

BODIAN-NISSL STAINING

FIXATION

⬇

SILVER IMPREGNATION
(PROTARGOL-S)

⬇

REDUCTION
(HYDROQUINONE/SODIUM SULFITE)

⬇

GOLD TONING
(CHLOROAURIC ACID)

⬇

DEVELOPMENT
(OXALIC ACID)

⬇

STABILIZATION
(SODIUM THIOSULFATE)

⬇

NISSL STAINING
(THIONIN, NEUTRAL RED)

⬇

MOUNTING
(COVERSLIPS--PERMOUNT,
MMEPS--CORN SYRUP)

first adapted for use with neuronal cultures grown on glass coverslips (Hightower and Gross, 1985). These cultured cells were treated in much the same manner as sections already affixed to a slide. The coverslips were removed from culture dishes, rinsed gently with phosphate buffered saline, and fixed for staining. Various fixatives were tested, including formalin (2, 5, and 10% aqueous solutions with 1% $CaCl_2$), glutaraldehyde (1, 3, and 6% in Millonig's phosphate buffer, pH 7.2-7.4), standard Bouin's (Clark, 1981) aged and unaged alcoholic Bouin's (Duboscq-Brasil) fluid (Clark, 1981), and Gregory's "improved synthetic alcoholic Bouin's" fixative (Gregory, 1980a). Fixation times were varied from 1 min to 48 hr. The Bodian procedure then followed thorough rinsing of the fixed slips.

Nissl Staining

Nissl staining followed Bodian processing and was achieved with either 0.25% thionin (Fisher Scientific, Springfield, NJ) or 0.1% neutral red (Sigma) by the methods of Humason (1979) and LaBossier and Glickstein (1976), respectively. In either case, the coverslips were then processed, without a water rinse, through an alcohol dehydration series (50, 70, 95% ethanol, 100% methanol, 50/50% methanol/xylene, 100% xylene), 10 sec at each step,

and subsequently mounted on microscope slides with Permount (Fisher Scientific).

Results

Evaluation of Fixatives

Proper fixation was discovered to be critical for adequate staining of cells with the Bodian procedure (Table 11). Fixation with glutaraldehyde in any of the tested concentrations yielded substantial nonspecific staining of background glial carpet cells, particularly of their nuclei (Fig 40*A*). A fine granular precipitate along cell processes was also occasionally observed, and this artifact could not be removed by rinsing. These effects could not be decreased by shortening the fixation time to as little as 1 min. Formalin solutions produced similar results (Fig 40*B*), although decreasing the formalin concentration to 5% and fixing for less than 1 hr partially reduced background staining.

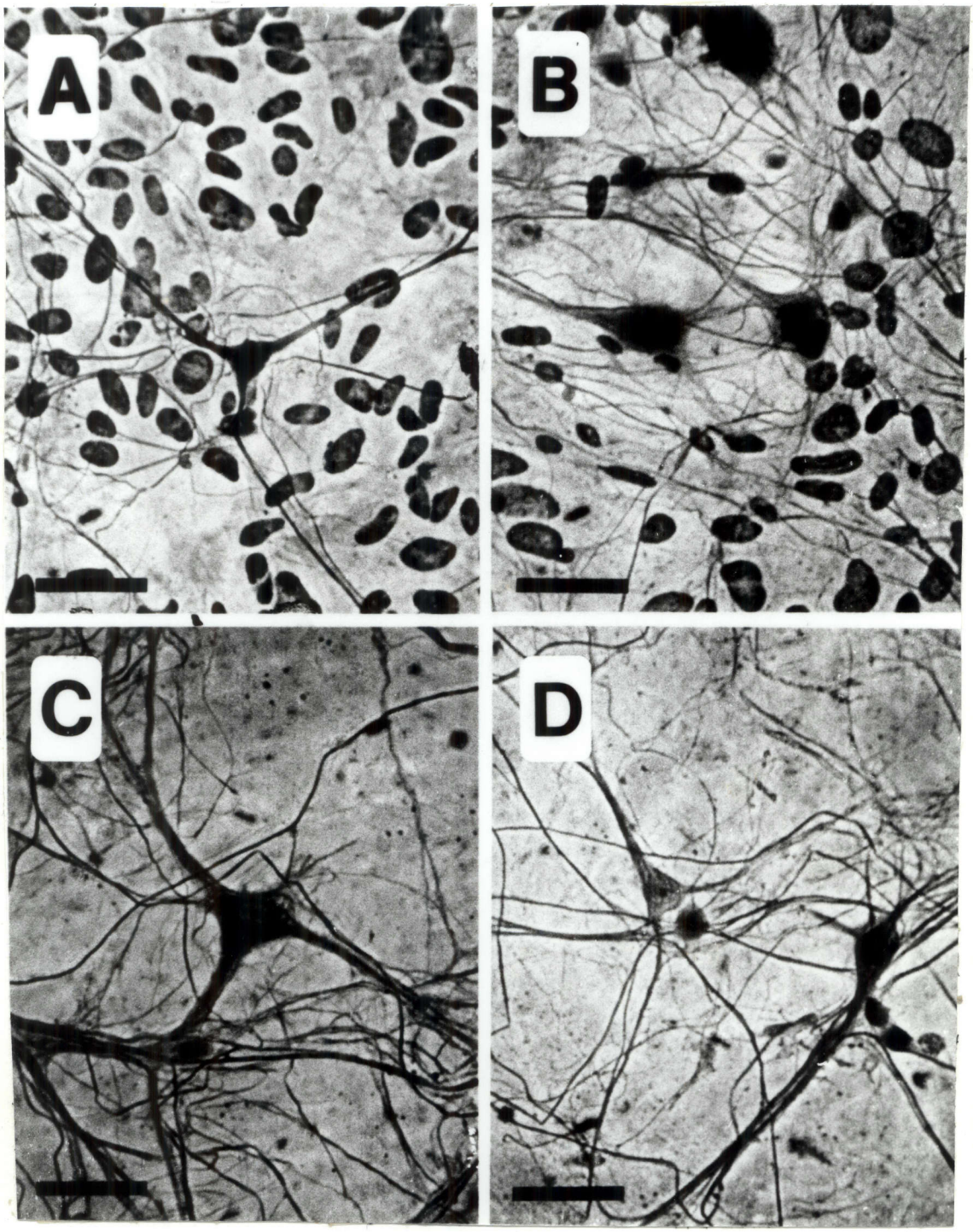
Fixation with standard Bouin's solution resulted in moderate to heavy glial staining, swelling of neuronal cytoplasm, and uneven staining of fine neuronal processes. Alcoholic Bouin's (Duboscq-Brasil) fluid did not improve neuronal staining or reduce that of the glial carpet; it did cause tissue shrinkage, resulting in significant fiber retraction and breakage. Fixation with "aged" alcoholic Bouin's solution (60°C, 40 d) resulted in generally good

Table 11. Comparison of fixative performance in Bodian staining

Fixative	Stain specificity[a]	Stain Uniformity[b]	Minimal Staining artifacts[c]	Tissue preservation[d]	Lot consistency[e]
Glutaraldehyde	-	+	-	+	+
Formalin	-	+	-	+	+
Bouin's	-	-	+	+	+
Alcoholic Bouin's	-	-	+	-	+
Aged alcoholic Bouin's	+	+	+	+	-
Gregory's	+	+	+	+	+

[a]Specificity: specific neuronal staining, with minimal glial staining
[b]Uniformity: even staining of neurons and fibers throughout a culture
[c]Minimal Artifacts: "clean" staining, with no precipitates or crystal formation
[d]Preservation: Maintenance of neuronal forms, minimal shrinkage or fiber retraction
[e]Lot reliability: uniform results with different "batches" of fixative

Figure 40. Bodian staining: fixation effects. *A, B,* Both glutaraldehyde and formalin resulted in staining of glial elements, particularly glial nuclei. *C,* Aged alcoholic Bouin's fixative produced generally good staining of neuronal processes, but different lots varied slightly, and 2-month aging was inconvenient. *D,* Gregory's synthetic aged alcoholic Bouin's solution allowed clean, even staining of neurons with little glial staining. Scale bars, 50 μM.


A
B
C
D

preservation and dark, even staining of neurons with little background staining (Fig 40*C*). However, staining intensity varied with different lots of fixative, and the long aging process was inconvenient.

These results with aged alcoholic Bouin's solution agreed well with the work of Gregory (1980a, 1980b) and his Bodian staining of sectioned insect ganglia. Fixation with his "improved synthetic aged alcoholic Bouin's solution" resulted in specific and consistent staining of the neuronal cultures (Fig 40*D*). Inclusion of formaldehyde in concentrations of 1-3% prevented the artifactual precipitation experienced with formalin-free solutions and the nonspecific staining associated with higher formaldehyde concentrations. As Gregory suggested, increasing ethyl acetate concentrations to 25% and omitting the less easily obtainable diethoxymethane resulted in no detectable differences in staining. Optimal fixation time with Gregory's solution was 1-24 hr.

Bodian Staining

Silver Impregnation

In order to make very fine neuronal processes visible, Protargol-S incubation for 24 hr at 37°C was optimal. However, dark somal staining resulted, and incubation time was reduced to 20 hr for more detailed circuit definition near perikarya. Concentrations of Protargol-S greater than

1.0% did not increase stain intensity. Copper shot was required for proper silver impregnation of neurons and for reduction of glial background staining (Coolidge and Howard, 1979), but differences in metal quantity and surface area were not critical. For 20 ml of staining solution, 3-10 gm of small copper shot was sufficient.

Silver Reduction

Timing of the reduction step greatly influenced staining and required a compromise between the desired intensity of the stain and its specificity for neurons. Formaldehyde in the reducing solution resulted in granular precipitates and inconsistent staining. Reduction in fresh 1% hydroquinone/5% sodium sulfite for 15 min resulted in generally reproducible, "clean" staining of adequate intensity.

Gold Toning, Stabilization

Gold toning with a 1% chloroauric acid resulted in more consistent results with less granularity than was achieved with gold chloride solutions. This step served to darken neurons and to "bleach" the background; toning duration was not critical between 10 and 30 min. After gold toning, "development" with 2% oxalic acid for 4-10 min was required for optimal darkening of the neuronal processes; the progress of this step was monitored by microscopic observation. Following development, adequate

stain stabilization and precipitate removal was achieved by "stain fixation" with 5% sodium thiosulfate for 5 min. Thorough rinsing of the plates with ultrapure water was required after each solution treatment.

Nissl Staining

After Bodian staining, aqueous solutions of thionin (0.25%) or neutral red (0.1%) were employed to counterstain the Nissl substance. Acidifiying the staining solution with glacial acetic acid (0.5%) as recommended by Sheehan and Hrapchak (1980) resulted in much more intense staining of the Nissl bodies, but unacidified solutions more clearly outlined the limits of somal cytoplasm and proximal dendrites. Nissl methods overstain the tissue; processing the coverslips through an alcohol series after staining serves to remove the excess dye, or "differentiate" the material as well as to dehydrate it in preparation for permanent mounting. Sheehan and Hrapchak recommended differentiation of thionin staining with 1:1 ethanol:dioxane. Similar results were obtained with a butanol or ethanol series. However, if ethanol was used as a dehydrant/differentiator, cultures had to be processed through the series quickly (30 sec/step). The use of neutral red as a Nissl counterstain resulted in excellent contrast between somata and processes, but dehydration with any alcohol series caused excessive destaining. Addition

of neutral red (0.1%) to the solutions of the series up to xylene and dehydration for only 10 sec/step resulted in satisfactory final stain intensity.

•

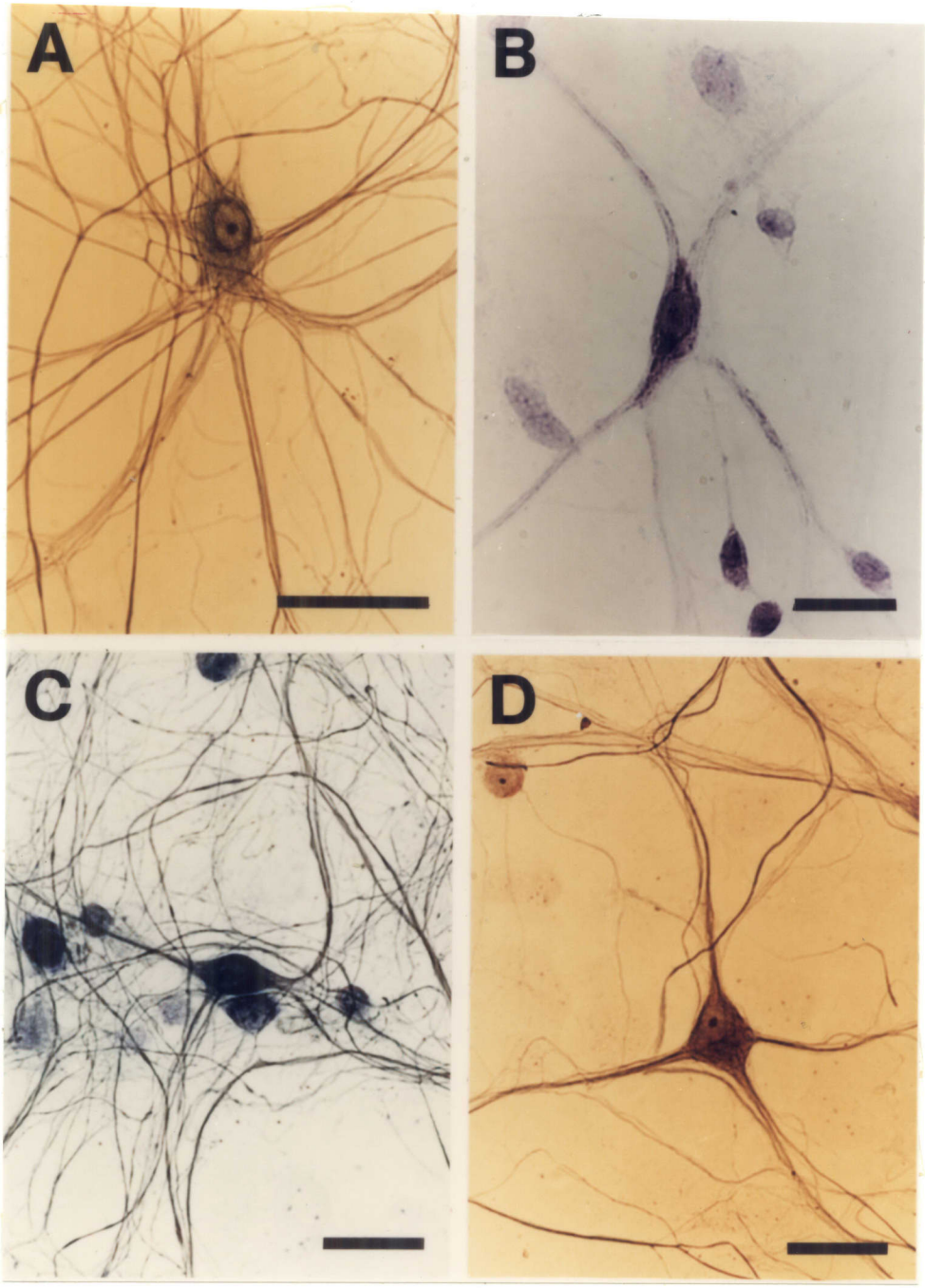
Bodian-Nissl Specificity, Reliability

The population of cells that stained with the Bodian or Nissl procedures were virtually the same: approximately 97% of cells stained with either procedure were also stained by the other (N = 1541 dual stained cells from a total of 1583 cells counted in 1 preselected field from each of 10 coverslips). More than 95% of living cells observed by phase contrast microscopy and identified morphologically as neurons were subsequently stained with this procedure (N = 2346 stained cells of 2420 cells identified as neurons by phase contrast microscopy, counted in 1 preselected field from each of 10 coverslips). Bodian staining revealed a dark fibrillar network in somata and processes, while Nissl counterstaining enhanced the three-dimensional structure of the cultures and delineated somal volume (Fig 41).

Bodian Intensification

Although axons and dendritic trunks were stained darkly by the Bodian procedure, the finest neuronal processes were only lightly stained. In his work with cultured *Xenopus* and chick neurons, Katz (1985) observed a similar lack of staining of fine processes and immature

Figure 41. Examples of Bodian and Nissl staining. *A*, The Bodian procedure produced excellent fiber staining, but somal definition was inadequate. Cells stained by the Nissl procedure (*B*, thionin) procedure produced perikaryal highlighting and somal volume definition. The combined Bodian-Nissl method (*C*, thionin Nissl, *D*, neutral red Nissl) resulted in both excellent fiber staining and somal definition. Scale bars, 50 μM.


A
B
C
D

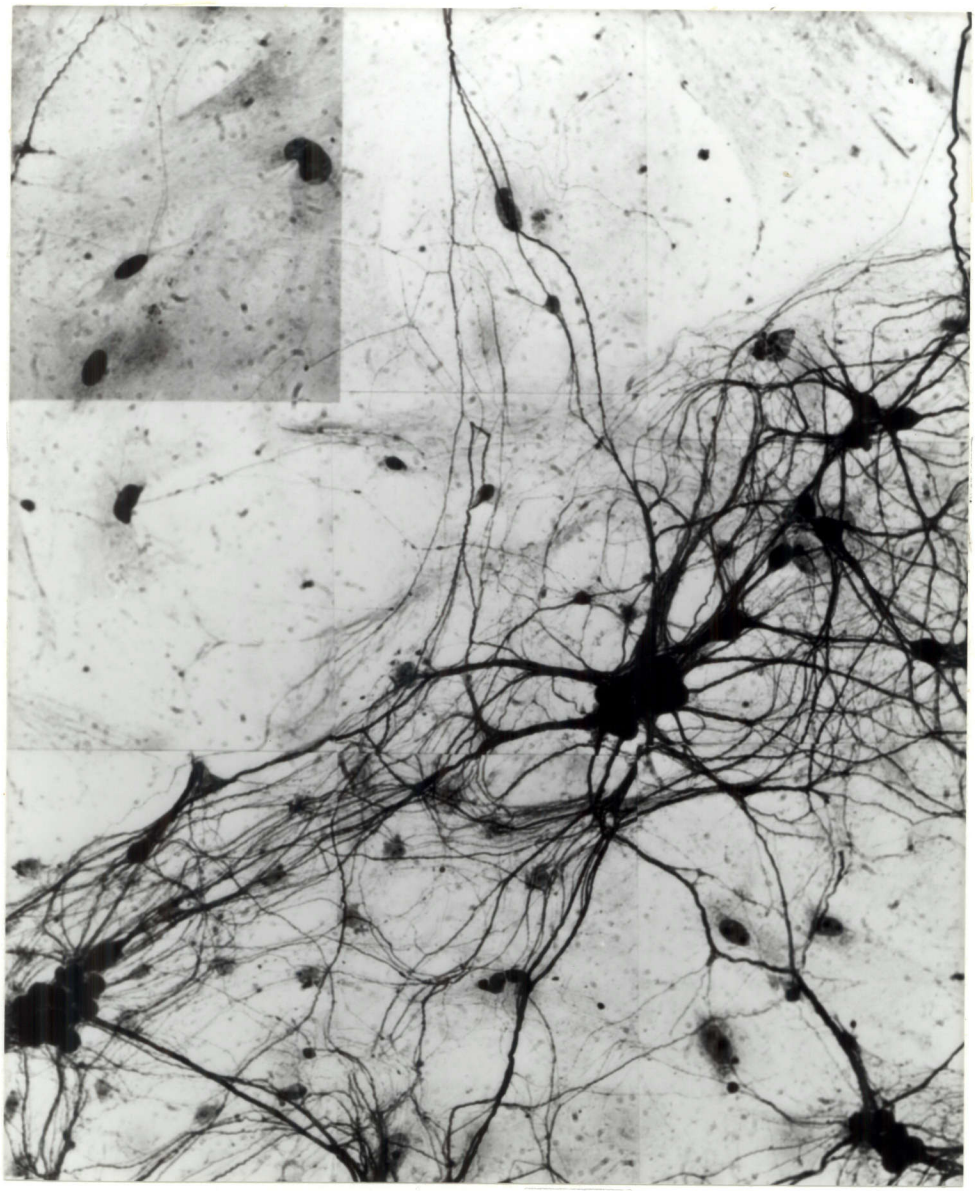
axons possessing few neurofilaments. He developed a poststaining intensification method based upon photographic enhancement procedures. Application of his protocol to mouse spinal cord cultures resulted in much better visualization of the fine neuropil (Fig 42), but nonspecific staining was increased, Nissl staining was obscured, and the neuronal somata often became opaque. Observation and photography of standard Bodian-stained cultures, followed by intensification and subsequent fine-fiber analysis was required to optimize collection of morphological data. The intensification procedure outlined by Katz was used unmodified with mouse spinal cord cultures, except that only a 1-2 min exposure to the intensification solution was required rather than the recommended 10 min.

Stain Modification for MMEP Mini-Cultures

Fixation

The staining procedure developed for use with monolayer cultures on coverslips required further modification to properly stain mini-cultures on MMEPs. The polysiloxane insulation layer underlying the neuronal monolayer could not withstand prolonged fixation with Gregory's solution, the fixative of choice for the Bodian procedure, nor any other solution containing picric acid or organic solvents. Insulation deterioration present prior

Figure 42. Enhancement of the Bodian stain using the Katz intensification method (Katz, 1985) with time modification resulted in very dark staining of fine neuronal processes. Note, however, the darkening of glial nuclei (arrows). The neuronal somata also became opaque. Scale bar, 100 μM.



to fixation (see Fig. 3) was so increased by exposure to these solutions that often, cells could not be seen on the MMEP surface. Sparing of the insulation layer required reduction of fixation time in Gregory's solution to 10 min.

Mimimization of Metal Deposition On Electrodes

The steps of the Bodian procedure were applied to MMEPs without modification, and the resulting stain quality was equal to that achieved with coverslip cultures. However, additional metal plating of the electrode tips was observed, sometimes obscuring a large area surrounding the conductor (Fig 43). Because this excessive metal deposition occurred during gold toning and development, the duration of these two steps was reduced to the minimum required for optimal staining. Adequate gold toning was achieved in 10 min, and development was reduced to 2-5 min, with careful microscopic monitoring. These measures reduced but did not eliminate metal deposition on the conductors. The intensification procedure could be applied to MMEP cultures, but it often resulted in unacceptable electrode plating.

Aqueous Mounting

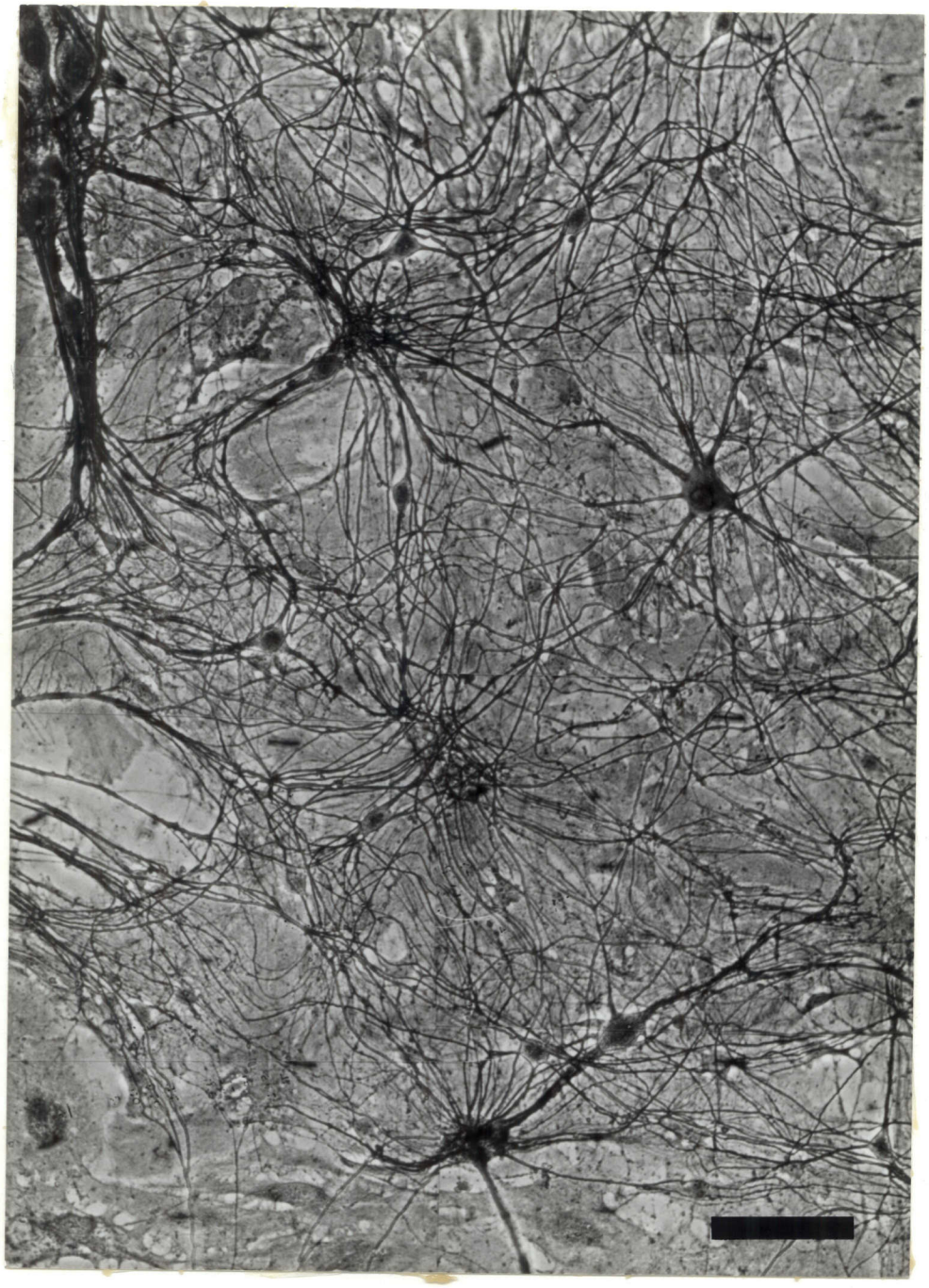
Following Nissl counterstaining, coverslips were dehydrated through an alcohol series to xylene and mounted on slides with Permount (Fisher Scientific). The MMEP insulation sensitivity to organic solvents precluded such

Figure 43. An extreme example of excessive metal deposition to a conductor during the Bodian staining procedure. These artefacts obscured morphological analysis of the near-electrode neuronal elements. Metal often plated excessively on some electrodes, whereas others plated only slightly (arrows). Reduction of the gold toning and developing steps partially mitigated but did not solve this problem. Scale bar, 100 μM.

dehydration and necessitated aqueous mounting. Both thionin and neutral red are water soluble dyes whose color leaches under aqueous conditions. Consequently, Nissl staining of MMEP cultures, particularly with neutral red, was not permanent and required overstaining prior to mounting as well as repeated restaining during morphological analysis.

Various aqueous mounting materials were tested in order to preserve stained MMEP cultures for long-term morphological analysis. Polymerizing mixtures were not employed because the ability to remove the coverslip for further histological processing was desired. Various mixtures of gelatin, gum arabic, and glycerin (Humason, 1979) were investigated, but the most convenient and acceptable mountant tested was white corn syrup (Karo, Best Foods, Englewood Cliffs, NJ) thinned to proper consistency with 95% ethanol, with glutaraldehyde (1%) added as a preservative. Unlike other formulations tested, corn syrup mounting, followed by a drying period (7-14 d, room temperature, or 24 hr, 37°C) resulted in almost total loss of the severe optical distortions caused by deterioration of the insulation material. Thus, cells which could not be visualized *in vitro* could be seen clearly and photographed after histological processing. Figures 7, 8, and 44 represent the staining quality achieved with mini-cultures on MMEPs.

Figure 44. A portion of a mini-culture on a MMEP stained by the Bodian-Nissl procedure. Note that the density of neuronal fibers is very high even in this low neuronal-density culture. Scale bar, 100 μM.



A Fade-Resistant Alternative to Classic Nissl Stains

A rapid Wright's staining procedure for blood elements (Camco Quik-Stain, Fisher Scientific, manufacturer's suggested method for smears) was tested as part of an adjunct project with neuronal/macrophage co-cultures. This stain showed specificity for the neuronal and macrophage populations in the cultures, with minimal staining of the glial carpet (Gardner et al., 1984). The presence of methylene blue in the prepared solution probably accounted for this specificity; Nissl (1894) referred to the use of methylene blue for neuronal staining in his classic discourse on the Nissl substance. The Quik-Stain procedure was much simpler than that of the true Nissl methods, and the resulting intensity rivalled that of thionin. In addition, cultures counterstained with the Wright's solution were less susceptible to aqueous color leaching and thus did not require repeated restaining. Therefore, Wright's staining became a method of choice for "pseudo-Nissl" counterstaining of MMEP cultures.

Discussion

The combined Bodian-Nissl staining procedure was developed for use with spinal cord monolayer cultures on glass coverslips by adaption of published tissue section staining methods. Extensive testing of fixatives was required for the selection of a formulation which would

satisfactorily preserve the tissue and not contribute to nonspecific staining. Further modifications of the staining procedure were required for its successful application on mini-cultures grown on MMEPs, including selection of a fixation protocol compatible with the MMEP insulation layer, the minimization of conductor plating during gold toning, the selection of aqueous mounting media, and the adaptation of a "pseudo-Nissl" method for greater counterstain permanence under aqueous conditions.

Under the appropriate conditions, the combined Bodian-Nissl procedure reliably stained the entire population of cells in the culture which exhibited neuronal morphological characteristics. Neuronal processes were stained darkly by the Bodian method, and Nissl counterstaining highlighted neuronal somata. Whenever visualization of very fine neurites was required, fiber staining could be increased using an intensification procedure. The combined Bodian-Nissl stain was employed in the first systematic morphological analysis of MMEP spinal cord mini-cultures (see Chapter 6) and became a routine histological protocol for MMEP cultures following electrophysiological studies.

CHAPTER 6

MORPHOLOGICAL ANALYSIS AND CORRELATION OF ACTIVITY WITH MORPHOLOGICAL VARIABLES

Introduction

The recording island mini-cultures survived well in culture, exhibited vigorous spontaneous activity, and responded in an organotypic fashion to the application of pharmacological agents. Development of the combined Bodian-Nissl staining method allowed identification the neurons in the mixed spinal cord cultures and offered the potential for visualization of the components of the network responsible for observed electrophysiological behavior. Consequently, Bodian/Nissl staining and morphological analysis of MMEP mini-cultures was planned to follow the monitoring of activity in order to correlate aspects of mini-culture structure with electrophysiological function.

Initial morphological analysis of spinal cord mini-cultures would include several tasks, beginning with the counting of neurons in recording island cultures and typing them by size and form. Secondly, cell reaggregation had been frequently observed in neuronal monolayers,

particularly in the restricted recording island mini-cultures, and this phenomenon would be quantified. In addition, large conditioning area cultures had been seeded and maintained with each recording island culture and these allowed the comparison of mini-culture network morphology with that of spatially more unrestricted cultures. Finally, electrophysiological activity was shown to vary with changes in the number of cells seeded and with age (see Chapter 3); it was of interest to determine how culture morphology would be altered by differences in these culture factors.

After studying the general features of the mini-culture neuronal network, a more detailed analysis of the neuronal elements near each electrode would be attempted. It was unknown whether the neurons and fibers present at the recording sites were representative of the general mini-culture population, so the number of neurons and neurites within the electrode craters would be determined and compared with that found in adjacent non-crater regions. The number of neuronal somata and processes within the vicinity of active conductors had not been systematically determined, so near-electrode cell body and fiber counts would be made and correlated with the presence of electrophysiological activity. It might then be possible to perform fiber tracing for short distances to assign a particular monitored spike to its probable source.

Materials and Methods

Neuronal Cell Counting

After Bodian/Nissl staining, all of the neuronal cells within the recording island were counted. Cells classed as neurons met the following morphological criteria: 1. dual staining by the Bodian/Nissl procedure; 2. presence in uppermost stratum of the culture; 3. possession of a bulging cell body and a darker, round nucleus; and 4. projection of compact processes, with no flattening or veil formation (Peacock et al., 1973; Fischbach and Nelson, 1977; Ransom et al., 1977; Hightower and Gross, 1985). All cell counting was performed using phase contrast microscopy at a magnification of 400. An ocular gridded reticule (50 μm/square) was employed to aid systematic counting, to allow estimation of neuronal size, and to permit accurate measurement of recording island diameter for calculations of cell density (expressed as neurons/mm^2 of culture surface area).

Neuronal Typing

Neurons were classified by size, number of visible processes extending from the somata, and aggregation (Rexed, 1964; Boehme, 1968; Matsushita, 1970; McClung and Castro, 1976; Wiksten, 1979; Brichta and Grant, 1985; Kato et al., 1985). The following six types of neurons were distinguished: 1. large multipolar neurons; 2. small

multipolar neurons; 3. large bipolar neurons; 4. small bipolar neurons; 5. round or unipolar neurons; and 6. cluster neurons (Table 12 for size, process, and aggregation specifications, Figs. 45 and 46 for pictorial examples of cell types). The first four neuronal classes represented the most distinct neuronal forms and were referred to as "morphologically differentiated" neurons. Neurons in a cell aggregate which possessed 2 or more distinct processes were were classed as bipolar or multipolar cells rather than as cluster cells (Fig. 46*B*).

Round and cluster neurons were small cells (<15 μm in diameter) which possessed only one or no visible processes. Only aggregation distinguished these two neuronal classes; round cells were dispersed, separate cells, whereas cluster cells were round neurons present in aggregates of 2 or more cells. These cells possibly: 1. possessed other processes which were not visible due to poor staining or tight cell aggregation; 2. were neurons which were not fully morphologically differentiated; or 3. were fully differentiated and represented a class of interneurons which also do not become bi- or multipolar *in situ*. Round and cluster cells were stained by the Bodian/Nissl procedure and met the other morphological criteria for neurons described above. In addition, even though somata were often not clearly associated with particular processes, large cables of neuronal-type processes often

Table 12. Features of the neuronal subtypes found in Bodian-stained MMEP mini-cultures

Neuron type	Number of processes	Diameter, largest axis (μm)	Present in an aggregate
Large multipolar	≥3	≥25	±
Small multipolar	≥3	≤25	±
Large bipolar	2	≥25	±
Small bipolar	2	≤25	±
Round	0-1	≤25	-
Cluster	0-1	≤25	+

Figure 45. Examples of the neuronal subtypes present in the mini-cultures. *A*, Large multipolar neuron. *B*, Large bipolar neuron. *C*, Small multipolar neuron. *D*, Small bipolar neuron. Scale bars, 50 μm.

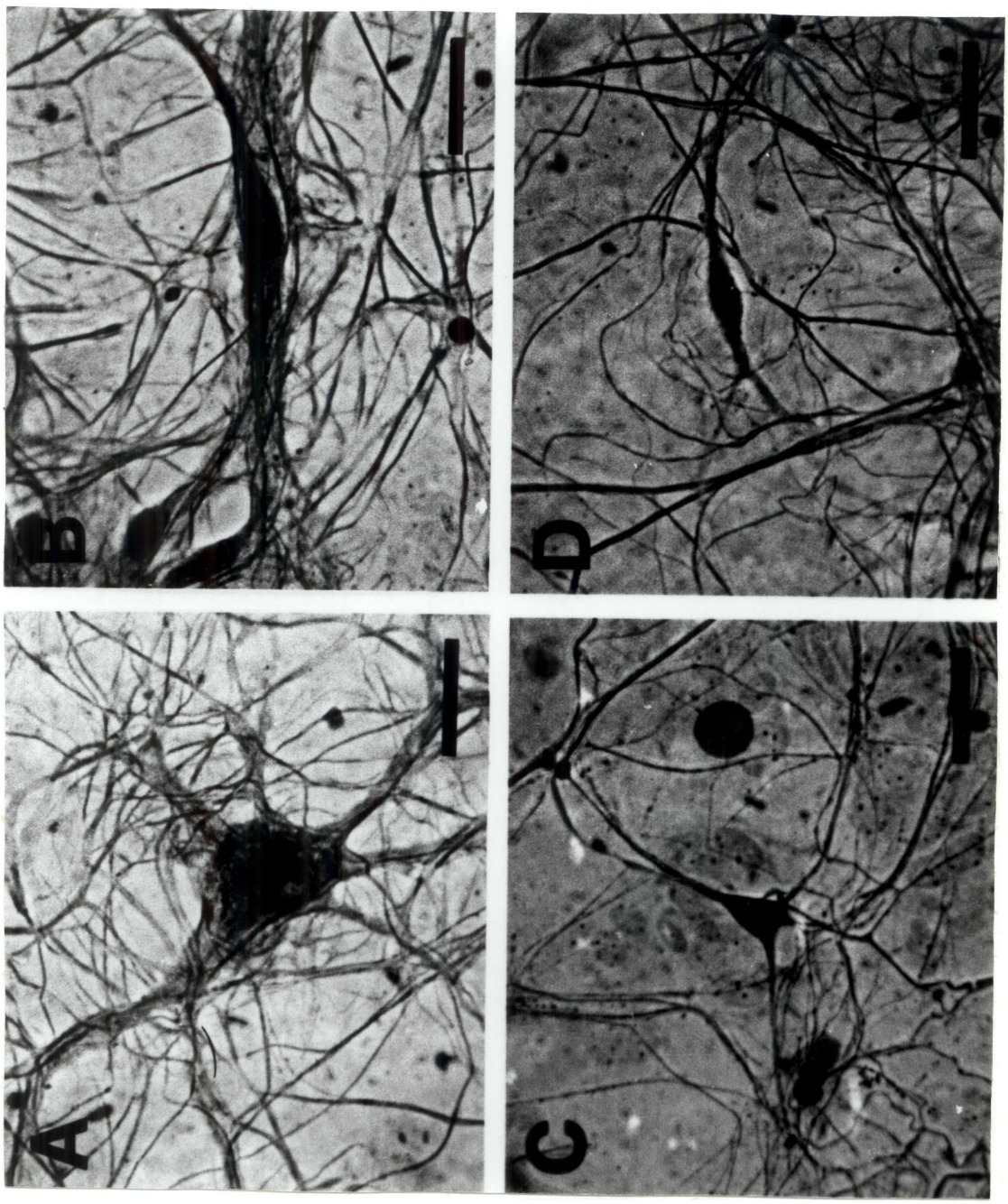
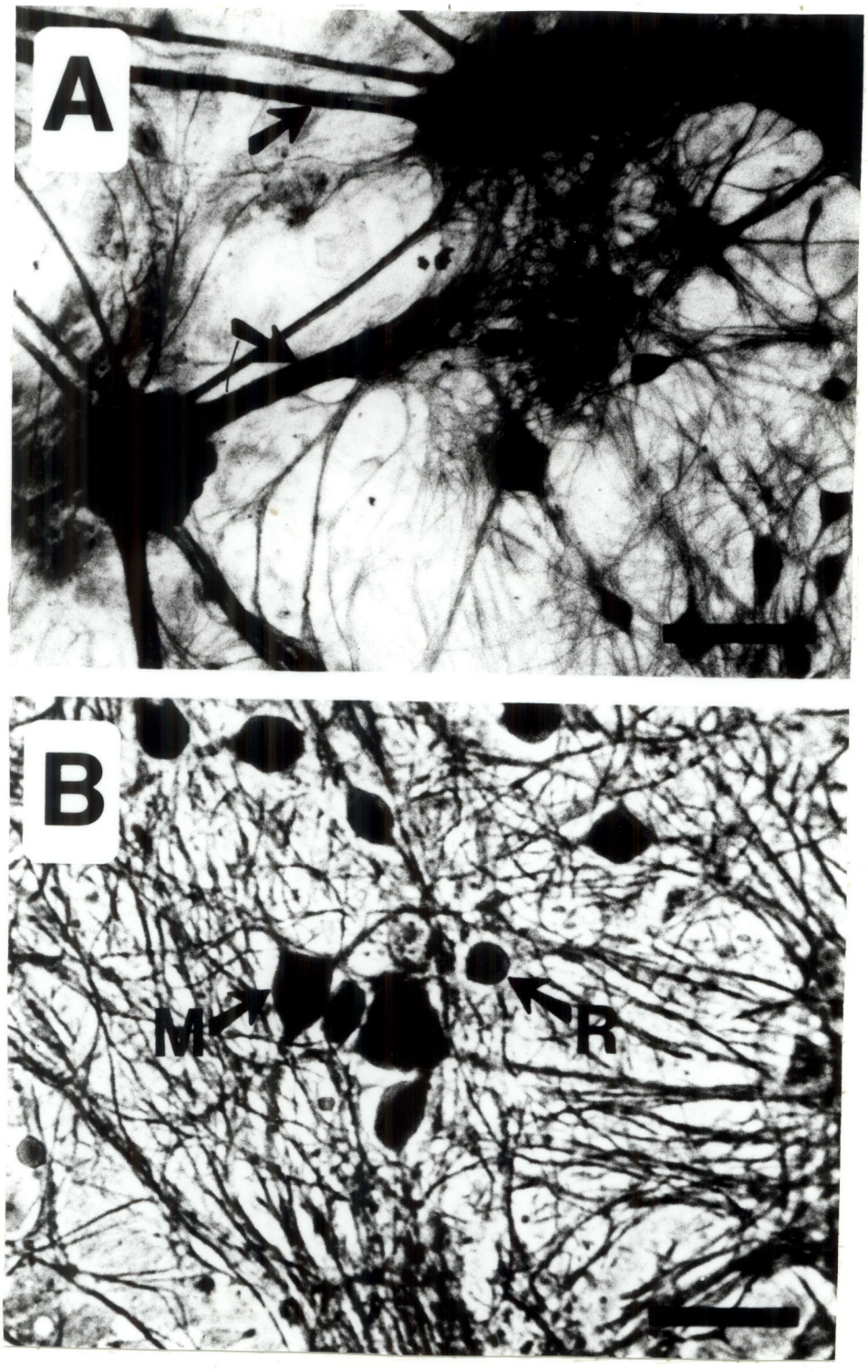

B
D
A
C

Figure 46. *A*, Large clusters of neurons were often interconnected by thick cables of neuronal processes (arrows). The cells of the such aggregates were classified as neurons because they stained darkly with the Bodian/Nissl procedure, were present in the uppermost stratum of the culture, possessed bulbous cell bodies. They were subtyped as cluster neurons because they exhibited no or only one distinguishable process. *B*, Although the small multipolar neuron (arrow) was part of this small cluster, it was classified as a multipolar neuron rather than a cluster cell. Round neurons (arrow, R) possessed no or only 1 discernable process but were separate from clusters. Scale bars, 50 μm.


A
B
M
R

extended from clusters of these cells to contact other single neurons and neuronal aggregates (Fig. 46A). Some small neurons of this type have been shown to be derived from dorsal root ganglia (Peacock et al., 1973). By employing immunocytochemical methods with spinal cord cultures, other researchers have determined that at least some grape-like clusters of cells are GABAergic (Kaduri et al., 1987) and probably represent classes of small interneurons. For these reasons, such cells were classified as neurons.

Aggregation Analysis

The number of cells comprising some very large clusters was estimated by adjusting the fine focus to different focal planes. Only distinct somata were counted, probably resulting in a systematic underestimation of the neurons in these clusters. The number of small clusters (aggregates of 2-5 neurons) and large clusters (aggregates of >5 neurons) were counted as an estimate of neuronal aggregation. The degree of clustering of somata was generally correlated with the extent of process fasciculation and cable formation.

Recording Island-Conditioning Area Comparisons

In order to compare the recording island morphological characteristics with those of less spatially restricted networks, morphological analyses of the conditioning areas

were performed. Cell and cluster enumeration and neuronal typing procedures were similar to those described for the recording island. However, the large size of the conditioning area precluded the enumeration of the entire cell population within the region. Therefore, a 1-2 mm^2 sample of the conditioning area was chosen at random prior to microscopic observation by marking the coverslip covering the stained culture using a circle template. This area was measured using an ocular micrometer, all of the cells within this circle were then counted and typed, and neuronal density was calculated. A cursory study of each entire conditioning area indicated that the sampled region was representative of the cells present in the area as a whole.

Correlations Between Morphology and Electrophysiology

The number of neurons contained within a 50 μm X 50 μm square region surrounding the center of each electrode crater was counted, and the neuronal processes within this region were also enumerated. Because extracellular electrodes have a severely limited signal acquisition distance (Snodderly, 1973), this "conductor population" of neurons and fibers constituted all of the components of the neuronal network which could have produced the monitored activity. This analysis represented the first effort to perform systematic cell-electrode coupling, the assignment

of particular electrical activity to its probable cellular source.

Several problems were encountered in the counting of neuronal processes. First, gold plating of the conductor tips also partially obscured the path of small processes crossing the electrode. Secondly, light microscopic resolution was insufficient for accurate fiber enumeration. Fiber counts performed at 400 magnification actually represented the number of single fibers or small fascicles; structures resolved as single processes were often shown to consist of multiple, tightly aggregated neurites when viewed in cross-section by transmission electron microscopy (Gross and Higgins, 1987). In addition, enumeration of >5 fibers/fascicles within a 50 μm X 50 μm area was difficult and inaccurate. Therefore, the maximum number of fibers assigned to any area was 6. This combination of unresolved multiple fibers in bundles and the upper limit for fiber counts resulted in systematic underestimation of the number of processes per unit area and precluded direct fiber/activity coupling studies. Nevertheless, such fiber/fascicle estimates were useful for initial investigations of process populations and for semi-quantitative comparisons between different MMEP cultures.

Crater and Intercrater Neuron and Fiber Distribution

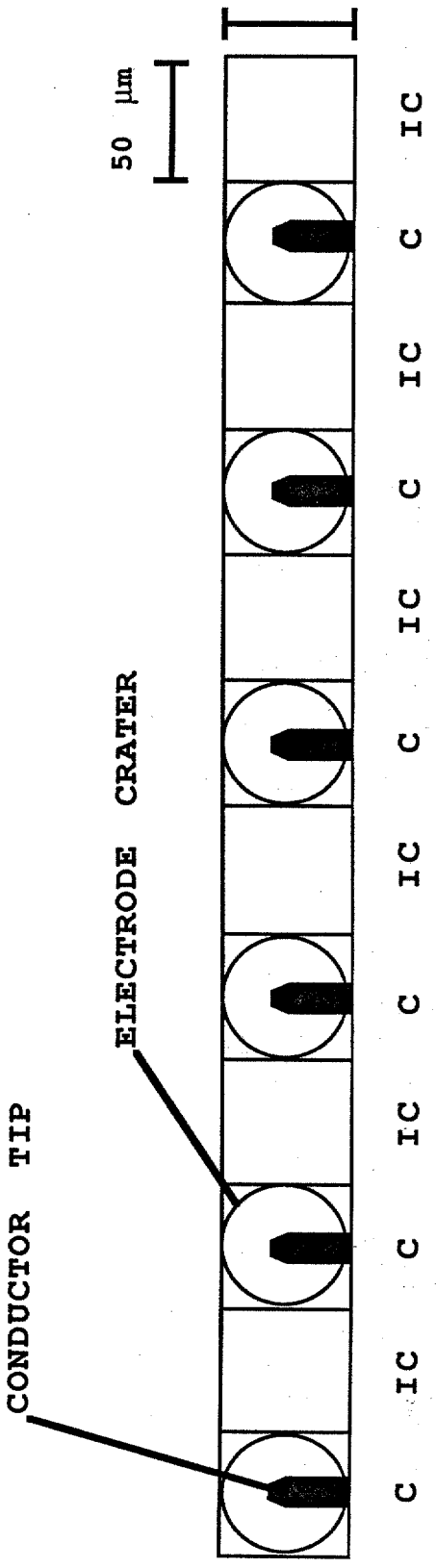
It was hypothesized that the laser deinsulation procedure might result in nonrandom neuronal adhesion and

process extension because it produced craters on the culture surface. Cells often adhere preferentially to surface irregularities (Wessells et al., 1973; Letourneau, 1975; Freshney, 1987), and this selective adhesion phenomenon could result in the concentration of neurons within craters. Alternatively, the gold plating of the conductors, especially with a cyanide-based plating solution, might produce toxic electrode tips, resulting in the positioning of the majority of cells and processes in the intercrater regions. In order to determine whether the crater-region neuronal components were representative of the mini-culture population, the average number of neuronal somata and processes/fascicles present in the 50 μm X 50 μm areas surrounding the electrode craters along a central 6-conductor row was compared with the same count of neurons and fibers in a region of equal size directly to the right of each crater (Fig. 47).

Minimization of Culture Variability

When the initial experiments for this project were performed, 3-5 MMEPs were routinely cultured per week, and electrophysiological and morphological data were collected over a period of several months from MMEPs of different culture dates. As was discussed in Chapter 3, the pooled data from these experiments exhibited such extreme variability in both electrophysiological behavior and

Figure 47. Schematic diagram depicting the determination of "on-crater" and "off-crater" neuronal components. All of the fibers and neuronal somata were enumerated in the 50 X 50 mm area surrounding each electrode crater in a central electrode row (a). These counts were then averaged to obtain values for the mean number on-crater cell bodies and fiber/fascicles. The neuronal somata and fibers were also enumerated the 50 X 50 mm areas to the right of each crater (b), and averaged to obtain the off-crater cell body and fiber/fascicle values. The calculated on-crater and off-crater values would be compared to determine whether the on-crater neuronal population was similar to that of the rest of the monolayer culture.


50 µm
CONDUCTOR TIP
ELECTRODE CRATER
C IC C IC C IC C IC C IC C IC

morphological characteristics that statistically significant generalizations concerning mini-cultures could not be made. Therefore, systematic analyses were performed on a set of 76 MMEP mini-cultures which were prepared, maintained, and assayed under controlled culture conditions (see Tables 1 and 2).

As described in detail in Chapter 3, the "controlled culture" MMEP experiments were assigned to SEED and AGE groups. The SEED file (containing data from MMEPs seeded with between 2.5 and 10 X 10^5 cells and assayed at 4 weeks) was used to investigate the effects of varying seeding density on electrophysiological and morphological development, while the AGE file (composed of data from MMEPs seeded with 6-8 X 10^5 cells and assayed during weeks 1, 2, 3, or 4) was used to assess culture age effects. These sets of "culture variable controlled" MMEP mini-cultures permitted morphological analyses following their electrophysiological assay and allowed the first correlations between mini-culture activity and morphology.

Results

Effects of Culture Variables on Neuronal Density

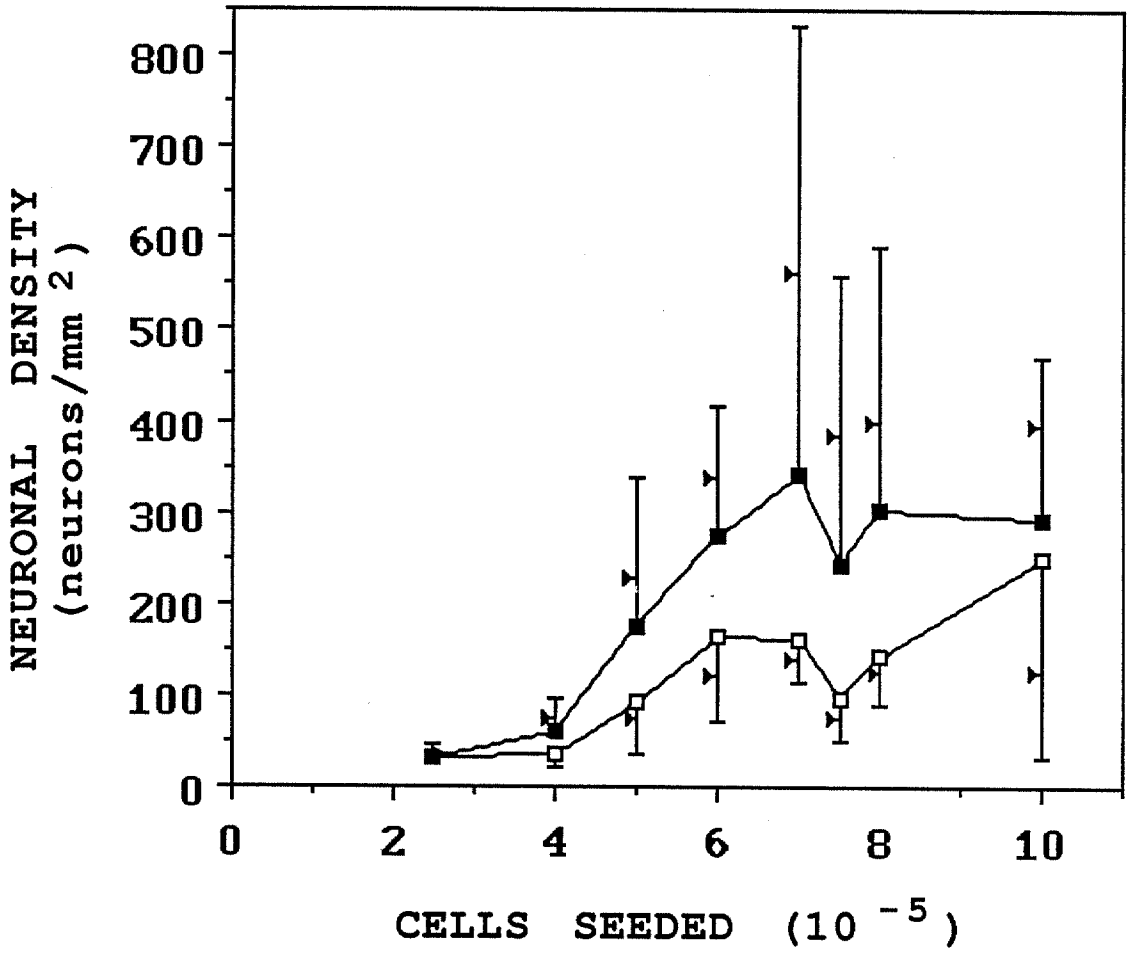
Neuronal Density as a Function Cell Seeding Concentration

Recording island neuronal density. Cultures from the SEED file were first used to establish the relationship

between the number of cells seeded and the recording island neuronal density at 4 weeks in culture (Fig. 48). MMEP cultures seeded with 2.5 or 4 X 10^5 cells displayed very low mean neuronal densities (<100 neurons/mm^2). Mean neuronal density was higher for cultures seeded with 5 X 10^5 cells and continued to rise to approximately 300 cells/mm^2 for plates seeded with 6 X 10^5 cells. Beyond this seeding density, however, no consistent increases in neuronal density were observed; seedings of between 6 and 8 X 10^5 cells resulted in average neuronal densities of 250-300 neurons/mm^2 at 4 weeks. It must be noted, however, that extreme variations in plate-to-plate neuronal density were observed within each seeding group. For example, the neuronal densities for MMEPs seeded with 8 X 10^5 cells varied ranged from less than 100 to over 900 neurons/mm^2.

Seedings of 6-8 X 10^5 cells onto MMEPs which contained a approximately 600 mm^2 of total adhesive surface area corresponded to a plating density of 1000-1300 cells/mm^2. The resulting average neuronal density at 4 weeks of 270 neurons/mm^2 for plates seeded in this range thus represented a plating/survival efficiency of 20-30%. It must be recalled, however, that neurons could not be distinguished from non-neurons in the seeding suspension, and the calculated number of cells seeded represented the total number of all cell types present (not neurons only). Thus,

Figure 48. *A*, Comparison plot of recording island and conditioning area mean neuronal densities as a function of the number of cells seeded in a constant seeding volume of 1 ml (SEED file data, assay age, 4 weeks). Lines connect mean values for each age group. Wide variability between individual experiments, particularly for the recording island cultures, was observed, as evidenced by the large error bars (bars, SD, arrows, SEM). This variability prohibited rigorous statistical comparisons between the recording island and conditioning area neuronal densities. However, the trend toward higher neuronal density on the recording island was evident at all seedings >2.5 X 10^5 cells. See Table 2 for the number of replicates associated with each seeding concentration.


NEURONAL DENSITY
(neurons/mm 2)
800
700
600
500
400
300
200
100
0
0
2
4
6
8
10
CELLS SEEDED (10 -5)

the true plating efficiency of the neuronal population was probably considerably higher.

Conditioning area neuronal density. The neuronal densities of the conditioning areas to either side of the recording island also varied with the number of cells seeded (Fig. 48). The mean neuronal densities for both the recording island and the conditioning area were similar for plates seeded with 2.5 or 4 X 10^5 cells. However, at higher seedings, the mean neuronal densities at 4 weeks diverged, with consistently higher neuronal densities observed on the recording islands.

While the recording island neuronal density function reached a plateau value of approximately 300 neurons/mm^2, the conditioning area function remained below 200 neurons/mm^2, with the exception of the last data point. This point, representing a mean neuronal density for MMEPs seeded with 1 X 10^6 cells, was calculated from only 3 MMEP experiments which yielded widely scattered data. It therefore did not carry sufficient weight to suggest that the conditioning area neuronal density function did not plateau. Although neuronal densities varied between cultures, the lower densities of the conditioning areas were consistently apparent. Thus, in contrast to the 20-30% average recording island culture plating/survival efficiency calculated for seedings of 6-8 X 10^6 cells, the conditioning region survival/plating efficiencies were

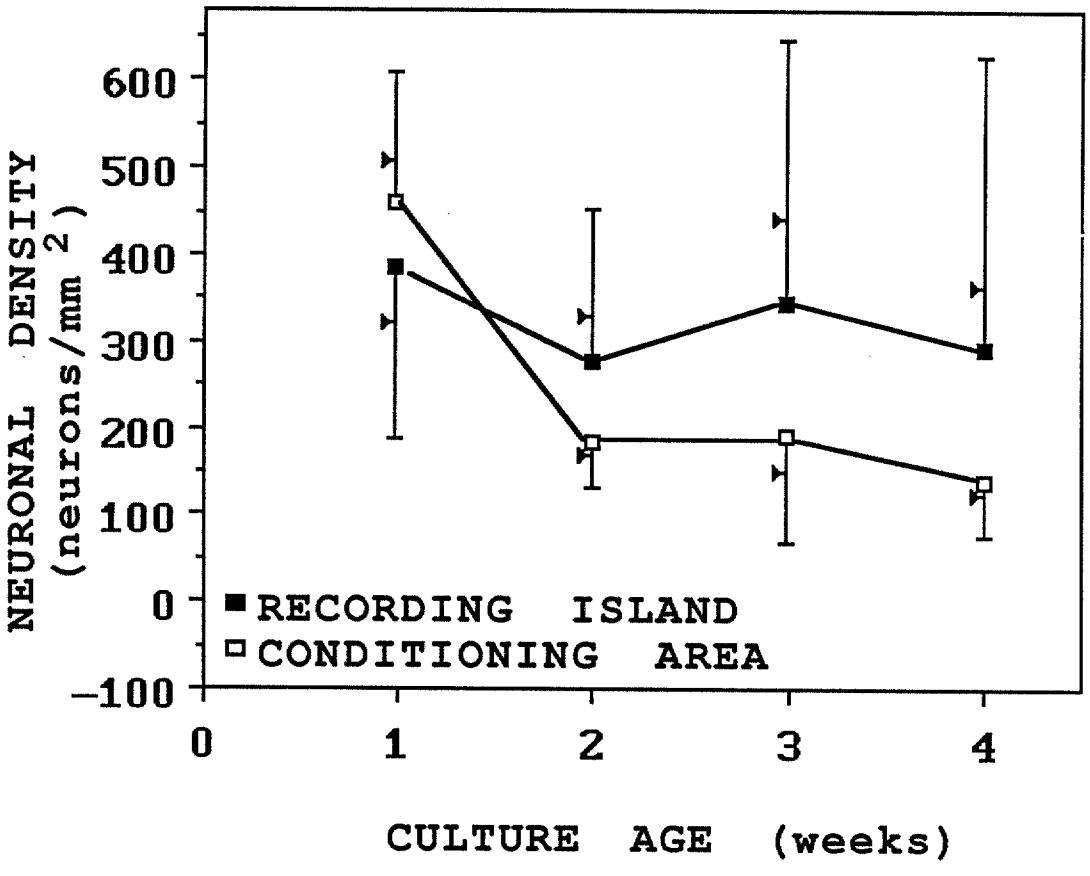
<20%. The discrepancy in neuronal densities between the two culture areas measured after 4 weeks in culture could have arisen from differences in initial neuronal adhesion or from differential neuronal survival. These two possibilities would be investigated by determining the effects of culture age on the neuronal densities of both regions.

Neuronal Density as a Function of Culture Age

The effect of culture age on recording island and conditioning area neuronal densities was investigated using the AGE file data (Fig. 49). Mean recording island neuronal density appeared to decrease only slightly with age, from 380 neurons/mm^2 at week 1 to approximately 350 neurons/mm^2 during weeks 3 and 4. Thus, the neurons present on the recording island at 1 week in culture were likely to continue to survive through week 4.

The mean conditioning region neuronal density was somewhat higher than that of the recording island during week 1 (approximately 450 cells/mm^2). However, while recording island neuronal density decreased only slightly with age, the mean conditioning area density decreased by more than 50% to <200 neurons/mm^2 during week 2 and reached a mean value of <150 neurons/mm^2 by week 4 (Fig. 49). Even though the neuronal densities calculated for individual MMEP experiments at each age varied substantially, the

Figure 49. Comparison plot of mean recording island and conditioning area neuronal densities as a function of culture age (AGE file data, all MMEPs seeded with 6-8 X 10^5 cells in a constant volume of 1 ml). Lines connect mean values for each age group. Considerable variability in neuronal density was apparent between MMEPs of the same age, as evidenced by large error bars (bars, SD, arrows, SEM). However, the mean recording island neuronal densities remained relatively stable between weeks 1 and 4. The average conditioning area neuronal density was actually higher than that of the recording island at week 1. However,the mean neuronal density of the conditioning region declined by >50% by week 2 and continued to decrease through week 4.


NEURONAL DENSITY
(neurons/mm 2)
600
500
400
300
200
100
0
−100
RECORDING ISLAND
CONDITIONING AREA
0
1
2
3
4
CULTURE AGE (weeks)

differential effect of age on the mean neuronal densities of recording island and conditioning region cultures was consistently observed. Thus, the initial adhesion and survival of neurons through week 1 was similar between the recording island and conditioning area cultures, but neurons survived in the mini-culture while many were lost from the conditioning regions between weeks 1 and 4.

Effects of Culture Variables on Neuronal Aggregation

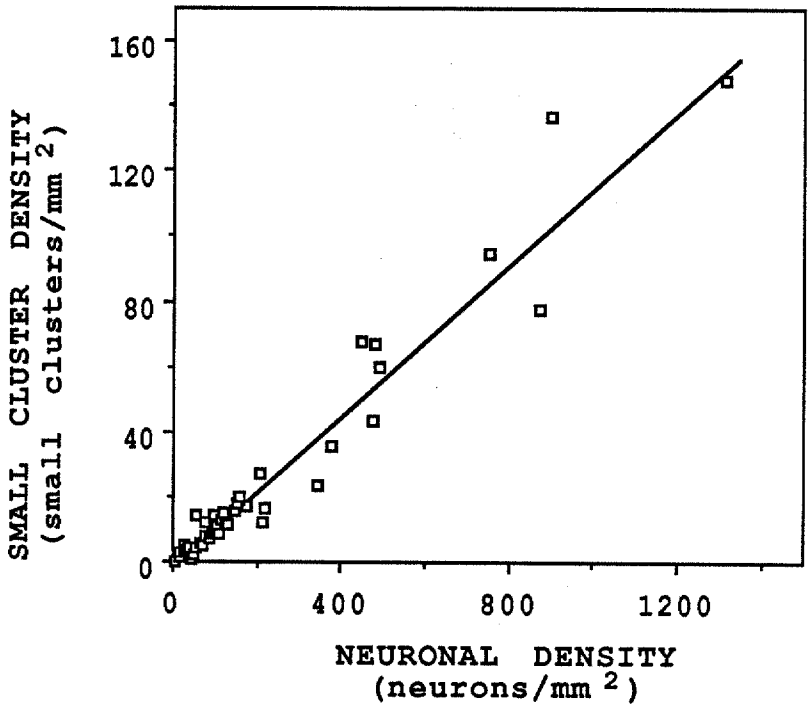
Effect of Neuronal Density on Aggregation

Even though MMEPs were flamed through the same mask, the diameters of the resulting recording island regions varied in diameter from 1.0-2.8 mm. In order to compare the degree of neuronal aggregation between recording island cultures of varying sizes, the counts of large and small clusters were normalized per unit of culture surface area (clusters/mm^2) and referred to as large and small cluster densities. As neuronal density increased, cluster densities also increased. The recording island density of small clusters varied linearly with increasing neuronal density (Fig. 50*A*). Large cluster density on the recording island also appeared to vary linearly with neuronal density, although greater scatter in this relationship was observed (Fig. 50*B*).

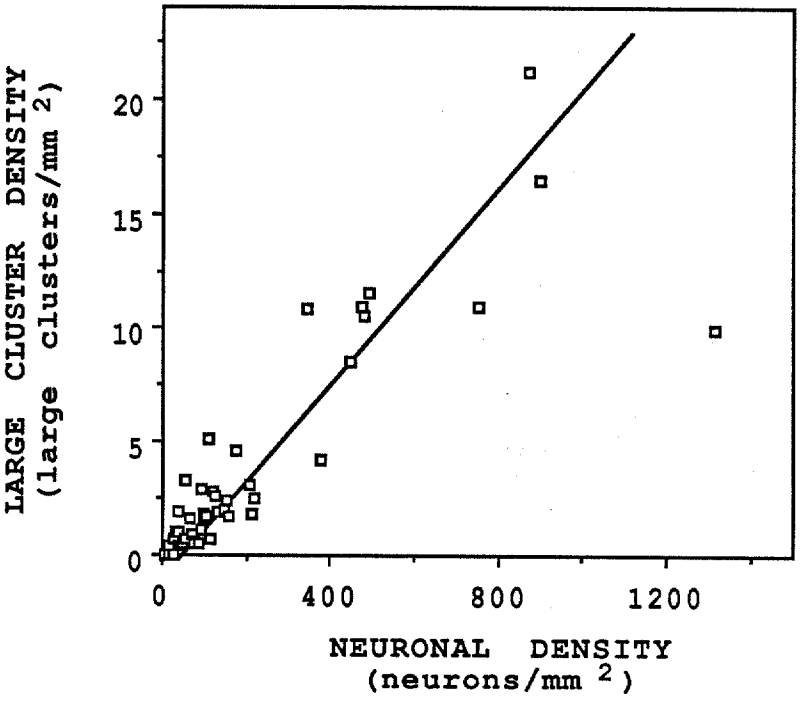
When comparing recording island and conditioning area cluster densities as a function of neuronal density,

Figure 50. Scatterplots of recording island cluster density as a function of neuronal density (SEED file data). Both small cluster density, aggregates of 2-5 neurons per mm^2 of culture area, (*A*) and large cluster density, aggregates of >5 neurons per mm^2, (*B*) appeared to vary as a linear function of the neuronal density.

A.


SMALL CLUSTER DENSITY
(small clusters/mm 2)
160
120
80
40
0
0
400
800
1200
NEURONAL DENSITY
(neurons/mm 2)

B.


LARGE CLUSTER DENSITY
(large clusters/mm 2)
20
15
10
5
0
0
400
800
1200
NEURONAL DENSITY
(neurons/mm 2)

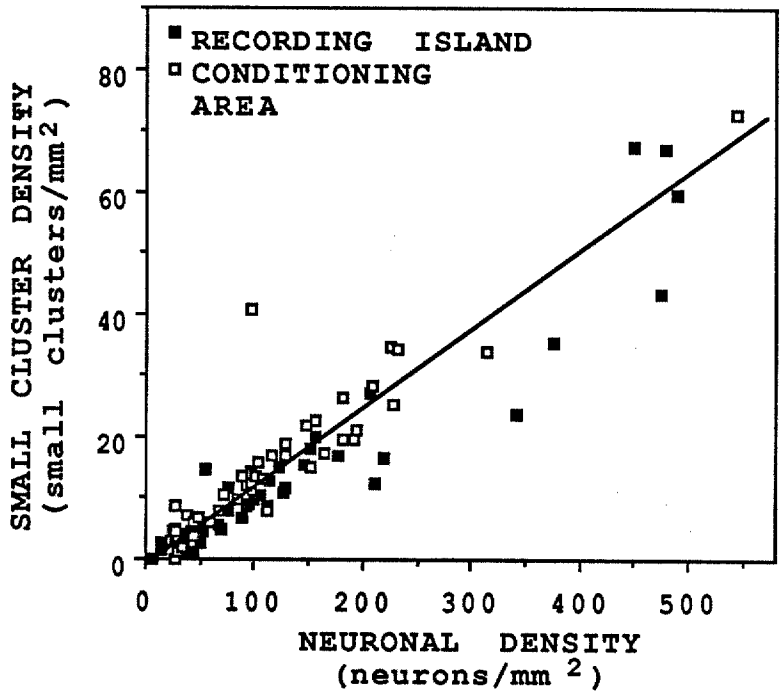
should be noted that the maximum conditioning area neuronal density encountered was less than 600 neurons/mm^2, while the maximum observed recording island neuronal density was almost 1400 neurons/mm^2. However, plots of recording island and conditioning area small cluster densities versus their corresponding neuronal densities were comparable at neuronal densities of up to 600 neurons/mm^2 (Fig. 51*A*). Large cluster densities were also comparable between the two regions, at least at neuronal densities of <300 neurons/mm^2 (Fig. 51*B*). Thus, the tendency toward neuronal reaggregation, as measured by cluster density, was similar in the recording and conditioning regions.

Effect of Culture Age on Aggregation

The plot of recording island large cluster density versus culture age using data from the AGE file showed that mean large cluster density declined from >10 large clusters/mm^2 at week 1 to approximately 5 clusters/mm^2 by week 4 (Fig. 52*A*). Conditioning area large cluster density decreased even more sharply, from a mean of 12 large clusters/mm^2 to approximately 2/mm^2 during the same time period (Fig. 52*B*). The mean density of small clusters on the recording island remained stable between weeks 1 and 4, at approximately 40 small clusters/mm^2 (Fig. 53*A*). The conditioning area small cluster density was comparable to that of the recording island during week 1, but declined to

Figure 51. Combined scatterplots of recording island and conditioning area small and large cluster densities as a function of neuronal density (SEED file data). The horizontal axis was limited to 600 neurons/mm^2 for comparison between the two culture areas because the maximum observed conditioning region neuronal density was 587 neurons/mm^2. *A*, Conditioning area and recording island small cluster densities were comparable at all neuronal densities. *B*, Conditioning region and recording island large cluster densities appeared to be comparable, at least at lower neuronal densities. However, greater variability in large cluster density was observed on both culture populations than was present with small cluster densities.

A.


RECORDING ISLAND
CONDITIONING AREA
SMALL CLUSTER DENSITY (small clusters/mm 2)
0
20
40
60
80
0
100
200
300
400
500
NEURONAL DENSITY (neurons/mm 2)

B.

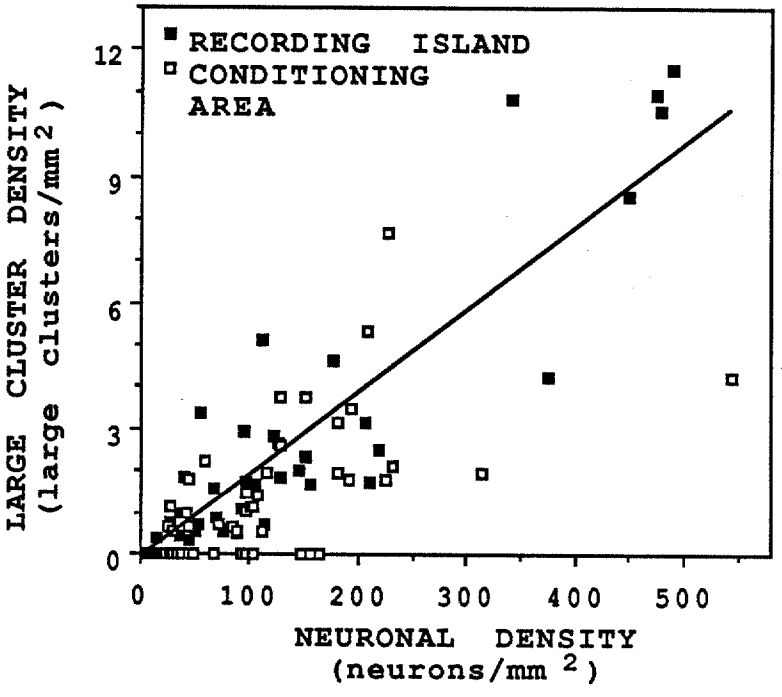
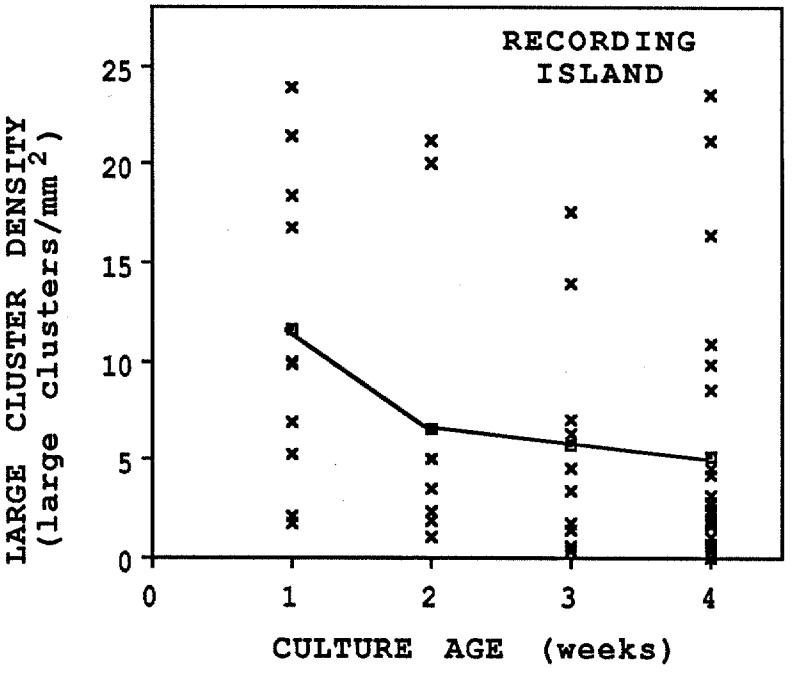

RECORDING ISLAND
CONDITIONING AREA
LARGE CLUSTER DENSITY (large clusters/mm 2)
0
3
6
9
12
0
100
200
300
400
500
NEURONAL DENSITY (neurons/mm 2)

Figure 52. Scatterplots of large cluster densities as a function of culture age (AGE data file). Lines connect the means of each age group. *A,* The mean large cluster density of the recording island declined by 55% between weeks 1 and 4. *B,* The mean conditioning area large cluster density decreased by 86% in the same time period. Although a trend toward reduced large cluster density, particularly in the conditioning area, was apparent, the wide variability between MMEP cultures of the same age made these differences nonsignificant.

A.


RECORDING
ISLAND
LARGE CLUSTER DENSITY
(large clusters/mm 2)
25
20
15
10
5
0
0
1
2
3
4
CULTURE AGE (weeks)

B.

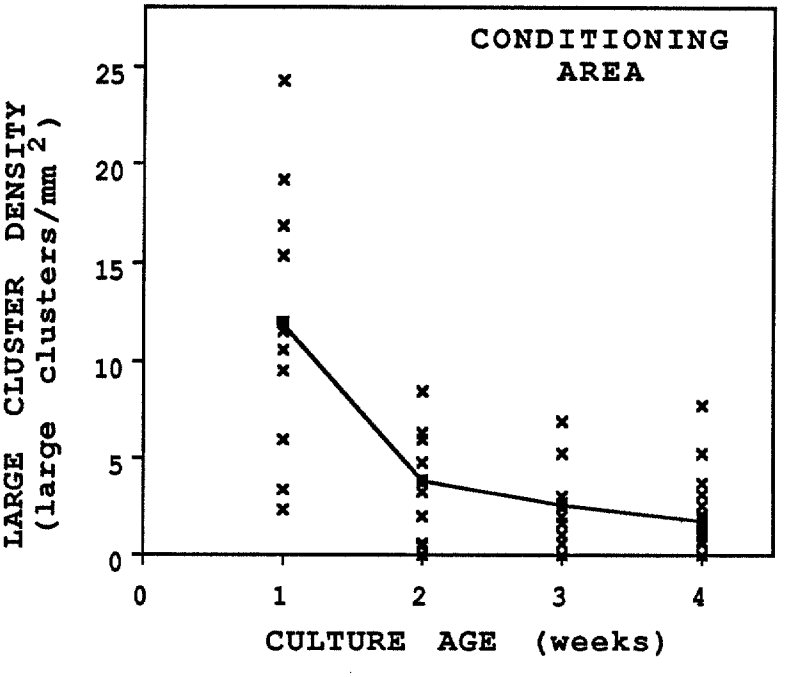
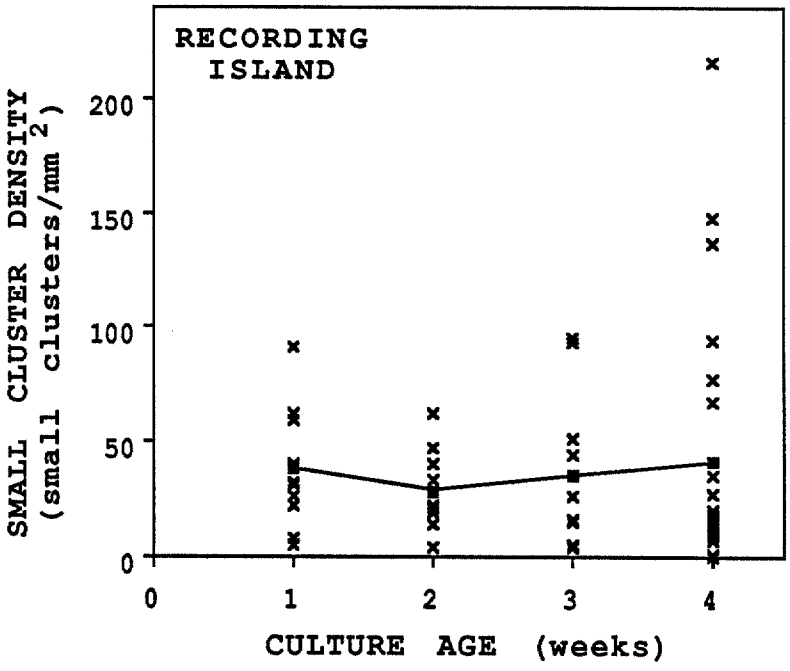
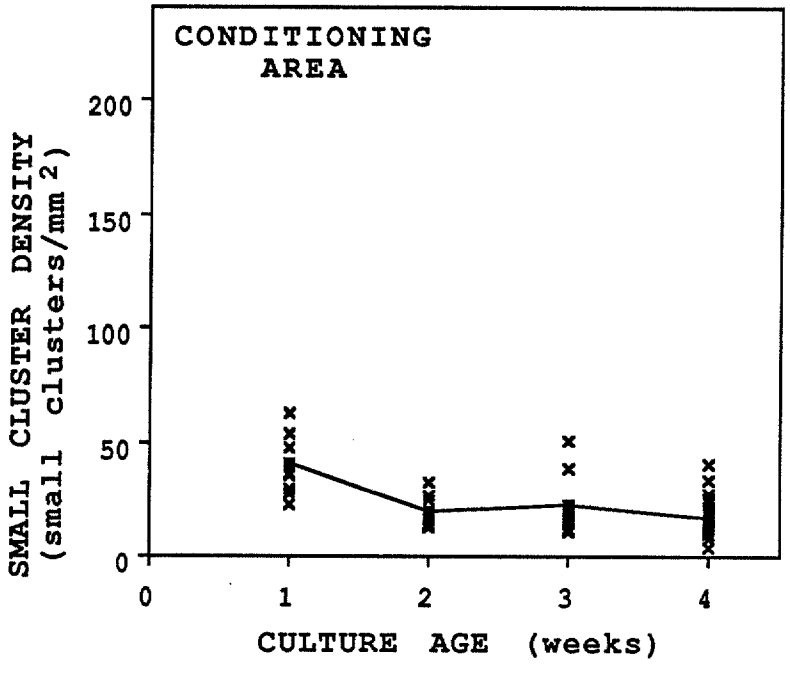

CONDITIONING
AREA
LARGE CLUSTER DENSITY
(large clusters/mm 2)
25
20
15
10
5
0
0
1
2
3
4
CULTURE AGE (weeks)

Figure 53. Scatterplots of small cluster densities as a function of culture age (AGE data file). Lines connect the means of each age group. *A*, The mean recording island cluster density remained stable throughout week 4. *B*, The mean conditioning area small cluster density was similar to that of the recording island during week 1 but exhibited a decline of 53% by week 4. However, due to wide variability between MMEP cultures of the same age group, these differences were not statistically significant.

A.


RECORDING
ISLAND
SMALL CLUSTER DENSITY
(small clusters/mm 2)
200
150
100
50
0
0
1
2
3
4
CULTURE AGE (weeks)

B.


CONDITIONING
AREA
SMALL CLUSTER DENSITY
(small clusters/mm 2)
200
150
100
50
0
0
1
2
3
4
CULTURE AGE (weeks)

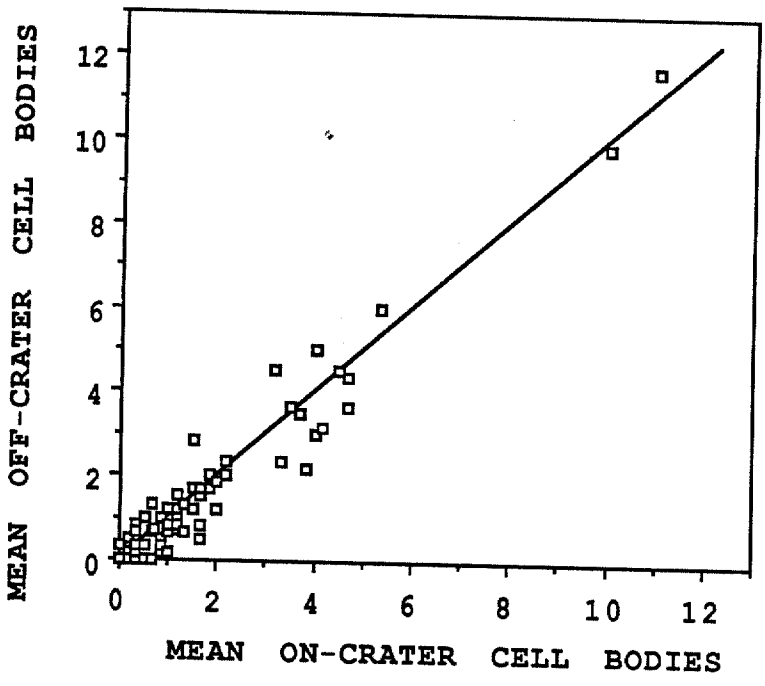
<25 small clusters/mm^2 by week 4 (Fig 53*B*). The observed differences between both the small and large cluster densities of the conditioning area and those of the recording island were probably at least partially attributable to the previously discussed decline in conditioning area neuronal density between 1 and 4 weeks in comparison to the relatively stable recording island neuronal density.

On-Crater and Off-Crater Somal and Fiber Populations

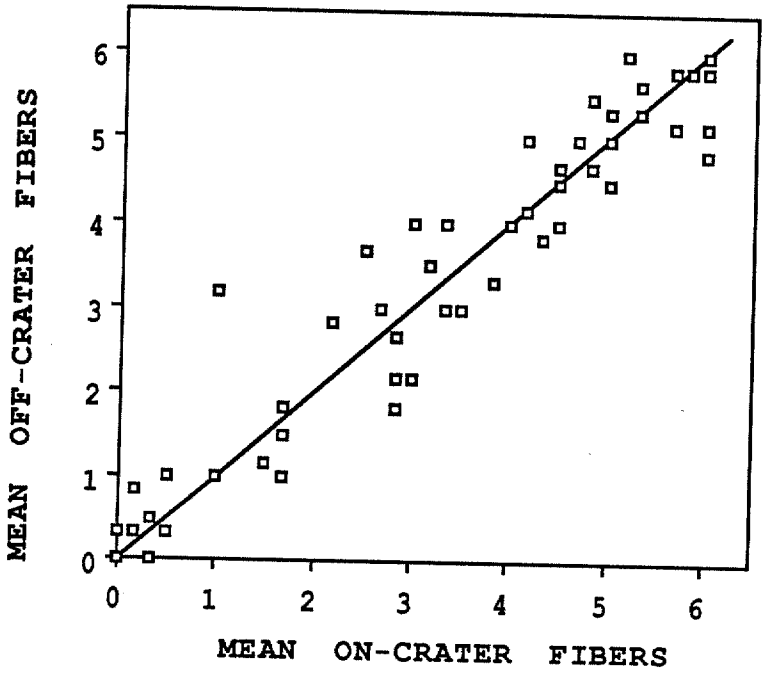
In order to determine if the population of neurons and fibers contacting the electrodes was similar to that of the rest of the mini-culture, the mean number of neuronal somata and fiber/fascicles counted in 50 X 50 μm electrode crater regions along a central 6-electrode row were compared with that of identical "off-crater" regions to the right of each electrode (See Fig. 47). The plot of mean off-crater cell bodies versus on-crater cell bodies revealed a one-to-one relationship (Fig. 54*A*). Likewise, plotting mean off-crater versus on-crater fiber/fascicles also revealed a one-to-one relationship (Fig. 54*B*). Thus, there was no evidence that the culture surface irregularities introduced by laser cratering and electrode plating resulted in differential neuronal adhesion and growth.

Figure 54. Scatterplot comparisons of off-crater and on-crater neuronal cell bodies and fibers (Each point represents the mean value for one MMEP experiment in the TOTAL data file, consisting of all 76 MMEPs of the experimental series described in Tables 1 and 2). *A*, The mean number of off-crater and on-crater neuronal somata for each recording island culture were very similar. *B*, Likewise, the mean fiber/fascicle counts in on- and off-crater regions were equivalent. Thus, counts of somata and fibers indicated that the neuronal population at the electrode sites was generally representative of the culture around the central recording matrix.

A.


MEAN OFF-CRATER CELL BODIES
0
2
4
6
8
10
12
MEAN ON-CRATER CELL BODIES

B.


MEAN OFF-CRATER FIBERS
0
1
2
3
4
5
6
MEAN ON-CRATER FIBERS

Neuronal Types as a Function Culture Variables

Cell Type Percentages as a Function Neuronal Density

The percentages of the various neuronal cell types found on the recording island were plotted against recording island neuronal density using data from the SEED file. As neuronal density increased, the percentages of the morphologically differentiated neurons (large and small bipolar and multipolar cells) decreased dramatically (Fig. 55). This was accompanied by an increase in the percentage of cluster cells to a plateau of approximately 70% (Fig. 56*A*). The percentage of round cells was similar at all neuronal densities (approximately 30%), although round cell percentages exhibited considerable variability at lower neuronal densities (Fig. 56*B*). The most numerous "morphologically differentiated" cell type present in recording island cultures was generally the small multipolar neuron, followed by the small bipolar neuron, the large multipolar neuron, and finally, the large bipolar neuron.

The percentages of morphologically differentiated cell types present in the conditioning regions exhibited the same rank order of prevalence as those of recording island cultures, with small multipolar neurons being the most numerous and large bipolar neurons the least often observed. The percentages of multipolar and bipolar

Figure 55. Scatterplots of the percentages of multipolar and bipolar neuronal cells present on recording island cultures as a function of neuronal density (SEED file data). *A*, Large multipolar neurons; *B*, Large bipolar neurons; *C*, Small multipolar neurons; *D*, Small bipolar neurons. The shape of of the plots indicated declines in these cell percentages as neuronal density increased.

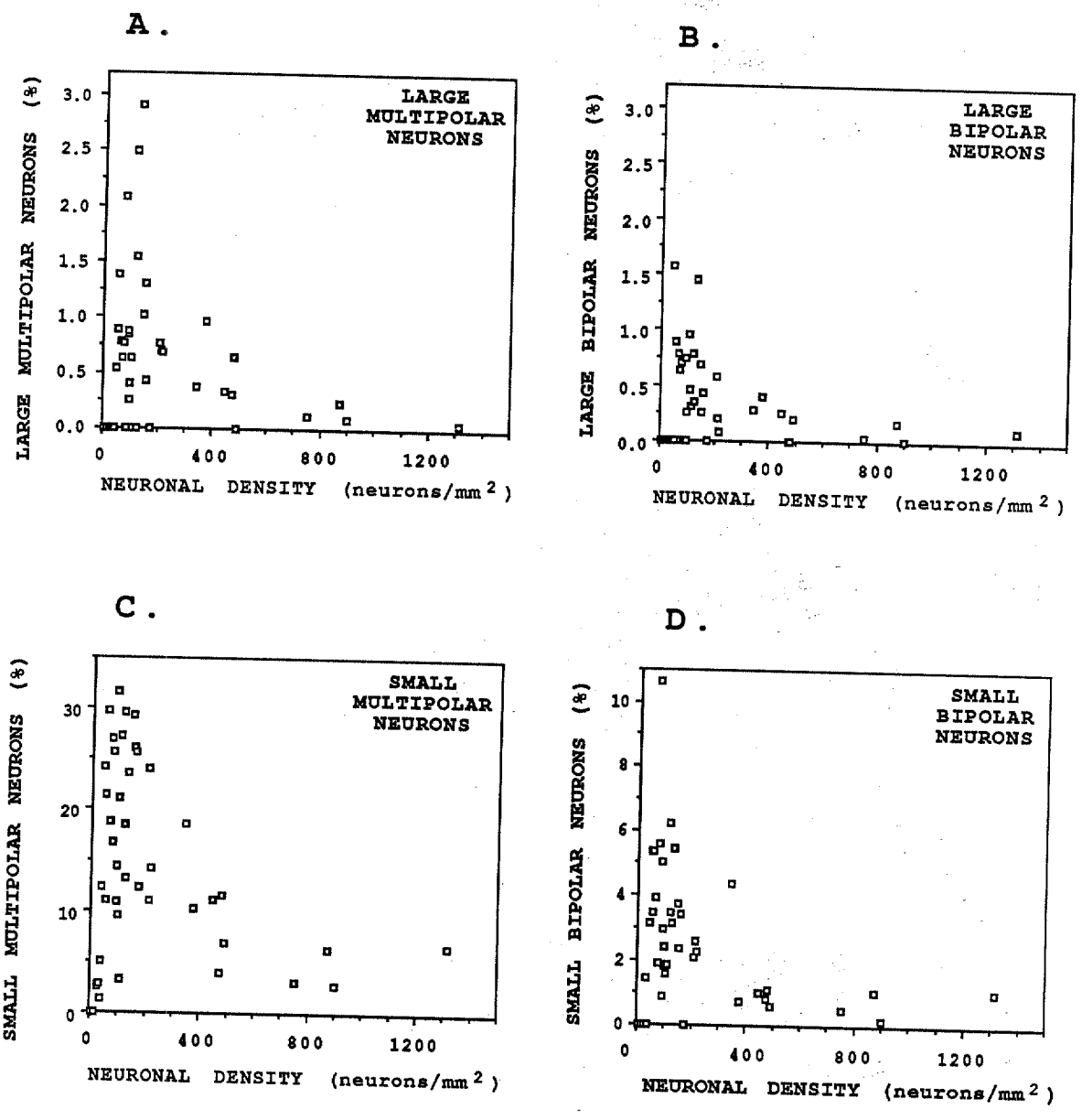
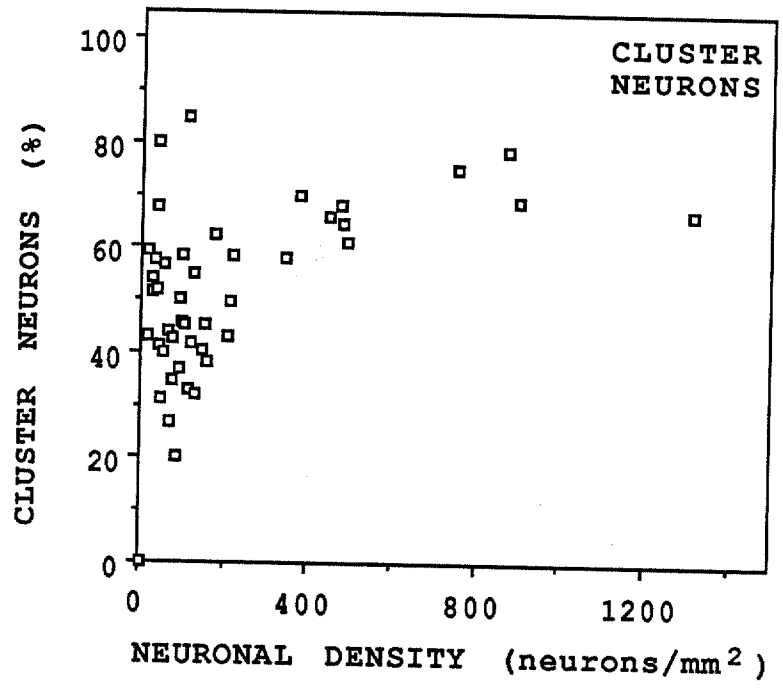
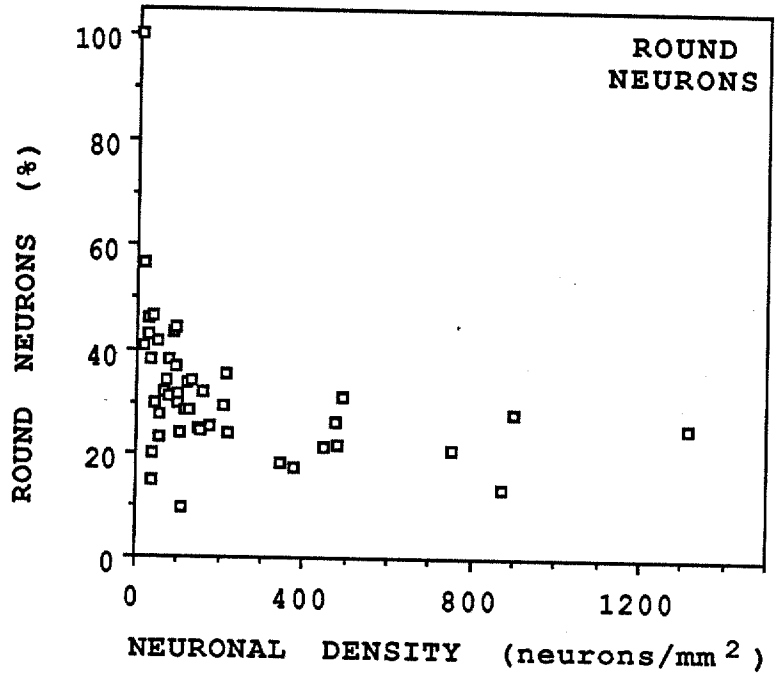

A.
LARGE MULTIPOLAR NEURONS
LARGE MULTIPOLAR NEURONS (%)
NEURONAL DENSITY (neurons/mm 2)
B.
LARGE BIPOLAR NEURONS
LARGE BIPOLAR NEURONS (%)
NEURONAL DENSITY (neurons/mm 2)
C.
SMALL MULTIPOLAR NEURONS
SMALL MULTIPOLAR NEURONS (%)
NEURONAL DENSITY (neurons/mm 2)
D.
SMALL BIPOLAR NEURONS
SMALL BIPOLAR NEURONS (%)
NEURONAL DENSITY (neurons/mm 2)

Figure 56. Scatterplots of the percentages of cluster cells and round cells present on the recording island regions as a function of neuronal density (SEED file data). *A*, The percentage of cluster neurons tended to increase with increasing neuronal density up to a plateau value of approximately 70%. *B*, The percentage of round neurons was relatively constant at all neuronal densities, comprising an average of approximately 20-30% of total neurons on recording island cultures. The percentages of both of these cell types varied considerably at low neuronal densities.

A.


CLUSTER NEURONS
CLUSTER NEURONS (%)
100
80
60
40
20
0
0
400
800
1200
NEURONAL DENSITY (neurons/mm 2)

B.


ROUND NEURONS
ROUND NEURONS (%)
100
80
60
40
20
0
0
400
800
1200
NEURONAL DENSITY (neurons/mm 2)

neurons on the conditioning areas declined dramatically with increasing neuronal density (Fig. 57), as they had on the recording island. However, the maximum observed percentages of these cell types were consistently higher on the conditioning areas than on the recording islands. Generally lower conditioning area cluster cell percentages (an apparent plateau value of 50% versus 70% for recording island cultures, Fig. 58*A*) accounted for this difference.

Round and cluster cells accounted for a vast majority of the neurons in almost all cultures. In cultures with high neuronal densities, as many as 95% or more of the neurons counted on the recording island and 90% on the conditioning areas were round and cluster cells (Fig. 59). The predominance of these cells masked any changes in the percentages of morphologically differentiated cell types resulting from increases in neuronal density. Therefore, in order to investigate differences in the populations of the morphologically defined neurons, percentages of these cells were calculated as a fraction of the total multipolar and bipolar neurons only, excluding round and cluster cells. These percentages were termed subtotal percentages.

The conditioning regions had displayed greater percentages of multipolar and bipolar cells than the recording islands. However, the subtotal percentages of these cell types were similar between the two culture regions. Therefore, differences in the neuronal

Figure 57. Scatterplots of the percentages of multipolar and bipolar neurons on conditioning areas as a function of neuronal density (SEED file data): *A*, Large multipolar neurons; *B*, Large bipolar neurons; *C*, small multipolar neurons; *D*, small bipolar neurons. The percentages of all of these cell types tended to decline with increased neuronal density, as was observed with recording island cultures. However, The observed percentages of these cell types was generally higher for the conditioning regions than those calculated for the recording island cultures (compare with Fig. 55).

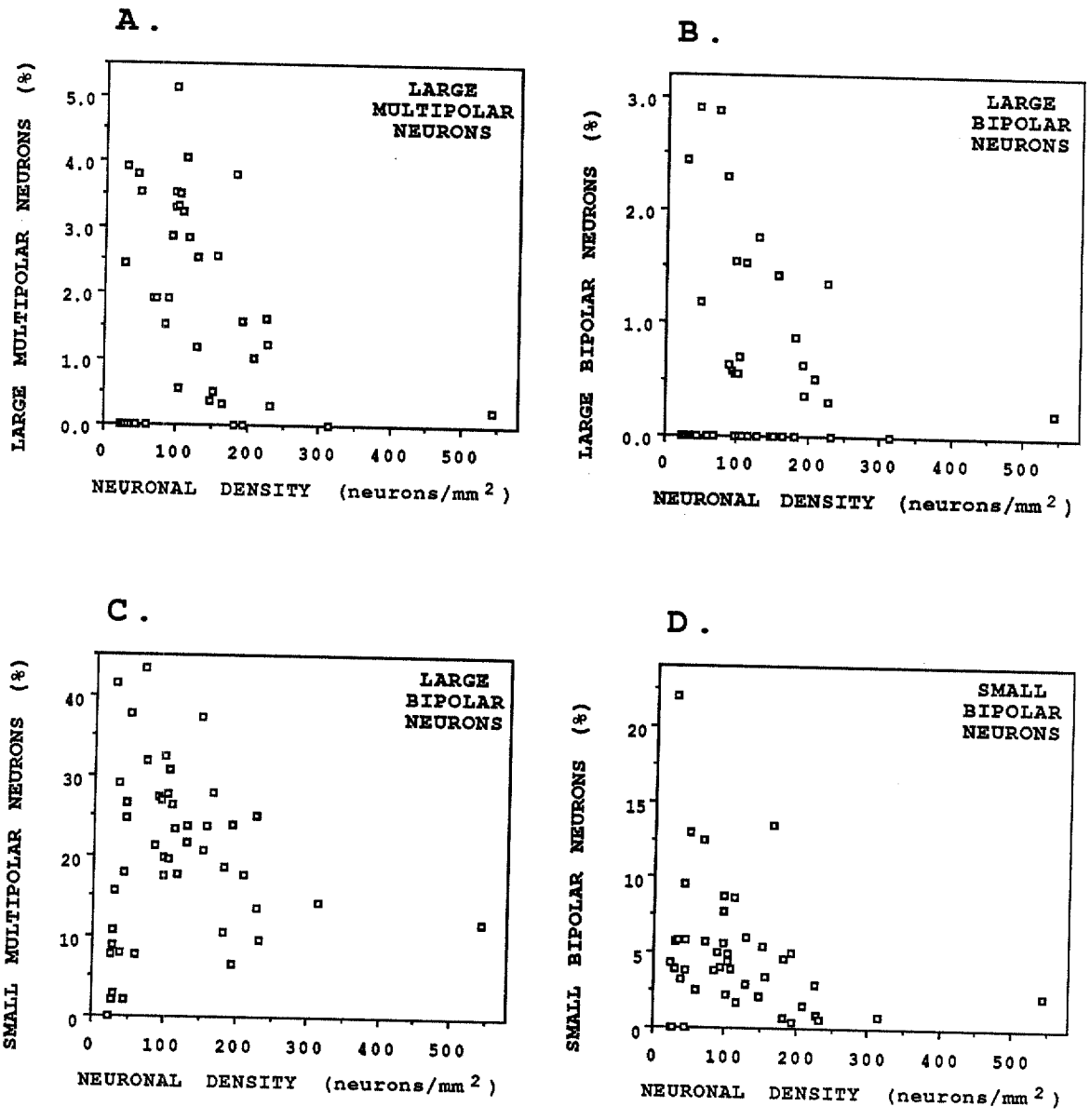
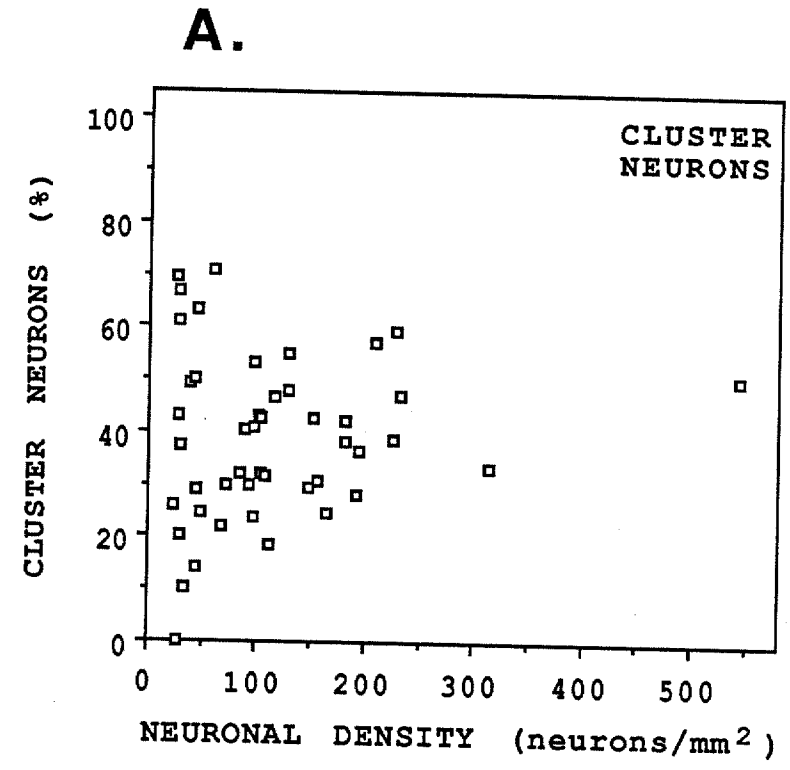

A.
LARGE MULTIPOLAR NEURONS (%)
LARGE MULTIPOLAR NEURONS
NEURONAL DENSITY (neurons/mm 2)
B.
LARGE BIPOLAR NEURONS (%)
LARGE BIPOLAR NEURONS
NEURONAL DENSITY (neurons/mm 2)
C.
SMALL MULTIPOLAR NEURONS (%)
LARGE BIPOLAR NEURONS
NEURONAL DENSITY (neurons/mm 2)
D.
SMALL BIPOLAR NEURONS (%)
SMALL BIPOLAR NEURONS
NEURONAL DENSITY (neurons/mm 2)

Figure 58. Scatterplots of the percentages of cluster cells and round cells present on the conditioning regions as a function of neuronal density (SEED file data). *A*, The percentage of cluster neurons in the conditioning regions did not reach the levels present on the recording island cultures. Although variable at lower neuronal densities, the conditioning area cluster cell percentage appeared to reach a relatively stable value of approximately 50% as neuronal density increased. This value was substantially lower than the plateau value of 70% cluster cells observed on recording island cultures (compare with Fig. 56a). *B*, The percentages of round neurons was variable in feeder area cultures but were generally slightly higher than those encountered on recording island cultures (see Fig. 56b).


A.
CLUSTER NEURONS
CLUSTER NEURONS (%)
100
80
60
40
20
0
0
100
200
300
400
500
NEURONAL DENSITY (neurons/mm 2)

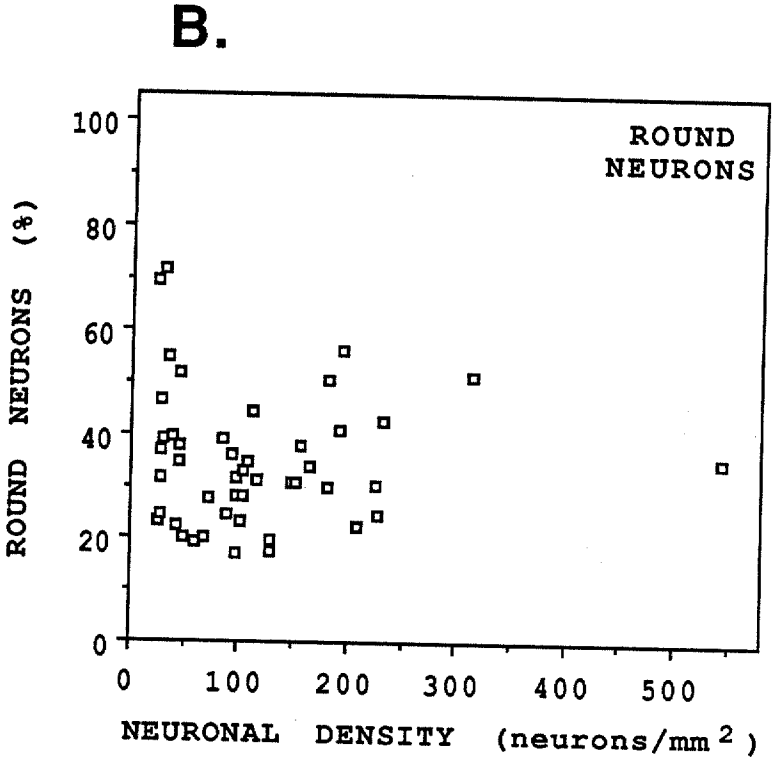
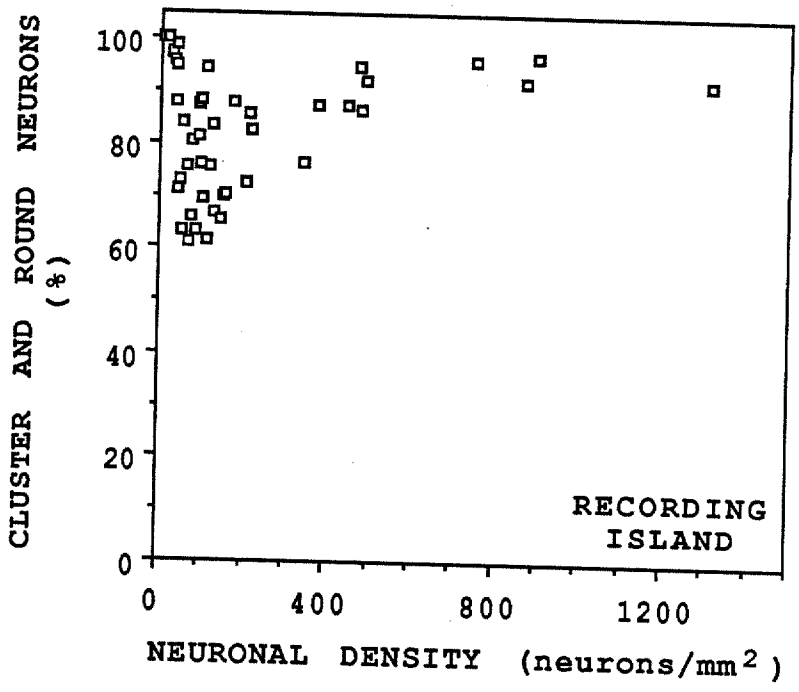
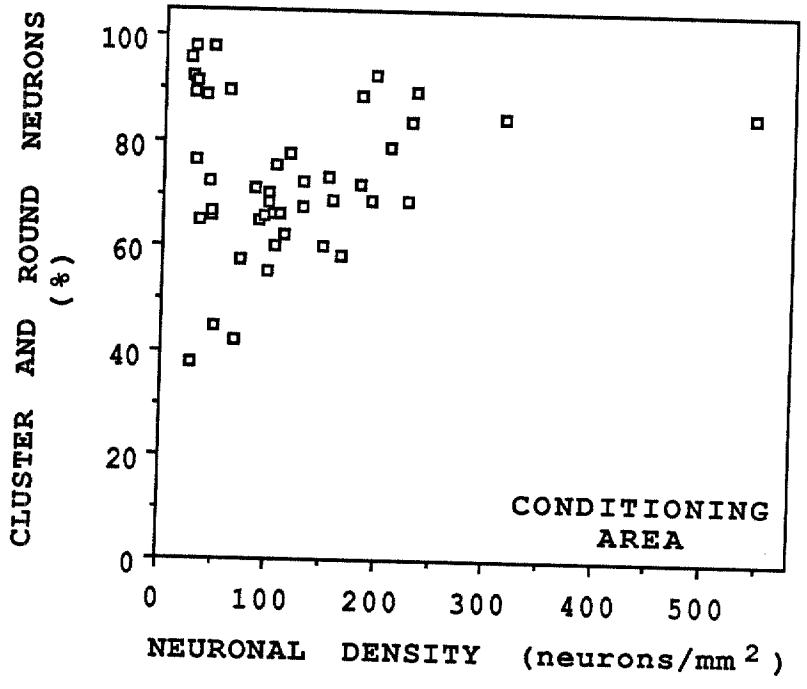

B.
ROUND NEURONS
ROUND NEURONS (%)
100
80
60
40
20
0
0
100
200
300
400
500
NEURONAL DENSITY (neurons/mm 2)

Figure 59. Percentages of the total "morphologically undifferentiated" neuronal types (cluster and round neurons) versus neuronal density (SEED file data). *A*, Recording island; *B*, Conditioning area. These cell types made up a vast majority of all of the neurons in culture, particularly on the recording islands. Their predominance masked differences in the percentages of other cell types, necessitating the calculation of subtotal percentages of multipolar and bipolar cells.

A.


CLUSTER AND ROUND NEURONS (%)
100
80
60
40
20
0
0
400
800
1200
NEURONAL DENSITY (neurons/mm2)
RECORDING ISLAND

B.


CLUSTER AND ROUND NEURONS (%)
100
80
60
40
20
0
0
100
200
300
400
500
NEURONAL DENSITY (neurons/mm2)
CONDITIONING AREA

populations between the two groups resulted from differences in the numbers of round and cluster cells. The recording island and conditioning area subtotal percentages of both large multipolar and large bipolar neurons exhibited a declining envelope as neuronal density increased (Figs. 60*A*, *B*, 61*A*, *B*). This decline was accompanied by relatively stable percentages of small multipolar and small bipolar neurons (Figs. 60*C*, *D*, 61*C*, *D*). Thus, increased neuronal density had a two-fold effect. First, it resulted in an increased percentage of cluster cells, with consequent decreases in the percentages of other cell types. However, by considering only the multipolar and bipolar cells in subtotal percentages, it was discovered that increased neuronal density also resulted in a selective decline in the proportion of large bipolar and multipolar cells.

Cell Type Percentages as a Function of Culture Age

The mean percentages of the neuronal cell types present in the recording island and conditioning area cultures remained relatively stable between weeks 1 and 4 (Figs. 62, 63), with one notable exception. A greater than four-fold increase in the mean percentage of large multipolar neurons on the conditioning area occurred between weeks 1 and 4 (Fig. 63*A*). The mean percentage of large multipolar neurons on the recording island also

Figure 60. Scatterplots of recording island subtotal percentages multipolar and bipolar neurons (calculated excluding round and cluster neurons from total neurons) versus neuronal density (SEED data file). *A*, The maximum percentages of large multipolar neurons declined with increasing neuronal density. *B*, Likewise, the maximum percentages of large bipolar neurons also declined as neuronal density increased. The subtotal percentage of small multipolar neurons (*C*) and small bipolar neurons (*D*) remained relatively constant. Therefore, with the masking effect of round and cluster cells was removed, it appeared that increased neuronal density was accompanied by a selective decrement in the percentages of larger neurons.

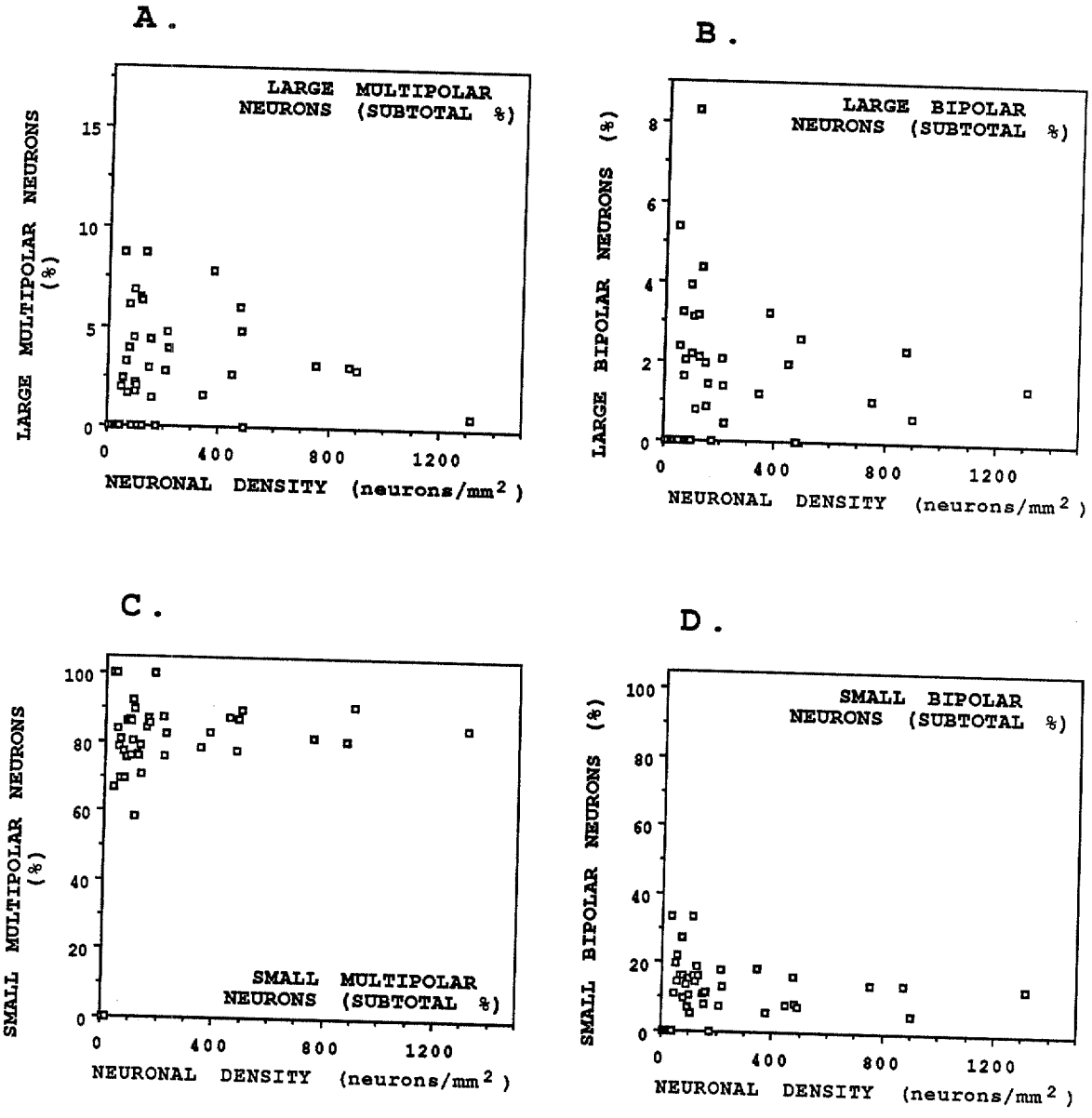
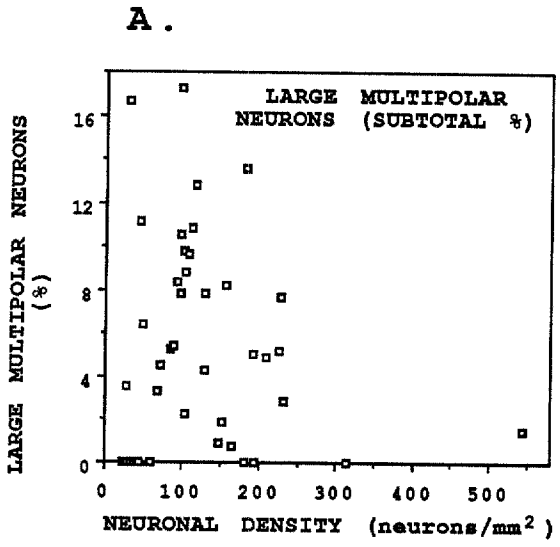
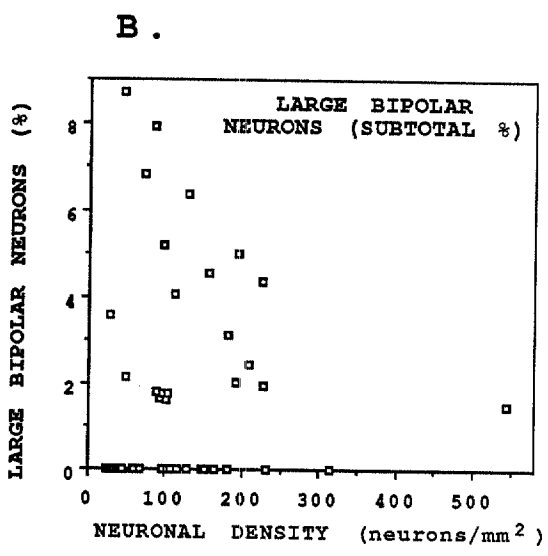
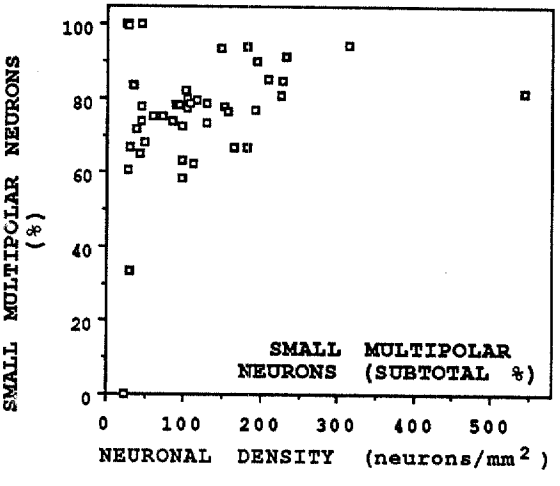

A.
LARGE MULTIPOLAR NEURONS (SUBTOTAL %)
LARGE MULTIPOLAR NEURONS (%)
NEURONAL DENSITY (neurons/mm2)
B.
LARGE BIPOLAR NEURONS (SUBTOTAL %)
LARGE BIPOLAR NEURONS (%)
NEURONAL DENSITY (neurons/mm2)
C.
SMALL MULTIPOLAR NEURONS (SUBTOTAL %)
SMALL MULTIPOLAR NEURONS (%)
NEURONAL DENSITY (neurons/mm2)
D.
SMALL BIPOLAR NEURONS (SUBTOTAL %)
SMALL BIPOLAR NEURONS (%)
NEURONAL DENSITY (neurons/mm2)

Figure 61. Scatterplots of the conditioning area subtotal percentages of multipolar and bipolar neurons (calculated excluding round and cluster neurons from total neurons) as a function of neuronal density (SEED data file). Conditioning area cultures exhibited similar changes in cell type percentages with increased neuronal density as did recording island cultures. *A, B,* Large multipolar and bipolar neuron percentages declined as neuronal density increased. Small multipolar percentages remained relatively constant (*C*), but small bipolar percentages appeared to decline (*D*). It should be noted that the multipolar and bipolar cell percentages for the conditioning region and the recording island were much more similar when the round and cluster cells were excluded from the total neuronal count (contrast Figs. 55 and 57 with Figs. 60 and 61).


A.
LARGE MULTIPOLAR NEURONS (SUBTOTAL %)
LARGE MULTIPOLAR NEURONS (%)
0
4
8
12
16
0
100
200
300
400
500
NEURONAL DENSITY (neurons/mm2)


B.
LARGE BIPOLAR NEURONS (SUBTOTAL %)
LARGE BIPOLAR NEURONS (%)
0
2
4
6
8
0
100
200
300
400
500
NEURONAL DENSITY (neurons/mm2)


C.
SMALL MULTIPOLAR NEURONS (SUBTOTAL %)
SMALL MULTIPOLAR NEURONS (%)
0
20
40
60
80
100
0
100
200
300
400
500
NEURONAL DENSITY (neurons/mm2)

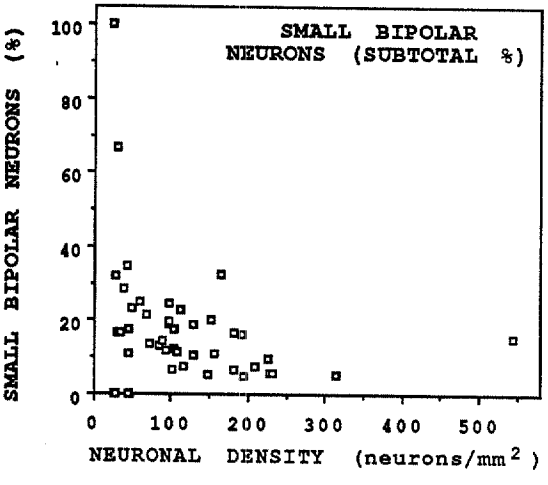

D.
SMALL BIPOLAR NEURONS (SUBTOTAL %)
SMALL BIPOLAR NEURONS (%)
0
20
40
60
80
100
0
100
200
300
400
500
NEURONAL DENSITY (neurons/mm2)

Figure 62. Scatterplots of the percentages of multipolar and bipolar neurons on recording island cultures versus culture age (AGE file data). Lines connect mean values for each age group. *A*, The mean percentage of large multipolar neurons doubled between weeks 1 and 4. The mean percentages of the other cell types (large bipolar neurons, *B*; small multipolar neurons, *C*; and small bipolar neurons, *D*) remained constant or decreased slightly. See Table 2

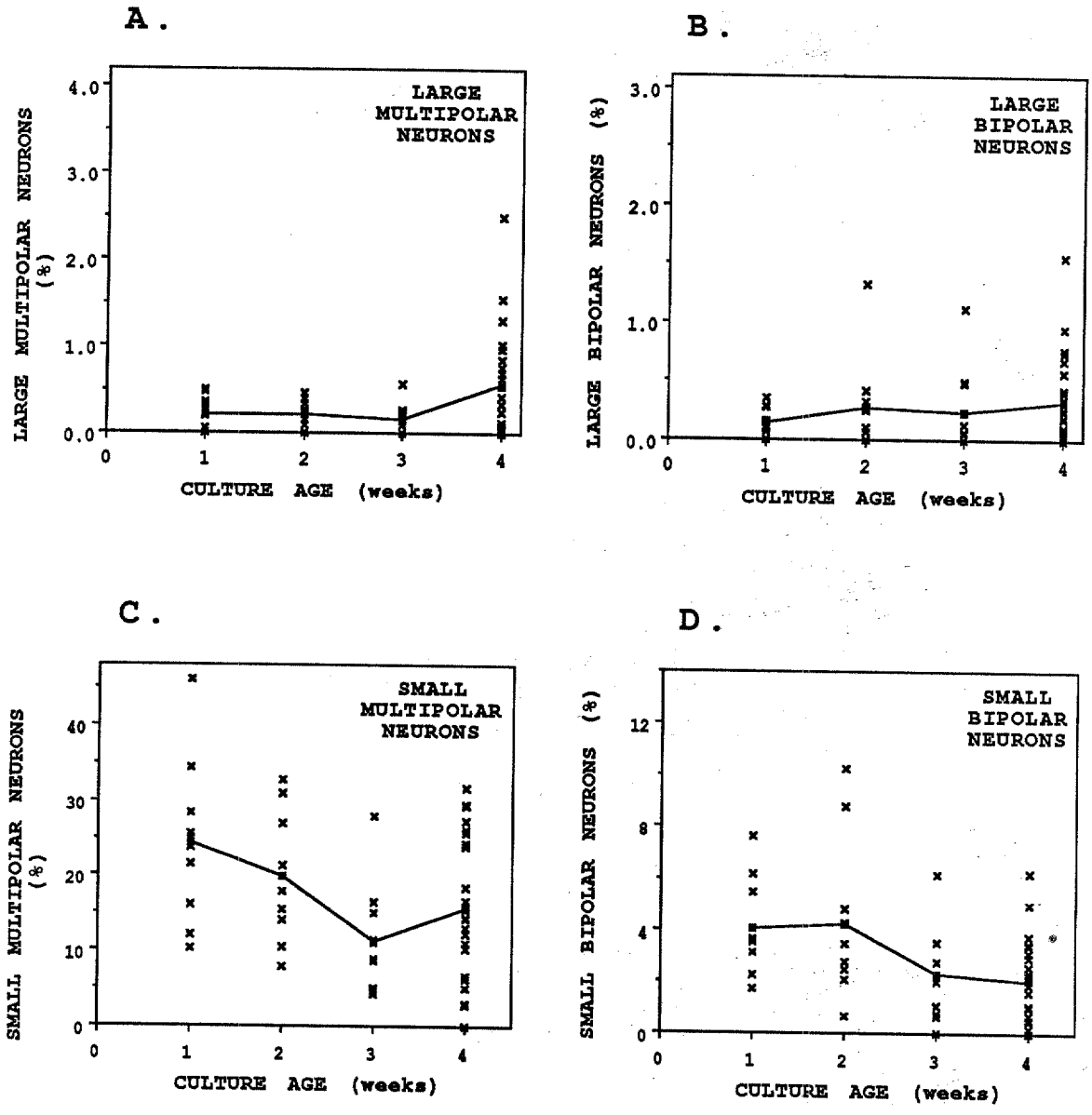
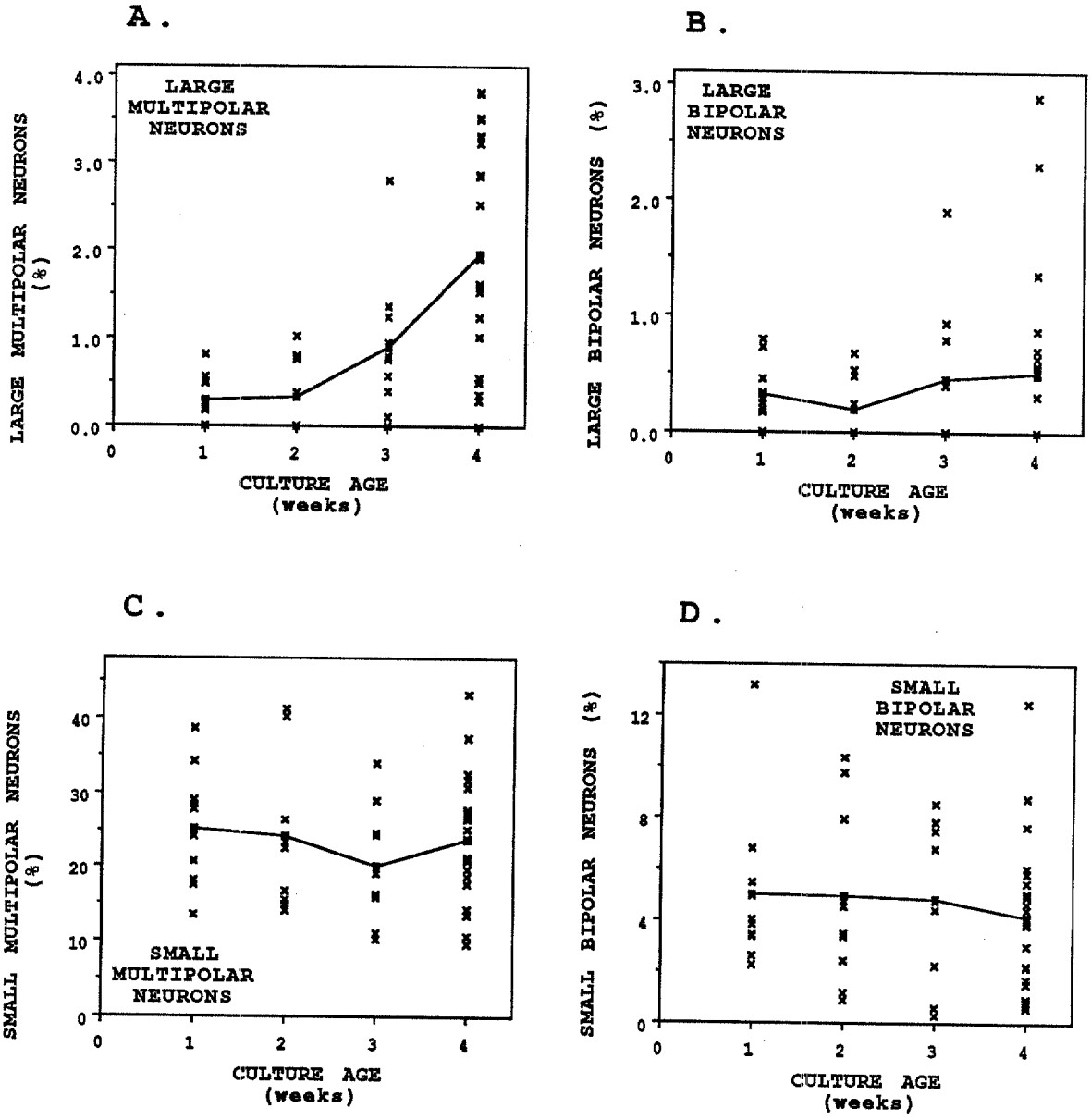

A.
LARGE MULTIPOLAR NEURONS
LARGE MULTIPOLAR NEURONS (%)
4.0
3.0
2.0
1.0
0.0
0
1
2
3
4
CULTURE AGE (weeks)
B.
LARGE BIPOLAR NEURONS
LARGE BIPOLAR NEURONS (%)
3.0
2.0
1.0
0.0
0
1
2
3
4
CULTURE AGE (weeks)
C.
SMALL MULTIPOLAR NEURONS
SMALL MULTIPOLAR NEURONS (%)
40
30
20
10
0
0
1
2
3
4
CULTURE AGE (weeks)
D.
SMALL BIPOLAR NEURONS
SMALL BIPOLAR NEURONS (%)
12
8
4
0
0
1
2
3
4
CULTURE AGE (weeks)

Figure 63. Scatterplots of the percentages of multipolar and bipolar neurons on conditioning regions versus culture age (AGE file data). Lines connect mean values for each age group. *A*, As with recording island cultures (see Fig. 62), the mean percentage of large multipolar neurons increased between weeks 1 and 4 in culture. However, this increase was much more pronounced on the conditioning regions (400% as opposed to 100% for recording island cultures). The other cell type percentages (large bipolar neurons, *B*; small multipolar neurons, *C*; and small bipolar neurons, *D*) remained relatively constant or declined slightly. See Table 2 for the number of replicates within each group.

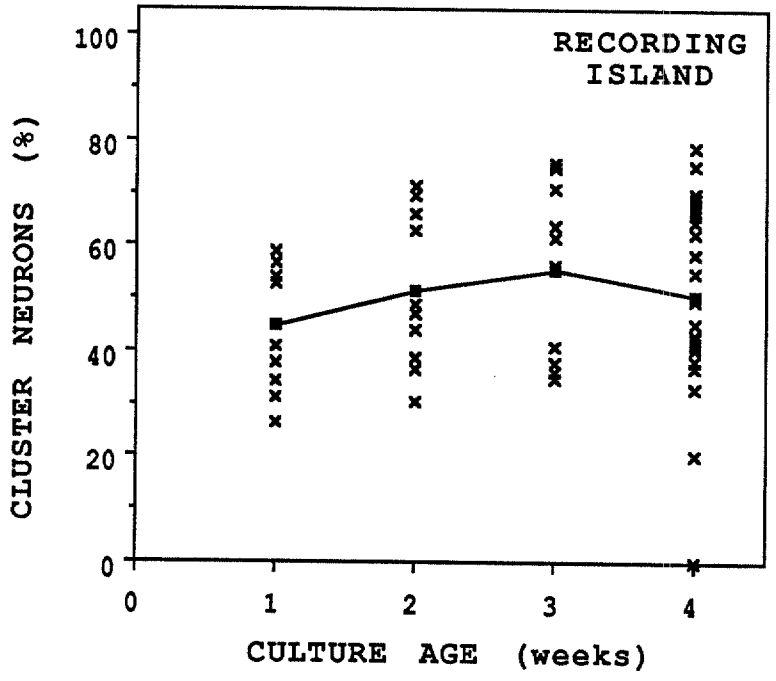

A.
LARGE MULTIPOLAR NEURONS
LARGE MULTIPOLAR NEURONS (%)
CULTURE AGE (weeks)
B.
LARGE BIPOLAR NEURONS
LARGE BIPOLAR NEURONS (%)
CULTURE AGE (weeks)
C.
SMALL MULTIPOLAR NEURONS
SMALL MULTIPOLAR NEURONS (%)
CULTURE AGE (weeks)
D.
SMALL BIPOLAR NEURONS
SMALL BIPOLAR NEURONS (%)
CULTURE AGE (weeks)

increased during this time, from approximately 0.25% at week 1 to approximately 0.5% at week 4 (Fig. 62*A*). At all culture ages, the percentages of multipolar and bipolar cell types on conditioning area tended to be slightly higher than those of the recording island cultures (contrast Figs. 62 and 63), while mean cluster cell percentages for the conditioning areas were lower than those of the recording island (Fig. 64). Neither the recording island nor the conditioning area cluster cell percentages changed substantially with age (Fig. 64). Thus, both the plots of cluster density and cluster cell percentages versus age (Figs. 52, 53, and 64) indicated that neuronal aggregation did not increase after week 1.

In order to study the effect of age on the percentages of multipolar and bipolar cells relative to each other without the masking effect of the large number of round and cluster cells, the subtotal percentages of the morphologically differentiated cell types were plotted versus culture age (Figs. 65, 66). The mean subtotal percentages of large multipolar and bipolar neurons increased between weeks 1 and 4 on both the recording islands and the conditioning areas, whereas those of small multipolar and bipolar neurons remained relatively constant or declined slightly. Because data scatter was large, the decreases in small bipolar and multipolar cell percentages were not statistically significant. It is possible,

Figure 64. Scatterplots of the percentages of cluster cells as a function of culture age (AGE file data). Lines connect mean values for each age group. The percentage of cluster cells on both the recording island (*A*) and the conditioning area (*B*) remained relatively constant between weeks 1 and 4. This supported cluster density data (Figs 52 and 53), which indicated that aggregation did not increase with culture age between weeks 1 and 4. See Table 2 for number of replicates within each group.

A.


RECORDING
ISLAND
CLUSTER NEURONS (%)
100
80
60
40
20
0
0
1
2
3
4
CULTURE AGE (weeks)

B.

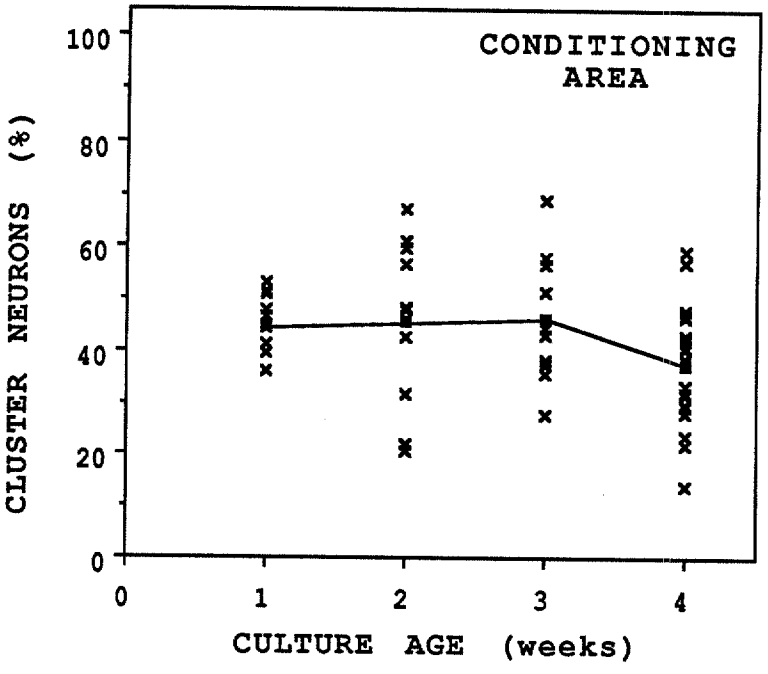
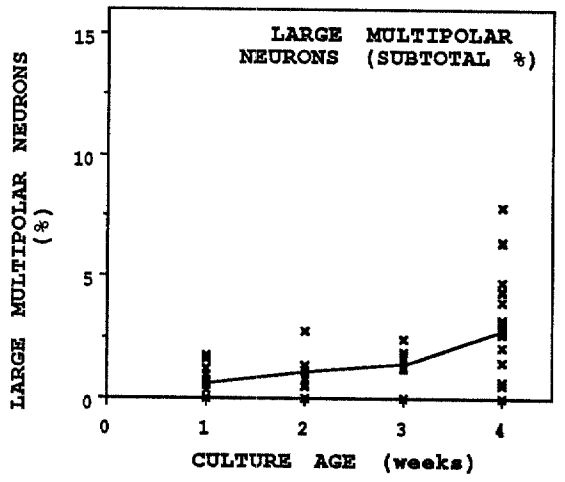
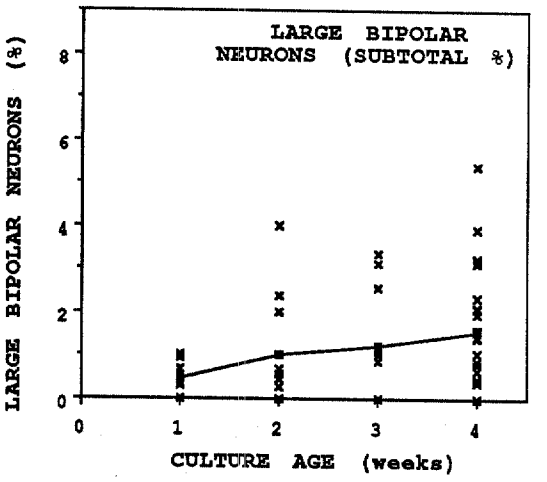

CONDITIONING
AREA
CLUSTER NEURONS (%)
100
80
60
40
20
0
0
1
2
3
4
CULTURE AGE (weeks)

Figure 65. Scatterplots of the subtotal percentages of the multipolar and bipolar neurons present on the recording island cultures as a function of culture age (subtotal percentages calculated excluding round and cluster cells from the total neurons, AGE file data). Lines connect mean values for each age group. *A, B,* The mean percentages of large multipolar and bipolar neurons increased between weeks 1 and 4. This increase was slightly more apparent in the subtotal percentages than in those plots which included the round and cluster cells in the neuronal count (contrast Fig. 65a with Fig. 62a). *C,* The average percentage of small multipolar neurons remained relatively constant. *D,* The mean percentage of small bipolar decreased slightly by the fourth week in culture.

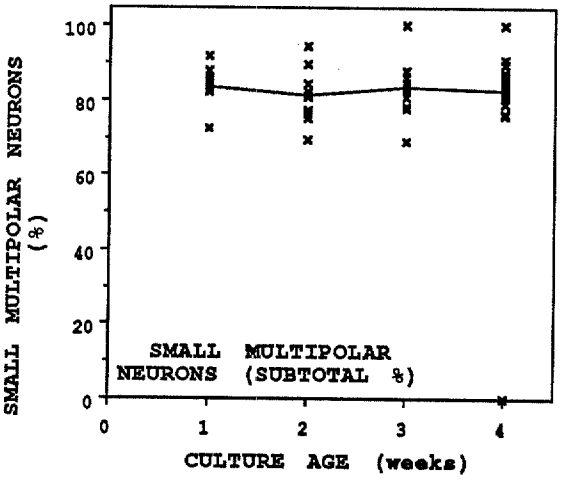
A.


LARGE MULTIPOLAR NEURONS (SUBTOTAL %)
LARGE MULTIPOLAR NEURONS (%)
CULTURE AGE (weeks)
0
5
10
15
1
2
3
4

B.


LARGE BIPOLAR NEURONS (SUBTOTAL %)
LARGE BIPOLAR NEURONS (%)
CULTURE AGE (weeks)
0
2
4
6
8
1
2
3
4

C.


SMALL MULTIPOLAR NEURONS (SUBTOTAL %)
SMALL MULTIPOLAR NEURONS (%)
CULTURE AGE (weeks)
0
20
40
60
80
100
1
2
3
4

D.

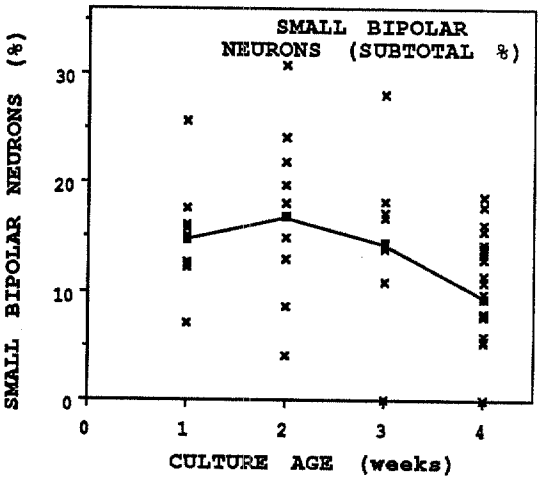
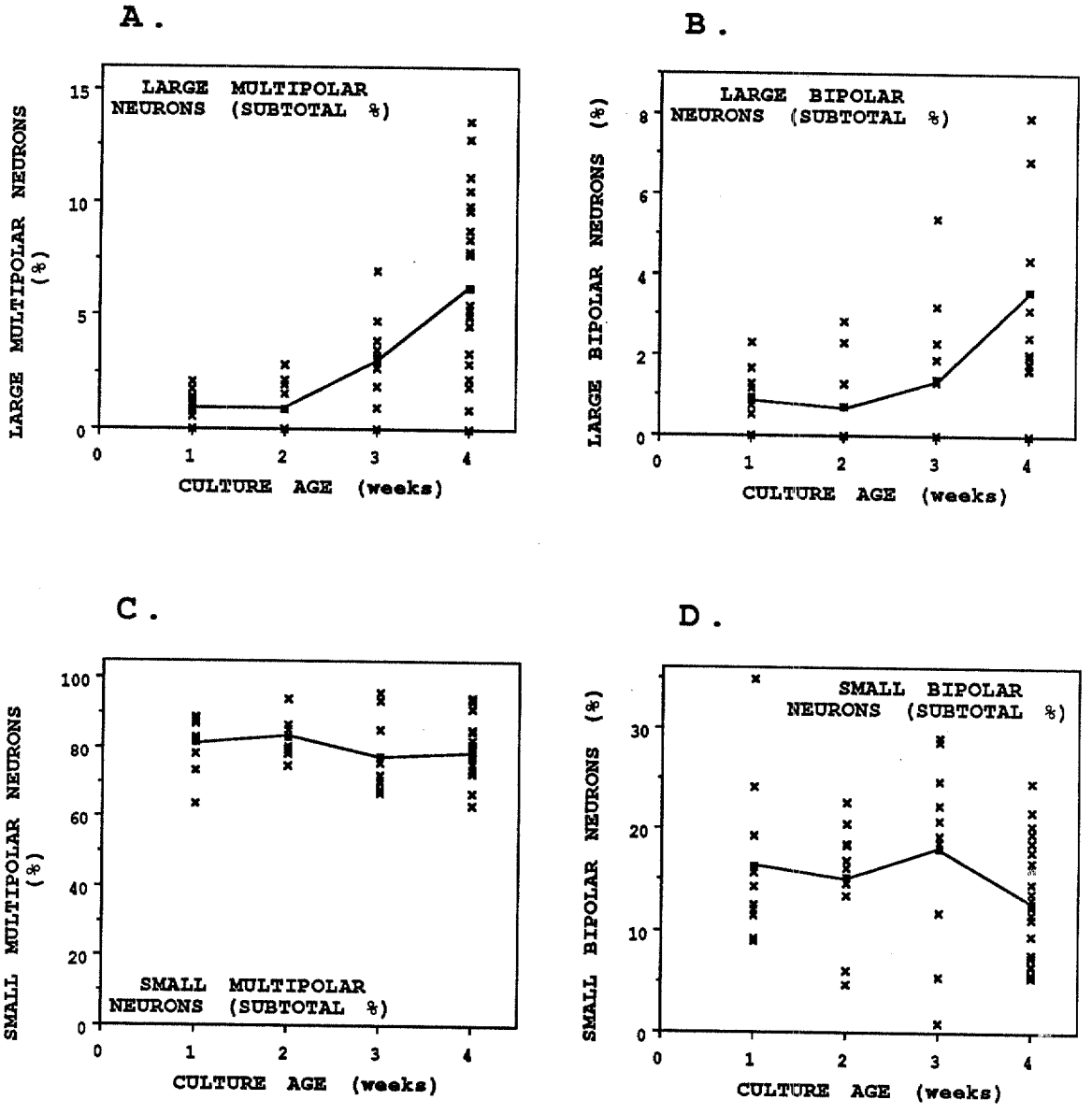

SMALL BIPOLAR NEURONS (SUBTOTAL %)
SMALL BIPOLAR NEURONS (%)
CULTURE AGE (weeks)
0
10
20
30
1
2
3
4

Figure 66. Subtotal percentages of the multipolar and bipolar neurons on the conditioning areas versus culture age. Lines connect mean values for each age group. *A, B,* The mean percentages of both the large multipolar and large bipolar neurons increased substantially during the 4-week culture period. It should be noted that the mean percentages of these cell types on the conditioning regions were more than double those calculated for recording island cultures (see Fig. 65a). *C, D,* The mean percentages of both small multipolar and small bipolar neurons remained relatively constant.


A.
LARGE MULTIPOLAR NEURONS (SUBTOTAL %)
LARGE MULTIPOLAR NEURONS (%)
CULTURE AGE (weeks)
B.
LARGE BIPOLAR NEURONS (SUBTOTAL %)
LARGE BIPOLAR NEURONS (%)
CULTURE AGE (weeks)
C.
SMALL MULTIPOLAR NEURONS (SUBTOTAL %)
SMALL MULTIPOLAR NEURONS (%)
CULTURE AGE (weeks)
D.
SMALL BIPOLAR NEURONS (SUBTOTAL %)
SMALL BIPOLAR NEURONS (%)
CULTURE AGE (weeks)

however, that the trend toward loss of small cells with concurrent increases in the percentages of larger cells resulted from growth of a portion of the smaller neurons during the first 4 weeks in culture.

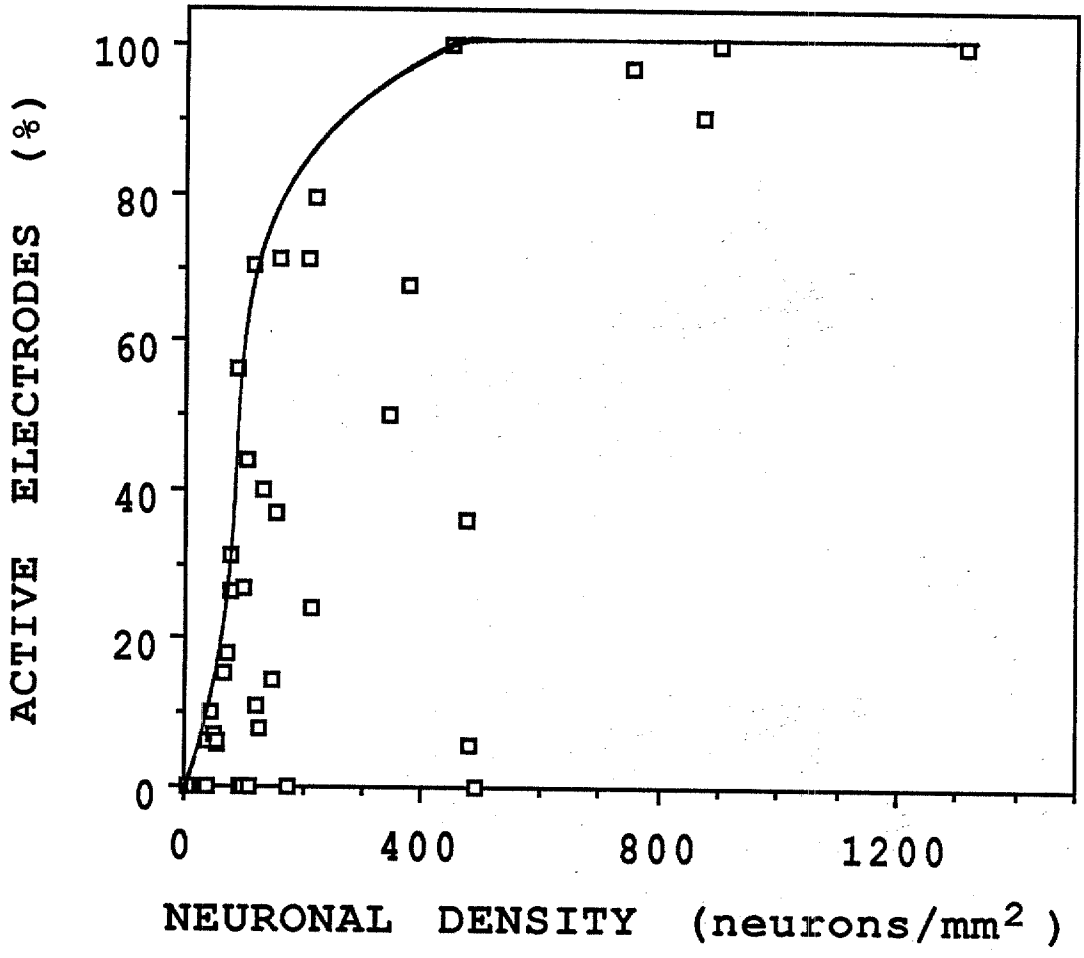
Effects of Morphological Variables on Activity

Activity as a Function of Neuronal Density

The mean percentage of active electrodes was shown to vary as a function the number of cells seeded (Fig. 19), but there was great variability in the percentage of electrodes active on individual MMEP experiments which were seeded with the same number of cells. Since a particular number of cells seeded produced a wide range of neuronal densities at week 4 (Fig. 48), it was hypothesized that levels of electrophysiological activity would vary more directly as a function of neuronal density rather than as a function of the number of cells seeded.

A sigmoidal relationship was revealed by plotting the percentage of active electrodes at week 4 against the calculated neuronal density (Fig. 67) using data from the SEED file. The steepest rise in the percentage of active electrodes occurred at approximately 100 neurons/mm^2, and the percentage of active electrodes continued to increase to reach 100% at a neuronal densities of 300-400/mm^2. The sigmoidal envelope formed represented maximum activity levels. Several MMEPs with densities greater than

Figure 67. Scattergram of the percentage of active electrodes versus neuronal density (SEED file data, assay age, 4 weeks). The curve represents the envelope of observed maximum activity levels. As neuronal density reached 100 neurons/mm^2, the percentage of active electrodes increased, reaching 100% levels at 400-500 neurons/mm^2.Some cultures of higher density failed to exhibit the ideal activity levels predicted by the envelope, indicating that factors other than neuronal density, such as culture health, probably influenced expression of maximum activity.


ACTIVE ELECTRODES (%)
100
80
60
40
20
0
0
400
800
1200
NEURONAL DENSITY (neurons/mm2)

100 neurons/mm^2 did not exhibit the high percentage of active electrodes predicted by the envelope, indicating that factors other than neuronal density (such as culture health) could influence the expression of maximum activity.

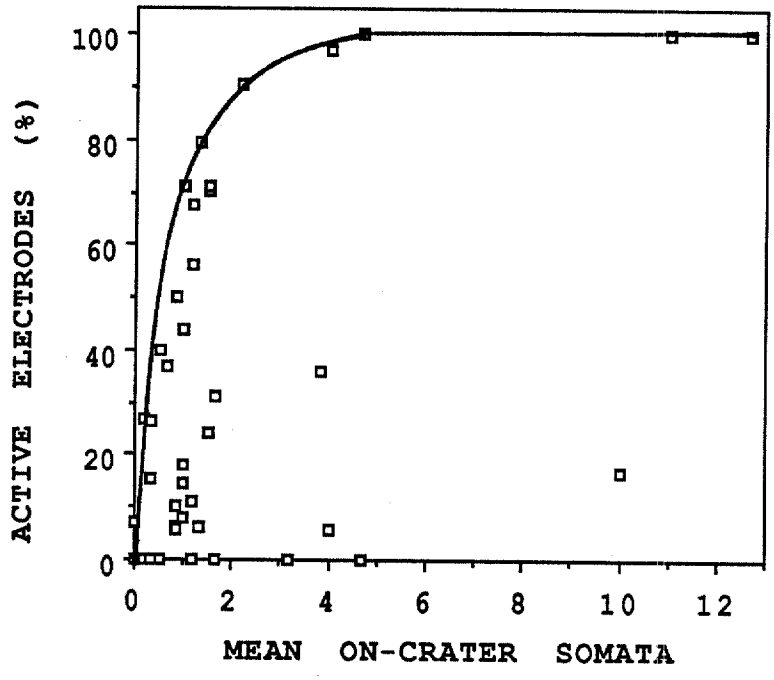
Activity as a Function of Near-Electrode Neuronal Elements

The envelope of the plot of on-crater cell bodies versus neuronal density was linear (Fig. 68*A*). It was not surprising, therefore, that the plot of per cent active electrodes versus mean on-crater somata revealed a sigmoidal relationship similar to that of the graph of per cent active electrodes versus neuronal density (Fig. 68*B*). The steepest rise in active electrodes occurred at approximately 1 neuron/crater, with 100% activity levels achieved with 4-5 neurons/crater. The 4-5 neurons/electrode at which maximal activity levels were achieved was associated with a neuronal density of approximately 500 neurons/mm^2, the neuronal density at which maximal activity was observed (Fig. 67).

The percentage of active electrodes increased as the mean fiber/fascicle count increased (Fig. 69*A*). However, there was considerable activity variability. A sigmoidal relationship was revealed by plotting the mean on-crater fiber/fascicles versus neuronal density (Fig. 69*B*). On-crater fiber/fascicle counts increased dramatically at a neuronal density of approximately 100 neurons/mm^2, the same

Figure 68. *A*, Scattergram of the percentage of active electrodes as a function of the mean number of neuronal cell bodies counted within the 50 X 50 μm crater regions (SEED file data, assay age, 4 weeks). The percentage of active electrodes increased with a mean on-crater neuronal soma value of 1. 100% levels were observed at on-crater cell body values of 4-5. The shape of the envelope curve was very similar to the plot of percent active electrodes versus neuronal density (see Fig. 67). This relationship was explained by the linear function which was exhibited by the plot of on-crater somata as a function of neuronal density (*B*).

A.


ACTIVE ELECTRODES (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
MEAN ON-CRATER SOMATA

B.

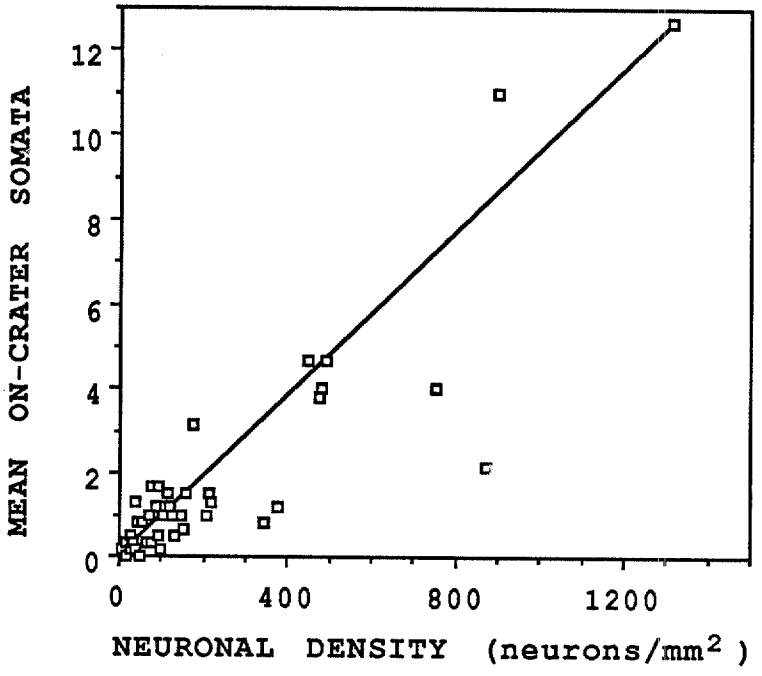
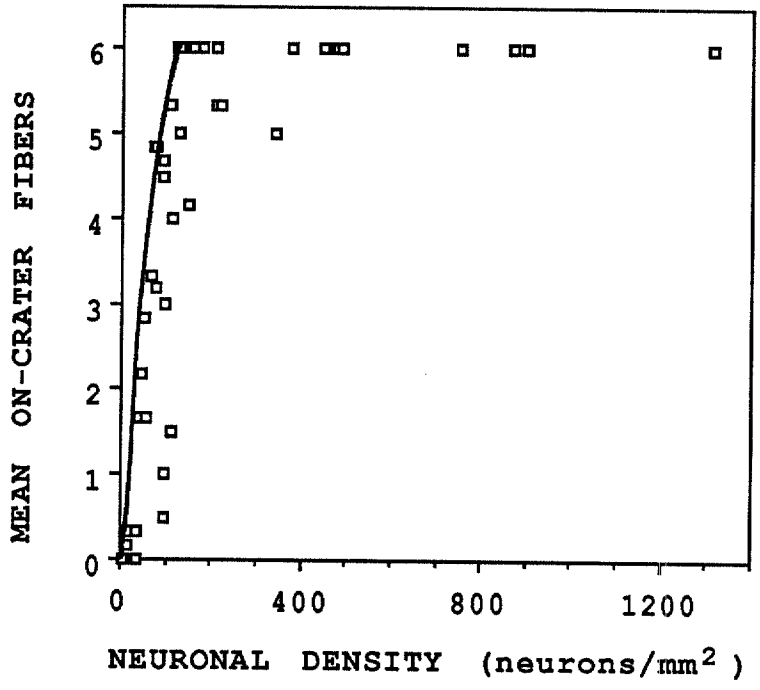
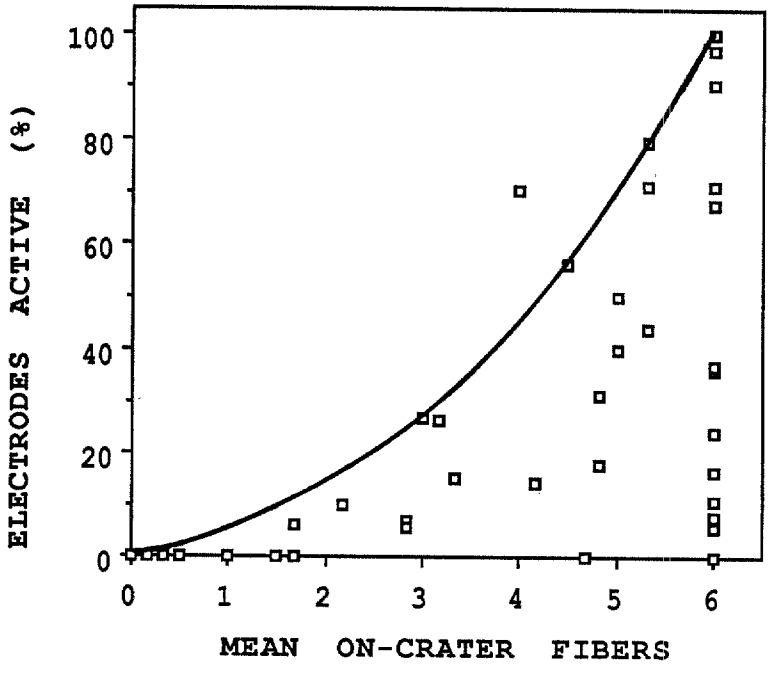

MEAN ON-CRATER SOMATA
12
10
8
6
4
2
0
0
400
800
1200
NEURONAL DENSITY (neurons/mm2)

Figure 69. *A*, Scatterplot plot of the mean number of fiber/fascicles present in the electrode crater regions as a function of neuronal density. The mean on-crater fiber/fascicle counts increased sharply at a neuronal density of approximately 100 neurons/mm^2, which was the same density at which the percentage of active electrodes dramatically increased (see Fig. 67). Unfortunately, neuronal process growth was extensive, and beyond the relatively low density of 100 neurons/mm^2, the mean fiber/fascicle counts reached the set counting limit of 6 fibers/electrode. *B*, Scattergram of the percentage of active electrodes versus mean on-crater fibers revealing a positive correlation between these two variables. However, there was considerable scatter in the data at higher mean fiber values, probably due in part to the previously described inaccuracy of fiber/fascicle counts. Curves represent envelope functions.

A.


MEAN ON-CRATER FIBERS
0
1
2
3
4
5
6
0
400
800
1200
NEURONAL DENSITY (neurons/mm2)

B.


ELECTRODES ACTIVE (%)
0
20
40
60
80
100
0
1
2
3
4
5
6
MEAN ON-CRATER FIBERS

neuronal density at which the percentage of active electrodes rapidly increased. The mean on-crater fiber counts reached the maximum value of 6 at 150 neurons/mm^2. A mean on-crater fiber value of 6 required that each electrode along the central row possess sufficient processes to be assigned the default value of 6. Therefore, beyond 150 neurons/mm^2, the number of fibers on each electrode exceeded that which could be accurately enumerated.

Correlation of Specific Activity Units with Particular Network Components: Cell-Electrode Coupling

Attempts to determine the "cell-of-origin" of a unit of activity monitored from a particular electrode were not often successful. On rare occasions, a high magnitude action potential of sufficient duration to be classified as a somal spike was observed and correlated with a single near-electrode neuron. More frequently, however, distinct somal spikes occurred on electrodes surrounded by several neurons or a neuronal cluster. Correlation of an axonal spike with the appropriate neuronal process was even more difficult. In fact, an electrode exhibiting a single distinguishable unit of activity was never observed which possessed a single fiber crossing it; morphological analysis always revealed at least 2-3 processes or a small cable at the electrode site. *Camera lucida* drawings of

neurites crossing conductors were attempted (Hightower et al., 1984), but tight process fasciculation prohibited unequivocal fiber tracing.

Discussion

Culture Variability

Even those MMEP cultures from the same culture dates, with controlled seeding densities or culture age exhibited marked variability. This variability was discussed in Chapter 3 as it related to differences in electrophysiological activity. Microscopic analyses of these same "culture variable controlled" cultures revealed substantial variation in morphological characteristics, including neuronal density and the percentages of the neuronal subtypes. Certain trends became apparent from plots of the data, but data scatter made quantitative statistical analysis impossible.

Culture Variable Effects on Neuronal Density

Neuronal density increased with increased cell seedings, up to a seeding of 6×10^5 cells. Thereafter, additional plated cells did not result in increases in the mean neuronal density observed at 4 weeks. Because recording island neuronal density remained relatively constant between weeks 1 and 4, the failure of additional cells to increase neuronal density must have resulted from

either a limitation of initial neuronal adhesion or a competition for survival during the first week.

Culture Variable Effects on Aggregation

As neuronal density increased, small and large cluster densities increased linearly, and recording island and conditioning region cultures of similar neuronal densities exhibited comparable cluster densities. Cursory observations of very young (1-d-old) cultures indicated that neurons were initially plated as single cells or groups of 2-3 cells. Aggregation became apparent shortly thereafter. Investigations of small and large cluster densities as a function of age indicated that aggregation was essentially complete by week 1; cluster densities did not increase between weeks 1 and 4. In fact, large cluster densities actually decreased in both the recording island and in the conditioning regions.

The decline in large cluster density might suggest a disaggregation of the neurons during development, but this hypothesis is in conflict with qualitative observation of cultures. Alternatively, the death of neurons within large aggregates might resulting in decreases in the number of large clusters. A third explanation for a large cluster density decline could involve the migration and coalescence of clusters, resulting in reduction in large cluster density without an actual reduction in cell aggregation.

Culture Variable Effects on Neuronal Type Percentages

The majority of the neuronal cell types present in spinal cord culture were round and cluster cells. The percentage of neurons which possessed classical differentiated neuronal morphologies was always low. As neuronal density increased, the percentage of cluster cells also increased, and the percentages of the "morphologically differentiated" neurons decreased dramatically.

The predominance of the round and cluster neurons masked the smaller changes in percentages of multipolar and bipolar neurons with increases in neuronal density. Therefore, subtotal percentages of the differentiated cell types were calculated, excluding round and cluster cells. Plotting subtotal percentages versus neuronal density revealed that the percentages of large neurons underwent specific decline while the small neurons were spared. It is possible that large neurons served as aggregation centers for neuronal clustering, were not visible within large clusters, and were thus not counted in high density cultures. Alternatively, the larger cells may have been eliminated because they were unable to compete for limited quantities of required nutritive or trophic factors.

The percentages of large multipolar and bipolar neurons increased slightly between weeks 1 and 4, whereas the percentages of the smaller neurons remained constant or declined slightly. This tendency was even more pronounced

when subtotal percentages of the morphologically differentiated neuronal subtypes were plotted against age. Although statistically inconclusive because of extreme culture variability, these data supported the hypothesis that a population of the smaller neurons grew in culture between weeks 1 and 4.

Comparison of Recording Islands and Conditioning Areas

The conditioning area morphological features were generally similar to those of recording island cultures. One notable exception was the sharp decline in conditioning area neuronal density between weeks 1 and 4, while the recording island population remained relatively stable. The mechanism for this difference between the two regions was unclear. As a result, conditioning areas observed at week 4 generally exhibited lower neuronal densities than the corresponding recording islands. A lower percentage of cluster cells and a higher percentage of morphologically differentiated cell types were generally present on conditioning areas. However, when subtotal percentages of morphologically differentiated neurons were compared, excluding round and cluster cells, the proportions of cell types present on the conditioning and recording areas were similar. Thus, the neuronal populations between the two culture regions appeared to differ only by the presence of a larger number of cluster neurons on the recording island.

These quantitative comparisons between recording island and conditioning area cultures supported previous qualitative observations. Recording island cultures had appeared to be of generally higher neuronal density, with greater cell aggregation. In addition, neurons in the recording island often appeared less healthy, with larger quantities of extracellular debris, rougher cell membranes, and more granular somata. Locating classic examples of large multipolar and bipolar neurons on recording islands which were suitable for photography was sometimes difficult. Systematic morphological analysis confirmed these initial impressions.

Electrophysiological/Morphological Correlations

The percentage of active electrodes increased as a sigmoidal function of neuronal density, with the steepest rise in activity occurring at a neuronal density of 100 neurons/mm^2. The critical density of 100 neurons/mm^2 for the monitoring of activity from a substantial number of electrode sites probably reflected the statistical probability that an active neuron or process would be positioned near one of the fixed electrodes. Vigorous, coordinated activity was monitored from MMEP mini-cultures of lower density, indicating that neurons in low-density culture could form functional networks. However, in such experiments, the number of active electrodes was always low. Thus, the activity of cultures with densities of less

than 100 neurons/mm^2 could not be effectively monitored with the MMEP technique.

The percentages of active electrodes also varied as a function of the average number of neuronal somata and processes near electrodes. Unfortunately, fiber counts suffered from both the poor resolution of individual processes in cables at the light microscopic level and the physical limitations on accurate enumeration of fibers in dense process nets. Consequently, accurate fiber/fascicle enumeration in cultures with neuronal densities of >150 neurons/mm^2 was impossible.

Attempts to correlate a specific unit of electrophysiological activity with its morphological source were rarely successful. Occasionally, a high-amplitude, long-duration somal spike could be unequivocally paired with a single neuronal cell body in the vicinity of the active electrode. However, an axonal spike was never monitored from a conductor with only one fiber in its vicinity. Tight process fasciculation prohibited the tracing over long distances of the fibers crossing electrodes.

CHAPTER 7

DISCUSSION, ANALYSIS, AND FUTURE EXPERIMENTS

Specific Aims

The specific aims of this project were: 1. to modify existing MMEP preparation protocols to produce optimally functional ITO MMEPS and to develop techniques for the production and maintenance of spinal cord monolayer mini-cultures; 2. to characterize the spontaneous electrical activity of these small neuronal networks; 3. to apply inhibitory and disinhibitory pharmacological agents to the mini-cultures in order to determine if their responses were "organotypic"; 4. to develop a histological method for the staining of neurons; 5. to perform initial morphological analyses of the mini-culture neuronal populations at the light microscopic level; and 6. to determine correlations between certain electrophysiological and morphological variables.

Data Summary

MMEP Preparation and Culture

The original MMEP insulation protocol which was developed for gold MMEPs was modified for use with the ITO

MMEPs. Optical deterioration of the polysiloxane insulation layer was eliminated, and a method for gold plating was developed to produce stable impedances of the ITO conductors for optimal signal monitoring.

Mini-cultures centered over the recording matrix were produced by the selective flaming of the insulation through a mask. Spinal cord mini-cultures with sharp growth boundaries centered over the recording matrix were thus produced. The production of mini-cultures represented a further level of simplification of cultured monolayer networks, and growing neuronal mini-networks required only minor modifications of the established culture preparation and maintenance procedure. Recording island cultures did require the co-culturing of larger "conditioning cultures" to condition the medium and to serve as "cell sinks" for reduction of recording island neuronal density.

Electrophysiological Analysis

Mini-cultures exhibited spontaneous activity which was similar to that monitored from larger, spatially unrestricted cultures. Activity from each MMEP was characterized by the percentage of functional electrodes active, number of distinguishable active units, and maximum signal amplitudes. Development of activity as a function of cell seeding concentration and culture age was assessed.

Since activity was monitored from 2 electrodes simultaneously, assessment of activity coordination was also performed. All activity was generally coordinated, but some exceptions were encountered. Of particular note were the alternating patterns of activity observed during a few experiments, which indicated that the mini-cultures were capable of complex network formation.

Pharmacology

Disinhibitory Agents

Burst frequency effects. Strychnine and bicuculline, antagonists of inhibitory neurotransmitters known to be present in the spinal cord *in situ*, were applied to spinal cord mini-cultures. Drug responses were complex, so burst analysis rather than the analysis of individual spikes was performed. Addition of either disinhibitor generally resulted in increased burst frequency, and dual application of both drugs usually produced a further burst frequency increases.

In a few experiments, MMEP cultures exhibiting initially high burst frequencies prior drug application displayed decreased frequencies following disinhibition. The reason for this atypical reaction was not clear. However, the coupled planar rotator model (Kowalski et al.,1988) predicts that various oscillator circuits within a network will entrain to a mean frequency upon

disinhibition. The results of these disinhibition studies represent the first experimental support for this theoretical prediction.

Burst regularity. In addition to the generally observed increases in burst frequency, each disinhibitor also tended to produce increases in burst regularity, especially after dual drug application. Disinhibition-induced regularity was independent of the effects of increased frequency. The profiles of the individual burst envelopes produced by the chart recorder also became more stereotyped.

Epileptiform activity. The mini-culture reaction to application of disinhibitory agents closely paralleled EEG tracings during epileptic seizures (Kandel and Schwartz, 1985). Disinhibitory drugs applied to certain brain areas *in situ* and to tissue slices have been shown to produce epileptic seizures (Kandel et al., 1961, Ayala et al., 1973). These agents have also been shown to produce heightened activity levels and regular burst patterns in brain slices, in spinal cord preparations, and even in larger dissociated spinal cord cultures (Crain, 1976; Grillner and Wallen, 1980; Alger, 1984; Cohen and Harris-Warrick, 1984). The fact that spinal cord mini-cultures exhibit the same type of reaction to disinhibition indicates that epileptiform activity is not limited to specific epileptogenic tissues. It also does not require

large neuronal networks. Thus,epileptiform activity may be a characteristic of all neural networks experiencing reduced inhibitory input

Traub and coworkers (1985), employing data from hippocampal slice preparations, postulated that only a few thousand neurons with proper interconnections would be required to produce epileptiform activity following disinhibition. The three components considered essential for epileptic activity synchronization in his model are: 1. the presence of intrinsic bursting; 2. a blockade of synaptic inhibition; and 3. the presence of excitatory chemical synaptic interconnections. All of these criteria are met by spinal cord monolayer networks after disinhibition, and results with these cultures substantiate the hypothesis that epileptiform activity is a general network phenomenon. In addition, the observation of such activity produced by adhesion island mini-cultures extends Traub's theoretical work, indicating that epileptogenesis requires a neuronal population of no more than a few hundred cells rather than the thousands proposed.

Inhibitory Agents

The inhibitory transmitters glycine and GABA were generally applied after disinhibitory agents and produced initial cessation of all activity with recovery of bursting at attenuated frequency within 1-2 min. The reactions of mini-cultures to both disinhibitory and inhibitory drugs

were similar to those observed in CNS tissue *in situ* and in isolated preparations (Homma and Rovianen, 1978; Lal, 1980; Cooper et al., 1982; Alger, 1984). Thus, at least in response to these agents, small populations of neurons exhibited *organotypic* behavior.

Histological Processing and Morphological Analysis

Bodian-Nissl Staining

A combination of the Bodian and Nissl procedures was adapted for use with monolayer cultures grown on MMEPs. The Bodian-Nissl method reliably stained neurons, highlighting the somata and staining the neuronal fibers darkly. This stain allowed the first morphological analysis of the neuronal components of the spinal cord mini-cultures.

Morphological Analysis

Mini-culture neurons were counted, percentages of different neuronal types were calculated, crater and inter-crater neuronal components were compared, and estimations of cell aggregation were made. Differences between recording island and conditioning area cultures were also assessed. These morphological characteristics were correlated with culture variables and with monitored activity. Because of the morphological complexity of the mini-cultures, anticipated correlation of a particular unit

of activity with the neuron or fiber producing it was accomplished only very rarely.

Impact of Research, Remaining Problems, and Future Experiments

Cell Culture

Culture Variability

Although spinal cord mini-cultures on MMEPs are now routinely produced, several improvements are necessary. The foremost problem encountered with mini-cultures was their wide range of variability in activity and morphological development. This variability was apparent even among cultures of the same culture date which were seeded with the same cell concentration from the same cell pool, were maintained with the same lots of medium and other chemicals, and were assayed on the same date. Activity levels of such "controlled" cultures sometimes ranged from 0-100% electrodes active, and morphological analysis revealed that neuronal densities varied from <100 to >1000 neurons/mm^2.

Variability as a function of culturing procedures. It is possible that a majority of mini-cultures contain similar network components with very similar interconnections which are determined by specific neuron-neuron interactions. Variability might then occur primarily as a result of slight differences in culture

technique. First, the flaming procedure, although effective in producing a hydrophilic surface, remains relatively primitive. It is very dependent on operator performance, and subtle differences in timing, flame temperature, and flame positioning could produce markedly different adhesive surfaces.

Neurons are also visibly stressed by the small changes in osmolarity, pH fluctuations, and hydrodynamic disturbances produced during routine medium changes. Further improvements in the technical consistency will be difficult to achieve, and variability arising from subtle differences in mini-culture production and maintenance may persist.

Variability as a function of dissociation. It is also likely that dissociation and random seeding of neurons may introduce some random network formation, despite a tendency toward specific interconnections. Subpopulations of neurons might survive or die based upon their ability to form necessary interconnections within an appropriate period of time. If variable development occurs as a result of dissociation and random seeding, even spinal cord cultures produced by a rigorously controlled protocol might be composed of different neuronal populations. They would thus be expected to exhibit different electrophysiological behavior and responsiveness to pharmacological agents. Treating these different mini-cultures as one experimental

group on the basis of their similar production and maintenance characteristics, as was done in this study, would then result in a data set with extreme variability. Development of a scheme for the classification of mini-cultures into different groups based on morphological and electrophysiological criteria is therefore essential.

Selection of mini-cultures as a means to reduce experimental variability. Since it has not yet been possible to adequately control mini-culture variability by simply controlling preparation and maintenance procedures, selection of MMEPs with particular features for use in certain experiments may be necessary. Cursory microscopic observation would allow the classification of cultures as "high," "medium," or "low" neuronal density, permitting subsequent elimination and routing of the cultures to different experiments. Likewise, a brief activity assay might allow electrophysiological categorization and selection. For example, a culture with high neuronal density exhibiting vigorous bursting activity would not be chosen for development of histological methods, montage fabrication, or cell-electrode correlations. However, such a culture would be ideal for preliminary investigation of the effects of a new pharmacological agent.

More sophisticated mini-culture selection. The limited number of MMEPs and the difficulty of culture has previously prohibited the production of large numbers of

mini-cultures. However, the new capability for on-site MMEP fabrication and the development of routine culturing methods has made the batch-preparation of mini-cultures feasible. Thus, in addition to the elimination of obviously unhealthy or otherwise defective mini-cultures and grouping of the remainder into broad utilization categories, it may be possible to establish a rapid battery of electrophysiological and pharmacological tests to select cultures for further experiments. For example, mini-culture reaction to drug Y might be tested on a group of cultures with moderate neuronal density which exhibited tonic single spike and bursting activity and which displayed high responsiveness to strychnine with a lesser response to bicuculline. In this manner, sophisticated studies could be performed with very similar MMEP cultures, possibly eliminating the problem of extreme data variability.

Neuronal Aggregation

Another problem encountered with mini-cultures was the clustering of neurons in aggregates and the grouping of neuronal processes into tight fascicles. The spinal cord cells were seeded as a primarily single-cell suspension, and neurons initially adhered to the plate separately. However, aggregation began within 24 h, and large clusters were present by the end of the first week in culture.

Particularly troublesome was the aggregation of cells at the periphery of the recording island. This effect probably resulted from random migration of glial cells to the recording island border, with cell stalling via prevention of further outward progress by the hydrophobic insulation surface. Neurons appeared to follow glial movement, with a subsequent cell "pile-up" at the periphery.

Substrate modification to reduce aggregation. Cell clustering and process fasciculation appear to be inherent properties of neurons observed both *in situ* and *in vitro* (Scheibel and Scheibel, 1970; Crain and Bornstein, 1972; Ransom et al., 1977; Seeds and Haffke, 1978; Akagawa and Barnstable, 1986; Anderson, 1988). Much of this behavior may result from the interactions of cell surface molecules. Cell-cell interactions may successfully compete with cell-surface interactions to produce aggregation. Therefore, any technique which raises the cellular affinity of the culture surface might minimize clustering. Such techniques could include chemical treatments of the culture surface to make it maximally hydrophilic. Alternatively, antibodies to cell-cell interaction molecules, adhered to the culture surface, might provide sufficient competition for neuron-neuron binding to reduce aggregation. One such promising molecule is anti-N-CAM (neuronal cell adhesion molecule, Edelman, 1984). Addition of such an agent may reduce

process fasciculation as well as somal aggregation (Mehrke et al., 1987).

Selective removal of cluster cells by laser irradiation. Total elimination of aggregation may not soon be feasible. However, the removal of some large neuronal clusters is possible. The UV laser which is used to deinsulate the MMEP conductor tips can be used to kill individual targeted neurons (Higgins et al., 1980; Gross et al., 1983). Thus, specific elimination of cells may be used to tailor the neural network with minimal damage to the other components.

Minimization of neuronal density to reduce clustering. Reduction of mini-culture neuronal density may also retard aggregation as well as facilitate mini-culture morphological analysis. However, the neurons of very low-density cultures often appeared unhealthy, and activity was frequently sparse and weak. Low-density cultures did not form an adequate glial carpet for stabilization of the overlying neuronal network. One solution to this problem would be the culturing of an astrocyte carpet (McCarthy and de Vellis, 1980) onto MMEP plates, followed by subsequent seeding of a low concentration of spinal cord cells onto the established carpet. Thus, cultures of very low neuronal density would have proper glial support for survival.

Limits to the reduction of neuronal density. Producing healthy spinal cord cultures of consistently low neuronal density may be feasible. However, investigation of the percentage of active electrodes as a function of neuronal density indicated that a density of at least 100 neurons/mm^2 was required in order to monitor activity from at least 30% of the functional electrodes. The relationship between activity and neuronal density appeared to be largely a function of the probability of monitoring a randomly positioned active unit at a fixed electrode site. Thus, a drastic reduction of mini-culture neuronal density may not allow effective MMEP monitoring of the resulting network.

Neuronal density reduction by non-random adhesion of neurons near electrodes. Approaches which may allow further decreases in mini-culture neuronal density without sacrificing activity levels would involve the selective growth of neurons on MMEPs in positions near the electrode sites. In fact, several experimental procedures are currently being considered by the Center for Network Neuroscience (CNNS) as prospective graduate student research projects. First, individual cells might be placed by micropipette onto the crater regions of a mini-culture. Neuronal survival under these conditions might require a pre-established a glial carpet. Another method to achieve neuronal placement near electrodes would involve

application of an electric field to the conductors to facilitate selective migration of neurons toward electrodes. The feasibility of this procedure is currently being tested, and negatively charged latex beads have been shown to cluster onto positively charged electrodes.

Neuronal Population Heterogeneity

Culturing of restricted spinal cord regions. The dissociation and culture of the whole spinal cord results in a very heterogeneous population of neurons. In addition, the dorsal root ganglia (DRGs) were difficult to remove during dissection, so the cultures are more properly referred to as spinal cord/DRG cultures. Several different preparation protocols might limit the variety of neurons in culture. First, DRGs should be removed; they could be cultured separately for the study of the sensory neurons in isolation. Secondly, it is possible to divide the cord into dorsal and ventral halves (Guthrie et al., 1987). Culturing the dorsal cord could provide a population of exclusively interneurons, whereas culturing of the ventral horn might produce networks of interneurons and motoneurons. It is also possible to prepare a fraction of spinal cord cells enriched with large motoneurons by gradient centrifugation (Berg and Fischbach, 1978). Unfortunately, survival of these isolated preparations, particularly of ventral horn cells, has not been good. However, modification of the culturing maintenance

procedures might allow study of limited neuronal populations of the spinal cord.

Culture simplification with specific toxins. Removal of neuronal populations can be achieved through the use of selective toxins. This research is in its infancy, but it has been determined that 6-hydroxydopamine kills dopaminergic neurons (Cooper et al., 1978) and that kainic acid in low concentrations can be used to remove cells with large numbers of glutamate receptors (Garthwaite et al., 1986; Rimvall et al., 1987). New drugs, or present drugs in proper dosages may soon be used for mini-culture network simplification.

Culturing of other brain regions

It is also desirable to compare the activity generated by spinal cord cultures with those of other brain regions. MMEP analysis of olfactory bulb cultures is already underway (Fracek et al., 1987). The cerebellum is another tissue being considered for culture on MMEPs because it is a large structure which can be easily dissected from neonatal mice. Culture procedures have already been developed for it (Aizenman and de Vellis, 1987), and a culture of almost 100% neurons (no glia) can be established. With the proper method, the cultured neuronal population consists almost entirely of one cell type, the

granule cell. Such a homogeneous population could be useful for neural modelling studies.

Electrophysiological Analysis and Pharmacological Testing

Data Collection and Measurement

Original electrophysiological analysis. In this study, features of the spikes generated by the mini-cultures were observed, and ranges of spike amplitude and duration were determined. Putative somal and axonal spikes were identified, the number of active units at each conductor site was estimated, and maximum signal amplitudes monitored from each electrode were noted. However, it was clear very early in the project that detailed spike analysis was too complex to be achieved manually. Thus, the investigation of monitored activity, particularly after pharmacological manipulation, relied on burst analysis.

The first burst parameters quantitatively studied were burst frequencies, periods, durations, and interburst intervals. These measurements for prolonged experiments were extremely time-consuming, and difficulties in accurately defining burst onsets and terminations were encountered frequently. To save chart paper, optimum speeds for determining burst parameters were not always selected. Nevertheless, these measurements allowed the first systematic characterization of electrophysiological

activity of MMEP mini-cultures and provided the quantitative data regarding their response to pharmacological agents.

Necessity for computer-assisted activity analysis. All electrophysiological analyses performed for this project were based on manual measurements of chart record data. Because each MMEP experiment provides an enormous quantity of electrophysiological data, more and better measurement of activity characteristics are required. This necessitates development of computer-assisted data acquisition and manipulation. Simple burst measurements would be provided quickly with a proper computer program, and more sophisticated measurements, such as burst areas, which are difficult or impossible to determine manually for a large quantities of data, could also be produced. Other laboratories have invested in data analysis systems and have begun to develop software (McGill and Dorfman, 1984; Schmidt, 1984; Greer and Field, 1986; Vibert et al., 1987). A computer could also be used for analysis of bursting patterns. Finally, computer programs would be able to differentiate individual spikes, allowing the first detailed analysis of spike patterns.

Plans for computer analysis. The CNNS presently possesses a Masscomp 5770 mainframe computer system dedicated to the uptake, storage, and analysis of electrophysiological data. Development of algorithms for

spike and burst analysis has been difficult, but all of the necessary programs are expected to be on-line shortly (Raymond et al., 1987). Computer assisted data analysis will permit immediate increases in experiment sophistication and complexity while allowing more research time to be spent in the design, and performance of experiments rather than in tedious manual measurement tasks.

Limitations of original electrophysiology equipment. The electrophysiological data presented in this dissertation was collected using a manual 2-probe data acquisition system. Only 2 electrodes could be monitored at one time, and assay time at each site was limited. Long-term monitoring of the activity of a single electrode necessitated the sacrifice of data from other sites. Coordination was assessed by comparing the activity of all electrodes against that of one conductor which was exhibiting vigorous activity. While this procedure was effective for general coordination studies, it was insufficient for the critical analysis of burst onsets which are necessary for determination of some network properties. When this project was initiated, data acquisition was limited to continuous chart records, which revealed the burst patterns, and pictures of oscilloscope tracings of representative and exceptional spike

characteristics. Due to their high cost, oscilloscope photographs were taken sparingly.

Equipment improvements for data acquisition. The design and acquisition of a new probe-amplifier system (Bak, Inc., Rockville, MD) now allows monitoring of 32 electrodes simultaneously. The experimenter can determine the location of active electrodes quickly with the aid of an activity light grid (Spectrum Scientific, Dallas, TX). Data from electrodes exhibiting the best activity can then be stored on the recently acquired 14-channel tape recorder (Racal Store 14DS, Sarasota, FL). These taped records may be replayed through an oscilloscope (for publication-quality photographs) or relayed to the computer (for spike or burst analysis).

Limitations of initial pharmacological experiments. In this study, all drugs were applied to the medium bath. Because no perfusion system was available and the cultures exhibited extreme sensitivity to rapid medium replacement, the drugs could not be removed. Thus, experiments with multiple drugs produced a complex "stacking" effect which stressed cultures and limited the number of agents which could be applied. The open chamber system also allowed evaporation of the medium which could not be corrected without changing activity patterns. This limited the monitoring time for each experiment to approximately 2-4 h.

Development of a new chamber. The CNNS has now developed a closed MMEP chamber designed for long-term monitoring of mini-cultures. It has a perfusion system for the continuous replenishment of conditioned medium and the delivery of pharmacological agents. Thus, maintenance of a controlled extracellular environment for prolonged periods is now possible, as is the design of complex studies with multiple drug applications and wash-outs between trials. Preliminary studies are very promising, indicating that a MMEP culture may be maintained in the chamber and monitored continuously for over a week.

Expanded pharmacological testing. Implementation of an extensive pharmacological protocol is essential, but drugs must be selected carefully based on pharmacological specificity. For example, many agents with primary effects on one neurotransmitter receptor system have been shown to directly affect another receptor subclass or to alter general cellular metabolism (Mayer and Westbrook, 1987). Data obtained with such agents must be interpreted with caution. In addition, bath application of drugs, either into an open chamber or through a closed-chamber perfusion system, can result in indirect as well as direct effects on the activity of a given cell.

Disinhibition as a means to decrease activity variability. Despite possible variations in inhibitory circuitry, the application of both GABA and glycine

antagonists could become a standard procedure for maximization of burst regularity. Dual disinhibition could be used to produce a regular activity pattern which is similar in all cultures, regardless of the form of previous spontaneous activity. This regular pattern of bursting could then be used as a base "network state" from which other experiments, such as further pharmacological testing or the investigation of stimulation effects, could be performed. In fact, Dingledine and his colleagues (1986) have already applied bicuculline or picrotoxin to the extracellular bath of hippocampal slices to reduce GABA inhibition. They then tested the effects of other pharmacological agents on the disinhibited system.

Histology and Morphological Analysis

Cell Typing of Bodian-Stained Cultures

The development of the Bodian/Nissl stain and its application to MMEP cultures allowed the distinguishing of all of the neurons present in the mini-networks and permitted morphological analysis at the light microscopic level. However, this initial analysis fell very short of the goal of complete morphological characterization of mini-cultures. For example, a set of typing criteria for neurons was developed from available literature (Rexed, 1964; Boehme, 1968; Matsushita, 1970; McClung and Castro, 1976; Wiksten, 1979; Brichta and Grant, 1985; Kato et al.,

1985) which specified two identifiable types of neurons, multipolar and bipolar, in two size ranges, small and large. Other neurons, which tended to be small and round, with no or only one discernable process, were assigned to default categories and called round cells if they were separate and cluster cells if they were present in aggregates. Unfortunately, round and cluster cells represented the vast majority of neurons present in the mini-cultures, sometimes making up 95% or more of the neuronal population.

Histologists and anatomists working with spinal cord sections rely on cell position as well as neuronal morphology and staining characteristics to assist them in making cell identifications. Numerous morphological and morphometric studies on sections of various spinal cord regions have been performed (Aitken and Bridger, 1961; Rexed, 1964; Boehme, 1968; Gwyn and Waldron, 1968; Matsushita, 1970; McClung and Castro, 1976; Wiksten, 1979; Brichta and Grant, 1985). Unfortunately, such studies are not immediately applicable to spinal cord cultures because dissociation eliminates positional information. For example, the presence of large numbers of small, round neurons has been described for different spinal cord regions *in situ*, (Rexed, 1964), but such cells in mixed spinal cord culture which originated from the substantia

gelatinosa could not be distinguished from similar cells of the ventral horn.

The largest cells of the spinal cord are alpha motoneurons (Aitken and Bridger, 1961; Rexed, 1964; Berg and Fischbach, 1978), and the largest multipolar cells in culture are therefore most likely motoneurons. Likewise, large, round, unipolar neuronal profiles which are derived from dorsal root ganglia are sometimes seen in culture (Baker et al., 1984). However, bipolar, medium and small multipolar neurons, and round cells may be found throughout the spinal cord, and simple morphological classification is not sufficient to determine probable cell origin or functional significance.

Classification of Neurons by Transmitter Production

It is imperative to classify neurons in dissociated culture not only by shape and size but also by origin or function. A few basic histochemical methods for neurotransmitter localization are available, such as the histofluorescence technique for catecholamines (Falck et al., 1962) and the acetylcholinesterase stain which labels cells producing the enzyme that degrades acetylcholine (Karnovsky and Roots, 1964). Many laboratories are also categorizing neurons by immunocytochemistry using antibodies to the transmitters themselves or to their associated enzymes (Westman et al., 1984; Madl et al., 1986; Meyer et al., 1986; Newton et al., 1986; Kaduri,

1987; Pourcho and Goebe, 1987; Hepler et al., 1988). Several of these antibodies have become commercially available.

Labelling of specific neuronal populations in culture would have many uses. For example, neurons labelled with antibodies to GABA would be identified a major class of inhibitory neurons. Cells labelled with anti-ChAT (choline acetyltransferase, the acetylcholine synthesis enzyme) would be defined as cholinergic, excitatory elements, and their origin could be narrowed to the areas in the intact spinal cord which contain cholinergic cells. With a battery of such immunocytochemical tests,combined with Bodian/Nissl staining, morphological analysis could be extended with the typing cells not only by size and the number of processes, but also by transmitter specificity.

Classification of Neurons by Cell Surface Markers

Other researchers are studying the cell surface markers of particular neuronal subpopulations with immunocytochemistry (Fields, 1979; Hockfield and McKay, 1985; Akagawa and Barnstable, 1986). This research is still in the early stages and has had less commercial success than neurotransmitter studies. The antigen bound by an antibody that labels a class of neurons is often undefined, so that surface markers are not as directly associated with a particular function as transmitter

markers. However, a series of antibodies to cell surface markers with identified distribution in the spinal cord would be very valuable in the study of dissociated cell culture. Such a series might help to identify the likely origin of certain cells in the cultured network and might thus indirectly indicate a function.

Network Tracing

In addition to the neuronal identification, analysis of interconnections is necessary. Extensive somal aggregation and process fasciculation made tracing of neuronal fibers for long distances impossible. However, modification of culture techniques to lower cell density and to reduce aggregation may partially solve these problems.

Computer-assisted tracing. Tracing neuronal projections of Bodian-stained cultures manually with the *camera lucida* technique provided some data (Hightower et al., 1984). However, it was quickly realized that computer assistance was required for efficient network tracing. Other laboratories have also experienced the need for computer-assisted morphological analysis (Mize, 1983; Street and Mize, 1983; Uylings et al.,1986; Upfold et al., 1987; Villa et al., 1987), and several systems have become commercially available. The acquisition of a neuronal tree reconstruction system (Eutectic Inc., Raleigh, NC) is now being considered. This system provides an interactive

program for tracing of fibers in three dimensions at the light microscopic level, allows for the linking of the process domain files encoded for individual neurons, and permits rotation of traced objects.

Tracing of process domains of selected neurons. The Bodian-Nissl procedure stains all of the neurons and their processes in the culture. Sometimes this results in an inability to unequivocally trace the processes of one component, and methods which label only one or a few selected neurons are desirable. Morphological studies of individual cultured neurons and their processes using the technique of intracellular injection of horseradish peroxidase have now commenced in this laboratory. Such a technique can be used to define the axonal and dendritic projections of a few neurons in mini-culture.

Scanning electron microscopy (SEM) for fiber analysis. SEM techniques can also be applied to mini-cultures to provide a three-dimensional surface view of the network. In some cases, elements in fascicles can be traced on SEM photographs. In addition, the glial carpet is easily distinguished and can be evaluated. It would be difficult to apply SEM to the detailed study of an entire mini-culture, but SEM analysis of cells at the crater sites would be possible. Initial SEM analysis indicated that the glial carpet often covered electrodes, and that neuronal fibers travelling under the carpet were

often responsible for monitored activity. Such data could only be obtained from SEM studies.

Development of a Battery of Histological Procedures

No one histological technique can be used to obtain complete morphological data. Therefore, several laboratories are currently developing combinations of procedures to provide comprehensive morphological analysis (Mize, 1983; Bienz et al., 1986; Wouters, 1987). General network components can be visualized by phase contrast microscopy. Intracellular injections of selected cells allows detailed tracing of dendritic domains. Histochemistry and immunocytochemistry aid in the functional subtyping of neurons, while Bodian-Nissl staining allows identification of all of the neurons in the network. Finally, SEM provides three-dimensional depiction of neurons, fibers, and glia, and can aid in cell-electrode coupling studies.

Conclusions

Although many difficulties and questions remain, the monolayer network may be the simplest functional system that can be obtained for the investigation of network properties of mammalian CNS neurons. It is especially well-suited for the correlation of network morphology with electrical activity. Pharmacological influences can also be readily assessed, and this should contribute to a

determination of basic rules that govern the behavior of small ensembles of neurons. In view of the rapid developments in neural modeling and the concomitant interest in the analysis of biological networks, this research is significant to both neurobiological and theoretical modeling studies.

Completion of this project contributed the development of the technique for the routine monitoring of monolayer mini-cultures on MMEPs. Initial electrophysiological analyses of mini-cultures were performed, and responses to certain pharmacological agents were studied. A method for histological processing of mini-cultures on MMEP was developed which allowed light microscopic morphological analysis. Culture variables, electrophysiological activity, and morphological characteristics were correlated. The data provided by this research exposed both the advantages and difficulties of studying neuronal networks grown on MMEPs and will serve as a springboard for the planning of future experiments with MMEP cultures.

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