OPTIMIZATION OF CELL CULTURE PROCEDURES FOR GROWING NEURAL NETWORKS ON MICROELECTRODE ARRAYS

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This thesis describes the development of an optimized method for culturing dissociated, monolayer neuronal networks from murine frontal cortex and midbrain. It is presented as a guidebook for use by cell culture specialists and laboratory personnel who require updated and complete procedures for use with microelectrode array (MEA) recording technology. Specific cell culture protocols, contamination prevention and control, as well common problems encountered within the cell culture facility, are discussed. This volume offers value and utility to the rapidly expanding fields of MEA recording and neuronal cell culture. Due to increasing interest in determining the mechanisms underlying Parkinson's disease, the newly developed procedures for mesencephalon isolation and culture on MEAs are an important research contribution.

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CHAPTER 1
OBJECTIVE, SPECIFIC AIMS, AND SIGNIFICANCE

Objective: Development of an optimized method for culturing dissociated, monolayer neural networks from murine frontal cortex and midbrain. This information is presented as a guidebook for use by cell culture specialists and other laboratory personnel who require updated and complete protocols for use with microelectrode array (MEA) recording technology.

Specific Aims:

1. Detailed procedures, delineating reliable guidelines for the production of consistent, high quality neuronal cultures are included. This practical summary, including specific protocols, is supported with information from the Center for Network Neuroscience (CNNS) at the University of North Texas, as well as corroborating literature from the scientific community.
2. Development of *de novo* protocols for culturing dopamine-enriched midbrain tissue is included, as well as a discussion of an *in vitro* model of Parkinson's disease.
3. Improvement of sterility protocols and contamination-free practices are presented, to obtain the highest possible efficiency and productivity in the cell culture laboratory.
4. Common problems encountered by the cell culturist are discussed, as well as potential solutions to them.

Significance: Increasing interest in the use of primary neuronal cell culture for neurotoxicity, drug development, biosensors, and basic neuroscience investigations, especially on MEAs, is evident. Such cultures have been demonstrated to be highly useful in these domains. These applications require efficient cell culture protocols and must maximize consistency of products from culture to culture. No specialized, detailed, and comprehensive guide to achieve a necessary level of reproducibility exists in the literature. This thesis offers value and utility to the rapidly

expanding fields of MEA recording and neuronal cell culture. In addition, continued interest in determining the mechanisms underlying Parkinson's disease makes the newly developed protocols for mesencephalon isolation and culture an important research contribution.

CHAPTER 2

INTRODUCTION

Since its first trials in 1885 by Wilhelm Roux, and subsequent establishment of a scientific methodology by Ross Harrison in 1907, the field of tissue culture has undergone great advances and become highly specialized (Hamburger, 1988; Harrison, 1907; Sanes, Reh, & Harris, 2006). Neuronal cell culture is perhaps one of the most diverse and challenging practices within this discipline. From single cell line research, to organotypic slice preparations, to primary monolayer networks, neural tissue culture has become a much tailored approach to studying a variety of neuroscientific topics.

The development of the patch clamp technique by Erwin Neher and Bert Sakmann in 1976 provided an important new platform for electrophysiological research (Sakmann & Neher, 1984). The patch clamp was an improvement upon the previously used voltage clamp method, pioneered by Kenneth Cole, George Marmount, Alan Hodgkin, & Andrew Huxley (Purves et al., 2001). However, patch clamp methodology simply allows for the investigation of single neurons. Although in theory, it is possible to analyze neuronal network behavior with multiple, simultaneous patch clamp recordings, such an undertaking has met with limited success. In practice, it is laborious, time consuming, and cannot provide stable recordings after a period of one to two hours (Potter & DeMarse, 2001; Otto et al., 2003).

In the late 1970s, prompted by the need to record data from many neurons simultaneously and the difficulty of using conventional microelectrodes for this purpose, a new methodology emerged. This approach utilized photoetched, substrate-integrated microelectrodes arranged in recording arrays to capture the action potential information traffic in tissue slices, reconstituted monolayer neural networks, or established sensory structures, such as retinae (Barres et al., 1988;

Gross et al., 1977; Gross, 1979; Pine, 1980). Of these three major levels of organization, the monolayer networks derived from dissociated embryonic tissue form the most intimate, non-destructive contact with the recording array, resulting in strong cell-surface adhesion, large signal-to-noise ratios, and stability over months (Gross et al., 1993; Potter & DeMarse, 2001; Shahaf & Marom, 2001). The monolayer networks growing on microelectrode arrays (MEAs) are now established as pharmacologically histiotypic, i.e., like the parent tissue (Xia & Gross, 2003; Gross & Gopal, 2006; Parviz & Gross, 2007). Given this progress and the nascent applications in the fields of toxicology, pharmacology, drug development, and biosensors, it is imperative to offer the highest level of reliability and reproducibility in production of these networks.

This thesis discusses effective frontal cortex and midbrain cell culture methodologies for use with MEA recording technology. Primary, dissociated neural cultures derived from embryonic mice are beneficial for this type of research. They offer an efficient use of animal resources and associated costs, theoretically yielding close to 1,000 MEA cultures from the embryos of one pregnant mouse. This reflects a substantial reduction in the number of research animals required for future drug development, toxicology, biosensors, and basic neuroscience studies by universities, industry, and government agencies. In addition, since neurons and glial cells alike are removed and plated simultaneously, they form a confluent supportive network which is, at least pharmacologically, representative of the parent tissue. Most effective for MEA research are low-density, monolayer networks, in which cell bodies and processes may be visualized (see Fig. 1). MEAs are fabricated from glass and transparent indium-tin oxide conductors, which allow for clear imaging of the cells microscopically, while continuously and simultaneously recording from 64 network sites. Since MEA studies require mature, electrically

active neuronal networks, cultures must be maintained *in vitro* for a period of weeks to months. This may seem impractical, since neural cultures do not possess many immunological defenses and are historically difficult to keep in contamination-free circumstances. However, with the proper materials and methodology, researchers have kept such cultures alive and experimentally active for up to and beyond one year in culture (Potter & DeMarse, 2001).

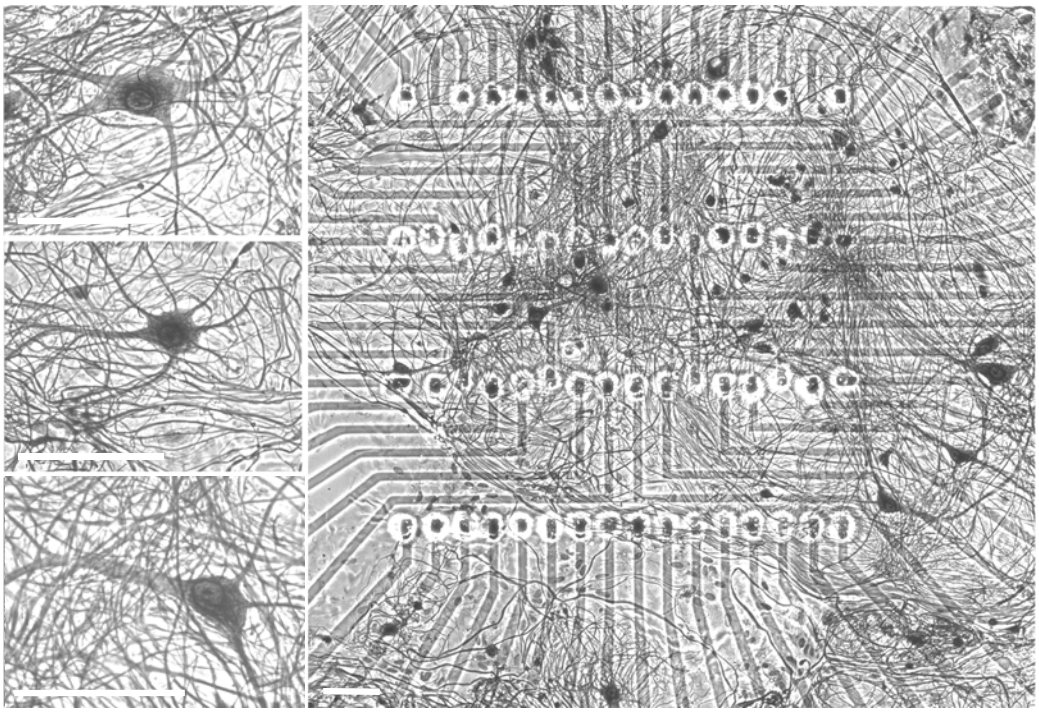

Figure 1. Mature, low density spinal cord network growing on a 64-electrode MEA at 96 days *in vitro*. Bodian stain, Center for Network Neuroscience (CNNS) archives.

No unifying guidebook exists in the literature for the culture and maintenance of dissociated monolayer neural networks, derived from embryonic mouse cortex, for use with MEA technology. Most often, terse protocols found in the literature involve culture of the hippocampal or spinal regions of pre- or post-natal rat central nervous systems. In addition, no comprehensive volume delineates clearly the problems most typically encountered by the cell culturist. The CNNS pioneered multisite recording with substrate-integrated thin film electrodes.

As a consequence, it is expected to provide cell culture protocols beyond the terse summaries provided in publications.

This thesis purports to not only offer clear instructions for the culture and maintenance of MEA networks; it also provides troubleshooting and experimental evidence as to why certain techniques may be selected. In addition, a chapter detailing a new method for culture and experimentation of a Parkinson's model, utilizing dopamine-enriched midbrain cultures, is included. Underlying themes of safety and sterility, as well as contamination contingencies, are offered throughout.

CHAPTER 3

FRONTAL CORTEX CELL CULTURE PROCEDURES

A well trained and proficient cell culture staff is critical to the overall success of neuroscience research efforts. Even minor variations from standard protocols may influence research findings. Regular communication between cell culture staff and researchers is essential to avoid procedure drifts and systemic errors. This is more critical when cell culture responsibilities are delegated to students and part-time staff.

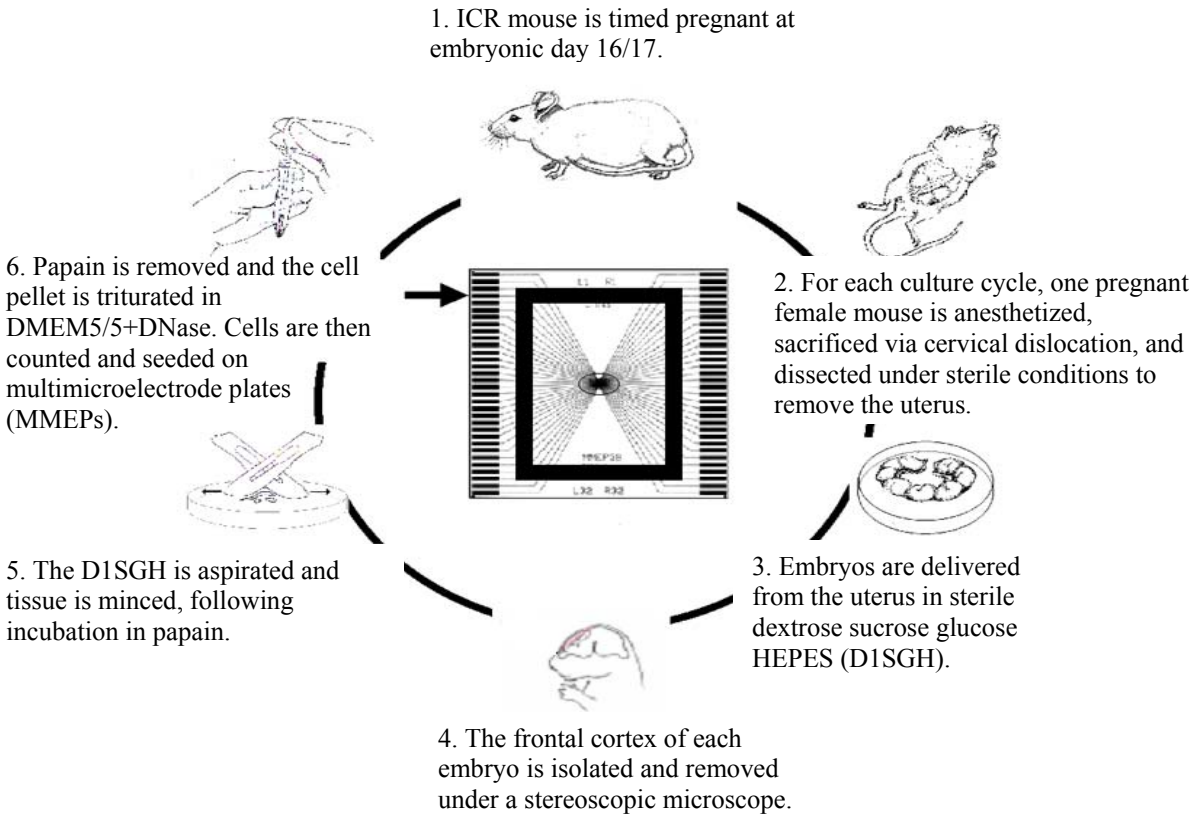

Figure 2. Steps involved in isolating and plating frontal cortex tissue from embryonic mice.

This chapter discusses the rationale and procedures for preparation, isolation, plating, and maintenance of primary frontal cortex cultures on microelectrode arrays (MEAs), isolated from embryonic mice. See Figure 2 for an overview of cell culture methods. Appendix B includes a

short-form, explicit protocol detailing the procedures described in this chapter. In addition, notes on the preparation, storage, and maintenance of cell culture reagents discussed herein are included in Chapter 5 and Appendix A.

Present Experimental Methods

The Center for Network Neuroscience (CNNS) has designed its own recording chamber and fabricates all MEAs in house with student labor. The general recording setup is shown in Figure 3. The components that impact cell culture are the MEAs containing the network, a rectangular gasket that holds the culture medium during network development, a stainless steel chamber block (used during electrophysiological investigation), and a heated cap to prevent condensation and allow maintenance of a 10% CO_2 in air environment for pH maintenance. Although most laboratories use 5% CO_2, 10% provides a greater buffering capacity and better pH stability when used in conjunction with the sodium bicarbonate concentrations in the media described herein.

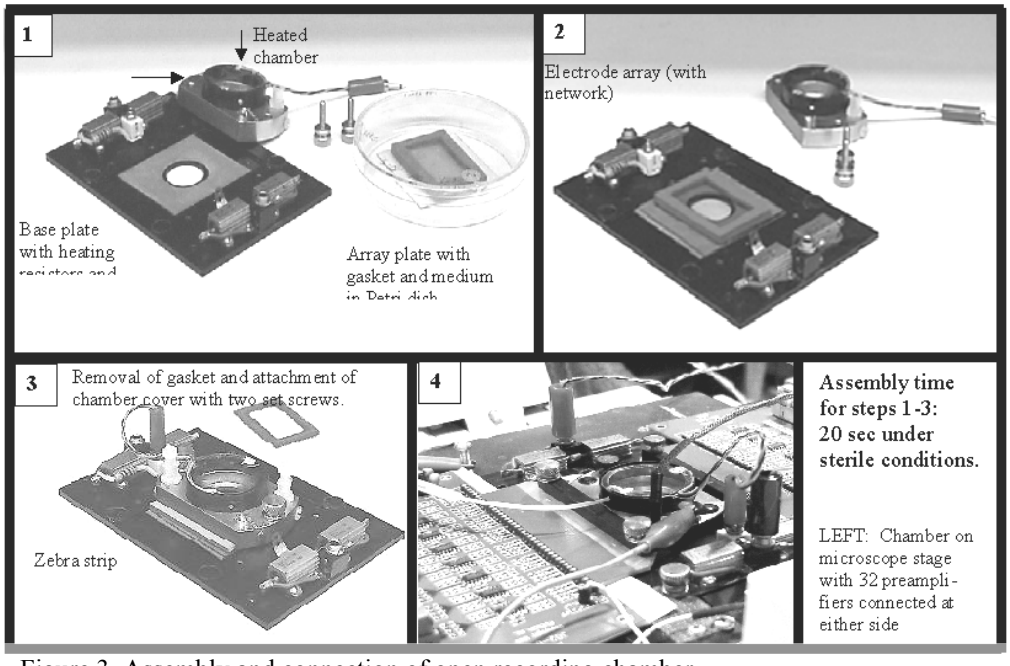

Figure 3. Assembly and connection of open recording chamber.

The essential substrate for electrophysiological recording consists of a multi- microelectrode plate (MMEP; CNNS, Denton, TX) and its attached neuronal culture. Except for the new 8-network plate, MMEPs (see Fig. 4) measure 5 cm x 5 cm square and are fabricated from 1 mm thick soda-lime glass plates covered with a 1,000 Å film of quartz. The indium-tin oxide conductors are photoetched into the surface of the MMEP and insulated by a 2 – 3 µm thick layer of polysiloxane resin. Microelectrodes at the recording area (matrix) of each MMEP are constructed by deinsulation of the insulating material in 15 – 20 µm diameter craters and subsequent electrolytic gold-plating of the exposed indium-tin oxide for decreased impedance. The recording

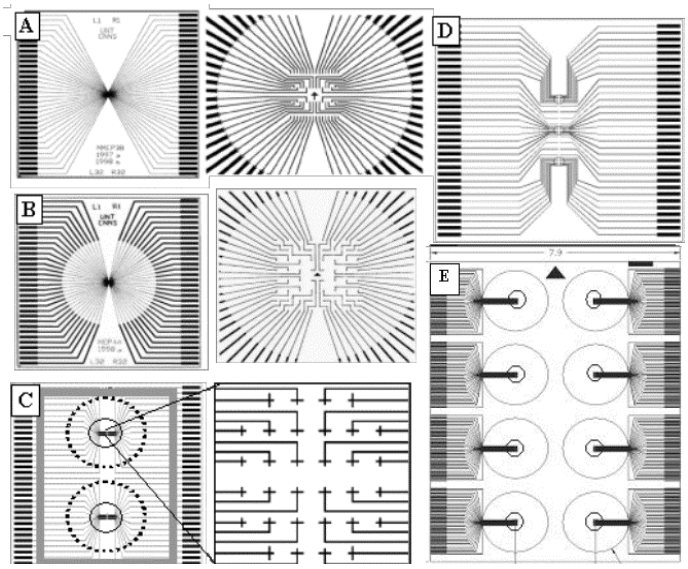

Figure 4. MMEPs in use by the CNNS. (A–D) measure 50 x 50 x 1.1 mm. Amplifier edge contacts are the same for all arrays. (E) 8-network plate (90 x 56 x 1.1 mm).

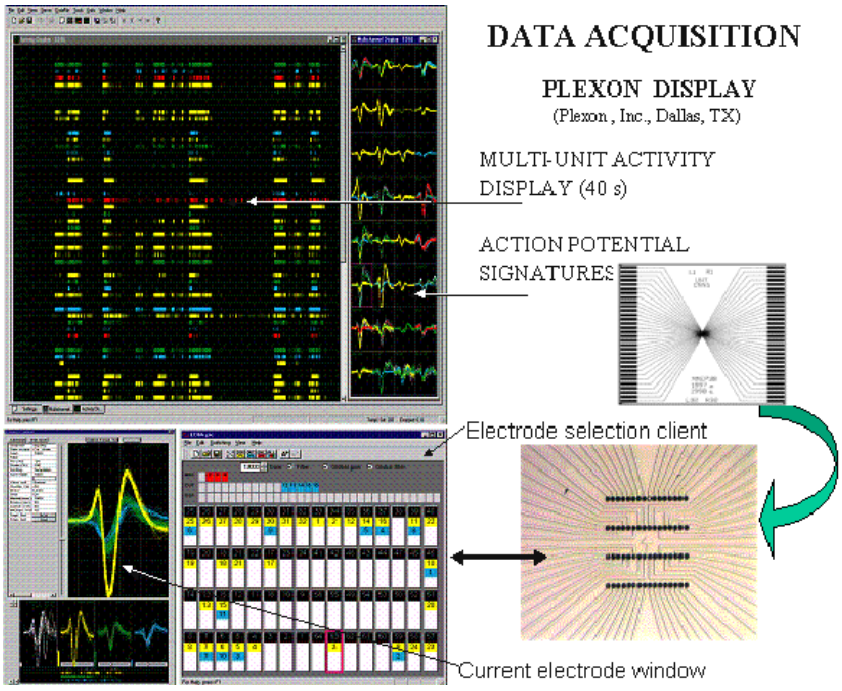

Figure 5. Digitized data displays of selected waveforms and spike raster plots. Up to 4 different units on one electrode can be discriminated and separated in real time.

area of each MMEP consists of 64 deinsulated, gold-plated microelectrodes in a recording area measuring approximately 1 mm^2.

Data retrieved on the electrophysiological recording station can be viewed, recorded, and manipulated via recording software (Plexon, Inc., Dallas, TX), which allows for assignment of neuronal signals and recording of a variety of variables, including time stamps and waveshapes (see Fig. 5). An embedded NACTAN software program (CNNS, Denton, TX) allows for real-time display of mean and total neuronal spike production as well as active unit counts in one minute time bins.

MMEP Surface Preparation

Two days prior to cell culture, MMEPs are prepared for seeding. Following removal of residual silicone grease (left from the previous experimental use of the MMEP) with the aid of a cell scraper and cotton, they are thoroughly cleaned with an enzymatic detergent and rinsed under deionized (DI) water. Rubber gaskets are degreased with a razor blade and soaked in 70% EtOH. Gaskets are then fitted with a thin line of silicone grease to form a water-tight seal upon adherence to the MMEPs. MMEPs and gaskets are autoclaved face-up for 25 minutes at 121°C. Note that silicone grease, although somewhat messy and cumbersome, is currently the only reliable method in place for providing a water-tight seal to hold in culture media. Efforts to replace silicone grease involve current developments with the use of Sylgard (Dow Corning, Midland, MI), and preliminary results are promising.

Surface preparation takes place one day prior to cell culture. During this procedure, the hydrophobic polysiloxane insulation material (synthesized from methyltrimethoxysilane) of the MMEP must be exposed to a torch flame to create a hydrophilic surface, sufficient for subsequent addition of adhesion molecules. Lucas, Czisny, & Gross (1986) demonstrated that

exposure of a hydrophobic surface, such as glass or MMEP insulation material, to an acetylene, butane, or propane flame increases wettability by more than 1200% over non-flamed surfaces. Although the exact mechanism of this form of surface modification is unknown, it is thought that the products of incomplete combustion, such as the hydronium ion H_3O^+, $C_3H_3^+$, and other cationic species, produce a positively charged surface (Bradley, 1969). Whereas the non-polar, unflamed surface of the polysiloxane resin cannot produce sufficient molecular bonding for adhesion, the flamed, hydrophilic surface layer can now accept adhesion molecules and subsequent cell suspensions. Precise flaming of the microelectrode matrix is achieved with the aid of a stainless steel flaming mask (see Fig. 6). If coverslips are to be used for histological or morphological studies, the flame may be passed over the entire glass surface to provide maximum coverage. This "whole-flamed" effect offers a greater surface area and ultimately results in a lower-density monolayer network, more suitable for micrographic viewing. Once flamed, MMEPs are fitted with greased gaskets and placed under a UV lamp for approximately ten minutes to insure sterility.

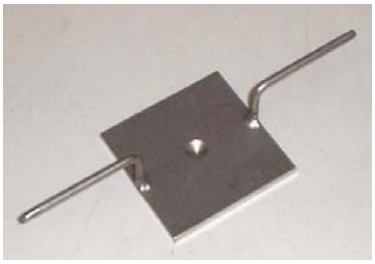

Figure 6. Stainless steel flaming mask with 2.5 mm diameter opening.

Addition of adhesion molecules prior to cell culture is a two-step process. It is extremely important that induced cell-surface adhesion be greater than naturally occurring cell-cell adhesion to avoid large aggregates of clumped cells (Zeng et al., 2007). These cell clumps are problematic in that they tend to promote retraction of networks from the adhesive surface.

One day prior to culture, 50 µL of a sterile 5 mg/L solution of 30,000-70,000 MW poly-D-lysine (PDL; Sigma Aldrich, St. Louis, MO) are applied to the flamed matrices of each MMEP under sterile conditions. PDL serves as a nonspecific attachment factor for *in vitro* cell

culture preparations. It is a positively charged polymer of the amino acid lysine, and it works by promoting favorable ionic interaction between the negatively charged cellular membranes and the cell culture surface. Although its absolute charge is positive, the negatively charged carbonyl oxygen can interact with the cations of the flamed resin or glass surface, while the positively charged sidechains are free to attach to the neuronal cell membranes. In short, PDL increases the quantity of positively charged binding sites for cellular adhesion.

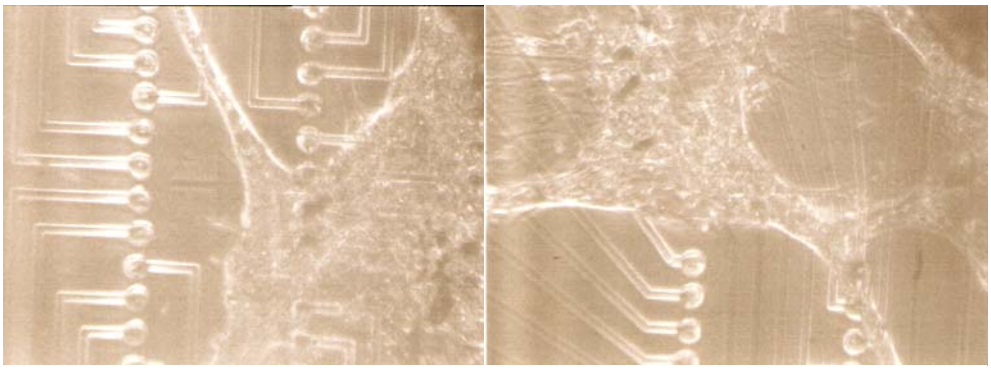

Figure 7. Retraction and subsequent loss of adhesion 3 months after PLL application. *200x magnification.*

Although flaming alone may provide an adequate substrate for adhesion of dissociated neural cells, polycationic substances such as PDL are necessary for promotion of neurite outgrowth. The stronger adhesion to the positively-charged poly-lysine surface promotes flattening of the growth cone and a preferred route, as compared to non-treated surfaces (Letourneau, 1975). In addition, experimentally evidenced increases in single-cell adhesion to PDL-modified flamed surfaces over unflamed surfaces appear to be related to decreased cell mobility after plating (Lucas, Czisny, & Gross, 1986). In fact, PDL has become the preferred molecular substrate for studies involving micropatterning of MEAs to promote directional growth of axons and dendrites within primary neuronal cultures (Chang, Brewer, & Wheeler, 2006; Sorkin et al., 2006). If PDL is not readily available, a 0.1% solution of poly-L-lysine (PLL; Sigma Aldrich, St. Louis, MO) may be substituted. However, the naturally occurring

L-isomer of this amino acid is readily digested by cells (French et al., 1997), and adhesion breakdown is evident after as little as two months in culture (see Fig. 7).

The MMEPs are stored overnight in a 37°C incubator under 10% CO_2 and 95% relative humidity to prevent PDL drying. The following morning, each MMEP is removed from the incubator and placed under a biohazard hood for rinsing. It is imperative that PDL be sufficiently rinsed from the culture surface, as the D-isomer (in solution) is known to induce toxicity upon entrance to living cells (Hüber et al., 1998). Should the PDL be allowed to air dry, evaporation of the solvent would form a crystal precipitate ring, as seen in Figure 8. In fact, simple aspiration of the PDL solution from the cell culture surface does not typically remove enough solute to prevent this precipitation. Therefore, the PDL must be rinsed from each matrix with ultra-pure water (UP H_2O) and removed with a sterile pipette. If this precautionary measure is not taken, it is likely that the precipitated PDL will resolubilize upon medium addition and produce a potentially toxic environment for cell development.

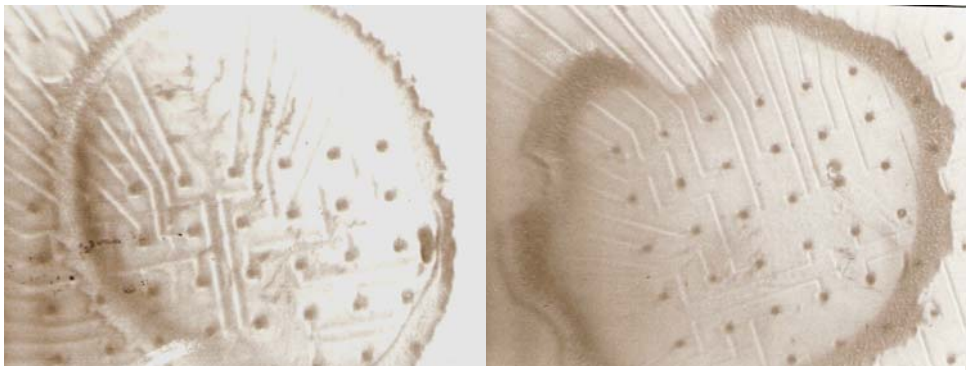

Figure 8. Rings of PDL precipitation on MMEP matrix caused by solution drying before aspiration. *100x magnification.*

The second step of surface adhesion promotion involves the use of laminin, the major non-collagenous glycoprotein of basement membranes. In addition to adhesion promotion, laminin is thought to play a key role in neuronal proliferation, migration, myelination, neurite outgrowth, and tissue survival both *in vivo* and *in vitro* (Colognato & Yurchenco, 2000).

Laminin also serves as an extracellular matrix adhesion molecule, promoting specific binding with integrins, e.g., growth cone receptors. This is important for cell culture preparations, because laminin-modified surfaces supplement endogenous supplies to stimulate axon growth within the adhesion island (Purves et al., 2001; Sanes, Reh, & Harris, 2006). Following aspiration of PDL, 80 µL of a 0.5 mg/mL solution of laminin (Roche, Indianapolis, IN) is diluted into 2 mL UP H_2O. Of this stock solution, 60 µL are applied to each matrix. Stock laminin may be diluted into culture media or phosphate-buffered saline (PBS), as an alternative to H_2O. However, to insure sterility of the mixture, UP H_2O may be autoclaved prior to use. This option is equally effective and does not pose a contamination risk. MMEPs are then incubated for at least 45 minutes prior to seeding in an effort to encourage proper adhesion. Cells must be plated the same day the laminin is applied, since the dilution breaks down relatively rapidly.

Sacrifice and Dissection

One timed-pregnant ICR mouse (Harlan Sprague Dawley, Inc.) is used for cell culture at embryonic day sixteen or seventeen (E16/17). It is important to note that at this embryonic age, a sufficiently mixed neuronal cell type is evident in the developing cortex. As characterized by Parnavelas (2000), in addition to excitatory pyramidal neurons, inhibitory non-pyramidal (i.e., GABAergic) cells have long since migrated from proliferative zones by this stage in development. Specifically, GABA-positive cortical neurons from the lateral ganglionic eminence manifest as early as E11.5, while more extensive inhibitory neurons (from the medial ganglionic eminence) are cortically evident at near E12.5. Therefore, cortical cultures prepared via this protocol are recognized as containing a mixed inhibitory and excitatory cell population, sufficient for complex synaptogenesis and electrophysiological behavior. It is very important to be able to accurately identify and maintain a short window in which embryonic development is

allowed to occur. Protocols using exact embryonic ages will always offer more reliable results than those with a broad window. For that reason, it is ideal for laboratories to breed and maintain a closely controlled murine colony.

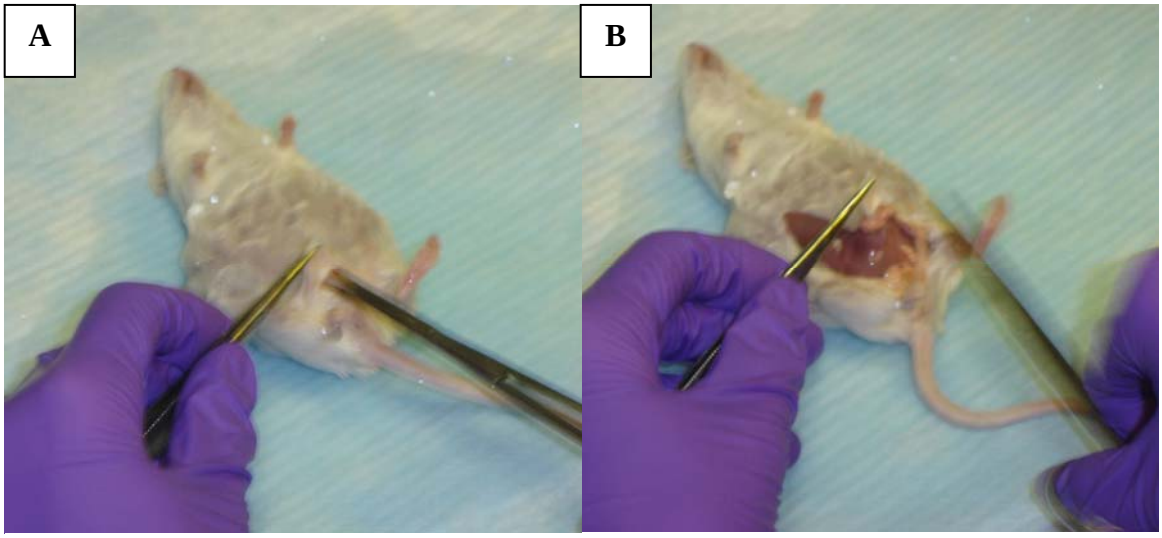

Figure 9. Removal of uterus via bikini cut procedure. (A) While skin is grasped with forceps, an incision is made superior to the vaginal opening. (B) A v-shaped cut is made through the skin and subcutaneous fat, extending to the distal ends of the thoracic cavity, exposing the uterus.

Following chloroform or halothane anesthesia and sacrifice via cervical dislocation, the uterus is removed under sterile conditions. See Figure 9 for a depiction of the "bikini cut" procedure for embryo removal. A typical dissection yields ten to fourteen embryos, which are immediately immersed in a cold (4°C) anesthetic bath of dextrose sucrose glucose HEPES (D1SGH). Further dissection is performed in this solution (see Fig. 10), which has been equilibrated at a pH of 7.35 and an

Figure 10. Placenta is removed and embryos are individually isolated in a cold anesthetic bath of D1SGH.

osmolarity of 320 mOsm. D1SGH is comprised of a mixture of physiological saline, sugar, and HEPES buffer. Sucrose in the solution aids in osmolarity maintenance, while glucose provides necessary nutritional content to supply the cells prior to introduction of culture media. HEPES buffer is important for tissue protection during transfer from petri dish to petri dish and exposure to environmental air.

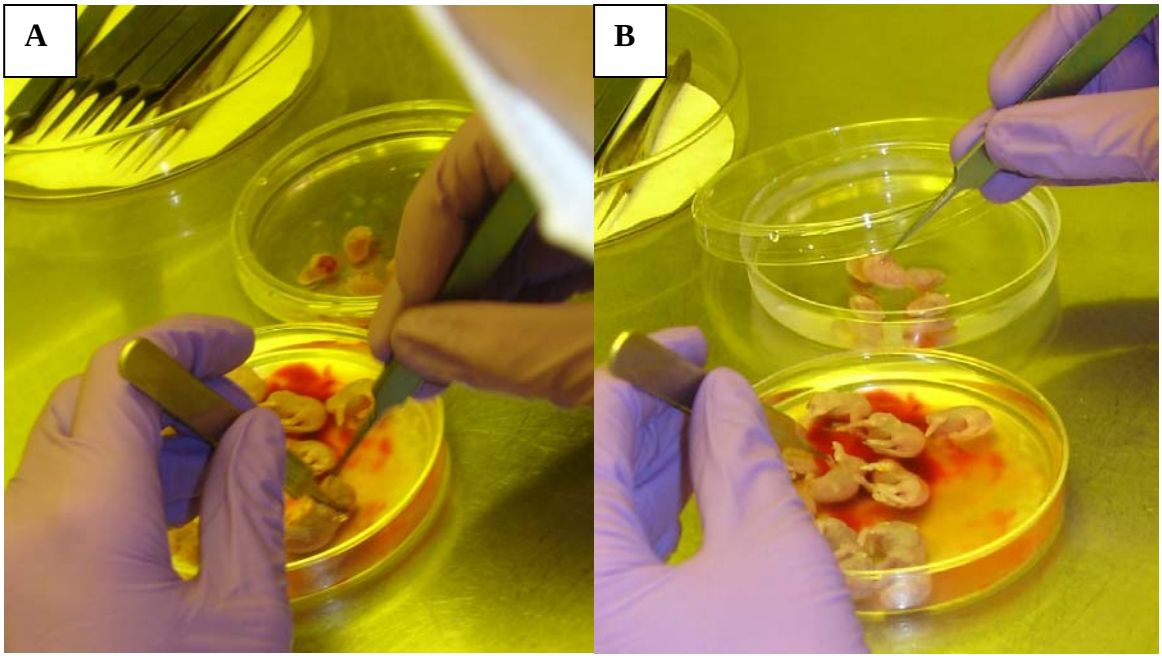

Figure 11. (A) Using a pair of sharp, fine-tipped forceps, embryos are decapitated. (B) Crania are transferred to a separate bath of D1SGH.

Under sterile conditions, with the aid of a stereoscopic microscope and a pair of fine-tipped forceps, embryos are decapitated (see Fig. 11) and skin and muscle tissue is removed to reveal the underlying skull. From the dorsal side, the skull is carefully removed, working rostrally to caudally. The intact brain is then lifted from the skull cavity. The meninges are removed from each brain to reveal the cerebral cortex, of which the frontal lobe is dissected in a trapezoidal pattern. Figure 12 contains micrograph images of microdissection. The frontal cortex is isolated from each remaining embryo and stored in a separate bath of D1SGH. See Appendix B for a detailed, step-by-step guide to embryo removal and frontal cortex isolation.

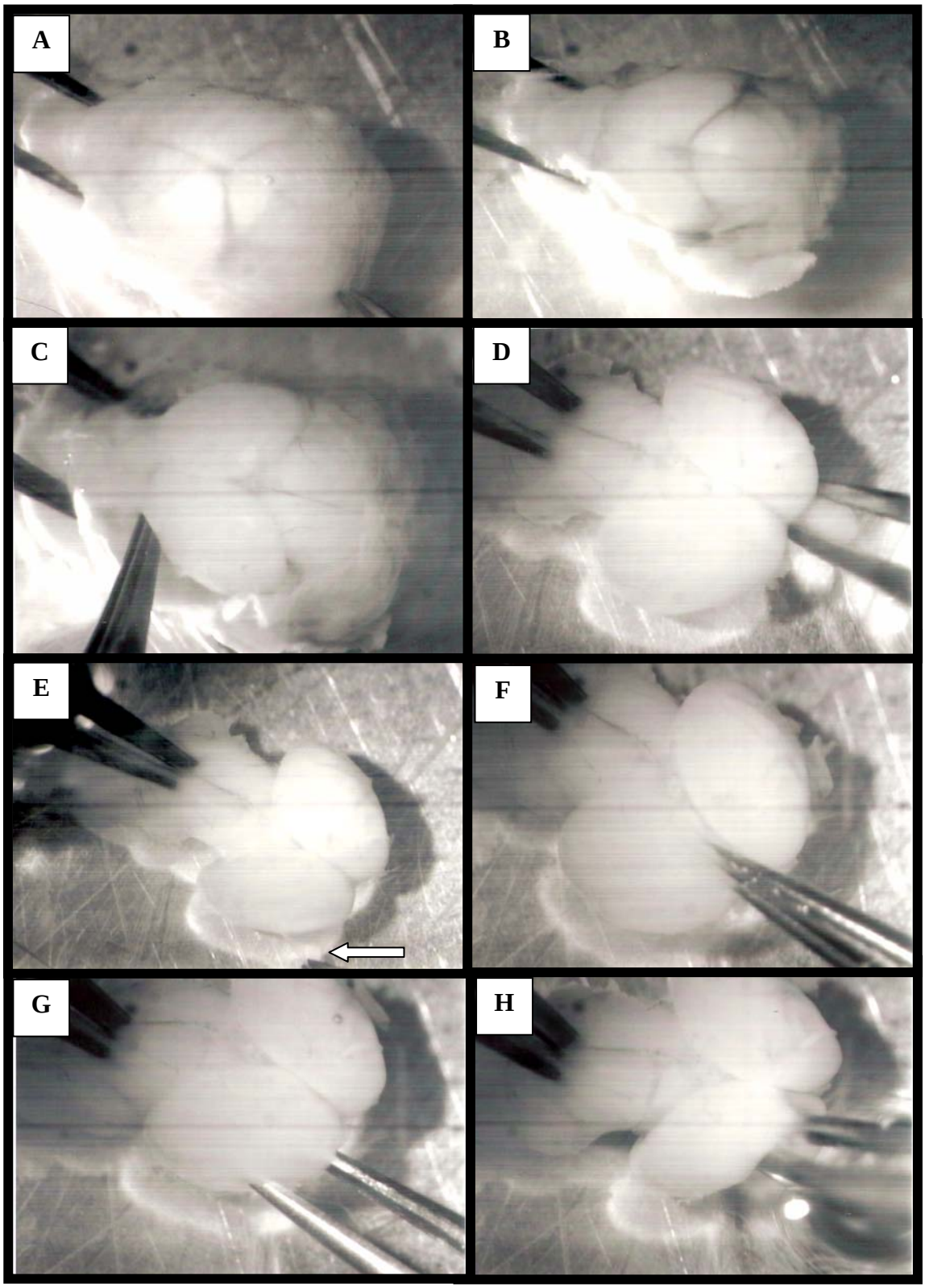

Figure 12. Embryonic frontal cortex microdissection. (A) Intact cranium. (B) Skin is removed. (C) Brain is lifted from skull cavity. (D) Olfactory bulbs are removed. (E) Arrow: meninges removal. (F-H) Frontal cortex isolation: cuts 1-3 of trapezoidal pattern.

Dissociation and Seeding

Following dissection, the D1SGH is aspirated from the petri dish, and the tissue is minced with two sterile scalpel blades. Use of a protease is necessary for optimal dissociation of cells as a first step, prior to mechanical dissociation by trituration. Papain is used to break down the extracellular matrix molecules that hold the cells together. In addition, it aids in loosening tissue by disrupting myelinated axon tracts. The frontal cortex tissue is enzymatically dissociated with the addition of 3 mL of a 1:14 papain:D1SGH solution and incubated at 37°C for five minutes, then at 22°C for five minutes. Papain is then removed via centrifugation with 50 µL DNase I in 5 mL DMEM5/5 at 900 rpm for four minutes. It is imperative that papain digestion be carefully timed to prevent total lysis of cells. The DNase I aids in preventing the free DNA—released by the proteolytic activity of papain—from inducing cell clumping during seeding. The supernatant is removed, and the cell pellet is triturated in a fresh 50 µL DNase I in 5 mL DMEM5/5 solution (see Chapter 5). It is important to note that tissues isolated from embryonic, as opposed to postnatal or perinatal mice, require less protease activity and gentler trituration to guarantee a large yield of viable cells (Malin, Davis, & Molliver, 2007). Cell density of the newly formed cell pool is calculated with a cytometer and adjusted to the desired level (90,000 – 110,000 cells/mL) with any necessary addition of DNase/DMEM5/5.

Once the proper density of the cell pool is established, cells are seeded onto the recording matrix area of each MMEP. Laminin is first removed with a fine-bore pipette, and 100 µL of cell suspension is seeded onto each matrix. Although laminin is not necessarily harmful to cells, the solvent may induce osmotic stress and should be aspirated completely prior to plating. The cultures are placed into incubation for approximately 2.5 hours before adhesion evaluation and initial feeding with 1 mL DMEM5/5. The following day, a full medium change is performed into

DMEM5/5 in an effort to remove any DNase and non-adhered cells and cell debris. Two to three days later, a full medium change into DMEM5 (a medium mixture containing 5% horse serum (HS)) is performed, to eliminate remaining fetal bovine serum (FBS) from the medium. See Table 1 for a summary schedule of medium changes and Chapter 5 for a complete description of culture media used throughout this procedure.

Table 1. Medium Change Schedule

Feeding Day/Time	Medium	Quantity	Purpose
Seeding	DMEM5/5 + DNase	100 µL/matrix	Plate cell suspension
2 hours post culture	DMEM 5/5	1 mL/MMEP* (addition)	Initial feeding
Culture day + 1	DMEM 5/5	3 mL/MMEP (full Δ)	Remove DNase
Culture day + 4	DMEM 5	3 mL/MMEP (full Δ)	Remove fetal bovine serum
Culture day + 8	DMEM 5	1.5 mL/MMEP (½ Δ)	Provide nutrition & remove cell debris/waste products
Life of culture (2x/week)	DMEM 5	1.5 mL/MMEP (½ Δ)	

*Standard MMEPs have a maximum volume of 3 mL. Specialized MMEPs or coverslips may hold more or less, and volumes should be adjusted accordingly.

The resulting cell culture consists of a monolayer neuronal network atop a confluent glial carpet layer encompassing the entire matrix of each MMEP. Cell cultures are maintained in a 37°C incubator under 10% CO_2 and 95% relative humidity for up to and beyond six months. They are not treated directly with antibiotics or fungicides, other than additions of $CuSO_4$ in incubator water reservoirs (see Chapter 6 for a discussion of contamination prevention and protocols). Nutrients are replenished and metabolic byproducts and/or cell debris are removed via bi-weekly half medium changes into DMEM5 for the life of the culture.

CHAPTER 4

DOPAMINE-ENRICHED MIDBRAIN CULTURE

Culturing primary neuronal networks from different regions of the mammalian brain is standard practice in many basic neuroscience laboratories. Expertise in identification of specific regions of interest is critical to establishing specialized cultures. For example, identification of the mesencephalon, specifically the dopamine-rich substantia nigra pars compacta (SNpc), may vary in difficulty depending on the model animal and the age at which the tissue is removed. The SNpc of an embryonic mouse is almost impossible to visually identify, because it has not yet developed the neuromelanin that gives it its unmistakable black color. However, using common external landmarks, the general area of the midbrain can be isolated. Targeting the SNpc for cell culture in E15-E18 embryonic mouse brains is essentially the equivalent of taking the midbrain and most of its accompanying cell populations. Primary cultures from this brain region form the basis for an experimental, *in vitro* model of Parkinson's disease that can be studied using electrophysiological methods.

Mesencephalon, Dopaminergic Neurons, and Parkinson's Disease

During mammalian embryonic development, involution of the neural tube forms three distinct brain regions, the forebrain, midbrain, and hindbrain. This will ultimately give rise to five regions in the adult brain, the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon (see Fig. 13). The area of interest, the mesencephalon, contains several gross anatomical structures that are rich in catecholaminergic cell bodies which are uniquely targeted in Parkinson's disease. Midbrain regions include the tegmentum (contains the rostral end of the reticular formation), the cerebral peduncles (important for voluntary motor function),

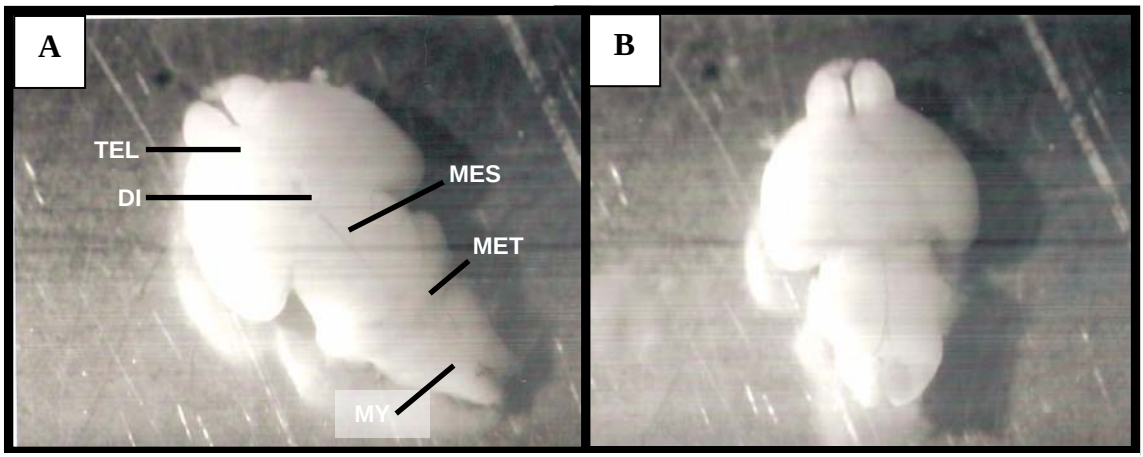

Figure 13. (A) Dorsal view of intact E16/17 murine brain. Telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon denoted. (B) Ventral view of same brain as in A.

the superior and inferior colliculi (important for visual and auditory function, respectively), the substantia nigra (SN) and the red nucleus (both important for motor function). The SN can be divided into three distinct regions: the pars compacta, pars reticulata, and pars lateralus. Along with the pallidal nuclei, the pars reticulata and lateralus form elements of the basal nucleus (includes the caudate, putamen, nucleus acumbens, globus pallidus, substantia nigra and subthalamic nucleus), which functions in modification of movement. Between the inhibitory input of the basal nuclei and the excitatory output of the cerebellum, smooth coordinated movement is possible.

Midbrain dopaminergic pathways originate in the inferior colliculus, SNpc, and the ventral tegmental area (VTA) at the level of the superior colliculus. Specific dopaminergic systems have been mapped by Dahlstrom and Fuxe within the mesencephalon, diencephalon, and telencephalon (DeArmond, Fusco, & Dewey, 1989). The two main dopaminergic pathways are the mesostriatal and mesolimbic systems. The majority of dopaminergic neurons in the central nervous system are found in the SN, the nearby VTA, and the arcuate nucleus of the hypothalamus. Dopaminergic projections from the VTA innervate the cerebral cortex, while SN nigrostriatal tracts terminate in the striatum.

The SN is named for the black pigment melanin which is formed along catecholaminergic pathways via auto-oxidation of tyrosine, the precursor to dopamine (Li et al., 2005). Neuromelanin is not present during embryonic or early postnatal development (Smythies, 1996). *In vitro*, exposure to oxygen may initiate auto-oxidation of tyrosine and subsequent formation of neuromelanin. Neuromelanin acts as a free radical sink for iron and other neurotoxic cations which tend to accumulate in dopaminergic neurons. This cell type is particularly vulnerable to oxidative stress due to its unusually high metabolic rate, linked to high spontaneous electrical activity, and limited enzymatic defenses, such as glutathione (Aguirre et al., 2006). Intracellular melanosomes represent a unique storage system capable of quenching and deactivating reactive oxygen species and storing them over a period of five to nine decades (Zucca et al., 2004). As neuromelanin accumulates, it can be visualized in the SN, and its accelerated depletion in the later decades of life represents pathology specific to Parkinson's disease.

The commonly recognized pathogenic process in Parkinson's disease advances slowly and follows a relatively stereotypic progression. The first degenerative processes in Parkinson's disease begin prior to the appearance of clinical symptoms. Sandyk and Iacono (1988) were among the first to suggest a relationship between the reticular system and the pathoetiology of Parkinson's disease. Clinical symptoms of Parkinson's disease are typically thought to be the result of dopamine deficiency; however, the dopamine hypothesis does not fully explain other frequently observed symptoms such as anosmia, reversible frontal dementia, depression, cognitive decline, change in affect, sleep disturbances, autonomic instability, and unexplained chronic pain syndrome (Wolters & Braak, 2006). Autopsy studies have provided evidence of characteristic topographic advance of Parkinson's disease neuropathology, with the first lesions

appearing in the dorsal motor nucleus of the vagal nerve. From there, they progress along an upward path through the basal nuclei of the mid- and forebrain and ultimately reach the cerebral cortex (Ballard et al., 2006; Halliday, Del Tredici, & Braak, 2006). This progression of neuropathology dictates the need to culture midbrain structures, not only to obtain dopamine-enriched neurons, but to include all neuronal tissue involved in Parkinson's disease pathology. It is known that cultured embryonic cells form a histiotypic monolayer network that is electrophysiologically similar to parent tissue, and the mixed neuronal milieu provides a better representation than single patch-clamp recordings.

The normal function of neuromelanin is to protect the dopaminergic neurons of the SN. However, in the early stages of Parkinson's disease, the dopaminergic cells become saturated, predominantly with Fe and other cations. As the disease progresses, the buffering capabilities of neuromelanin reach a critical saturation point and neuromelanin-containing dopaminergic neurons die. When this occurs, toxins are released into the extracellular environment, causing microglial activation (see Fig. 14 for an example of such activation). In addition to the loss of dopaminergic neurons, inflammatory factors induced by chronic microglial activation interfere with the function of serotonergic pathways, which compensate for dopamine loss in the early stages of Parkinson's disease. The characteristic Parkinson's tremor is evidence of increased serotonin activation.

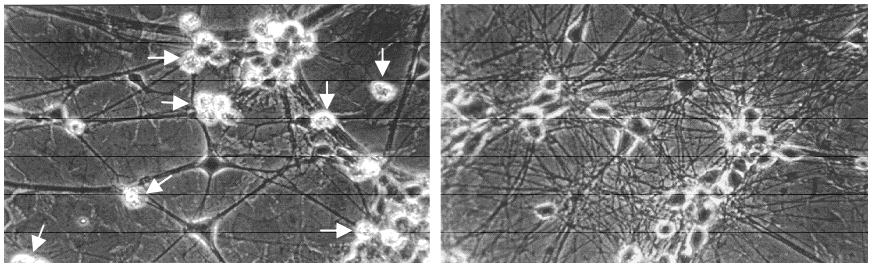

Figure 14. Microglia activation in culture. Phase bright microglia are visible (arrows). Right: Magnified center region of micrograph.

In Vitro Parkinson's Model

The dopamine-enriched cell culture model established in this laboratory uses primary neural cultures, offering a distinct advantage over those plated from pure cell lines. It is known that glial cells from the mesencephalon mediate dopaminergic neuronal differentiation and survival (Engele, Schubert, & Bohn, 1991). In fact, co-culture of SN neurons with glia from other brain regions is less effective in promoting cell viability than mixed neuron-glia cultures derived from the SN as a whole (O'Malley et al., 1992).

Although controversy exists regarding the most appropriate embryonic age at which to isolate midbrain tissue, it is believed that cultures prepared from E14-E18 brains offer the most promising experimental results. This is because dopaminergic neurons have differentiated at this stage of development, although axonal projections to the striatum have not been completely established. Therefore, tissue damage due to axotomy is limited and cell yields are generally higher than in cultures prepared from perinatal or postnatal mice (Lyng, Snyder-Keller, & Seegal, 2007).

Although there is no perfect animal model of Parkinson's disease, the approach delineated in this chapter mimics certain hallmark defects of this pathology. There are several substances, when used *in vivo* or *in vitro*, which are known to selectively damage dopaminergic neurons and induce similar pathology as that seen in Parkinsonian brains. Specifically, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium (MPP+), rotenone, and paraquat have demonstrated the ability to inhibit cellular respiration. MPP+ is thought to inhibit the first enzyme of cellular respiration, NADPH oxidase, and induces necrotic neuronal death due to a decline in ATP synthesis (Hartley et al., 1994). Free radicals may also be involved in MPP+ toxicity. MPTP is endogenously converted to MPP+ by monoamine oxidase B

in glial cells. Therefore, MPP+ is a better choice as a pharmacological agent for mesencephalic cultures due to its ease of use and ability for more precise quantification (Gao et al., 2003). Using MPP+ bypasses the requirement for glial conversion of MPTP and allows for a more accurate estimation of dosages.

The dopamine transporter is necessary for MPP+ entry into dopaminergic neurons (Ramsay & Singer, 1986). Additions of MPP+ to primary midbrain cultures induce oxygen free radical-mediated damage to dopaminergic cells specifically within the SN, while sparing nearby dopamine-containing neurons of the VTA. MPP+ is a relatively weak neurotoxin known to target complex I (NADH dehydrogenase) of the electron transport chain, disrupting oxidative phosphorylation in dopamine-containing cells. The MPP+ model of Parkinson's disease has been extensively utilized *in vivo*, and when used in conjunction with microelectrode array (MEA) technology, it provides a well-validated format for studying the electrophysiological activity of normal and pathological dopaminergic neurons (Ulanowska et al., 2007; Tian et al., 2006; Watanabe, Himeda, & Araki, 2005; Goralski & Renton, 2004; Boada et al., 2000).

Midbrain Culture Protocol

Cell culture of the murine embryonic mesencephalon follows many of the same general protocols as that of frontal cortex in these animals. Like in cortical dissection, midbrain is removed at E16 or 17. Multi-microelectrode plates (MMEPs), gaskets, and coverslips are prepared according to procedures delineated in Chapter 3. Following surface preparation, sacrifice is performed, and embryos are removed. After embryo isolation and decapitation, microdissection is conducted.

When removing the skin and skull, it is important to work carefully so as not to puncture the underlying brain. Once exposed, the brain is removed gently. Ensure that the brain can be

isolated without damage to forebrain or hindbrain structures. This aids in midbrain isolation, because it allows for more tissue to grasp while making incisions.

Once the brain is removed and transferred to a fresh petri dish filled with dextrose sucrose glucose HEPES (D1SGH), the mesencephalon is separated from the surrounding structures. If the same brains are used for frontal cortex and midbrain isolation, the procedure is slightly more difficult. In this case, the brain is grasped by forceps through the hindbrain, using the non-dominant hand. The cortical structures are then removed from the forebrain and midbrain manipulation is carried out. If the frontal cortical structures are intact prior to midbrain dissection, they may be gripped by forceps throughout the dissection (see Fig. 15).

The brain is ablated using a No. 3 scalpel and 15 blade at the junction between the mesencephalon and metencephalon, releasing the entire hindbrain. Next, the meninges are completely removed from the dorsal to the ventral midbrain using fine-tipped forceps. Then, a scalpel cut is made just inferior to the cortical structures, separating the telencephalon and relatively concealed diencephalon from the mesencephalon. Further incisions may be made at this point to isolate the ventral region containing the SNpc from the dorsal structures. It is important to utilize as many brains as possible when preparing midbrain cultures, because historically, cell yields are relatively low (Smeyne & Smeyne, 2002).

Isolated tissue is transferred to a small petri dish containing a thin layer of D1SGH. Dissociation and seeding are carried out in a separate biohazard hood within a clean room. Following aspiration of D1SGH with a sterile pipette, mincing of the tissue is performed with two sterile scalpel blades until a viscous, homogenous mass is produced. The partially dissociated tissue is bathed in 3 mL of papain solution (see Appendix A) and incubated at 37°C for 15 minutes. To remove papain, the mixture is suspended in approximately 5 mL

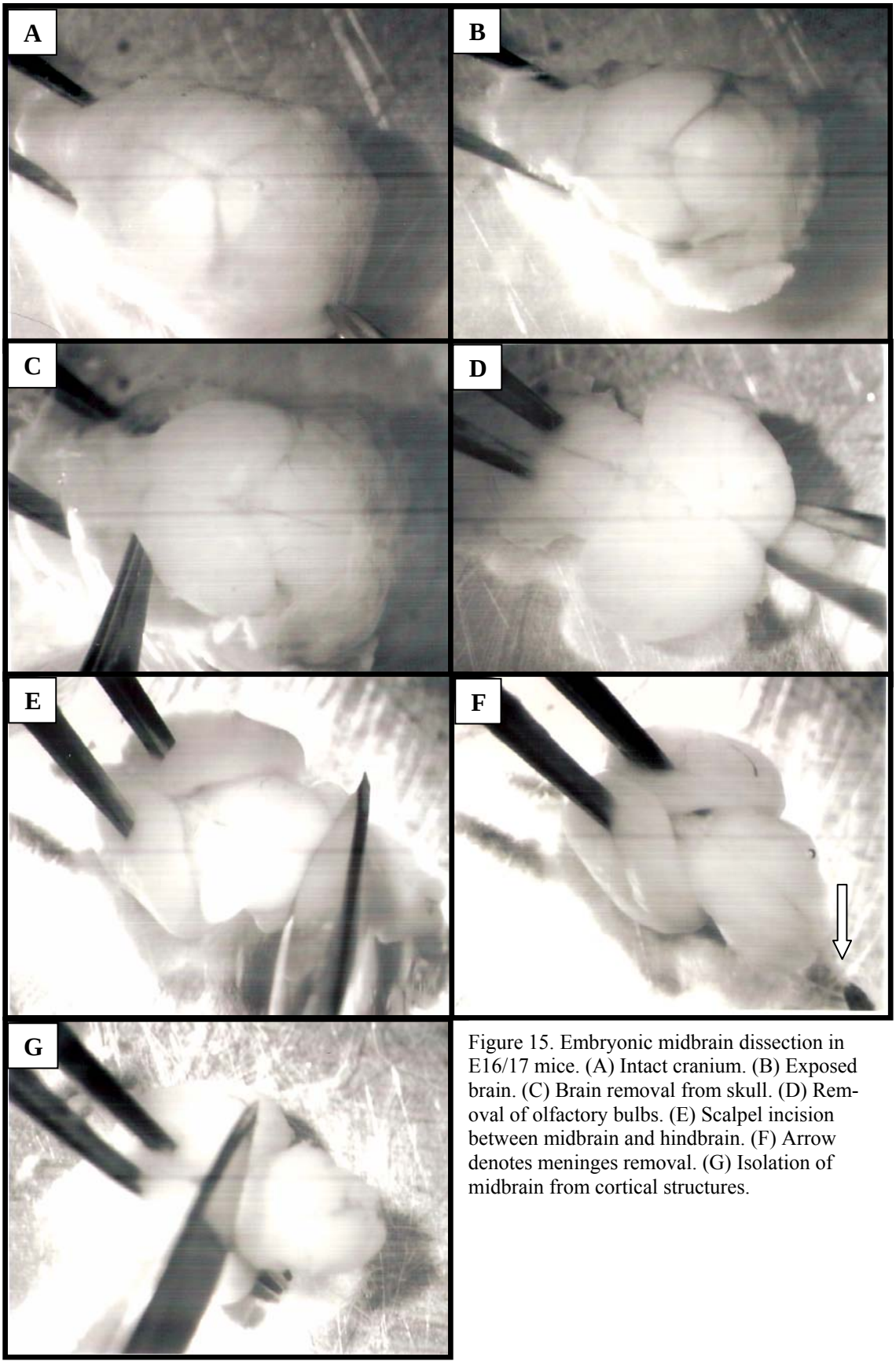

Figure 15. Embryonic midbrain dissection in E16/17 mice. (A) Intact cranium. (B) Exposed brain. (C) Brain removal from skull. (D) Removal of olfactory bulbs. (E) Scalpel incision between midbrain and hindbrain. (F) Arrow denotes meninges removal. (G) Isolation of midbrain from cortical structures.

DMEM5/5+DNase and spun down in a 3-4,000 rpm centrifuge for approximately 10-15 minutes. The supernatant is discarded, and the cell pellet is triturated in a small quantity of DMEM5/5+DNase (no more than 2-3 mL). Adding too much media to the cell suspension at this point may result in lower-than-anticipated cell counts. Because the pellet is large and the suspension solution is minimal, care should be exercised during trituration. Bubbles must not be introduced to the solution, and over-trituration should be avoided. Although the cells are separated during this process, the solution will remain turbid in appearance.

Cells are counted on a cytometer and overall cell pool dilution is calculated to 150,000 cells/mL, if possible. As mentioned previously, the cell yield in midbrain is significantly lower than frontal cortex, and a typical seeding of 10 brains yields approximately 20 MMEPs and 8 coverslips. Neuronal cultures are plated according to frontal cortex instructions (see Chapter 3). Cells are maintained in DMEM5/5 for the first 3-4 days after plating, and DMEM5 for the life of the culture.

Electrophysiological Characterization

Dopaminergic neurons possess unique electrophysiological characteristics. In general, they exhibit slow, irregular spiking with intermittent bursting. Grace et al. (2007) describes two modes of firing typically seen in dopaminergic cells *in vivo*: “single spike firing” and “bursting spike firing.” Dopamine-containing neurons have been characterized as being spontaneously active, exhibiting a characteristic pattern that oscillates between regular or irregular single spikes and rapid bursts. Firing rates and patterns have been categorized by Mameli-Engvall et al. (2006) into four categories: low firing/low bursting, low firing/high bursting, high firing/low bursting, and high firing/high bursting. This crude categorization will be further explored and improved with the aid of MEA technology.

Figure 16. Top: Single waveshape (action potential) signatures and multichannel display of all selected waveshapes across 64 electrodes. Bottom: Raster display of network spike and burst activity. Note the pattern variability and limited coordination among channels, as well as the tonic pacemaker activity of one neuron (red, third row).

In Center for Network Neuroscience (CNNS) midbrain cultures, tonic firing is frequently seen. However, there may be modulation of dopaminergic influence by the presence of GABAergic inhibition, preventing some neurons from firing spontaneously in native conditions (Grace et al., 2007). Mitochondrial dysfunction in dopaminergic neurons can decrease electrical activity, leaving them susceptible to degeneration. Liss et al. (2005) noted a concentration-dependent increase in KATP channel conductance in dopaminergic neurons from midbrain slices following MPP+ additions. Reportedly, this feature mediates much of the decreased activity and apparent neurotoxicity associated with MPP+. This is consistent with preliminary results observed at the CNNS, using the MEA *in vitro* model of Parkinson's disease.

Early recordings using these dopamine-enriched cultures show robust electrophysiological activity, characterized by high spike and burst activity of individual signals, which are less coordinated with the overall network than those in frontal cortex cultures. Periodically, embedded synchronized global network bursting is apparent. See Figure 16 for a summary characterization of early midbrain culture recordings.

A brief summary of sigmoidal dose-response curves (Fig. 17), generated from experiments using dopamine-enriched cultures, has shown greater consistency of IC_{50} values vs. frontal cortex cultures. At this time, IC_{50} values for MPP+ in midbrain fall in the range of 5 µM to 15 µM (Fig. 18). Additional studies are underway to quantify dopamine neurons present in frontal cortex vs. midbrain

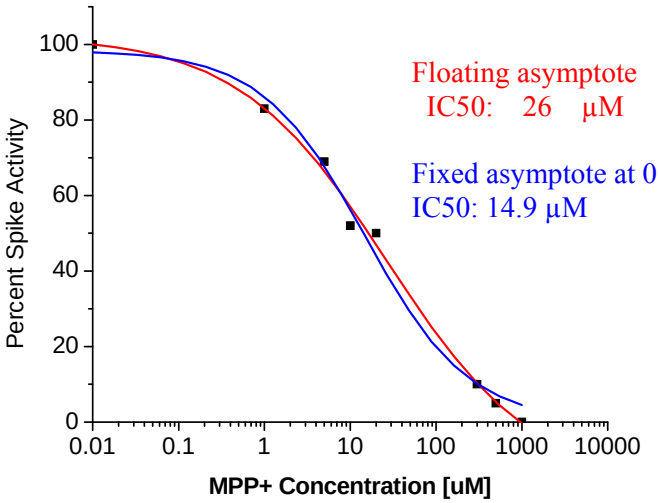

Figure 17. Preliminary dose-response data of MPP+ additions to midbrain cultures.

using staining techniques (Fig. 19). Optimistically, this will allow for continued evaluation of MPP+ as a cell culture model for Parkinson's disease using MMEP technology.

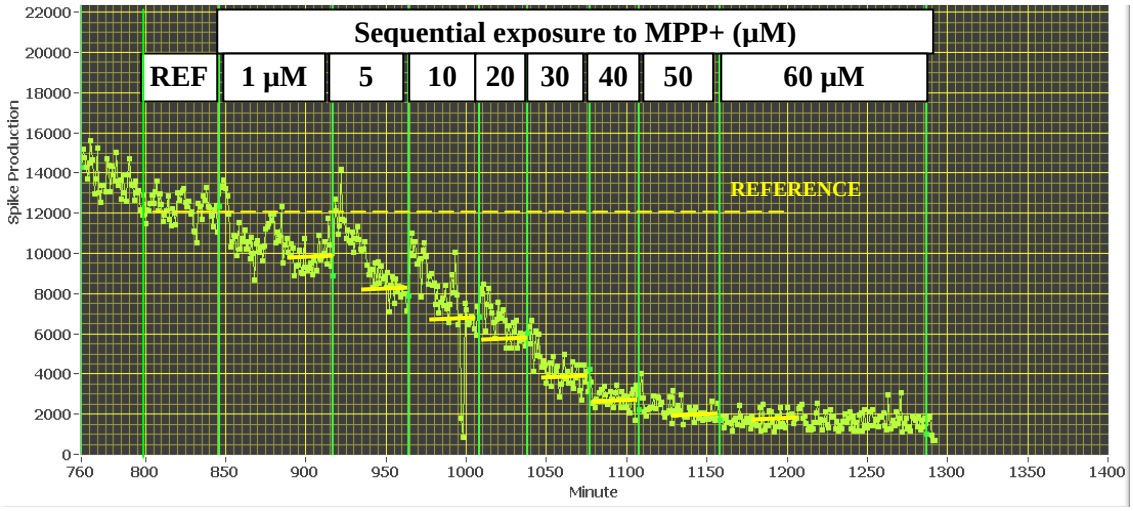

Figure 18. Example of MPP+ concentration-dependent activity loss in a mesencephalon culture. The dashed horizontal line represents the reference activity from which all activity decreases are determined. Short solid lines indicate the approximate activity plateau values used for calculation of dose-response curves.

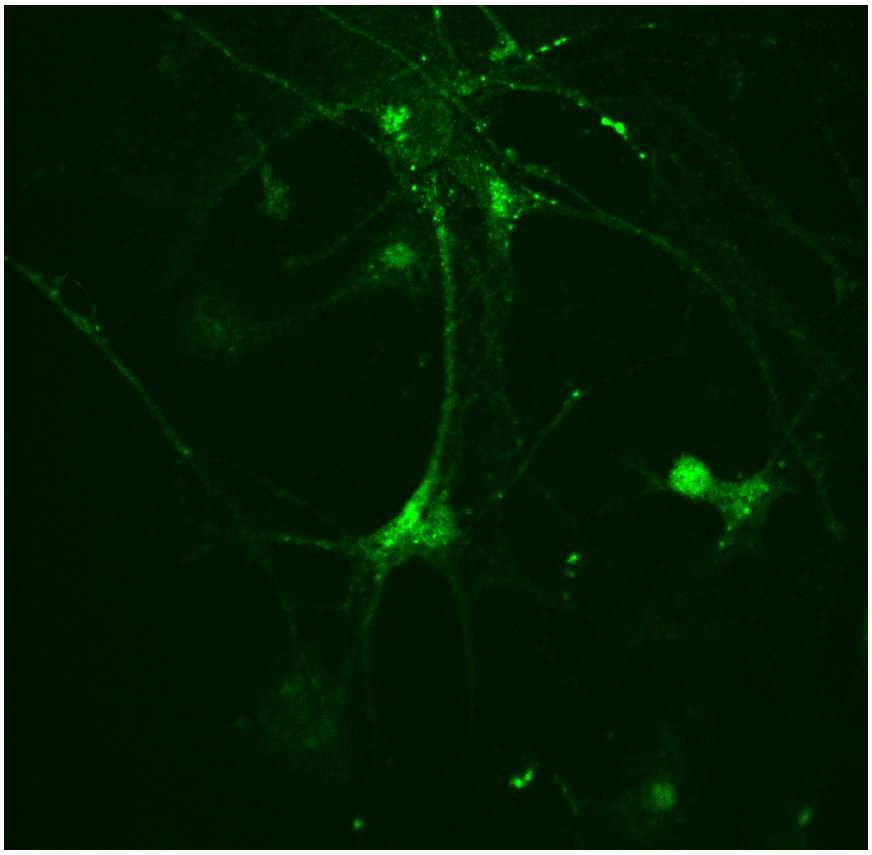

Figure 19. Early tyrosine hydroxylase immunofluorescent staining of catacholaminergic neurons in dopamine-enriched midbrain culture.

CHAPTER 5

SOLUTION DEFINITIONS AND PREPARATORY COMMENTS

The use of many chemicals and solutions is necessary for effective operation of the cell culture facility. The Center for Network Neuroscience (CNNS) adheres strictly to an experimentally-modified protocol for selection, preparation, and use of specific cell culture reagents and chemicals. Rationale for use of many of the solutions mentioned here is offered in Chapter 3. Specific culture preparation protocols which correspond with relevant solutions are offered in Chapters 3 and 4, as well as Appendix B. This chapter primarily serves to provide in-depth instruction on the selection, preparation, storage, and proper usage of chemicals and reagents which may directly or indirectly contact cell cultures. Please see Appendix A for a short-form solution formula index, including supplier catalogue numbers.

Surface Preparation Solutions

As mentioned in Chapter 3, primary cell culture is impossible to achieve without a satisfactory level of cell-to-surface adhesion. Glass and multi-microelectrode plate (MMEP) insulation material (polysiloxane) are hydrophobic culture surfaces, to which cell suspensions will not readily adhere. A torch flame prepares the surface for subsequent addition of adhesion molecules by supplying it with positively charged ions.

Following flaming, polylysine (PDL or PLL) is applied to the recording matrix area of each MMEP. The D-isomer (PDL) is the preferred form, since it is not naturally occurring, and cells do not possess enzymes to digest it. Poly-D-lysine hydrobromide is available in different lengths. In the CNNS, the 30,000-70,000 MW formula is preferred, since it is less viscous in solution and easier to mix. Many laboratories prefer the 70,000-150,000 MW formula, however, because it offers more positively charged attachment sites per molecule.

Ease of solvation is an important factor since PDL cannot be filter sterilized. Both molecular weight formulas mentioned previously are available in a cell culture-tested and γ-irradiated form, so contamination should not be a concern if mixing can be performed in a sterile manner. The PDL solution is prepared in a 0.005 mg/mL concentration. When preparing solutions, it is important to use ultra-pure water (UP H_2O; 18.3 MOhms resistivity) in mixtures that may directly contact cells. UP H_2O can be autoclaved for sterility. The PDL and UP H_2O are mixed carefully by hand under a sterile biohazard hood. Stir-bars and plates should be avoided unless they can be thoroughly decontaminated prior to use.

Once in solution, the PDL mixture is dispensed into 5 mL aliquots and immediately frozen at -10°C. Sterile solutions may be stored for up to two years. The HBr present causes PDL to form a crystalline structure in its frozen, solid state. Excess PDL per used aliquot should not be refrozen, but instead discarded to prevent damage to the aminos by ice crystals. A typical seeding cycle requires about 1-2 aliquot(s), so PDL has a relatively long shelf-life and utility.

When applying PDL, it is important to return MMEPs and coverslips to a high-humidity incubated environment as soon as possible following dispensing. PDL is toxic to cells, so ease of removal is paramount following an incubation period. If PDL is allowed to dry, the precipitate will become adhered to the surface, and form a crystal ring (see Fig. 8, Chapter 3) which can negatively affect cell health and viability. The CNNS uses a much lower concentration of PDL than that recommended by the chemical supplier (Sigma suggests 50 mg/mL), but the incubation period is significantly extended (from 5 minutes/culture to 1 day). This is beneficial when working with large numbers of cultures. After one day of incubation, PDL is rinsed from the MMEP or coverslip surface with sterile UP H_2O. Trials have shown that even if PDL is aspirated

while it is still wet, toxic precipitates may form. Therefore, rinsing prior to aspiration is required for the health of the cultures.

In lieu of PDL, poly-L-lysine (PLL) may be used as an attachment factor. The L-isomer is the naturally occurring form and is significantly less toxic to cells. However, it is also more readily digested by naturally occurring cell enzymes than PDL. PLL may be a preferred adhesion molecule for cultures with limited storage prior to experimental use. After about 60 days *in vitro*, however, significant adhesion breakdown is evident (see Fig. 7, Chapter 3). PLL is purchased from the manufacturer in sterile 0.1% solution. It is stored at 4°C in a stock bottle. Since small quantities are generally used multiple times throughout the life of the solution, it is recommended that each retrieval be passed through a sterile 0.22 μm syringe filter prior to application to prevent contamination.

One day following PDL or PLL application, the solution is rinsed and aspirated, and laminin may be applied to the surface. Laminin serves as the final attachment factor for the best possible growth and maintenance of cells in culture. It is available from the manufacturer (Roche) in a sterile 0.5 mg/mL in 0.15 M NaCl, 2 mM EDTA, and 0.05 M Tris-HCl solution with a pH of 7.4. The solution may be stored at -10°C prior to preparing sterile 160 μL aliquots into plastic v-vials. Aliquots may be stored at 4°C for up to three months and should not be filtered or refrozen.

For surface modification, 80 μL laminin is combined with 2 mL sterile UP H_2O. The manufacturer recommends dilution into culture media; however, this presents a potential risk for introduction of contamination. Since UP H_2O may be autoclaved, this mixture is equally effective and insures sterility. After mixing thoroughly, 60 μL of the laminin mixture is dispensed per MMEP matrix. This number is doubled for complete coverage of whole-flamed

coverslips. The laminin dilution breaks down rapidly, so it should be mixed immediately prior to use. It is beneficial to calculate the precise quantity required per seeding cycle, as unused portions are to be discarded. If treated MMEPs are not used the same day, they should be cleaned and reprocessed prior to next use. Laminin-coated MMEPs and coverslips should be incubated for at least 45 minutes prior to delivery of cells, to allow for maximum adhesion. Prior to dispensing the cell suspension, laminin should be completely aspirated. There is no need for rinsing, as laminin is an endogenous adhesion molecule and does not present a toxicity risk. However, the high water concentration of the mixture may cause the extracellular environment to be extremely hypoosmotic, and potentially lyse the cells within the newly plated culture. Therefore, thorough aspiration of the laminin mixture is necessary prior to seeding.

Dissection Solution

All microdissection following embryo removal is performed in a cold bath of dextrose sucrose glucose HEPES (D1SGH). D1SGH is made from three stock solutions, which should be prepared in advance and stored for ease of use. The first component is 20x saline, which is prepared by combining 160 g sodium chloride, 8 g potassium chloride, 1.8 g sodium phosphate dibasic, 0.6 g potassium phosphate monobasic, and 1 L UP H_2O. The solution is mixed on a stir-plate and filtered through a double-membraned vacuum filter unit (see instructions in next paragraph). Aliquots are prepared into 50 mL plastic centrifuge tubes and stored at 4°C until use. The second component is sucrose/glucose, which is prepared by combining 125 g glucose, 60 g sucrose, and 1 L UP H2O on a stir-plate. The solution is filtered according to the above-mentioned methods and 50 mL aliquots are prepared into plastic centrifuge tubes and stored at 4°C. The last stock solution in D1SGH is HEPES, which is made up of 58.5 g HEPES

and 1 L UP H_2O. HEPES is mixed, filtered, and stored according to directions for 20x saline and sucrose/glucose.

To prepare D1SGH, combine 50 mL 20x saline, 50 mL sucrose/glucose, 50 mL HEPES, and 850 mL UP H_2O in a 1 L capacity flask and mix on a stir-plate. The mixture is brought to a pH of 7.35 by adding HCl or NaOH and an osmolarity of 290-300 mOsm by adding UP H_2O or sucrose. Under a biohazard hood, the mixture is poured into a sterile vacuum filter unit containing a double membrane, 0.2 μm above and 0.45 μm below. The two vacuum hoses should also include a dry filter between them to prevent possible line contamination. Before turning off the vacuum pump, it is important to remove the hose from the filter unit as a defense against contamination by back-flow of air. The filter pack is always poured from the opposite spout of the hose attachment. (Note: unless otherwise mentioned, this is the preferred filtration method for virtually all solutions that require sterilization by vacuum filtration.) This preparation makes 8 sterile bottles at 125 mL each. D1SGH is stored at 4°C prior to use.

D1SGH serves as a cold anesthetic bath for embryo isolation and dissection. It also acts as a modified cerebrospinal fluid mixture to simulate the neuronal environment. It is important to maintain the osmolarity and pH of the solution by keeping bottle caps tightly closed when not in use. In addition, the temperature must be maintained to insure humane handling and sacrifice of the embryos.

As soon as the embryos are released from the mouse, they are cascaded with a stream of EtOH followed by D1SGH. They are then immersed in a petri dish filled with D1SGH and remain in this solution throughout the process. During microdissection, fresh D1SGH is poured into a series of petri dishes as distinct stages of the dissection process are completed (e.g., following removal from the uterus, decapitation, brain removal, and specific tissue isolation). If

clouding of the solution occurs (via blood, bodily fluid, or excess tissue buildup in the dish), tissue is transferred to a sterile petri dish filled with fresh D1SGH so that optical clarity is maintained through the stereoscopic microscope.

Neural Tissue Dissociation Solution

Dissociation of isolated tissue is achieved via mechanical and enzymatic means. After tissue is sufficiently minced with two sterile scalpel blades, incubation in protease is necessary to sufficiently disrupt the extracellular matrix proteins holding the cells together. In addition, established, myelinated axon tracts require the aid of chemical disruption, as mechanical means (mincing and trituration) are typically not sufficient to break apart individual cells. The CNNS uses papain, a cysteine protease present in papaya. It is obtained from the supplier (Roche) as a frozen 100 mg/10 mL solution. Papain (10 mL) is thawed and mixed with 140 mL D1SGH under sterile conditions. It cannot be filter sterilized, so working under a biohazard hood is necessary. Once in solution, 3 mL aliquots are prepared in cryovials and stored at -10°C.

About one hour prior to use, the papain mixture is transferred to a 37°C incubator to equilibrate at the enzyme's optimum temperature. Following tissue isolation and first-stage mechanical dissociation with sterile scalpel blades, 3 mL papain is added to the tissue. Frontal cortex tissue is incubated at 37°C for 5 minutes, followed by 22°C for 5 minutes. Mesencephalic tissue is incubated at 37°C for 15 minutes. After incubation, the solution is transferred to a centrifuge tube with added culture media and spun down. The supernatant must be removed nearly completely following centrifugation to remove all remaining papain and protect the cells from proteolytic damage. Second-stage tissue dissociation via trituration continues after this step.

Culture Media

Precise electrophysiological and pharmacological investigations require the use of defined, reliable culture media. The question regarding the use of serum in culture media is important for any laboratory to seriously consider. Serum-free media is often considered a more appropriate choice during electrophysiological investigation of pharmacology and toxicology, since serum albumin is relatively a non-specific binding agent. However, during the growth and development of long-term *in vitro* cultures, serum is an essential nutritional component to general culture media.

Historically, cultures prepared in serum-free media in the CNNS simply have not exhibited the same longevity or ease of maintenance as those prepared in defined media containing serum. Cultures grown in serum-free media tend to be more sensitive to pH, temperature, and osmolarity shifts, as well as mechanical forces. In addition, sensitivity to antibiotics and other chemical agents is common without the buffering ability of serum albumin present. It is always recommended that cell culture reagents offered by specific suppliers be systematically tested in the cell culture laboratory to insure consistency of results. In addition, each time a serum lot is expended, new lots should be stringently tested prior to introduction into the cell culture pool.

Dulbecco's modified eagle medium (DMEM) is the base for all medium formulations used with frontal cortex and midbrain cultures. It contains a mixture of inorganic salts, amino acids, and vitamins that must be supplemented to produce DMEM stock, the stock solution for the various culture solution formulations. DMEM stock is then used as a base to produce DMEM5 and DMEM5/5, the essential culture media used in the CNNS cell culture facility. See Table 2 for a full description of DMEM components.

DMEM stock is a serum-free, minimally nutritive base that is used in making DMEM5 and DMEM5/5. It can also serve as a medium and wash for use during pharmacological manipulations on the electrophysiological recording platform. DMEM requires the addition of a sodium bicarbonate buffer for pH maintenance during storage and incubation under 10% CO_2. Glucose is added to provide minimal nutrition at the stock stage, instead of adding it to serum-mixed media alone. To prepare, mix 1.75 g glucose,
3.7 g sodium bicarbonate, 10 g DMEM powder, and 1 L UP H_2O on a stir-plate. Carefully adjust the pH using HCl or NaOH to a final value of 7.3-7.4. Osmolarity is adjusted by adding UP H_2O or sucrose until a level of 290-300 mOsm is reached. DMEM stock is filtered using methods described above, and stored at 4°C until use. Following filtration, sterile methods must be employed when handling the stock solution.

DMEM5/5 contains 5% fetal bovine serum (FBS) and 5% horse serum (HS), although it is likely that vacuum filtration lowers the final concentrations to some extent. Serum is required to maintain the health of neuronal and glial cells alike. FBS is an important addition to culture media used in the initial seeding and early medium changes. However, around 4 days *in vitro*, it should be removed via a full medium change to prevent neuronal toxicity. Cultures are additionally supplemented with 0.5% B-27. Originally developed as a serum substitute for hippocampal cultures, B-27 has been shown to improve cell health and viability in cortical cultures, as well as those prepared from substantia nigra and striatum (Brewer et al., 1993). Ascorbic acid is added as an anti-oxidant. The efficacy of this supplement has not yet been validated.

To prepare, mix 5 mL HS, 5 mL FBS, 500 µL B-27, 200 µL vitamin C, and 89 mL DMEM stock under a biohazard hood. Caution should be exercised when working with serum as

it tends to aerate easily and form a large foam layer. Mixing is achieved by slowly inverting the solution in a bottle. Next, the solution is passed through a sterile vacuum filter. Disposable, bottle-top filter units are preferred, as the glucose and serum concentrations in DMEM-mixed media provide an ideal environment for growth of contaminants, such as fungi and bacteria. Limiting exposure to potential sources of contamination are imperative at this stage of medium preparation and storage.

Multiple bottles of DMEM5/5 may be prepared at once, although aliquots should be prepared at 100 mL or smaller to minimize exposure during use. DMEM-mixed media is stored at 4°C until one day prior to use. At this time, media is transferred to a 37°C, 10% CO_2, and 95% relative humidity incubator. The exterior of the bottle should be sprayed with EtOH prior to incubator entry. The bottle cap should be loosened while inside the incubator to allow for CO_2 to contact the sodium bicarbonate buffer.

For trituration, dilution, and plating of isolated cells, DNase I is added to the DMEM5/5. During enzymatic and mechanical dissociation of tissue, some cells are disrupted and their DNA leaks into the cell suspension. This “sticky” DNA causes cell clumping and subsequent difficulty during trituration. Adding DNase I to the plating medium reduces free DNA in the solution and provides greater trituration efficacy with less cell death due to over-trituration and introduction of bubbles to the media. DNase I is obtained from the supplier (Roche) in powder form at 100 g. It is mixed with 25 mL phosphate buffered saline (PBS; see Appendix A for instructions on preparing PBS) under sterile conditions. Aliquots are prepared into cryovials and stored at -10 ºC. Prior to cell culture, 300 µL DNase I mixture are added to 30 mL DMEM5/5 and moved to a 37°C, 10% CO_2, and 95% relative humidity incubator. This solution is used during cell pool preparation and seeding.

Table 2. DMEM Formulation	
Inorganic Salts	
Description	mg/L
$CaCl_2$ (anhydrous)	200.00
$Fe(NO_3)\cdot 9H_2O$	0.10
KCl	400.00
$MgSO_4$ (anhydrous)	97.67
NaCl	6400.00
$NaH_2PO_4\cdot H_2O$	125.00
Amino Acids	
Description	mg/L
L-Arginine HCl	84.00
L-Cystine 2HCl	62.57
L-Glutamine	584.00
Glycine	30.00
L-Histidine HCL$\cdot H_2O$	42.00
L-Isoleucine	104.80
L-Leucine	104.80
L-Lysine HCl	146.20
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.20
L-Tryptophan	16.00
L-Tryosine 2Na$\cdot 2H_2O$	103.79
L-Valine	93.60
Vitamins	
Description	mg/L
D-Ca Pantothenate	4.00
Choline Chloride	4.00
Folic Acid	4.00
Myo-Inositol	7.00
Niacinamide	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl	4.00
Other	
Description	mg/L
D-Glucose	1000.00
Phenol Red (Sodium)	15.90
Sodium Pyruvate	110.00

DMEM5 is the cell culture media used most often in the CNNS cell culture facility. It is comprised of essentially the same substrates as DMEM5/5, minus the FBS. DMEM5 is used for long term maintenance of cell cultures and may be used as a wash during experimental investigations. Bi-weekly half medium changes into DMEM5 are essential for replenishing

neurons and glia of necessary growth factors, vitamins, amino acids, and sugars, while simultaneously ridding cultures of toxic metabolic products, such as lactate and ammonium. Full medium changes after those initially described in Table 1 should be avoided, so as not to undermine medium conditioning by glial cells.

DMEM5 is prepared according to instructions provided for DMEM5/5 preparation. The following reagents are mixed, vacuum filtered, and stored as specified: 5 mL HS, 500 µL B-27, 200 µL vitamin C, and 94 mL DMEM stock. In addition, 1 mL L-glutamine (as prepared in Appendix A) is added to each bottle of media immediately prior to use. L-glutamine is known to be one of the most essential components of culture media for cell health, but also one of the most volatile (Minamoto et al., 1991). Because it rapidly breaks down both in culture and in media storage, it is added at the last stage in media preparation, immediately prior to medium changes. DMEM5 is used for the life of the culture (see Table 1 for medium change schedule, describing use of DMEM5/5, DMEM5/5+DNase, and DMEM5).

CHAPTER 6

COMMON PROBLEM ENCOUNTERED IN THE CELL CULTURE LABORATORY

Primary neural networks obtained from embryonic murine tissue are known to be spontaneously active, exhibiting complex electrophysiological spike and burst patterns (Martinoia et al., 2005; Bettencourt et al., 2007; Rubinsky et al., 2007). However, networks must reach a particular stage of development in culture before electrophysiological recording of such dynamic activity is possible. After a general time frame of about three weeks in culture, sufficient synaptic connections are thought to have formed and strengthened to elicit a stereotypic shift from single spike patterns to complex bursting (Chen et al., 2006). Problems are seen in the Center for Network Neuroscience (CNNS) facility when cultures are utilized experimentally after too few days *in vitro*. Action potentials are generally smaller, spikes are less organized with regard to overall network activity, and general spike and burst rates are more sluggish than those of older, more established cultures.

Relating morphology of networks maintained in the cell culture facility to general neuronal health and predicted electrophysiological ability can be challenging. Knowing when cultures are mature enough for experimental use is not the only problem faced by cell culture personnel and researchers. Many problems have been addressed throughout the body of this thesis. The currently pressing issues of osmolarity control, crystal precipitates in culture media, and general contamination prevention and contingency are discussed in this chapter.

Osmolarity Control

Osmolarity is maintained via ultra-pure water (UP H_2O) additions during medium changes. Care should be taken to systematically test the osmolarity of the cultures, because many complex factors can influence it. For example, should the surface area of the water reservoir of

the culture incubator be diminished, the relative humidity inside of the incubator will drop; subsequently, the culture media may evaporate slightly. If cell cultures are not handled carefully and media is allowed to spill out of the gasket, the osmolarity may rise dramatically. Over time, osmolarity drift is inevitable. It is advisable to initially add 50-100 µL UP H_2O to every 3 mL volume culture to counter this drift. Quantities should be adjusted according to actual evaporation rates. Additions larger than 100 µL at one time may induce osmotic shock and should be avoided.

Media Precipitates

Within the past few years, the CNNS cell culture facility has been struggling to determine what causes a seemingly random aggregation of stereotypic crystalline structures in the culture media. Systematic analysis of cultures plated with and without adhesion factors provides evidence that these crystal precipitates form in the absence of initial surface preparation molecules. In addition, media in any form (Dulbecco's modified eagle medium (DMEM) stock without serum or serum additives, DMEM5 with horse serum (HS), and DMEM5/5 with HS and fetal bovine serum (FBS)) cannot develop these specific crystal precipitates in the absence of cells.

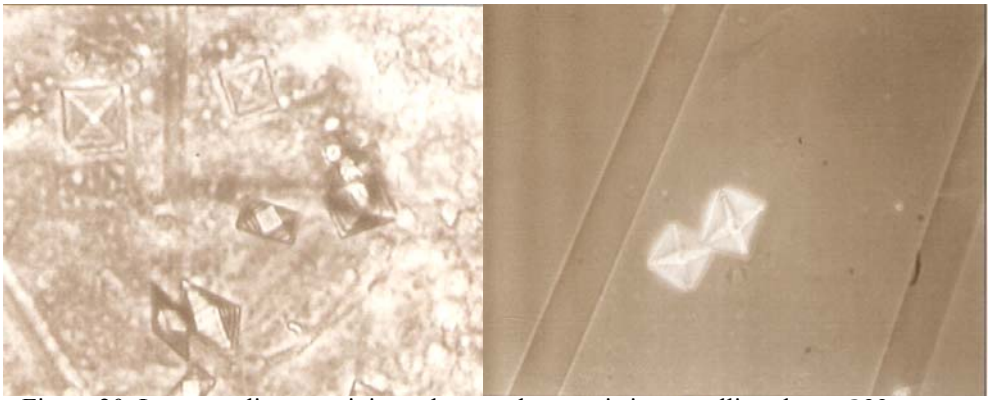

Figure 20. Large medium precipitates have a characteristic crystalline shape. *200x magnification.*

It appears as though some biochemical interaction between the culture media and the neuronal network itself gives rise to these precipitates. Since they are insoluble in water, NaCl and other water-soluble substances do not appear to be the source of the crystal structures. In fact, culture medium conditions are maintained at a relatively constant osmolarity, much lower than that necessary to form NaCl crystal aggregates. The serum present in the media is rich in calcium, and calcium precipitations are not uncommon in serum-rich media. However, the stereotypic crystalline structure of these complexes (see Fig. 20) has a strikingly similar morphology to that of calcium oxalate monohydrate, the major component of kidney stones (Wesson et al., 1998).

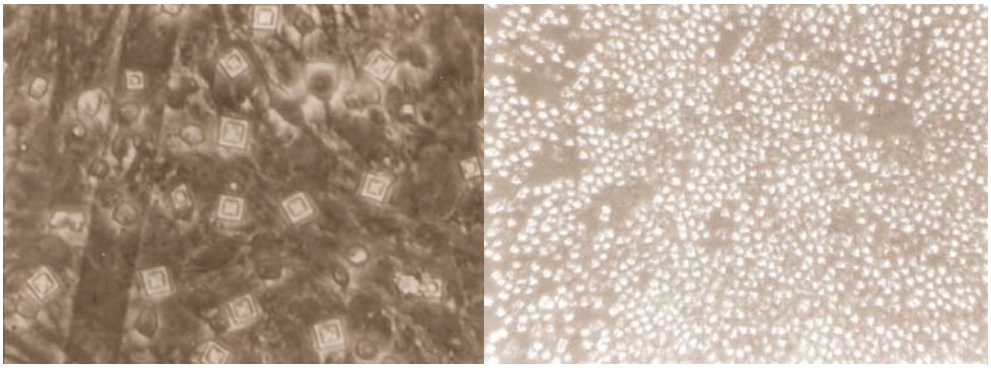

Figure 21. Increase in crystal number appears to be correlated with high osmolarity levels, when crystals are initially present in the media. Large fields of crystals (right) dramatically reduce electrophysiological activity and cell health. *200x magnification.*

Although it is hypothesized to be the source of these precipitate crystals, it is unknown how or why calcium oxalate may form in neuronal cultures. In fact, there exists no mention of such structures in primary neuronal cultures in the literature. It seems unlikely that this phenomenon is solely observed in the CNNS; instead, it is assumed that reports of these specific crystals are absent due to an inability to definitively isolate, identify, and reproduce these structures. Experimentally forced calcium precipitation as insoluble oxalate crystals is a laborious task that has been used to determine the level of calcium present in sera (Sendroy, 1944). The apparently random, natural (though potentially pathological) development of

supposed calcium oxalate precipitation in media is not well understood, although it appears as though increased osmolarity may lead to an increase in crystal number and size.

Calcium oxalate in mammals is generally associated with kidney stone formation. Oxalate poisoning may occur by ingesting large quantities of oxalic acid (Sanz & Reig, 1992) or via oxalosis, a condition following kidney failure, in which large quantities of oxalate are released into the blood and spread to the eyes, bones, muscles, heart, and brain (Akhan et al., 1995). Calcium oxalate precipitation is also caused by ethylene glycol poisoning. When ethylene glycol is consumed, it is metabolized into other toxins, such as glyceraldehydes, glycolic acid, glyoxalin acid, and finally, oxalic acid. Oxalic acid combines with calcium to form calcium oxalate crystals (Barceloux et al., 1999). Oxalate crystal formation is very dangerous to animals, and may cause burning sensations, swelling, choking, digestive upset, difficulty breathing, coma, and/or death (Chaplin, 1977).

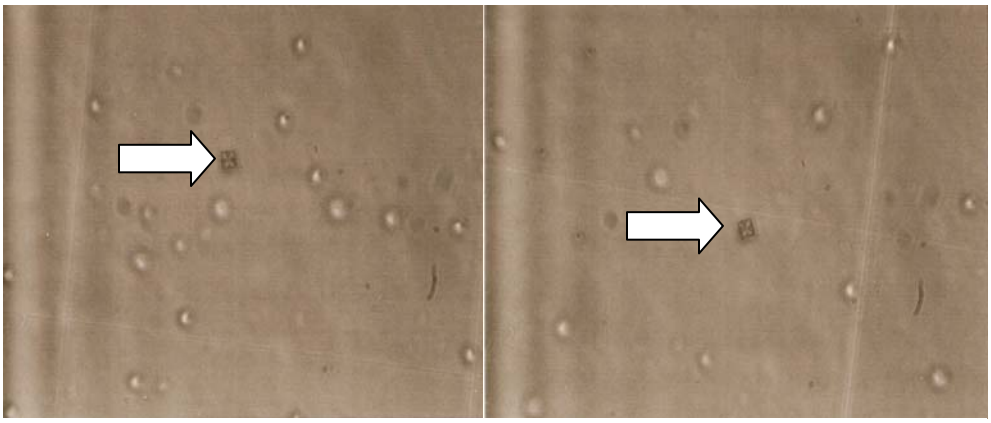

Figure 22. Small, newly formed crystal precipitates in media on cell counting chamber (arrows). Round, phase-bright circles are dissociated cells. *200x magnification.*

Crystal formation in neuronal culture media does appear to have a toxic effect. Cell health declines in proportion to the number of developing crystals. Although electrophysiological recording is not perceptibly affected when a small number of crystal precipitates is present, osmolarity should be tightly controlled in cultures where crystals are found in an effort to protect

cell health and viability (see Fig. 21). Recently, a supposed calcium oxalate crystal was observed to be present on the cytometer during cell counting (see Fig. 22). Until this point, it was believed that crystal precipitation took days to weeks to fully develop.

Contamination

Contamination is perhaps the most threatening risk to the cell culture facility. Although many advanced technologies exist to aid in the prevention of contamination, such as separate air handling systems and self-decontaminating incubators, measures can be taken to prevent or control contamination on virtually any laboratory budget. Different facilities encounter unique problems with regard to culture contamination. Since the CNNS does not work with primary cell lines, cross-contamination of lines is not a concern. Instead, fungal and bacterial infection prevention is paramount, as mixed-neural network cultures contain little immunological defenses and are often stored in the long term (weeks to months). In addition, antibiotic-free culture methodology is absolutely essential for electrophysiological investigation of pharmacology and toxicity using the multielectrode array (MEA) platform.

Perhaps the most common cause of contamination is poor laboratory practice. Researchers should pay careful attention to always abide by sterility guidelines set forth in the cell culture facility. Care and respect of the facility equipment, products, and reagents which may encounter cells at any stage of preparation or storage is crucial. First and foremost, laboratory personnel should focus on sterility in all arenas, including appropriate culture facility attire and hygiene, solution preparation and handling, decontamination of equipment, and actual handling of cell cultures. Contaminated cultures are virtually useless in research practices, and large amounts of time, effort, and monetary resources are wasted when a high level of vigilance with regard to sterile procedures is not maintained by each individual with facility access. In short,

complex and specialized research efforts cannot exist without the relatively simple, often overlooked, and critical understanding that sterility is key to successful cell culture and maintenance.

The first line of protection against bacterial and fungal agents is an understanding of the concepts of permissive versus hostile environments. The laboratory should be an extremely hostile environment for unwanted microorganisms, while allowing the healthy growth and maintenance of cultured neurons. This balance can be hard to achieve, especially when dealing with fungal contamination, as fungicides often target eukaryotic cells in similar ways. Antibiotic agents are generally safe for use with cultured neural cells, although they may have dramatic effects on native-state cellular activity and electrophysiological response to pharmaceutical agents.

General organization of the cell culture facility greatly reduces contamination risk. Three main vent hoods and six incubators are used in the CNNS cell culture laboratory. Mouse sacrifice and dissection takes place under a biohazard hood with sash to prevent airborne contaminants from entering the room. Microdissection is performed under a separate laminar flow hood outfitted with a stereoscopic microscope and camera mount (see Fig. 23). Tissue preparation, medium changes, and solution preparation takes place under a separate biohazard hood. All hoods are outfitted with air filters and UV decontamination lights. The latter hood is housed within the culture facility's adjacent clean room. This separate clean room is maintained at the highest level of sterility. In addition to the biohazard hood, it contains five incubators, a microscope station, and storage for supplies. The clean room is fitted with an overhead UV decontamination lamp and charcoal filter to absorb ozone.

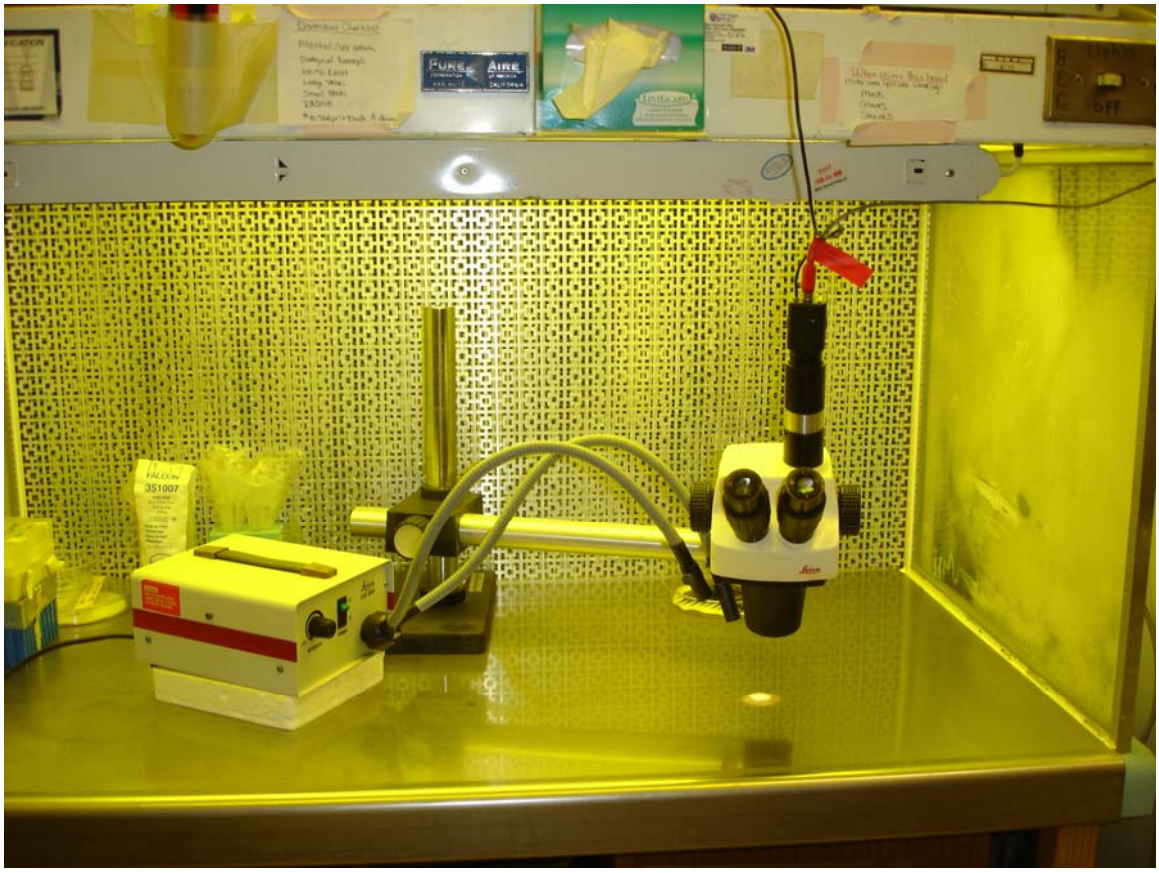

Figure 23. Open vent hood for microdissection of embryonic mouse brains. Separate hoods are necessary for mouse sacrifice, microdissection, and media preparation and culture handling in an effort to prevent cross-contamination.

Four of the clean room's five incubators are used for culture storage and maintenance. They each hold nine copper-plated shelves and a reservoir for humidification water. Cultures are rotated within the incubators on a regular basis, to allow for monthly decontaminations. Although the incubators possess an auto-decontamination feature, the walls and doors are manually cleaned with 70% EtOH prior to decontamination. In addition, the incubator shelves are autoclaved separately before the cycle is run. After decontamination, fresh incubator water is added. It is important to only use deionized water (DI H_2O) that has been autoclaved within the incubators. As a precautionary measure, 500 mL $CuSO_4$ (see Appendix A) are added to 2.5 L DI H_2O. This mixture is added to the incubator reservoir to maintain humidity while simultaneously

preventing microbial growth. Care should be exercised when working with and disposing of $CuSO_4$-tainted water, as it is toxic to both prokaryotic and eukaryotic organisms. The fifth incubator in the clean room specifically houses cell culture solutions. Here, culture media and reagents are equilibrated prior to use.

A separate incubator is maintained outside of the clean room for researcher's media and solutions, as well as short-term culture storage. Since this incubator is used by many individuals for multiple purposes, it must be decontaminated on a regular basis. It is important to separately house cell culture reagents and solutions that are used by researchers and miscellaneous personnel in the laboratory. Cross-contamination is extremely common when an uncontrolled number of individuals are in direct contact with critical cell culture reagents. It is also important to control reentry of cultures to the clean room incubators. If a culture is viewed under the microscope within the clean room under sterile conditions, it is generally permissible to return it to its appropriate storage incubator. If a culture is removed from the clean room, however, it must not, under any circumstances, be returned to a clean room incubator and risk contaminating the other cultures housed within. Instead, it should be transferred to the external incubator prior to use.

All personnel are required to carefully and completely abide by protocols for proper clean room hygiene and attire. This includes thoroughly washing hands with antimicrobial soap prior to wearing gloves. In addition, a sterile laboratory coat, cap, mask, sleeves, and booties must be worn prior to entry in the clean room. Gloves, sleeves, and booties are generously sprayed with 70% EtOH prior to entry. While working in the clean room for extended periods of time, gloves are sprayed periodically with EtOH. During critical dissection and tissue isolation procedures, gloves are changed during each stage.

Equipment and surfaces should be handled with care and regularly decontaminated. Solutions of 2% bleach and 70% EtOH are maintained in spray bottles for ease of surface decontamination. Floors are regularly mopped with a mixture of antimicrobial soap and bleach. The clean room and hoods are UV sterilized regularly. Autoclaved materials are transferred to a sterile drying oven to reduce the risk of bacterial or fungal contamination within the residual water from autoclaving. Tools are properly wrapped to prevent contamination in storage.

All objects entering the hood (other than culture reagents) are sprayed generously with 70% EtOH and placed under UV decontamination prior to use. It is important to note, however, that ultraviolet radiation alone is not always sufficient to completely eradicate microbial organisms. UV cannot reach all areas of the room or biohazard hood. In addition, studies show that repeated exposure to irradiation may increase chances of survival of UV-resistant bacterial mutants (Bower & Daeschel, 1999; Rames et al., 1997).

The CNNS produces all primary cultures in the absence of antibiotic agents. Studies have shown that beta-lactam antibiotics commonly used in cell culture, such as penicillin and streptomycin, can induce electrophysiological and pharmacological response pattern changes *in vitro*. Such antibiotics have been shown to dramatically reorganize native state global bursting and reduce spike and burst rates in culture, even under concentrations recommended for use in cell culture applications. In addition, shifts in dose-response curves have been observed during pharmacological investigations under cultures treated with antibiotics, implying a sensitization to some drugs, especially GABA agonists (Hollmuller, Rijal, & Gross, 2005; Lebedeva, 2001; Fujimoto, Munakata, & Akaike, 1995).

Due to the overwhelming evidence that antibiotic agents have a significant effect on cellular function *in vitro*, pretreatment should be avoided. In rare cases, contaminated cultures

may require rescue with antibiotics. In this situation, ampicillin or gentamicin are the preferred agents for use, as they are gentler and produce less perceptible effects on electrophysiological activity and pharmacological sensitivity (Hollmuller, Rijal, & Gross, 2005). Because periodic contamination cannot always be avoided, contingencies should be put into effect as soon as contamination is spotted.

Fungal contamination is perhaps the most common and easily recognized form of microbial growth. Dermatophytes can be easily transferred to cell cultures, reagents, or equipment if sterile practices are not strictly followed. Fungi may be slow or fast growing in culture and culture media. Microscopically, it is observed as filamentous growth, with outward budding of hyphae (see Fig. 24). If left untreated, fungal contamination becomes visible to the naked eye as a white, yellow, or green growth. In cultures, media will become gelled prior to developing a stereotypic "fuzzy" layer of mold at the air interface. In media, fungus may be perceived as a change in the viscosity of the solution. As soon as fungal growth is recognized, all affected cultures should be disposed of immediately. It is impractical to treat mold or yeast-contaminated cultures with fungicides, since such agents will dramatically decrease viability of all eukaryotic organisms, including cultured neurons. As a general rule, petri dish lids should never be removed from cultures within the clean room, except under the biohazard hood during medium changes.

Infected cultures are removed and taken to a sink on the opposite side of the culture facility. All affected media is saturated with a solution of bleach and soap, to eradicate fungus while preventing airborne spore contamination. Cultures found to be

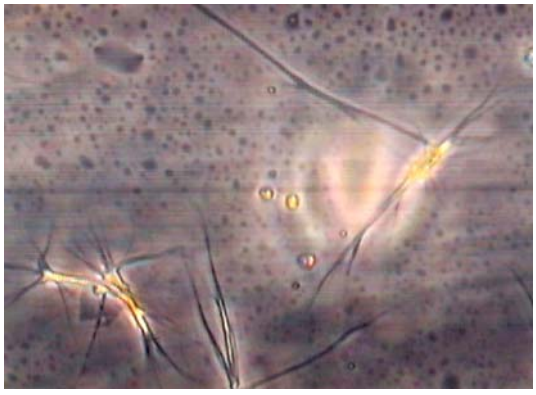

Figure 24. Fungus isolated from contaminated media. *200x magnification.*

clear of fungus which were housed in an incubator alongside affected cultures should be quarantined for at least one month to insure sterility. Contaminated media, supplies, and incubators should be discarded or thoroughly sterilized.

In an effort to prevent or contain contamination wherever possible, cultures should receive medium changes, osmolarity checks, and general maintenance in a "clean-to-dirty" fashion. This means that newly seeded cultures should always be attended to first, followed by older cultures, and finally, cultures that present a contamination risk. During medium changes, no more than four cultures should be handled with the same pipette, in an effort to prevent spread of infection.

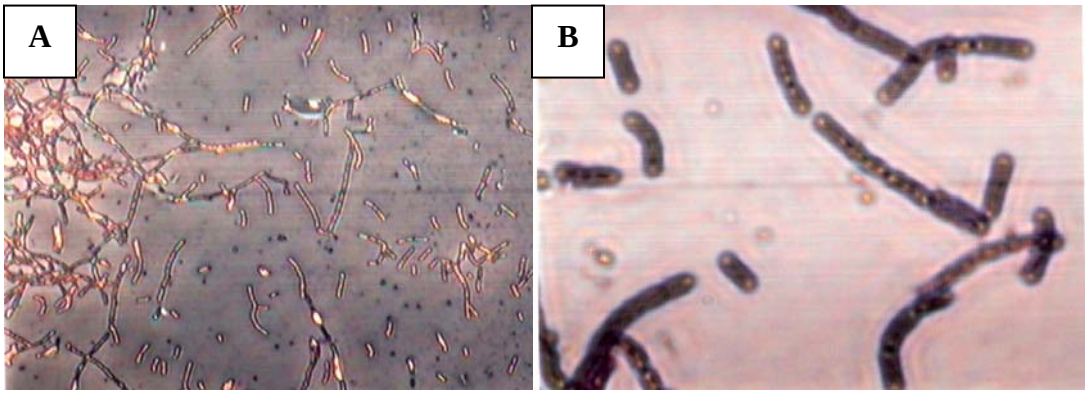

Figure 25. Gram-positive chains of *Bacillus sp.*, isolated from contaminated media, cultured on brain and heart infusion. (A) *200x magnification*. (B) *800x magnification*.

Bacterial contamination is often harder to recognize, as it is primarily only microscopically evident (see Fig. 25). Cultures should be evaluated under the microscope on a regular basis. Should a culture reach turbidity that is recognizable by the naked eye, it is likely that bacterial contamination has been present for a long time and spread to other cultures within the incubator. Brownian movement of precipitates and solid culture additives, as well as cell debris, is often mistaken for bacteria. Therefore, sampling of culture media (prior to use and following conditioning by cells) is recommended. Sterility tests utilizing brain and heart

infusions in nutrient broth or agar are an additional preventative measure that may detect infected solutions prior to interaction with cells. Cell culture media is a permissive environment for any microbial growth, as it is rich in sugars, amino acids, and vitamins. Care should be exercised when handling media and sera to insure sterility of solutions as well as culture surfaces. If media is spilled on a solid surface, it should be immediately cleaned with bleach or EtOH. Given the resilient and opportunistic nature of microbes, it is imperative to appreciate that this diverse and often fastidious group of organisms will flourish under even seemingly sterile conditions. Therefore, care should be taken to always remain vigilant within the cell culture facility.

APPENDIX A

SOLUTION INDEX

Stock Solutions

20X SALINE
- 160 g NaCl (Sigma #S9625)
- 8 g KCl (Sigma #P8041)
- 1.8 g Na_2HPO_4 (Sigma #S5136)
- 0.6 g KH_2PO_4 (Sigma # P5379)
- 1 L UP H_2O (Barnstead ultra pure water system)

- Mix and filter for sterility.
- Prepare 50 mL aliquots in plastic centrifuge tubes.
- Store at 4 ºC.

B-27
(Gibco #17504-044)
- Store at -10 ºC.

COPPER SULFATE ($CuSO_4$)
- 10 g $CuSO_4$ (Sigma #C8027)
- 500 mL DI H_2O

- Mix and autoclave in glass bottle at 121 ºC.
- Add to incubator reservoirs along with 2.5 L DI H_2O for a final volume of 3 L.

D1SGH
- 50 mL sucrose/glucose (see solution definition)
- 50 mL 20x saline (see solution definition)
- 50 mL HEPES (see solution definition)
- 850 mL UP H_2O (Barnstead)

- Bring pH to 7.35 using HCl or NaOH.
- Bring osmolarity to 290-300 mOsm using sucrose or UP H_2O.
- Filter for sterility.
- Prepare sterile aliquots into 125 mL glass bottles and store at 4 ºC.

DMEM STOCK
- 1.75 g $C_6H_{12}O_6$ (Sigma #G7021)
- 3.7 g $NaHCO_3$ (Sigma #S5761)
- 10 g DMEM (Hyclone #SH30002.02)
- 1 L UP H_2O (Barnstead)

- Bring pH to 7.3-7.4 using HCl or NaOH.
- Bring osmolarity to 290-300 mOsm using sucrose or UP H_2O.
- Filter for sterility.
- Store at 4 ºC in 1 L glass bottle.

DNase I
- 100 mg DNase I (Roche #104159)
- 25 mL PBS (see solution definition)

- Mix and filter for sterility.
- Prepare sterile aliquots into 600 µL cryovials.
- Store at -10 ºC.

FETAL BOVINE SERUM (FBS)
(Gibco #10082-147)
- Thaw and dispense into 50 mL aliquots in plastic centrifuge tubes under sterile conditions.
- Store at -10 ºC.

HEPES
  - 58.5 g HEPES (Sigma #H4034)
  - 1 L UP H_2O (Barnstead)
- Mix thoroughly and filter for sterility.
- Prepare 50 mL aliquots into plastic centrifuge tubes.
- Store at 4 ºC.

HORSE SERUM (HS)
(Gibco #26050-088)
- Thaw and dispense into 50 mL aliquots in plastic centrifuge tubes under sterile conditions.
- Store at -10 ºC.

L-GLUTAMINE
  - 5 g L-glutamine (Sigma #G6392)
  - 200 mL UP H_2O (Barnstead)
- Mix thoroughly and filter for sterility.
- Prepare 15 mL aliquots in plastic centrifuge tubes.
- Store at -10 ºC.

LAMININ
(Roche #1243217)
- Thaw and dispense into 160 µL aliquots in plastic v-vials under sterile conditions.
- Store at 4 ºC.

PAPAIN
  - 140 mL D1SGH (see solution definition)
  - 10 mL papain (Roche #108014)
- Mix under sterile conditions in biohazard hood.
- Prepare sterile aliquots in 3 mL cryovials.
- Store at -10 ºC.

PHOSPHATE BUFFERED SALINE (PBS)
  - 0.04 g KCl (Sigma #P8041)
  - 0.04 g KH_2PO_4 (Sigma #P5379)
  - 1.6 g NaCl (Sigma #S9625)
  - 0.43 g Na_2HPO_4 (Sigma #S5136)

- 200 mL UP H_2O (Barnstead)

- Mix thoroughly and filter for sterility.
- Prepare 50 mL aliquots into plastic centrifuge tubes.
- Store at -10 ºC.

POLY-D-LYSINE (PDL)

- 5 mg PDL (Sigma #P7280)
- 1 L UP H_2O (Barnstead)

- Mix under sterile conditions in biohazard hood.
- Prepare sterile aliquots in 5 mL cryovials.
- Store at -10 ºC.

POLY-L-LYSINE (PLL)
(Sigma #P4707)

- Store at 4 ºC.
- Syringe filter prior to each use.

SUCROSE/GLUCOSE

- 125 g $C_6H_{12}O_6$ (Sigma #G7021)
- 60 g $C_{12}H_{22}O_{11}$ (Sigma #S1888)
- 1 L UP H_2O (Barnstead)

- Mix thoroughly and filter for sterility.
- Prepare 50 mL aliquots in plastic centrifuge tubes.
- Store at 4 ºC.

VITAMIN C

- 2 g L-ascorbic acid (Sigma #A0278)
- 50 mL UP H_2O (Barnstead)

- Filter for sterility.
- Prepare sterile 5 mL aliquots in plastic vials.
- Store at 4 ºC.

Surface Preparation Solutions

PDL or PLL

- Apply approximately 60 µL per matrix following flaming.
- PDL must be thawed prior to use and cannot be refrozen.
- PLL should be syringe filtered prior to use.
- Store MMEPs at 37°C, under 95% relative humidity to prevent drying and precipitation.

LAMININ

- 80 µL laminin
- 2 mL UP H_2O

- Vortex in centrifuge tube.
- Apply 60 µL laminin/UP H_2O mixture to each MMEP matrix (x2 for coverslips).
- Incubate for at least 45 minutes prior to seeding.

Dissection Solution

D1SGH

- Maintain at 4°C throughout use.
- Completely immerse embryos and tissue throughout dissection.

Neural Tissue Dissociation Solution

PAPAIN

- Incubate aliquot at 37°C for at least 1 hour prior to use.
- Time papain digestion carefully to avoid lysis of cells.

Culture Media

DMEM5/5

- 5 mL horse serum
- 5 mL fetal bovine serum
- 500 µL B-27
- 200 µL vitamin C
- 89 mL DMEM stock

- Mix and filter for sterility.
- Store in sterile 100 mL glass bottle at 4°C.
- One day prior to use, transfer to 37°C, 10% CO_2, and 95% relative humidity incubator.
- For use with initial medium changes of newly seeded cultures.

DMEM5/5+DNase I

- 300 µL DNase I
- 30 mL DMEM5/5

- Mix in sterile centrifuge tube.
- Store at 37°C, under 10% CO_2 and 95% relative humidity prior to use.
- Use for trituration, cell dilution, and seeding.

DMEM5

  - 5 mL horse serum
  - 500 µL B-27
  - 200 µL vitamin C
  - 94 mL DMEM stock
  - 1 mL L-glutamine*

- Mix and filter to insure sterility.
- Store in sterile 100 mL bottle at 4°C.
- One day prior to use, transfer to 37°C, 10% CO_2, and 95% relative humidity incubator.
- *L-glutamine should be thawed and added immediately prior to feeding with this solution.
- Use for medium changes throughout the life of the culture.

APPENDIX B

ABBREVIATED CELL CULTURE PROTOCOL FOR FRONTAL CORTEX (QUICK REFERENCE GUIDE)

Note: See reference figures in Chapter 3 for visual aid in following protocol.

Material Preparation: Culture Day -2

1. Remove excess silicone grease from MMEPs with the aid of a cell scraper and cotton.
2. Clean MMEPs with an enzymatic detergent, and rinse under deionized (DI) water.
3. Degrease rubber gaskets with a razor blade, and soak them in 70% EtOH.
4. Fit gaskets with a thin line of silicone grease to form a water-tight seal upon adherence to MMEPs.
5. Clean coverslips with 70% EtOH.
6. Autoclave MMEPs, coverslips, and gaskets for 25 minutes at 121°C.
7. Store all sterile materials under a biohazard or laminar flow hood overnight.

Surface Preparation: Culture Day -1

1. Flame recording area of MMEPs using a butane torch and flaming mask.
2. Flame coverslips across the entire surface with a sweeping motion.
3. Fit MMEPs with greased gaskets, insuring that a proper seal is made.
4. Place all materials under a UV lamp for approximately ten minutes to insure sterility.
5. Transfer MMEPs and coverslips to individual, sterile petri dishes.
6. Label dishes with identifying information (e.g., MMEP number, tissue type, seeding date, and any special instructions or descriptions).
7. Add 50-100 μL PDL solution to the recording area (matrices) of each MMEP. Double or triple the quantity added to coverslips to cover the entire flamed surface.
8. Store MMEPs and coverslips in a sterile culture incubator at 37°C, under 10% CO_2 and 95% relative humidity.

Surface Preparation: Culture Day

1. Remove MMEPs and coverslips from the incubator and place under a biohazard hood.
2. Rinse PDL from each surface using autoclaved UP H_2O and sterile pipettes.
3. Apply 60 µL laminin solution to each MMEP matrix (doubled or tripled for each coverslip).
4. Return MMEPs and coverslips to the incubator and allow to sit undisturbed for at least 45 minutes prior to seeding cells.

Mouse Sacrifice: Culture Day

1. Within a laminar flow hood, place E16/17 timed-pregnant mouse into a chloroform or halothane equilibrated chamber.
2. Anesthetize until no perceptible motor activity is apparent.
3. Remove mouse, and place dorsal side-up onto a stack of clean absorbent bench pads.
4. Using a blunt object such as a pen or screwdriver, perform a cervical dislocation to insure complete paralysis and sacrifice by:
    a. Applying firm pressure on the cervical spine with the non-dominant hand, and
    b. Pulling the tail with the dominant hand until a complete dislocation of the spinal column is perceived.

Embryo Dissection: Culture Day

1. Place mouse ventral side-up.
2. Remove any urine or fecal matter released during sacrifice with a clean wipe.

3. Fully saturate the fur and skin, using 1.5% bleach in a squeeze bottle, from anus to neck.
4. Follow with 70% EtOH.
5. Using a pair of sterile forceps, grasp the skin just superior to the vaginal opening, while simultaneously making a lateral incision with a pair of sterile, fine dissection scissors.
6. Insure that all dermal layers, subcutaneous fat, and the abdominal wall are punctured, being careful not to disrupt the uterine embryonic sacs.
7. Extend incision in a V-shape to the distal regions of the lower thoracic area (bikini cut).
8. Fold back excess skin and tissue to reveal the uterine structure.
9. With forceps, carefully lift uterus (between embryos to minimize damage), and cut away at connective tissue with a dissection scissor until the entire uterus is freed.
10. Quickly rinse uterus under 70% EtOH, followed by a D1SGH rinse to remove blood and body fluids.
11. Immerse uterus in a petri dish containing a cold (4°C) anesthetic bath of D1SGH, and transfer to a sterile dissection area.
12. Release each embryo from uterus and interior embryonic sac using a pair of fine-tipped forceps. Ensure that umbilical cord has been severed and placental sac removed.
13. Transfer each embryo to a fresh petri dish filled with cold D1SGH.
14. Decapitation should be performed quickly. With the non-dominant hand, squeeze a pair of forceps around the neck of the embryo. With the dominant hand, simultaneously pass a close-tipped pair of sharp forceps firmly across the neck, ventrally to dorsally.
15. Transfer the released crania to a fresh petri dish filled with D1SGH.

Microdissection: Culture Day

1. Arrange petri dish beneath a stereoscopic microscope for the remainder of the dissection.
2. One embryo at a time, grip the skull by placing a pair of forceps through the ocular cavities with the non-dominant hand.
3. Working rostrally to caudally, remove the skin and underlying muscle tissue with a pair of fine-tipped forceps.
4. Puncture the cartilaginous skull between hemispheres where the cortical lobes meet.
5. Peel back the skull to reveal the underlying brain.
6. Carefully release the olfactory bulbs with close-tipped forceps.
7. Remove the brain by slowly working closed forceps caudally, from an inferior position.
8. Release the hindbrain, and transfer brain to a fresh petri dish filled with D1SGH.
9. Gently remove the meninges from the frontal lobe of each brain, being careful not to puncture the cortical tissue.
10. To remove the frontal cortex, grip the hindbrain or midbrain with the non-dominant hand.
11. With the dominant hand, hold a pair of forceps at a vertical angle, and puncture the most superior region of one hemisphere, distal to the midline.
12. Squeeze the forceps to produce a half-v-shaped cut.
13. Opposite this cut, close to the midline of the same hemisphere, produce another incision to complete the v-shape.
14. Perpendicular to the v-incision, with forceps pointing downward, produce a third cut to ablate the tissue.
15. Using a fine-bore pipette, transfer the tissue to a smaller petri dish, filled with a thin layer of D1SGH. This petri dish should only house isolated frontal cortex tissue.

16. On the opposite hemisphere, perform the same technique to remove the frontal cortex.
17. Repeat with remaining embryos as necessary.

Tissue Dissociation & Seeding: Culture Day

1. Transfer tissue to a sterile biohazard hood.
2. Aspirate D1SGH from the petri dish, being careful not to remove any tissue.
3. Using two sterile scalpel blades, mince the frontal cortex tissue until individual tissue pieces are no longer visible.
4. Pour papain (protease) solution over the scalpel blades, into the petri dish.
5. Gently swirl the petri dish to insure that all tissue is free in the solution, and not adhered to the bottom of the dish.
6. Incubate in a 37°C incubator for 5 minutes, and at 22°C for another 5 minutes.
7. Remove all tissue and papain with a large-bore transfer pipette and gently transfer to a 15 mL centrifuge tube.
8. Rinse petri dish with DNase/DMEM5/5 solution to remove all tissue, and transfer to the centrifuge tube. Top off with DNase/DMEM5/5 solution.
9. Balance centrifuge, and spin at 900 rpm for four minutes.
10. Following centrifugation, carefully remove and dispose of all supernatant to reveal the cell pellet.
11. Add fresh DNase/DMEM5/5 solution to a level of approximately 5 mL.
12. Using a fine-bore pipette, triturate until all or the majority of cells are in solution. Be careful not to introduce air bubbles into the trituration procedure, because they will damage cells and produce a lowered cell density.

13. Add a 10 µL sample of cells to each side of a cytometer (or according to instruction), and sample viable cells until an accurate count is made. Viable cells are seen as phase-bright round spheres under a standard compound light microscope.
14. Calculate cell pool volume based on desired density and cell count. Add DNase/DMEM5/5 to achieve desired cell pool volume.
15. Aspirate laminin from each MMEP or coverslip using a fine-bore pipette.
16. Add 100 µL cell pool to each MMEP matrix and 250 µL to each coverslip.
17. Return cultures to incubator and allow to sit undisturbed for 2-2.5 hours to promote cell-surface adhesion.
18. After 2-2.5 hours, add 1 mL DMEM5/5 to each culture and return to incubation overnight.

Medium Changes: Life of the Cultures

1. The day after culture, perform a full medium change into 3 mL DMEM 5/5 to remove any DNase from the seeding procedure.
2. Four days following initial seeding, perform a second full medium change into DMEM5 to remove fetal bovine serum.
3. For the life of the cultures, perform half medium changes into DMEM5. In addition, add 100 µL ultra-pure H_2O to each culture during each medium change to maintain osmolarity levels between 290 and 320 mOsm. Water additions should be adjusted according to regular osmolarity readings.

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